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Review Article

Lactose Intolerance in Sub-Saharan Africa

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Summary: Lactose is a disaccharide mainly found in dairy and dairy-containing products which yields D-galactose and D-glucose on hydrolysis. Lactose intolerance (LI) is characterized by abdominal pain, bloating, flatulence, nausea and/or diarrhoea following ingestion of dairy or lactose-containing meals. LI affects about 75% of the world's population though the condition is poorly recognized despite being of great public health significance in sub-Saharan Africa (ssA). The aim of the review is to highlight the epidemiology, types, pathophysiology, genetics, diagnosis, management and prevention of LI. Literature search was performed using Pubmed, Crossref and Google Scholar data bases for the terms lactose, lactose consumption + lactose intolerance, sub-saharan Africa. The high prevalence of LI in most countries of ssA is a major cause for concern with malnutrition as an independent entity serving as a key contributor especially among children. Differential diagnosis for LI poses a huge challenge due to disparity in presentation of symptoms among individuals and unavailability of testing in most routine laboratories in ssA. Though wide variation in prevalence of LI exists between countries and regions within ssA, recognizing regional patterns of LI is important to guide prevention, diagnosis and management of the condition. There is also need for increased laboratory vigilance and preparedness to tackle the silent epidemic especially in ssA.

Keywords*: Lactose Intolerance, Epidemiology, Pathogenesis, Diagnosis, sub-Saharan Africa.*

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INTRODUCTION

Lactase is an enzyme produced by cells situated in the microvilli of the small intestine which hydrolyses dietary lactose into galactose and glucose for transport across the cell membrane. In the presence of osmotic pressure and deficiency or absence of lactase, unabsorbed lactose causes an influx of fluid into the bowel lumen, and as a result, unabsorbed lactose then enters the colon and is used as a substrate by intestinal bacteria, producing short-chain fatty acids and gas via fermentation. Since the fatty acids cannot be absorbed by the colonic mucosa, more fluid is drawn into the bowel. A proportion of the lactose can be absorbed, but the overall result of ingestion is a substantial rise of gas and fluid in the bowel, causing the symptoms of lactose intolerance (LI) (Misselwitz *et al*., 2019). Although lactase expression is not up-regulated by lactose ingestion, tolerance could be induced by adaptation of the intestinal flora (Deng *et al*., 2015).

Symptoms of LI usually occur between 30 minutes and a few hours after the ingestion of lactose though severity of symptoms can be influenced by the amount of lactose consumed, degree of lactase deficiency, and the form of food substance in which the lactose is ingested; typically, the more lactose consumed, the more frequent or severe the symptoms (Jansson-Knodell *et al*., 2020). LI denotes the emergence of gastrointestinal symptoms predominantly, abdominal cramps, nausea, vomiting, bloating and/or diarrhoea, after the consumption of lactose-rich food items (Shafi and Hussain, 2022). Some individuals may experience dyspepsia, flatulence, borborygmi or abdominal distension post consumption (Misselwitz *et al*., 2019). Less commonly it may present with a range of systemic symptoms like urinary difficulties, loss of concentration, headaches, muscle and joint pain, fatigue and mouth ulcers though it is unclear whether these atypical symptoms are related to the presence of functional diseases or directly due to lactose ingestion (Deng *et al*., 2015). In the general population, symptoms are often non-specific, mild and vary between individuals (Bridges, 2018; Misselwitz *et al*., 2019).

The prevalence of LI varies with ethnicity and is related to the use of dairy products in the diet which subsequently results in genetic selection of individuals with the ability to digest lactose. Globally, the estimated prevalence of lactose intolerance exceeds 65% (Catanzaro *et al*., 2021) with about

70% of the world's population having primary lactase deficiency (Heyman, 2006) and approximately 75% losing the ability to digest lactose at some point, while others digest lactose into adulthood (Sambasivarao *et al*., 2022). The prevalence of LI in ssA countries is between 34-100% (figure 1). Despite the high prevalence worldwide, adopting a standard diagnostic procedure remains a major challenge. Differences in diagnostic criteria and methodology have obscured the global adoption of a "gold standard" in the diagnosis of LI. The review aims to highlight the epidemiology, types, pathophysiology, genetics, prevention, management and guide laboratory diagnosis of LI in ssA.

Figure 1:
Estimated Prevalence of Lactose Intolerance in ssA

EPIDEMIOLOGY

LI is the commonest form of food intolerance that does not involve immunological mechanisms and reported to allegedly affect more than two-thirds of the world's population (Queiroz *et al*., 2019). Most individuals are born with the ability to digest lactose which is the main source of nutrition until weaning and can ingest up to 20g of lactose or 12oz of milk daily without symptoms (Sambasivarao *et al*., 2022). Depending on the ethnic group, the enzyme activity gradually decreases from 2-5 years (Shafi and Hussain, 2022) or during the first 10 years of life except among those with a highly conserved mutation in the promoter region of the lactase gene (Storhaug *et al*., 2017). Variations in prevalence have been reported not only within countries but also between countries (Storhaug *et al*., 2017). The prevalence of LI varies according to ethnic characteristics and age with most causes being genetically determined (Queiroz *et al*., 2019; Shafi and Husain, 2022). Epidemiological studies report highest rates of LI among populations that historically consumed agricultural products as the main source of food since their early stages of survival (Wortmann *et al*., 2013). High prevalence of LI in certain populations is explained by two hypotheses. The first hypothesis upholds that alleles for lactase enzyme persistence were rare until the beginning of the consumption of dairy products, and unfermented milk and as a result, natural selection increased those allele frequencies. The second hypothesis advocates that alleles of LCT gene for a persistent phenotype which favored the acquisition of the habit of consuming milk and its derivatives were already present from the outset (Burger *et al*., 2007; Itan *et al*., 2009; Krüttli *et al*., 2014).

TYPES

There are various forms of lactase deficiency that can result in lactose intolerance; developmental lactase deficiency, congenital lactase deficiency, primary lactase deficiency and secondary lactase deficiency.

a. **Developmental Lactase Deficiency;** This is a type of lactase deficiency that occurs in pre-term/premature infants of less than 34 weeks of gestation due to underdevelopment of the infant intestines resulting in inability to hydrolyze lactose (Talia and Kiran, 2023). It largely arises from congenital deficiency of lactase and other disaccharidases, typically temporary and rapidly improves as the intestinal mucosa matures (Coutis, 2013).
b. **Congenital Lactase Deficiency;** It is otherwise known as alactasia and occurs due to the inheritance of the two defective alleles of the lactase (LCT) gene and the condition worsens due to the loss of nutritional components, and often leads to delay in growth, dehydration, and alkalosis (Shafi and Husain, 2022). It is a life-long genetic condition involving the complete deficiency of lactase expression from birth, despite having an otherwise normal intestinal mucosa. Congenital lactase deficiency is an extremely rare disorder with an unknown incidence reported in only a few infants (Heyman, 2006). Fewer than 50 cases have been reported globally with a higher frequency in Finland, where about 1 in 60,000 newborns are affected by this disorder (Shafi and Husain, 2022). Affected newborn infants present with intractable diarrhoea as soon as human milk or lactose-containing formula is introduced and if not recognized and treated quickly, the condition becomes life-threatening due to dehydration and electrolyte loss risk. Treatment is simply removal and substitution of lactose from the diet with a commercial lactose-free formula (Heyman 2006).

c. **Primary Lactase Deficiency:** Primary lactase deficiency (PLD) is regarded as the commonest "genetic disease" and predominant cause of LI caused by the non-persistence of β-galactosidase (Bayless *et al*., 2017). About 70% of the world's population have PLD resulting from genetically programmed decrease in lactase synthesis after weaning (Heine *et al*., 2017; Misselwitz *et al*., 2019), with global estimates reporting a prevalence of about 80% in the Black population (Cantazaro *et al*., 2021). The age of onset of PLD differs among populations due to decrease in lactase enzyme activity with increase in age (Heyman,

2006). The enzyme activity usually begins to decrease during childhood and symptoms manifest in adolescence or early adulthood (Talia and Kiran, 2023) though acute development is also possible.

d. Secondary Lactase Deficiency; The deficiency arises due to medical conditions mainly influencing the intestinal tract hence infections that affect the microvilli result in the loss of enzyme synthesis since the enzyme is secreted from the edge of the duodenum (Shafi and Husain, 2022). The ephemeral nature of SLD is caused by modification of the intestinal mucosa which leads to lactase expression reduction and lactase deficiency with resultant lactose malabsorption which may resolve after one to two months but may be permanent if caused by a long-term underlying condition (Heyman, 2006)

Medical conditions that may cause secondary hypolactasia include Crohn disease, enteropathies, bacterial, actinic or viral enteritis, celiac disease, severe malnutrition, inflammatory bowel diseases, certain medications that can cause villous atrophy such as chemotherapeutic drugs, antibiotics like neomycin, aminoglycosides, kanamycin, tetracycline and polymycin (Fassio *et al*., 2018; Asfari *et al*., 2020).

BIOCHEMICAL AND GENETIC PERSPECTIVES

a. The Unique Nature of Lactose Sugar; Lactose is the major disaccharide carbohydrate in milk occurring as β-D-Galactopyranosyl-(1→4)-D-glucose. Hydrolysis of lactose in the intestinal tract produces galactose and glucose that are absorbed into the enterocytes as sources of energy and structural elements. It has variable concentration depending on the species but exclusively found in milk of mammals. Human milk contains about 7g of lactose while other mammals such as cow and sheep contain 4.8g each and goat has 4.1g per 100 mL of milk respectively (Rossi *et al*., 1997).

b. The Lactase-Phlorizin Hydrolase (LPH) Enzyme: Lactose hydrolysis is catalyzed by lactase-phlorizin hydrolase (LPH) (EC 3.2.1.108–EC 3.2.1.62), located in the brush border membrane of small-intestinal enterocytes. LPH is expressed only in the small intestine and restricted to absorptive villi enterocytes. The site restriction control of LPH in the enterocytes is revealed by a tightly controlled pattern along the proximal-distal axis in all mammals with high levels in mid-jejunum and low levels in the duodenum and distal ileum (Naim, 2001). The enzyme is a trans-membrane glycoprotein with molecular weight of 160 kDa, having a C-terminus (26 amino acid) intra-cellularly and an N-terminus at the luminal surface of the lipid bilayer of the microvillus membrane of enterocytes. The trans-membrane-spanning region constitutes of 19 hydrophobic amino acids short sequence. It is a multifunctional specific enzyme catalyzing primarily lactose and other substrates such as phlorizin, cellobiose, lactosylceramide, cellotriose, and flavonoid glucosides. The enzyme has a four-fold internal homology designated as I, II, III and IV domains assumed to be due to two independent duplication events during evolution (Boll *et al*., 1991; Montgomery *et al*., 2007). Domains I and II are not glycosylated and observed to regulate protein folding in the endoplasmic reticulum having no enzymatic activity while III and IV are deeply glycosylated containing a glutamate residue in each nucleophile at the two active sites. The amino acid sequence around the active site glutamic acid (E) in domain III is PIYITENG while that of domain IV is PIYVTENG. First, embryonic LPH (195 kDa) is synthesized in the endoplasmic reticulum and undergoes co-translational, dolichol-dependent, high-mannose glycosylation, yielding a molecular mass of 215 kDa. In the golgi, complex glycosylation of domains III and IV occur, yielding a structure of 220kDa (N-glycosylations in asparagine and O-glycosylations in threonines and serines). The glycosylation plays a role in the enzymatic activity as well as folding and ease of intracellular transport (Naim and Lentz, 1992). The subsequent cleavage of a small N-terminal pro-enzyme and of domains I and II protect the remaining molecule, which is inserted into the microvillus membrane. The final extracellular cleavage by pancreatic proteases re-orients the pro-enzyme producing the mature LPH enzyme (Segurel and Bon, 2017).

c. Biochemical Variability of Lactose in Milk: Milk contains the primary carbohydrate, lactose, synthesized by epithelial cells of mammary glands in mammals essentially for the development and nutrition of infants. The onset of lactose synthesis and its composition in milk varies between species and throughout lactation. Little is documented about the precursors, genes, proteins and ions that regulate lactose synthesis (Mattar *et al*., 2012). Human infants receive about 40% of their caloric requirements from the approximately 70 g/d of lactose they consume in the first six months of life (Segurel and Bon, 2017).

Lactose variability has become an interesting aspect in milk production and constitutes over 80% of total carbohydrate of most placental mammal comprising of glucose and galactose. There is a negative relationship between the content of lactose and fat in milk, this variability ensures the offspring receive a steady source of calories accordingly (Buller and Grand, 1990).

d. Genetic Basis of Enzymatic Defect in LI: It is believed that humans have the ability to digest milk lactose because they possess the β-galactosidase enzyme, lactase-phlorizin hydrolase (LPH). In majority of humans after weaning, the levels of the enzyme declines and the condition is referred to as lactase non-persistence (LNP) while few individuals that sustain high levels of LPH can digest milk into adult hood and the condition is called lactase persistence (LP) (Priehodova *et al*., 2017). LP alleles have spread through migration and show strong signals of selection (Gerbault, 2013). Though LNP is a familial condition for humans, domestication of animals for diary purposes contributed to LP alleles spread among early populations (Wiley, 2020). However, populations who consume dairy products with reduced lactose content have lower incidences being reported.

The LPH enzyme, with highest levels of activity during the lactation period, is encrypted by the lactase (LCT) gene, located on chromosome 2q21 mainly expressed in the apical part of microvilli within the brush border membrane of enterocytes (Montgomery *et al*., 1991). LP can be independently caused by single nucleotide polymorphisms (SNPs) ranging from 5 to 18 or more in a regulatory region

called minichromosome maintenance complex component 6 (MCM6), which is located upstream of the LCT gene (Troelsen *et al*., 2003; Fang *et al*., 2012). The percentage LP haplotype globally is approximately 35%, with the lowest frequencies present in ssA and southeast Asia (Hassan *et al*., 2016).

Migration events have played a role in the geographic distribution of LP-associated variants in Africa (Campbell and Ranciaro, 2021) with the LI phenotype polymorphism responsible for natural selection in many communities (Queiroz *et al*., 2019). LI though common in pastoralist populations from Africa (~50% in Fulani, ~90% in Tutsi), has an estimated prevalence between 5%–20% among West African agriculturalists (Deng *et al*., 2015). The major variants C/G-13907 and T/G-13915, among the Beja of East Africa, show remarkable frequencies in Sudanese populations, especially those of pastoralists, in line with the historical links and nomadic populations bidirectional migration between East Africa and Arabia. The C/T-13910 variant, commonly linked with European populations is also uniquely present among the Fulani (Hassan *et al*., 2016). The C14010 allele which is thought to have originated from eastern Africa is present in Bantu- and Khoisan speaking pastoralist groups in southern Africa (Campbell and Ranciaro, 2021).

e. Polymorphic Defect of LCT gene in LI: Located on chromosome 2q21 and comprising 17 exons, the human LCT gene encodes 1927 amino acids from the initiation to the stop codon and covers about 49kb with a resultant mRNA of more than 6kb thereby forming a complete translation product. Initially, several genetic variants were identified within the coding region and the 50-flanking region but observed to show no significance (Boll *et al*., 1991). Certain mammals including man, mouse, rat and pig are reported to have identical sequence of their first 100 bp of the proximal LCT promoter with similar regulatory pattern. The binding sites for GATA, hepatocyte nuclear factor 1-α (HNF1-α), caudal type homeobox 2 (Cdx-2) and transcription factors are positioned relative to the transcriptional start site (Boudreau *et al*., 2002; Anguita-Ruiz *et al*., 2020). Other transcription factors such as HOXC11 (homeobox C11), HNF-3, FREAC-2/3 (fork-head box F2) and C/EBP (CCAAT/enhancer binding protein) are reported to interact with the LCT 50-flanking sequence with some in the more distal loci (Mitchelmore *et al*., 2000; Anguita-Ruiz *et al*., 2020). Exon 17 of MCM6, function as a regulatory enhancer of LCT in the cell cycle ending 3.5 kb from the start site of the human LCT gene. The transcriptional start site of the MCM6 gene is located approximately 39 kb 5' of the LCT transcriptional start site (Harvey *et al*., 1996; Mattar *et al*., 2012).

f. Lactase Phenotypes and Epigenetics Alterations: The LI phenotype is a polymorphism naturally selected to promote survival of most populations globally (Queiroz *et al*., 2019). The LP mutation was first reported in 2002 with its alleles recording 14 kb upstream of the LCT gene and not within, or immediately upstream, of it. The reported variants in Middle East and Africa were i) -13910:C>T (rs4988235), ii) -13907:C>G (rs41525747), iii) -13915: T>G (rs41380347), iv) -14009: T>G (rs869051967) and v) -14010: G>C (rs145946881) with variable frequencies (Enattah *et al*., 2002; Ingram *et al*., 2007; Anguita-Ruiz *et al*., 2020). Additional eighteen genetic SNPs markers were also recorded that map the MCM6 and are also associated with LP.

Ingram *et al*., (2006) reported that neither the -13910*T allele nor the A haplotype (LCT core haplotype), upon which it resides, account for lactase persistence even after resequencing the 13.9 kb region. The association of the phenotype of LI with these genotypes has recently been confirmed for African populations (Ranciaro *et al*., 2014). Other mechanisms including epigenetic modifications in histone proteins and DNA may be responsible for LNP and LI in addition to gene mutations (Cantazaro *et al*., 2021).

PATHOPHYSIOLOGY AND DISEASE PRESENTATION

For several decades, detection, description, and diagnosis of lactose malabsorption has been multidirectional and non-specific which has resulted in confusion among physicians and patients. Evidently, it is not possible to make a definitive diagnosis based on clinical presentation of LI alone due to the poor association between self-reported lactose intolerance and the occurrence of symptoms even after ingestion of lactose in patients with lactase deficiency (Deng *et al*., 2015).

Lactose intolerance is the manifestation of a physiologic disorder known as lactose malabsorption which is due to a disequilibrium between quantity of ingested lactose and ability to hydrolyze the disaccharide by lactase (Heyman, 2006). Two physiological processes are involved; firstly, the increased osmotic load increases the intestinal water content. Secondly, lactose is readily fermented by the colonic microbiome leading to production of gas and short chain fatty acids primarily carbon dioxide (CO2), hydrogen (H2), and methane (CH4) though these biological processes are present also for other poorly-absorbed, fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) that are found abundantly in diets. The unabsorbed lactose being present in the intestinal tract as a result of lactase deficiency has effects that can lead to symptoms of lactose intolerance such as osmotic or secretory diarrhoea and gas in susceptible individuals (Deng *et al*., 2015).

Although the link between infectious diarrhoea and secondary LI has been reported, the epidemiological data to validate this linkage is surprisingly rare (Harvey *et al*., 2018). A review of studies that clearly reported the global burden of secondary LI in relation to other diseases like childhood diarrhoea and pneumonia in children younger than five years showed the highest incidence and severity burden for these diseases were present in Africa and Asia though the studies were not performed in these regions (Walker *et al*., 2013).

SCREENING METHODS/DIAGNOSIS

There are numerous screening methods for diagnosing LI. Diagnostic investigations available include physiological challenges/tests, genetic and endoscopic investigations.

i. **Lactose Tolerance Test (LTT):** This is an indirect method based on the fact that lactose is broken down into

glucose and galactose by lactase (Law et. al., 2010). The method is therefore influenced by physiologic response to glucose (Sendino *et al*., 2020). Serial draws of blood samples are taken after ingestion of standard 50g lactose in adults. In children, 2g/Kg body weight is given, at a maximum of 25g (Scrimshaw and Murry, 1988). Blood glucose levels are checked at baseline, 60- and 120-minutes following ingestion of lactose. Blood glucose levels rising by less than 20mg/dL (<1.1mmol/L) compared to the baseline in addition to presentation of such symptoms as; bloating, diarrheoa and abdominal pain are diagnostic of LI (Alihashemi et. al., 2020).

ii. **Hydrogen Breath Test (HBT):** It is the commonest indirect approach for diagnosing LI. The intestinal flora ferments undigested lactose to methane, carbon dioxide and hydrogen gas which are eliminated via the lungs. HBT is based on the increase in expired hydrogen gas after lactose challenge. A standard dose of lactose, usually 20-50g is given orally to the patient (Misselwitz *et al*. 2019). Baseline respiratory/expired hydrogen gas is recorded followed by recording changes at 30 minutes interval (Bridges, 2018). Expired hydrogen gas levels greater than 20ppm (parts per million) compared to baseline within 3hrs of ingestion is diagnostic of lactose intolerance (Sendino *et al*., 2020 and Alihashemi *et al*., 2020). HBT has a sensitivity and specificity of 78% and 98% respectively (Gasbarrini *et al*., 2009). Thus, having the most diagnostic efficiency. HBT is not recommended for subjects with baseline hydrogen gas >20ppm. The method is greatly affected by colonic flora (Szilagyi *et al*., 2009) and as a result, bacterial overgrowth may lead to false positive results. On the other hand, intake of antimicrobials few days to the test procedure and subject's inability to produce hydrogen gas may result in false negative results and subjects must refrain from cigarette smoking and exercise 2hrs to the test in order to prevent hyperventilation and ensure accuracy of the results.

iii. **Endoscopic Biopsy:** The method involves assessment of lactase activity in a biopsy specimen of jejunum and duodenum (Bridges, 2018; Sendino *et al*., 2020) for the diagnosis of primary and secondary lactase deficiency (Matter *et al*., 2012; Deng *et al*., 2015; Bridges, 2018). The method has an estimated diagnostic sensitivity and specificity of 96% and 100% respectively (Kuokkanen *et al*., 2006 and Marton *et al*., 2012). Low lactase activity in the small bowel (jejuna/duodenal) biopsy is confirmatory for lactose intolerance (Scrimshaw and Murry, 1988).

The limitations of endoscopic biopsies are that no assessment of symptoms is made which impacts on the clinical relevance of these investigations because, only a proportion of subjects with lactase deficiency develop abdominal symptoms after ingesting glucose. The invasiveness of the test poses a huge limitation due to inhomogeneous expression of lactase across the epithelium (Deng *et al*., 2015) thereby making assay unavailable in many service laboratories (Harvey *et al*., 2018)

iv. **Genetic Testing:** Single nucleotide polymorphism cytosine (C)/thymine (T) upstream of the lactase gene is considered in the genetic testing for hereditary lactase persistence. The C/C genotype is lactose intolerant while the C/T or T/T genotypes are lactose tolerant. This method is costly compared to those described above. It involves the use of genotyping to assess lactase deficiency and often based on C/T-13910 polymorphism though other possible polymorphisms resulting in lactase deficiency can be found. The limitation of this method is it can only identify subjects with primary cause of gene under expression, but not hypolactasia caused by other conditions (Szilagyi *et al*., 2007; Bridges, 2018).

v. **Faecal pH Test:** This is a non-specific marker for lactose (or other carbohydrate) malabsorption. A pH of < 6.0 suggests lactose intolerance. The test is mostly recommended for infants below 2 years of age because of the high rate of false negative results.

vi. **Faecal Reducing Substances:** This is an indirect test for lactose (or other carbohydrate) malabsorption occasionally considered in the context of secondary lactose intolerance where a gastroscopy is being performed to determine an underlying cause (e.g., coeliac disease, Crohn's disease, protracted diarrhoea). Absence of the corresponding enzyme indicates a positive test. Nonetheless, if an individual has not ingested lactose recently a false negative report can be obtained. Inappropriate stool collection may make results of reducing substances inaccurate (Harvey *et al*., 2018).

vii. **Quick Lactose Intolerant Test (QLIT):** QLIT consists in execution of mucosal biopsies at the post-bulbar duodenum level and their subsequent incubation with lactose on a test plate. The incubation confirms the presence or absence of lactase activity. if there is a slight hypolactasia, there will be a light blue-coloured reaction; if lactase activity is present, a dark blue–coloured reaction occurs; if no staining develops, it is indicative of severe hypolactasia (Kuokkanen *et al*., 2006). The method, despite its high sensitivity, has some limitations, including high cost and invasiveness, and the size of biopsies which, if shorter or larger than 2 mm, may give false-positive or false-negative hypolactasia, due to patchy expression of lactase. Due to the bioptic nature, the method is conditioned by the patient's clinical conditions and coagulation (Mattar *et al*., 2013; Misselwitz *et al*., 2019).

Some quick test kits/point of care testing devices for diagnosis of LI are commercially available which support diagnosis by detecting activity of lactase from a biopsy specimen taken via gastroscopy from the upper small intestinal mucosa. The principle is based on a simple color change which is compared with a reference colour scale incorporated in the product package, lactase activity is detectable in 20 minutes. Severe and mild hypolactasia can easily be differentiated by this approach. The test uses a positive lactase control for daily verification of the performance of this test.

viii. **Gaxilose Test:** This non-invasive test consists of administration of a synthetic disaccharide that has a structure similar to lactose i.e. Gaxilose (4-O-β-D-galactopyranosyl-D-xylose). Similar to lactose, Gaxilose is also metabolized by lactase in the intestine and a molecule of galactose and one of xylose are derived, which are absorbed by enterocytes. Subsequently, measurement of xylose in the blood and urine, is carried out to quantify lactase activity (Hermida *et al*., 2006). The Gaxilose test is easy to use, non-invasive, well tolerated and does not cause discomfort to the subjects (Aragón *et al*., 2014; Monslave-Hernando *et al*., 2018).

If a dietary challenge proves inconclusive, alternative investigations and diagnoses should be considered, including stool examination if parasitic infestation is suspected and blood tests such as anti-tissue transglutaminase antibody, total immunoglobulin A concentration and quantitative immunoglobulins if coeliac disease or immunodeficiency is queried. These investigations along with others should be readily available in ssA to increase vigilance and preparedness of laboratories in the sub region.

Till date, no specific ideal diagnostic conditions or gold standard test has been independently singled out for LI region. Therefore, a combination of the above tests, though may be more costly and invasive, have been suggested to be most reliable. Despite the challenges, laboratory diagnosis for LI can still be performed with minimal resources. Hence the need for incorporating LI laboratory testing into clinical routine practice in the ssA sub region.

MANAGEMENT AND PREVENTION

Lactose intolerance (LI), although has a genetic basis, is also connected to culture and cultural dietary practices (Li *et al*, 2023) which is the basis of the expedience of examining the situation in ssA and the need for health care professionals to be vigilant. Inadequate indigenous population studies exist in most of SSA where dietary patterns vary from the rest of the world, especially from Caucasians leading to insufficient information that will inform epidemiological studies in ssA. Traditionally, low milk and milk products intake and high calorie intake is prevalent in most ssA. LI appears to parallel the increased consumption of milk and milk products with the embracing of Western Diet with some African regions consuming high calorie diet and low milk, presenting with low prevalence of LI (Redvers, 2019).

Although the medical basis of milk intake is essentially to ensure bone health and prevention of osteoporosis, calcium may be obtained from alternative sources to avoid LI. Understanding the above determinants are important for the management and prevention of LI and will involve understanding of population genetics, need for cultural balance and decreased frequent milk product consumption. Eliminating milk from modern ssA diet is not feasible, but a pragmatic approach including the reduction of the quantity of milk product consumed among others may be helpful. Indigenous food rich in calorie and micronutrient sources, and low in milk and milk products should be promoted. Importantly, as milk cannot be completely avoided, consumption should be proportionate to an individual's residual intestinal lactase activity. In addition to the laboratory medicine perspective, public health awareness must be raised. These will constitute the collective pragmatic approach to managing and preventing LI, although commercially produced enzymes are now available for individuals genetically deficient in lactase.

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Review Article

The Global Perspective of Human Metapneumovirus

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Summary: Globally, human metapneumovirus (hMPV) constitutes a more significant proportion of acute respiratory tract infections (ARTIs), a primary cause of morbidity and mortality in children. A range of viruses, such as influenza viruses, respiratory syncytial virus (RSV), picornaviruses, coronaviruses, parainfluenza viruses, and adenoviruses, have been linked to various respiratory syndromes across all age categories. Notably, some of these viruses circulate and co-infect individuals, increasing the possibility of resulting in complex interactions that may affect disease severity, immune response, and epidemiology. It is also known that the majority of children contract hMPV by the age of 5, with the most severe cases found in infants, comprising both symptomatic and asymptomatic infections. Although hMPV is widely common, no approved vaccines or specific antiviral therapies are available, highlighting the need for more research into focused treatments and vaccine development. Improvements in molecular diagnostics have increased detection rates, though difficulties persist in disease monitoring and management. The 2024-2025 current outbreak in China commenced in December 2024 and has experienced a rapid rise in cases, especially among children aged 14 and younger. Additionally, other countries like the United Kingdom (UK), France, and Germany have reported cases of the virus, reflecting its extensive spread. Consequently, an understanding of the epidemiology of hMPV is essential for creating effective targeted interventions. This review provides a global perspective of hMPV and points out knowledge gaps to drive future research initiatives in its management, prevention, and control.

Keywords*: Human metapneumovirus, Respiratory viruses, Epidemiology, Public Health.*

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INTRODUCTION

Human metapneumovirus (hMPV), although being a newly described virus, was first isolated in 2001 in the Netherlands from the nasopharyngeal aspirates (taken over 20 years) of 28 hospitalised children and infants with ARTIs (acute respiratory tract infections), with signs and symptoms similar to RSV (respiratory syncytial virus infection) (Mullins *et al*., 2004; Haas *et al*., 2013). However, findings from retrospective studies indicate that the virus has been in circulation since 1958 (Kroll & Weinberg, 2011) and has been found worldwide (Wolf *et al*., 2003; Vinci *et al*., 2018) (Table 1).

hMPV, a lipid-enveloped, negative-sense, single-stranded RNA virus, belongs to the subfamily Pneumovirinae within the Pneumoviridae (formerly Paramyxoviridae) family (van den Hoogen *et al*., 2004; Vinci *et al*., 2018; Uddin & Thomas, 2021). Other members of this subfamily include respiratory syncytial virus (RSV) and avian pneumovirus (van den Hoogen *et al*., 2004). Based on genetic differences, the virus is divided into two major groups (A and B) and four primary subtypes (A1, A2, B1, B2) (Boivin *et al*., 2004). The genome of hMPV is around 13 kb and consists of eight open reading frames (ORFs) that encode nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion glycoprotein (F), transcription enhancer protein (M2), small hydrophobic protein (HP), adhesive glycoprotein (G), and large polymerase protein (L) (Kamau *et al*., 2020).

The virus exhibits significant genetic diversity globally due to its evolving genome and regional circulation patterns (Kim *et al*., 2016). This is evident by the genome-wide analysis of 103 complete genomes, which identified recombination events and divergent selection pressures across genes, particularly in the G gene (Kim *et al*., 2016). Despite sparse hMPV genome sequence data and limited information on genome-wide diversity in Africa, the transmission of the virus shows strong local and regional clustering in Africa, with genotypes often circulating within subregions such as East Africa (Kenya), West Africa (Mali, Gambia), and Southern Africa (Zambia, South Africa) (Kamau *et al*., 2020; Oketch *et al*., 2021). This clustering

suggests localised outbreaks and limited intercontinental spread (Oketch *et al*., 2021).

TRANSMISSION AND PATHOGENESIS:

The disease is transmitted through direct inhalation of infected droplets or indirectly via fomites (Boivin *et al*., 2007). Upon entry into the host's respiratory tract, the virus binds to epithelial cells through its G and F glycoproteins. While the G protein helps in attaching to the virion of the host cell, the F protein facilitates the entry of the virus by promoting membrane fusion, which is subsequently followed by replication within the cytoplasm. This viral replication leads to cytopathic effects, including the formation of syncytia and the shedding of cells, which in turn contribute to inflammation and obstruction of the airways (Ballegeer and Saelens, 2020).

The pathogenesis of hMPV involves both direct damage caused by the virus and the response of the host's immune system. The infection leads to disruption of epithelial cells, excessive mucus production, and the infiltration of inflammatory cells, notably neutrophils and macrophages. The innate immune response is marked by the release of proinflammatory cytokines, such as IL-6 and IL-8, as well as interferons (Ballegeer and Saelens, 2020). However, hMPV has evolved strategies to evade or diminish these immune responses, including the disruption of Toll-like receptor signaling pathways (Ballegeer and Saelens, 2020). hMPV has been observed to mainly impact young children, the elderly, and immunocompromised individuals, presenting with symptoms that vary from mild upper respiratory infections to serious bronchiolitis and pneumonia (Deffrasnes *et al*., 2007). In young children, common symptoms include fever, runny nose, cough, pharyngitis, otitis, wheezing, and hypoxia, while adults typically experience cough, nasal congestion, hoarseness, sore throat, and fever (Schildgen *et al*., 2011). Nevertheless, the specific factors that confer protection and the immunopathological mechanisms underlying the severity of the disease are still not well understood.

DIAGNOSIS AND DIAGNOSTIC ADVANCES:

The infection can be diagnosed using various methods, such as culture, nucleic acid amplification tests (NAAT), antigen detection, and serological tests. Immunofluorescence also serves as a promising method for diagnosing hMPV infection, but it has not been implemented in clinical practice (Chiu *et al*., 2007). Due to its slow growth in conventional cell culture and mild cytopathic effects, virus culture is usually difficult (Haas *et al*., 2013). However, identifying viral RNA using NAAT techniques like reverse transcriptase-PCR (RT-PCR) is the most sensitive approach for hMPV diagnosis (Gray *et al*., 2006; Haas *et al*., 2013). Particularly, RT-PCR has established itself as the gold standard diagnostic method due to its exceptional sensitivity and specificity, as well as its capability to differentiate between the two hMPV genotypes, A and B (Haas *et al*., 2013). Also, multiplex PCR assays have shown concurrent identification of hMPV along with a range of other respiratory viruses, such as respiratory syncytial virus (RSV), influenza, adenovirus, and parainfluenza viruses (Yoshida *et al*., 2010). This capability is especially beneficial in clinical environments where co-infections are prevalent and symptoms often overlap (Yoshida *et al*., 2010; Popowitch *et al*., 2022). Currently, commercially available respiratory pathogen panels, including those from BioFire, GenMark, and Luminex, are extensively utilized in both hospitals and research laboratories (Popowitch *et al*., 2022).

TREATMENT:

Currently, there are no treatments and vaccines for this disease except supportive measures such as oxygen therapy, bronchodilators, corticosteroids, and mechanical ventilation (Yoshida *et al*., 2010). However, ribavirin and intravenous immunoglobulins have shown in-vitro activity but are not standard treatments (Khan *et al*., 2024). Research has been conducted on monoclonal antibodies (mAbs) that target the F protein, with several showing neutralizing effects in animal studies. Nevertheless, there are currently no mAbs approved for clinical application against hMPV (Proenca-Modena *et al*., 2011). Consequently, prevention relies on hygiene practices such as frequent hand washing, sanitizing surfaces, wearing masks in crowded areas during peak seasons, and maintaining social distancing when feeling unwell (Khan *et al*., 2024).

GLOBAL EPIDEMIOLOGY AND BURDEN OF HUMAN METAPNEUMOVIRUS:

Since its first isolation, cases of hMPV have been recorded in individuals of all ages across North America, South America, Europe, Africa, Asia, and Oceania, with seasonal variations in these regions (WHO, 2025). For instance, Rodriguez *et al*. (2020) reported that hMPV displays a seasonal distribution, with peaks noted at the end of winter and in spring, following RSV and Flu in temperate areas. The study further reported that the virus circulates alongside the parainfluenza virus throughout winter and into late spring (Rodriguez *et al*., 2020). In contrast, many other regions experience year-round circulation with reduced activity in late spring, summer, and fall (Kahn, 2006). However, the onset of the COVID-19 pandemic disrupted its circulation. While enveloped viruses continued to circulate from the summer of 2020, hMPV and RSV were not among the dominant strains. In the summer of 2021, hMPV and RSV were again in circulation and caused two epidemic peaks, the second of which started in the autumn (Kivit *et al*., 2022; Piñana *et al*., 2023). For instance, in China, there has been a surge surpassing pre-pandemic levels (World Health Organization, 2024). Also, reports from the UK Health Security Agency (UKHSA) indicated a slight increase in HMPV positivity, reaching approximately 4.5% among the respiratory samples analyzed in the United Kingdom (UK GOV, 2025).

Similarly, Japan saw a notable increase in hospital admissions related to hMPV from July 2022 to June 2023 following the relaxation of COVID-19 restrictions (Fukuda *et al*., 2023). According to seroprevalence studies, around 90 to 100% of children get infected with hMPV by the age of 5 to 10 years (Arnott *et al*., 2011; Banerjee *et al*., 2011; Uddin & Thomas, 2021). Furthermore, the PERCH study, which identified the leading cause of severe pneumonia in hospitalised children across seven countries in Africa and Asia over two years, found viruses to be the predominant (61%) cause (O'Brien *et al*., 2019). This study also found that hMPV is third after RSV and Rhinoviruses (O'Brien *et al*., 2019). Additionally, a 2018 systematic review reported that the disease accounts for 6.1–6.4% of hospital

admissions related to ALRI (acute lower respiratory tract infections) in patients under 20 years old globally (Wang *et al*., 2021). The findings of this review further revealed that between 1990 and 2015, the mean hMPV-associated ALRI incidence was 22.1 per 1000 children per year in children aged 0–59 months in settings with high child mortality, and 18.9 in settings with low child mortality (Wang *et al*., 2021).

Although, in Africa, the true burden of hMPV is not known due to underreporting, studies conducted in countries like South Africa, Kenya, Uganda, Yemen, and Senegal (Table 1) have revealed that the virus is a notable cause of lower respiratory tract infections (LRTIs), often co-circulating with RSV and influenza viruses (Kahn, 2006). For instance, Ramocha *et al*. (2021) discovered that hMPV is responsible for 4.7% of LRTIs and severe acute respiratory infections (SARI) in African children under five years, with the case fatality rate estimated at 1.3%. Similarly, Owor *et al*. (2016) reported that the hospitalization incidence in Kenyan children under five due to hMPV ranged from 1.2% to 8.7% annually, with children under one year mostly affected. Malnourishment, HIV infection, and other immunosuppressive conditions further contribute to the risk of severe disease in African populations (Madhi *et al*., 2007; Groome *et al*., 2015).

Table 1:
Historical Overview of Human Metapneumovirus

Continent	Countries	Year of isolation/ detection	Outbreaks/incidences observed	Key findings	References
Europe	Netherlands	2001	Initial isolation of hMPV from respiratory samples of Dutch children.	First identified in 2001; the prototype strain was isolated.	(van den Hoogen *et al.,* 2004)
	United Kingdom	2000-2001	Sentinel general practices in England and Wales gathered samples from patients of every age exhibiting influenza-like illnesses (ILI) during the winters of 2000-2001.	hMPV was identified in 9 (2.2%) patients, and appeared to be associated with community-acquired ARTI.	(Stockton *et al.,* 2002)
	Finland	2001	The detection of hMPV by PCR in ten (8%) of 132 consecutive children admitted to Turku Hospital, Finland, for acute expiratory wheezing.	hMPV accounted for 8% of the total patients investigated. Co-infection with other respiratory viruses was also reported.	(Jartti *et al.,* 2002)
	Spain	2000s	Reported cases among pediatric populations; seasonal peaks like RSV.	hMPV prevalence rates was reported alongside RSV and other respiratory viruses.	(Kahn, 2006)
	Italy	2000-2002	The study examined nasal swab specimens from 90 infants with acute respiratory tract infections in Pisa, Italy, throughout three respiratory virus seasons.	The incidence of infection varied in each of the 3 years, with the rates of positivity for hMPV being 7% in 2001 but 37 and 43% in 2000 and 2002, respectively.	(Maggi *et al.,* 2003)
	Austria	2000-2007	Yearly surveys showed that seasonal hMPV activity varied substantially from year to year, with the majority of hMPV infections occurring in winter or spring.	The hMPV seasonality showed a biennial pattern of alternate winter versus spring activity.	(Aberle *et al.,* 2008)
	Switzerland	2004-2008	A total of 3,934 Nasopharyngeal aspirates (NPAs) were tested for hMPV, of which 198 (5%) were positive.	hMPV epidemics follow a biannual variation in the study.	(Heininger *et al.,* 2009)
South America	Argentina	2004	Prevalence of 20.3% among respiratory infections in children under one year old.	Local strains show high genetic similarity with those from neighbouring countries.	(Kahn, 2006)
	Brazil	2000s	hMPV was detected in pediatric cases; seasonal patterns were observed.	Similar trends in respiratory infections were noted across various age groups.	(Kahn, 2006)
North America	United States	2002	The first documented study of the occurrence of hMPV in the United States.	The study confirms that hMPV infection occurs in young adults as well as elderly persons.	(Falsey *et al.,* 2003)
	Canada	2000-2002	Children aged <5 years and elderly subjects aged 165 years represent 35.1% and 45.9% of the hMPV-infected cases, respectively.	In hospitalised children, the most frequent diagnoses were pneumonitis (66.7%) and bronchiolitis (58.3%), whereas bronchitis and/or bronchospasm (60%) and pneumonitis (40%) were most commonly seen in elderly subjects.	(Boivin *et al.,* 2002)

Asia	Thailand	2001-2002	The study investigated the prevalence of hMPV in Thai children by RT-PCR, using primers specific for the N gene.	The results showed a prevalence of hMPV in 4.2% of the patients tested.	(Thanasugarn *et al.,* 2003)
	Israel	2002	A high prevalence of anti-hMPV antibodies among young children in southern Israel. By age 2 years, >50% of children have been exposed to the virus.	Studies indicated circulation and genetic diversity.	(Wolf *et al.,* 2003)
	Japan	2000s	Frequent transmission with China noted.	Close phylogenetic relationship with strains from China; regional transmission dynamics observed.	(Liu *et al.,* 2019)
	India	2003	The study suggests the importance of hMPV in causing mild and severe respiratory infections among children, especially infants in India.	First detection of hMPV in Children with ARIs in India.	(Rao *et al.,* 2004)
	Singapore	2005-2007	The first attempt to assess the importance of hMPV among the pediatric population in Singapore.	An estimated infection rate of 5% observed in this study indicates that hMPV is a notable contributor to illness in Singapore's children.	(Loo *et al.,* 2007)
	China	2013-2017	Increasing incidence, especially in Guangzhou; peak detection rates around 11% in 2017.	Emergence of B1 lineage; substantial seasonal patterns.	(Liu *et al.,* 2019)
	Malaysia	2010–2012	hMPV is an important, although relatively infrequent, cause of respiratory virus infection in hospitalised children in Malaysia, with prematurity and asthma as the commonest predisposing conditions.	The prevalence rate of hMPV between 2010 and 2012 was 1.1%, and it contributed 6.5% of confirmed viral respiratory infections.	(Nor'e *et al.,* 2014)
	Vietnam	2009	Hospitalized Vietnamese children with ARIs were investigated for 13 viral pathogens using multiplex-PCR	hMPV accounted for 4.5% of the confirmed viral respiratory pathogens.	(Yoshida *et al.,* 2010)
Oceania	Australia	2001	Three hMPV isolates were identified from 200 nasopharyngeal aspirate samples collected in 2001 from children with respiratory illness in Brisbane hospitals. These samples, initially negative for common respiratory viruses via antigen testing and culture, were screened using PCR. The PCR results confirmed hMPV presence, with sequences showing 100% homology to known hMPV strains.	The first report of the presence of hMPV infection in Australian children and described a new viral respiratory syndrome.	(Nissen *et al.,* 2002)
Africa	Yemen	2002-2003	This is the first report of hMPV from the Arabian Peninsula and confirms its importance as a cause of ARI in Yemen.	hMPV had marked seasonal variations with an RSV peak in December and January and an hMPV peak in February and March.	(Al-Sonboli *et al.,* 2005)
	Kenya	2007	The study was on the viral aetiology of severe pneumonia among Kenyan young infants and children.	hMPV accounted for 3.0% of the total sample.	(Berkley *et al.,* 2010)
	Uganda	2008	The study investigated the presence of viruses associated with (ILI) in Uganda.	hMPV accounted for 1.4% of the total sample.	(Balinandi *et al.,* 2013)
	South Africa	2009-2012	The description of LRTI hospitalizations among South African children aged <5 years.	hMPV (5%) was detected alongside other respiratory pathogens during outbreaks.	(Cohen *et al.,* 2015)
	Senegal	2012-2016	hMPV detection rates in the different age groups varied significantly with the children under 5 years group accounting for 71.7% of positive patients.	The temporal distribution pattern for hMPV infection showed a clear seasonal pattern with higher activity during the rainy period (July-September). Phylogenetic analyses revealed that hMPV specimens circulating in Senegal were distributed into the two main genetic lineages, A and B.	(Jallow *et al.,* 2019)

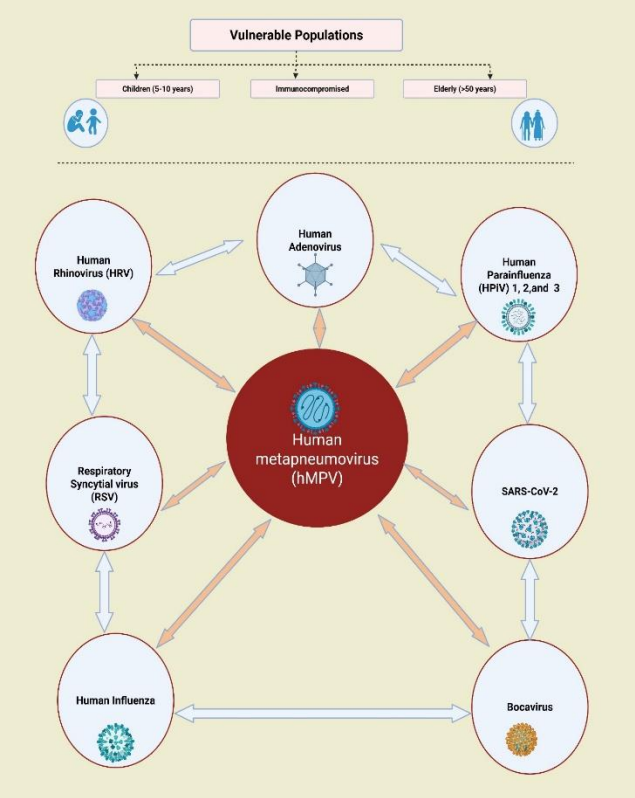

Figure 1:
Interrelationship between human metapneumovirus and other respiratory viruses

HUMAN METAPNEUMOVIRUS AND OTHER RESPIRATORY VIRUSES:

hMPV usually circulates and co-infects people with other respiratory viruses (including RSV, influenza viruses (types A and B), parainfluenza viruses, rhinoviruses, bocaviruses, and adenoviruses), resulting in complex interactions that may affect disease severity, immune response, and epidemiology (Etemadi *et al*., 2019; Jallow *et al*., 2019; Yew *et al*., 2019) (Figure 1). Semple *et al*. (2005) indicated that co-infections of hMPV and RSV may lead to more severe respiratory diseases, especially in children and those with weakened immune systems. Conversely, an earlier study by Bosis *et al*. (2004) observed that a small group of children co-infected with hMPV and RSV or influenza viruses showed no signs of greater disease severity.

KNOWLEDGE GAPS IN HMPV DIAGNOSIS, TREATMENT, AND PREVENTION:

Despite two decades of research since the identification of hMPV, several critical knowledge gaps persist, hindering advancements in its diagnosis, treatment, and prevention. Addressing these gaps is vital for informing public health strategies, driving innovation in clinical management, and ultimately reducing the global disease burden associated with hMPV infections. A huge gap in knowledge exists regarding the pathogenesis of hMPV and the corresponding host immune responses. While it is established that hMPV can disrupt innate immune signaling, particularly by inhibiting interferon production, the precise viral-host interactions that influence the severity of the disease are not well understood (Ballegeer and Saelens, 2020). Also, current epidemiological insights into hMPV are primarily from hospital-based studies, which may not accurately reflect the actual incidence and impact of the disease within the community. There is therefore a pressing need for longitudinal, population-based studies that will focus on geographic and socioeconomic factors as well as other risk factors to enhance our understanding of the transmission dynamics of hMPV.

As revealed by the reviewed studies, the lack of specific antiviral treatments for hMPV constitutes a considerable challenge in patient management. Future research should focus on the development of targeted therapies, such as small-molecule inhibitors, immunomodulators, and monoclonal antibodies (Proenca-Modena *et al*., 2011). There is an urgent requirement for well-structured clinical trials to assess the safety and effectiveness of these treatments across diverse patient groups (Li *et al*., 2024).

Although promising vaccine candidates in the preclinical phase exist, no licensed vaccine for hMPV is currently available. Research efforts should aim at developing broadly protective and long-lasting vaccines that can effectively address the virus's genetic diversity (Li *et al*., 2024).

While molecular diagnostics have become the standard for detecting hMPV, their accessibility remains limited in low-resource environments, where the disease burden is often the highest. It is vital to develop cost-effective, rapid, and point-of-care diagnostic tools that retain high sensitivity and specificity to support global surveillance and outbreak management

DISCUSSION

Human metapneumovirus has gained increased attention in the post-COVID era due to its role in respiratory infections, particularly in vulnerable populations. Although hMPV was not as widely recognized as COVID-19 or influenza viruses initially during the pandemic era due to stringent control measures suppressing its spread along with other viruses like RSV, however, it poses great risks now that many countries have relaxed their infection control strategies.

Studies also suggest that hMPV is associated with a substantial morbidity affecting both pediatric and adult populations. The virus usually presents its epidemic peak in late winter and early spring in pre-pandemic seasons, but its epidemic nature in 2020 was subtly interrupted by the SARS-CoV-2 pandemic (Piñana *et al*., 2023). Furthermore, according to Piñana *et al*. (2023), two unexpected epidemic peaks occurred in the summer and autumn of 2021, with the latter associated with an increase in both the virus's prevalence and the median age of affected pediatric patients.

CONCLUSION

The hMPV is an important respiratory pathogen with clinical similarities to RSV and influenza but with distinct epidemiological characteristics. Given its global impact, especially among vulnerable populations, focused public health strategies and research are vital to mitigate its burden. Additionally, genomic surveillance is essential in monitoring hMPV evolution by enabling the identification of new variants and linking these discoveries to the clinical attributes of confirmed cases, which is important for public health purposes. Furthermore, there is the need for the incorporation of molecular assay as a routine diagnostic in

the hospitals as this can improve the diagnosis and management of respiratory tract infections among the vulnerable populations, particularly children. This will ensure the development of effective treatment, prevention, and control strategies against this respiratory virus.

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Full-length Research Article

Characterization of the Clinical Phenotype and Reproductive Hormones of Polycystic Ovary Syndrome in Nigerian Population

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Summary: This study described the peculiarity of the clinical phenotypes and the pattern of reproductive hormones among women with Polycystic Ovary Syndrome (PCOS) in the Nigerian populace. A total of 90 consented volunteers consisting of 45 PCOS and 45 controls were recruited. The diagnosis of PCOS was established using the International PCOS guidelines 2018. Demographic, anthropometric, and clinicopathological data were obtained from each participant. Hormonal assay was done using the electrochemiluminescence (ECL) technology (Roche Diagnostics, Switzerland). Statistical analysis was done using the statistical package for the social sciences (SPSS) version 25. Polycystic ovaries (PCO) are the most popular feature of PCOS, observed in more than 90% of PCOS subjects, followed by oligomenorrhea (68 %), while hirsutism and acne were found in about 50% of the cases. The study shows 62 % of PCOS subjects had phenotype D, 52 % Phenotype C, 40 % Phenotype B, while 38 % had Phenotype A. Increased levels of Anti-Mullerian hormone (AMH), Testosterone, Prolactin, Luteinizing hormone (LH) and LH: FSH were observed in PCOS with median (95% Confidence interval) of 4.98(3.4-7.1), 32.5(19.75-53.0), 401.6(230.9-623.0), 9.23(5.4-16.6) and 2.03(1.48-3.58), respectively compared to control [1.43(0.8-2.4), 19.0(12.3-29.5), 252.2(196.3-337.0), 6.36(4.0-12.6) and 1.47(0.7-2.4), respectively] ($p<0.05$). The serum levels of follicle-stimulating hormone (FSH), estradiol, and sex hormone binding globulin (SHBG) were lower in PCOS with values of 5.21(3.6-5.9), 70.45(50.5-145.9) and 44.84(27.3-75.7), respectively compared to controls [6.0(4.4-7.9), 104.0(64.6-216.6) and 74.05(54.0-96.8), respectively] ($P<0.05$). FSH, LH:FSH, AMH, testosterone, estradiol, SHBG, and prolactin show significant odd ratio with risk analysis in PCOS ($p<0.05$). There existed a negative correlation between the hormones (Estradiol and AMH) and the PCOS phenotypes (estimated Spearman's rho were -.310 and -.348, respectively) ($p<0.05$). Phenotype D and C characterized by ovulatory dysfunction, polycystic ovary morphology and hyperandrogenism are the two predominant phenotypes of PCOS in our study population. This is accompanied by marked changes in hormonal pattern among PCOS subjects, particularly steroids and follicular hormones. Modulating this phenotype-hormonal interplay may support or improve PCOS management in the study population.

Keywords: *Polycystic Ovarian Syndrome, Reproductive Hormones, Clinical Phenotype.*

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INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is a group of symptoms associated with endocrine disorders and has been described as the main endocrine disorder in women, with an associated increased risk for infertility and a myriad of metabolic conditions (Conway *et al*., 2014). The global prevalence of PCOS has been reported to range from 6% to 10% (Bozdag, 2016). Different diagnostic criteria are believed to have contributed to the variation in the prevalence of PCOS reported across various populations.

The diagnosis of PCOS depends on clinical, morphological, and biochemical criteria. Stein and Leventhal first described polycystic ovaries by linking ovulatory dysfunction with morphologic changes of the ovaries to define the PCOS (Stein and Leventhal) Since 2003, a threshold of 12 follicles (measuring 2–9 mm in diameter) per whole ovary has been largely used, which now seems outdated. Under Rotterdam criteria, PCO morphology is defined as a follicle number per ovary of ≥ 12 and/or an ovarian volume of >10 cc in at least one ovary, however, the 2014 Androgen Excess and PCOS Society task force recommended the use of ≥25 follicles and/or a

volume of >10 cc (Dewailly, *et al*., 2014), and in a more recent data, the International PCOS Guideline for diagnosis of PCOS revised the criteria for definition of PCO morphology and thus, recommended ≥20 antral follicles (2-9 mm) per ovary and/or an ovarian volume ≥10 mL as a diagnostic threshold using a transducer frequency ≥8 MHz (Teede, *et al*., 2018). Four phenotypic classifications of PCOS have been recommended as follows: Phenotype A: Hyperandrogenism (HA) (clinical or biochemical presence) + Ovulatory Dysfunction (OD) + Polycystic Ovary (PCO) morphology; Phenotype B: HA + OD; Phenotype C: HA + PCO; and Phenotype D: OD + PCO (Teede, *et al*., 2018).

The most common clinical symptoms of PCOS include menstrual disorders such as oligomenorrhea or amenorrhea, infertility, high levels of masculinizing hormones manifested by acne and hirsutism, and metabolic syndrome which appear as a tendency towards central obesity and other symptoms associated with insulin resistance [Kabel, 2016]. Available data on PCOS pathophysiology suggests the role of different factors, including androgen excess, obesity, insulin resistance, environmental factors, genetic, and epigenetics (Adewuni *et al*, 2022, Bednarska and Siejka 2017, Ganie *et al*., 2019). The hypothalamic-pituitary unit and the ovaries feedback communication is an important component of the woman reproductive cycle, and estradiol and progesterone play a major role in these feedback communications. Women with PCOS experience an increase in hypothalamic Gonadotropin-releasing hormone pulses frequency and this result in an increased LH/FSH ratio (Lewandowski *et al*., 2011) In women, pituitary gonadotropins regulate oocyte development, folliculogenesis, and the development and maintenance of the corpus luteum. Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) drive the synthesis of the traditional gonadal sex steroid hormones: estradiol from granulosa cells, androstenedione from theca cells, and progesterone from luteinized granulosa cells (Garg and Berga, 2020)

The heterogeneity of PCOS may well reflect multiple pathophysiological mechanisms, however, the exact etiology and pathogenesis, especially in our study population have not been well-established.

MATERIALS AND METHODS

Study Population: The study involved a total of 90 consented volunteers (50 PCOS and 40 controls). PCOS diagnosis was done using the criteria defined by International PCOS guidelines 2018, requiring the presence of any two of (1) Oligomenorrhea – and/or anovulation, (2) Clinical and/or biochemical signs of hyperandrogenism, and (3) Polycystic ovary morphology (presence of 12 or more follicles in each ovary, 2-9 mm in diameter and/or increased ovarian volume>10mL). Apparently, healthy age-matched women with no PCOS were recruited as controls.

Ethical Approval: Ethical approval was granted by the Health Research Ethics Committee, Lagos State Ministry of Health Service Commission (No. LSHSC/2222/VOL.I/64).

Demographic and Clinical Data: General demographic and clinicopathological details were obtained from the participants using structured questionnaires. Anthropometric data such as height, weight, waist circumference, and hip circumference were measured by conventional methods, and the body mass index (BMI) was calculated as weight (kg)/height^2 (m2), while the waist-hip ratio (WHR) was calculated as the measurements of the waistline/hipline ratio.

Sampling and Hormonal Assay: Five mL of venous blood was collected into plain bottle and centrifuged for 10 mins at 3000rpm to separate serum for hormonal analysis. LH, FSH, AMH, Testosterone, Progesterone, Estradiol, Dehydroepiandrosterone sulfate (DHEAS), SHBG and Prolactin were assayed using electrochemiluminescence (ECL) technology (Roche Diagnostics, Switzerland).

Statistical analysis: Statistical data analysis was performed using IBM SPSS version 25 (IBM Illinois, USA). Data were analyzed using student's t-test, Mann-Whitney U, ANOVA and Logistic regression, where appropriate. A p-value < 0.05 was considered statistically significant. The calculated sample size was 37, however, to increase the power of the study, the actual PCOS sample size used was 50.

RESULTS

The demographic and anthropometric characteristics of the PCOS and controls were presented in Table 1. The mean weight, BMI, waist circumference, and waist-hip ratio were higher in PCOS (77.70±15.7, 29.57±6.0, 0.93±0.1 and 0.87±0.1, respectively) compared to the controls (68.43±11.5, 26.19±4.4, 0.87±0.1 and 0.83±0.1, respectively) p<0.05. Figure 1 shows the clinical characteristics distribution among the study population. 58 % had hirsutism, 94 % showed polycystic ovaries (PCO) morphology on Transvaginal scan, 68 % had Oligo/amenorrhea, 46 % had acne, and 8 % had Acanthosis nigricans.

Table 1:
Comparison of Demographic and Anthropometric Parameters in the Study Population

Parameter	PCOS (Mean±SD)	Control (Mean±SD)	t-value	p-value
Age (Years)	29.44±5.0	30.83±4.0	-1.367	0.175
Age at menarche (Years)	13.15±2.2	14.13±2.3	-1.131	0.264
Weight (Kg)	77.70±15.7	68.43±11.5	2.942	**0.004***
Height (m)	1.62±0.1	1.72±0.6	-1.124	0.264
BMI (Kg/m^2)	29.57±6.0	26.19±4.4	2.810	**0.006***
Waist circumference (m)	0.93±0.1	0.87±0.1	2.666	**0.026***
Hip-circumference (m)	1.07±0.1	1.04±0.1	1.369	0.175
Waist hip ratio (WHR)	0.87±0.1	0.83±0.1	2.304	**0.024***

*Difference is statistically significant, p<0.05. student's t-test

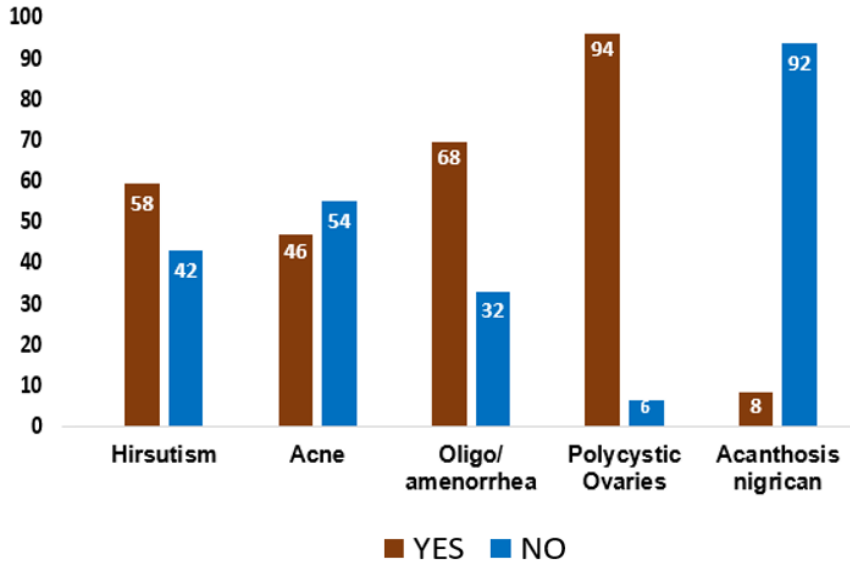

Figure 1:
Characteristics of Clinical Symptoms among PCOS Subjects.

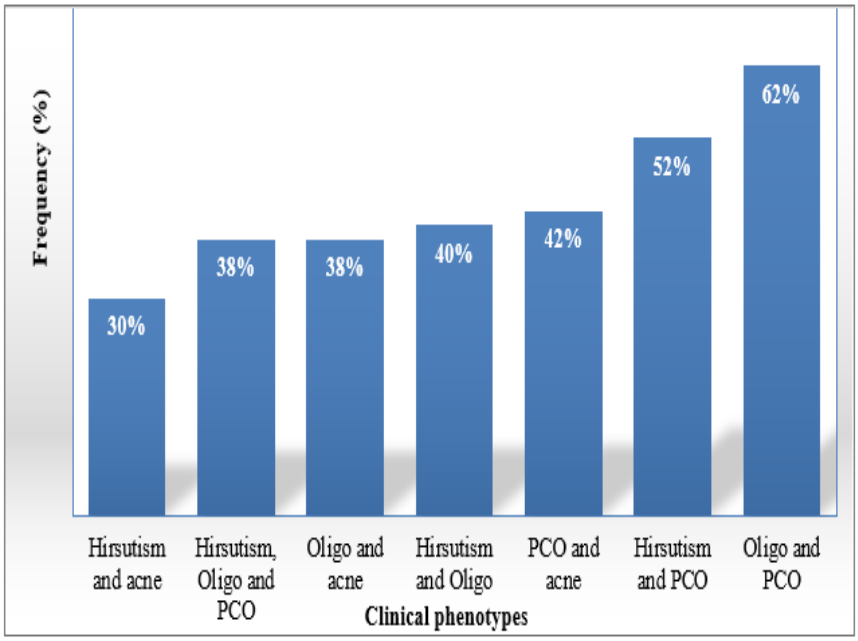

Figure 2:
Distribution of Clinical Phenotypes of PCOS in the Study Population.

Table 2:
Risk Analysis of Significant Hormonal Parameters in PCOS

Parameter	Odd ratio	95% CI	p-value
FSH	0.854	0.730-0.999	**0.049***
LH	1.037	0.986 – 1.090	0.156
LH/FSH ratio	1.691	1.162- 2.461	**0.006***
AMH	1.823	1.374 – 2.42	**0.001***
SHBG	0.987	0.977-0.998	**0.021***
Testosterone	224.8	8.418 – 6002.3	**0.001***
Estradiol	0.993	0.987-0.999	**0.021***
Prolactin	1.003	1.001-1.005	**0.016***

**Odd ratio is significantly significant, $p<0.05$*

The clinical phenotypes among the PCOS group are described in figure 2; the study shows that 62 % of cases had both PCO and Oligomenorrhea (phenotype D), 52 % had both Hirsutism and PCO (Phenotype C), 40 % had both hirsutism and Oligomenorrhea (Phenotype B), while 38 % had a combination of Hirsutism, Oligomenorrhea and PCO (Phenotype A). Table 2 shows the risk analysis of significant hormonal parameters in PCOS. (FSH, LH: FSH, AMH, testosterone, estradiol, SHBG, and prolactin) show significant odd ratio ($p<0.05$). Table 3 shows the anthropometric and hormonal comparison between the four phenotypes (A, B, C and D). The mean WHR is higher in Phenotype A (0.94 ± 0.14) compared to phenotype D (0.83 ± 0.07), $p \leq 0.05$. Figures 3a and 3b show the comparison of gonadotropins (FSH and LH) and the LH:FSH ratio. FSH (mIU/ml) was lower in PCOS [5.21(3.6-5.9)] than in control group [6.0(4.4-7.9)] ($p<0.05$). LH and LH:FSH ratio were higher in PCOS [9.23(5.4-16.6) and 2.04(1.5-3.6), respectively] than in the control group [6.36(4.0-12.6) and 1.47(0.7-2.4), respectively] ($p<0.05$). The Anti-Mullerian hormone (AMH) was higher in PCOS [4.98(3.4-7.1)] than in the control group [1.43(0.8-2.4)] ($p<0.05$) (Figure 3c).

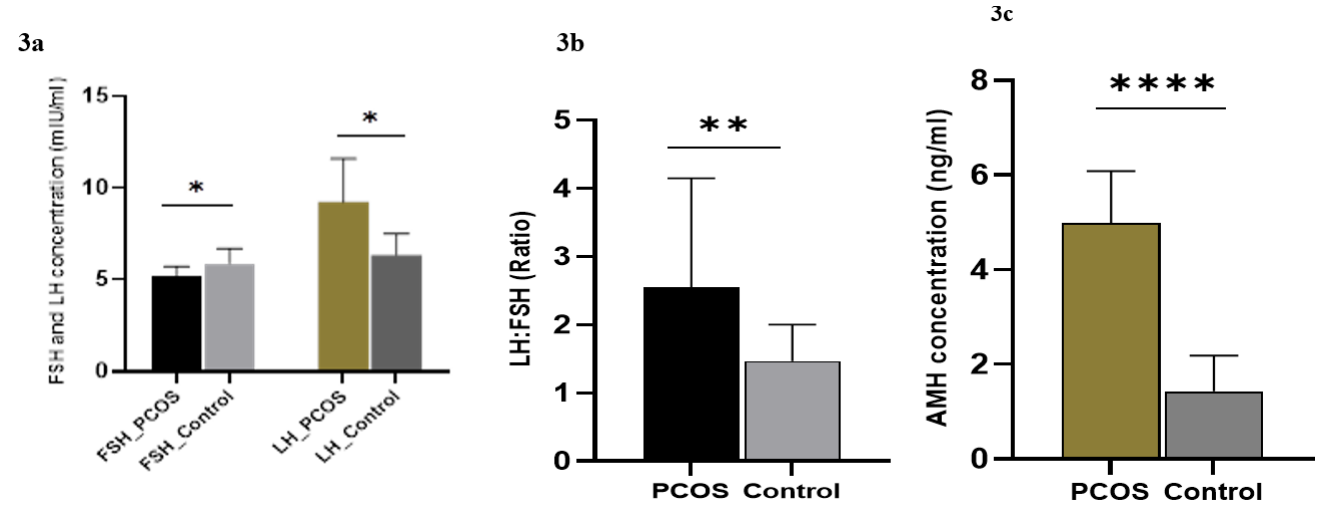

Figure 3:
Comparison of Gonadotropin and Anti-mullerian Hormones in PCOS and Controls. Values are presented as median (Q1 – Q3), using Mann-Whitney U test. Q1: 25% percentile, Q3: 75% percentile. Statistical significance is at *P<0.05, **P<0.01, ****P<0.0001

Table 3:
Anthropometric and hormonal comparison between the four phenotypes (A, B, C and D

Parameter	Phenotype A (Mean ± SD)	Phenotype B (Mean ± SD)	Phenotype C (Mean ± SD)	Phenotype D (Mean ± SD)	F-Value	P - Value
Age	30.25 ± 2.66	29.80 ± 5.61	30.57 ± 4.33	28.00 ± 5.88	0.814	0.493
Weight (kg)	85.50 ± 23.63	80.25 ± 12.10	74.24 ± 13.71	75.52 ± 14.58	1.095	0.361
Height (m)	1.66 ± 0.06	1.61 ± 0.03	1.62 ± 0.08	1.62 ± 0.07	1.235	0.308
BMI	30.89 ± 7.84	31.25 ± 5.06	28.40 ± 4.79	28.96 ± 6.57	0.616	0.608
WC (m)	0.98 ± 0.16	0.96 ± 0.13	0.94 ± 0.15	0.88 ± 0.12	1.207	0.318
HC (m)	1.05 ± 0.14	1.11 ± 0.07	1.05 ± 0.13	1.06 ± 0.08	0.691	0.562
WHR	0.94 ± 0.14	0.86 ± 0.07	0.89 ± 0.08	0.83 ± 0.07	3.216	**0.031***
FSH (mIU/ml)	5.48 ± 3.11	6.10 ± 2.42	5.12 ± 2.69	4.58 ± 1.76	1.191	0.324
LH (mIU/ml)	16.31 ± 5.97	15.36 ± 6.41	10.92 ± 2.90	10.39 ± 8.34	1.079	0.368
AMH (ng/ml)	6.64 ± 2.80	8.39 ± 4.45	5.77 ± 3.35	4.6 ± 3.44	2.542	0.068
LH:FSH (Ratio)	2.88 ± 1.48	2.72 ± 1.20	2.34 ± 1.9	2.46 ± 1.63	0.237	0.870
DHEAS (ug/dl)	123.56 ± 55.92	144.90 ± 53.76	189.33 ± 134.92	143.11 ± 60.59	0.678	0.570
SHBG (nmol/l)	37.02 ± 6.84	37.79 ± 16.83	73.96 ± 54.15	52.09 ± 36.87	2.430	0.077
Testosterone (ng/dl)	0.44 ± 0.27	0.40 ± 0.20	0.43 ± 0.11	0.28 ± 0.14	1.103	0.357
Estradiol (pg/ml)	141.17 ± 67.56	72.07 ± 32.76	114.57 ± 72.89	81.48 ± 57.62	1.911	0.141
Prolactin (uIU/ml)	459.0 ± 107.23	247.51 ± 113.99	658.97 ± 160.83	594.81 ± 110.13	1.800	0.160
Progesterone (ng/ml)	0.92 ± 0.34	0.93 ± 0.56	3.11 ± 1.37	3.85 ± 1.81	0.909	0.444

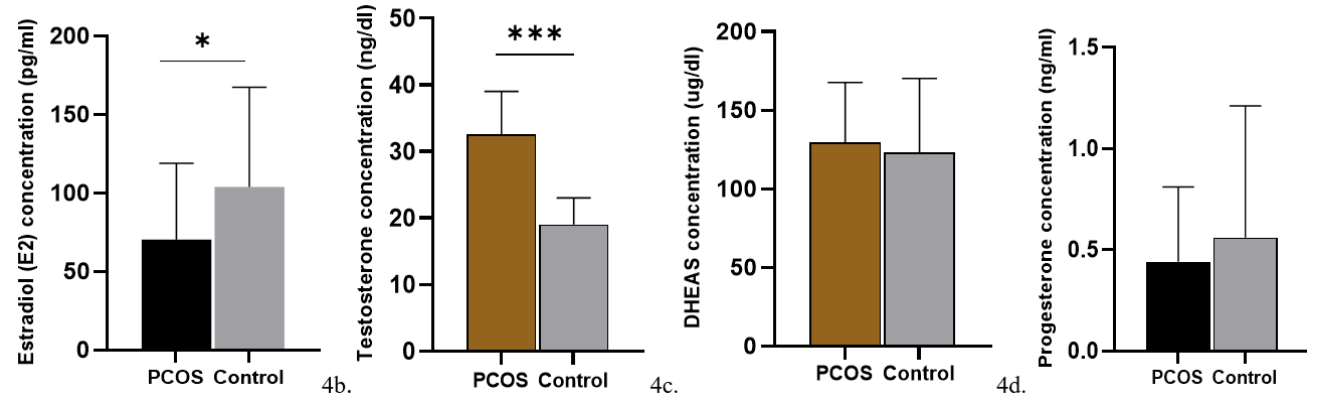

Figure 4:
Comparison of Steroid Hormones in PCOS and Controls. Values are presented as median (Q1 – Q3), using Mann-Whitney U test. Q1: 25% percentile, Q3: 75% percentile. Statistical significance is at *P<0.05, ***P<0.001

Figure 4a and 4b show the estradiol and testosterone levels respectively. Estradiol was lower in PCOS [70.45(50.5-145.9)] than in the control group [104.0(64.6-216.6)] (p<0.05), and testosterone was higher in PCOS [32.5(19.7-53.0)] than in control [19.0(12.3-29.5)] (p<0.05). There was no significant difference in the progesterone and DHEAS concentrations between PCOS and control respectively (Figure 4c and 4d). SHBG was lower in PCOS [44.85(27.3-75.7)] than in control [74.05(54.0-96.8)] (p<0.05) (Figure 5a). However, prolactin was higher in PCOS [401.6(230.9-623.0)] than in control [252.2(196.3-337.0)] (p<0.05) (Figure 5b).

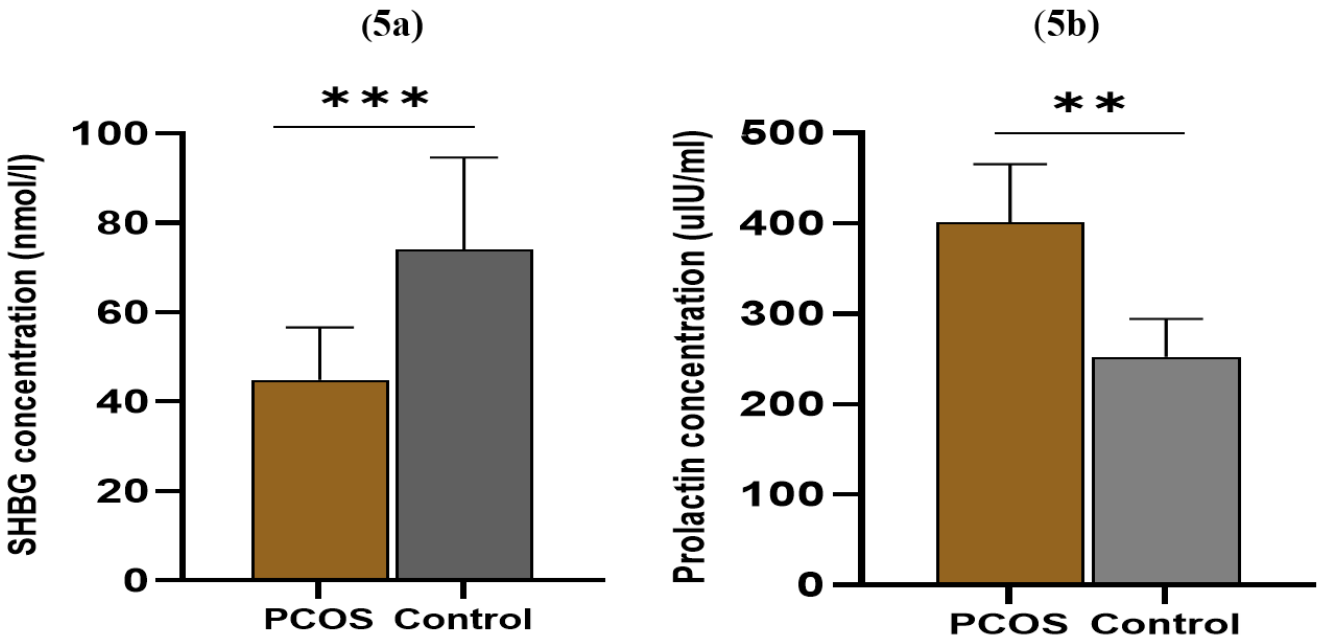

Figure 5:
Comparison of transport hormone (SHBG) and Prolactin serum levels in PCOS and control. Values are presented as median (Q1 – Q3), using Mann-Whitney U test. Q1: 25% percentile, Q3: 75% percentile. Statistical significance is at **P<0.01, ***P<0.001, ****P<0.0001

The Spearman's correlation shows a negative correlation between Estradiol and PCOS phenotypes (estimated Spearman's rho was -.310) ($p<0.05$) (Table 4). Similarly, there existed a negative correlation between AMH and PCOS phenotypes A, B, C and D (estimated Spearman's rho was -.348) ($p<0.05$) (Table 4).

Table 4:
Spearman's correlation between the Hormones and PCOS Phenotypes

Parameter	Spearman's rho	p-value
FSH	-.226	.115
LH	-.261	0.067
AMH	-.348	**.013***
SHBG	.216	.132
Testosterone	-.180	.212
Estradiol	-.310	**.028***
DHEAS	.039	.787
Progesterone	.216	.131
Prolactin	.148	.304

DISCUSSION

The understanding of the clinical phenotypes and the hormonal pattern in PCOS is necessary for proper patient classification and management. The observed phenotypes in this study were consistent with the hormonal patterns. This study demonstrated that polycystic ovary (PCO) morphology is a single popular predicting clinical feature of PCOS in Nigeria population followed by oligomenorrhea. The phenotypic occurrence of both ovulatory dysfunction and PCO morphology (Phenotype D) is the commonest phenotype of PCOS in the study population followed by hirsutism and PCO (Phenotype C). This is contrary to reports of a combination of hirsutism, oligomenorrhea and PCO (Phenotype A) described as the most prevalent in some other population (Sachdeva *et al*., 2019). The phenotypic group A is characterized by a higher prevalence of hyperandrogenism, insulin resistance, obesity, abnormal lipid profile, and metabolic syndrome, thereby presenting an increased risk of adverse metabolic and cardiovascular outcomes compared to other phenotypes especially phenotype D which has been described to represent a milder form of PCOS and considered the least severe phenotype (Sachdeva *et al*., 2019, Lizneva *et al*., 2016)

The observed elevated waist-hip ratio, which is a marker of obesity, corroborates the high prevalence of metabolic disorder in Phenotype A compared to phenotype D and other phenotypes. The elevated WHR in Phenotype A potentially indicates a higher amount of abdominal fat and, by extension, an increased risk of metabolic issues. This could explain the higher incidence of metabolic complications associated with phenotype A compared to phenotype D with lower waist-hip ratio. PCOS is a multifaceted disorder with various etiologic factors that might induce or complicate PCOS phenotypic characteristics and hormonal patterns. The occurrence of mostly phenotype D in the study population could be because of an interplay of unique hormonal, genetic and environmental factors with inherent compensatory mechanisms to prevent adverse PCOS-associated health conditions.

The observed hypogonadotropic FSH in this study suggests a follicular dysfunction in the study population and this obviously contributes to the clinical feature of oligomenorrhea/ anovulation. Similarly, the increased LH and LH: FSH are indications of the hypothalamic-pituitary ovarian axis dysfunction which favors the pulsatile release of Luteinizing hormone. The alterations in gonadotropins and ovarian steroidogenesis are major mechanisms proposed to be involved in PCOS pathophysiology (Dumesic *et al*., 2015). FSH stimulates follicular development and maturation, and its biological effects are felt on the maturation and function of the granulosa cells in the ovary. A consistent elevation of GnRH pulse frequency has been described to cause an increase in LH pulse frequency and amplitude with normal or low follicle-stimulating hormone (FSH) secretion, which results in an elevated LH:FSH ratio (McCartney *et al*., 2002, Morshed *et al*. 2021). Furthermore, observed increased AMH in the

PCOS group alludes to the presence of small (antral) follicles observed in PCOS. Naturally, AMH protein expression begins at the primary follicle stage and highest expression is detected in the pre-antral and small antral follicles, thus, elevated AMH reported in this study is an indication of presence of more antral follicles in PCOS. This suggests follicular maturation arrest in PCOS group, leading to lack of matured follicles necessary for ovulation. Thus, the elevated level of AMH together with low FSH observed in this study distinctly show follicular/ovulatory dysfunction in the PCOS study population. This agrees with similar studies which reported a higher serum level of AMH in women with PCOS compared with the control group (Parahuleva *et al*., 2013, Desforges-Bullet *et al*., 2010). While it is likely that elevated AMH contributes to the PCOS pathogenesis, the cause(s) of its elevated level remain unknown. However, LH has been reported to increase AMH production 4-fold in granulosa cells of PCOS ovaries but not of normal ovaries Pellatt *et al*. (2007).

In addition to the gonadotropins, estrogen and testosterone are two key sex steroids that have also been implicated in PCOS pathophysiology. Findings from this study showed increased testosterone and reduced estradiol concentration in PCOS. These findings indicate a physiological imbalance in steroid homeostasis, and this explains the clinical androgenicity observed in the study population. The elevated testosterone level could either be due to the increased testosterone production from the thecal cell because of LH stimulation or an accumulation of testosterone due to defects in its conversion to estradiol (because of possible decreased aromatase activity). In the normal ovary, estradiol is produced in the granulosa cell from testosterone through the catalytic action of aromatase enzyme and, FSH has been described as the primary inducer of aromatase activity and estradiol production in granulosa cell (Hobeika *et al*., 2020) The elevated level of testosterone observed in this study is mainly attributed to the ovarian theca cell origin with little or no involvement from the adrenal gland, as there was no difference in DHEAS between the PCOS and the control groups. DHEAS is gotten from the DHEA produced by the adrenal cortex and when sulfated through DHEA sulfotransferase it is released to the circulation as DHEA sulfate (DHEAS) (Goodarzi *et al*., 2015). These adrenal precursor androgens function as pre-hormones contributing largely to the amount of the more potent androgens, Testosterone, and dihydrotestosterone (DHT).

The plasma level and biological actions of sex steroids are regulated by SHBG, and sex steroids are transported by SHBG in the plasma with a high affinity for testosterone. Thus, low level of SHBG is often related to manifestations of hyperandrogenemia [22]. This baseline knowledge is consistent with the findings in this study; the serum SHBG level was lower in PCOS group compared to the control. The implication of this low serum SHBG is increase in bioavailable androgens and hyperandrogenemia which characterize PCOS pathogenesis.

Obviously, imbalance in gonadotropins and sex steroids is a major hormonal risk factor that contribute to phenotypic expression of PCOS in our study population. This is further reflected in the two predominant phenotypes (phenotype D and C) characterized by ovulatory dysfunction (oligomenorrhea), polycystic ovary morphology and hyperandrogenism (hirsutism) in our study population. The phenotypic characterisation of patients with PCOS helps in better understanding of the pathophysiology of PCOS and in predicting adverse metabolic and cardiovascular outcomes unique to our population. This will help in providing appropriate treatment options and modulating the hormones may support or improve PCOS management.

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Ethical Statement

This study obtained informed consent from all the study participants and ethical approval was granted by the Health Research Ethics Committee, Lagos State Ministry of Health Service Commission (No. LSHSC/2222/VOL.I/64).

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Full-Length Research Article

Assessment of Some Genetic Thrombophilias in Nigerian Patients with Venous Thromboembolism

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Summary: Venous thromboembolism (VTE) is a leading cause of mortality globally, resulting from genetic risk factors and/or acquired risk factors like oral contraceptives, smoking, diabetes, immobilization, cancer, trauma, fracture, and surgical procedures. This study aims to assess the involvement and prevalence of some genetic thrombophilias in Nigerian VTE patients. A total of 107 participants were recruited, comprising 67 individuals with VTE from three tertiary hospitals in Southwest Nigeria and 50 apparently healthy controls. Parameters included the absolute platelet count, prothrombin time (PT), activated partial thromboplastin time (APTT), D-dimer, protein C, protein S, and antithrombin levels. The VTE patients had significantly lower mean platelet counts, protein C and S antigenic concentrations, and protein S activity ($p<0.05$), with elevated mean PT and D-dimer levels ($p<0.05$). Protein C antigen and activity levels were reduced in 6.8% and 2.5% of participants, respectively, indicating deficiencies, while protein S antigen and activity were reduced in 1.7% and 0.9%. One (0.9%) participant had reduced antithrombin III level. Seven participants with protein S or C deficiencies experienced recurrent thrombosis. The study identifies type I antithrombin III deficiency and types I and II protein C and S deficiencies as genetic risk factors for VTE. The prolonged PT and elevated D-dimer levels observed in this study challenged the assumption of consistently reduced PT in VTE patients.

Keywords*: Genetic thrombophilia, protein C and S deficiency, Protein S deficiency, antithrombin deficiency, venous thromboembolism.*

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INTRODUCTION

Venous thromboembolism account for a notable incidence of thrombotic complication with genetic and/or acquired risk factors leading to an increasing yearly mortality rate of a 1 in 2 deaths per 1000 individual worldwide (Horner & Mahan, 2017; Wendelboe & Raskob, 2018). Genetic or inherited thrombophilias primarily result from inheritance of genetic mutations; they include antithrombin deficiency, protein C or S deficiency, factor V Leiden mutation (activated protein C resistance), histidine-rich glycoprotein deficiency and prothrombin-related thrombophilia (Dautaj *et al*.,2019), while the secondary (acquired) thrombophilia can be obtained through heparin-induced thrombocytopenia, antiphospholipid antibody syndrome, neoplasia, oral contraceptive use, obesity, smoking and surgery (Dautaj *et al*.,2019). These deficiencies could occur to affect the functionality, concentration of the proteins or both, resulting in their categorization into types based on deficiencies.

Type I protein C deficiency occurs because of reduced level of protein C, while Type II deficiency results from production of an altered molecule with decreasing levels of activity (Gupta & Patibandla, 2023). Type I protein S deficiency is characterized by a quantitative defect that shows low levels of total protein S (TPS) and free protein S (FPS). Type II (also known as type IIb) deficiency occurs when protein S activity is decreased but TPS and FPS antigen levels are normal. Type III (also known as Type IIa) deficiency is characterized by a quantitative defect that shows normal levels of TPS but reduced levels of FPS and protein S activity (Gupta *et al*., 2022). Also, quantitative (type I) or qualitative (type II) antithrombin deficiency is also present. Type II is further classified into two subtypes: type IIa, which is less frequent but more thrombophilic due to mutations in the thrombin-binding site, and type IIb, which is more common but less thrombogenic due to a failure in the heparin-binding region of the AT. There is also a pleiotropic type IIc deficit (Patnaik & Moll,2008).

The precise prevalence of inherited thrombophilia remains a subject of ongoing investigation as new studies continue to emerge, and this prevalence varies among different racial groups. Additionally, the situation is further complicated by the existence of numerous unidentified

genetic abnormalities, contributing to a significant number of unexplained venous thromboembolism (VTE) cases observed in families where no identifiable genetic defects have been identified (Ashraf *et al*., 2019). Pulmonary embolism has been reported to have an estimated annual incidence of two to three cases per 1000 in the United States population with 7-30 % prevalence in autopsy series (Ashraf *et al*., 2019; Turetz *et al*., 2018), 0.2-6.0% in Asia, 0.14%-61.5% in Africa (Danwang *et al*., 2017) all of which results in high mortality rate if left untreated. Furthermore, the incidence of deep venous thrombosis has been reported as 1 in 1000 yearly in United Kingdom and United state of America population with a resulting mortality rate of 5-10% (Siddiquie *et al*., 2018), Asian countries have reported a 0.15-1.35 % incidence rate in post-operative patients with a population –wide incidence of 15-20%, and Africa reporting 2.4-9.6% prevalence across post-operative and pregnant women (Danwang *et al*., 2017; Adeleye and Ogun, 2016). Despite the reported incidence of venous thromboembolic disorders in Nigeria which ranges from 2.4-9.6 % in post-operative cases and 380- 448 cases per 100,000 pregnant women (Adeleye and Ogun, 2016), there has been dearth of information on the existence and prevalence of genetic thrombophilias. These being significant contributors in the recurrence and complication of the thrombotic conditions; hence there is a need for adequate information that will serve as a guide in proper diagnosis and management of the patients which prompted the need for this study.

MATERIALS AND METHODS

Study Design: This is a descriptive-quantitative, cross-sectional study using convenience sampling technique.

Study Population and Sites: A total of 107 individuals participated in this study, including 67 patients with venous thromboembolism (VTE) comprising 61 individuals with deep vein thrombosis (DVT) and six with pulmonary embolism (PE). These patients were recruited with Ethical approvals from the hematology clinics of three tertiary hospitals in Southwest Nigeria: LAUTECH Teaching Hospital, Osogbo (LTH/EC/2019/05/415); University College Hospital, Ibadan (UI/EC/21/0623); and Federal Teaching Hospital, Ido-Ekiti (ERC/2019/02/13/1015). Informed consent was obtained from all participants. Fifty apparently healthy, age- and sex-matched individuals served as controls. Patients with cancer, liver cirrhosis, pregnancy, or those on anticoagulant therapy were excluded from the study.

Sample and Data Collection: Clinical histories were obtained using a pre-tested, structured, interviewer-administered questionnaire. Five millilitres of blood were collected from each participant: 2 ml dispensed in EDTA bottles for platelet count and 3 ml in trisodium citrate vacutainer tubes, which were centrifuged at 2000g for 15 minutes to isolate platelet-poor plasma. Plasma was stored at -20°C in labeled sterile cryovial tubes for assays, including prothrombin time (PT), activated partial thromboplastin time (APTT), D-dimer, Protein S, Protein C, and Antithrombin III.

Methods: Platelet counts were measured using a Sysmex KX-2IN autoanalyzer (Sysmex, 1999). PT and APTT assays were performed on a Coagulometer (Unitron Bio Medical, India) using Diagen Diagnostic reagents (UK). D-dimer levels were assessed using Tina-Quant D-dimer Gen 2 reagent on a Cobas C111 analyzer (Roche). Protein C, Protein S, and Antithrombin III antigenic assays were conducted using ELISA (Biorex Diagnostic Reagents, UK), with optical density measured at 450 nm using a microtiter plate reader. Blood pressure was measured using the Oscillometric technique by Bakris *et al*. (2016).

Protein S and Protein C Activity Assays: Protein S activity was measured using a clotting-based assay (Biorex Diagnostic, UK). Two millilitres each of Protein S APTT reagent and calcium chloride were prewarmed in a coagulometer (Unitron Bio Medical, India) at 37°C. A 1:10 dilution of the sample in buffer was prepared, and 500 µL was pipetted into a clean test tube. Fifty microliters each of Protein S-deficient plasma, Protein S activator, and prewarmed APTT reagent were sequentially added to each solution, followed by incubation for 3 minutes. Prewarmed calcium chloride (50 µL) was then added, and the clotting time was recorded. Calibration curves provided by the manufacturer were used to derive Protein S activity from the sample time, following the manufacturer's instructions.
For Protein C assay, samples were diluted in imidazole buffer (1:10), and 100 µL of the diluted sample was mixed with 25 µL each of Protein C activator and APTT reagent sequentially. After 3 minutes of incubation at 37°C, 50 µL of calcium chloride was added, and the clotting time recorded. Protein C concentration was calculated using a standard curve per the manufacturer's guidelines.

Antithrombin III Activity Assay: Antithrombin III activity was measured using a chromogenic method with reagents from Coamatic Chromogenix, Diapharma, UK. Plasma was diluted 1:9 in heparin buffer, and 200 µL of the diluted sample was incubated with 200 µL of Factor Xa reagent at 37°C for 90 seconds. Chromogen S-2765 (200 µL) was added, and absorbance was measured spectrophotometrically at 405 nm. Results were read against a standard curve prepared according to manufacturer's instruction to determine antithrombin activity.

Data Analysis: Data were analyzed using IBM SPSS version 25.0. Categorical variables were summarized using frequencies and proportions, while continuous variables were summarized using means and standard deviations. Chi-square tests assessed associations between categorical variables, and t-tests analyzed continuous variables. Statistical significance was determined at $p < 0.05$.

RESULTS

Table 1 represents the demographic features of all the participants; the mean±standard deviation (SD) age for the test subjects and controls is 43.32±15.16 and 40.12±11.41 with a t-value of 1.687 and no significant difference ($p>0.05$) observed among the two groups.

Table 1:
The Sociodemographic Characteristics of the test and control subjects.

Characteristics	Test	Control	X^2 value	p-value	t-value
Age group (years)	**Frequency (%)**	**Frequency (%)**			
≤20	1(1.5)	1 (2)	0.299	0.061	
21-30	5(7.5)	7(14.0)			
31-40	25(37.3)	22(44.0)			
41-50	9(13.4)	11(22.0)			
> 50	27(40.2)	9(18.0)			
Age (mean±SD years)	43.32±15.16	40.12±11.41		0.091	1.687
Blood Pressure (mean±SDmmHg)					
Systolic	123.98±10.10	121.59±8.69		0.061	1.063
Diastolic	80.30±6.39	77.56±4.46		0.001*	3.516
Sex					
Male	34(50.7)	24(48.0)	0.180	0.671	
Female	33(49.2)	26(52.0)			

* Statistically significant at p≤0.05
LTH- LAUTECH Teaching Hospital; UCH- University College Hospital (UCH); FETHI- Federal Teaching Hospital, Ido-Ekiti, Nigeria.

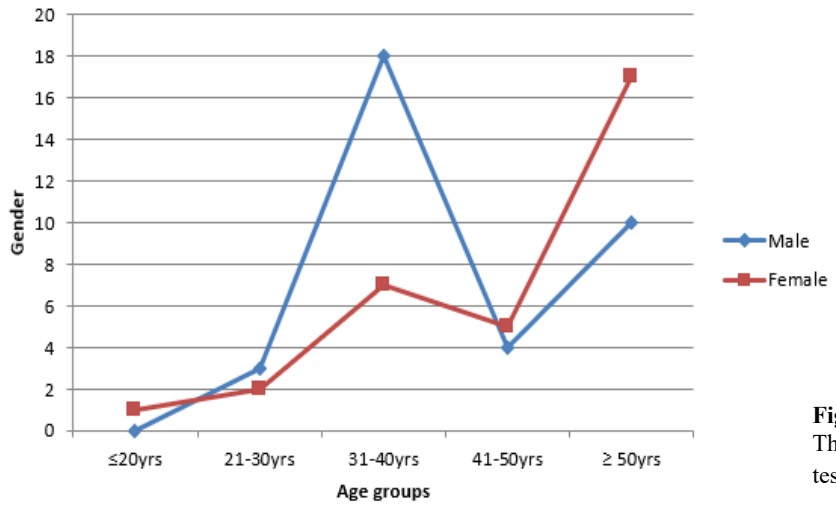

Figure 1:
The age group distribution across the gender of the test and control participants

The distribution of the age group within the test subjects shows participants above fifty years with the highest population frequency followed closely by those in their thirties and those below twenty years with the lowest frequency.
The diastolic blood pressure in the test group is significantly higher than that of the control group ($p<0.05$) while no difference exists in the systolic blood pressure. In addition, the gender frequency in Table 1 revealed no significant difference ($p>0.05$) among the gender when compared across each group of the participants.

Figure 1 displayed line plot of the age group distribution across the gender of the subject participants where the male individuals within age group 31years to 40 years had the significantly highest frequency compared to their female counter parts and other groups ($p<0.05$), they were followed by the female in those above 50 years and the male below 20 years had the lowest frequency.

The case participants portray all the subjects with pulmonary embolism as females, their clinical history showed approximately 3%, 34% and 7% of those with deep venous thrombosis had experienced miscarriage, hypertension and smoking ($p<0.05$) (Figure 2a); while Figure 2b shows 3, 5 and 2 individuals out of the 6 with pulmonary embolism have histories of miscarriage, hypertension and smoking respectively. From the questionnaire administered, all the subjects had history of lower limb pain, none of the female participants was pregnant at the time of sample collection as they tested negative to serum human chorionic gonadotrophin (HCG) antigen test while all those with pulmonary embolism had experienced chest pain at a time.

Table 2 displayed the mean ± SD of all parameters assessed between the test and control participants. In the table the absolute platelet count was significantly lower in the tests than in the control ($p<0.05$) whereas the PCV and APTT were also lower in the test group but not statistically significant ($p>0.05$). The mean ± SD prothrombin time was significantly prolonged in the test subjects, the D-dimer was higher as well in the same group ($p<0.05$). In addition, the mean ± SD protein C and S antigenic concentrations and protein S activity level were significantly reduced in tests compared with the controls ($p<0.05$) while protein C activity level which was also lower in the test was not statistically significant ($p>0.05$). The table further reveals lower values of antithrombin III antigenic and activity level among the tests than the controls but at an insignificant statistical level ($p>0.05$).

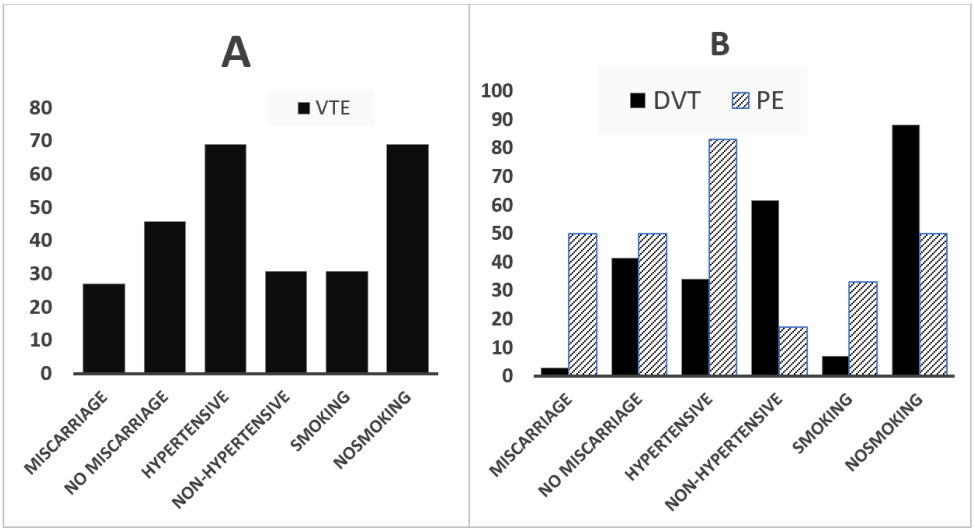

Figure 2:
(a) Distribution by Clinical History of test subjects with Venous Thromboembolism type (b) Distribution by Clinical History of test subjects with Deep Vein thrombosis (DVT) and Pulmonary Embolism (PE)

Table 2:
Mean ± SD of PCV, Absolute platelet count and other basic coagulation parameters in Tests and Controls.

Parameters (Mean ± SD)	Tests	Controls	T -value	p-value
Absolute Platelet Count (X 10^9/L)	196.90±66.40	329.04±93.66	11.509	0.001*
Prothrombin Time (seconds)	21.55±11.82	14.37±3.02	5.880	0.001*
APTT (seconds)	39.85±14.47	41.69±5.18	1.198	0.233
D-dimer (µg FEU/mL)	1.06±0.55	0.41±0.05	11.836	0.001*
Protein C Antigen (ug/ml)	4.19±1.00	4.88±0.51	6.143	0.001*
Protein C Activity (U/ml)	1.32±0.50	1.34±0.30	0.293	0.770
Protein S Antigen (ug/ml)	20.86±6.53	25.60±6.62	5.096	0.001*
Protein S Activity (%)	112.53±22.61	119.93±13.53	2.809	0.005*
Antithrombin III Antigen (g/L)	0.61±0.62	0.65±0.63	0.482	0.630
Antithrombin III Activity (%)	93.65±13.69	97.19±15.55	1.709	0.089

*- Statistically significant at p≤0.05
Mean ± SD – Mean ± Standard deviation; PCV- Packed cell volume; APTT- Activated partial thromboplastin time.

Table 3:
Prevalence of Some Genetic Thrombophilia markers among the test and control participants

	Variable	Tests (%) (n-67)	Controls (%) (n-50)	X^2 value	p-value
Platelet (X10^9/L)	160-400 (Normal)	48(41.0)	50(42.7)	64.471	0.001*
	<160 (Reduced)	19(16.22)	0		
PT (seconds)	14-16 (Normal)	32(27.3)	50(42.7)	53.193	0.001*
	>16 (Prolonged)	27(23.1)	0		
	<14 (Reduced)	8(6.8)	0		
APTT (seconds)	28-41 (Normal)	27(23.1)	49(41.8)	43.690	0.001*
	>41 (Prolonged)	8(6.8)	1(0.9)		
	<41 (Reduced)	32(27.3)			
D-dimer (µgFEU/mL)	<0.48 (Normal)	7(6.0)	50(42.7)	124.832	0.001*
	>0.48 (Increased)	60(51.2)			
Protein C Antigen (ug/ml)	3.9- 4.9 (Normal)	59(50.4)	50.0(42.7)	18.579	0.001*
	<3.9 (Reduced)	8(6.8)	0		
Protein C Activity (U/ml)	0.9-1.33 (Normal)	64(54.6)	50 (42.7)	20.930	0.001*
	<0.9 (Reduced)	3(2.5)	0		
Protein S Antigen (ug/ml)	15-25 (Normal)	65(55.5)	50 (42.7)	5.799	0.055
	<15 (Reduced)	2(1.7)	0		
Protein S Activity (%)	65-120 Normal	66(56.3)	50(42.7)	3.047	0.218
	<65 (Reduced)	1(0.9)	0		
AT III Antigen (g/L)	0.3-0.65 Normal	64(54.6)	50(42.7)	1.005	0.605
	>0.65 (Increased)	2(1.7)	0		
	<0.3 (Reduced)	1(0.9)	0		
AT III Activity (%)	Normal (75-110)	100(100.0)	50(42.7)		

*- Statistically significant at p≤0.05
PT- Prothrombin Time; APTT- Activated partial thromboplastin time, AT III- Antithrombin III.

Some notable results on the prevalence of indicative parameters of genetic thrombophilias in this study indicate a significant difference in the distribution of the test participants when compared with controls ($p<0.05$) in the platelet values, PT, APTT and D-dimer which displayed the prevalence of baseline genetic thrombophilias among the test and control participants (Table 3). The test subjects with reduced absolute platelet count are significantly lesser in distribution and percentage than those with normal platelet values, this occurrence is also observed in the distribution based on PT level where those with reduced values where significantly lower in number than those with prolonged and normal PT values. Test subjects with prolonged PT values (41%) are closer in distribution to those with normal values (47%). However, subjects with prolonged APTT level are significantly lower in distribution than those with reduced and normal values in which the latter groups do not differ excessively amongst themselves. Notably, the D-dimer level is increased in majority of the test subjects with 89% of the entire test population having higher level of the marker.

The protein C and protein S antigen and activity levels all have frequencies and percentages of test participants with reduced values at both significant ($p>0.05$) and insignificant statistical differences ($p<0.05$). Importantly, 6.8% and 2.5% of the entire study population, had reduced protein C antigen and activity levels respectively; while 1.7% and 0.9% had reduced protein S antigen and activity levels. From the population with reduced protein S and C parameters, 7 subjects had recurrent thrombotic condition. Also, antithrombin III antigen assessment had one (0.9%) individual with reduced level while all other participants had normal activity level of the marker.

DISCUSSION

The association of genetic thrombophilias with mortality is often underreported in Africa due to under-diagnosis and limited resources. Despite scarce studies in this region, some have identified these disorders even in unsuspecting populations (Adeyemo *et al*., 2012; Abdi and Osman, 2017). Participants in this study had a mean age above 40 years, reflecting age as a thromboembolism risk factor linked to thickened venous valves with aging (Yusuf *et al*., 2013). While some research suggests venous thromboembolism (VTE) incidence rises after 60 years (Yusuf *et al*., 2013; White *et al*, 2021), others highlight age as a key factor from 40 years onward, especially in younger hospitalized populations17,18. This study observed a high VTE incidence in individuals over 40 years, suggesting an earlier onset in the studied region which is likely influenced by lifestyle, reduced mobility, and genetic factors diminishing fibrinolytic activities.

Notably, males aged 31–40 years formed the largest group, suggesting a higher risk in this age range, potentially linked to lifestyle-related factors. Despite controls for age and sex, no significant differences were noted between genders, possibly due to both experiencing acquired and genetic VTE risk factors. While reproductive hormonal changes have been linked to thromboembolism in females, studies show males may face greater recurrence and severity of thrombotic events (Olié *et al*., 2012; Bamisaye *et al*., 2021; Albertsen *et al*., 2022; Pastori *et al*., 2023) thus aligning with this study where males aged 31–40 years had the highest VTE occurrence.

This study revealed that most participants exhibited normal systolic blood pressure, suggesting there was no significant association identified between elevated systolic blood pressure and the development or severity of VTE in the absence of other risk factors. However, lower systolic blood pressure has been linked to VTE risk, aligning with Virchow's triad, where circulatory stasis triggers endothelial hypoxemia, promoting adhesion molecule expression and activation of the extrinsic coagulation pathway (Ghouse *et al*., 2023). Diastolic blood pressure in our VTE participants was significantly higher than in controls, this is consistent with Anders *et al*. (2010), who associated high diastolic pressures with increased VTE risk. Furthermore, this study found deep venous thrombosis (DVT) as the predominant form of VTE, with pulmonary embolism (PE) observed in 9% of cases, aligning with reports that PE complicates 30-40% of DVT cases (García-Fuster *et al*., 2014; Center for Disease and Control, 2023). The PE cases were associated with smoking and miscarriage histories which emphasizes their role as risk factors. Notably, some DVT cases lacked identifiable risk factors, indicating a potential role of genetic predispositions or other contributors (Ageno *et al*., 2008; Zhang *et al*., 2016; Pastori *et al*., 2023).

The VTE group exhibited significantly reduced mean platelet counts compared to controls ($p<0.05$) which suggests impaired clot formation potentially contributing to the condition. Variable platelet counts in thrombotic crises have been documented in VTE conditions (Di Micco *et al*., 2018; Lim *et al*,2019; Bamisaye *et al*., 2020). Prolonged prothrombin time in the VTE participants indicates clotting factor consumption during thrombotic episodes, while the non-significant APTT difference suggests controlled intrinsic pathway involvement. Elevated D-dimer levels observed reflect active fibrinolysis, though D-dimer are nonspecific markers of various conditions (Wypasek and Undas, 2018; Bamisaye *et al*., 2020).

Protein C and S antigen levels, along with Protein S activity, were significantly reduced in the VTE group. Protein S, in conjunction with protein C, inhibits coagulation; its decreased activity suggests a type II deficiency, disrupting coagulation and elevating VTE risk (Fasola *et al*.,2021).

Notably, this study found that 6.8% and 2.5% of participants exhibited reduced level of protein C antigen and activity, indicating the presence of type I and type II protein C deficiencies in the region. These findings highlight the existence of genetic thrombophilia associated with protein C deficiencies, thereby emphasising the need to investigate specific gene mutations. A case study of a patient with a portal vein thrombus linked to gastro-oesophageal reflux affirmed the presence of type II protein C deficiency in the studied region. Corroborating studies by Okoye *et al*. (2019) found a 29.4% prevalence of the protein C deficiencies which are the type I and 27.35% type II, while Imoru *et al*. (2015) reported 29% among Nigerian women with a history of miscarriage. A Benin Republic study also found a 9.5% prevalence among individuals with venous thrombophilia, challenging prior assumptions of rarity in Africa (Houenassi *et al*., 2011).

Furthermore, the Type I deficiency of protein S was identified in 1.7% of participants, with type II protein S deficiency found in 0.9% which is consistent with previous studies in Nigeria where reduced free protein S has been linked to HIV (44.5%), miscarriages (1.3%), and ischemic stroke (6%) in Black Africans (Bello *et al*., 2021). Contrastingly, a local study reported non-association between deficiencies of protein C and S with preeclampsia, suggesting limited relevance to this condition (Okoye *et al*., 2017).

Additionally, one participant (0.9%) with recurrent venous thrombosis exhibited reduced antithrombin III (AT III) antigen levels, indicating type I deficiency, while all participants showed normal AT III activity levels, ruling out types II and III deficiencies. Similar studies in Nigeria have observed lower antithrombin values in 14% of sickle cell anemia cases and among blood donors (Onyemelukwe and Jibril,1992; Osunkalu *et al*., 2015), supporting the presence of type I AT III deficiency in this region.

In conclusion, this study revealed the prevalence of types I and II protein C as well as protein S deficiencies with type I antithrombin III deficiency which are genetic thrombophilias and genetic risk factors for venous thromboembolism (VTE). The patients often presented with prolonged prothrombin time and elevated D-dimer levels, contradicting assumptions of consistently reduced prothrombin time in VTE cases. Also, VTE occurs irrespective of age or gender in this region. Further research is recommended to identify the specific gene mutations and conduct genomic studies to improve management and treatment strategies which was a limitation of the study.

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Authors' contributions

Conceptualization: E.O.B and E.O.O; Methodology, E.O.B., PO and E.O.O.; Software, E.O.B.; Validation, E.O.B and E.O.O; Formal Analysis, E.O.B. and P.O; Investigation, E.O.B, P.O. and E.O.O; Resources, E.O.O.; Data Curation, E.O.B, P.O. and E.O.O; Writing, Review and Editing, E.O.B, P.O. and E.O.O; Supervision, E.O.O.

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Full-length Research Article

Radiographic Evaluation of Posterior Tibial Slope Angle: Its Relationship with Socio-Demographic Factors and Effects on Knee Function

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Summary: Major activities in life depend on the proper function of the knees, which in turn is determined by the posterior tibial slope angle (PTSA) of the knee. However, beyond certain degrees of PTSA the likelihood of the risk for tears in the anterior cruciate ligament (ACL), posterior cruciate ligament (PCL) and menisci increases, which consequently impairs knee joint functions. The objectives of this study were to determine PTSA, its relationship with sociodemographic factors and effect on subjective knee joint function using Tegner activity scale. This was a prospective cross-sectional observational study that was conducted in the Radiology Department of a Tertiary hospital over a 6-moths period. PTSA of the lateral radiographs of 152 subjects were evaluated. Tegner activity scale was administered on each subject to subjectively ascertain knee joint function. Chi square and Pearson's correlation were used to analyze the data. Mean PTSA for the right and left knees were 11.03 ± 0.25^0 (SEM) and 11.02 ± 0.26^0 (SEM). Age and body mass index (BMI) significantly correlated with right PTSA ($r = 0.404$, $P = 0.000$ and $r = -0.853$, $P = 0.000$) and left PTSA ($r = 0.408$, $P =$ and $r = -0.818$, $P = 0.000$). Mean Tegner activity scale was 8.47 ± 0.38 (SEM) and the mean BMI was 24.06 ± 0.29 kg/m^2 (SEM). It is concluded that mean PTSA in this study is similar to that obtained in other populations and has a significant relationship with age and BMI, and maintaining normal weight significantly positively affects PTSA to ensure optimal knee functions. This study offers regional data to assist Orthopaedic surgeons on knee ligaments evaluation and reconstruction procedures.

Keywords: *Posterior tibial slope, lateral knee radiograph, knee function, tibia, knee biomechanics.*

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INTRODUCTION

A great proportion of our daily activities in life depends on a perfectly functional knee joint. The knee function includes weight bearing, absorbing and adjusting pressures and blows during working, running, jumping and maintaining the position (Karimi *et al*., 2017; Al Badwi *et al*., 2023). Posterior tibial slope angle (PTSA) plays a crucial role in the stability and biomechanics of the knee joint. Changes in this angle has a significant effect on knee function and various pathologies in the knee have also been reported to be associated with it (Karimi *et al*., 2017; Hassa *et al*., 2023).

The increase in PTSA is accompanied by an increased risk of anterior cruciate ligament (ACL) tear, knee instability, progressive loosening of the tibio-fibular joint gap as a result of decreased collateral ligament tension during flexion, increased likelihood of medial meniscal posterior root tears and the development of spontaneous osteonecrosis of the knee (Koga *et al*., 2022; Aljuhani *et al*., 2020; Karimi *et al*., 2017; Hassa *et al*., 2023). On a beneficial note, increased PTSA ensures the attainment of a full range of motion in the knee of an individual which enhances excellent performance of general physical activity. At the other end of the spectrum, a reduced PTSA increases the strain on the posterior cruciate ligament following total knee arthroplasty and may be a contributing factor in failed PCL surgeries (Aljuhani *et al*., 2020; Thirunarayanan *et al*., 2021; Shelburne *et al*., 2011; Pangaud *et al*., 2020). Poorly managed PCL strain or injury results in the development of medial femoral osteoarthritis and patellar osteoarthritis (Yang *et al*., 2023).

The essence of evaluating PTSA is due to the rising number of knee reconstruction surgeries globally, especially knee joint replacements and the important role of maintaining normal lower limb angles to maintain the fundamental functions of the joints (Karimi *et al*., 2017). Evaluation of PTSA has major applications in total knee arthroplasty (TKA), high tibial osteotomy (HTO) and

anterior cruciate ligament (ACL) reconstruction surgery (Chen *et al*., 2022). An increase in the medial and lateral PTSA by 10 leads to a 1.24-fold and 1.17-fold increase in graft failure, respectively, and the risk of graft failure is substantial in high PTSA which is extremely undesirable. To achieve perfect outcomes in knee function following surgeries, PTSA needs to be precise for a given population (Kasman *et al*., 2023). Socio-demographics such as ethnicity, age, gender and body mass index are important factors that play important roles in the modification of the PTSA and by implication, knee joint function (Bisicchia *et al*., 2017).

Tegner activity scale is a standardized method for grading knee function and it requires individuals to select their level of sports participation which best describes their current level of activity. Although originally utilized for patients with ACL injury, it has been modified for normal populations. In addition, it significantly correlates with other equally reliable knee function evaluation tests (McHugh *et al*., 2020). Patient-reported-outcome-measures (PROMs) such as Tegner activity scale, have been demonstrated to effectively differentiate favourable outcomes, in terms of knee joint functions, and unfavourable outcomes (Hung *et al*., 2024).

There is a significant correlation between conventional radiograph evaluated PTSA and 3-D evaluated PTSA, employing either a CT scan or MRI, with an average error of about 3.400. Moreover, the costs of CT and MRI scans are expensive for clients and besides, they are not readily available in many climes (Bisicchia *et al*., 2017).

The findings of this study will be of immense assistance to orthopaedic surgeons by way of identifying patients that may be at a great risk of ACL & PCL injury and tailor PTSA reconstruction that is within the normal range to obtain optimal knee function. Therefore, it is pertinent for Orthopaedic surgeons to have knowledge of the average PTSA of indigenes in this locality.

This study was aimed at determining Posterior tibial slope angle, its relationship with sociodemographic factors and effect on subjective knee joint function using Tegner activity scale.

MATERIALS AND METHODS

Study design: This was a prospective cross-sectional observational study that was conducted in the Radiology Department of a tertiary health facility located in the South-south region of Nigeria. The five-month study commenced from June 2023 to November 2023. The study population consisted of the patients requested to conduct knee radiographs in the Radiology Department. Approval for this study was obtained from the institutional review board (IRB), and all subjects involved gave consent before inclusion in the study.

Exclusion criteria: Below 18 years of age, previous and recent bone fractures around the knee, congenital bone anomalies, bone tumours, osteomyelitis, obvious lower limb soft tissue swelling, knee osteoarthritis, surgical implants around the knee, diagnosed ACL injuries, previous knee joint surgery and presence of foreign bodies around the evaluated knee radiograph.

Sampling technique and sample size: Purposive sampling technique was utilised for the study. One hundred and fifty-two subjects were subsequently recruited.

Clinical assessment: Medical history including body mass index (BMI) was obtained from the consenting subjects. Tegner activity scale evaluation questionnaires were administered to the subjects to record their knee function scores (McHugh *et al*., 2020). The knees of the subjects were clinically assessed, and anterior drawer test and pivot shift test were done to rule out ACL injury or deficiency (Singh *et al*., 2023).

Radiographic technique and PTSA evaluation: The radiographic technique was standardized for all the subjects. Antero-posterior and lateral view radiographs with superimposed condyles were obtained from the subjects using BRIVO XR575 X-ray machine in the Radiology Department, but only the lateral views were utilized for this study.

To acquire the lateral view radiographs, the subjects were positioned with the knees flexed at approximately 25 - 30^0. The central x-ray beam was directed vertically towards the medial aspects of the knee joint with a cephalad angulation of about 5 - 7^0 (Singh *et al*., 2023).

Measurement of the PTSA was done manually using the anterior tibial cortex method on a digital viewer. A straight line was drawn through the anterior cortex of the tibial shaft and it was extended proximally to be intersected by a second straight line drawn tangential to the proximal tibial articular surface connecting the anterior and posterior ends of the tibial plateau. A further straight line was drawn from the point of intersection, perpendicular to the anterior cortical line. The angle between this perpendicular line and the tangential line along the tibial plateau is the PTSA (Figure 1). The authors who measured the posterior tibial slope angles of the subjects' lateral knee radiographs were different from the ones who administered the Tegner activity scale questionnaire.

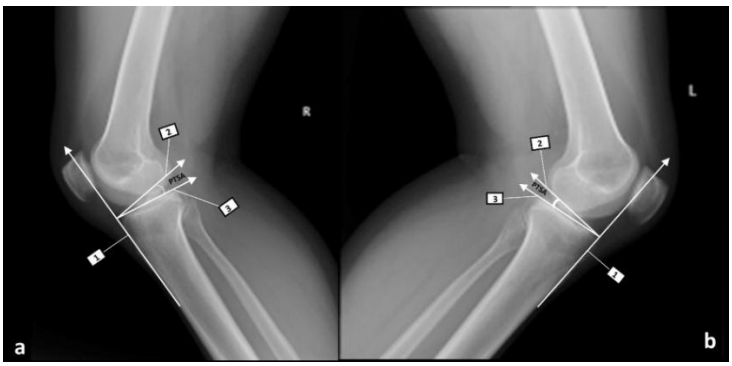

Plate 1:
Lateral radiographs of the right knee (a) and left knee (b). 1 – Anterior cortical line. 2 – Line perpendicular to line 1. 3 – Tangential line to the tibial plateau. PTSA – Posterior tibial slope angle

Statistical analysis: The statistical packages for social sciences version 23 (SPSS Inc., Chicago, Il, USA) was employed to analyze the data obtained. The values were reported as means ± standard error of mean (SEM). Appropriate tables were the means of displaying results where applicable. Chi square analysis and ANOVA were done to assess the relationship of all the variables with right and left PTSA, respectively and to measure the differences in the values of these variables. P value < 0.05 was considered statistically significant

RESULTS

The study group consisted of 41 males and 111 females. Most of the subjects were married (78.95%), had tertiary education (65.13%) and employed (61.33%). Few subjects were widowed (6.58%) and divorced (1.97%), about one-third of the subjects had secondary education (34.87%) and slightly above one-third of the subjects were unemployed (38.67%). No subject had primary education (0%). Gender (P = 0.416 and P = 0.62), was not significantly associated with Right PTSA and Left PTSA (Table 1).

Right PTSA and left PTSA ranges from 4.00^0 to 16.00^0, respectively. The least age of the subjects was 18 years while the oldest was 61 years. Age significantly correlated with right PTSA (r = 0.404, P = 0.000) and left PTSA (r = 0.408, P = 0.000) and in both instances the correlation strength was moderate. BMI also significantly correlated with both the right PTSA (r = -0.853, P = 0.000) and left PTSA (r = -0.818, P = 0.000) negatively but with a high strength respectively. Tegner activity scale ranged from 5.32 to 9.10 with a mean value of 8.47±0.38 (SEM) (Table 2).

The 34 – 49 years age group had the highest frequency (57.90%) while ≥50 years age group had the least frequency of subjects (11.84%). The right PTSA and left PTSA progressively increased with the age groups and the difference in the mean values of the angles at these different age groups were significant (P = 0.002, respectively). The highest Tegner activity scale was 7.83±0.28 (SEM) and it was noted in the 34 – 49 years age group while the least, which was 7.21±0.56 (SEM), was noted in the 18 - 33 years age group (Table 3).

Table 1:
Association of categorical sociodemographic variables with right and left PTSA

			Right PTSA (^{0})		Left PTSA (^{0})	
		n, (%)	df	P value	df	P value
Gender	**Male**	41 (26.97)	11	0.416	12	0.662
	Female	111 (73.03)				
Marital status	**Married**	120(78.95)	22	0.058	36	0.066
	Single	19 (12.50)				
	Divorced	3 (1.97)				
	Widowed	10 (6.58)				
Educational status	**Primary**	0 (0)	11	0.668	12	0.144
	Secondary	53 (34.87)				
	Tertiary	99 (65.13)				
Employment status	**Employed**	92 (61.33)	11	0.054	12	0.038
	Unemployed	60 (38.67)				

(*) – P value less than 0.05 is significant; PTSA – Posterior tibial slope angle.

Most of the subjects had normal weight (42.76%) while few of the subjects were obese (24.34%). Obese subjects were noted to have the least mean right PTSA ($8.64±0.30^0$ (SEM)) and mean left PTSA ($8.86±0.27^0$ (SEM)) and the difference in the mean values of the right and left PTSA in the various BMI groups were significant (P = 0.000, respectively). Tegner activity scale was highest in the normal weight group (8.39±0.81 (SEM)) (Table 4).

Table 2:
Mean and correlation of continuous socio-demographic variables and Tegner activity scale with right and left PTSA

				RIGHT PTSA (^{0})		LEFT PTSA (^{0})	
	Min	Max	Mean±SEM	Correlation coefficient (r)	P value	Correlation coefficient (r)	P value
Age (years)	18.00	61.00	36.25±1.03	0.404	0.000*	0.408	0.000*
BMI (kg/m^2)	19.54	34.11	24.06±0.29	-0.853	0.000*	-0.818	0.000*
Tegner activity scale	5.32	9.10	8.47±0.38	-0.249	0.072	-0.305	0.063
Right PTSA (^{0})	4.00	16.00	11.03±0.25	NA	NA	0.929	0.000*
Left PTSA (^{0})	4.00	16.00	11.02±0.26	0.929	0.000*	NA	NA

(*) – P value less than 0.05 is significant; BMI – Body mass index; PTSA – Posterior tibial slope angle

Table 3:
Distribution and analysis of variance of the continuous socio-demographic variables and Tegner activity scale in the Age groups

		Age (years)	BMI (kg/m^2)	Right PTSA (^{0})	Left PTSA (^{0})	Tegner Activity Scale
AGE GROUPS	n, (%)	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
18 – 33 years	46 (30.26)	28.83±0.85	27.70±0.67	10.56±0.56	10.67±0.56	7.21±0.56
34 – 49 years	88 (57.90)	42.83±0.61	26.23±0.36	12.49±0.28	12.57±0.31	7.83±0.28
≥50 years	18 (11.84)	53.68±1.10	23.01±0.70	12.74±0.70	12.70±0.60	7.69±0.66
P value		0.000*	0.083	0.002*	0.002*	0.174

(*) – P value less than 0.05 is significant; BMI – Body mass index; PTSA – Posterior tibial slope angle

Table 4:
Distribution and analysis of variance of the continuous socio-demographic variables and Tegner activity scale in the BMI groups

		BMI (kg/m²)	Age (years)	Right PTSA (^{0})	Left PTSA (^{0})	Tegner Activity Scale
BMI GROUPS	n, (%)	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Normal weight (18.50 – 24.99) kg/m²	65 (42.76)	22.12±0.19	48.23±1.45	14.51±0.15	14.54±0.24	8.39±0.81
Overweight (25.00 – 29.99) kg/m²	50 (32.90)	28.47±0.21	49.98±1.59	10.19±0.29	10.17±0.27	8.00±0.25
Obese (≥ 30.00) Kg/m²	37 (24.34)	31.41±0.18	37.50±2.15	8.64±0.30	8.86±0.27	6.80±0.30
P value		0.000*	0.000*	0.000*	0.000*	0.066

(*) – P value less than 0.05 is significant; BMI – Body mass index; PTSA – Posterior tibial slope angle

DISCUSSION

PTSA in conjunction with preserved anterior cruciate ligaments play preponderant roles in providing normal knee joint range of motion and biomechanics (Hassa *et al*., 2023). In this study the mean PTSA for the right and left knees were 11.03 ± 0.25^0 and 11.02 ± 0.26^0. A plethora of research findings fairly aligned with our results and these included; Singh *et al.,* (2023) in India (right PTSA - $12.76^0\pm2.35^0$ and left PTSA - 12.55 ± 2.46^0, with overall mean PTSA of 13.60 ± 3.50^0), Aljuhani *et al.,* (2020) whose retrospective cohort study evaluated 524 knee radiographs of Saudi indigenes (right PTSA - 13.60 ± 3.40^0 and left PTSA - 13.70 ± 3.30^0, with an overall mean PTSA of 13.60 ± 3.40^0), Saidapur *et al.,* (2023) in India, whose study involved 153 osteoarthritic subjects, also noted an overall mean PTSA of 11.50 ± 1.34^0, Katchy *et al.,* (2019) in South-East Nigeria, also observed that the overall mean PTSA in 265 retrospectively evaluated knee radiographs of Igbos was 11.90 ± 3.40^0 and Kavak *et al.,* (2024) in Turkey, who employed three methods to measure PTSA, realized that the right PTSA (11.08 ± 2.04^0) and left PTSA (10.99 ± 2.26^0) were also similar to our findings using the anterior tibial cortex technique. However, Thirunarayanan *et al.,* (2021), who evaluated osteoarthritic knees of Indians, observed an overall mean PTSA value (14.05^0) that was higher than the findings of this study and also that of Saidapur *et al.,* (2023), who similarly evaluated the knee radiographs of Indians with osteoarthritis of the knee. Osteoarthritic degeneration has been postulated as one of the possible pathogenetic factors that lead to an increase in PTSA values (Thirunarayanan *et al.,* 2021).

Some of the studies that utilized 3-D imaging for the evaluation of PTSA, such as those of Karimi *et al.,* (2017) in Iran, who evaluated 132 knee MRI images and Hassa *et al*., (2023) whose study involved the assessment of 89 knee CT images of Turkey citizens, observed lower PTSA values compared to the findings of this study (medial PTSA - 7.78 ± 2.48^0 and 8.97 ± 2.93^0; lateral PTSA - 6.85 ± 2.24^0 and 8.35 ± 2.53^0, respectively). However, a study conducted by Endo *et al*., (2020) that had to do with 62 MRI-evaluated knee images of Japanese college students who had a mean age of 21.1 years, demonstrated congruity with the results of this study as they reported that the overall mean medial PTSA for the dominant and non-dominant knees were $9.70^0\pm2.10^0$ and $10.10^0\pm1.80^0$ while the overall mean lateral PTSA for the dominant and non-dominant knees were $9.60^0\pm2.10^0$ and $9.60^0\pm1.80^0$.

Kasman *et al.,* (2023) in Turkey, conducted a clinical outcome follow-up on patients who had arthroscopic ACL reconstruction with hamstring tendons, and demonstrated that the mean Tegner-Lysholm knee score for those with PTSA above 10^0 (88.20 ± 8.80^0) was insignificantly higher than those whose PTSA was below 10^0 (85.60 ± 9.10^0). The subjects whose PTSA was above 10^0 had a mean value of 11.10 ± 0.80^0 which was similar to the mean values of the right and left PTSA in this study, and this probably suggests that knee function is excellent at this PTSA with reduced risk for a knee ligament tear (Kasman *et al.,* 2023).

The subjects in this study had a high overall mean Tegner activity scale of 8.47±0.38 which was attained with a mean PTSA of 11.03 ± 0.25^0 and 11.02 ± 0.26^0 for the right and left knee respectively. It can be propounded that knee activity and functions are likely utmost at the mean PTSA for the population within the metropolis of this study. Hung *et al*., (2024) observed that medial tibial slope and lateral tibial slope had a significantly negative correlation with Lysholm score (r = -0.300, P = 0.000 and r = -0.366, P = 0.000) and Tegner score (r = -0.328, P = 0.000 and r = -0.383, P = 0.000), respectively. They inferred that individuals with a high slope angle usually experience low functional outcomes evidenced by their low score in the Lysholm score scale. Elaborating on the probable pathogenesis of this observation, Singh *et al.,* (2023) succinctly noticed that when the mean PTSA rises to a certain level a plateau sets in, in terms of general physical activity and the permissible range of motion in the knee, followed by a decline.

Li *et al*., (2023) observed in their study that mean PTSA in subjects with bilateral ACL tears was 11.17^0 while those with unilateral ACL tears was 9.72^0 and the difference of these mean values was significant (P = 0.005). Even though the mean PTSA in this study was similar to their value we could not determine the presence of ligament tears in the knees of our subjects.

Age and BMI were relevant socio-demographic factors in this study since they both had a significant relationship with right and left PTSA. Singh *et al.,* (2023) who similarly observed a significant correlation between age and PTSA however, found out that as age advanced the PTSA significantly reduced at the right and left knees (P < 0.001, respectively). In this study, a positive correlation was rather noticed between both variables. Bone cortical thickness reduces with age, especially after 50 years, and this can be accelerated by unhealthy lifestyle (Vári *et al*., 2023). It can thus be propounded that age affects the thickness of the tibial plateau, which is a major determinant of the posterior slope orientation and consequently, PTSA.

The mean BMI of the subjects in this study was observed to exhibit a significant negative correlation with PTSA such that as the BMI increases, PTSA reduces. When an individual's BMI rises, a proportional hike in biochemical

bone markers occurs, which induces a rapid decline in bone resorption markers while bone formation markers increase with heightened osteoblastic stimulation. [U] These biochemical alterations, coupled with the mechanical effect of the increased lean mass volume of the body weight on bone, result in an elevation in the bone mineral density (which increases tibial bone cortical thickness and reduces PTSA). Even in menopause, higher BMI decelerates bone loss (Vári *et al*., 2023; Rinonapoli *et al.,* 2021). Buttressing this postulation, it was observed that a weight loss of about 14% or more within a three to four months period results in significant bone loss and this is more profound in women than men (Shapses and Riedt, 2006). In alliance with our findings, Singh *et al.,* (2023) lucidly demonstrated that a rise in BMI was significantly associated with a reduction of PTSA (P = 0.001). BMI is a modifiable sociodemographic factor which can possibly be adjusted to fit into the desired PTSA of a patient for optimal knee joint function. Kizilgoz *et al.,* (2019) in Turkey, on the other hand, demonstrated that BMI increased linearly with the increase in medial and lateral PTSA. However, Karimi *et al*., (2017), Katchy *et al*., (2019), de Sousa Filho *et al*., (2021), and Fares *et al*., (2023) all reported that PTSA had no significant relationship with socio-demographics of their subjects.

It is pertinent to note that sequel to total knee arthroplasty, the reconstructed PTSA predominantly influences knee stability and the tension in ACL and PCL. Furthermore, postoperative stiffness, abnormal femoral rollback and polyethylene wear can be a consequence of an abnormal PTSA (Aljuhani *et al.,* 2020).

Racial homogeneity of the recruited subjects was not maintained, since most of them where from multiple tribes in the country and also were indigenes of other countries. This means that our findings might not be specific for the population in the locality of this study. However, the mean PTSA in this study was observed to have a good Tegner activity scale. The utilization of MRI, which is the gold standard imaging modality for the evaluation of the knee (Aljuhani *et al.,* 2020), would have been suitable to determine both medial and tibial PTSA in individuals with ligament tears or to objectively rule out subjects with knee ligament tears.

It is concluded that mean PTSA in this study is similar to that obtained in other populations and has a significant relationship with age and BMI, and maintaining normal weight significantly positively affects PTSA to ensure optimal knee functions. This study offers regional data to assist Orthopaedic surgeons on knee ligaments evaluation and reconstruction procedures.

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Full-Length Research Article

Exploring Factors Influencing Cohabitation among Osun State University Students, Nigeria: Implications for Family Demography and Sociological Practices

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Summary: Cohabitation represents a significant shift in family structures as they undergo demographic and economic transitions. This practice is a common phenomenon among Nigerian university undergraduates. Therefore, this study examined the demographic factors that determine cohabitation practices among undergraduates in Osun State, Nigeria. Guided by the individualization theory, the research design adopted was a survey, and questionnaire and in-depth interview instruments were used to collect the data. A sample of 400 respondents was included in the quantitative analysis, while 18 participants were included in the qualitative aspects of the analysis. Descriptive (frequency and percentages) and bivariate (chi-square test and correlation) were used to analyze the quantitative data, while the qualitative data were content analyzed. The findings revealed that a majority of the respondents have high (45.3%) knowledge of cohabitation practice, and pre-marital cohabitation (56.5%) is one of the major dimensions of cohabitation reported by the respondents. Determining factors adduced for engaging in cohabitation were the urge to be a 'baby mama' (77.5%) and economic benefits (74.5%); while 73.0% of them have a regular pattern of practicing cohabitation. About 42.0% of them have good perception, and less than 55% have a negative attitude towards cohabitation. A significant age difference in knowledge of cohabitation ($\chi 2 = 96.96$, $\rho<0.01$) and knowledge of cohabitation was positively correlated with attitude ($r = 0.611$, $p<0.01$) and perception ($r = 0.528$, $p<0.01$) among the respondents. Similarly, perception of cohabitation was positively correlated with attitude towards cohabitation ($r = 0.187$, $p < 0.01$). The implication of this finding is that high knowledge will lead to high perception and positive attitude towards disengaging from cohabitation practices. Adolescents and youths should be enlightened about the negative consequences of cohabitation relationships that may jeopardize their future endeavours.

Keywords*: Cohabitation, dimensions, knowledge, pattern, perception, university students*

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INTRODUCTION

Cohabitation refers to a living arrangement where two individuals of opposite sex reside together and maintain an intimate relationship without being legally married (Gold, 2012; Foran *et al*., 2022; Lamela *et al*., 2015). Marriage in Nigeria is customarily arranged through an extended process that includes ceremonies and negotiations. Marriage is a sign of social acceptance in traditional society, indicating one's position of maturity and responsibility (Perelli-Harris *et al*., 2014; Obarisiagbon, 2023; Avogo *et al*., 2019). There are spiritual, legal, social, economic, and family implications to marriage. Couples who live together without having a legally recognised marriage ceremony are viewed as having disregarded long-standing norms of culture. Cohabitation was uncommon in traditional society, and those who did so were looked down upon until they officially married. Various factors, including education, religion, urbanisation, ethnicity, generation, and marriage laws, are considered when establishing a legal marriage among Nigerian couples (Perelli-Harris *et al*., 2014; Avogo *et al*., 2019; Moore *et al*., 2013;). Youth in African countries such as in Nigeria and Kenya are confronted with challenging decisions: either to embrace and uphold customary marriage customs, or to embrace modern values, mores, and lifestyles, or even to find a means of reconciling the two (Nungo *et al*., 2025; Nii-Amoo Dodoo *et al*., 2006; Avogo *et al*., 2019; Moore *et al*., 2013). A paradigm shift in marriage and family life has occurred in recent decades. Examples include the transition from extended to nuclear households, parent-arranged marriages to individual mate selection, large to small family sizes, and rural to urban places of residence (Alebiosu, 2020). In the 21st century, many young people choose to live together without getting married instead of delaying marriage. Adults employed in metropolitan settings also practiced cohabitation. According to the 2018 Nigeria Demographic and Health Survey (NDHS), about 2.3% of respondents who were female, and 2.7% of respondents who were male reported they were

cohabiting (NDHS, 2018). These statistics show a rise in cohabitation compared to a 2008 NDHS survey that found 1.0% of men and 0.5% of women lived together (NDHS, 2018; NDHS, 2008).

Social scientists such as demographers and sociologists treat cohabitation as a distinct occurrence; not only has it displaced marriage, but also it has represented a structural change in family relationships (Perelli-Harris *et al*., 2018; Perelli-Harris *et al*., 2019; Duyilemi *et al*., 2018; Scent *et al*., 2019; Adeyera *et al*., 2023; Odimegwu *et al*., 2018). Although in the past, cohabitation before marriage was not viewed as the right thing to do, it is now sometimes seen as a "necessity," since some people do it out of preparation for marriage and others do it out of convenience. For instance, in Nigerian cultural and traditional society, it is against the norms and values of the society to allow a young unmarried couple to live together, especially where they do not have any family affinity (Odimegwu *et al*., 2018; Obarisiagbon, 2023; Lawal *et al*., 2021; Muhinat, 2022; Obikeze *et al*., 2018; Iyekolo, 2021). However, the rising prevalence of cohabitation in many developed countries has placed the phenomenon at the forefront of discussion and debate on family change. Contemporary cohabitation, which dates primarily from the 1960s and 1970s, has attracted much attention as a demographic and social innovation (Gold, 2012). Yet, cohabitation prior to marriage has been consistently associated with a poorer quality of marital communication, lower marital satisfaction and higher levels of domestic violence, even though cohabitation was an obscure practice, and seen as taboo throughout the 19th century until the 1970s (Duyilemi *et al*., 2018; Scent *et al*., 2019). Non-marital unions have become common, because the meaning of the family has been altered by individualistic social values which have progressively matured since the late 1940s (Kalu *et al*., 2021; Aborisade, 2021). Notwithstanding, the field of family sociology and demography interests hinges on cohabitation as an informal co-residential union that is less well defined and documented than marriage, unlike its traditional marriage counterpart. The role of cohabitation in the modern family is the focus of demographical and sociological attention, in particular, how closely it resembles marriage or such premarital statuses as dating and formal engagement (Gold, 2012; Foran *et al*., 2021). Cohabitation has been structured into a policy debate regarding legal provision for cohabiters, and creating a suitable platform for cohabiting unions that will aid them in building a firm union and rearing their children (Gold, 2012; Avogo *et al*., 2019).

A change in family structure is, diplomatically a central and contentious subject in both developed (Britain, the United States, Europe and Australia) (Foran *et al*., 2021; Perelli-Harris *et al*., 2024) and developing nations (Nigeria, South Africa and Kenya) (Avogo *et al*., 2019; Moore *et al*., 2013). In Nigeria, the increase in cohabitation is one of the most significant shifts in family demographics of the past century and has become common among undergraduate students in Nigerian higher education institutions (Alebiosu, 2020). The increase in population of undergraduate students and the inability of the government to adequately provide the needed social infrastructures and funding of higher education in Nigeria has led to risky coping mechanisms among the students (Adeyera *et al*., 2023; Odimegwu *et al*., 2018; Obarisiagbon, 2023; Lawal *et al*., 2018). The increase in the number of undergraduate students and the inability of the school authorities to provide adequate hostel accommodation has led to an increase in cohabitation among the student population in Nigerian public universities (Adeyera *et al*., 2023). Government policy on students' hostel accommodation, which encourages private developers, has created an inability of the institutions to build and expand new hostels for the student population, and this has forced students to look outside for accommodation (Alo, 2008). The majority of students who live together do not allow their parents to know about it. This therefore exposes the students to all forms of risk and harm as they continue to cohabit. Students who live with their partners are often faced with domestic violence and sexual exploitation by their partners, including by outsiders or individuals that they are not acquainted with (Adeoye *et al*., 2012).

The constraint of hostel accommodation within the universities has led to a deviant form of cohabitation known as 'campus marriage, or 'couple's life' among students (Baranowska-Rataj, 2014; Shields-Dutton, 2016). This concept of 'campus marriage or couples' life' among students explains how students of the opposite sex come to an agreement to live together and share things in common, without any traditional or legal authorization (Iyekolo, 2021; Kalu *et al*., 2021). These forms of lifestyles over time in recent years have threatened the values, beliefs and sanctity of the institution of marriage and family. Cohabitation has inevitably come to stay with all its supposed positive and negative consequences; although similar to marriage, cohabitation has some distinct functions from marriage (Odimegwu *et al*., 2018; Lawal *et al*., 2021). The participants in a cohabitation setting are not immune from the various problems that besiege it, problems such as the sacrifice of the primary aim of being in school, an unwanted pregnancy, the use of contraceptives by female students, abortion, domestic violence, sharing of domestic chores, and the dangers inherent in such practices (Avogo *et al*., 2019; Perelli-Harris *et al*., 2018). Frequently, Nigerian society frowns on unmarried adults cohabiting within the society, without paying adequate attention to young adults/adolescents who are in tertiary institutions living together in the same society (Ogunsola, 2004). Most tertiary institutions make laws to control indecent dressing among students but do not make any reference to cohabitation among them. Some of the consequences that are often associated with cohabitation include unprotected sex, unwanted pregnancies, engaging in abortion, sexually transmitted diseases, mistrust of either partner, lack of privacy, poor academic performance, or a financial crisis involving one partner (Ogunsola, 2004). Conversely, the practice of cohabitation has serious health implications, more especially for females who are often at the receiving end of the dangers and consequences that come up in the aftermath of cohabitation. For instance, a female student may use incorrect contraceptive pills that can affect some of her vital organs, and if a pregnancy occurs, they are more likely to seek an abortion as a way to avoid discrimination and stigmatization (Amato *et al*., 2007; Beck *et al*., 2002). In addition, female students are more likely to visit 'quack; doctors who are not licensed practitioners for abortions and other health related issues (Amato *et al*., 2007). Some of the female students might be advised not to have another

abortion since they already experienced several, and this will lead to them giving birth to an unwanted, unplanned baby, which could mean the end of their academic pursuit. Therefore, the objective of this study was to explore the factors that influences cohabitation practices among Nigerian undergraduate students in Okuku campus of Osun State University. The specific objectives were to: examine the knowledge of cohabitation, attitudes towards cohabitation, perceptions of cohabitation, types and dimensions of cohabitation, pattern of cohabitation, social problems associated with cohabitation, and factors associated with cohabitation, among students at Osun State University. These objectives formed the central base of this research.

MATERIALS AND METHODS

Study design: This exploratory study employed a survey research design, appropriate for its non-experimental nature. The approach facilitated the collection of opinions from a representative sample of the target population, enabling informed conclusions, inferences, and generalizations.

Study location: The study was conducted in Osun State, South-Western Nigeria, whose capital is Oshogbo. The state comprises three senatorial districts and 30 local government areas and hosts several tertiary institutions, including Osun State University, located in the semi-rural Okuku community. This area is primarily agrarian, with farming as the dominant occupation. The university operates six campuses—Oshogbo, Okuku, Ipetu-Ijesha, Ejigbo, Ikire, and Ifetedo. The Okuku campus was purposively selected due to its large student population and concentration of adolescents (15–19 years) and youths (18–30 years). It houses two faculties: Social Sciences, with four departments (Sociology, Political Science, Geography, and Economics) and an estimated 2,000 students; and Management Sciences, with five departments (Accounting, Banking and Finance, Business Administration, Human Resource Management, and Entrepreneurship), comprising approximately 3,500 students.

Data collection and study population: Data were gathered using structured questionnaires and in-depth interviews. The study population consisted of students enrolled in both faculties. Simple random sampling was used to select 400 students from a sampling frame of 5,000 registered students, spanning Levels 100 to 400. The sample size determination followed Lemeshow *et al.*'s (1990) formula, which yielded a baseline of 384 and was expanded to 400 to enhance generalizability.

Purposive sampling was employed to recruit 18 participants for qualitative interviews. These participants were currently engaged in cohabitation practices and consented to participate voluntarily. Given the sensitive nature of the topic, non-probability sampling was deemed appropriate to ensure ethical engagement and informed consent. Ethical considerations were thoroughly observed during respondent selection across both faculties. A total of 400 questionnaires were administered and successfully retrieved by the principal investigator. This 100% response rate was achieved by encouraging immediate completion and collection at various student locations, including hostels and campus facilities. Questionnaires were distributed daily to maximize coverage. In-depth interviews explored the patterns, dimensions, and lived experiences of cohabitation among student participants. The fieldwork was conducted between June and July 2017, incorporating both quantitative and qualitative data collection methods.

Statistical analysis: Quantitative data were analyzed using descriptive statistics (frequencies and percentages) and bivariate analyses (chi-square tests and correlations), executed with SPSS version 21. Descriptive analyses highlighted core data features, while bivariate techniques assessed associations and relationships among study variables. Qualitative data from interviews were transcribed, translated verbatim, systematically sorted, and subjected to content analysis.

RESULTS

Table 1 presents the socio-demographic characteristics of the study sample. Of the respondents, 46.2% were male and 53.8% were female. The majority (76.0%) fell within the 20–24 age group, followed by 13.5% aged 25–30 years and 10.5% aged 15–19 years. In terms of religious affiliation, most respondents identified as Christian (63.5%), while 30.5% were Muslim and 6.0% practiced Traditional African religions. Ethnically, the sample comprised 42.5% Igbo, 41.3% Yoruba, and 16.2% Hausa respondents. Regarding parental occupation, 69.0% of respondents reported that their fathers were employed, and 62.2% indicated their mothers were self-employed..

Knowledge of cohabitation practice among university students: Figure 1 illustrates respondents' levels of knowledge regarding cohabitation. The results indicate that a majority of respondents possess a high level of awareness and understanding of cohabitation practices.

Perspectives on Cohabitation Practices Among University Students: Figure 2 presents respondents' perspectives on cohabitation practices. A majority (73.8%) perceived cohabitation as "not married but living together," indicating widespread recognition of its informal nature. Additionally, 12.0% viewed cohabitation as a "conjugal relationship supported by parents," while 7.9% considered it a "long-term relationship that resembles marriage." Only 6.3% interpreted cohabitation as equivalent to marriage between two people. These varied perspectives suggest diverse understandings of cohabitation among university students, shaped by cultural expectations, personal experiences, and social context.

Qualitative findings further revealed that participants gained their understanding of cohabitation through interactions with individuals in their community who were actively engaged in such relationships. In relation to this, one participant in the in-depth interview (IDI) sessions remarked that:

> *I do not know what a cohabitation relationship was until I got admission into the university. I see students – males and females staying together as couple…that is why they are called 'couple's life'. A lot of young people in this campus often engage in cohabitation*

*relationships. They believe that it is a way to draw a deeper intimate relationship between themselves that will secure marriage position for them after graduation. Often times, both male and female enjoy the cohabitation union since they mutually benefit from it (**Female student/20 years/IDI**).*

Table 1.
Distribution of Socio-Demographic Characteristics of Respondents

Variables		N	%
Sex	Male	185	46.2
	Female	215	53.8
Age at last birthday	15 – 19 years	42	10.5
	20 – 24 years	304	76.0
	25 – 30 years	54	13.5
Religion	Christianity	254	63.5
	Islam	122	30.5
	Traditional religion	24	6.0
Ethnic Group	Yoruba	165	41.3
	Igbo	170	42.5
	Hausa	65	16.2
Marital Status	Single	376	94.0
	Married	24	6.0
Average Monthly Stipend	Less than ₦10,000	60	15.0
	₦10,000 – ₦14,000	205	51.2
	₦15,000 – ₦19,000	42	10.5
	₦20,000 and above	93	23.3
Level of study	100 level	102	25.4
	200 level	69	17.3
	300 level	160	40.0
	400 level	69	17.3
Years spent in the University	One year	108	27.0
	Two years	89	22.3
	Three years	134	33.4
	Four years	69	17.3
Family type	Monogamous	243	60.8
	Polygamous	157	39.2
Who do you live with?	Both parents	298	74.5
	Father only	18	4.5
	Mother only	78	19.5
	No response	5	1.5
Father level of education	No formal schooling	24	6.0
	Primary school	18	4.5
	Secondary school	22	5.5
	Tertiary school	318	79.5
	Vocational school	18	4.5
Mother level of education	No formal schooling	24	6.0
	Primary school	18	4.5
	Secondary school	5	14.5
	Tertiary school	228	57.0
	Vocational school	72	18.0
Father/Guardian occupational stat	Employed	276	69.0
	Unemployed	24	6.0
	Self employed	100	25.0
Mother/Guardia occupational status	Employed	115	28.8
	Unemployed	36	9.0
	Self employed	249	62.2
	Total	**400**	**100**

Respondents who expressed a strong preference for cohabitation relationships frequently encouraged their partners to participate in such arrangements. These relationships typically begin as temporary living situations, which gradually evolve into more permanent cohabitation. Over time, one partner often applies increasing pressure on the other to formalize the arrangement by moving in for the duration of their university education. This dynamic was highlighted in one of the in-depth interview (IDI) sessions, which affirmed that:

*I got involved in cohabitation relationship in the university and I encouraged my partner to cohabit with me after some months we met. We stayed together temporary and much later as our intimate relationship was going deeper, I mounted pressure on her to stay with me permanently till we graduate from the university together. This is as a result that I want her to be very close to me and we can therefore be honest with each other throughout our stay in the university. I felt this type of cohabitation union is likely to lead us to a longer relationship that will end in future marriage when we have finished our studies (**Male student/19 years/IDI**).*

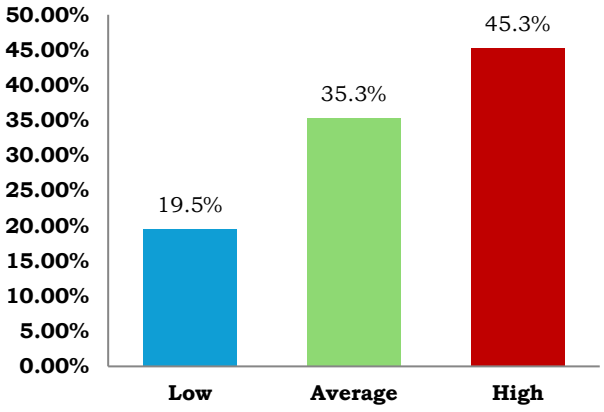


Figure 1:
Bar chart showing Respondents' Level of Knowledge of Cohabitation

Types of Cohabitation Relationships Among University Students: Figure 3 illustrates the various types of cohabitation relationships reported by respondents. A majority (60.5%) identified pre-marital cohabitation as the most prevalent form among university students. Other forms of cohabitation were cited less frequently, including alternative relationships (3.8%), limited cohabitation (8.5%), convenient cohabitation (13.5%), and substitute cohabitation (13.8%). These findings suggest that students primarily view cohabitation as a precursor to formal union rather than as an alternative or substitute to marriage.

In addition, the qualitative data reinforced the findings from the quantitative analysis. This alignment was echoed by a female participant during one of the in-depth interview (IDI) sessions, who remarked:

*University students engage in cohabitation because they feel that it will end up in marriage. So, they cohabit to stay together and build an intimate relationship as the so-called 'husband' and 'wife'. Majorly, young people in the university cohabit to build a 'long-time picture' of a marital union for the future; but it does not always end in marriage. They often broke up after staying together in a year or less. There could be reasons for their break-up which they never disclose to anyone (**Female student/22 years/IDI**).*

Additionally, a male participant in the IDI sessions corroborated the preceding response, stating:

*I engage in cohabitation, and I felt that I do not want my girlfriend to have another relationship with another boy in my university, as this will hurt me a lot. So, I discussed with her and she agreed to move in with me. I am more comfortable seeing her every day in my hostel room and I believe we will get married as soon as we are done with our studies. She benefits from me and I also benefit from her…it is a mutual understanding for both of us to stay together. However, we do not discuss our pre-marital affairs or cohabitation relationships with families and relatives because they will frown and be mad at us cohabiting together (**Male student/20 years/IDI**).*

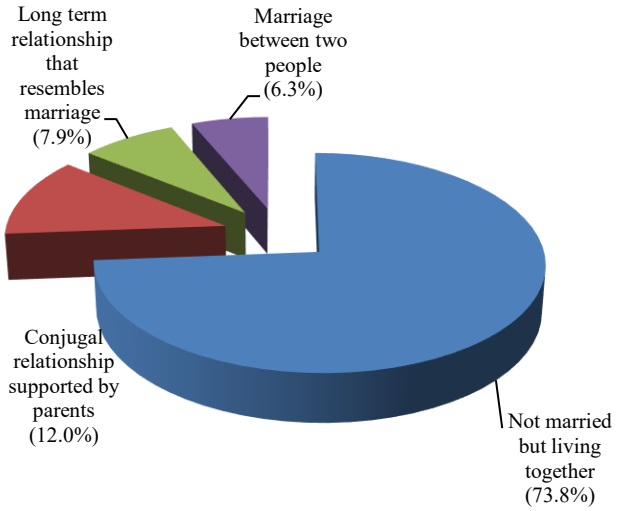


Figure *2*:
Respondents' perspectives on cohabitation practices

However, findings from the qualitative data further revealed that participants acquired their knowledge from other individuals who practice cohabitation within their community. Regarding this, one of the participants in the in-depth interview (IDI) sessions commented that:

I do not know what a cohabitation relationship was until I got admission into the university. I see students – males and females staying together as couple…that is why they are called 'couple's life'. A lot of young people in this campus often engage in cohabitation relationships. They believe that it is a way to draw a deeper intimate relationship between themselves that will secure marriage position for them after graduation. Often times, both male and female enjoy the cohabitation union since they mutually benefit from it (Female student/20 years/IDI).

Respondents who love cohabitation relationships encouraged their partners to be involved with them in such relationships. Such relationships often start with temporary cohabitation, and after a long time, one of the partners will keep mounting pressure on the other partner to move in with him or her throughout their stay in the university. One of the IDI sessions affirmed that:

I got involved in cohabitation relationship in the university and I encouraged my partner to cohabit with me after some months we met. We stayed together temporary and much later as our intimate relationship was going deeper, I mounted pressure on her to stay with me permanently till we graduate from the university together. This is as a result that I want her to be very close to me and we can therefore be honest with each other throughout our stay in the university. I felt this type of cohabitation union is likely to lead us to a longer relationship that will end in future marriage when we have finished our studies (Male student/19 years/IDI).

Types of cohabitation relationships prevalent among university students: The figure 3 below showed types of cohabitation relationships prevalent among respondents. A majority (60.5%) of them mentioned that pre-marital cohabitation relationships is more prevalent among university students than other type of relationships (Alternative – 3.8%; Limited – 8.5%; Convenient – 13.5%; and Substitute – 13.8%).

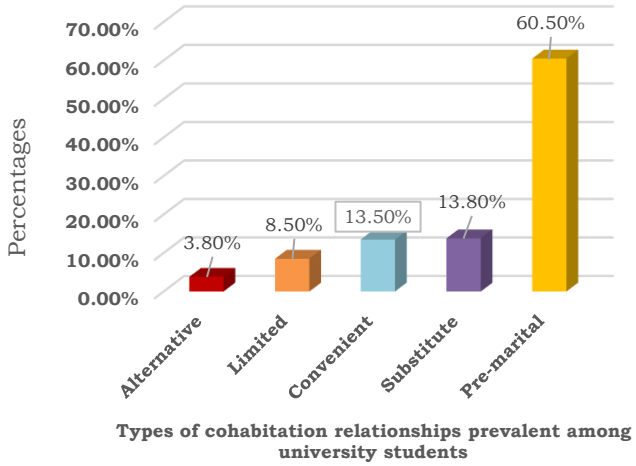


Figure *3*.
Bar chart showing the types of cohabitation relationships prevalent among university students**.**

Also, the findings from the qualitative data buttressed the quantitative data findings, and one of the female participants in the IDI sessions asserted that:

University students engage in cohabitation because they feel that it will end up in marriage. So, they cohabit to stay together and build an intimate relationship as the so-called 'husband' and 'wife'. Majorly, young people in the university cohabit to build a 'long-time picture' of a marital union for the future; but it does not always end in marriage. They often broke up after staying together in a year or less. There could be reasons for their break-up which they never disclose to anyone (Female student/22 years/IDI).

Also, another male participant in the IDI sessions corroborated the above response as below:

I engage in cohabitation and I felt that I do not want my girlfriend to have another relationship with another boy in my university, as this will hurt me a lot. So, I discussed with her and she agreed to move in with me. I am more comfortable seeing her every day in my hostel room and I believe we will get married as soon as we are done with our studies. She

benefits from me and I also benefit from her…it is a mutual understanding for both of us to stay together. However, we do not discuss our pre-marital affairs or cohabitation relationships with families and relatives because they will frown and be mad at us for cohabiting together (Male student/20 years/IDI).

Dimensions of cohabitation practices among university students: Respondents were examined on the dimensions of cohabitation practised, and Figure 4 below illustrates the dimensions of cohabitation relationships practiced among respondents. A majority (63.6%) of the respondents indicated that cohabitation practices among university students have a positive dimension compared with those (36.4%) with the notion of negative dimensions regarding cohabitation practices

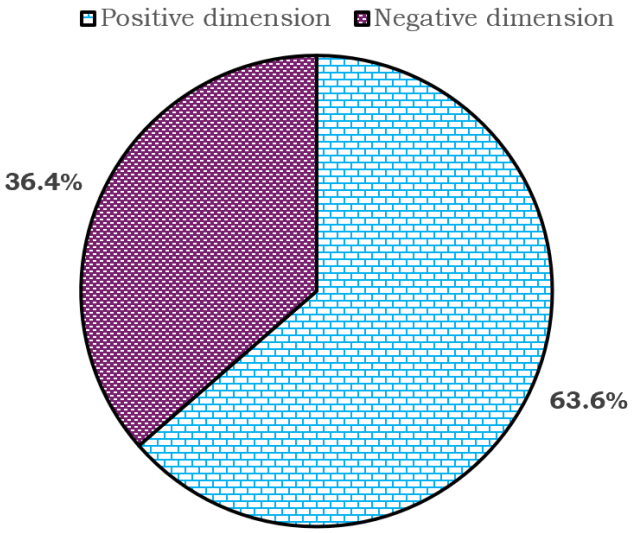


Figure *4*.
Pie-chart illustrating the dimensions of cohabitation practices among respondents

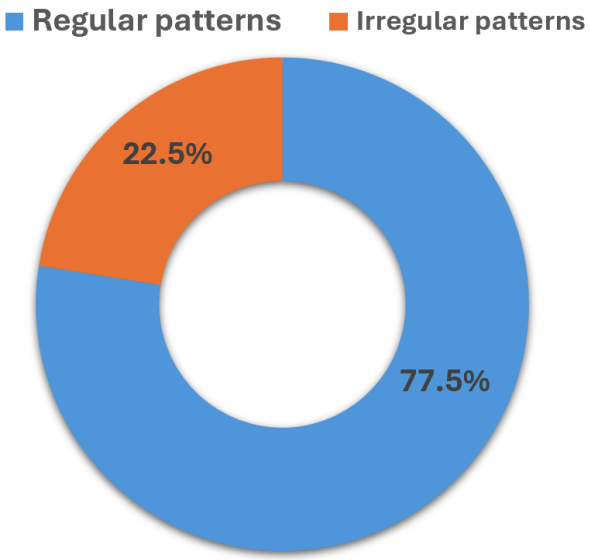


Figure *5*
Pie-chart showing the patterns of cohabitation practices among respondents

Patterns of Cohabitation Practices: Figure 5 illustrates the reported patterns of cohabitation practices among respondents. A significant majority (77.5%) described their involvement in regular cohabitation arrangements, indicating consistency and stability in their living situations. In contrast, 22.5% reported irregular cohabitation practices, reflecting episodic or non-continuous patterns of living together. These findings suggest that cohabitation among university students is largely structured and sustained, though a notable proportion engage in more transient or informal arrangements.

Qualitative findings further illuminated the patterns of cohabitation practices among participants. One respondent in the IDI sessions remarked:

*Cohabitation among young persons in the university is an individual choice…some people have started cohabiting from home before getting admission to the university. While others decided and chose on their own to cohabit when they get to school. So, I believe that it is an individual choice to cohabit or not (**Male student/20 years/IDI**).*

Reasons for Patterns of Cohabitation Practices: Respondents identified several factors that influence the observed patterns of cohabitation among university students in contemporary society. As shown in Figure 6, the majority cited age as a primary reason shaping cohabitation practices. Additional factors included educational status (21.4%), employment status (10.4%), and marital status (5.9%), suggesting that demographic and socio-economic characteristics play a significant role in students' cohabitation behaviors.

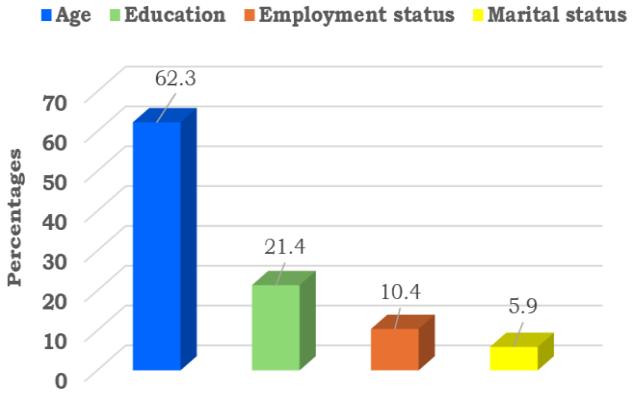


Figure 6
Reasons for the patterns of cohabitation practices among respondents

Also, the finding from the qualitative data buttressed the quantitative data finding above, and one of the female participants in the IDI sessions emphasised that:

*One of the major reasons why young people in the university cohabit is as a result of low socio-economic reasons such as lack of financial capacity or non-employment levels. Based on these reasons mentioned above, several young persons in the university will agree to cohabit in order to share their responsibilities among themselves, and since they are already planning to extend their cohabitation relationships to marriage in the future (**Female student/22 years/ IDI**).*

Table 2. Distribution of Respondents' Attitude towards Cohabitation Relationships and Practices

Variables	Responses	N	%
Do you have approval attitude towards cohabitation relationships and practices among university students?	Yes	235	58.8
	No	141	35.2
	No response	24	6.0
Do you have disapproval attitude towards cohabitation relationships and practices among university students?	Yes	168	42.0
	No	208	52.0
	No response	24	6.0
Do you have defensive attitude towards your personal experience of your involvement in cohabitation relationships and practices?	Yes	163	40.8
	No	213	53.2
	No response	24	6.0
Based on your attitude and experience towards cohabitation relationships and practices, will you recommend the cohabitation practices to your age cohorts in the university?	Yes	151	37.8
	No	225	56.2
	No response	24	6.0
Respondents' attitude towards cohabitation relationships and practices	Positive attitude	168	42.0
	Negative attitude	232	58.0
	Total	**400**	**100**

Source: Fieldwork, 2017.

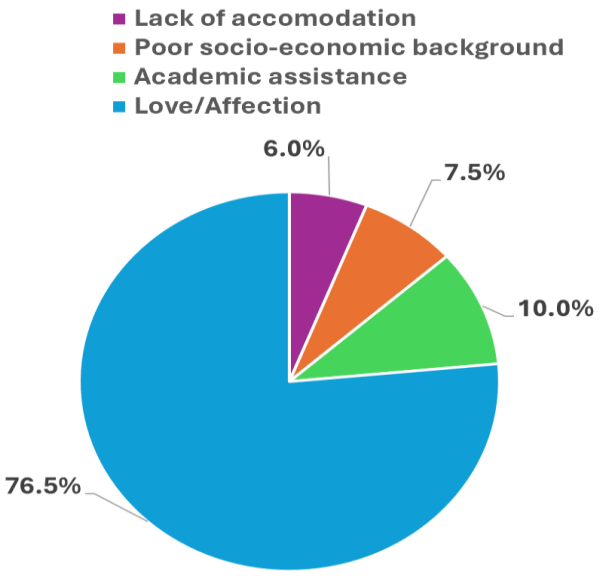
Table 3. Distribution of Respondents' Perception of Cohabitation Relationships and Practices

		N	%
Perception of cohabitation relationships and practices as not a good path for future marriage	Low perception	265	66.3
	Moderate perception	117	29.2
	High perception	18	4.5
Perception of cohabitation relationships and practices as trial marriage	Low perception	352	88.0
	Moderate perception	30	7.5
	High perception	18	4.5
Do you perceive that cohabitation relationships and practices can lead to marriage?	Yes	211	52.8
	No	165	41.2
	No response	24	6.0
	Total	**400**	**100**

Source: Fieldwork, 2017.

Attitudes toward cohabitation relationships and practices: Table 2 below shows the respondents' attitude towards cohabitation relationships and practices among university students. A majority of the respondents (58.8%) indicated a 'Yes' affirmation, having an approval attitude towards cohabitation relationships and practices among university students. About 52.0% of respondents indicated a 'No' reply affirmation of a disapproval attitude towards cohabitation relationships and practices among university students. Also, 53.2% of respondents indicated a 'No' reply as affirmation of a defensive attitude towards their personal experience of their involvement in cohabitation relationships and practices, and 56.2% of respondents indicated a 'No' reply as affirmation of their attitude and experiences towards cohabitation relationships and practices that they would not recommend cohabitation relationships and practices to their age cohorts in the university. A majority of the respondents display negative attitudes (58.0%) towards cohabitation relationships and practices (Table 2).

Perception of cohabitation relationship and practices: Also, table 3 below shows the respondents' perception of cohabitation relationships and practices among university students. A majority, 66.3% of the respondents, reported a low perception of cohabitation relationships and practices as not a good path for future marriage. A majority (88.0%) of respondents mentioned a low perception of cohabitation relationships and practices as a trial marriage. Furthermore, 52.8% of respondents indicated a 'Yes' reply in affirmation, perceiving that cohabitation relationships and practices can lead to marriage.



Factors associated with cohabitation relationships and practices: Figure 7 showed the factors associated with cohabitation relationships and practices among university students. Factors such as lack of accommodation (6.0%), poor socio-economic background (7.5%), academic assistance (10.0%), and love/affection (76.5%) were mentioned by the respondents that are associated with cohabitation relationships and practices among university students.

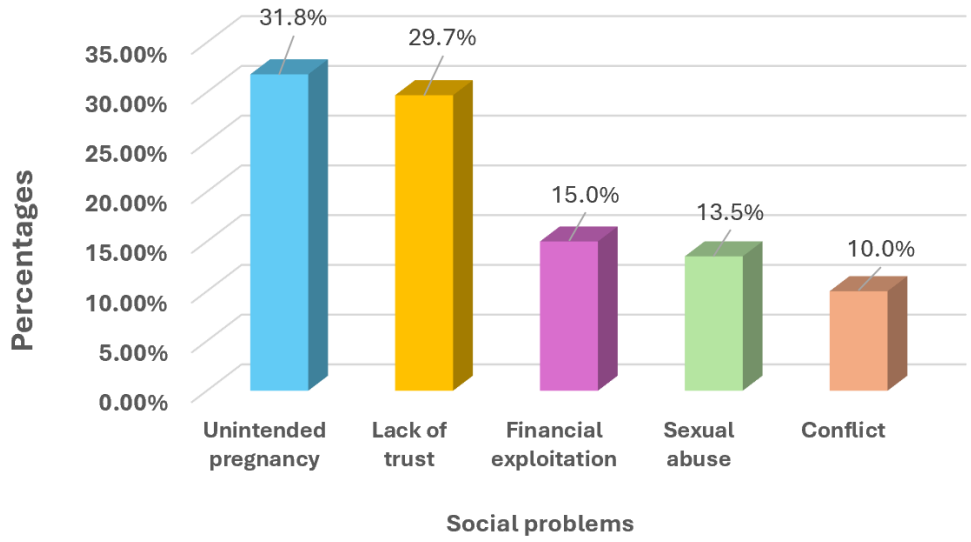


Figure 8.
Factors associated with cohabitation relationships and practices among university students

Table 4a.
Chi-Square Analysis of the Association Between Socio-Demographic Factors and Respondents' Knowledge of Cohabitation Relationships and Practices

	Variables	Low	Average	High	Total	χ^2	ρ
Gender	Male	60 (15.0%)	27 (6.8%)	98 (24.5%)	185		
	Female	18 (4.5%)	114 (28.5%)	83 (20.8%)	215	75.72	0.001
Age in years	15 – 19 years	78 (19.5%)	36 (9.0%)	06 (1.5%)	42		
	20 – 24 years	00 (0.0%)	99 (24.8%)	48 (12.0%)	304		
	25 – 30 years	00 (0.0%)	06 (1.5%)	127 (31.8%)	54	96.96	0.000
Marital status	Single	78 (19.5%)	141 (35.2%)	157 (39.2%)	219		
	Married	00 (0.0%)	00 (0.0%)	24 (6.0%)	24	30.89	0.000
Average monthly stipend	Less than ₦10,000	30 (7.5%)	24 (6.0%)	06 (1.5%)	60		
	₦10,000 – ₦14,000	36 (9.0%)	78 (19.5%)	42 (10.5%)	205		
	₦15,000 – ₦19,000	00 (0.0%)	00 (0.0%)	91 (22.8%)	42	100.44	3.020
	Above ₦20,000	12 (3.0%)	39 (9.8%)	42 (10.5%)	93		
Level of study	100 level	20 (16.4%)	54 (44.3%)	48 (39.3%)	122		
	200 level	10 (14.5%)	16 (23.2%)	43 (62.3%)	69		
	300 level	58 (48.3%)	12 (10.0%)	50 (41.7%)	120	320.60	2.120
	400 level	20 (22.5%)	49 (55.0%)	20 (22.5%)	89		

Source: *Fieldwork, 2017*

Social problems associated with cohabitation relationships and practices: Figure 8 showed the social problems associated with cohabitation relationships and practices among university students. A majority of the respondents reported unintended pregnancy (31.8%), lack of trust (29.7%) and financial exploitation (15.0%) as social problems associated with cohabitation relationships and practices that occurred among university students. Also, sexual abuse (13.5%) and conflict (10.0%) were also mentioned by the respondents as social problems associated with cohabitation relationships

Bivariate analysis of socio-demographic factors and cohabitation knowledge: Table 4a presents the bivariate analysis of socio-demographic factors associated with respondents' knowledge of cohabitation. A higher proportion of female (28.5%) and male (24.5%) respondents reported average to high levels of knowledge about cohabitation. The Chi-square test revealed a statistically significant association between gender and knowledge of cohabitation ($\chi^2 = 75.72$, $p < 0.001$). Respondents aged 25–30 years (31.8%) demonstrated high knowledge levels, with a significant association observed between age group and knowledge ($\chi^2 = 96.96$, $p < 0.001$). Similarly, a majority of single respondents (39.2%) reported high knowledge, and marital status was significantly associated with knowledge of cohabitation. In contrast, only 22.8% of respondents receiving a monthly stipend of ₦10,000–₦14,000 indicated high knowledge levels. However, the Chi-square test showed no significant association between monthly stipend and knowledge of cohabitation ($\chi^2 = 100.44$, $p > 0.05$). Furthermore, while 62.3% of 200-level students reported high knowledge, there was no statistically significant association between level of study and knowledge of cohabitation ($\chi^2 = 320.60$, $p > 0.05$) (Table 4a).

Also, table 4b below shows the socio-demographic factors associated with respondents' attitude towards cohabitation. A majority of the females (58.8%) have a negative attitude towards cohabitation relationships and practices, and the Chi-square test shows a significant association of respondents' attitude towards cohabitation with gender differences ($\chi^2 = 9.48$, $\rho < 0.001$). Respondents who are aged 20-24 years (68.2%) exhibited higher levels of a negative attitude towards cohabitation relationships and practices, and the Chi-square test shows a significant association with age ($\chi^2 = 90.62$, $\rho = 0.000$). Fifty-nine percent of respondents who are single asserted a negative attitude towards cohabitation relationships and practices, and the Chi-square test shows a significant association with marital status ($\chi^2 = 31.40$, $\rho = 0.000$) (Table 4b).

Table 4b.
Chi-square Analysis showing the association of Socio-demographic factors and Respondents' Attitude towards Cohabitation Relationships and Practices among University Students.

	Variables	Positive	Negative	Total	χ^2	ρ
Gender	Male	96 (48.9%)	100 (51.0%)	196	9.48	0.001
	Female	84 (41.2%)	120 (58.8%)	204		
Age in years	15 – 19 years	30 (71.4%)	12 (28.6%)	42	90.62	0.000
	20 – 24 years	91 (31.8%)	195 (68.2%)	286		
	25 – 30 years	48 (66.7%)	24 (33.3%)	72		
Marital status	Single	144 (41.1%)	206 (58.9%)	350	31.40	0.000
	Married	24 (48.0%)	26 (52.0%)	50		
Average monthly stipend	Less than ₦10,000	24 (44.4%)	30 (55.6%)	54	58.99	0.000
	₦10,000 – ₦14,000	73 (36.7%)	126 (63.3%)	199		
	₦15,000 – ₦19,000	42 (63.6%)	24 (36.4%)	66		
	Above ₦20,000	30 (37.0%)	51 (63.0%)	81		
Level of study	100 level	30 (29.4%)	72 (70.6%)	102	26.63	0.000
	200 level	45 (48.4%)	48 (51.6%)	93		
	300 level	70 (51.5%)	66 (48.5%)	136		
	400 level	24 (34.8%)	45 (65.2%)	69		

Source: Fieldwork, 2017

Table 4c.
Chi-square Analysis showing the association of Socio-demographic factors and Respondents' Perception towards Cohabitation Relationships and Practices among University Students.

	Variables	Poor	Moderate	Good	Total	χ^2	ρ
Gender	Male	85 (43.8%)	21 (10.8%)	88 (45.4%)	194		
	Female	15 (7.3%)	113 (54.9%)	78 (37.8%)	206	118.62	0.001
Age in years	15 – 19 years	09 (18.8%)	27 (56.2%)	12 (25.0%)	48	19.96	0.000
	20 – 24 years	85 (21.5%)	89 (22.5%)	118 (30.9%)	292		
	25 – 30 years	09 (15.0%)	21 (35.0%)	30 (50.0%)	60		
Marital status	Single	97 (24.6%)	131 (33.5%)	139 (35.6%)	367		0.000
	Married	03 (0.0%)	03 (0.0%)	27 (6.3%)	33	35.53	
Average monthly stipend	Less than ₦10,000	30 (51.7%)	20 (34.5%)	08 (13.8%)	58		
	₦10,000 – ₦14,000	54 (26.5%)	88 (43.1%)	62 (30.4%)	204	110.39	0.000
	₦15,000 – ₦19,000	01 (2.2%)	20 (43.5%)	25 (54.3%)	46		
	Above ₦20,000	14 (15.2%)	08 (8.7%)	70 (76.1%)	92		
Level of study	100 level	02 (1.9%)	36 (34.6%)	66 (63.4%)	104		
	200 level	22 (31.9%)	23 (33.3%)	24 (34.8%)	69	265.86	0.000
	300 level	72 (46.8%)	12 (7.8%)	70 (45.4%)	154		
	400 level	02 (2.7%)	68 (93.2%)	03 (4.1%)	73		

Source: Fieldwork, 2017

Respondents with average monthly stipend of ₦15,000–₦19,000 (in Nigerian naira) reported a positive attitude towards cohabitation relationships and practices, and the Chi-square test shows a significant association with average monthly stipend and respondents' attitude towards cohabitation relationships and practices ($\chi^2 = 58.99$, $\rho < 0.000$). About 71% of respondents who are in their 100 level of study showed a negative attitude towards cohabitation relationships and practices, and the Chi-square test shows a significant association with level of study ($\chi^2 = 26.63$, $\rho = 0.000$) (Table 4b).

Similarly, table 4c below shows the socio-demographic factors associated with respondents' perception towards cohabitation relationships and practices. The findings showed that a majority of the female respondents (54.9%) showed a moderate perception towards cohabitation and the Chi-square test showed a significant association of perception of cohabitation with gender differences ($\chi^2 = 118.62$, $\rho < 0.001$).

Respondents who are aged 15-19 years (56.2%) exhibited a moderate perception of cohabitation, and the Chi-square test showed a significant association of perception of cohabitation with age ($\chi^2 = 19.96$, $\rho = 0.000$) (Table 4c). About thirty-six percent of the respondents who are single have a good perception towards cohabitation relationships and practices, and the Chi-square test showed a significant association of perception of cohabitation with marital status ($\chi^2 = 35.53$, $\rho = 0.000$). A majority (76.1%) of the respondents with above ₦20,000 have a good perception towards cohabitation relationships and practices, and the Chi-square showed a significant association of perception of cohabitation and average monthly stipend ($\chi^2 = 110.39$, $\rho = 0.000$) (Table 4c). A majority (93.2%) of the respondents who are in their 400 level of study showed moderate perception towards cohabitation and the Chi-square test shows a significant association of perception of cohabitation and level of study ($\chi^2 = 265.86$, $\rho = 0.000$) (Table 4c). Also, the findings from the qualitative data buttressed the

quantitative data findings above, and one of the male participants in the IDI sessions indicated that:

Young persons, especially the male individuals, have positive attitude towards cohabitation relationships and practices. They engaged in and enjoyed cohabiting, most especially the younger male folks, because most females are engaged in domestic chores for them such as cleaning, washing, cooking and so on. The male encouraged themselves to be part of cohabitation relationship as they exhibit masculinity behaviour during cohabitation relationships and practices…I was introduced into cohabitation relationships and practices by a close friend of mine. And since then, I have been living together with my girlfriend at a rented hostel outside the university campus. She helps me a lot with domestic chores and supports me emotionally ***(Male student /23 years/IDI)****.*

Another participant mentioned that:

I am into cohabitation relationship for three years since my 100 level. My boyfriend talked me into it and I could not say no to him for fear of losing him to another girl. I moved in to his house and we live together since our 100 level of study. I feel comfortable with it because he cannot cheat on me and I also cannot cheat on him either. My personal perception is that female folks are more likely to engage in cohabitation relationships and practices more than their male counterparts to protect their relationship from breaking-up. Although our families are not aware of our cohabitation relationships and practices, but we live and do as couples do and everyone at the university campus are aware of our relationship and our staying together as 'husband and wife' ***(Female student/22 years/IDI)****.*

Table 5:
Inter-correlation of knowledge, attitude and perception of cohabitation relationships and practices among university students

S/No	Variables	1	2	3
1	Knowledge of cohabitation	1	–	–
2	Attitude towards cohabitation	0.611**	1	–
3	Perception of cohabitation	0.528**	0.187**	1

Source: Fieldwork, 2017

Inter-Correlation of Knowledge, Attitudes, and Perceptions Regarding Cohabitation Relationships and Practices: Table 5 shows the inter-correlation of the respondents' knowledge, attitude and perception of cohabitation relationships and practices among university students. The findings revealed that respondents' knowledge was positively correlated with attitude ($r = 0.611$, $p < 0.01$) and perception ($r = 0.528$, $p < 0.01$) towards cohabitation relationships and practices. Also, respondents' perception are positively correlated with attitude ($r = 0.187$, $p < 0.01$) towards cohabitation relationships and practices. This implies that high knowledge will lead to positive attitude and high perception towards cohabitation relationships and practices among university students. Therefore, the findings further revealed that respondents' knowledge, attitude and perception of cohabitation relationships and practices are inter-related, which further implied that high knowledge would lead to positive attitude and high perception that may likely discourage university students from engaging in cohabitation relationships and practices.

DISCUSSION

We explored the factors that influence the cohabitation relationships among students of Osun State University, Nigeria. The findings of the recent study indicated that the knowledge of cohabitation among undergraduate students in Osun State University was 45.3%. This figure is similar to the one reported for universities in Ghana (Gyasi-Gyamerah *et al*., 2023) and Kenya (Hattori *et al*., 2007). Thus, the cohabitation knowledge levels among the respondents were comparable to that reported in more developed countries, which is lower (Hattori *et al*., 2007; Axinn *et al*., 1993). Such trends of lower knowledge of cohabitation relations before indulging into such practices, if not checked, are bound to result in rising cases of being predisposed to early sexual debut, sexually transmitted infections (STIs), human immunodeficiency virus (HIV), sexual abuse, gender violence, abortions, and illegitimate children (children born out of wedlock) (Pierce *et al*., 2020; Gevers *et al*., 2013; Posel *et al*., 2013). One interesting aspect of the knowledge trends showed that undergraduate students have different perspectives towards cohabitation practices, as they viewed these as cultural values and norms that exist among young people aged 15-24 years, as they see it as living together not being married, or having a conjugal relationship supported by parents, and a long-term relationship that resembles marriage, as well as viewing it as a marriage between young people (Hattori *et al*., 2007; Gevers *et al*., 2013).

Several studies have laid emphasis that these kind of perspectives evolving around young persons had severely compromised their life orientations and core values, particularly when having to bargain about education standards, particularly among female students (Brown *et al*., 2023; Osuafor *et al*., 2018). This is seen when female students remain behind in the hostels washing clothes and cooking for their cohabiting male partners, who never miss their lectures and school activities (Brown *et al*., 2023; Pleasence *et al*., 2012). Also, the knowledge of the cohabitation practice is average to high, as a result of their orientations from their family values and background, where they are not allowed to engage in such practices. This implies that the respondents have a better understanding of what cohabitation is, but they do not know that unmarried people living together in the same house are practicing cohabitation. Within the university, the term 'couple's life' is used instead of cohabitation and it is used to refer to an unmarried male and female who are living together. This study collaborated with the summation of the studies of Zhang *et al*. (2022) and Uprety (2023), that cohabitation practices are usually copied among young people who are exposed to such relationships within their environs. This was mostly disturbing, given that the findings of this study indicated that those who are into cohabitation relationships acquired their knowledge from their counterparts who are

practicing cohabitation relationships within their environs (Uprety, 2023; Cho *et al*., 2016).

Moreover, partners who love cohabitation relationships often lure their partners into such practices, which often begins with temporary living together for few days. Then, much later, one of the partners will create a platform of escalating pressure on the other partner to move in with him or her throughout their stay in the university. Such partners who are in a relationship and do not succumb to such peer pressure are made to suffer stigma, discrimination, and shame by their partners, peers and friends, and the wider school community. This is in line with a few studies, such as in the published works of Ezumah *et al*. (2021) and Adeyera *et al*. (2023), who mentioned that young people, especially females, who refused a cohabitation relationship were subjected to discrimination and stigmatization by friends, as well as leading to abruptly broken relationships (Scent *et al*., 2019; Argentova *et al*., 2018). Despite the high levels of knowledge and perspectives towards cohabitation practices, pre-marital cohabitation relationships were predominantly prevalent among the respondents, while convenient and substitute cohabitation practices were to an extent largely mentioned by the respondents. The findings showed that there were mixed feelings among undergraduate students as to the types of cohabitation relationships that were prevalent among university students. However, the effect of cohabitation on marital success showed equal proportions holding contradicting perspectives.

The predominant types of cohabitation relationships being practised by undergraduate students is not adequately studied, as this varies across persons' identity and cultural orientations (Argentova *et al*., 2018; Vigil *et al*., 2022; Vaingankar *et al*., 2020; Afifi *et al*., 2024). However, another study's findings revealed that cohabitation and its practices were not an option in a traditional African family and as such, it is frowned upon by families, religious bodies, and society (Alo, 2008; Brown *et al*., 2023). However, in contemporary families, there is a growing approval of cohabitation among young Nigerian adults, and university students are not an exception (Lawal *et al*., 2021; Muhinat, 2022; Obikeze *et al*., 2018; Iyekolo, 2021; Kalu *et al*., 2021; Aborisade, 2021; Alo, 2008). The findings of the present study indicate that there were striking similarities between the study subjects and findings of some studies in developed countries such as in the United States (Ogunsola, 2004; Manning *et al*., 2014), United Kingdom (Perelli-Harris *et al*., 2018; Manning *et al*., 2019) and across Europe (Perelli-Harris *et al*., 2014; Perelli-Harris *et al*., 2018) as well as parts of Asian countries (Pelikh *et al*., 2022).

However, the study revealed that the majority of respondents perceived cohabitation practices positively, in contrast to those who expressed negative views. This finding suggests that most respondents perceive cohabitation among university students in a favorable light, identifying benefits or constructive aspects to the practice. These 'positive dimensions' may reflect beliefs that cohabitation fosters emotional intimacy, enhances relationship experience, promotes shared responsibilities, or provides practical solutions to challenges like accommodation shortages (Muhinat, 2022; Obikeze *et al*., 2018; Iyekolo, 2021; Kalu *et al*., 2021). In contrast, the 36.4% who view cohabitation negatively likely associate it with social risks such as emotional instability, exploitation, academic distraction, or moral concerns. This split highlights a nuanced outlook: while cohabitation is widely accepted or even normalized within the university context, a significant minority still questions its social and developmental implications. It may also underscore generational shifts in relationship norms, shaped by exposure, personal values, and socio-economic pressures (Vigl *et al*., 2022; Ghosh, 2021).

The findings of this study reveal that cohabitation among university students is a multifaceted practice shaped by a range of personal, relational, socio-economic, and institutional factors. The meaning and experience of cohabitation varied considerably across respondents, reflecting diverse motivations—from intimacy, autonomy, and accommodation challenges to relationship experimentation and peer influence. Consistent with studies conducted in the United States and Europe (Manning *et al*., 2014; Manning *et al*., 2019), respondents in this study commonly perceived cohabitation as a transitional stage in dating relationships, rather than as an alternative to marriage. The dimensions and patterns of cohabitation observed—whether regular or irregular, trial-based, or convenience-driven—mirrored global trends in student relationship behavior, thereby supporting the view that young adults increasingly negotiate partnerships outside traditional marital frameworks. These findings align closely with the individualisation theory, which argues that individuals in contemporary society pursue self-directed life paths that prioritize personal autonomy, choice, and emotional fulfilment (Beck *et al*., 2002). Within the university context, cohabitation emerges as a site of this individualising process: students make strategic decisions about living arrangements that reflect their values, needs, and emerging identities. The flexibility and fluidity of these arrangements underscore a departure from rigid societal norms in favour of personal agency.

Nevertheless, the study also highlights context-specific drivers of cohabitation in African university settings, where economic and environmental conditions exert substantial influence. Socio-economic factors such as parental occupation, income, and residence—as well as limited institutional housing—were significant in shaping cohabitation choices, echoing findings by Akokuwebe *et al*. (2016), Maharaj *et al*. (2005), and Tamuno-Opubo *et al*. (2021). These factors reinforce the notion that cohabitation is not solely a relational choice but also a coping strategy in response to structural constraints. In sum, the study's synthesis of global and local findings demonstrates that while university students engage in cohabitation for various reasons, their practices are rooted in a broader social evolution characterized by increased individual autonomy, reduced parental oversight, and shifting conceptions of intimacy and commitment. The individualisation theory offers a compelling lens through which to interpret these patterns, capturing the dynamic interplay between personal aspiration and structural limitation in students' lived experiences.

However, this study's findings have shown that age, education, employment, and marital statuses were the major factors for the patterns of cohabitation relationships among the respondents. Similarly, few studies have mentioned socio-cultural factors, such as age, sex, education, marital

status, and family history of cohabitation, as the major basis for the dimensions and patterns of cohabitation practices among university students (Tamuno-Opubo *et al*., 2021; Akokuwebe *et al*., 2015; Maharaj *et al*., 2005; Mohlatlole *et al*., 2018; Akokuwebe *et al*., 2019; Akokuwebe *et al*., 2023). It is important to note that a majority of the respondents indicated a positive approval attitude towards cohabitation relationships and practices among university students. Studies conducted in the East and South of sub-Saharan African countries show that marriages that are preceded by living together have 60% to 100% higher disruption rates than marriages without premarital cohabitation (Obikeze *et al*., 2018; Iyekolo, 2021; Kalu *et al*., 2021; Aborisade, 2021). Cohabitation is considered as a half-way house for people who do not want the extent of personal and social obligation that marriage represents. Other studies have shown that cohabitation experiences have affected the quality of marriage (Manning *et al*., 2019; Akokuwebe *et al*., 2016). Marriages in which at least one spouse is an ex-cohabiter are on average more likely to end up in divorce than are marriages in which neither partner experienced premarital cohabitation (Tamuno-Opubo *et al*., 2021). Partners who cohabited before marriage reported lower levels of commitment to marriage as an institution (Scent *et al*., 2019; Alo, 2008).

Cohabitation may consequently have far-reaching negative impacts in the lives of young adults later in life (Gold, 2012; Perelli-Harris *et al*., 2014). Similarly, this study's findings showed that university students may harbour negative attitudes and a low perception towards cohabitation relationships and practices, but their engagement in such relationships is largely prejudiced by various influences. Also, this study's findings revealed that factors such as lack of accommodation, poor socio-economic background, academic assistance, and love/affection influence university students to engage in cohabitation relationships and practices. However other studies mentioned that cultural and social norms (Pierce *et al*., 2020), parental influences and attitudes (Adeoye *et al*., 2012), religious teaching (Axinn *et al*., 1993), fear of commitment (Gold, 2012), risk perception (Scent *et al*., 2019), financial considerations (Alebiosu, 2020), generational shifts and communication (Adeyera *et al*., 2023) and conflict (Odimegwu *et al*., 2018) may negatively or positively influence cohabitation relationships and practices among university students. Thus, traditional norms often emphasize marriage as the ideal relationship structure, and cohabitation, being less conventional, might be viewed with scepticism. Social expectations around marriage and family also play a role, and in societies where marriage is highly valued, cohabitation may be seen as a deviation (Wu, 2000; Oppenheimer, 2003).

Consequently, parental influence and attitudes significantly impact young people's views, as parents hold conservative beliefs, and their children may internalize these norms. Subsequently, parental disagreements and disapproval of cohabitation can lead to negative perceptions among university students. Also, religious teachings often prioritize marriage, where the majority of religious communities often frown upon pre-marital cohabitation relationships and practices. Studies have shown that young people who adhere strictly to religious principles may perceive cohabitation as morally obnoxious (Brown *et al*., 2023; Scent *et al*., 2019). Furthermore, cohabitation lacks a formal commitment compared to marriage, and individuals fear that it may hinder a long-term commitment, or lead to instability, as the ideal of a 'trial marriage' through cohabitation may not resonate with every individual. Moreover, studies have suggested that cohabiting partners face higher risks of relationship dissolution and divorce, as fear of potential breakup or uncertainty may contribute to negative attitudes and low perception among the university students (Zhang *et al*., 2022; Posel *et al*., 2013).

Economic factors play a major role where cohabitation often lacks legal protections and financial benefits associated with marriage, and some young people may view cohabitation as financially risky. Importantly, the millennials and Gen Z generations tend to prioritize individual autonomy and flexibility, as they may resist societal pressure to conform to traditional norms (Gold, 2012; Posel *et al*., 2013). This generational shifts can lead to more casual attitudes toward cohabitation, but it can also create tension with older generations. Notably, cohabiting partners face unique challenges, as miscommunication and unresolved conflicts can strain relationships, as well as negative experiences may shape attitudes (Scent *et al*., 2019; Axinn *et al*., 1993). Hence, attitudes towards cohabitation are multifaceted, influenced by cultural, familial, religious, and personal factors; while some young persons may view it positively, but others remain cautious due to perceived risks and societal norms (Pleasence *et al*., 2012; Cho *et al*., 2016). This study's findings showed that a majority of the respondents mentioned unintended pregnancy, lack of trust, financial exploitation, sexual abuse, and conflict are social problems associated with cohabitation relationships and practices among university students.

Our findings revealed a significant association between knowledge and socio-demographic factors towards cohabitation relationships and practices among university students. We found that socio-demographic factors such as gender (female), age (15–30 years) and marital status (single) were found to be associated with knowledge among university students towards cohabitation relationships and practices. This finding is in accordance with other studies that showed a substantial association between respondents' knowledge and socio-demographic factors (Pleasence *et al*., 2012; Zhang *et al*., 2022). In recent years, the knowledge of cohabitation relationships and practices has translated into various patterns of marriage and family formation, which have undergone significant transformations (Gold, 2012; Scent *et al*., 2019). Thus, cohabitation, in particular, has become a noteworthy phenomenon that has been observed as both a postponement of marriage and an alternative form of coupling among university students. For instance, in South Africa, cohabitation is predominantly seen among persons in the 20-40 years age group and its prevalence has grown by about 50% (Moore *et al*., 2013; Osuafor *et al*., 2018).

Among young unmarried women, approximately 17.9% of White women and 17.1% of African women are presently cohabiting with a partner (Gevers *et al*., 2013). Nevertheless, the respondents' attitudes was found to be associated with socio-demographic factors (gender, age, marital status, average monthly stipend, and level of study) among university students. The findings showed that more

than half of the respondents had a negative attitude towards university students living together even if they do not intend to get married, that it is advisable to live together before marriage to determine the compliance of future partners, that cohabitation is associated with some levels of negative attitudes. Studies have shown that cohabitation may be prevalent among university students who are more liberal and less religious, and they perceive it as a usual way of starting their first union and as a trial marriage (Gold, 2012; Scent *et al*., 2019).

Respondents' perceptions were found to be associated with socio-demographic factors (gender, age, marital status, average monthly stipend, and level of study) among university students. This is in line with the works of Foran *et al*. (2021) and Afifi *et al*. (2024). Thus, several studies have documented that perceptions in cohabitation relationships and practices determine how one approaches, engages in, and reacts to relationships such as the cohabitation type (Scent *et al*., 2019; Vaingankar *et al*., 2020). Perceptions of cohabitation relationships will enable one to initiate and nurture healthy relationships and also to have the idea that rejecting a cohabitation relationship or practice is not a reflection of one's lack of value as a person (Lawal *et al*., 2021; Muhinat, 2022). The findings from the intercorrelation analysis revealed that respondents' knowledge will stimulate a positive attitude and high perception towards discouraging university students from engaging in cohabitation relationships and practices (Alebiosu, 2020; NDHS, 2008).

Furthermore, this study draws from an intensive investigation of the relationship experiences, practices and values of people who are not living with a partner. It is worth mentioning that, over the past five decades, unmarried cohabitation has become widely accepted and even normative across societies in developed and developing countries, as support for cohabitation has increased among many groups, from teenagers to elders (Gold, 2012; Obikeze *et al*., 2018). In fact, most first co-residential romantic unions are cohabitations rather than marriages. Yet, while the majority of university students will enter a cohabiting union, most of such cohabiting unions do not lead to marriage, and the share of those engaging in serial cohabitation are rising (Moore *et al*., 2013). Whereas many young individuals in the university in the 20th century viewed cohabitation as a stepping stone to marriage, in the 21st century cohabitation increasingly serves as an intensive form of dating – at least at its inception (Gold, 2012; Posel *et al*., 2013).

The pathways into marriage from cohabitation differ in important ways by social class, highlighting how union formation contributes to growing levels of inequality. Hence, regardless of whether the university students are deeply involved in cohabitation relationships, several studies have shown that cohabitation unions have observed greater predisposition to relationship toxicity (Scent *et al*., 2019; Argentova *et al*., 2018). Also, the findings from the intercorrelation analysis have shown that respondents' knowledge was positively correlated with attitudes that kindle a stronger perception towards cohabitation relationships and practices. This infers that high knowledge will lead to a positive attitude and high perception towards cohabitation relationships and practices (Lawal *et al*., 2021; Muhinat, 2022). Therefore, the findings of this study further revealed that respondents' knowledge, attitude and perception of cohabitation relationships and practices are interrelated, as adequate and correct knowledge will lead to a positive attitude and high perception that may likely discourage university students from engaging in cohabitation relationships and practices (Alebiosu, 2020).

Studies have shown that the inconsistencies concerning age cohorts in awareness and approaches to cohabitation relationships and practices among university students may reflect either variations in attitudes as people mature, or modifications between groups that will be taken over time owing to the changed involvements of those born in different decades (Afifi *et al*., 2024; Manning *et al*., 2019). Other studies suggested that university students moving in together before getting involved is linked with lower marital settlement, commitment, and confidence, worse interaction, and higher odds of separation (Wu, 2000; Manning *et al*., 2019). Cohabitation continues to rise, but there is a lack of knowledge about expectations about cohabitation and the association between expectations and subsequent cohabitation. The consequence of living together ahead of marriage is attached to the predisposition for some couples to make less of a commitment to each other, or feel less pleased with their planning (Alebiosu, 2020; Brown *et al*., 2023). University students who choose to cohabit may have different expectations than their partners about the shift. This study findings underline the significance of contemplating not only just behaviour but also individuals' anticipations for recognizing development of the union and more largely, family change. Thus, cohabitation has surpassed marriage as the most common union experiences in young adulthood, as the typical relationship experience in young adulthood, with the majority having cohabited but are not yet married (Posel *et al*., 2013; Ezumah *et al*., 2021).

Several studies have mentioned in the past that cohabitation typically served as a stepping stone to marriage, but this appears to have changed (Ghosh, 2021; Perelli-Harris *et al*., 2015). Presently, cohabitation does not essentially serve as the pathway to marital life and bliss, and combined with this 'decoupling' of cohabitation and marriage, a growing proportion of young adults have stayed with several cohabiting partners (Pelikh *et al*., 2022; Akokuwebe *et al*., 2015). Although general behavioural trends (knowledge, attitude and perceptions) regarding cohabitation relationships and practices are clear, yet little is known about how university students view their relationship prospects in a climate in which cohabitation is more common than marriage. Several studies have shown that behavioural patterns of union formation are of limited utility for understanding this issue, and young university students sometimes comprise a group in which only half have entered into marriage by their late twenties (Adeoye *et al*., 2012; Kalu *et al*., 2021). Hence, concentrating on expectations is key as it offers perception into selected alternative unions. In addition, there is the possibility of growing disconnection from opportunities and interactive patterns in settings with great operational restrictions (such as economic indecision and commitment), such as a generation who came of age in the Great Recession (Gyasi-Gyamerah *et al*., 2023; Hattori *et al*., 2007).

The study offers critical insights into the evolving family dynamics among Nigerian university students. The increasing prevalence of cohabitation—driven by economic

considerations and shifting cultural aspirations (such as the desire to become a 'baby mama')—signals a notable shift away from traditional pathways to union formation, reflecting broader societal transformation. These emerging patterns carry significant implications for family demography, including potential delays in formal marriage, shifts in fertility timing, and the rise of non-traditional household structures, all of which underscore the gravity of the phenomenon. From a sociological standpoint, the observed positive relationships among knowledge, perception, and attitudes toward cohabitation indicate a growing normalization of informal unions among young adults. The study applies the theory of individualisation to elucidate how relationship decisions are increasingly shaped by personal autonomy, self-realisation, and economic pragmatism, rather than by conventional societal expectations. This theoretical lens situates the findings within the context of broader transitions in family behaviour, emphasizing a movement toward individualized life choices. The findings highlight the urgent need to develop youth-centered family policies and sociological frameworks that reflect and respond to the evolving relational norms within contemporary sub-Saharan Africa.

Strengths and Limitations of the Study

A key strength of this study lies in its use of primary data, integrating both qualitative and quantitative methods. This mixed-methods approach distinguishes it from prior studies on cohabitation among students, which have relied mainly on qualitative data alone. By examining individual-level characteristics, this study provides contextually grounded insights into a historically underrepresented population, thereby enhancing the relevance and engagement of the findings for scholarly audiences. However, several limitations warrant consideration. First, the data were drawn from a cross-sectional sample of students from a single campus within a multi-campus institution, limiting the generalizability of the findings. Due to the study's restriction to a single campus within a multi-campus university, the analysis does not support conclusions regarding temporal associations or broader institutional patterns. This awareness of potential limitations is crucial for cautious interpretation of the findings. Additionally, the sample consisted of students living off-campus due to the lack of university-provided accommodation, which introduces potential recall bias in responses. Moreover, the research team applied basic descriptive and inferential statistical methods, which limit cross-campus comparisons, particularly owing to potential inconsistencies in the recoding and renaming of variables to align with the statistical software. Lastly, the study focused exclusively on undergraduate students aged 15 to 30 years, therefore, the findings may not apply to older student populations.

This study highlights the high prevalence of cohabitation among undergraduate students at Osun State University, primarily driven by inadequate housing, interpersonal challenges, privacy concerns, and the desire for intimacy. The lack of on-campus accommodation—particularly for female students—emerges as a key structural factor encouraging cohabitation. Off-campus living arrangements further limit institutional oversight, increasing students' vulnerability to various forms of abuse and socio-emotional risks. Findings indicate that students often perceive cohabitation as a trial marriage or pathway to future relational stability. However, such relationships frequently involve unintended consequences, including unplanned pregnancies, financial exploitation, emotional trauma, and conflict. Adolescents in particular are drawn to cohabitation as a symbol of independence, yet they may lack the maturity and support systems to navigate its complexities.

This study underscores the pressing issue of the remarkably high rate of cohabitation among undergraduate students at Osun State University. The urgent need for adequate housing, interpersonal difficulties, the pursuit of privacy, and the desire for intimate companionship primarily drive this. A particularly critical factor is the lack of sufficient on-campus accommodation, especially for female students, which prompts many to enter cohabitation arrangements as a practical solution. Off-campus living further reduces institutional oversight, thereby heightening students' exposure to various forms of abuse and socio-emotional risks. The findings reveal that many students view cohabitation as a trial marriage or a stepping stone toward future relational stability. Nevertheless, these arrangements often carry unintended consequences such as unplanned pregnancies, emotional trauma, financial exploitation, and interpersonal conflict. Adolescents, in particular, may be drawn to cohabitation as a symbol of independence; however, they often lack the maturity and support systems necessary to manage the associated complexities. In light of these insights, the study advocates for a series of targeted interventions. Institutions are encouraged to integrate cohabitation education into orientation programmes to promote informed decision-making. Parents must actively monitor their children's living situations to achieve this outcome. University authorities should also prioritise the development of affordable on-campus hostels, particularly for female students, to reduce vulnerabilities related to cohabitation. Furthermore, the establishment of collaborative oversight mechanisms involving both university administrators and local community leaders could help regulate student conduct off-campus and promote safer living environments. Lastly, students must be made aware of, and encouraged to utilise, available support services—including social counselling—to navigate emotional distress and foster resilience throughout their academic journey

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Full-length Research Article

An Evaluation of the Genetic Variability of *Tilapia guineensis* Populations Based on Microsatellite Markers

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Summary: Improvement of aquaculture production, conservation and stock management of T. guineensis species are important. The genetic variation of this species, as well as its cross-breeding pattern and growth performance were assessed. One hundred and fifty live samples with average weights of TG (21.90g and 31.60g) for females and males, respectively, were collected from three different water bodies. DNA was extracted using the phenol-chloroform isoamyl alcohol method from randomly selected fish populations. The quality and quantity of DNA were determined using a spectrophotometer. Microsatellites were amplified, and PAGE electrophoresis was analyzed using PopGene version 3.6 software. The DNA concentrations ranged from 100.36 ng/l to 3889.40 ng/l with purity values of 1.69 to 2.00. The partial regions of the gene fish populations amplified were polymorphic, with an average of two allele differences in frequency. The values of gene diversity, polymorphic information content, and inbreeding coefficient were 0.41, 0.30, and -0.41, respectively. Observed heterozygosity was higher than expected heterozygosity in the fish populations. The genetic variations observed among the crossbreeds emphasized the importance of selective breeding for fast growth by increasing productivity, but pure lines should be maintained so that they form part of the baseline population and avoid inbreeding over time.

Keywords: *T. guineensis, genetic variation, cross-breeding, growth performance.*

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INTRODUCTION

Genetic tools are being used to significantly increase production in world aquaculture as well as to conserve and protect. The progress of DNA-based markers has had an innovative impact on gene mapping and, more generally, on all of animal and plant genetics. According to Nazari and Pourkazemi, (2023), different molecular tagging methods have been designed to study fish species characterization and population structure. Microsatellite, or Simple Sequence Repeats (SSR), has been among the tagging methods (Chen *et al*., 2024). The use of molecular genetic techniques such as SSR in fisheries research has increased due to the increasing availability of such techniques in research labs.

The increasing importance of fish farming has necessitated advancements in the technology required to secure the early and fundamental prerequisites for productive aquaculture, namely the production of fish seed for stocking. According to Sanda *et al*. (2024), the status of genetics in aquaculture would positively impact sustainability in the development of fish breeding programs in several developing countries, especially in Africa. Population and quantitative genetics are critical components in assessing genetic diversity or variation, which characterize naturally occurring genetic differences across individuals of the same species (Salgotra and Chauhan, 2023). According to Vieira *et al*. (2025), evaluating genetic diversity and genetic relationship base population is critical for successful control over generations, reducing problems associated with genetic potential loss for selective breeding. According to Mojekwu and Hoareau (2024), detected microsatellite loci might be recommended for characterizing *Tilapia species* in Nigerian water bodies. Such information has implications for future bloodstock selection and breeding management and for further analysis of interactions between different populations of *Tilapia species*. As a result, the goal of this study was to use microsatellite markers to describe *Tilapia guineensis* of both broodstock and crossbreed in order to improve the quantity and quality of fish seeds for hatchery operations for aquaculture purposes.

MATERIALS AND METHODS

Sample Locations: Live fish samples (broodstocks) (50 fish samples from each location) were collected from three locations in Southwestern Nigeria, namely, Ondo (On), Oyan (Oy), and Lagos (La) Lagoons. The broodstocks were collected from Mahin (Ondo) (O) Lagoon, Oyan Lake (Oy), and Lagos Lagoons (L). Ondo Lagoon with coordinates N 5055′05″ and E40590′ 2″; N ′ and E3015′20′ and N6029′ 24″ and E3023′58″, respectively (Fig. 1).

Collection and Paring of Broodstocks: The broodstocks were collected with oxygenated double nylon and transported to Badore NIOMR, Lagos, and acclimated for 2 weeks. The average weight of females and males of 21.9g and 31.6 g, respectively, were paired (each strain of the locations was crossed together) in a ratio of 1:2 in the triplicate: pure strains (La x La; On x On; Oy x Oy) and crossed breeds (La x On; Oy x On; La x Oy), respectively. The spawning and hatching periods occurred. After the fry's absorption of yoke, the fry was transferred into a white tank for culturing; they were fed with 45% COPPENS FEED intensively for 4 weeks.

DNA extraction: A total of 180 fish samples (DNA) were extracted from the caudal fin tissue of both the broodstock and randomly selected offspring (F1 generation) using a modified chlorophenol / isoamyl / alcohol protocol according to Sambrook and Russell (2001) on the bench at the biotechnology laboratory of NIOMR, Badore Outstation, Nigeria. Electrophoresis on 0.8% ethidium bromide-stained agarose gel (Plates 1a and 1b) was used to assess the integrity of the isolated DNA. A nano-drop spectrophotometer (Shimadzu Corporation Japan, MODEL UV-1800, 2000 series) at an absorbance of 260/280nm was used to check the concentration of the DNA. It was diluted to a final concentration of 50–100 ng/µl in highly purified water for amplification. The isolate was stored at -20°C prior to PCR amplification.

PCR Amplification: Nine microsatellite primers (Ukenye *et al*; 2022), were used to characterize and investigate the genetic variation between and among broodstocks from different locations, as well as the first filial generation (F1) offspring from cross-breeding. The polymerase chain reaction was carried out on a gradient thermal cycler (Biorad, module 10-8731) to determine the annealing temperature of each primer, which ranged from 55oC to 65oC (Table 1).

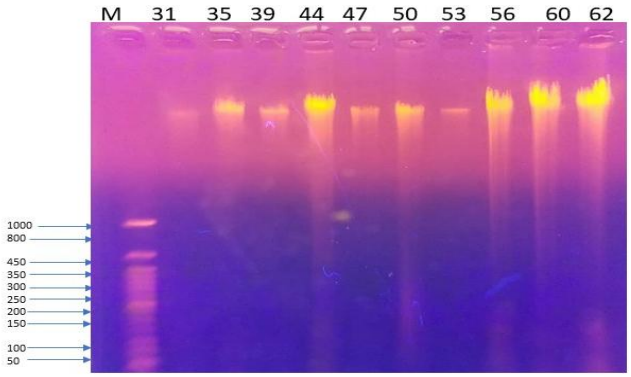

Plate 1a:
Agarose Gel Electrophoresis from randomly selected DNA of *T. guineensis.*

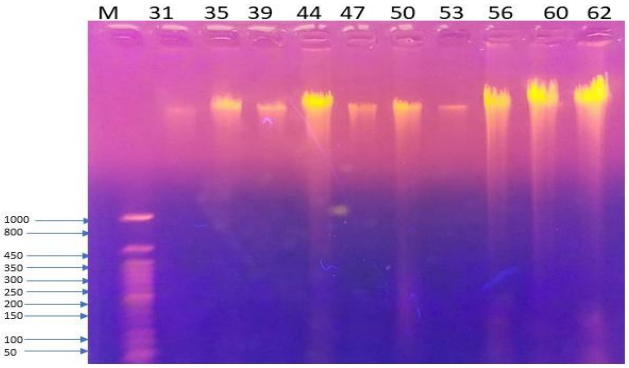

Plate 1b:
Agarose Gel Electrophoresis from randomly selected DNA of *T. guineensis.*

Table 1:
SSR Primer Code, Sequence, Annealing Temperature and Band Size

Primer code	Sequence	Annealing Temperature (ºC)	Molecular size (bp)
GM 211	Forward 5' GCAAGTTGAGAGGCTACTGT 3'	60	178–398
	Reverse 3' AAACAACCCACAACCTTAGTT 3'		
UNH 995	Forward 5' CATCATCACAGAGACAGATTAGA 3'	55	180-350
	Reverse 3 ' GCAGCACAACCACAGTGCTA 3'		
UNH 123	Forward 5' CATCATCACAGACAGATTAGA 3'	55	145-208
	Reverse 3' GATTGAGATTTCATTCAAG 3'		
UNH 207	Forward 5' ACACAACAAGCAGATGGAGAC 3'	55	140-220
	Reverse 3' CAGGTGTGCAAAGCAGAAGC 3'		
UNH 146	Forward 5' CCACTCTGCCTGCCCTCTAT 3'	55	130-300
	Reverse 3' AGCTGCGTCAAACTCTCAAAAG 3'		
GM 538	Forward 5' CAGCATGTTGTCTGGATCTTG 3'	60	140-300
	Reverse 3' TTTGTTGCTGTGGTCTGTTCTT 3'		
GM 531	Forward 5' AAAGCCAACGGTCTGAATTG 3'	55	140-190
	Reverse 3'AGCAGAGGACACCCCTCAT 3'		
UNH 104	Forward 5' GCAGTTATTTGTGGTCACTA 3'	55	170-250
	Reverse 3' GGTATATGTCTAACTGAAATCC 3'		
UNH 185	Forward 5' CAGACACACTAGACACATTCTA 3'	55	120-150
	Reverse 3' GTGTTTCCATGTGTCTGTAC 3'		

Lee *et al.* (2005) and Saad *et al.* (2013)

Each PCR tube received a total volume of 25µl of PCR components, which included 2.5µl of 10 x PCR buffer, 1µl of 25mM MgCl2, 1µl of each primer (forward and reverse), 1µl of DMSO, 2µl of 2.5mM DMSO, 0.1µl of 5u/ul Taq DNA polymerase, 3µl of 10ng/l of DNA, and 13.4µl of nuclease-free water. The ingredients were run on a thermocycler (Biorad, module 170-8731) for amplification. The PCR condition is: 96oC for 2 minutes (initial denaturation); 30 cycles of 94oC for 30 seconds (denaturation); 55oC (optimal temperature of each primer, which varies for each of the primers) at 30 seconds; 72oC for 30 seconds, followed by a final extension of 72oC for 6 minutes.

Polyacrylamide gel electrophoresis: 6% of polyacrylamide gel was prepared by mixing 7.5ml of acrylamide (instant page buffer), 2.5ml of TBE buffer (Tris-borate/EDTA electrophoresis buffer), 50µl of TEMED, and 500µl of ammonium per sulphate (APS) in 40ml of distilled water, giving a total volume of 50ml. A total 8µl volume of solutions, which consist 6µl of PCR product and 2µl of 6x loading dye, were loaded into the wells created by the combs. A 1 kb base pair (bp) ladder was used as the size standard, which was loaded alongside the PCR products on the gel. The gels were then viewed with a UV transilluminator (SpectrolineR TC 312 E), and the pictures were taken using a camera (Alpha Imager) (Plate).

Statistical Analysis: Population genetics data generated was analyzed using PopGene version 3.6 software to obtain the number of alleles per Simple Sequence Repeat (SSR) locus (Na), effective number of alleles (Ne), Shannon information index (I), observed heterozygosity (Ho), expected heterozygosity (He), and Nei's Pairwise genetic distance.

RESULTS

DNA purity quantification: Concentration and purity values are shown in Table 2, with mean concentration values ranging from 100.36 ng/l to 3889.40 ng/l and purity values ranging from 1.69 to 2.00 using a nano-spectrophotometer.

Genetic Variability of *Tilapia guineensis* among Microsatellite Loci: A total of 18 alleles were found in the study of 9 populations of both *Tilapia guineensis* (broodstocks, pure lines, and crosses). The mean average number of alleles per locus generated by each marker was 2, with variations in the frequency of alleles. Table 3 shows the allelic diversity of the coding region of *Tilapia guineensis* with the same alleles as two (2). Locus UNH 146 had the highest number of allele frequencies (0.95), while Locus UNH 185 had the lowest allele frequency (0.45). The level of diversity revealed by the studied loci ranged from 0.04 to 0.50, with an average of 0.39. The PIC value of each primer is evaluated on the basis of its alleles. The value for *Tilapia guineensis* varied greatly for all the tested Simple Sequence Repeat (SSR) loci, from 0.04 to 0.38 with an average of 0.30 (Table 4). The highest PIC value of 0.38 was obtained for UNH 123 and UNH 104, followed by UNH 185 and UNH 211 (0.37), UNH 995 (0.36), GM538 (0.31), UNH 146 (0.30), UNH 207 (0.20), and GM 531 (0.04), respectively, with an average value of 0.30 of major allele frequency.

Table 2:
DNA concentration (ng/µl) and ratio of absorbance (260/280nm) of DNA extracted from fish population broodstocks, pure lines and crossbreed lines of *T. guineensis*

Sample source	Species	Concentration (ng/l)	Purity	Mean Purity(X^{-1})	Mean ±SD
Lagos Broodstock	*T. guineensis*	830.87 - 2036.24	1.76 - 1.93	1.88	1.88±0.06
Lagos (Pure line)	*T. guineensis*	100.36 - 2459.85	1.57 - 1.93	1.85	1.85±0.10
Lagos X Ondo (Cross breed)	*T. guineensis*	573.79 - 1666.40	1.70 - 1.96	1.69	1.69±0.27
Ondo Broodstock	*T. guineensis*	1319.40-3889.40	1.75 - 1.91	1.86	1.86±0.05
Ondo (Pure line)	*T. guineensis*	873.02-1031	1.50 - 1.95	1.88	1.88±0.98
Lagos X Oyan (Cross breed)	*T. guineensis*	704.67-835.93	1.68-1.98	2.00	2.00±0.11
Oyan Broodstock	*T. guineensis*	672.04-935.30	1.89 – 2.02	1.94	1.94±0.58
Oyan (Pure line)	*T. guineensis*	830.52-949.30	1.65 – 2.03	1.87	1,87±0.54
Oyan X Ondo (Cross breed)	*T.guineensis*	546.20-635.29	1.78 – 1.95	1.83	1.83±0.36

Table 3:
Characteristics of SSR loci analyzed for *Tilapia guineensis*

Maker	Freq	Sample size	NA	Gene Diversity	PIC
GM 538	0.88	180	2	0.38	0.31
GM 531	0.85	180	2	0.40	0.04
UNH 104	0.69	180	2	0.50	0.38
UNH 123	0.86	180	2	0.49	0.38
UNH 185	0.45	180	2	0.23	0.37
UNH 207	0.91	180	2	0.49	0.20
UNH 211	0.68	180	2	0.38	0.37
UNH 146	0.95	180	2	0.47	0.30
UNH 995	0.88	180	2	0.39	0.36
Means	**0.79**	**180**	**2**	**0.41**	**0.30**

Legend: **Freq** -Major allele frequency, **NA** – Number of alleles, **PIC** – Polymorphic Information content

Table 4:
Locus Specific Indices of Genetic Diversity in the *Tilapia guineensis* Population

Locus	Na	Fis	Heterozygosity	Fit	Inbreeding coefficient	Fst	D
GM 538	2	0.061	0.311	0.181	0.185	0.127	-0.59
GM 531	2	-0.049	0.040	-0.020	-0.017	0.027	-0.59
UNH 104	2	-1.000	1.000	-1.000	-1.000	0.000	-0.48
UNH 123	2	-0.988	0.994	-0.987	-0.988	0.000	-0.60
UNH 185	2	-0.336	0.506	-0.030	-0.019	0.244	-0.59
UNH 207	2	-0.378	0.264	-0.150	-0.150	0.165	-0.62
UNH 211	2	-0.868	0.916	-0.850	-0.844	0.025	-0.40
UNH 146	2	-0.393	0.466	-0.249	-0.241	0.104	-0.48
UNH 995	2	-0.278	0.426	-0.075	-0.086	0.276	-0.63
Mean	**2**	**1.63**	**0.387**	**0.65**	**-0.412**	**0.086**	**-0.55**

Na - Number of alleles, **Ne** – effective number of alleles, **Fit** – Maker Fitness, **Fst**- Maker Fixation Index, **Fis**- Inbreeding co-efficient of individual and **D**- Heterozygote deficiency calculated as D = (Ho-He)/He. **Ho** – Observed heterozygosity and **He**- Expected heterozygosity

DISCUSSION

The used of the phenol-chloroform isoamyl alcohol protocol for the extraction of the DNA, which proved to be good for obtaining pure DNA from the fish caudal fin. This finding is in agreement with the results obtained in a comparative study of an improved method of DNA extraction from fish fins and fish scales (Tayyab, 2021), in which repeated DNA phenol chloroform isoamyl alcohol showed no sign of degradation and spectrophotometer absorbance at 260/280 indicating a good DNA template for PCR analysis.

In the present study, nine (9) microsatellite markers were utilized to characterize and investigate the genetic variation in *Tilapia guineensis* populations for both broodstocks and their crosses, with the aim of having information in the base population for crossbreeding, minimizing inbreeding, and improving the seed quality among the sample's populations for breeding and conservation programs (Kumari *et al*; 2025)..

This study revealed an average Polymorphism information Content (PIC) which suggested that the makers are moderately informative, indicating that they have good merit to distinguish different *Tilapia species*. This is in line with the report of Marques, *et al*. (2023), which stated that the PIC values ranged from 0.25 to 0.50, which is somewhat informative.

The number of alleles in these fish populations studied shows the variation in the number of alleles across multiple loci within the populations. This is similar to the study conducted by Amoussou *et al*. (2025), in which the number of alleles ranges from 2 to 4 in the same locus positions on the chromosomes of Nile Tilapia (Oreochromis niloticus) in Volta Lake, Ghana, using microsatellite makers.

A total of 18 alleles were also observed in this study on fish populations, which is incomparable with the report of Sadler *et al*. (2023), who reported 75 allele frequencies in Oreochromis niloticus populations. The low gene variation encountered in this study may suggest there is a need for an increase in the diversity of these species through selective breeding and conservation programs.

In qualitative terms, a marker is considered polymorphic if it has at least two alleles, and the most frequent allele has a frequency range of 50% to 99% (Rezk *et al*., 2024). This is in accordance with this study, in which the average means allele frequencies of SSR markers used for *Tilapia guineensis* (81%). This finding suggests that there is a relatively high percentage of allele frequency among the fish populations, which can lead to genetic drift or new mutations within the populations.

The average means of gene diversity and inbreeding coefficient of the populations using microsatellite markers is relatively informative when compared with other markers such as ISSR (Hesamzadeh Hejazi., 2024). The gene diversity based on locus specifies that there is genetic differentiation between populations; this implies that there are a range of different inherited traits within a species. This is in agreement with the results obtained by Ekerette *et al*. (2024) on genetic variations of Oreochromis niloticus.

In this present study, the fish populations showed negative values of inbreeding co-efficient, which implies excess heterozygosity among the populations in disparity to what is expected in the intensive production of selective growth traits, which can lead to homozygosity among species. This is in accordance with the research findings of Böhne *et al*. (2023), who observed excess heterozygosity in a natural population of the West African cichlid fish Pelvicachromis taeniatus, which showed clear kin mating in experiments but no inbreeding depression. It is nevertheless not in agreement with Ukenye *et al*. (2022), in which some of the loci showed positive inbreeding co-efficient among *Tilapia guineensis* populations in some coastal water in Nigeria.

This present study deviated from the Hardy-Weinberg equilibrium principle, in which there are differences in genotype and allele frequency among the fish populations (Singh *et al*; 2024). These differences could be the result of forces such as mutations, natural selection, non-random mating, genetic drift, and gene flows. Therefore, microsatellite variation at most estimated loci was more informative in characterizing *Tilapia species* differences than the ISSR markers (Chang *et al*; 2024).

In conclusion, these results will provide useful information for genetic variations of T. guineensis and therefore provide the needed genetic information for effective decision-making toward the management of these fishes for improvement on aquacultural purposes. This information may be needed for effective management strategies by the government to plan toward managing the fisheries resources.

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Full-Length Research Article

Species- and Tissue-Specific Variations in Cholesterol Content among Wild and Domesticated Birds in Nsukka, Nigeria

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Summary: Avian species exhibit variations in adaptive features, including differences in lipid metabolism, which are essential for successful survival and reproduction in unique habitats. However, there is a lack of information on species- and tissue-specific variations in cholesterol metabolism among avian species living in similar habitats. This study aimed to investigate potential variations in tissue cholesterol levels in adult males of the wild, white-breasted crow (*Corvus albus*, Passeriformes) and the local and exotic (broiler) breeds of domestic chicken (*Gallus gallus domesticus*, Galliformes) avian species found in Nsukka, Nigeria. These species have diverse diets and behaviours. Tissues from the brain, liver, gizzard, heart, and breast muscle were dissected and used for total lipid extraction and cholesterol quantification. The results showed significant species- and tissue-specific differences in cholesterol levels among the examined birds ($p < 0.001$). Moreover, the brain and liver had notably higher cholesterol content compared to other tissues ($p < 0.001$ in most cases). Lastly, multiple comparisons showed that domesticated species generally exhibited higher cholesterol levels than wild species. These findings support the hypothesis of species- and tissue-specific differences in cholesterol metabolism in avian species inhabiting similar environments but differing in diets and behaviours.

Keywords: *birds; cholesterol; diet; lipid metabolism; tissues*

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INTRODUCTION

Species survival and reproductive success require the development of unique adaptive characteristics to secure optimal shelter, food, defend territories, evade predators, find mating partners, and ultimately produce offspring for the next generation (Bevan *et al*., 1997; Broggi *et al*., 2011; Birn-Jeffery *et al*., 2012; Blix *et al*., 2016; Furness *et al*., 2022; Kerschbaumer *et al*., 2023; Pap *et al*., 2024). However, adaptive features vary depending on species, varieties, strains, diet, habitat, sex, and the presence, type or absence of predators. While morphological adaptations are often discussed, biochemical and physiological adaptations, including the incorporation of necessary lipids, play an even greater role in successful environmental adaptations.

Cholesterol is a crucial lipid found in living organisms, serving as a key component of cellular and nuclear membranes. It helps maintain the fluidity of the phospholipid bilayer, regulating the transport of nutrients, gases, ions, synthesized molecules, and water (Bastiaanse *et al*., 1997; Zhang *et al*., 2018; Frangos *et al*., 2023). Additionally, cholesterol plays a vital role in the myelin sheath and Schwann cells surrounding neuronal axons, aiding in the conduction of nerve signals (Mouritsen & Zuckermann, 2004; Poitelon *et al*., 2020; Barnes-Vélez *et al*., 2022). The vertebrate liver synthesizes and stores over 70% of the body's cholesterol, with the remaining 30% being produced in other cells or obtained from the diet (Jeske & Dietschy, 1980). Cholesterol synthesized in the liver is transported in the blood as low-density lipoprotein-cholesterol (LDL-C) to other organs for various functions, such as membrane incorporation, bile acid synthesis, and hormone production (Gustafsson *et al*., 1977; Rone *et al*., 2009; Miller & Bose, 2011; Ikonen & Zhou, 2021).

In contrast, the brain synthesizes its own cholesterol and there is no evidence to suggest that plasma LDL-borne cholesterol can cross the blood-brain barrier (Björkhem & Meaney, 2004). Tissue cholesterol content varies based on species, diet, tissue type, habitat, and sex (Lorenz *et al*., 1938; Musacchia, 1953; Mancinelli *et al*., 2022; Teekell *et al*., 1974; Al-ruwaili *et al*., 2014; Punam *et al*., 2024; Palmisano *et al*., 2018; Robinson *et al*., 2021; Conlon *et al*., 2023; Gould *et al*., 1953; Tomkins *et al*., 1953; Frantz *et al*., 1954; Jeske & Dietschy, 1980). For example, it has been shown that temperate mammals and birds have higher cholesterol levels compared to their tropical counterparts (Calhoon *et al*., 2014). Despite these findings, a comprehensive comparative study on tissue cholesterol composition among avian species with similar habitats but differing diets, behaviors, and breeding cycles is lacking. Such research could provide valuable insights into species- and tissue-specific cholesterol metabolism.

To address this gap, we conducted a study, quantifying cholesterol levels in the brain, liver, breast muscle, heart, and gizzard of adult males from three avian species: local and exotic breeds of domestic chicken (*Gallus gallus domesticus*) and the white-breasted crows (*Corvus albus*). These birds were chosen for their genetic, dietary, and microhabitat differences. We hypothesized that birds inhabiting different microhabitats and with varied diets would have different intra- and inter-tissue cholesterol levels.

MATERIALS AND METHODS

Birds: A total of twenty (20) adult male birds (n=5/species/breed) were used for this study. The local and exotic (broiler) breeds of domestic chicken (*Gallus gallus domesticus*) were purchased from breeders at a local market, while the white-breasted crow (*Corvus albus*) was trapped by experienced local hunters using baited traps that inflicted no physical harm on the birds. The presence of external morphological features such as the comb in domestic chickens and the presence of testes upon dissection were used to confirm sexual maturity in male birds. Female birds were excluded from this study to prevent potential confounding effects of sex hormones on the data generated. Birds were sampled during their respective breeding seasons when mature males were more likely to have pronounced testis and other external sexual characteristics, which enhanced sex identification.

Tissue Extraction: Each bird was humanely euthanized with an overdose intramuscular injection of pentobarbital sodium. Once under permanent anesthesia (confirmed when there were no signs of breathing and the bird became unresponsive to a strong toe pinch), birds were pinned to a dissecting board, defeathered around the chest and abdomen, and samples of the liver, brain, heart, gizzard, and breast muscle were quickly dissected. To extract the brain, an incision was made in the skin to expose the skull, followed by incisions in the midline of the skull and then sideways through the top of the eye sockets to the base of the beak. Each half of the skull bone was gently pulled apart to expose the brain; the optic chiasma was cut to release the brain. All brain samples were washed thoroughly in 0.1 M PBS (pH 7.4), dried with filter paper.

Extraction of total lipids: Total lipids were extracted following the method by Folch *et al*. (1957). Each tissue was first dissected into a tissue blender and blended until a uniform homogenate was achieved, and for every 10 g of tissues, 10 mL of a chloroform-methanol solution (2:1, v/v) was added to the blender, mixed thoroughly, and transferred to a test tube. The blender was rinsed with an additional 10 mL of chloroform-methanol solution, and the rinse was transferred to the same test tube. The test tube was tightly sealed, shaken for 10 minutes, and left at room temperature for 45 minutes. The contents of the tube were filtered through filter paper into a test tube. Then, 0.2% CaCl2 (0.2 times the volume of the filtrate) was added to the filtrate, homogenized, and allowed to stand until two layers formed. The upper layer was aspirated and discarded, while the lower lipid-containing layer was preserved for quantification of total cholesterol.

Quantification of total cholesterol: Before quantifying total cholesterol in each sample, the lipid sample was dissolved in 2 mL of chloroform. The analytical method, which employed the Liebermann-Burchard reagent as described by Kim and Goldberg (1969), was used to quantify total cholesterol concentrations from extracted lipid samples. To quantify total cholesterol, 3 mL of Liebermann-Burchard reagent was added to 0.2 mL of each sample, gently mixed, and left in the dark until a blue-green coloration developed. For the blank, 3 mL of the Liebermann-Buchard reagent was added to 0.2 mL of chloroform. A standard solution containing 0.4 mg of cholesterol was also prepared. The blank was used to calibrate the spectrophotometer, and the optical density measured at 550 nm.

Statistical analysis: To assess if different avian species found in Nsukka regulate tissue cholesterol differently, One-way analysis of variance was used to compare intra- and inter-tissue total cholesterol among birds (SPSS, version 18.0 for Windows, IBM Statistics). Post hoc tests, Duncan and LSD, were used to identify significant differences in intra-species differences and inter-species differences in tissue cholesterol levels. Statistical significance was considered as $p \leq 0.05$ and the results were presented as mean ± standard error of the mean.

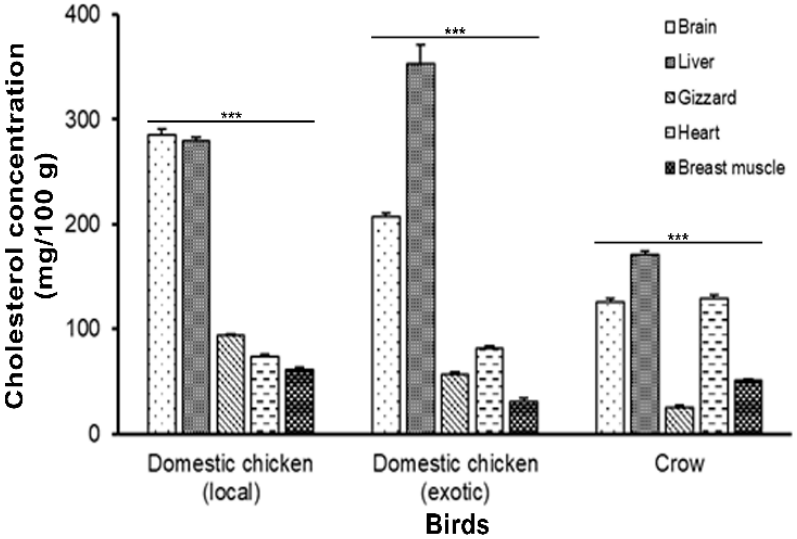

Figure 1:
Intra-species variations in tissue cholesterol content among adult in male avian species found in Nsukka. Plotted values are Mean ± SEM. Error bars: SEM. Top right: Legends. Bars with distinct patterns represent cholesterols in different tissues in a bird. Asterisks represent significant level (n = 5 tissues/species, One-way ANOVA, $p \leq 0.001$).

The amount of cholesterol varies in different birds and their tissues

RESULTS

Intra-specific variations in tissue cholesterol contents: To assess potential intra-specific variations in tissue cholesterol metabolism, we analysed the mean differences in tissue cholesterol levels in three avian species found in Nsukka, Nigeria. The results show that there were significant differences in tissue cholesterol levels in all birds examined (Figure 1).

Table 1:
Multiple comparisons of intra-species differences in tissue cholesterol levels.

Dependent variable	(i) Tissues	(j)Tissues	Mean difference (i-j)	SEM	Sig.	Lower bound	Upper bound
Crow	Brain	Gizzard	100.74*	3.96	<0.001	92.48	109.00
		Heart	-4.15ns	3.96	0.307	-12.41	4.11
		Liver	-45.24*	3.96	0.001	-53.50	-36.98
		Breast muscle	74.54*	3.96	0.001	66.28	82.80
	Gizzard	Brain	-100.74*	3.96	0.001	-109.00	-92.48
		Heart	-104.89*	3.96	0.001	-113.15	-96.63
		Liver	-145.99*	3.96	0.001	-154.25	-137.73
		Breast muscle	-26.20*	3.96	0.001	-34.46	-17.94
	Heart	Brain	4.15 ns	3.96	0.307	-4.11	12.41
		Gizzard	104.89*	3.96	0.001	96.63	113.15
		Liver	-41.10*	3.96	0.001	-49.36	-32.84
		Breast muscle	78.69*	3.96	0.001	70.43	86.95
	Liver	Brain	45.24*	3.96	0.001	36.98	53.50
		Gizzard	145.99*	3.96	0.001	137.73	154.25
		Heart	41.10*	3.96	0.001	32.84	49.36
		Breast muscle	119.78*	3.96	0.001	111.52	128.04
	Breast muscle	Brain	-74.54*	3.96	0.001	-82.80	-66.28
		Gizzard	26.20*	3.96	0.001	17.94	34.46
		Heart	-78.69*	3.96	0.001	-86.95	-70.43
		Liver	-119.03*	3.96	0.001	-128.04	-111.52
Domestic chicken (local)	Brain	Gizzard	191.03*	5.15	0.001	180.30	201.77
		Heart	211.25*	5.15	0.001	200.51	221.99
		Liver	4.9 ns	5.15	0.353	-5.84	15.64
		Breast muscle	223.24*	5.15	0.001	212.50	233.98
	Gizzard	Brain	-191.03*	5.15	0.001	-201.77	-180.29
		Heart	20.21*	5.15	0.001	9.47	30.95
		Liver	-186.13*	5.15	0.001	-196.87	-175.39
		Breast muscle	32.20*	5.15	0.001	21.46	42.94
	Heart	Brain	-211.25*	5.15	0.001	-221.99	-200.51
		Gizzard	-20.21*	5.15	0.001	-30.95	-9.47
		Liver	-206.35*	5.15	0.001	-217.09	-195.61
		Breast muscle	11.99*	5.15	0.030	1.25	22.73
	Liver	Brain	-4.90 ns	5.15	0.353	-15.64	5.84
		Gizzard	186.13*	5.15	0.001	175.35	196.87
		Heart	206.35*	5.15	0.001	195.61	217.09
		Breast muscle	218.34*	5.15	0.001	207.6	229.08
	Breast muscle	Brain	-223.24*	5.15	0.001	-233.98	-212.50
		Gizzard	-32.20*	5.15	0.001	-42.94	-21.46
		Heart	-11.99*	5.15	0.030	-22.73	-1.25
		Liver	-218.34*	5.15	0.001	-229.08	-207.60
Domestic chicken (Broiler)	Brain	Gizzard	151.09*	11.93	0.001	126.21	175.96
		Heart	126.28*	11.93	0.001	101.41	151.16
		Liver	-145.44*	11.93	0.001	-170.32	-120.56
		Breast muscle	176.60*	11.93	0.001	151.72	201.47
	Gizzard	Brain	-151.09*	11.93	0.001	-175.96	-126.21
		Heart	-24.80 ns	11.93	0.051	-49.68	0.07
		Liver	-296.53*	11.93	0.001	-321.41	-271.65
		Breast muscle	25.51*	11.93	0.045	0.64	50.39
	Heart	Brain	-126.28*	11.93	0.001	-151.16	-101.41
		Gizzard	24.80 ns	11.93	0.051	-0.72	49.68
		Liver	-271.73*	11.93	0.001	-296.60	-246.85
		Breast muscle	50.32*	11.93	0.001	25.44	75.19
	Liver	Brain	145.44*	11.93	0.001	120.57	170.32
		Gizzard	296.53*	11.93	0.001	271.65	321.41
		Heart	271.73*	11.93	0.001	246.85	296.60
		Breast muscle	322.04*	11.93	0.001	297.17	346.92
	Breast muscle	Brain	-176.60*	11.93	0.001	-201.47	-151.72
		Gizzard	-25.51*	11.93	0.045	-50.39	-0.64
		Heart	-50.32*	11.93	0.001	-75.19	-25.44
		Liver	-322.04*	11.93	0.001	-346.92	-297.17

Note: Values are expressed as mean ± SEM (n = 5 birds/species, One-way ANOVA, followed by LSD separation). *: significant, ns: Not significant.

Additionally, the brain and liver maintained significantly higher cholesterol levels compared to other tissues in all species studied, except for the crow that had heart cholesterol levels similar to the brain (129.52±3.32 mg/100 g in the heart vs 125.37±3.40 mg/100 g in the brain). For instance, there was a cholesterol concentration of 125.37±3.4 mg/100 g in the crow brain; 207.53±3.33 mg/100 g in the broiler; and 284.66±6.60 mg/100 g in the local chicken On the other hand, the gizzard (24.63±2.18 mg/100 g in the crow; 56.44±2.63 mg/100 g in the broiler; and 93.63±2.04 mg/100 g in the local chicken) and breast muscle (30.93±3.02 mg/100 g in the broiler; 50.83±1.37 mg/100 g in the crow; and 41.43±2.04 mg/100 g in the local domestic chicken) had the least amount of cholesterol compared to other tissues, except for the domestic chicken gizzard that had a higher cholesterol content (93.63±2.04 mg/100 g) compared to the heart (73.42±2.53 mg/100 g) and breast muscles (61.43±2.04 mg/100 g). The multiple species-specific comparisons of cholesterol levels revealed significant differences between most tissues, except for the brain vs heart in the crow (Table 1, p = 0.307), brain vs liver in the domestic chicken (p = 0.353), and heart vs gizzard in the exotic breed (broiler) (p = 0.051), which were not significantly different.

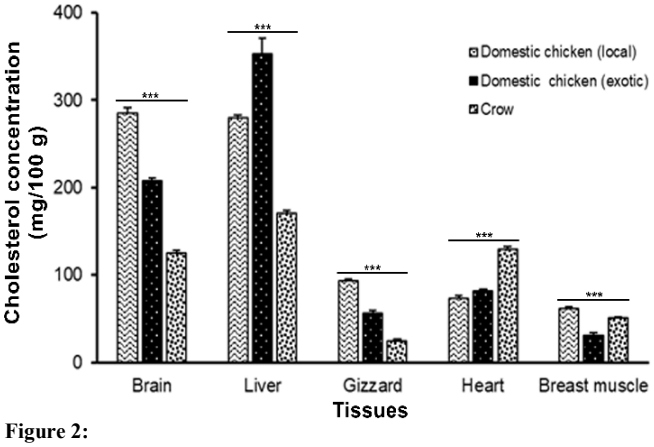

Figure 2:
Inter-species differences in tissue cholesterol levels in a specific tissue in Nsukka.
Plotted values are Mean ± SEM. **Error bars**: SEM. **Top right**: Legends. Bars with different patterns represent different species for a particular tissue. Asterisks represent significant level (n = 5 birds/species, One-way ANOVA, $p \leq 0.001$).

Table 2:
Multiple comparisons of inter-species variations in tissue cholesterol contents.

Dependent variable	(I) Species	(J) Species	Mean difference (I-J)	SEM	Sig.	Lower bound	Upper bound
Brain	Crow	Domestic chicken (local)	-159.29*	6.64	<0.001	-173.77	-144.81
		Domestic chicken (Broiler)	-82.16*	6.64	<0.001	-96.64	-67.67
	Domestic chicken (local)	Crow	159.29*	6.64	<0.001	144.81	173.77
		Domestic chicken (Broiler)	77.13*	6.64	<0.001	62.65	91.62
	Domestic chicken (Broiler)	Crow	82.16*	6.64	<0.001	67.67	-62.65
		Domestic chicken (local)	-77.13*	6.64	<0.001	-91.62	-61.92
		Domestic chicken (Broiler)	-69.00*	6.64	<0.001	-76.08	-24.73
Liver	Crow	Domestic chicken (local)	-109.15*	15.08	<0.001	-142.01	-76.28
		Domestic chicken (Broiler)	182.36*	15.08	<0.001	-215.22	-149.49
	Domestic chicken (local)	Crow	109.15*	15.08	<0.001	76.28	142.01
		Domestic chicken (Broiler)	-73.21*	15.08	<0.001	-106.08	-40.34
	Domestic chicken (Broiler)	Crow	182.36*	15.08	<0.001	149.49	215.22
		Domestic chicken (local)	73.21*	15.08	<0.001	40.34	106.08
Gizzard	Crow	Domestic chicken (local)	-69.00*	3.25	<0.001	-76.03	-61.92
		Domestic chicken (Broiler)	-31.81*	3.25	<0.001	-38.90	-24.73
	Domestic chicken (local)	Crow	69.00*	3.25	<0.001	61.92	76.08
		Domestic chicken (Broiler)	37.19*	3.25	<0.001	30.11	44.27
	Domestic chicken (Broiler)	Crow	31.81*	3.25	<0.001	24.73	38.90
		Domestic chicken (local)	-37.19*	3.25	<0.001	-44.27	-30.11
Heart	Crow	Domestic chicken (local)	56.10*	3.87	<0.001	47.67	64.54
		Domestic chicken (Broiler)	48.27*	3.87	<0.001	39.84	56.71
	Domestic chicken (local)	Crow	-56.10*	3.87	<0.001	-64.54	-47.67
		Domestic chicken (Broiler)	-7.83	3.87	0.066	-16.26	0.60
	Domestic chicken (Broiler)	Crow	-48.27*	3.87	<0.001	-56.71	-39.84
		Domestic chicken (local)	7.83	3.87	0.066	-0.60	16.26
Breast muscle	Crow	Domestic chicken (local)	-10.59*	3.17	0.006	-17.51	-3.68
		Domestic chicken (Broiler)	19.90*	3.17	<0.001	12.99	26.82
	Domestic chicken (local)	Crow	10.59*	3.17	0.006	3.68	17.51
		Domestic chicken (Broiler)	30.50*	3.17	<0.001	23.58	37.41
	Domestic chicken (Broiler)	Crow	-19.90*	3.17	<0.001	-26.82	-12.99
		Domestic chicken (local)	-30.50*	3.17	<0.001	-37.41	-23.58

Note: Values are expressed as mean ± SEM (n = 5 birds/species, One-way ANOVA, followed by LSD separation). *: significant, ns: Not significant.

Inter-specific variations in tissue cholesterol levels: To evaluate the possibility that avian species inhabiting similar environments, differing in diets and behaviours regulated cholesterol metabolism in the same tissue differently, this compared cholesterol levels in each of the brain, liver, heart gizzard, and breast muscle among the crow, domestic local and exotic chickens. The results show significant difference in tissue-specific cholesterol levels among the species examined (Figure 2, n = 5 birds/species, One-way ANOVA, $p < 0.001$ for all comparisons), with the local and exotic chickens having higher cholesterol levels in all tissues except in the heart where the crow had significantly higher cholesterol levels (129.52±3.32 mg/100 g in the crow heart, one-way ANOVA, $p < 0.001$). For instance, the brain cholesterol concentration was 284.66±6.6 mg/100 g in the local chicken; 207.53±3.33 mg/100 gin the broiler; and 125.37±3.40 mg/100 g in the crow, whereas the liver cholesterol concentration was 352.97±17.98 mg/100 g in the broiler; 279.76±2.82 mg/100 g in the local chicken; and 170.62±3.14 mg/100 g in the crow. On the other hand, the lowest cholesterol concentrations were found in the breast muscles (30.93±3.02 mg/100 g in the broiler; 50.83±1.37 mg/100 g in the crow, and 61.43±2.04 mg/100 g in the local domestic chicken). Additionally, except for the higher gizzard cholesterol level in the local domestic chicken, the birds examined had comparable gizzard cholesterol concentration to levels found in the breast muscle. The multiple comparisons of inter-specific variations in tissue cholesterol contents revealed significant differences for all pairs of tissues compared except for the broiler vs domestic comparison in the heart (Table 2, $p = 0.066$).

DISCUSSION

This study investigated the possibility that tissue cholesterol content varied within and among avian species that differ in diet, behaviour and micro-habitat preferences. Analysis of the tissue cholesterol content in three avian species revealed both significant intra- and inter-species differences. Liver and brain tissues recorded relatively higher cholesterol levels compared to the heart, gizzard, and breast muscle in all the species examined. Furthermore, we reported that the two breeds of domestic chickens exhibited higher cholesterol levels in all examined tissues relative to other species. Lastly, the exotic breed had higher cholesterol levels in all tissues compared with the local breed of the domestic chicken.

Although we recorded intra- and inter-species differences in brain and liver cholesterol levels like previous studies, the observed cholesterol levels appeared to be higher than previous reports in various chicken strains. For instance, whereas we recorded liver cholesterol levels of 174.21±4.22, 170.62 ± 3.15, 279.76±2.82, and 352.97±17.97 for the quail, crow, local domestic chicken, and the exotic chicken respectively, Konjufca *et al*. (1997) reported liver and muscle cholesterol levels of 143 mg/100g and 46 mg/100g in males of the fast-growing Ross 208 broiler strain raised on a corn and soybean-based diet. On the other hand, Al-ruwaili *et al*. (2014) found strain-specific differences in thigh, breast, and liver cholesterol contents of four broiler strains including Ross. However, the liver cholesterol level recorded by Al-ruwaili and colleagues was greater than that recorded by Konjufca *et al*. (1997) in the same strain (160.37 vs 143 mg/100g), suggesting that tissue cholesterol levels may vary even within the same strain (Ross). The breast muscle and liver cholesterol levels recorded in our current study were higher compared to values in Al-ruwaili *et al*. (2014) for the Ross, Lohman, Hubbard, and the local (Baladi) broiler strains. However, the Baladi strain had higher breast muscle cholesterol compared to the local and exotic chickens in our study (92.55 mg/100g vs 61.42±2.04 mg/100g and 30.93±3.02 mg/100g). In male cross-bred chicks, a protein-rich diet increased cholesterol synthesis in the liver and intestine compared to other organs (Yeh and Leveille 1971). Although we do not have precise information about the diets of our bird samples, it is likely that genetic, habitat, seasonal, and dietary differences could explain the discrepancies between our findings and previous studies. For instance, diet supplementation with 2% cholesterol increased the liver free cholesterol levels in White Leghorns (Teekell *et al*., 1974). In dogs fed a diet supplemented with 1 g of cholesterol for seven days, liver cholesterol levels strongly increased in cholesterol-fed dogs compared to controls (315 vs 246 mg/100g) (Gould *et al*., 1953). These findings support our thinking that diet could be one of the major factors that influenced tissue cholesterol levels recorded in the present study and that irrespective of genetics, diet has a strong impact on the modulation of cholesterol homeostasis in animals. The crow and quail in this study subsist on unique diets. Whereas the crow feeds mainly on waste food scraps and occasionally, dead rodents and chicken eggs the quail feeds on dry seed, insects, and fresh and dry tubers (cassava, potato, and yam). An examination of variations in cholesterol content in other wild and domesticated birds may help resolve the idea that domesticated species may have relatively higher levels of tissue cholesterol compared to their wild counterparts.

The current study also reports a higher brain cholesterol level compared to the heart, gizzard, and breast muscle. An animal's body contains, on average, about 2100 mg/kg of body weight of cholesterol and in humans and mice, 15% and 23% of cholesterol are found in the central nervous system (Dietschy, 2009). The brain synthesizes its cholesterol from astrocytes and oligodendrocytes and there is no evidence of uptake of the plasma-borne LDL-C across the blood-brain-barrier even in newborn lambs (Cavender *et al*., 1995). In humans, the average amount of free cholesterol in the central nervous system (CNS) is around 23 mg/g, the highest of all tissues (Dietschy & Turley, 2004). As we do not have extant data on the brain cholesterol content of birds, these studies suggest that at baseline physiological conditions, the brain may have cholesterol levels like the liver, but greater than other internal organs. Put together these studies and our current findings support unique but conserved tissue-specific mechanisms for cholesterol homeostasis across different avian species inhabiting the same or different environments. The range of gizzard cholesterol levels recorded in our study does not compare well with previous studies. For instance, cooked gizzard and roasted heart giblets had cholesterol contents of 72.68 and 213.18 mg/100g respectively (Pereira *et al*., 2002), while Antunes *et al*. (2018) recorded a value of 176.8 mg/100g for the ostrich gizzard. On the other hand, we recorded gizzard cholesterol values ranging from 24.63 mg/100g in the crow to 93.63 mg/100g in the local domestic chicken, whereas cholesterol values in the heart ranged from

51.98±3.61 mg/100g in the quail to 129.52±3.32 mg/100g in the crow. These discrepancies could be due to genetic, nutritional, environmental differences, or an interplay of factors. A recent study found sex differences in liver cholesterol levels of males and females of the Ongole cross breed cattle and the Kacang goat, respectively, with males of both species having higher liver cholesterol levels compared to females (Susanto *et al*., 2022). Since all subjects in our study were males, we do not know the extent to which sex differences impacted our findings. However, Rule *et al*. (2002) reported a higher cholesterol content in chicken breast muscle compared to the longissimus dorsi and semitendinosus muscles in the bison.

In conclusion, we present evidence supporting intra- and inter-species differences in tissue cholesterol metabolism among various avian species inhabiting similar environments, but unique in genetics, diet, and behaviour. More extensive studies are recommended for other wild and domesticated species to further reveal the extent to which differences in genetics, habitat, diet, sex, and behaviour impact on tissue-specific cholesterol metabolism.

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Author contributions
CN Asogwa: Conceptualization, ran wet experiments, design, statistical analysis, and drafting the manuscript, and correspondence. CA Ezema: Statistical analysis, investigation, writing. DA Osibe: Sample collection, design, writing the manuscript.CI Ugwu: Sample collection, collation, supervision.

Ethics statement
Permission to conduct this investigation was obtained from the Faculty of Biological Sciences Ethics Committee on Animal Experiments. This Committee's guidelines are based on the regulations for animal welfare in Nigeria which ensures humane treatment and management of experimental animals.

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Full-Length Research Article

Body Temperatures, Respiration, Heart Rate and Haematological Parameters of Four Species of Captive Monkeys in Nigeria during the Rainy Season

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Summary: Monkeys are phylogenetically close to humans and are therefore used in a lot of research models. This study aimed to determine the basic physiological and haematological parameters in four different species of captive monkeys during the rainy season. Fourteen adult monkeys at the University of Ibadan Zoological Garden were used for this study (3 - *Cercocebus sebaeus*, 3 - *Cercopithecus mona*, 3- *Erythrocebus patas* and 5- *Papio Anubis*). Dry-bulb temperature and relative humidity were recorded concurrently with the measurement of rectal temperature (RT), body surface temperatures (BST), respiratory (RR) and heart rates (HR). Blood sample were collected for the evaluation of haematological parameters. The dry-bulb temperature and relative humidity were 25.9°C and 75%, respectively. The *Erythrocebus patas* monkey had mean RT value of 38.23 ± 0.2°C, while the *Cercocebus sebaeus* monkey had 38.87 ± 0.7°C when compared to that of *Papio Anubis* and *Cercopithecus mona* monkey (38.4 ± 0.3°C and 38.53 ± 0.6°C, respectively). The mean RR value of 40.0 ± 2.3 cycle/min in *Erythrocebus patas* monkey was significantly ($p < 0.05$) higher than 27.33 ± 1.3 cycles/min and 21.60 ± 1.9 cycles/min in *Papio Anubis* and *Cercocebus sebaeus* monkey, respectively. The mean bias difference of rectal temperature versus eye temperature (0.8643 ± 0.749) was significantly ($p < 0.05$) lower than the mean bias differences of the rectal temperature versus forehead (2.19 ± 0.75), but was not statistically ($p > 0.05$) different from the value of 1.471 ± 0.9 obtained in the comparison of rectal temperature versus base of the tail temperature. The haematological parameters were not significantly different among the species. In conclusion, the basic thermoregulatory apparatus and haematological parameters of the different species of monkeys were not significantly different and the values recorded were within the established range for the monkey.

Keywords*: Monkey; Rectal temperature; Body surface temperature, Haematology.*

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INTRODUCTION

At high temperatures, monkeys increased evaporative heat loss through increased respiration and sweating rate (Mahiney, 1980; Walters *et al*., 2004). The interaction between ambient temperature and relative humidity has been linked to alterations in thermal balance in animals (Adair, 1985; Habeeb *et al*., 2018, 2023). Thermoregulation in animals under varying weather conditions varies in depth depending on the innate ability of the animal to regulate its thermal balance. The effectiveness of individual animals to regulate body temperature in extreme thermal conditions is important for their survival (Mota-Roja *et al*., 2021).

The ability of the monkeys in captivity for thermoregulation may be significantly influenced by their adaptation to captivity. In order to ensure the thermal comfort of captive monkeys, understanding of their physiological responses in the different seasons, with varied climate parameters, is very important. Primates in captivity are challenged with the great demand of maintaining an optimal body temperature within a physiologically acceptable range in a thermally changing environment (Thompson *et al*., 2014).

Thermal challenges may be more prevalent in primates than previously thought, especially with the advent of increasing global warming. Prolonged birth interval in free-

ranging baboons has been linked to changes in climate conditions in different seasons of the year (Thompson *et al*., 2014). It has been reported that vevet monkeys cope well in the heat conditions by the use of behavioural changes to aid their thermoregulation capability (Mcfarland *et al*., 2020).
Basic clinical and haematological variables are conveniently used as index of health status in different animals (Koo *et al*., 2019; Yu *et al*., 2019; Ayo *et al*., 2023; Bakker *et al*., 2023). It is imperative for clinicians in the zoo and the wild to be cautious in the interpretations of basic physiological and haematological variables; alterations in these variables have been linked to diurnal and circadian fluctuations and also seasonal effects (Ake *et al*., 2023a; Coskun *et al*., 2023; Iconaru *et al*., 2024). According to Park *et al*. (2016) there is no established link between changing environmental parameters and the values of haematological parameters recorded, suggesting that haematological values obtained in particular region can be used in another region. There is the need to review the long-established values of haematological parameters, due to evolving laboratory methods and the different methods of restrain applied. Age, sex and living conditions have been reported to affect haematological values (Rosenblum and Coulston, 1981; Koga *et al*., 2005; Nakayama *et al*., 2017). Species difference may also be a factor that influences the values (Ogunro *et al*., 2019). Therefore, the aim of the study was to determine the physiological and haematological parameters of four different species of captive monkeys during the rainy season.

MATERIALS AND METHODS

Animals and management: Fourteen adults' caged monkeys were used for this study (3 - *Cercocebus sebaeus*, 3 - *Cercopithecus mona*, 3- *Erythrocebus patas* and 5- *Papio Anubis*). The monkeys were kept at the University of Ibadan Zoo, Oyo state, Nigeria. This work is part of the Humboldt Research Hub project and Ethical approval was obtained from the Nigerian Veterinary Research Institute (AEC/03/116/22). Additionally, approval for the method and personnel to bleed the monkeys was granted by the Board of the Zoological Garden, University of Ibadan. The study was conducted during the rainy season. All the animals were apparently healthy at time of sampling.

Environmental parameters: The recording of environmental parameters was done concurrently with the physiological measurements. Dry-bulb temperature was recorded using a wet- and dry-bulb thermometer (Haryana, India), and relative humidity was calculated using Osmond's hygrometric table (Narindra Scientific industries, Haryana, India).

Evaluation of rectal and body surface temperatures: The measurement of rectal temperature (RT) from each monkey was recorded after the effect of the administered anesthetic agents has been established, using a standard digital clinical thermometer (Krusser Thermometer® Amazon, Berlin, Germany) inserted at 5 cm deep into the rectum via the anus. The recording of the body surface temperature (BST) (eye, forehead and base of the tail temperatures) was done using infrared thermometer (Model: JXB-181, Nanjing, Jiangsu, China). The distance of the animal from the infra-red thermometer camera was < 0.5 m for each temperature measurement.
Measurement of heart and respiratory rates: Evaluation of heart rate (HR) and respiratory rate (RR) was measured in all the monkeys, using stethoscope to count the number of beats per minute. The RR was measured by observing and counting the number of respiratory flank movements for one minute for each monkey.

Blood sample collection: The monkeys were darted at close range (1-5m) with a blow pipe delivering anaesthetic (ketamine hydrochloride, Ketanil®, Wildlife Pharmaceuticals, Windsor, USA) at 10mg/kg body weight of the monkey. Phlebotomy was via cephalic or tibial venipuncture using 21-gauge needle and a 5-mL syringe in each sedated animal. The volume of blood collected was 4mLs, placed in tubes with potassium ethylenediaminetetraacetic acid (EDTA) was used for the evaluation of haematological parameters.

Determination of packed cell volume (PCV): The PCV of each blood sample was determined using the microhaematocrit method described by Schalm (2010).

Determination of haemoglobin concentration: Haemoglobin concentration was determined by cyanmethaemoglobin method described by Schalm (2010). The absorbance of cyanmethaemoglobin was then determined spectrophotometrically.

Determination of red blood cell (RBC) count: The RBC count was determined by the haemocytometer method.

Determination of white blood cell count: The total white blood cell (leucocytes) count was determined using the haemocytometer method.

Determination of differential leucocyte count: A drop of blood from the EDTA blood sample was placed on a clean glass slide; it was fixed with methanol and then stained with Giemsa. One hundred cells were identified morphologically and counted and the number of each leukocyte type was expressed as a percentage of the total WBC from which the absolute leukocyte values were calculated.

Haematological indices: The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated from the PCV, RBC and HB values obtained earlier using the following formulas:

$$\text{MCV (fl)} = \frac{\text{PCV (\%) X 10}}{\text{RBC (X } 10^{12} \text{ /µL)}}$$

$$\text{MCH (pg)} = \frac{\text{Hb (g/dl) X 10}}{\text{RBC (X } 10^{12} \text{/µL)}}$$

$$\text{MCHC (g/dl)} = \frac{\text{Hb (g/dl) X 100}}{\text{PCV (\%)}}$$

Statistical analysis

The data were expressed as standard error of the mean (Mean ± SEM) and were subjected to normality test (Kolmogorov-Smirnov test). One-way ANOVA was used to compare the mean differences among the groups and subjected to Tukey's post-hoc test to determine the means differences. Values of $p < 0.05$ were considered significant.

RESULTS

The prevailing environmental temperature and relative humidity recorded during the study period are shown in Figure 1. The dry-bulb temperature measured was 25.9℃, while relative humidity recorded was 75%. Table 1 shows the basic physiological parameters of four species of monkeys during the rainy season. The rectal temperature of the four different species of monkey ranged from 37.4 – 39.6℃. The *Cercocebus sebaeus* monkey had highest mean RT ETs values of 38.87 ± 0.7℃ and 37.87 ± 0.3℃ when compared to others, though not significantly different. The mean FTs of the four monkeys were within the range of 36.2 – 37.9℃.

The mean RR value of 40.0 ± 2.3 cycle/min in *Erythrocebus patas* monkey was significantly ($p < 0.05$) higher than 27.33 ± 1.3 cycles/min and 21.60 ± 1.9 cycles/min in *Papio Anubis* and *Cercocebus sebaeus* monkey, respectively. The mean RR value of 27.33 ± 1.3 cycles/min in *Papio Anubis* monkey was significantly ($p < 0.05$) higher than the values recorded in *Cercopithecus mona* and *Cercocebus sebaeus* monkey. The HR values in the four monkeys range from 66 – 130 beats/min.

The Bland-Altman comparisons of rectal temperature with body surface temperatures in the four species of monkey during the rainy season are shown in Figure 2. The upper limit and lower limit of agreement for the rectal temperature versus eye temperature measurement were -0.6037 and 2.332, respectively. The rectal temperature versus forehead temperature level of agreement, the value for the upper limit of agreement was 3.669 while the value for the lower limit of agreement was 0.7172. The limits of agreement for the rectal temperature versus base of the tail temperature were 3.264 and -0.3207 for the upper and lower points, respectively.

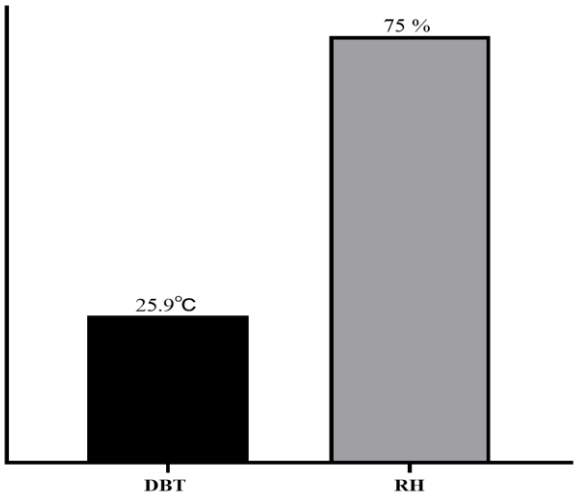

Figure 1:
Dry-bulb temperature and relative humidity recorded during data collection in the rainy season

Figure 3 shows the statistical comparisons between the mean bias differences of rectal temperature with the body surface temperatures in the four species of monkey. The mean bias difference of rectal temperature versus eye temperature (0.8643 ± 0.749) was significantly ($p < 0.05$) lower than the mean bias differences of the rectal temperature versus forehead (2.19 ± 0.75), but was not statistically ($p > 0.05$) different from the value of 1.471 ± 0.9 obtained in the comparison of rectal temperature versus base of the tail temperature. There was no significant ($p > 0.05$) difference between the mean bias differences of the rectal temperature versus forehead and rectal temperature versus base of the tail temperature.

Table 1:
Basic clinical parameters of four species of Monkeys during the rainy season (mean ± SEM)

Parameters	*Cercocebus sebaeus*	*Cercopithecus mona*	*Erythrocebus patas*	*Papio Anubis*
RT (℃)	38.40 ± 0.3	38.87 ± 0.7	38.23 ± 0.2	38.53 ± 0.6
	(39.1 – 37.8)	(39.6 – 37.5)	(38.6 – 37.5)	(39.6 – 37.4)
ET (℃)	37.76 ± 0.2	37.87 ± 0.3	37.17 ± 0.5	37.63 ± 0.1
	(38.3 – 37.2)	(38.4 – 37.6)	(38.2 – 36.6)	(37.7 – 37.5)
FT(℃)	36.36 ± 0.1	36.30 ± 0.1	36.30 ± 0.1	36.20 ± 0.0
	(36.7 – 36.2)	(36.4 – 36.2)	(36.5 – 36.2)	(36.2 – 36.2)
BT (℃)	37.20 ± 0.3	37.03 ± 0.1	37.00 ± 0.5	36.73 ± 0.4
	(37.9 – 36.4)	(37.2 – 36.9)	(37.9 – 36.4)	(37.4 – 36.2)
RR (cycles/min)	21.60 ± 1.9[a]	21.33 ± 3.5[a]	40.0 ± 2.3[b]	27.33 ± 1.3[a,c]
	(26.0 – 15.0)	(28.0 – 16.0)	(44.0 – 36.0)	(30.0 – 26.0)
HR (beats/min)	106.0 ± 7.6	90.33 ± 16.8	90.33 ± 4.3	91.33 ± 19.6
	(128.0 – 88.0)	(124.0 – 73.0)	(96.0 – 82.0)	(130.0 – 66.0)

Maximum – minimum in parenthesis
RT = rectal temperature, ET = eye temperature, FT = forehead temperature, BT = base of the tail temperature,
RR = respiratory rate, HR = heart rate

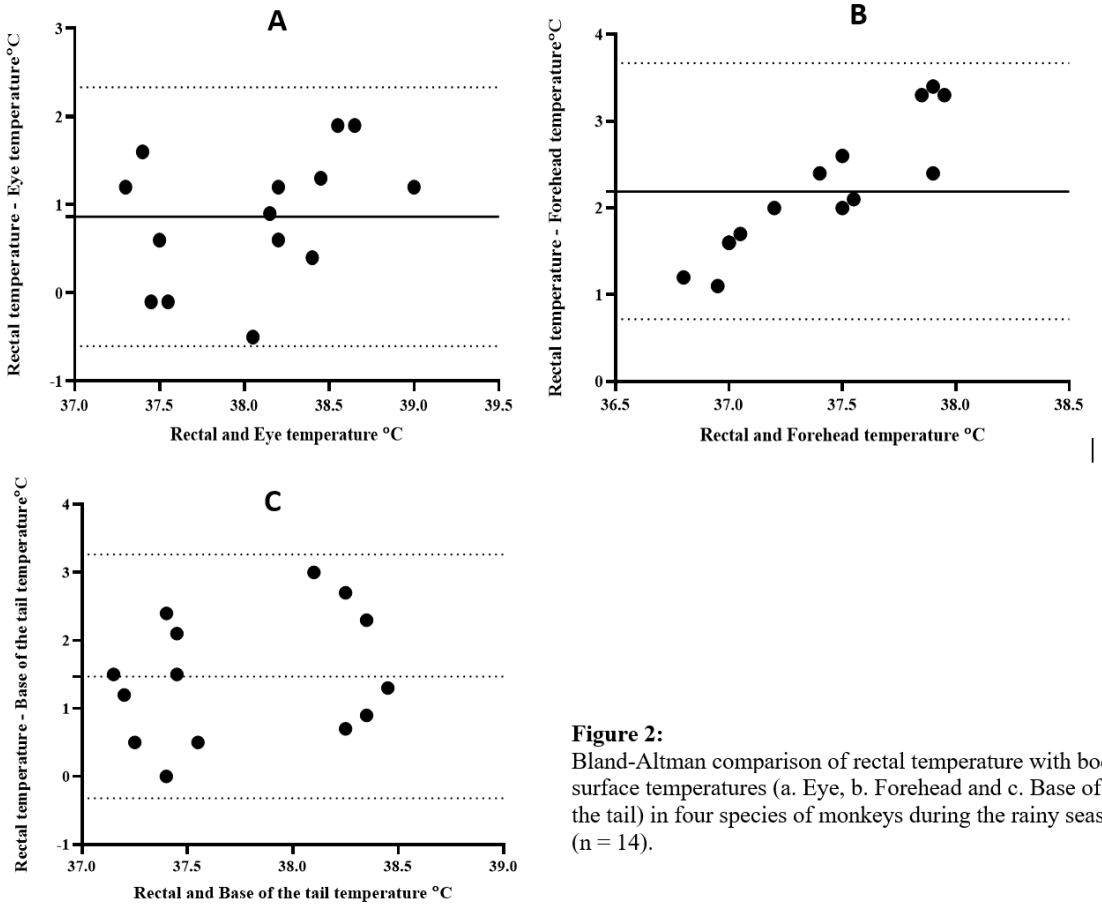

Figure 2:
Bland-Altman comparison of rectal temperature with body surface temperatures (a. Eye, b. Forehead and c. Base of the tail) in four species of monkeys during the rainy season (n = 14).

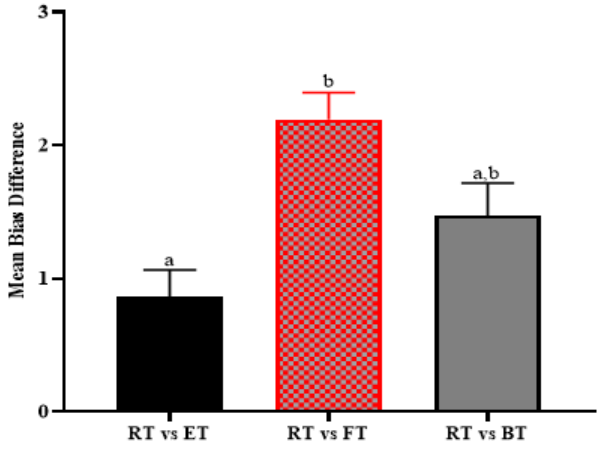

Figure 3:
Mean bias differences in rectal temperature versus body surface temperatures in of four species of monkeys during the rainy season. (Mean ± SEM, n = 14). Different superscripts alphabets indicate significant ($p < 0.05$) difference.
Note: RT = rectal temperature, ET = eye temperature, FT = forehead temperature, BT = Base of the tail

The comparisons of the rectal and body surface temperatures using receiver operating characteristic curve analyses are presented in Figure 4. The comparison between rectal temperature and eye temperature had area under curve of 0.8138 ± 0.08 while that of rectal temperature versus forehead temperature and rectal temperature versus base of the tail temperature had area under curve of 1.0 ± 0.0 each. Table 2 shows the relationships of rectal temperature versus respiratory rate and versus heart rate in the monkeys. The correlation coefficient values obtained in rectal temperature versus respiratory rate and versus heart rate were not significantly ($p > 0.05$) different.

The erythrocytic parameters of four species of Monkeys during the rainy season are shown in Table 3. The mean PCV and Hb values in the *Cercocebus sebaeus* (46.33 ± 8.7% and 15.43 ± 2.9g/dL, respectively) were the highest, it was not significantly ($p > 0.05$) different to the values recorded in the other species.

The value of RBC ranged from 2.2 to 7.0 x 106/µL in the Monkeys. The highest mean RBC value of 5.26 ± 0.4 x 106/µL was recorded in *Cercocebus sebaeus*. The mean RBC values were not significantly ($p > 0.05$) different. The MCV, MCH, MCHC and RDW values ranges in the four species of monkeys are 80 – 100 fl, 26 – 32.3 pg, 33.3 – 33.5 g/dL and 13.6 – 15.2 fl, respectively. The leukocytes values in four different species of monkeys are shown in Table 4. The highest mean TWBC (8.77 ± 2.4 x 103/µL) was recorded in the *Cercopithecus mona* species. The values of the TWBC obtained in the monkeys were not significantly different.

The Neutrophil values ranges are 1.1 – 3.4 x 103/µL, 2.0 – 9.3 x 103/µL, 0.8 – 1.4 x 103/µL and 2.2 – 6.9 in the four species of monkeys. *Cercopithecus mona* monkey had the widest Neutrophil value range of 2.0 – 9.3 x 103/µL while *Erythrocebus patas* had the highest Lymphocyte value of 2.1 ± 0.2 x 103/µL. The highest mean platelet count was recorded in *Cercopithecus mona* (291.70 ± 39.8 g/dL). The platelets values recorded in the monkeys were not significantly ($p > 0.05$) different.

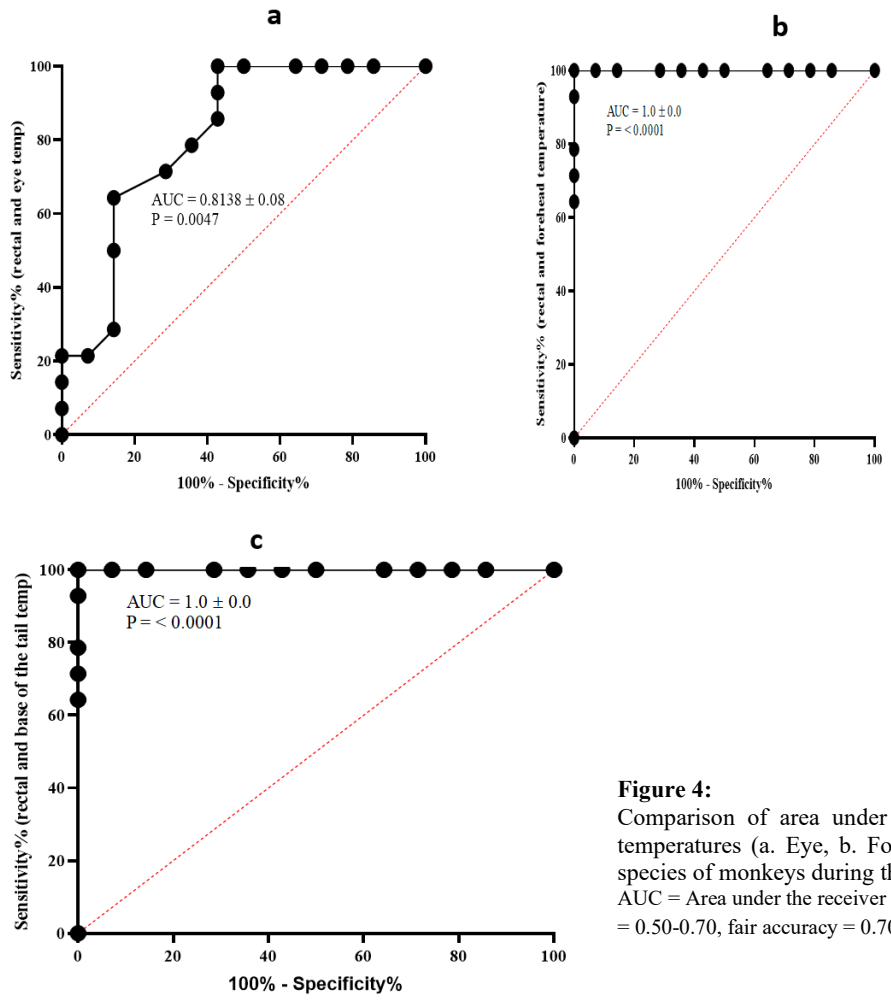

Figure 4:
Comparison of area under the curve of rectal and body surface temperatures (a. Eye, b. Forehead and c. Base of the tail) of four species of monkeys during the rainy season (n = 14).
AUC = Area under the receiver operating characteristic curve. Low accuracy = 0.50-0.70, fair accuracy = 0.70-0.90, and high accuracy = > 0.90

Table 2:
Correlation coefficient of rectal temperature with respiratory and heart rates in in of four species of monkeys during the rainy season

Parameters	Correlation coefficient (r)			
	Cercocebus sebaeus (n = 3)	*Cercopithecus mona* (n = 3)	*Erythrocebus patas* (n = 3)	*Papio Anubis* (n = 5)
RT vs RR	0.1474^{ns}	0.8386^{ns}	0.9966^{ns}	0.5424^{ns}
RT vs HR	0.5216^{ns}	0.9216^{ns}	0.2833^{ns}	-0.5280^{ns}
RR vs HR	-0.7670^{ns}	0.9843^{ns}	0.2035^{ns}	0.0774^{ns}

ns = p>0.05

Table 3:
Erythrocytic parameters of four species of monkeys during the rainy season (mean ± SEM) during the rainy season (mean ± SEM)

Parameters	*Cercocebus sebaeus*	*Cercopithecus mona*	*Erythrocebus patas*	*Papio Anubis*
PCV (%)	46.33 ± 8.7 (61.0 – 31.0)	38.33 ± 7.8 (54.0 – 30.0)	40.00 ± 1.0 (42.0 – 39.0)	36.80 ± 6.5 (57.0 – 17.0)
Hb (g/dL)	15.43 ± 2.9 (20.3 – 10.3)	12.77 ± 2.6 (18.0 – 10.0)	13.33 ± 0.3 (14.0 -13.0)	12.28 ± 2.1 (19.0 – 5.7)
RBC (x 10^6/µL)	5.26 ± 1.0 (7.0 – 3.4)	4.37 ± 0.9 (6.2 – 3.4)	4.56 ± 0.1 (4.7 – 4.5)	4.156 ± 0.6 (5.9 – 2.2)
MCV (fl)	86.0 ± 1.2 (88.0 – 84.0)	85.33 ± 1.5 (88.0 – 83.0)	87.33 ± 1.5 (90.0 – 85.0)	88.40 ± 3.3 (100.0 – 80.0)
MCH(pg)	29.53 ± 0.4 (30.3 – 29.0)	29.27 ± 0.1 (29.4 – 29.1)	29.20 ± 0.4 (29.9 – 28.6)	29.0 ± 1.0 (32.3 – 26.0)
MCHC (g/dL)	33.30 ± 0.0 (33.4 – 33.3)	33.30 ± 0.0 (33.3 – 33.3)	33.3 ± 0.0 (33.3 – 33.3)	33.36 ± 0.04 (33.5 – 33.3)
RDW (fl)	14.40 ± 0.1 (14.6 – 14.2)	14.27 ± 0.2 (14.6 – 13.9)	14.07 ± 0.1 (14.2 – 13.8)	14.18 ± 0.3) (15.2 – 13.6)

Maximum – minimum in parenthesis
PCV = Packed cell volume, Hb = Haemoglobin concentration, RBC = Red blood cell count, MCV = Mean corpuscular volume, MCH = Mean corpuscular haemoglobin, MCHC =Mean corpuscular haemoglobin concentration.

Table 4:
Leukocytes parameters of four species of monkeys during the rainy season (mean ± SEM) during the rainy season (mean ± SEM)

Parameters	*Cercocebus sebaeus*	*Cercopithecus mona*	*Erythrocebus patas*	*Papio Anubis*
TWBC (x 10^3/µL)	4.97 ± 1.9 (8.7 – 2.4)	8.77 ± 2.4 (13.5 – 5.6)	3.88 ± 0.3 (4.2 – 3.2)	7.80 ± 1.9 (15.1 – 4.3)
NEU (x 10^3/µL)	1.9 ± 0.6 (3.4 – 1.1)	4.8 ± 2.3 (9.3 – 2.0)	1.2 ± 0.2 (1.4 – 0.8)	3.5 ± 0.9 (6.9 – 2.2)
LYM (x 10^3/µL)	1.8 ± 0.1 (1.9 – 1.6)	1.5 ± 0.4 (2.2 – 0.9)	2.1 ± 0.2 (2.5 – 1.7)	1.6 ± 0.1 (1.9 – 1.4)
MON (x 10^3/µL)	0.1 ± 0.04 (0.2 – 0.1)	0.2 ± 0.10 (0.3 – 0.1)	0.1 ± 0.01 (0.1 – 0.1)	0.2 ± 0.04 (0.3 – 0.1)
EOS (x 10^3/µL)	0.01 ± 0.00 (0.01 – 0.01)	0.01 ± 0.00 (0.01 – 0.01)	0.01 ± 0.00 (0.01 –0.01)	0.01 ± 0.00 (0.01 – 0.01)
BAS (x 10^3/µL)	0.0 ± 0.0 (0.0 – 0.0)	0.0 ± 0.0 (0.0 – 0.0)	0.0 ± 0.0 (0.0 – 0.0)	0.0 ± 0.0 (0.0 – 0.0)
Platelets (x 10^3/µL)	237.67 ± 79.0 (384.0 – 113.0)	291.70 ± 39.8 (369.0 – 237.4)	173.67 ± 9.9 (185.0 – 154.0)	212.6 ± 40.2 (332.0 – 82.0)
MPV (fl)	8.00 ± 0.1 (8.1 -7.9)	8.27 ± 0.7 (9.6 – 7.3)	8.53 ± 0.4 (9.2 – 7.8)	7.6 ± 0.1 (8.0 – 7.3)

Maximum – minimum in parenthesis
TWBC = Total white blood cell count, NEU = Neutrophil count, LYM = Lymphocyte count, MON = Monocyte count, EOS = Eosinophil count, BAS = Basophil count, MPV = Mean platelet volume.

DISCUSSION

The prevailing environmental parameters, dry-bulb temperature and relative humidity obtained in the current study during the rainy season are characteristic of the season. Low air temperature and high relative humidity have little impact on thermoregulatory mechanisms of animals because at low air temperature the body homeostatic apparatus does little to keep the body temperature within the normal range. Stitt and Hardy (1971) reported the thermoneutral zone of monkey to be within the range of 25 - 35°C. The value of ambient temperature recorded in this study was largely within the established thermoneutral zone. Basic clinical parameters of monkeys in the captive are very important basis for the evaluation of the wellbeing and health status of the animals (Pasciu *et al*., 2023). Seasons of the year, hours of the day have been reported to exact significant influence on these parameters and therefore their interpretation in drawing inference on the state of the health and wellbeing of the monkeys must be carried out with the cognizance of the impacts of these factors. The results of the environmental parameters indicated high relative humidity and low ambient temperature which are characteristic of the rainy season (Oke *et al*., 2021). As the ambient temperature increases, the body temperatures tend to also increase, but rectal temperature is maintained within a limited range for homeostatic purposes. The values of rectal temperature obtained in the four species of monkey in the captive are a reflection of the thermoregulatory capacities of the animals. Normal rectal temperature range of 38.0 – 39.5°C has been reported in Macaca fascicularis hand caught and restrained for examination. The results of the current study show that the rectal temperature ranges of *Cercocebus sebaeus* (37.8 – 39.1°C), *Cercopithecus mona* (37.5 – 39.6°C), *Erythrocebus patas* (37.5 – 38.6°C) and *Papio Anubis* (37.4 – 39.6°C) were all close to the range of normal rectal temperature reported in cynomolgus macaques (Laffins *et al*., 2017). The findings indicate that the rectal temperature of the four species of monkey recorded in this study were normal and could serve as basis for further studies.

The results of the body surface temperature recorded in the eye, forehead and base of the tail regions of the body varied significantly across the different regions of the body. The values of the body surface temperatures recorded in the monkeys were within the range of 34 to 37°C reported by Baker *et al*. (1976) in undisturbed animals. Body surface temperatures are largely influenced by the prevailing environmental parameters, but some regions of the body are much influenced than others (Thompson *et al*., 2017; Ake *et al*., 2023b). The eye temperature in particular has been considered reliable, stable and with reduced influence from environmental parameters when compared to other parts of the body (da Costa *et al*., 2024). The findings of the study indicate that the eye temperature in the different species of monkeys was the closest to the rectal temperature. The result is in agreement with the previous studies that the eye temperature was stable and could be a reliable alternative to the rectal temperature (Ake *et al*., 2023b). The base of the tail temperature was also not too far from the rectal temperature; apparently the anatomical structures around the tail and the root of the tail close to the rectal orifice of the monkey are much more exposed to the direct effect of air temperature when compared with other non-primate animals. Finding reliable and accurate alternative to rectal temperature measurement requires further studies and applications of mathematical models in establishing the validity of the method.

The relationships between the rectal temperature and body surface temperature were not significant in this study. Respiratory and heart rates have been reported in previous studies to have some degree of association with increase in body temperature (Jensen and Brabrand, 2015; Heal *et al*., 2022). The meteorological conditions prevailing during the data collection were within the thermoneutral comfort for the monkeys, therefore does not require additional responses from the respiratory or cardiovascular system to maintain adequate thermoregulatory responses. The cardiorespiratory values recorded might have been influenced by the chemical use for restraint. The combination of ketamine and xylazine has been previously reported to reduce respiratory rate and the heart rate in monkeys (Hernández-Godínez *et al*., 2019; Archer *et al*., 2025). This must be taken into perspective, especially when

recording the basic physiological parameters of monkeys requires the use of anesthetic agents.

Established haematological values are of great importance for clinical diagnosis, determination of treatment outcomes, diseases prevention and for biomedical research purposes (Haile *et al*., 2023). The findings in the haematological parameters evaluated in the study apparently showed no species significant difference. The results are consistent with the reports from several studies (Nakayama *et al*., 2017; Koo *et al*., 2019; Ogunro *et al*., 2019; Perez-Brigido *et al*., 2021; Bakker *et al*., 2023). Several other studies in monkeys have indicated the influence of age, sex and especially living conditions on haematological parameters (Sugimoto *et al*., 1986; Xie *et al*., 2014; Yu *et al*., 2019). Sugimoto *et al*. (1986) reported larger percentage of Lymphocyte compared to the percentage of Neutrophil in Monkeys from birth to about 4 years of age but the relationship is inverted as the monkeys become adults. The reverse is true in the adult monkeys used in this study especially in *Cercocebus sebaeus*, *Erythrocebus patas* and *Papio Anubis*. The higher percentage of Neutrophil compared to percentage of Lymphocytes was only recorded in *Cercopithecus mona*. Some other studies have also reported varied values in the percentage of Neutrophil to that of Lymphocytes in different species of Monkeys (Schuurman *et al*., 2004; Capitanio *et al*., 2023). The values of haematological parameters obtained in this study will add to the existing clinical measurement in different species of monkeys in the captive, which will aid practicing clinicians and researchers to draw inference which could help in arriving at a sound clinical judgment and interpretation, respectively. In conclusion, the basic thermoregulatory apparatus of the different species of monkeys were not significantly different and the values recorded were within the established range for the monkey in the tropical region. The eye temperature was the closest to the rectal temperature and can serve as a reliable and valid alternative. Haematological parameters were not significantly different across the species studied.

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Full-Length Research Article

Maternal Environmental Temperature During Gestation Affects Renal Function Indices and Oxidative Stress in the Kidney of Offspring of Wistar Rats

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Summary: Maternal exposure to increased temperature reduces birth weight. Low birth weight is related to an adult-onset of non-communicable diseases. In this study, the effects of maternal exposure to various environmental temperatures during gestation on renal function and oxidative stress indices in offspring of Wistar rats were assessed. Fifteen pregnant Wistar rats were divided into three groups (n=5). The animals were housed in specialized thermoregulatory cages at 25^0C, 32^0C and 39^0C respectively. The animals were allowed to deliver spontaneously. At 12 weeks of age, the offspring were euthanized. Urea concentrations in serum, urine and creatinine clearance were assessed as renal function indices. Renal superoxide dismutase (SOD), catalase and malondialdehyde (MDA) activities were estimated as indices of oxidative stress. Histopathology and histomorphometry of the kidney were also assessed. Concentrations of urine creatinine and creatinine clearance of the temperature group 39°C were significantly higher than those of the 25°C and 32°C groups in both male and female offspring. Catalase and SOD activities in the kidneys of the male offspring were significantly decreased in temperature group 32°C when compared to the temperature groups 25°C and 39°C. Meanwhile, the MDA level was significantly higher in the 32^0C group. In the female offspring, exposure to 25°C significantly increased the concentration of serum creatinine, urea and renal MDA levels compared to other temperature groups. However, SOD activities were lowered in the 25^0C group. In conclusion, maternal environmental temperature during gestation affects renal function and oxidative stress indices in offspring of Wistar rats. These effects may be influenced by the sex of the offspring.

Keywords: *Environmental Temperature, kidneys, offspring, serum creatinine, serum urea, urine creatinine*

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INTRODUCTION

Maternal exposure to various environmental factors, such as temperature and stress, increases the susceptibility of offspring to various diseases that manifest either in childhood or adulthood (Brown, 2011). Alterations in body homeostasis have been discovered to alter metabolic changes in many nutrients in pregnant women, which can affect neonatal development (Sharma *et al*., 2013). According to Basu and Samet (2002), extreme temperatures have a detrimental impact on human health because they overburden the body's ability to self-regulate. The thermoregulatory and sympathetic nervous systems of fetuses and infants are not well developed, resulting in their extreme sensitivity to hot temperatures (Knobel & Holditch-Davis, 2007). Previous studies have demonstrated that the health of the fetus in its post-uterine life is negatively impacted by intense heat throughout pregnancy and the early postpartum period (Hanson & Gluckman, 2014).

The theory of fetal origins posits that the length of gestation has a major influence on an individual's developmental health and well-being from birth to maturity (Almond and Currie, 2011). According to O'Donnell and Meaney (2016), the two main aspects of fetal origin are genetic programming and latency, which refers to the possibility that conditions resulting from fetal effects won't become evident until much later in life for a particular individual.

Chronic kidney disease (CKD) in adults can be programmed by an adverse in-utero environment (Tain & Hsu, 2017). Furthermore, low birth weight (LBW) is linked to an elevated risk of adult-onset disorders, such as renal function dysfunction, and has been utilized as a clinical surrogate for a poor intrauterine environment (Calkins & Devaskar, 2011). Luyckx *et al*., (2011) in their study noted that decreased number of nephrons due to impaired nephrogenesis is an hypothesized mechanism that associates LBW with eventual hypertension and CKD risk. Additionally, there is a positive link between kidney weight and the number of glomeruli. The clinical symptoms include reduced glomerular filtration rate, azotemia, proteinuria, and glomerulosclerosis up to adulthood (Wang *et al*., 2015). In the same study, Wang *et al*., postulated that a decrease in

the number of nephrons induces glomerular hyperfiltration and compensatory hypertrophy, potentially at the cost of increasing intraglomerular pressures and eventual glomerulosclerosis, which leads to CKD via a separate, as-yet unknown mechanism. Sweating and inadequate water intake can cause electrolyte imbalance, and dehydration. According to Rampatzis *et al*. (2013), renal function may be hampered by compensatory physiological mechanisms such as circulatory adaptation and thermoregulation. Several factors including maternal and fetal starvation, exposure to glucocorticoids from the mother, and renin-angiotensin contributes to the loss of nephrons during fetal development (Wang *et al*. 2015).

In this study, we seek to investigate the effect of maternal exposure to various environmental temperatures during gestation on the serum analyte and renal function indices of the F1 offspring.

MATERIALS AND METHODS

Experimental Animal: Fifteen female Wistar rats were obtained from the Department of Physiology, Federal University of Technology, Akure. The animals were housed in standard, well-ventilated wooden cages in the departmental animal holding facility. They had free access to food (vital feed) and water. After two weeks of acclimatization, animals in proestrus were exposed to mature male overnight and the presence of sperm in their vaginal smear was taken as the first day of gestation.
After confirmation of gestation, the animals were randomly divided into three different temperature groups of five animals in each group. Group 1 were exposed to temperature of 25^0C while animals in Groups 2 and 3 were exposed to 32^0C and 39^0C temperature respectively. The animals were allowed to deliver spontaneously, immediately after the delivery the dams with their pups were moved into well ventilated cages under standard laboratory temperature. The study was conducted by the International Ethical Norms on Animal Care and Use as contained in NIH publication/80-23, revised in 2010. This study was carried out at the Department of Physiology, School of Basic Medical Sciences, Federal University of Technology, Akure, Nigeria.

Collection of Urine from Offspring: The offspring were allowed to grow to adulthood (12 weeks of age). A 24-hour urine sample was thereafter collected from the offspring to measure urine creatinine concentration, urinary urea concentration and creatinine clearance at PND 12 weeks. A specialized metabolic cage was used to collect urine. The female offspring were placed in the metabolic cage during their proestrous stage. The urine samples were centrifuged at 4000g for 10 minutes. The supernatant was aspirated and kept in a freezer for further assays.

Collection of Blood: Blood samples were collected from the animals via retro-orbital sinus into plain sample bottles. The blood samples were centrifuged immediately at 4000g for 10 minutes. The serum was aspirated and kept in a freezer for further assays.

Sacrifice of animals: At the end of the study (PND 12 weeks), the animals were euthanized via cervical dislocation under sodium thiopental anaesthesia (50 mg/kg, i.p.). The kidney of each animal was harvested and weighed with the dry weight recorded before being immersed in phosphate buffer solutions. The left kidney was put in phosphate buffer while the right kidney went into the formal-saline. The left kidney put in the phosphate buffer was homogenized using a homogenizer, after which the sample was centrifuged at 10,000g for 10 minutes in cold centrifuge. The supernatant was removed and stored in a refrigerator for analysis of oxidative stress indices. The biochemical analysis was done within 72hours of sample collection.

Biochemical analysis

Determination of Creatinine Concentration in Serum and Urine: Creatinine levels were determined using Jaffe method with creatinine kit from Fortress Diagnostics Limited, United Kingdom. Creatinine reacts with picric acid in an alkaline medium to form a deep yellow complex. The amount of complex formed is directly proportional to the level of creatinine in the sample.

Determination of Creatinine Clearance: This is measured using both the serum creatinine and urine creatinine as described in manufacturer manual (Fortress Diagnostic Limited, United Kingdom).

Determination of serum and urine urea: Urea levels were determined using Urea Kinetic assay (Fortress Diagnostics Limited, United Kingdom). Urea is hydrolyzed in presence of urease to produce ammonia and CO2. The ammonia produced combines with 2-oxoglutarate and NADH in presence of GLDH to yield glutamate and NAD.
Urea +H2O+2H+ Urease 2 NH+4 + CO2
NH4+ + 2-oxoglutarate + NADH GLDH H2O + NAD++ Glutamate
The relationship between the urea concentration and the decrease in absorbance resulting from a decrease in NADH content over time is proportional. This approach determines ammonia generated by various breakdown processes.

Determination of Protein Concentration: The protein contents of the different samples were ascertained using the Biuret method, which was slightly modified by adding potassium iodide to the reagent to stop the precipitation of Cu2+ ions as cuprous oxide, as reported by Gornal *et al*. in 1949.

Determination of Oxidative Stress (LPO Assessment): By quantifying the thiobarbituric acid reactive compounds (TBARS) generated during lipid peroxidation, lipid peroxidation was ascertained. This was done using the procedure outlined by Beuge and Aust in 1978.

Determination of Catalase Activity: Catalase activity was measured using Claiborne's (1985) technique. The technique is based on the absorbance loss that occurs when catalase breaks hydrogen peroxide, which is seen at 240 nm. Although hydrogen peroxide doesn't have a maximum absorbance at this wavelength, its absorbance and concentration can be correlated sufficiently for use in a quantitative experiment. The employed extinction coefficient was 0.0436 mM-1cm-1.

Determination of Superoxide Dismutase (SOD) Activity: Using Misra and Fridovich's approach (Misra and Fridovich, 1972), the amount of SOD activity was measured. This reaction serves as the foundation for a straightforward assay for this dismutase because SOD can prevent the autoxidation of epinephrine at pH 10.2. The oxidation of epinephrine to adrenochrome was triggered by the superoxide radical produced by the xanthine oxidase process. The yield of adrenochrome produced per superoxide injected rose as the pH and epinephrine concentration increased.

Histopathology of the kidney: Normal paraffin wax embedding procedures were performed on tissues preserved in 10% neutral buffered formalin. Hematoxylin and Eosin (H&E) staining was performed routinely to obtain sections that were 5 µm thick and assessed for histological alterations. A digital bright field microscope was used to examine the sections, and a photomicrograph was taken.

Histomorphometry: Photomicrographs were obtained at x100 magnification and imported unto Image J software. Glomeruli present per field were identified and counted using the Image J cell counter tool as previously described (Ebokaiwe *et al*. 2018).

Statistical Analysis: Standard Error of Mean (SEM) ± mean was used to express the data. For statistical analysis, GraphPad Prism version 8.02® (LA Jolla, CA, USA) was utilized. Two-way ANOVA was used to examine the values, which were shown as the mean ± SEM. At a significance threshold of $p<0.05$, the Tukey's multiple comparisons test was employed to identify significant differences between the means.

RESULTS

Effect of maternal exposure to various environmental temperature during gestation on creatinine concentration in serum and urine in offspring of Wistar rats: The statistical analysis of serum creatinine concentrations in offspring following maternal exposure to varying environmental temperatures revealed significant effects based on sex [$F(1, 24) = 1500$, $P < 0.0001$], interaction [$F(2, 24) = 123.7$, $P < 0.0001$], and treatment [$F(2, 24) = 106.8$, $P < 0.0001$] (Fig. 1).

Post hoc analysis indicated that the serum creatinine concentration in male offspring exposed to 39°C was significantly higher ($P < 0.05$) compared to those at 25°C and 32°C. In contrast, female offspring exhibited significantly reduced serum creatinine concentrations at 25°C, 32°C, and 39°C when compared to their male counterparts at the same temperatures.

Additionally, the statistical analysis of urine creatinine concentrations in offspring following maternal exposure to different environmental temperatures demonstrated significant effects related to sex [$F(1, 24) = 84.3$, $P < 0.0001$], interaction [$F(2, 24) = 14.17$, $P < 0.0001$], and treatment [$F(2, 24) = 205.5$, $P < 0.0001$] (Fig. 2).

The post hoc analysis revealed a significant increase ($P < 0.05$) in urine creatinine concentration in male offspring at 32℃ and 39℃ compared to those at 25℃. Conversely, urine creatinine concentration was significantly lower ($P < 0.05$) in the kidneys of female offspring at 25℃ and 32℃ when compared to 25℃ male offspring.

Additionally, there was a significant increase ($P < 0.05$) in urine creatinine concentration in female offspring at 32℃ and 39℃ relative to those at 25℃. Notably, the urine creatinine concentration was significantly higher ($P < 0.05$) in 39℃ female offspring compared to their 32℃ counterparts

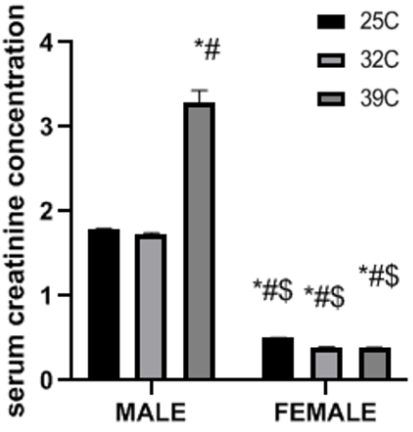

Figure 1:
Effect of maternal exposure to various environmental temperature during gestation on serum creatinine concentration of male and female offspring of Wistar rats. Values are mean ± SEM for 5 offspring per temperature group. $P < 0.05$. *P<0.05 compared to 25^0C male group; #P<0.05 compared to 32^0C male group; $P<0.05 compared to 39^0C male group;

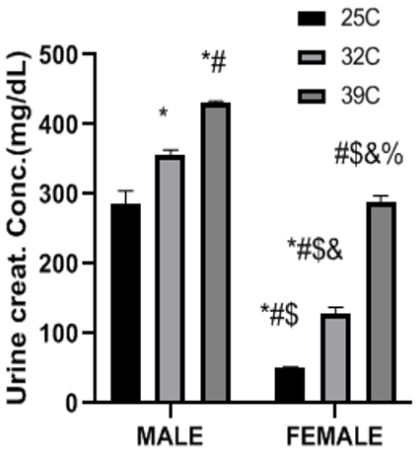

Figure 2:
Effect of maternal exposure to various environmental temperature during gestation on urine creatinine concentration in male and female offspring of Wistar rats. Values are mean ± SEM for 5 offspring per temperature group. $P < 0.05$. *P<0.05 compared to 25^0C male group; #P<0.05 compared to 32^0C male group; $P<0.05 compared to 39^0C male group; &P<0.05 compared to 25^0C female group; %P<0.05 compared to 32^0C female group

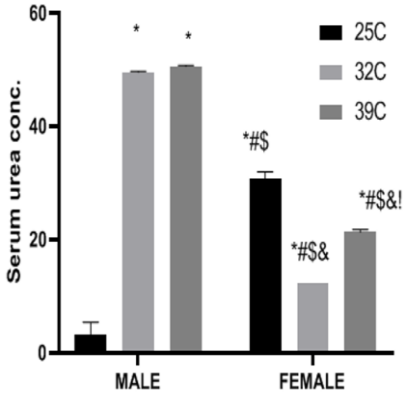

Figure 3:
Effect of maternal exposure to various environmental temperature during gestation on serum urea concentration in male and female offspring of Wistar rats. Values are mean ± SEM for 5 offspring per temperature group. $P < 0.05$..*P<0.05 compared to 25^0C male group; #P<0.05 compared to 32^0C male group; $P<0.05 compared to 39^0C male group; &P<0.05 compared to 25^0C Female group; !P<0.05 compared to 32^0C Female group

Effect of maternal exposure to various environmental temperature during gestation on Urea concentration in serum and urine in offspring of Wistar rats; The statistical analysis of serum urea concentration in Wistar offspring following maternal exposure to various environmental temperatures revealed significant effects based on sex [$F(1, 24) = 206.0$, $P < 0.0001$], interaction [$F(2, 24) = 509.7$, $P < 0.0001$], and treatments [$F(2, 24) = 160.2$, $P < 0.0001$] (see Fig. 3).

Post hoc analysis indicated a significant increase ($P < 0.05$) in serum urea concentration for male offspring at 32°C and 39°C compared to those at 25°C. Additionally, the serum urea concentration of male offspring at 32°C was significantly reduced ($P < 0.05$) when compared to that of male offspring at 39°C and female offspring at 25°C.

For female offspring, serum urea concentrations at both 39°C and 32°C were significantly lower ($P < 0.05$) compared to the male offspring at 32°C, 39°C, and the female offspring at 25°C. However, there was a significant increase ($P < 0.05$) in the serum urea concentration of female offspring at 39°C compared to those at 32°C.

Furthermore, the statistical analysis of urine urea concentration in the offspring, following maternal exposure to different environmental temperatures, showed no significant effects due to interaction [$F(2, 24) = 3.133$, $P = 0.0602$] and treatment [$F(2, 24) = 3.166$, $P = 0.0602$]. In contrast, the effects of sex on urine urea concentration were significant [$F(1, 24) = 10550$, $P < 0.0001$] (see Fig. 4).

The post hoc analysis demonstrated that urine urea concentration was significantly higher ($P < 0.05$) in male offspring at 39°C compared to those at 25°C. Conversely, the urine urea concentration in female offspring at 25°C was significantly higher ($P < 0.05$) than that in male offspring at 25°C, 32°C, and 39°C. Additionally, there was a significant increase ($P < 0.05$) in urine urea concentration for female offspring at 32°C when compared to the male offspring at 25°C, 32°C, and 39°C. Similarly, female offspring at 39°C exhibited a significant increase ($P < 0.05$) in urine urea concentration compared to male offspring at 25°C, 32°C, and 39°C.

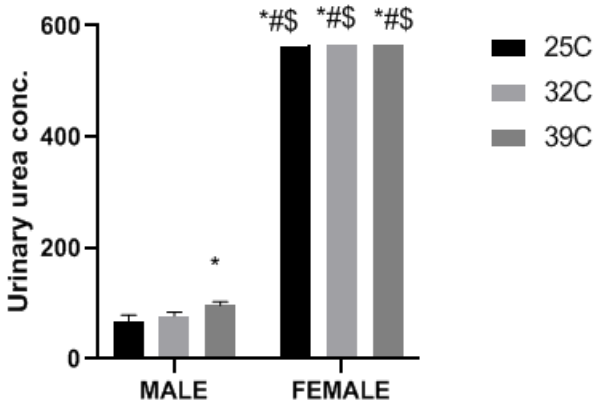

Fig. 4:
Effect of maternal exposure to various environmental temperature during gestation on urinary urea concentration of male and female offspring of Wistar rats. Values are mean ± SEM for 5 offspring per temperature group. $P < 0.05$. *P<0.05 compared to 25^0C male group; #P<0.05 compared to 32^0C male group; $P<0.05 compared to 39^0C male group

Effect of maternal exposure to various environmental temperature during gestation on creatinine clearance in offspring of Wistar rats

The statistical analysis of creatinine clearance in offspring following maternal exposure to various environmental temperatures revealed significant effects related to sex [$F(1, 24) = 39.85$, $P < 0.0001$], interaction [$F(2, 24) = 49.52$, $P < 0.0001$], and treatment [$F(2, 24) = 308.2$, $P < 0.0001$] (Fig. 5).

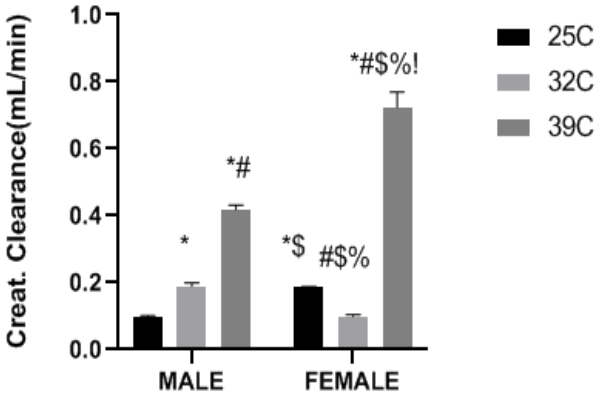

Figure. 5:
Effect of maternal exposure to various environmental temperature during gestation on creatinine clearance in male and female offspring of Wistar rats. Values are mean ± SEM for 5 animals per temperature group. $P < 0.05$. *P<0.05 compared to 25^0C male group; #P<0.05 compared to 32^0C male group; $P<0.05 compared to 39^0C male group; %P<0.05 compared to 25^0C Female group; !P<0.05 compared to 32^0C Female group.

Post hoc analysis indicated that creatinine clearance was significantly higher ($P < 0.05$) in male offspring at 32°C and 39°C compared to male offspring at 25°C, with a notable increase ($P < 0.05$) in creatinine clearance in 39°C male offspring relative to those at 32°C. Furthermore, the analysis revealed a significant increase ($P < 0.05$) in the creatinine clearance of female offspring at 25°C when compared to male offspring at 25°C, alongside a significant decrease ($P < 0.05$) relative to 39°C male offspring.

Additionally, the post hoc analysis showed that creatinine clearance in female offspring at 32°C was significantly lower ($P < 0.05$) compared to 32°C and 39°C male offspring as well as 25°C female offspring. In contrast, there was a significant increase ($P < 0.05$) in creatinine clearance levels for female offspring at 39°C when compared to 25°C, 32°C, and 39°C male offspring, as well as 25°C and 32°C female offspring.

Effect of maternal exposure to various environmental temperature during gestation on Malondialdehyde levels in the kidney of offspring of Wistar rats: The statistical analysis of renal malondialdehyde levels in offspring of Wistar rats, following maternal exposure to varying environmental temperatures, indicated a significant impact of sex [$F(1, 24) = 6927$, $P < 0.0001$], interaction [$F(2, 24) = 156.9$, $P < 0.0001$], and treatment [$F(2, 24) = 7.912$, $P = 0.0023$] (Fig. 6).

Post hoc analysis revealed that malondialdehyde levels (MDA) were significantly elevated ($P < 0.05$) in the kidneys of male offspring at 32°C and 39°C compared to those at 25°C, with a notable decrease ($P < 0.05$) when comparing

39°C male offspring to 32°C male offspring. Additionally, a significant decrease ($P < 0.05$) was observed in 32°C female offspring compared to those at 25°C. Furthermore, there was a significant increase ($P < 0.05$) when comparing female offspring at 25°C, 32°C, and 39°C to their male counterparts at the same temperatures.

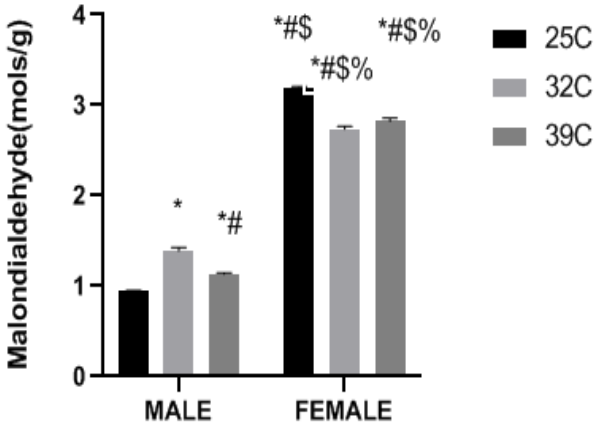

Figure 6:
Effect of maternal exposure to various environmental temperature during gestation on Malondialdehyde levels in male and female offspring of Wistar rats. Values are mean ± SEM for 5 animals per temperature group. $P < 0.05$. *P<0.05 compared to 25^0C male group; #P<0.05 compared to 32^0C male group; $P<0.05 compared to 39^0C male group; %P<0.05 compared to 25^0C female group.

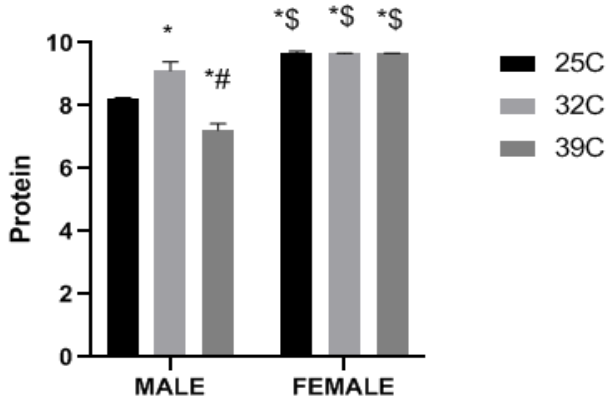

Figure 7:
Effect of maternal exposure to various environmental temperature during gestation on protein levels in male and female offspring of Wistar rats. Values are mean ± SEM for 5 animals per temperature group. $P < 0.05$. *P<0.05 compared to 25^0C male group; #P<0.05 compared to 32^0C male group $P<0.05 compared to 39^0C male group.

Effect of maternal exposure to various environmental temperature during gestation on protein levels in the kidney of offspring of Wistar rats: The statistical analysis of protein levels in Wistar rat offspring following maternal exposure to various temperatures revealed significant effects related to sex [$F(1, 24) = 157.4$, $P < 0.0001$], interaction [$F(2, 24) = 21.54$, $P < 0.0001$], and treatment [$F(2, 24) = 21.34$, $P < 0.0001$] (Fig. 7).

Post hoc analysis indicated that the protein levels in male offspring from the 32°C temperature group were significantly increased ($P < 0.05$) compared to those from the 25°C group. Conversely, the protein levels in the male offspring from the 39°C group were significantly decreased ($P < 0.05$) when compared to both the 25°C and 32°C male offspring. Furthermore, the post hoc analysis of protein levels in female offspring from the 25°C, 32°C, and 39°C groups showed a significant increase ($P < 0.05$) when compared to the male offspring from the 25°C and 39°C groups.

Effect of maternal exposure to various environmental temperatures during gestation on Superoxide Dismutase (SOD) activities in the kidney of offspring of Wistar rats
The statistical analysis of superoxide dismutase (SOD) activities in the offspring of Wistar rats, following maternal exposure to various environmental temperatures, revealed significant effects related to sex [$F(1, 24) = 430.4$, $P < 0.0001$], interactions [$F(2, 24) = 17.35$, $P < 0.0001$], and treatment conditions [$F(2, 24) = 12.11$, $P = 0.0002$] (see Figure 8).

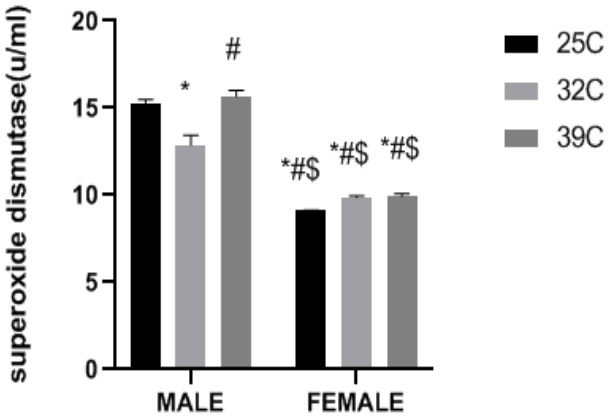

Figure 8:
Effect of maternal exposure to various environmental temperature during gestation on Superoxide Dismutase (SOD) levels in male and female offspring of Wistar rats. Values are mean ± SEM for 5 animals per temperature group. $P < 0.05$. #P<0.05 compared to 32^0C male group; *P<0.05 compared to 25^0C male group; $P<0.05 compared to 39^0C male group.

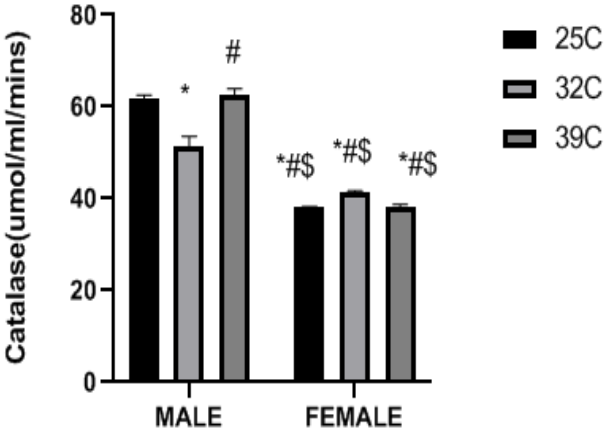

Fig. 9:
Effect of maternal exposure to various environmental temperature during gestation on catalase activity in male and female offspring of Wistar rats. Values are mean ± SEM for 5 animals per temperature group. $P < 0.05$. *P<0.05 compared to 25^0C male group; #P<0.05 compared to 32^0C male group; $P<0.05 compared to 39^0C male group.

Post hoc analysis indicated that SOD activities were significantly higher ($p < 0.05$) in male offspring from the 25°C and 39°C temperature groups compared to those from the 32°C group. In contrast, female offspring from the 25°C, 32°C, and 39°C groups exhibited a significant decrease ($p < 0.05$) in SOD levels when compared to all male offspring from the respective temperature groups.

Effect of maternal exposure to various environmental temperatures during gestation on catalase activities in the kidney of offspring of Wistar rats

The statistical analysis of catalase activities in offspring following maternal exposure to different environmental temperatures revealed a significant effect based on sex [$F(1, 24) = 409.8$, $P < 0.0001$], interaction [$F(2, 24) = 24.34$, $P < 0.0001$], and treatment (temperature) [$F(2, 24) = 7.436$, $P = 0.0031$] (Fig. 9).

Post hoc analysis indicated that catalase activity in male offspring at 32°C was significantly decreased ($p < 0.05$) compared to those at 25°C. In contrast, there was a significant increase ($p < 0.05$) in the catalase activity of male offspring at 39°C when compared to those at 32°C. However, no significant difference in catalase activity was observed between male offspring at 25°C and 39°C.

In female offspring, catalase activity was significantly reduced ($p < 0.05$) in all temperature groups (25°C, 32°C, and 39°C) when compared to their male counterparts at the respective temperatures.

Effect of Environmental Temperature during Gestation on the Histopathology and Histomorphometry of Kidney (Glomeruli Cells) in the Offspring of Wistar Rats: Histological examination of the kidney appears largely normal across all groups. In the renal cortex, renal corpuscles are distinctly outlined, with glomeruli displaying a normal appearance and surrounded by well-defined Bowman's space. The parenchyma of the renal cortex is populated by proximal and distal convoluted tubules, both of which exhibit normal morphology with intact cuboidal epithelial lining (Fig. 10).

Statistical analysis of the histomorphometry of the kidney in offspring subjected to maternal exposure to varying environmental temperatures revealed no significant effects based on sex [$F(1, 24) = 5.317$, $P = 0.301$] or treatment [$F(2, 24) = 0.6725$, $P = 0.5198$]. However, a significant interaction effect was observed [$F(2, 24) = 9.157$, $P = 0.0011$] (Fig. 11).

Post hoc analysis indicated a significant decrease ($P < 0.05$) in the glomeruli cell count of male offspring at 39°C when compared to their counterparts at 32°C. Additionally, female offspring at both 25°C and 39°C exhibited a significant increase in glomeruli cell count compared to the male offspring at 39°C.

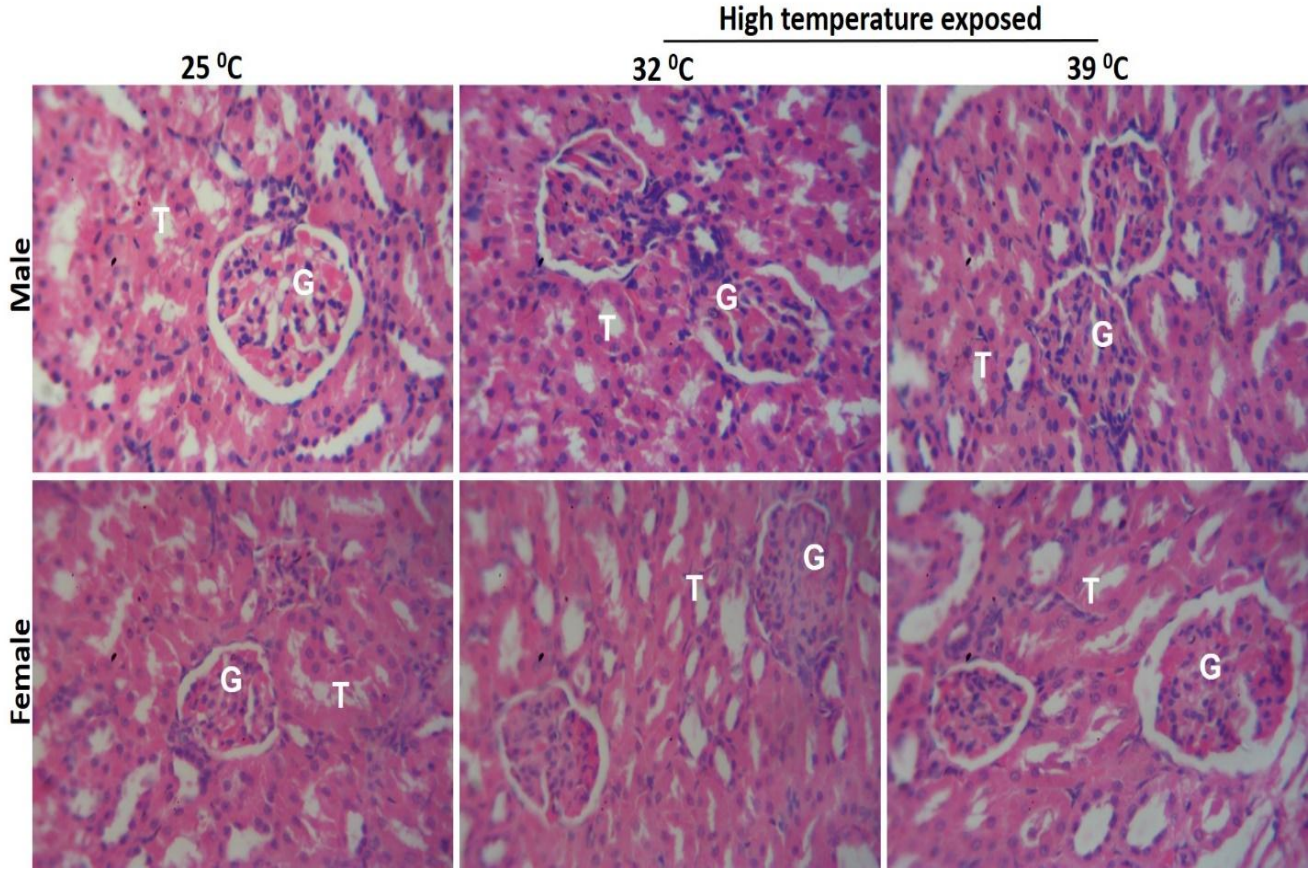

Plate. 10:
Histology of renal cortex of control and high temperature-exposed rats. H&E x 400. G – glomerulus; T – tubules.

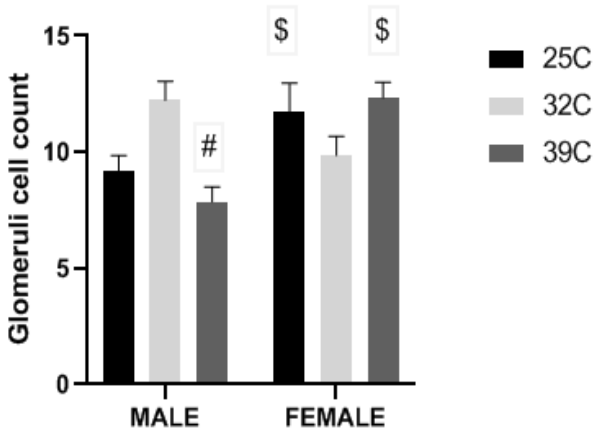

Fig. 11:
Effect of environmental temperature during gestation on histomorphometry of the kidney of offspring of Wistar rats. Values are mean ± SEM for 5 offspring per temperature group. P < 0.05. *P<0.05 compared to 25°C male group; #P<0.05 compared to 32°C male group; $P<0.05 compared to 39°C male group

Effect of maternal exposure to various environmental temperature during gestation on birth body weight of the offspring of Wistar rats.

The statistical analysis of birth weight suggest a significant reduction in birth in the male offspring exposed to 32°C and 39°C when compared with the 25°C male offspring. However, there no significant change in the birth weight of the female offspring exposed to various environmental temperatures.

However, in adulthood the significant reduction in body weight was only observed in male offspring exposed to 39°C during prenatal life.

Table 1:
The bdy weight of offspring following prenatal exposure to various environmental temperature

	Male Offspring			Female offspring		
Weight/ group	25ºC	32ºC	39ºC	25ºC	32ºC	39ºC
Birth weight (g)	4.3± 0.1	3.7±0 .02	3.3±0. 02*	2.2± 0.2	2.22± 0.1	1.9±0 .21
Weight at 12 weeks PND (g)	187± 2.4	179± 3.1	167±2. 46*	178± 2.2	181± 2.4	176± 3.4

*P<0.05 compared to 25°C group

DISCUSSION

This study provides insights into how prenatal elevated temperatures affect biochemical markers in both male and female offspring. The findings for male offspring raise important questions about the mechanisms behind increased serum creatinine and urea levels. Elevated serum creatinine, urea, and creatinine clearance levels suggest a potential metabolic response to higher temperatures, which may involve increased production rates despite the kidneys' ability to effectively clear these substances (Gounden et ak., 2024). References to heightened urea production or impaired clearance, as noted by Higgins 2016 and Gouden 2024, indicate a complex interplay between metabolism and kidney function that appears to vary with environmental stressors. This raises intriguing implications regarding how males might be physiologically adapting—or struggling to adapt—to higher prenatal-thermal conditions.

In contrast, the response observed in female offspring, where increased urine creatinine levels and clearance positively correlate with rising temperatures, suggests that their kidneys function more efficiently when exposed to this condition in utero. Increased urine creatinine levels and clearance in female offspring, positively correlating with rising temperatures, suggest a heightened efficiency of renal function n due to prenatal exposure to high environmental temperature. This contrasts with the generally more limited adaptive responses observed in male offspring. This might indicate sex-specific responses to prenatal environmental stress, potentially linked to hormonal differences or inherent physiological characteristics. Further exploration of these findings could involve examining the underlying mechanisms for these differing responses and how these metabolic changes might affect long-term health outcomes for both sexes.

In addition, increased MDA Level with a corresponding reduction in SOD and catalase activities in the male offspring of 32°C and 39°C suggest an increased exposure to oxidative stress in this tissue. However, in the female offspring, there was a reduction in MDA, SOD and catalase activities, this suggests a significant decrease in oxidative stress in this group. The study by Daenen *et al*. (2019) highlights the intricate relationship between oxidative stress and renal health. The results reinforce the idea that oxidative stress significantly influences the progression of renal disease, particularly due to the kidneys' high metabolic activity and vulnerability to oxidative damage.

Moreover, male offspring displayed significant variations in glomerular cell counts when compared with female offspring exposed to high environmental temperature during prenatal life Furthermore, the lack of significant changes in glomerular cell numbers in females points to possible sex-specific protective mechanisms against the adverse effects of thermal stress during gestation. The pattern of distribution of glomerular cell count suggests a possible association between the occurrence of oxidative stress and glomerular cell count. The reduced cell counts may be secondary to an increase in cell death associated with oxidative stress.

The study also highlights the physiological adaptations that occur during heat exposure. The hyperplastic response observed in male offspring at 32°C may reflect an evolutionary adaptation aimed at maintaining homeostasis. This mechanism might have been programmed during intra-uterine life in this offspring. However, the significant decrease in glomerular cell counts in the 39°C group raises concerns about the potential long-term implications for renal function, particularly under extreme temperature conditions. Exposure to a high environmental temperature as been previously reported to cause an increase in the occurrence of kidney diseases (Hansen *et al*. 2008; de Lorenzo and Liaño, 2017). The connection between heat exposure and conditions such as Acute Kidney Injury (AKI), Chronic Kidney Disease (CKD), and kidney stones underscores the urgency of understanding the effects of prenatal heat stress on kidney health.

In summary, the findings illustrate the complex interplay between gestational environmental factors, oxidative stress, and sexual dimorphism in renal responses. Understanding these relationships is crucial for developing preventative measures and therapeutic strategies to mitigate renal diseases linked to temperature fluctuations and oxidative stress exposure, especially in a changing climate where heat exposure is becoming more prevalent.

In conclusion, maternal exposure to increase environmental temperature during gestation might result in altered serum and urine analyte,glomeruli cell count and increased oxidative stress in the kidney of the offspring in a Wistar rats model.

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Full-length Research Article

Gastroprotective Activity of Low-Dose Vanadium in Streptozotocin-Induced Diabetic Rats: Roles of Gastric Acid, Mucous Cells and Oxidative Stress

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Summary: Vanadium, a heavy metal with insulin-mimetic properties and gastro-protective potentials, has been reported to protect the stomach of healthy rats against aggressive agents like acetic acid. In this study, we investigated the gastroprotective effects of low-dose sodium metavanadate ($NaVO_3$) in experimental diabetic rats. One hundred male Wistar rats (100-130g) were randomized into two experiments (normal and diseased) with 5 major groups (n=10) each. First experiment included normal (control) and non-diabetic groups treated with varying doses (20mg/kg/p.o, 40mg/kg/p.o, 60mg/kg/p.o and 80mg/kg/p.o) of sodium metavanadate ($NaVO_3$) only. The second experiment included diabetes-induced (65mg/kg/i.p Streptozotocin-STZ) and diabetic groups concomitantly treated with the same doses of sodium metavanadate as in the first experiment. Body weight and blood glucose level (BGL) were measured weekly. After 8 weeks of treatment, gastric acid secretion (GAS) was determined by the continuous perfusion method. Gastric tissue malondialdehyde (MDA), reduced glutathione (GSH), sulfhydryl, nitric oxide levels, Na+/K+ and H^+-K^+-ATPase pump activities were assessed spectrophotometrically. Gastric tissue histological examination and immunohistochemistry expression of gastric MUC5AC were evaluated. Data were analyzed using two-way ANOVA and were significant at $p < 0.05$. The BGL was significantly decreased in 20 and 40 mg/kg $NaVO_3$-treated groups in both non-diabetic and diabetic Wistar groups. Basal GAS significantly decreased in NaVO3-treated diabetic groups. Stimulation with acetylcholine significantly decreased GAS in NaVO3-non-diabetic treated groups. Gastric MDA and GSH were significantly reduced in 60 and 80mg/kg-$NaVO_3$ non-diabetic treated groups. Gastric sulfhydryl and nitric oxide levels were significantly reduced in 20 and 40mg/kg-NaVO3 non-diabetic treated groups. Treatment with $NaVO_3$ in diabetic groups significantly decreased gastric MDA and sulfhydryl but increased GSH and nitric oxide levels. Gastric H^+- K^+ ATPase and Na^+-K^+ ATPase pump activities significantly decreased in diabetic groups treated with 20, 40 and 60mg/kg-$NaVO_3$ compared with the untreated diabetes group. Gastric MUC5AC expression in $NaVO_3$ non-diabetic and $NaVO_3$-treated diabetic groups significantly increased compared with control and diabetes alone, respectively. Sodium metavanadate treatment dose-dependently reduced blood glucose levels and improved body weight in diabetic rats. It also modulated gastric acid secretion via the suppression of H^+/K^+-ATPase and Na^+/K^+-ATPase activities, reduced oxidative stress markers, enhanced antioxidant defences, and increased expression of gastric MUC5AC.

Keywords*:* Diabetes, vanadium, gastric acid, H+/K+ pump activities, MUC5AC.

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INTRODUCTION

The gastrointestinal tract is exposed to various substances as it is a route of entry into the body hence, its functions and secretions can be modulated by these substances. Gastric acid secretion is a major function of the gastrointestinal system that can be affected by external factors (heavy metals, drugs, etc.) through dose and route of administration of such stimulus and the synergistic action of other stimuli (body weight, hormones, neurotransmitters etc.) (Jaishankar *et al*., 2014; Omayone *et al*., 2020; Balali-Mood *et al*., 2021). These factors initiate a response from the nervous and peripheral regulating systems. Acetylcholine and gastrin primarily affect acid secretion by stimulating histamine release from the enterochromaffin-like (ECL) cell of the gastric mucosa to stimulate the parietal cell or by somatostatin, inhibiting histamine release as a response from the regulating systems. These independent pathways converge to activate/deactivate the gastric acid pump "H^+-K^+ - ATPase" responsible for the production of gastric acid (Prinz *et al*., 1992; Kim *et al*., 2021). Sodium potassium ATPase (Na+/K+ ATPase) and H+/K+ ATPase are classified as P-type ATPases because they form a high-

energy phosphorylated intermediate during the catalytic cycle (Morth *et al*., 2009). The possibility of a relationship between the two ATPases is consistent with findings that these pumps can substitute for each other in maintaining intracellular ionic homeostasis (Faraj *et al*., 2021). The potassium ions required for the H+/K+ ATPase to function are supplied by the Na+/K+ ATPase. Gastric acid secretion is facilitated by the H+/K+ ATPase's ability to exchange the potassium that the Na+/K+ ATPase pumps into the cell for hydrogen ions. The Na+/K+ ATPase prepares the way for the H+/K+ ATPase to secrete gastric acids. The high viscosity mucus that forms a protective mucous layer at the stomach lumen-surface epithelium is predominantly made up of mucins and numerous other glycoproteins. The primary gastric mucins in human stomachs are the cell surface mucin, MUC1, and the secreted mucins, MUC5AC and MUC6 (Muthupalani *et al*., 2019). Sulfhydryl groups and nitric oxide (NO) play significant roles in regulating gastric functions, particularly influencing the activity of parietal cells, the process of blood flow, mucus secretion, and response to chemicals/irritants (Nagl *et al*., 2007; Akinade *et al*., 2022).

Vanadium compounds have been a source of interest to researchers because of their potential as therapeutic agents for the treatment of various health issues, including diabetes, atherosclerosis, and cancer (Treviño *et al*., 2019; Gilbert *et al*., 2023). Vanadium has been reported to maintain mucosa integrity by decreasing gastric acid output and enhancing mucus activity in the pyloric ligation ulcer model (Suthar *et al*., 2007). It is also suggested to have protective activities against gastric ulceration by acting as a proton pump inhibitor, enhancing antioxidant enzyme activities as well as mucosal blood flow via increased NO mechanism (Omayone *et al*., 2016; 2020). The disturbances of the gastrointestinal tract caused by autonomic gastrointestinal neuropathy, which is a consequence of diabetes mellitus complications, manifest as gastroparesis, enteropathy, and cholecystoparesis. This is a major reason for the focus on the functional state of the stomach for early diagnosis of digestive changes that can aggravate the clinical course of the existing pathology (Sirchak *et al*., 2021).

There is a dearth of information on acid and mucus secretory responses in diabetic rats during vanadium treatment. This study investigated the gastric acid and mucus secretory responses of nondiabetic and diabetic rats treated with varying doses of sodium metavanadate.

MATERIALS AND METHODS

Animals: One hundred Male Wistar rats (110-130g) were used in the study. All rats received pellet chow and water *ad libitum*.

Induction of experimental diabetes: A single intraperitoneal injection of streptozotocin (STZ) at a dose of 65 mg/kg was used to induce diabetes (Junod *et al*. 1969). Animal with a fasting blood glucose concentration above 200 mg/dL was considered diabetic after 72 hours of induction.

Experimental design: The experimental protocol was reviewed and approved by the Animal Care and Use regulation Comittee of the University of Ibadan with Ethical approval number: UI-ACUREC/19/0025. The rats were randomly divided into 5 groups of 10 rats in 2 experiments.
Experiment 1 (non-diabetic): Group I- Control (normal rats); Group II- administered 20mg/kg/p.o $NaVO_3$; Group III- administered 40mg/kg/p.o $NaVO_3$; Group IV- administered 60mg/kg/p.o $NaVO_3$ and Group V- administered 80mg/kg/p.o $NaVO_3$.
Experiment 2 (diabetic): Group I- Diabetes alone (Diabetic untreated rats); Group II- Diabetic treated with 20mg/kg/p.o $NaVO_3$; Group III- Diabetic treated with 40mg/kg/p.o $NaVO_3$; Group IV- Diabetic treated with 60mg/kg/p.o $NaVO_3$ and Group V- Diabetic treated with 80mg/kg/p.o $NaVO_3$. Sodium metavanadate ($NaVO_3$) was administered daily by gavage for 8 weeks.
The body weight and fasting blood glucose level (BGL) of rats was measured daily. At the last day of the experiment, rats were fasted for 24 hours, gastric acid secretion was determined using the continuous perfusion method of Ghosh and Schild (1958), modified by Amure and Ginsburg (1964).
A midline laparotomy was made to expose the stomach and duodenum after the rats had been anaesthetized with pentobarbital (35 mg/kg i.p). A semi-transection was made at the junction of the pylorus with the duodenum where a pyloric cannula was inserted and ligated to collect gastric contents. The stomach was gently rinsed with isotonic saline (pH 7.0, 37^0C) that was introduced through an orogastric cannula until gastric effluent was clear. Thereafter, the animal was perfused at a rate of 1mL/minute and gastric acid was collected via the pyloric cannula at 10 minutes intervals. Gastric acid secretion was allowed to stabilize for about 50 minutes and the mean acidity of three gastric secretions was termed basal acid output. After the basal output collection, Acetylcholine was administered (0.5 mg/kg i.m) for the stimulated acid secretory response (Skliarov, 1995). To determine acidity, 10 mls of the stomach perfusate was titrated against 0.025M sodium hydroxide (NaOH) solution with phenolphthalein as indicator (Salami *et al*., 2017).

Biochemical assays: After STZ injections, blood samples from the rats were collected from the tail of both control and diabetic animals for evaluation of blood glucose levels using the glucose oxidase method. After the 8 weeks, stomach tissues were collected and stored in 4 volumes of ice-cold 0.1M phosphate buffer, pH 7.4 and homogenized. The resulting homogenates were centrifuged at 10,000g at 4^0C for 10 minutes and the supernatant was collected and processed for biochemical estimations of; lipid peroxidation (MDA) using the method of Varshney and Kale (1990), reduced glutathione (GSH) using the method of Beutler *et al* (1963), total sulfhydryl group using the method of Sedlak and Lindsay (1968), nitrite levels using the method of Ignarro *et al* (1987), gastric Na^+/K^+ and Proton-Pump ATPse activities using the method of Ronner *et al* (1977).

Histology of the Gastric tissue: A small section of the stomach was cut and fixed in phosphate buffer formalin. Hematoxylin and eosin (H&E) staining was done after tissue processing and slide mounting. The stained sections underwent morphological evaluation, and a microphotograph was taken to reveal any pathology or microscopic alterations (Salami *et al.,* 2018).

Immunohistochemistry method: Immunohistochemistry procedure as described by Salami *et al* (2024) for gastric MUC5AC with slight modification using 2-step plus Poly-HRP Anti Mouse/Rabbit IgG Detection System with DAB solution (Catalog number: E-IR-R217 from Elabscience Biotechnology®, China). Briefly, the processed histology waxed gastric samples were subsequently dewaxed using absolute xylene for and afterward, hydration in different progressing ethanol concentrations with 70% as the lowest. Antigen retrieval was performed on the hydrated slides with citrate buffer solution (pH 6.0) Endogenous peroxide was according to manufacturer's instructions on the kit (E-AB-51447) and incubated in a humidifying chamber. The slides were rinsed afterwards while Goat serum (E-1R-R217A) was added onto the slides to prevent nonspecific binding and returned into humidifying chamber and the gastric tissues were probed with primary antibodies [Gastric MUC5AC antibody (E-AB-40037)]. They were then incubated for 2 hours at room temperature and with a secondary antibody (E-1R-R217B) with drops of substrate diaminobenzidine (DAB) was added at room temperature in the dark. The slides were rinsed with deionized water and slides were immersed in haematoxylin before ethanol and then xylene. The dried slides had a DPX mountant applied on it before a cover slip was affixed on it. Sections were observed with a light microscope (Leica LAS-EZ®) using Leica software application suite version 3.4 equipped with a digital camera.

Statistical analysis: The data were expressed as Mean ± SEM and were analyzed using a Two-Way ANOVA by means of Graphpad prism version 8.0 (GraphPad software, San Diego, CA). Differences were considered significant at $p < 0.05$.

RESULTS

Effect of sodium metavanadate on the Body Weight of Normal and Diabetic Rats: Sodium metavanadate treatment in normal rats decreased percentage body weight compared with control (Figure 1). Diabetes caused a significant reduction in percentage increase in body weight but were significantly increased by sodium metavanadate treatment (Figure 2).

Effect of sodium metavanadate on the Blood Glucose Levels of Normal and Diabetic Rats: Blood glucose levels significantly decreased in the non-diabetic 20mg/kg and 40mg/kg sodium metavanadate treated groups (83.31±0.52, 82.22±0.47) compared with control (87.29±0.64) after 8 weeks of experimentation in the healthy rats (Figure 3). The 20 and 40mg/kg sodium metavanadate treated diabetic groups had a significantly lower blood glucose level (372.7±43.63, 374±44.52) while there was no significant difference in the blood glucose level of the 60 and 80mg/kg sodium metavanadate treated diabetic groups (465±57.89, 390.2±48.64) compared with Diabetes alone group (452.3±52.85) (Figure 4).

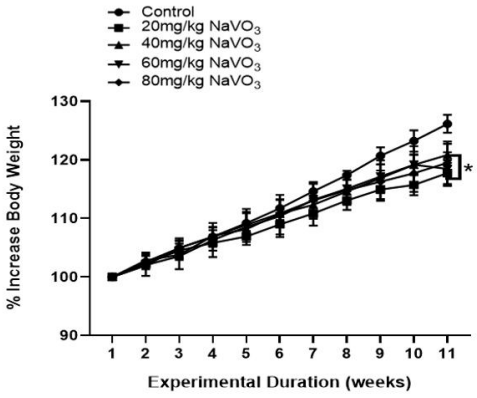


Figure 1
Effect of sodium metavanadate on percentage increase in body weight of normal (not-diabetic) rats. Values are represented as Mean ± SEM, *significant difference compared with control.

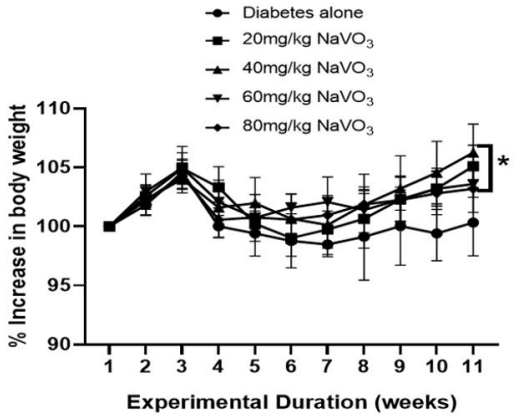


Figure 2
Effect of sodium metavanadate on percentage increase in body weight of diabetic rats. Values are represented as Mean ± SEM, *significant difference compared with diabetes alone. p=0.05

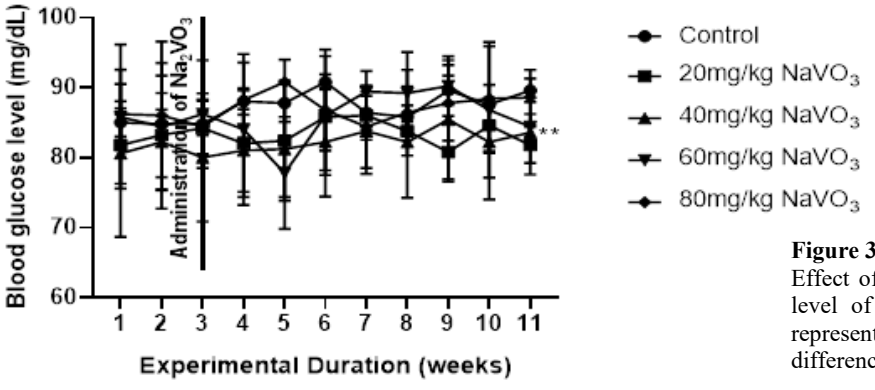


Figure 3:
Effect of sodium metavanadate on blood glucose level of normal (non-diabetic) rats. Values are represented as Mean ± SEM, *significant difference compared with control. p=0.05

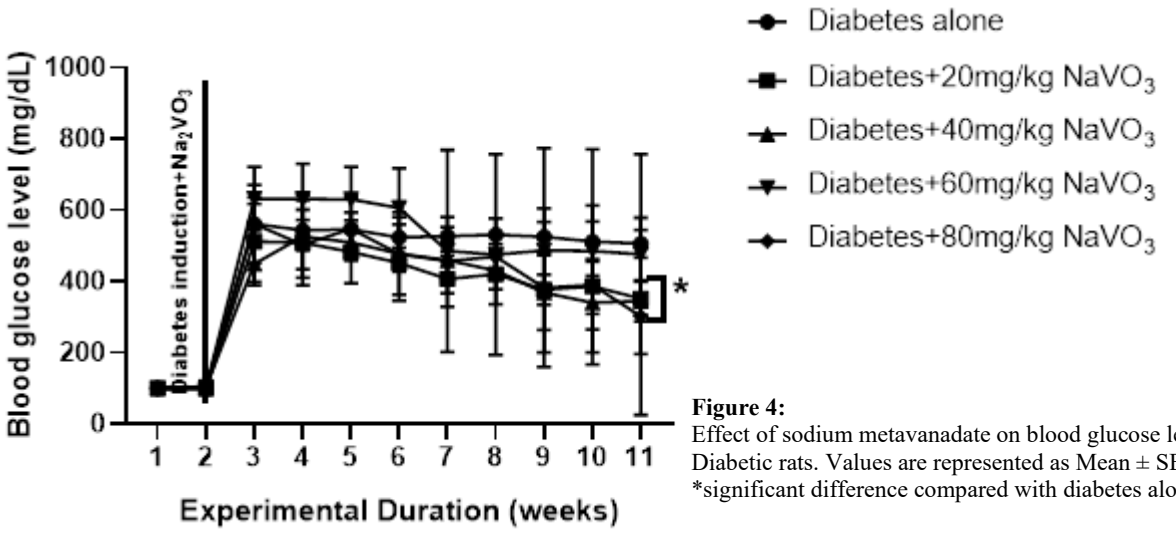


Figure 4:
Effect of sodium metavanadate on blood glucose level of Diabetic rats. Values are represented as Mean ± SEM, *significant difference compared with diabetes alone p=0.05

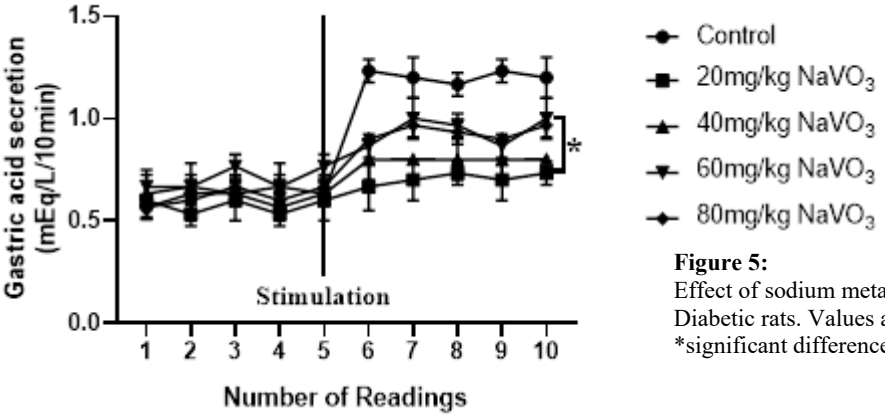


Figure 5:
Effect of sodium metavanadate on blood glucose level of Diabetic rats. Values are represented as Mean ± SEM, *significant difference compared with diabetes alone p=0.05

Figure 5:
Time-course of basal and acetylcholine-stimulated gastric acid secretion in Sodium metavanadate treated normal (not-diabetic) rats at 8 weeks**.** Values are represented as Mean ± SEM, *significant difference compared with control. p=0.05

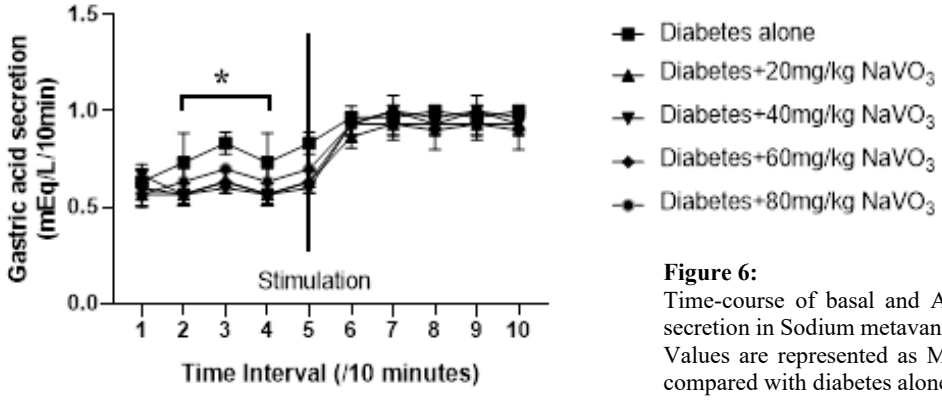


Figure 6:
Time-course of basal and Acetylcholine-stimulated gastric acid secretion in Sodium metavanadate treated diabetic rats at 8 weeks. Values are represented as Mean ± SEM, *significant difference compared with diabetes alone. p=0.05

Effect of sodium metavanadate on Basal/Stimulated Gastric Acid Secretion in Normal and Diabetic Rats: The action of sodium metavanadate on both basal and acetylcholine-stimulated gastric acid secretion in healthy rats is illustrated in Figure 5. There was no significant difference in the basal gastric acid secretion across the groups while stimulation with acetylcholine resulted in significant increase in gastric acid secretion. This was significantly decreased in groups administered sodium metavanadate (0.64±0.02, 0.72±0.03, 0.82±0.04, 0.78±0.05) compared with the Control group (0.91±0.10) (Figure 5).

Figure 6 shows the action of sodium metavanadate in diabetic rats on both basal and acetylcholine-stimulated gastric acid secretion. There was significant decrease in the basal gastric acid secretion of the groups treated with sodium metavanadate while stimulation with acetylcholine caused no significant difference in gastric acid secretion in groups treated with sodium metavanadate compared with the diabetic alone group (Figure. 6).

Effect of sodium metavanadate on Gastric MDA, GSH, Sulfhydryl and NO in Normal and Diabetic Rats: Gastric MDA significantly increased only in the 60 and 80mg/kg $NaVO_3$ treated non-diabetic groups compared to control. Gastric GSH activity significantly increased in the 20mg/kg $NaVO_3$ and decreased in the 60 and 80mg/kg $NaVO_3$ treated non-diabetic groups compared with control. Gastric sulfhydryl and nitrite levels significantly reduced in the 20 and 40mg/kg $NaVO_3$ treated non-diabetic groups compared with control (Table 1).

The 40, 60 and 80mg/kg $NaVO_3$ treated diabetic rats had a significantly lower MDA with a corresponding increase in GSH levels. Decrease in sulfhydryl levels and increase in nitrite levels of the 40, 60 and 80mg/kg $NaVO_3$ treated diabetic rats compared with diabetes alone (Table 1).

Effect of sodium metavanadate on Gastric Hydrogen Potassium ATPase (H^+/K^+ATPase) Pump Normal and Diabetic Rats: There was significant decrease in the activity of gastric H^+/K^+ATPase pump in groups administered sodium metavanadate (2103±8.73, 2511±32.19, 2252±55.15, 2357±43) compared with the control group (2747±41.12) at 8 weeks (Figure. 7). Gastric H^+/K^+ATPase pump activity in groups administered 20, 40 and 60mg/kg sodium metavanadate has a significant decrease compared with the Diabetic alone group while H^+/K^+ATPase pump activity was significantly increased in the 80mg/kg sodium metavanadate group (Figure. 8).

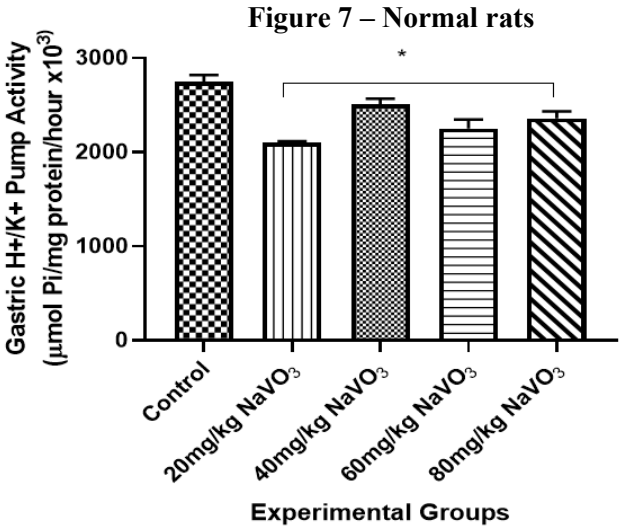
Effect of sodium metavanadate on Gastric Sodium Potassium ATPase (Na^+/K^+ATPase) Pump Normal and Diabetic Rats: There was no significant diifference in the activity of gastric Na^+/K^+ATPase pump in groups administered sodium metavanadate compared with the control group (Figure. 9). Gastric Na^+/K^+ATPase pump activity in groups administered 20, 40 and 60mg/kg sodium metavanadate has a significant decrease compared with the diabetic alone group while Na^+/K^+ATPase pump activity was significantly increased in the 80mg/kg sodium metavanadate group (Figure. 10).

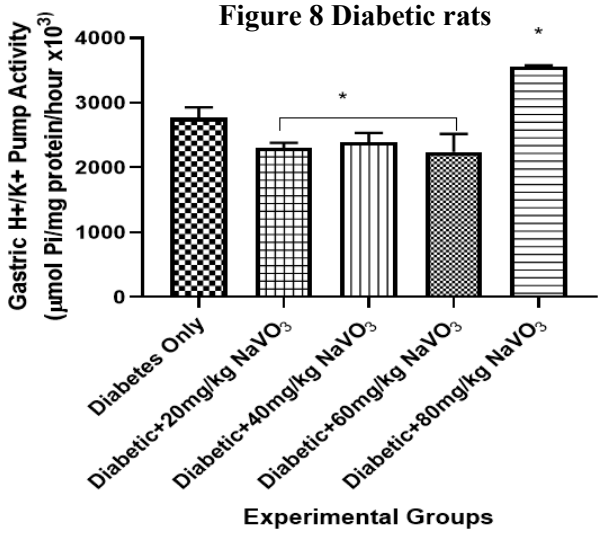
Effect of sodium metavanadate on gastric tissue histology in normal and diabetic rats: Mild sloughing off of the surface mucous cells was observed in the 60mg/kg $NaVO_3$ exposed group compared with the control (Plate 1). The histological architecture of the diabetic group showed degenerative damage compared with the NaVO3-treated groups.

Table 1:
Gastric MDA, GSH, Sulfhydryl and NO in Normal and Diabetic Rats treated with varying doses of Sodium metavandate.

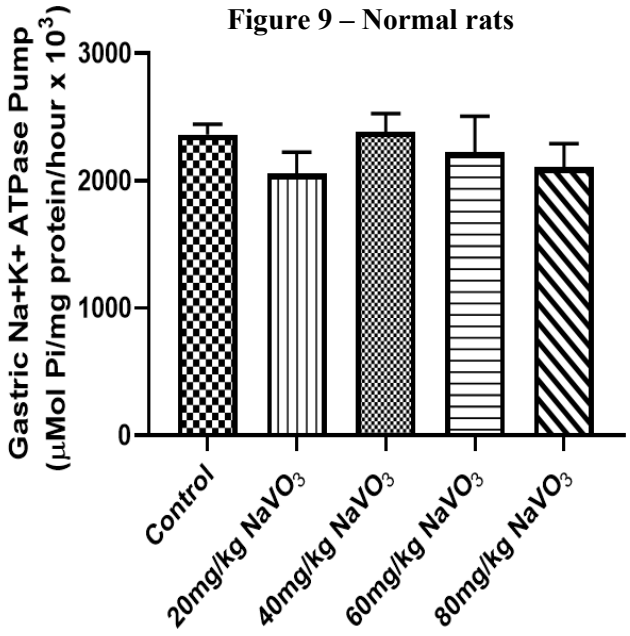
Healthy Rats	**Experimental Groups**				
Biochemical Parameters	Control	20mg/kg $NaVO_3$	40mg/kg $NaVO_3$	60mg/kg $NaVO_3$	80mg/kg $NaVO_3$
MDA (µmol/L)	1.17±0.08	1.33±0.11	1.34±0.04	1.96±0.14*	1.75±0.12*
GSH (mmol/L)	2.84±0.05	3.14±0.07*	2.70±0.04	1.95±0.07*	1.8±0.01*
Sulfhydryl (nM)	0.14±0.01	0.06±0.01*	0.07±0.01*	0.10±0.02	0.17±0.01
Nitrite (µg/g tissue)	197.8±4.17	122.3±13.19*	129.8±8.19*	192.3±5.28	169.8±5.28
Diabetic Rats	**Experimental Groups**				
Biochemical Parameters	Diabetes alone	Diabetes+20 mg/kg $NaVO_3$	Diabetes+40mg/kg $NaVO_3$	Diabetes+60mg/kg $NaVO_3$	Diabetes+80mg/kg $NaVO_3$
MDA (µmol/L)	5.97±0.41	5.15±0.25	1.89±0.25#	2.69±0.11#	1.97±0.25#
GSH (mmol/L)	1.98±0.01	2.66±0.03#	2.85±0.02#	2.78±0.06#	2.73±0.11#
Sulfhydryl (nM)	0.08±0.00	0.12±0.01#	0.06±0.00#	0.05±0.00#	0.04±0.00#
Nitrite (µg/g tissue)	79.64±1.81	115.6±6.94#	114.4±3.19#	178.5±6.11#	172.6±12.64#

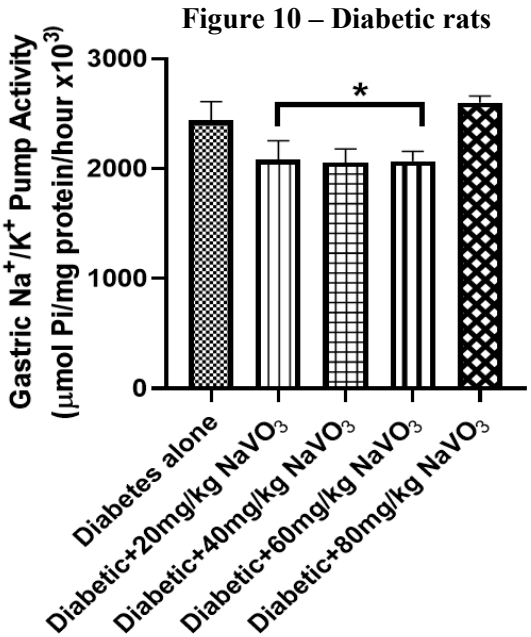
*Values are represented as Mean ± SEM, *significant difference when compared with control, #significant difference when compared with Diabetes alone. p=0.05*





Effect of sodium metavanadate on gastric H^+/K^+ATPase pump activity in normal rats (Figure 7) and Diabetic rats (Figure 8). Values are represented as Mean ± SEM, *significant difference compared with control. p=0.05





Effect of sodium metavanadate on gastric Na^+/K^+ATPase pump activity in normal (Figure 9) and Diabetic rats (Figure 10). Values are represented as Mean ± SEM, *significant difference compared with control. p=0.05

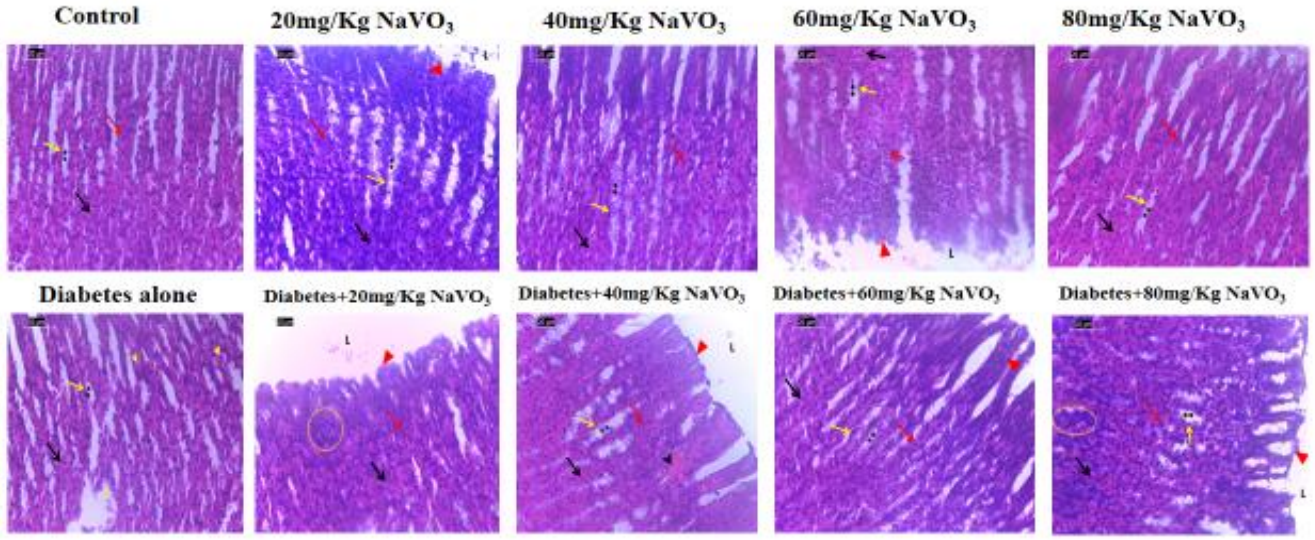


Plate 1
Effect of vanadium on the histology of gastric tissue in normal and diabetic rats
(** = gastric gland; black arrow = parietal cells; red arrow = chief cells; yellow arrow = mucous neck cells; red triangle = epithelial lining; red circle = inflammatory cells). H & E x400

Effect of sodium metavanadate on *Gastric MUC5AC expression* in normal and diabetic rats.

The level of gastric MUC5AC expression in the $NaVO_3$ exposed groups significantly increased compared with control in normal (non-diabetic) rats, as represented in plate 2. Expression of MUC5AC was significantly increased in the $NaVO_3$-treated diabetic groups compared with diabetes alone (Plate 2).

DISCUSSION

Vanadium has been a subject of interest for researchers because of the various effects it has, which can be beneficial or adverse. Its role in the management of diabetes mellitus as reported gave it a popularity that has increased the curiosity about this element and its role in the body (Dai *et al.,* 1994; Gilbert *et al.,* 2023). The decrease in body weight of the normal (not-diabetic) $NaVO_3$ treated groups corresponds with the work of Domingo *et al.,* (1996) where sodium metavanadate administered for 64 days decreased body and epididymis weights (Domingo *et al.,* 1996; Dabros *et al.,* 2006). A decrease in body weight gain is frequently observed as an indicator of diabetes mellitus. This weight loss is often associated with the development of diabetes as observed in the diabetic rats (Markholst *et al.,* 1993). However, treatment with $NaVO_3$ increased the body weight of the diabetic rats, indicating the potential therapeutic effect of $NaVO_3$ in mitigating diabetes-induced changes.

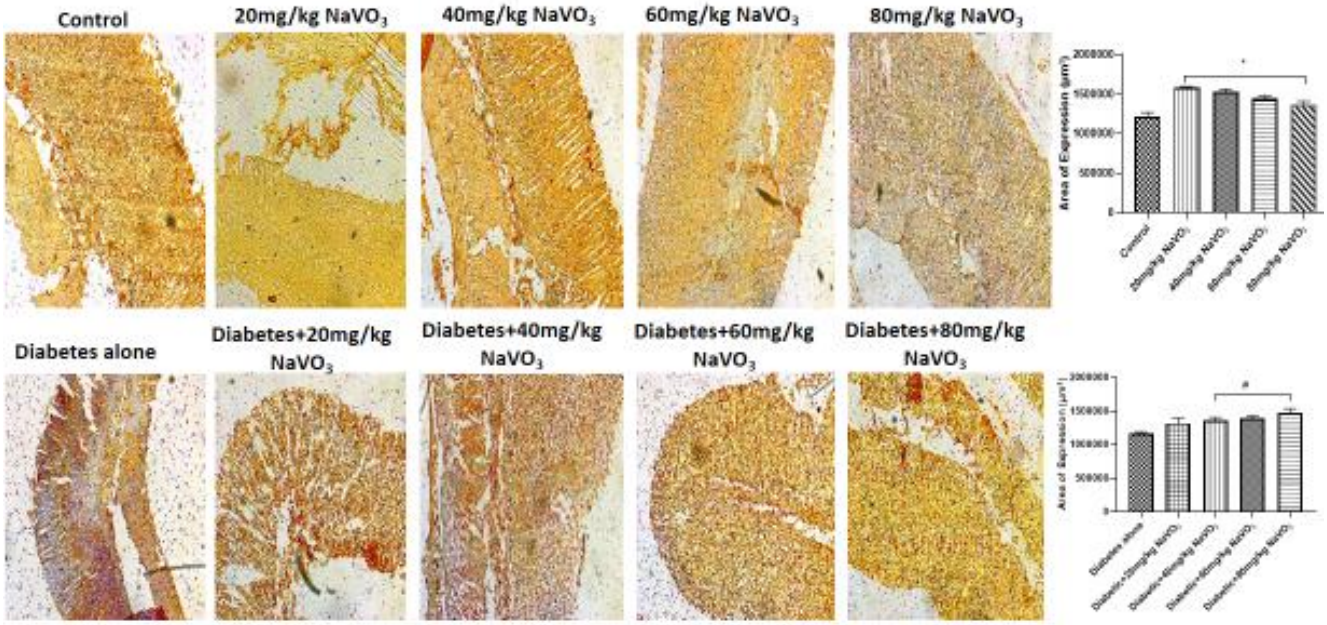


Plate 2
Effect of NaVO3 on expression of gastric MUC5AC in non-diabetic and diabetic rats (x100). The intensity of MUC5AC immunoreactivity is seen within the gastric mucosa and quantified in relation to the area of expression.

Blood glucose level was reduced in 20 and 40 mg/kg sodium metavanadate treated groups to corroborate previous studies on the glucose lowering properties of vanadium (Brichard *et al.,* 1990, Meyerovitch *et al.,* 1991; Domingo and Gomez 2016; Adam *et al.,* 2017; Dayanand *et al.,* 2024).

The secretion of gastric juice is affected by various stimulatory and inhibitory factors arising from the central nervous system and within the gastrointestinal system itself (Yao and Forte, 2003), and one of such stimulatory factors is acetylcholine, which is of the neural pathway; others include histamine and gastrin. Acetylcholine can be released by vagal and intramucosal reflex stimulation and then act directly on the parietal cell (Szelenyi, and Vergin, 1980; Furness *et al.,* 2020). This can be mediated via an increase in cytosolic levels of calcium ions, but strong synergism exists between histamine and either gastrin or acetylcholine through post-receptor interaction between the distinct pathways. All these pathways converge on and modulate the activity of the luminal enzyme, H^+/K^+ ATPase, ultimately responsible for acid secretion. (Schubert and Shamburek, 1990). Gastric H^+/K^+ ATPase is known to transport H^+ against the concentration gradient and it's the final step of acid secretion, which suggests that an inhibition of the pump will be more effective in suppressing gastric acid secretion than the receptor antagonist (Schubert and Peura, 2008). However, gastric Na^+/K^+ ATPase establishes the electrochemical gradient that is necessary for H^+/K^+ ATPase to function properly (Morth *et al.,* 2009). Hence, the decrease in the activity of gastric Na^+/K^+ ATPase in the NaVO3-treated diabetic groups explains the observed modulated reduction of gastric H^+/K^+ ATPase, unlike the diabetic untreated group, leading to the reduction in gastric acid secretion observed in this study. Disturbances to the gastrointestinal tract functions are common in diabetes mellitus. Streptozotocin (STZ) induced diabetes exhibits different levels of acid output, which are obviously dependent on the time interval after diabetes induction in rat studies (Takeuchi *et al.,* 1994). Administration of acetylcholine increased gastric acid secretion in non-diabetic experimental animals in this study; however, exposure to sodium metavanadate inhibited this increase. Activity of the gastric H^+/K^+ ATPase pump was reduced as an indication of inhibition of the activity of the cholinergic pathways and its direct effect on the parietal cells. Omayone *et al.* (2016) reported that vanadium reduced basal and histamine-stimulated gastric acid secretion in pylorus ligated animals through its probable role as a proton pump inhibitor. Basal gastric acid secretion possesses a complex, multifactorial control system. Adrenergic agonists, serotonin, secretin and somatostatin are the potent endogenous inhibitors of gastric secretion (Bech, 1986), therefore, the reduction in basal gastric acid secretion of $NaVO_3$ treated diabetic groups might probably be due to the effect of sodium metavanadate in modulating any of these pathway (Ozcelikay *et al.,* 1993; Adewoye *et al.,* 2007). Tashima *et al*. (2000) explained that vagus nerve stimulation diminished rather than increased the acid output among diabetic rats. Acetylcholine stimulation of gastric acid secretion in diabetic animals treated with sodium metavanadate resulted in no response as observed in the secretion of gastric acid which suggests the dysfunction of the cholinergic system via the vagal nerve due to the diabetic vagal neuropathy (Chang *et al.,* 2002). Burghen *et al*. (1992) pointed out that diabetic children or adolescents, especially uncontrolled patients, were at risk of developing peptic ulcer diseases. Sodium metavanadate action as a potential therapeutic agent for the management of diabetes mellitus can be coupled with its role as a proton pump inhibitor.

Heavy metals can activate lipid peroxidation in the gastric tissue, especially concerning the dose and duration of exposure (Balali-Mood *et al.,* 2021). This occurs through various mechanisms, including disrupting the gastric mucosal barrier, promoting inflammation, and generating reactive oxygen species (ROS), leading to oxidative damage

and potentially contributing to gastric tissue damage (Fernandes *et al.,* 2012). Vanadium at the lower doses (20 and 40mg/kg $NaVO_3$) posed no threat to lipids, unlike the higher doses (60 and 80mg/kg $NaVO_3$) that increased the levels of the marker of oxidation (MDA) in the gastric tissues. This might have accounted for the mild sloughing off of the surface mucous cells in the histology of the normal rats. Diabetes mellitus is a disease characterized by hyperglycaemia, depletion of antioxidants (increased generation of reactive oxygen species and decreased antioxidant levels in the body) and alteration in lipid metabolism (Hink *et al*., 2001). This was corroborated by the increase in MDA levels in the diabetic rats, while treatment with vanadium reduced lipid peroxidation. Reduced glutathione (GSH) is a very important antioxidant that protects gastric tissues against oxidative damage, especially lipid peroxidation (Kwiecien *et al.,* 2014). GSH can be oxidized to glutathione disulfide (GSSG) when it reacts with reactive oxygen or nitrogen species, and it can also be involved in the reduction of disulfide bonds (formed when sulfhydryl groups are oxidized) (Fitzpatrick *et al*., 2011). The increased GSH activity explains the protective effect of vanadium on the gastric mucosa at lower doses of vanadium in healthy rats. The diabetic rats had a decrease in GSH activity, but treatment with vanadium at all dosages increased antioxidant activity. This might have caused an improved mucosal protection and reduced damage in mucosal architecture as seen in the histology of treatment groups. To prevent acid, inflammation, and other irritants from damaging the stomach mucosa, sulfhydryl groups are essential. They are antioxidants, aid in mucus secretion, and take part in a number of cellular communication pathways that support the health of the stomach (Komolafe *et al.,* 2025). Nitric Oxide (NO) as a signalling molecule reduces gastric acid secretion by increasing the levels of cGMP in the parietal cells (Berg *et al.,* 2005), reduces inflammation, and influences gastric blood flow and mucus secretion (MacNicol and Pearson, 2021). The oxidative inactivation of NO is often measured as either nitrite (NO^{2-}) or nitrate (NO^{3-}) due to the difficulty of measuring NO levels in biological fluids. The reduction in the Sulfhydryl and NO levels in the groups with lower doses of vanadium (20 and 40 mg/kg $NaVO_3$) corroborates the results seen in the gastric acid secretion and antioxidant studies in the normal rats. This is an indication that NO is being rapidly consumed by molecules (GSH and sulfhydryl) or pathways that is helping in reducing oxidation and inhibiting acid secretion as described by Atakisi *et al.,* (2010).

The increase in the expression of MUC5AC corroborates the rapid use of NO to mediate the increase in gastric mucus release. Diabetes is associated with reduced NO, leading to endothelial dysfunction, disturbances to blood flow, and inflammation. Vanadium as a treatment increased nitrite levels in the diabetic rats, and this resulted in a decrease in gastric acid secretion and an increase in MUC5AC expression, which helped in the preservation of the histological architecture observed in this study.

In conclusion, this study provides compelling evidence for the dual role of sodium metavanadate ($NaVO_3$) in metabolic, gastric regulation and protection, particularly in the context of diabetes mellitus. $NaVO_3$ improved body weight and lowered blood glucose levels in diabetic rats, supporting its insulin-mimetic properties. It also modulated gastric acid secretion by inhibiting H^+/K^+ ATPase activity, potentially through its impact on the Na^+/K^+ ATPase activity, cholinergic pathway and vagal nerve function. Additionally, $NaVO_3$ treatment reduced oxidative damage by lowering malondialdehyde (MDA) levels and enhancing antioxidant markers such as glutathione (GSH), sulfhydryl groups, and nitric oxide (NO). However, higher doses of $NaVO_3$ in non-diabetic rats triggered mild gastric mucosal alterations, indicating possible oxidative stress. Importantly, the upregulation of gastric MUC5AC expression across treated groups suggests enhanced mucosal protection. These findings position $NaVO_3$ as a promising candidate for adjunct diabetes therapy, with added gastroprotective benefits. Nonetheless, its dose-dependent effects highlight the importance of cautious therapeutic application and the need for further studies to explore its long-term safety and mechanistic pathways.

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Full-Length Research Article

Hesperidin Nanoparticles Prevent Scopolamine-Induced Cognition Impairment Through Amplification of Antioxidant Defense System and Cholinergic Neurotransmission in Mice

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Summary: The rapid increase in the aging population and age-linked cognitive impairment, as well as dementia of Alzheimer's type, are becoming more prevalent globally. Genetic and environmental interactions played a key role in dementia pathology. Oxidative stress and cholinergic disruptions are well-linked with dementia. Hence, phytochemicals with neuroprotective and antioxidant properties could help ameliorate mitochondrial dysfunction and toxic effects misfolded amyloid-beta and tau proteins in dementia of Alzheimer's type. Previous studies have alluded to the beneficial action of hesperidin in mild cognitive impairment, but delivery could be better enhanced in nanoparticulate form. Hence, this study sought to investigate the memory-enhancing ability of hesperidin nanoparticles (HES_) on scopolamine-induced memory impairment in mice. Mice were randomly assigned into 6 groups (n=6) and treated as follows; vehicle only, vehicle + SCOP (1mg/kg, i.p.), HES (1,10 and 50mg/kg, p.o., respectively) + SCOP and donepezil (1mg/kg; p.o.) + SCOP for 14 consecutive days followed by behavioral assessment for memory function using open field test, Y-maze, novel object recognition and Morris water maze for locomotion, working, cognition and spatial learning, respectively. The animals were euthanized and brain samples were collected for biochemical assays (oxidative stress markers and acetylcholinesterase activity). SCOP or HES administration did not affect locomotor activity; however, SCOP reduced the percentage of alternation behaviour in the Y-maze and discrimination index in NOR tests with no significant change in escape latency time in the MWM task, indicative of working memory, cognition and spatial learning impairment. In contrast, the pre-administration of HES produced a dose-dependent and significant increase in working memory, cognition and spatial learning abilities. Similarly, HES_ pretreatment reduced scopolamine-induced increases in lipid peroxidation and acetylcholinesterase activity and deficit in antioxidant enzyme activity in the hippocampus and prefrontal cortex caused by SCOP. The results of the present study further showed the potential of hesperidin in nanoparticle form in the enhancement of memory formation through the amplification of antioxidant defense and cholinergic neurotransmission.

Keywords: *Alzheimer of dementia type; hesperidin nanoparticles; oxidative stress; scopolamine; memory.*

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INTRODUCTION

Cognitive impairment is a decline in memory and other cognitive functions and can also manifest as an underlying condition including Alzheimer's disease (AD) and aging. Cognition is a mental action and the part of the central nervous system that plays a pivotal role in normal cognition is the brain's cholinergic system. The cholinergic system has also been implicated in the pathology of AD, age-related cognitive decline, and mild cognitive impairment (Ishola *et al*., 2020; Sultzer *et al*., 2022). AD, the most common form of dementia, is a genetic and sporadic neurodegenerative disease characterized by the progressive loss of neurons, leading to severe impairments in cognitive functions (Briggs *et al*., 2016). According to WHO (2023), more than 20 million people worldwide between the ages of 65 years and above have AD and over 60% of them reside in low and middle-income countries (WHO, 2023). Furthermore, evidence from various studies has linked oxidative stress to brain aging and AD as oxidative stress mediates an increase in amyloid precursor protein (APP) expression and secretase activities, which contribute to the pathological hallmark of AD: amyloid-β peptide accumulation and associated neurodegeneration (Ishola *et al*., 2017; Cheignon *et al*., 2018; Ionescu-Tucker *et al*., 2021; Chae *et al*., 2022). The general pathology of cognitive deficits is damage to neuronal tissue, of which oxidative stress plays a significant role. AD continues to pose a global healthcare challenge,

placing heavy economic and social strains on families and societies, despite progress in understanding its underlying mechanisms.

Despite significant pharmacological advancements, definitive cures for these diseases are still lacking, prompting increased interest in naturally-derived substances for potential preventive benefits. The potential of antioxidants (especially from natural origin) to mitigate cognitive aging has caused an emerging interest in their therapeutic potential for disease management (Morris *et al*., 2002; Tamilselvam *et al*., 2013; Almukainzi *et al*., 2024). Flavonoids, found in various plant-based foods (fruits, vegetables and leaves), offer medicinal benefits, including antioxidant, and anti-inflammatory properties. Reactive oxygen species (ROS)-driven oxidative stress in the hippocampus and prefrontal cortex underlies aging-related cognitive decline and AD development, making antioxidants a potential therapeutic target (Ishola *et al*., 2020; Chae *et al*., 2022). Hence, this study was designed to investigate the potential benefit of hesperidin in scopolamine-induced memory impairment in mice. Hesperidin is a flavonoid found in citrus fruits such as oranges and possesses a variety of biological activities including antioxidant properties (Almukainzi *et al*., 2024), anti-inflammatory properties (Tamilselvam *et al*., 2013), and anti-apoptotic activities (Ikram *et al*., 2019). Thus, making hesperidin a potential and promising neuroprotective agent (Hong and An, 2018; Moghaddam and Zare, 2018; Almukainzi *et al*., 2024). Despite hesperidin's therapeutic potential, its limited bioavailability and solubility restrict its absorption, emphasizing the need for a targeted delivery mechanism to optimize its therapeutic efficacy. Hence, in this study, hesperidin nanoparticles were used to improve its potential therapeutic benefits in scopolamine-induced cognitive impairment.

Scopolamine acts by blocking muscarinic receptors, impairing learning and memory in animal studies, making it a useful research tool for investigating the memory-enhancing properties of new therapeutic agent (Ishola *et al*., 2020; Chae *et al*., 2022; Ishola *et al*., 2023; Al-Tawarah *et al*., 2023). Evidence from previous studies has shown that scopolamine disrupts short-term or long-term spatial working memory, however, acetylcholinesterase inhibitors (AChEIs), like donepezil, reverse its activity (Sheng *et al*., 2018; Ishola *et al*., 2020; Chae *et al*., 2022). This underscores the urgent need for safe and effective AD treatments.

MATERIALS AND METHODS

Materials: Donepezil, scopolamine, ethanol, and hesperidin were obtained from Sigma Aldrich (MO, USA), and other reagents used in this experiment are of analytical grade.

Synthesis of water-dispersible Hesperidin Nanoparticles: The micro-emulsion method described by Ali *et al* was employed to synthesise hesperidin nanoparticles (HES) with some modifications. In a typical synthesis, 1 g (0.012 mol) of polyvinyl acetate (PVAc) was added to 15 ml of distilled water and heated to about 65 °C to dissolve the polyvinyl acetate. Then, 0.25 g of the PVAc solution was measured, added to 30 mL of dichloromethane, followed by 20 mL of acetone to form homogeneous solution and lastly 0.25g (0.00041 mol) of bulk hesperidin was added. The mixture was then sonicated for 30 mins to form an emulsion. The primary emulsion was then injected into 10 ml of the prepared Bovine serum albumin (BSA) solution (0.015mmol) and sonicated for about 15 mins. To disperse the final oil/water emulsion and remove the residual organic solvent in the solution, 15 ml of deionized water was added and stirred overnight. The mixture was centrifuged at 6000 rpm for 30 mins. The supernatant was discarded and the obtained nanoparticles were washed with deionized water and centrifuged at 6000 rpm for 5 mins. The resulting nanoparticles were dried and kept at room temperature.

Laboratory animals: Adult mice used in this study were purchased from the Laboratory Animal Centre, College of Medicine, University of Lagos, Lagos State, Nigeria. The animals were housed in well-aerated plastic cages and well-fed (Livestock Feeds, Lagos, Nigeria) and water ad libitum. This study was carried out following ethical approval obtained from the Animal Care and Use Research Ethic Committee of the College of Medicine, University of Lagos (CMUL/ACUREC/01/20/7810). In accordance with the ARRIVE (Animal Research: Reporting of in vivo experiments) guidelines for reporting animal research.

Experimental Procedure: Mice were randomly assigned into 6 groups (n=6) and treated as follows; groups 1 and 2 received normal saline (10 ml/kg, p.o.), respectively, group 3- donepezil (1mg/kg; p.o.) and groups 4-6 received graded doses of HES (1,10 and 50mg/kg, p.o., respectively) for fourteen (14) days. Scopolamine (SCOP) administration commenced from day 3 to day 14, mice in groups 2 to 6 received SCOP (1mg/kg, i.p) one hour after pretreatment with vehicle or graded doses HES. Thereafter, animals were subjected to behavioral tests.

Behavioral Assessments

Open field Test (OFT): The Open Field Test (OFT) assesses locomotion, anxiety, and exploration in laboratory animals using a 96cm × 96cm × 45cm wooden arena divided into 16 squares (18 × 18cm) by black lines (Ishola *et al*., 2019). Each mouse placed at the centre point of the apparatus was allowed an acclimatization period of 60 s. Afterwards, the total number of rearing, line crosses and grooming behaviours were recorded for 5 mins. The apparatus was cleaned with 10 % ethanol and allowed to dry after each mouse.

Y-Maze Test: The Y Maze Test is a behavioral assay used to measure spontaneous exploration and short-term memory capabilities (Ishola *et al*., 2020). The Y-maze apparatus is Y- shaped (wooden) with arms labelled A, B, and C. Each animal was placed in the mid-point of the maze, and the number of arm entries and spontaneous alternations (sequence of entries [ABC, BAC, CBA]) were observed and recorded.

$$\% \text{ Spontaneous alternation: } \frac{\text{Number of alternation}}{\text{Number of entries- 2}} \times 100$$

Novel Object Recognition test (NORT): The Novel object recognition test is used to evaluate memory performance in mice based on the natural tendency of the mice to explore novel objects. This test was carried out using an open field area (60 cm x 50cm x 40cm). The animals were allowed to interact with the familiar object for 5 minutes before testing. This test was in two phases, namely, the trial phase and the test phase. During the trial phase, each mouse was positioned in the middle of two identical objects (A and B) in the open field arena for 5 minutes. Afterwards, the animals were returned to their cages for 1 hour. In the test phase, object B was replaced with object C, which was novel to the mice and different from objects A and B. Then, the mouse was left to explore objects A and C for a period of 5 minutes. The apparatus was cleaned after each test, and the time spent exploring each object was recorded in both phases. The preference index was calculated as the difference in exploration time of novel objects and familiar objects divided by the total amount of time spent with both objects (Ennaceur, 2010)

$$\text{Preference index (PI)} = \frac{T1}{T1 + T2}$$

Morris Water Maze Task (MWM): MWM is a behavioral test protocol used to assess spatial memory and learning in laboratory animals. The apparatus is made of a circular black water tank (110 cm diameter and 60 cm height) to a depth of 30 cm. The tank was partitioned into four sections (North, East, West, and South) with a submerged platform in the Southwest quadrant serving as an escape route. The mice were allowed a maximum of 60 s to find the hidden/submerged platform and were allowed to stay on it for 10 s. The escape latency time (ELT), defined as the time it takes for the mouse to locate the escape platform, was recorded. Mouse unable to locate the platform within 1 minute were gently guided to it and allowed a 10-s recovery time. Three trials were conducted each day for four days (days 10-13). On the 5th day (day 14), a probe test was done, during which the escape platform was removed from the tank, before placing the animal into it and each animal was given 30 s to search for the platform. The time spent around the area where the platform was initially located was recorded (Ishola *et al*., 2013; Ishola *et al*., 2019).

Dissection: After the probe test, the animals in each group were anaesthetized with ketamine, then perfused with cold normal saline and brain was rapidly removed, and hippocampus was then dissected on iced pack, weighed and kept in 0.1× PBS (pH 7.4) at −20 °C until biochemical analysis.

Biochemical Analysis: The extent of lipid peroxidation was evaluated by measuring MDA levels through the TBA spectrophotometric assay procedure as described by Ishola *et al*. (2017). The content of reduced glutathione (GSH) in brain tissue was determined as non-protein sulphydryl, following a previously described protocol by Sedlak and Lindsay (1968). The activity of superoxide dismutase (SOD) was assayed according to the method described by Nauseef *et al*. (2014). Catalase activity was also determined according to the method described by Sinha *et al*. (1972) while the acetylcholinesterase (AChE) activity in the hippocampal homogenate was quantified using the protocol of Ellman *et al*. (1959).

RESULTS

Characterization of Hesperidin: The FTIR spectra of hesperidin nanoparticles capped with PVAc is shown in Figure 1. The absorption band at 3414 and 1644 cm-1 correspond to O–H stretching vibration, C–H stretching, respectively while 1603 and 1517cm-1 are attributed to aromatic C=C stretch. At 1439, 1297, and 1096 cm-1, the peaks observed are attributed to C-H bend, C-O-C stretching of aryl ether, and C-O stretching of secondary alcohol respectively. The peak at 2919 cm-1 was attributed to C–H stretching from the PVAc. The SEM images revealed that the nano hesperidin is composed of agglomerated granules in the 2 to 10 µm size, as shown in Plate 1.

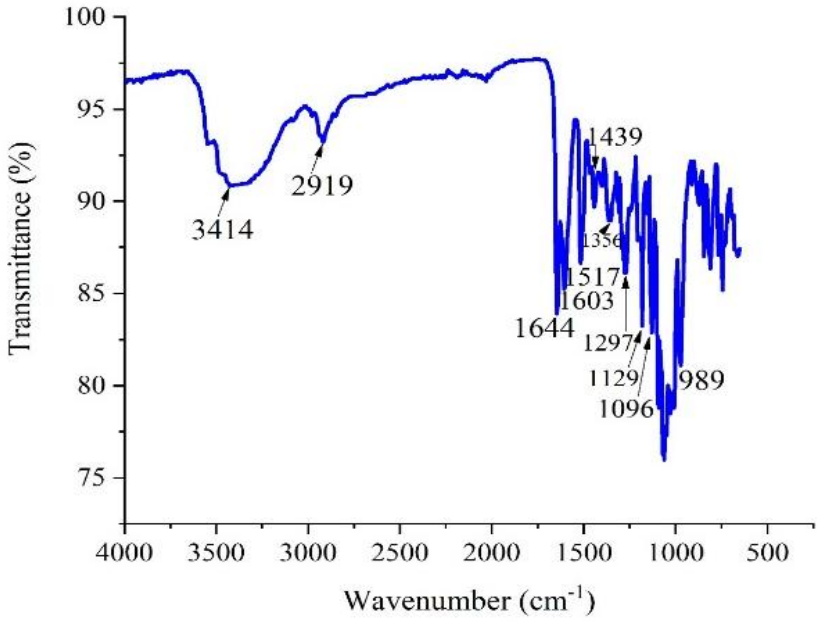

Figure 1:
FTIR spectrum of hesperidin nanoparticles capped with polyvinyl acetate.

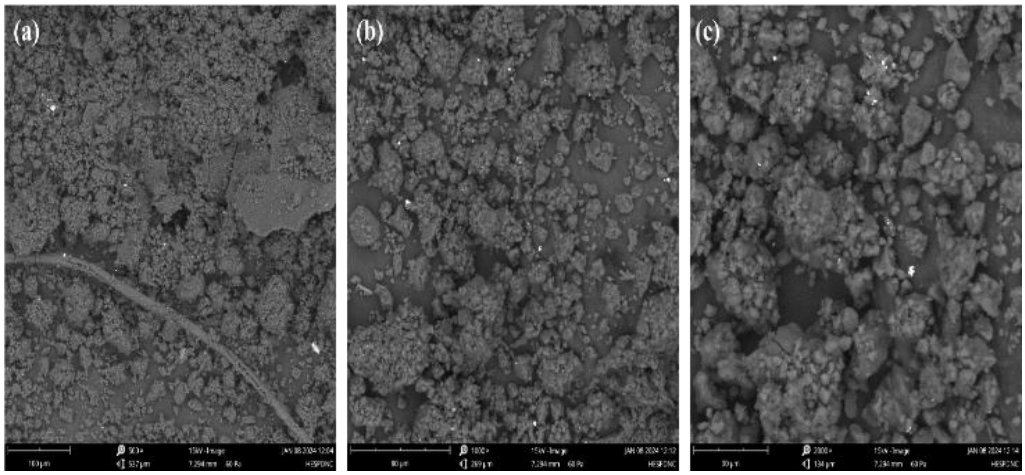

Plate 1:
SEM images at different magnifications (a) 100 µm, (b) 80 µm and (c) 30 µm

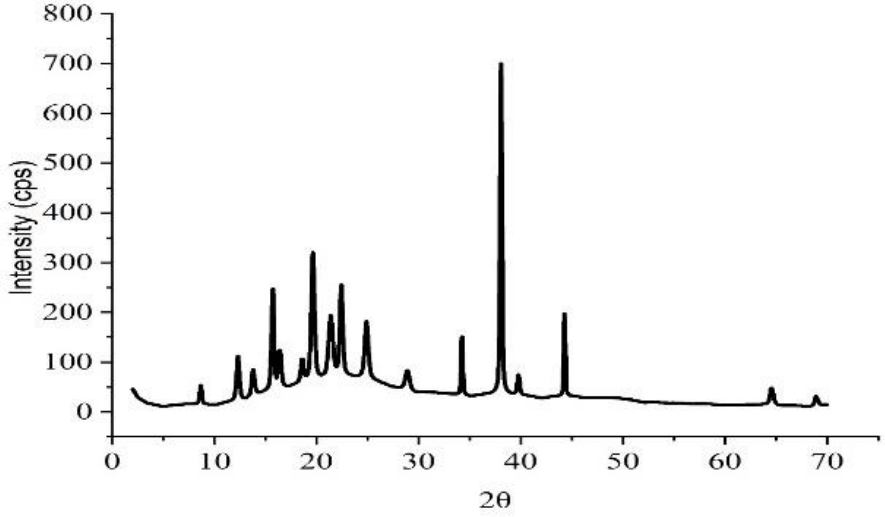

Plate 2
The p-XRD patterns of hesperidin nanoparticles

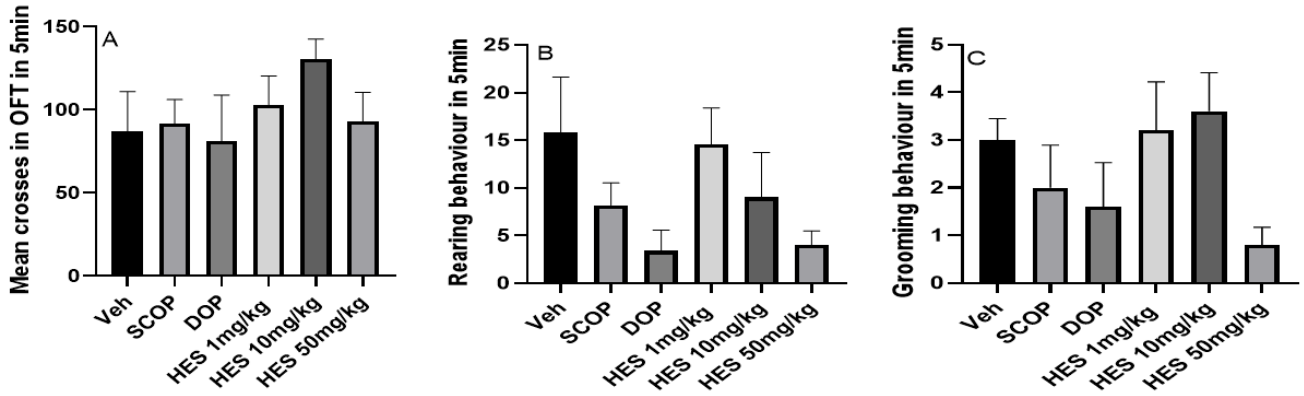

Figure 2A-C:
Effect of hesperidin nanoparticles on spontaneous motor activity (a) number of crosses, (b) number of rearing behaviour, and (c) number of grooming behaviour in open field test. Values are presented as mean±SEM (n=5). P>0.05 versus vehicle normal control, *p>0.05* versus vehicle-scopolamine treated group

Plate 2 shows the p-XRD patterns characterized by prominent peaks appearing at 12,1 15.5, 17.2, 19.6, 22.4, 23.4, 25,6, and 29.2 indicating the crystallinity of the compound. This result is similar to those reported by Ali *et al*., (2019).

Effect of HES on Locomotion: One way ANOVA showed no significant effect of treatment on mean number of crosses [F(5,30)=0.82,P=0.55] (Fig. 2A), rearing behaviour [F(5,30)=1.92,P=0.13] (Figure 2B) and grooming [F(5,30)=1.88,P=0.14] (Figure 2C) in the open field test. Dunnet post hoc multiple comparison tests showed no significant difference between scopolamine treated and control, as well as HES treated mice.

Effect of HES on Working memory of Mice: One way ANOVA showed significant [F(5,30)=17.37,P<0.0001] effect of vehicle, scopolamine and HES treatment on percent alternation behaviour in Y-MAZE task. Vehicle treated showed significant discriminative percent compared

to scopolamine treated (p<0.001). the decrease in spatial working memory caused by scopolamine was dose dependently reversed by HES (1, 10 and 50mg/kg) as shown in Figure 3.

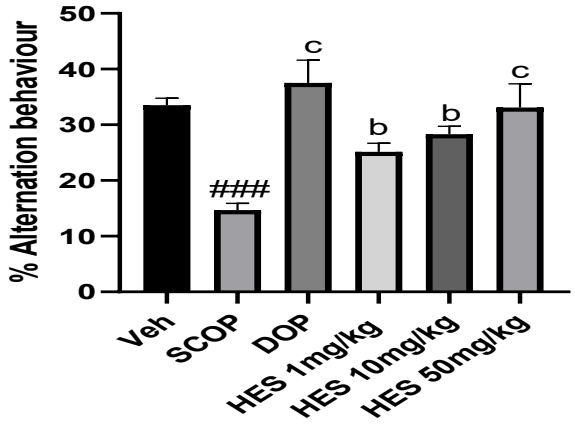

Figure 3:
Effect of hesperidin nanoparticle on working memory in Y-maze. Values are presented as mean±SEM (n=5). ###p<0.001 versus vehicle normal control, bp<0.01; cp<0.001 versus vehicle-scopolamine treated group

Effect of HES on exploratory time in mice subjected to NORT: One way ANOVA and multiple comparison tests revealed no significant effect of treatments on time spent interacting with the three similar objects in familiarization phase [F(5,24)=2.19,P=0.0885] (Figure 4a) indicative of no preference for all the objects. In the test trial, scopolamine significantly reduced the time spent by the animals exploring the novel object compared with the familiar objects indicative of a reduced preference index. However, the pretreatment of mice with HES (1, 10 and 50mg/kg) or donepezil significantly increased the preference index when compared with scopolamine-vehicle treated [F(5,24)=3.98,P=0.009] (Fig. 4b) in NORT.

Effect of HES on Escape latency (MWM): Two-way ANOVA results showed that the number of training days [F (3, 96)=51.70, p= 0.0001), the treatments [F(5,96)=7.30, p< 0.0001), and their interaction [F(15,96)=2.67,p=0.002) had a significant impact on the escape latency (Figure7a). On days 3-4 of training, the scopolamine-vehicle treated group exhibited significant prolongation of escape latency compared to the vehicle only treated animals. Donepezil administration significantly reduced escape latency during training days 3 and 4 compared to scopolamine-vehicle treated. HES (1, 10 and 50mg/kg) administration significantly decreased the time spent locating the escape platform. In the probe trial, HES (1, 10 and 50mg/kg) significantly increased the time spent by the animals at the target quadrant in comparison to scopolamine-vehicle treated [F(5,24)=3.17,P=0.02] (Figure 7b). Interestingly, scopolamine-vehicle treated mice exhibited a reduced time spent in the target zone in comparison with the vehicle control group. Donepezil significantly prolonged the time spent by the animals in the target zone in the probe test.

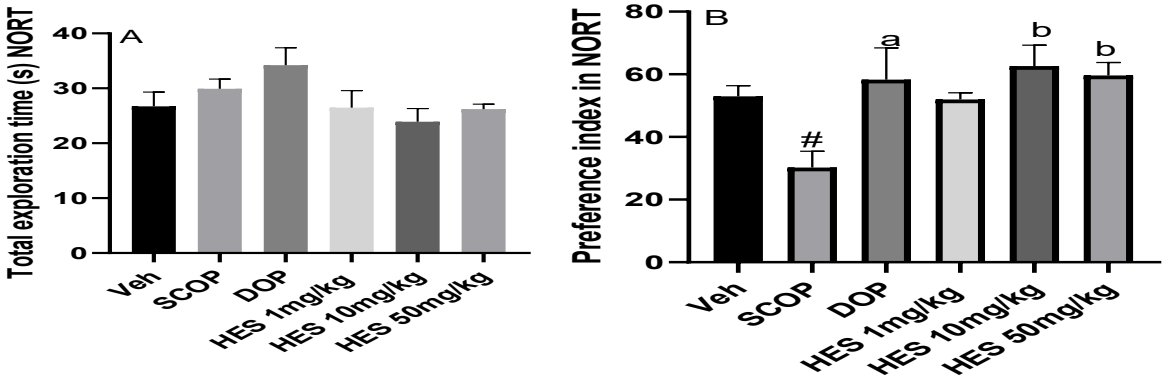

Figure 4A-B:
Effect of hesperidin nanoparticle on objects exploration time in (a) familiarization phase and (b) preference index in test phase in NORT in mice. Values are presented as mean±SEM (n=5). ###p<0.001 versus vehicle normal control, bp<0.01; cp<0.001 versus vehicle-scopolamine treated group.

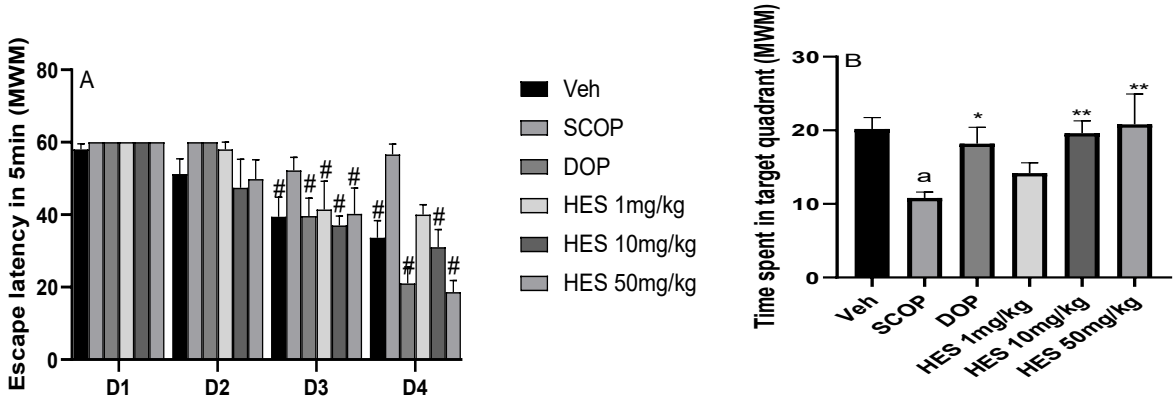

Figure 5A-B:
Effect of hesperidin nanoparticles on (A) escape latency and (b) time spent at target quadrant in MWM test. Values are presented as mean±SEM (n=5). #p<0.05 versus session 1, ap<0.01; cp<0.05 versus vehicle-control treated group, **p<0.01 versus vehicle-scopolamine treated.

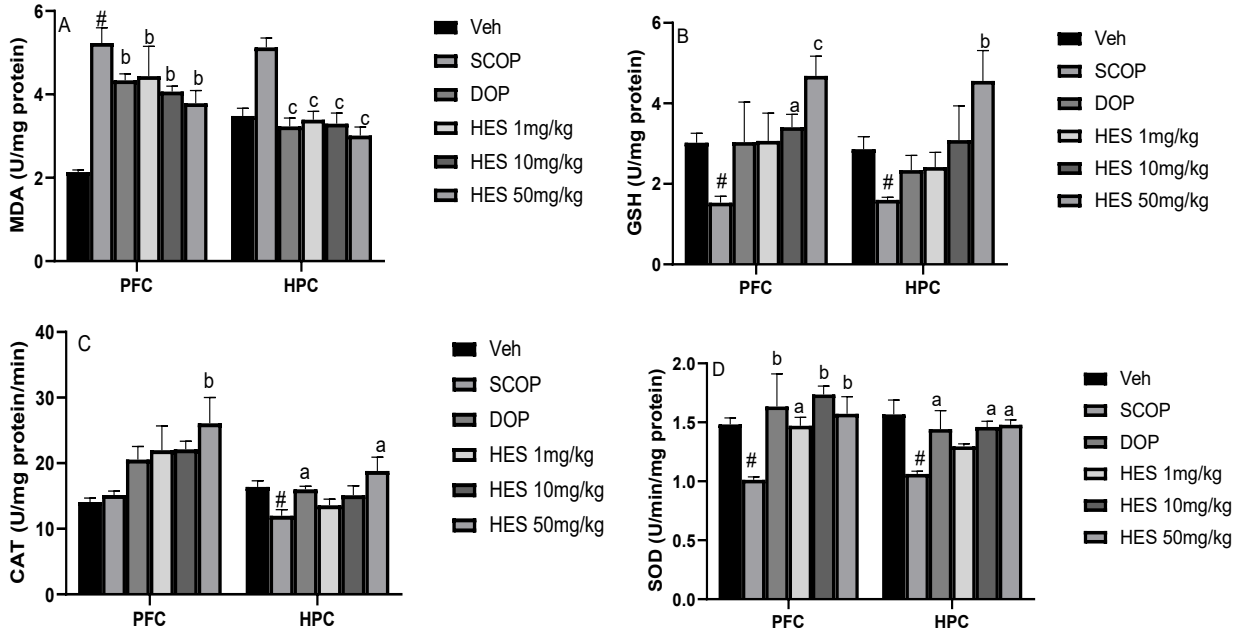

Figure 6A-D:
Effect of hesperidin nanoparticle on scopolamine-induced oxidative stress markers (A) MDA, (B) GSH, (C) catalase and (D) SOD in discrete brain regions of the prefrontal cortex (PFC) and hippocampus (HPC). Values are presented as mean±SEM (n=5). $^{\#}p<0.05$ versus vehicle-control treated, $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$ versus scopolamine-vehicle treated.

HES protected against Scopolamine-induced oxidative stress in the hippocampus and PFC of mice: Two-way ANOVA results showed the effect of treatment on MDA levels in the PFC and hippocampus [F (1,48)=5.73, p= 0.02), the treatments [F(5,48)=14.05, p< 0.0001), and their interaction [F(5,48)=4.94,p=0.001) had a significant impact on MDA level (Figure 8a). Scopolamine-vehicle treated caused significant increase in MDA level. However, HES_ (1, 10 and 50mg/kg) or donepezil administration significantly attenuated MDA generation in the PFC and HPC. Figure 8b showed the effect of treatments on GSH activity in the PFC and HPC [F (1,48)=0.99, p= 0.32), the treatments [F(5,48)=6.62, p< 0.001), and their interaction [F(5,48)=0.15,p=0.97) had a significant impact on GSH activity. Scopolamine-vehicle treated caused a significant decrease in GSH activity in the PFC and HPC. However, HES (1, 10 and 50mg/kg) or donepezil administration significantly reversed the deficit in GSH activity caused by scopolamine in the PFC and HPC.

Two-way ANOVA revealed significant effect of treatment on catalase activity in the PFC and hippocampus [F (1,48)=17.56, p= 0.001), the treatments [F(5,48)=5.03, p= 0.0009), and their interaction [F(5,48)=2.08,p=0.08) (Figure 8c). Scopolamine-vehicle treated caused significant reduction in catalase activities in the PFC and HPC. However, HES_(1, 10 and 50mg/kg) or donepezil administration significantly improved catalase activities in the PFC and HPC.

Also, a significant decrease in SOD activities in the PFC and HPC following scopolamine treatment was observed when compared with vehicle treated group. Conversely, HES_ (1, 10 and 50mg/kg) or donepezil administration significantly reversed the decrease in SOD activity in the PFC and HPC caused scopolamine administration. Moreover, two-way ANOVA revealed significant effect of HES_ pretreatment and scopolamine treatment on SOD activity in the PFC and hippocampus [F (1,48)=2.28,p=0.14), the treatments [F(5,48)=5.03, p= 0.0009), and HES × scopolamine interaction [F(5,48)=0.78,p=0.57) (Figure 8d).

Effect of HES on AChE activities in the hippocampus and PFC of mice: In this study, scopolamine administration significantly increased AChE activities in the PFC and HPC when compared with vehicle-treated group. However, HES (1, 10 and 50mg/kg) or donepezil administration significantly reversed the increase in AChE activities in the PFC and HPC caused scopolamine treatment. Moreover, two-way ANOVA revealed significant effect of HES pretreatment and scopolamine treatment on AChE activities in the PFC and HPC [F (1,48)=42.57,p<0.0001), the treatments [F(5,48)=8.04, p<0.0001), and HES × scopolamine interaction [F(5,48)=1.17,p=0.33) (Figure 7).

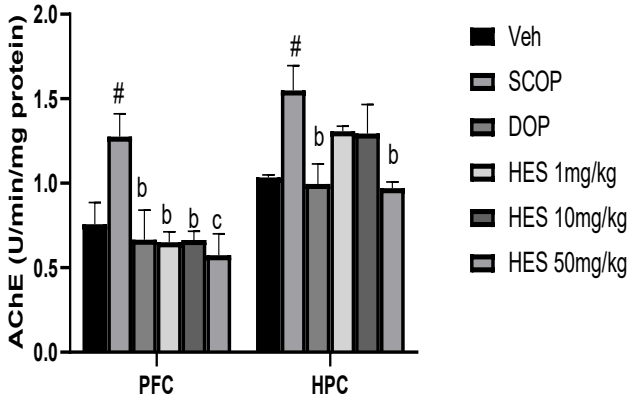

Figure 7:
Effect of hesperidin nanoparticles on scopolamine-induced acetylcholinesterase activities in in the prefrontal cortex (PFC) and hippocampus (HPC). Values are presented as mean±SEM (n=5). $^{\#}p<0.05$ versus vehicle-control treated, $^{b}p<0.01$; $^{c}p<0.001$ versus scopolamine-vehicle treated.

DISCUSSION

Results from the behavioral and biochemical analysis of the mouse hippocampus and prefrontal cortex showed that hesperidin nanoparticles used in this study ameliorated scopolamine-induced memory impairment, cholinergic dysfunction, oxidative stress, and lipid peroxidation in mouse models of AD (Ishola *et al*., 2023; Amoah *et al*., 2023). HES (10, 50mg/kg) increased spontaneous alternation in Y-maze, decreased escape latency time in MWM, and increased preference index in NORT which were altered by scopolamine. In addition, HES ameliorated the scopolamine-induced increase in AChE activities in the hippocampus and prefrontal cortex of mice.

In AD, short-term memory deficit occurs at the early stages and then progresses to loss of long-term memory (Götz *et al*., 2018). Animal models of AD, including Y-maze, Morris water maze, NORT are used to measure spatial memory deficit.

The Y-maze test is used to assess spatial recognition and spontaneous alternation behaviour in rodents. This spontaneous alternation behaviour reflects the rodents' innate tendency to explore novel environments (Kraeuter *et al*., 2019). This test also provides insights into hippocampal integrity, cognitive deficits, and the therapeutic potential of novel cognitive-enhancing agents (Choi *et al*., 2023). In line with previous studies, findings from this study showed that scopolamine (1mg/kg) reduced spontaneous alternation behaviour of mice subjected to Y-maze test which denotes spatial working memory impairment (Choi *et al*., 2023; Ishola *et al*., 2020). However, pretreatment with HES (10 and 50mg/kg) inhibited this effect, evidenced by an increase in percentage alternation, indicative of the cognitive enhancing properties of HES. Also, to rule out the locomotor activity of scopolamine on the mice, the animals were subjected to open field test, and the results showed that scopolamine treatment had no significant effect on the number of line crosses.

To further investigate the possible memory enhancing property of HES, the mice were subjected to NORT. The NORT is more relatable to human memory studies, making it a valuable tool for understanding human cognition as it relies on the rodents' natural predisposition for exploring the unfamiliar (Lueptow, 2017). The time taken to explore the new object serves as an index of recognition memory (Leger *et al*., 2013). Mice treated with scopolamine only, showed decrease in time spent exploring the novel object evidenced by a reduced preference index which was similar to findings from other studies (Wahid *et al*., 2022; Cheon *et al*., 2021). But, pretreatment with HES_ (10 and 50mg/kg) ameliorated this effect, increasing the preference index. Interestingly, the effect of HES_ was similar to that of donepezil (anticholinesterase inhibitor) which served as the standard reference drug, further suggestive of the cognitive-enhancing property of hesperidin nanoparticles.

In addition, the effect of HES_ on scopolamine-induced decline in spatial learning and memory function was evaluated by carrying out Morris water maze test. In MWM, the time spent locating the hidden platform (escape latency) and the time spent exploring the platform area are used to assess spatial learning and memory function (Lee *et al*., 2018). Scopolamine treatment has been reported to cause an increase in escape latency and reduced time spent in the escape platform area, which implies spatial learning and memory impairments (Hindam *et al*., 2020; Ishola *et al*., 2020). Similarly, in this present study, Scopolamine treated group spent more time swimming (increased escape latency) and a reduced time in the escape platform area during the probe test. But, pretreatment with HES ameliorated this effect, suggestive of the potential of HES_ in improving spatial learning and memory in mice.

Furthermore, the effect of hesperidin nanoparticles on scopolamine induced oxidative damage in mice hippocampal and PFC brain region was investigated. Findings from literature have reported that oxidative stress is one of the hallmark of neurodegenerative diseases pathogenesis and it can further exacerbate neurodegenerative disorder. In AD, oxidative stress contributes to AD's progression by fostering amyloid-β (Aβ) accumulation, tau protein hyperphosphorylation, and neuronal damage (Chen and Zhong, 2014). Results from this study showed that GSH, SOD and catalase activities were decreased and the levels of MDA were increased in the hippocampal and PFC region of mice exposed to scopolamine. However, pretreatment with HES reversed these effects leading to an increase in GSH, SOD and catalase levels with a marked reduction in MDA level, indicative of the neuroprotective and antioxidant properties of HES.

We further evaluated the effect of HES_ against scopolamine-induced acetylcholine hydrolysis as increased acetylcholinesterase activity would invariably lead to a cascade of events leading to cholinergic dysfunction (Garabadu *et al*., 2019). Cholinergic system dysfunction, particularly ACh depletion, disrupts cognitive processes (learning and memory) and this has been implicated in AD. Scopolamine is used to induce loss of cholinergic function in the hippocampal and Prefrontal cortex brain region characterized by increased acetylcholinesterase activity (Ishola *et al*., 2023; Ishola *et al*., 2020). By previous findings, scopolamine treatment caused a surge in AChE activities in the himpocampus and PFC of mice. However, HES pretreatment attenuated this effect resulting in the inhibition of ACh hydrolysis in the hippocampus and PFC, which further suggest the neuroprotective effect and memory-enhancing effect of hesperidin nanoparticles.

In conclusion, HES showed promising potential as a memory-enhancing agent against scopolamine-induced learning and memory deficits possibly through the enhancement of antioxidant defense mechanisms and cholinergic signaling.

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Full-length Research Article

Therapeutic Potential of Tadalafil in Doxorubicin-Induced Pulmonary and Haematological Toxicities in Wistar Rats

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Summary: Doxorubicin (DOX) therapy is associated with pulmonary toxicity and hematotoxicity as its off-target side effects. In this study, tadalafil (TAD) was investigated for its chemopreventive potential against DOX-induced pulmonary toxicity and hematotoxicity in 48 male Wistar rats that were divided into 8 groups of 6 rats/group and orally pretreated with 2.5 mg/kg/day, 5.0 mg/kg/day, and 10 mg/kg/day TAD 1 hour before intraperitoneal injection of 2.5 mg/kg DOX on alternate days for 12 days after which the rats were humanely sacrificed. Blood samples for haematological and biochemical endpoints, and lung tissue samples for oxidative stress markers, pro-inflammatory cytokine assays, and histopathology were collected. The rats' body weights were measured at the start and end of the experiments. Results showed that DOX toxicity was associated with significant ($p<0.0001$) weight loss, with corresponding significant ($p<0.05$) reduction in the relative lung weight. DOX intoxication was also associated with leukopenia, thrombocytopenia, lymphocytopenia, myelocytosis, and neutrophilia, suggesting bone marrow suppression, while it induced significant ($p<0.0001$) decreases in the serum bicarbonate and pH levels, as well as an increase in iCa^{2+} levels. DOX intoxication was also associated with profound ($p<0.0001$) increases in the lung tissue oxidative stress markers. However, oral TAD pretreatment did not significantly ($p>0.05$) improve DOX-associated weight loss but reversed the decrease in relative lung weight. TAD pretreatments also profoundly ($p<0.001$, $p<0.0001$) reversed both the DOX-induced haematological and biochemical alterations. Overall, TAD may have potential protective effects on DOX-induced pulmonary and haematological dysfunctions, highlighting the chemopreventive potential of TAD, which was probably mediated via antioxidant and/or free radical scavenging and anti-inflammatory mechanisms.

Keywords: Doxorubicin, pulmonary function biomarkers, complete blood count, pro-inflammatory cytokines, oxidative stress markers, Tadalafil.

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INTRODUCTION

Doxorubicin (DOX), a broad-spectrum anthracycline cytotoxic antibiotic, is an effective and potent antitumoral drug used for the treatment of diverse hematological and solid tumors such as soft and solid tissue tumors such as the sarcomas, cancers of the breast, ovary, bladder and the thyroid, lymphoblastic leukemia, acute myeloblastic leukemia, Hodgkin lymphoma, and small cell lung cancer (Qi *et al.*, 2020; Johnson-Arbor *et al.*, 2024; Kudelkina *et al.*, 2025). DOX's precise mechanisms of cytotoxic action are complex and debatable. However, there is a consensus in the scientific community that DOX's cytotoxic mechanisms include inhibition of cell proliferation, oxidative stress induction, free radical generation, inhibition of RNA and DNA synthesis via topoisomerase II enzyme inhibition, induction of inflammation, cell death acceleration mainly via the induction of autophagy and apoptosis (Jabłońska-Trypuć *et al.*, 2018; Rawat *et al.*, 2021; Zhao *et al.*, 2023; Wang *et al.*, 2024). Its clinical use, however, is limited by the multi-organ off-target side effects that it causes, resulting from its multi-directional cytotoxic effects and non-specificity to cancerous cells (Xing *et al.*, 2022). The DOX-mediated multi-organ toxicities include cardiotoxicity, hepatotoxicity, nephrotoxicity, pulmonary toxicity, myelosuppression, and gonadotoxicity (Qi *et al.*, 2020; Zhao *et al.*, 2023).

Pulmonary toxicity and hematotoxicity are well-recognised and documented off-target side effects of prolonged DOX chemotherapy (Vershoore *et al.*, 1987;

Eisenbens *et al.*, 2001; Nevadunsky *et al.*, 2013). These off-target side effects have limited the clinical use of DOX in human cancer therapy (Guzel *et al.*, 2021).

Although the precise causative role of DOX in pulmonary toxicity remains poorly understood, oxidative stress signalling remains the foremost etiopathogenetic mechanism of this toxicity (Skeoch *et al.*, 2018). Oxidative stress induces intracellular accumulation of reactive oxygen species (ROS), including superoxides that can cause lipid peroxidation and injury to proteins and DNA (Qi *et al.*, 2020; Mazzotta *et al.*, 2016). More so, DOX is known to significantly decrease the endogenous antioxidant profile, further destabilizing the antioxidant/oxidant homeostasis (Guzel *et al.*, 2021). Cumulatively, these result in the disturbances of pulmonary tissue and bone marrow antioxidant/oxidant homeostasis to cause pulmonary toxicity and hematotoxicity (Jablońska-Trypuć *et al.*, 2018; Guzel and Tekremur, 2021). Given the above, managing ROS accumulation and maintaining antioxidant/oxidant balance homeostasis is a potentially critical therapeutic strategy for DOX-induced pulmonary toxicity and hematotoxicity (Guzel and Tekremur, 2021).

Tadalafil (TAD) is a selective, long-acting phosphodiesterase type 5 inhibitor that is widely known for its beneficial use in the clinical management of erectile dysfunction (ED) and pulmonary artery hypertension (PAH) (Klinger, 2011; Duvanti et al., 2017; Lee et al., 2023). TAD is also documented to be an effective antioxidant (Duvanti *et al.*, 2017; Sheweita *et al.*, 2020; Yeni *et al.*, 2022).
Despite being recognized for over a few decades, there are little or no therapeutic remedies available to either prevent the onset or treat these off-target side effects when they arise in the course of DOX therapy. Thus, this is the basis for this current study, which evaluates the TAD's ameliorating potential against DOX-induced pulmonary and haematological toxicities in young adult male Wistar rats.

MATERIALS AND METHODS

Care and Use of experimental rats: Adult male Wistar Albino rats (aged 10-12 weeks old and body weight: 180-200 g) were procured from Bayo Farms, Sango-Ota, Ogun State, after obtaining an institutional ethical approval (with the reference number: LASU/23/REC/083) from the Lagos State University Research Ethics Committee (LASU-REC), Main Campus, Ojo, Lagos State, Nigeria. The rats were acclimatized in the Lagos State University College of Medicine (LASUCOM) Animal House for two (2) weeks. The rats were cared for and handled in line with global best practices guiding the Use and Handling of Experimental Animals as stipulated by the National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011). Standard rat chow and potable tap water were made freely available for the rats and maintained at standard laboratory conditions throughout the study.

Body Weight Measurement: A rodent digital weighing scale (Model: Virgo Electronic Compact Scale®, New Delhi, India) was used to take the rat body weights at the beginning and end of the study. The rat weights obtained were in grams (g).

Drug pre-treatment and experimental induction of DOX-induced pulmonary and haematological toxicities in the treated rats: Pulmonary and haematological toxicities were induced with DOX using the method earlier described by Adeneye *et al.* (2021). Briefly described, the experimental rats were randomly allocated into eight (8) groups of six (6) rats per group, such that the weight variations within and between groups do not exceed ± 20% of the average weights of the sample population.

The drug treatment of each group is in Table 1. The experimental rats were pretreated with oral sterile water, silymarin (Silybon-140®, Micro Labs Limited, 92 Sipcot Hosur-635126, India), and tadalafil (Honnonil®, Lyn-Edge Pharmaceutical Limited, Chevron Alternative Route, Poroku, Lekki, Lagos State, Nigeria) one hour before administering 2.5 mg/kg DOX (Oncodox-50®, Cipla Limited, Plot No. 5, S-103 Verna, Goa403-722, India) given intraperitoneally. The choice of drug doses used was based on the results of the preliminary studies and the literature searches.

Table 1.
Experimental Groups and Respective Treatment Protocols

Groups	Treatment Protocols
Group I	10 ml/kg/day of sterile water given *p.o.* daily + 1 ml/kg/day of sterile water given *i.p.* on alternate days for 12 days
Group II	10 ml/kg/day sterile water given *p.o.* daily + 2.5 mg/kg/day *DOX* in sterile water given *i.p.* on alternate days for 12 days
Group III	20 mg/kg/day silymarin in sterile water given *p.o.* daily + 1 ml/kg/day sterile water *i.p.* on alternate days for 12 days
Group IV	20 mg/kg/day silymarin in sterile water given *p.o.* daily + 2.5 mg/kg/day *DOX* in sterile water given *i.p.* on alternate days for 12 days
Group V	5 mg/kg/day tadalafil in sterile water given *p.o.* daily + 1 ml/kg/day sterile water given *i.p.* on alternate days for 12 days
Group VI	2.5 mg/kg/day tadalafil in sterile water given *p.o.* daily + 2.5 mg/kg/day *DOX* in sterile water given *i.p.* on alternate days for 12 days
Group VII	5 mg/kg/day tadalafil in sterile water given *p.o.* daily + 2.5 mg/kg/day *DOX* in sterile water given *i.p.* on alternate days for 12 days
Group VIII	10 mg/kg/day tadalafil in sterile water given *p.o.* + 2.5 mg/kg/day *DOX* in sterile water given *i.p.* on alternate days for 12 days

Blood Samples and Tissues Collection: Twenty-four (24) hours after the last DOX injection on day 12 of the treatment, treated rats were fasted overnight and humanely sacrificed under light inhaled halothane anesthesia for whole blood sample collection directly from the heart with fine 21G hypodermic needle and 5 ml syringe without causing damage to the heart tissues. A long surgical incision was made on the ventral surface of the thorax and abdomen and gently retracted to expose the abdominal organs. The liver and kidneys were identified, carefully dissected en bloc, and weighed on a digital weighing balance with the weight values expressed in grams (g).
At the end of the experiment, the rat carcasses were evacuated and duly processed by the trained and certified Animal House Attendants.

Calculation of percentage weight changes (%Δwt): The percentage weight change (%Δwt) was calculated as a ratio of the difference between the final and initial body weights and the initial body weight multiplied by 100 which is expressed mathematically in the equation:

$$\{[final\ body\ weight\ (g) - initial\ body\ weight\ (g)] / [initial\ body\ weight\ (g)]\} \times 100$$

Calculation of relative liver and kidney weights

The respective relative kidney weight was calculated as the ratio of the absolute weight (g) of both kidneys and the final rat body weight (g) multiplied by 100. This is expressed mathematically as:

$$\{[absolute\ organ\ weight\ (g)] / [final\ rat\ weight]\}\ x\ 100$$

Blood sample collection and determination of pulmonary function test: For each rat, 4-5 ml of whole blood was collected directly from the heart chamber using a 21G needle mounted on a 5 ml syringe. 2 ml of the fresh blood sample was collected into EDTA-treated sample blood and placed on auto stirrer to prevent the blood from clotting was used for the complete blood count test. The remaining blood was collected into a plain sample bottle and allowed to stand at 4ºC for 4 hours before being centrifuged at 5000 revolutions/min for 5 minutes to separate the sera from the other clotted blood components. Then, the serum was separated and used for serum pH, bicarbonate (HCO_3^-), and ionized calcium (Ca^{2+}) estimation. The bio-assays were carried out using standard analytical procedures and the Manufacturer's instructions on the enclosed leaflets in the commercial diagnostic test kits (Randox Diagnostics Test Kits®, Randox Laboratories Ltd, Crumlin, United Kingdom).

Lung tissue antioxidant enzymatic assays and pro inflammatory markers: After the rats were sacrificed humanely under inhaled light halothane anesthesia, the lungs were dissected en bloc and rinsed briskly in ice-cold 1.15% KCl solution to preserve the tissue's oxidative enzyme activities before being kept in a clean sample bottle that was in an ice-pack-filled cooler. The tissues were homogenized using a mechanical homogenizer with a Teflon ice-cold sodium phosphate buffer (0.1M, pH 7.4). The tissue homogenates were centrifuged at 5000 revolutions per minute for 10 min at 4˚C. The supernatants were used for the determination of activities of the tissue oxidative stress markers (GSH, MDA, CAT, SOD, GST, and GPx) as described by Olorundare *et al.* (2020, 2021). The antioxidant enzyme activities were expressed as U/ mg protein.

The pro-inflammatory markers: interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) levels in the rat lung tissues were also determined using rat-specific commercial ELISA kits sourced from ElabScience (14780 Memorial Drive, Suite 105, Houston, Texas, 77079, USA).

Histopathological studies of the lung tissues: The remaining one-half of the dissected lung tissues was preserved in 10% formo-saline solution. The tissue slide preparation and reading were done using the procedures earlier described by Olorundare *et al*. (2020).

Statistical Analysis of Data

The average body weight, body weight changes, and biochemical assays were expressed as mean ± standard deviation (S.D.) and mean ± standard error of the mean (S.E.M.) of six observations, respectively. The statistical analysis using a One-way analysis of variance followed by Tukey's post hoc test on GraphPad Prism version 5 was adopted. The levels of statistical significance were at $p<0.05$, $p<0.001$, and $p<0.0001$.

RESULTS

Modulatory impact of TAD pre-treatment on DOX-induced alterations in body weight dynamics in rats: Table 2 shows that alternate-day intraperitoneal injections with DOX at 2.5 mg/kg for 12 days caused the most significant ($p<0.0001$) weight losses in the final average body weight and percentage body weight changes (137.40 ± 20.68g, and -28.22 ± 04.22%, respectively) in the DOX-only treated (Group II) rats when compared with the untreated control (Group I) rats (182.50 ± 22.60 g, and 05.05 ± 03.85%, respectively) (Table 2).

Daily oral pretreatments with SIL (used as a standard antioxidant drug) and graded TAD (2.5 mg/kg body weight/day, 5 mg/kg body weight/day, and 10 mg/kg body weight/day) were also associated with losses in the final average body weight and percentage body weight changes (143.10 ± 25.71 g and -16.37 ± 22.07%; 158.50 ± 24.75 g and -22.78 ± 06.82%; 154.00 ± 26.05 g and -21.63 ± 06.61%; 150.10 ± 17.88 g and -25.00 ± 03.22%, respectively) with the most significant further weight losses recorded in the 10 mg/kg body weight/day TAD pretreated, DOX intoxicated rats (Table 2).

Table 2. Effects of DOX intoxication, and graded oral TAD pre-treatment on the mean initial body weights (initial Wt.), mean final body weight (final Wt.) and percentage body weight changes (%ΔWt) in treated rats.

Groups	initial Wt. (g)	final Wt. (g)	%ΔWt
I	173.0 ± 21.2	182.5 ± 22.6	05.1 ± 03.9
II	192.2 ± 16.6	137.4 ± 20.7^{c}	-28.2 ± 04.2^{c-}
III	193.7 ± 05.9	207.2 ± 12.1$^{a+, a\#}$	07.10 ± 06.08$^{c\#}$
IV	186.8 ± 05.6	143.1 ± 25.7^{c}	16.37 ± 22.02^{c-}
V	200.7 ± 22.8	218.8 ± 18.6$^{a+, c\#}$	05.80 ± 4.41$^{c\#}$
VI	204.7 ± 21.6	158.5 ± 24.8^{c-}	22.78 ± 06.82^{c}
VII	195.5 ± 19.5	154.0 ± 26.1^{b-}	21.63 ± 06.61^{c-}
VIII	199.7 ± 20.2	150.1 ± 17.9^{c-}	25.00 ± 03.22^{c-}

$^{b-}$ and $^{c-}$ represent significant decreases at $p<0.001$ and $p<0.0001$, respectively, when compared to untreated normal control (Group I) values while $^{a+}$ and $^{c+}$ represent significant increases at $p<0.05$ and $p<0.0001$, respectively, when compared to untreated normal control (Group I) values; $^{a\#}$ and $^{c\#}$ represent significant increases at $p<0.05$ and $p<0.0001$, respectively, when compared to untreated DOX intoxicated (Group II) values.

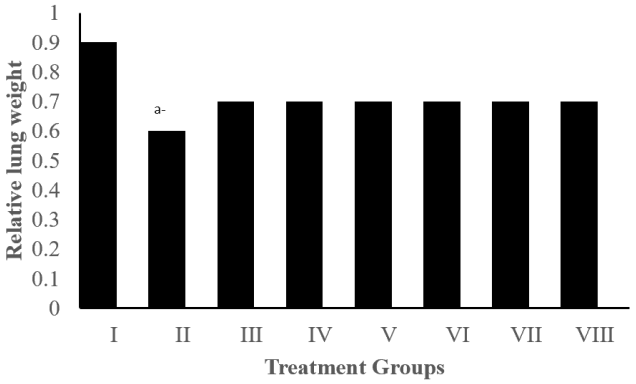

Figure 1.
Effect of DOX intoxication and graded tadalafil (TAD) and silymarin (SIL) pretreatments on the relative lung weight of treated rats
[a-] represents a significant decrease at p<0.005 when compared to the untreated normal control (Group I) value.

TAD ameliorating influence on doxorubicin-induced pulmonary weight alterations in rats: On the relative lung weight, DOX intoxication caused a significant decrease ($p<0.05$) in the relative lung weight (0.6 ± 0.1) (Figure 1). However, oral SIL and TAD pre-treatments significantly ($p<0.05$) reversed the DOX-induced reduction in the relative lung weight and restored it to control values (0.7 ± 0.1, 0.7 ± 0.1, 0.7 ± 0.1, and 0.7 ± 0.1, respectively) (Figure 1).

Tadalafil's ameliorative effects on doxorubicin-altered pulmonary function parameters: A focus on acid-base and calcium homeostasis in treated rats: Repeated intraperitoneal injections of 2.5 mg/kg body weight DOX resulted in significant ($p<0.001$, $p<0.0001$) decreases in the serum bicarbonate (HCO_3^-) and pH levels when compared to untreated control (Group I) values (Table 3). DOX intoxication also caused a significant ($p<0.05$) increase in the ionized calcium (iCa^{2+}) level when compared to untreated control (Group I) values (Table 3).

Oral SIL and TAD pretreatments resulted in significant increases ($p<0.05$, $p<0.001$, and $p<0.0001$) in the serum HCO3- and pH levels when compared with the values obtained for untreated DOX intoxicated (Group II) rats (Table 3). Conversely, oral SIL and TAD pre-treatment significantly ($p<0.05$ and $p<0.001$) increased the circulating iCa^{2+} when compared to the untreated control (Group I) values but not significantly ($p>0.05$) different from those of untreated DOX intoxicated (Group II) rats (Table 3).

Table 3.
Effects of DOX intoxication, and graded oral TAD pre-treatment on the lung function parameters (HCO_3^- and pH) and ionized calcium ion (iCa^{2+}) in treated rats

Groups	HCO_3 (mmol/L)	iCa^{2+} (mmol/L)	pH
I	23.7 ± 0.9	1.2 ± 0.0	7.5 ± 0.0
II	$18.4 \pm 2.4^{b-}$	$1.6 \pm 0.1^{a+}$	$7.4 \pm 0.1^{a-}$
III	$25.0 \pm 0.6^{b\#}$	$1.2 \pm 0.0^{a*}$	7.5 ± 0.0
IV	$23.5 \pm 1.5^{b\#}$	$1.2 \pm 0.0^{a*}$	$7.6 \pm 0.1^{a\#}$
V	$28.1 \pm 0.8^{b\#}$	$1.2 \pm 0.0^{a*}$	7.5 ± 0.0
VI	20.7±1.9	$1.2±0.1^{a*}$	7.5±0.0
VII	$23.0±1.7^{b\#}$	$1.3±0.1^{a*}$	$7.6±0.1^{b*}$
VIII	$25.6±1.0^{b\#}$	$1.3±0.0^{a*}$	$7.6±0.0^{b*}$

[a+] represents a significant increase at p<0.05 while [a-] and [b-] represent significant decreases at p<0.05 and p<0.001, respectively, when compared to untreated normal control (Group I) values. [a] and [b*] represent significant decreases at p<0.05 and p<0.001, respectively, while [a#] and [b#] represent significant increases at p<0.05 and p<0.001, respectively, when compared to untreated DOX intoxicated control (Group II) values*

Navigating the oxidative storm: TAD's therapeutic compass guiding pulmonary antioxidant defenses against DOX-induced imbalance in treated rats: The effects of DOX intoxication and oral SIL and TAD pre-treatments on the lung tissue antioxidant profile are in Table 4. Repeated alternate-day treatments with intraperitoneal injections of 2.5 mg/kg body weight DOX for 12 days resulted in a significant ($p<0.0001$) decrease in the lung tissue SOD, CAT, GST, and GPx activities and a remarkable ($p<0.0001$) increase and decrease in the pulmonary tissue MDA and GSH levels, respectively when compared to untreated control (Group I) values (Table 4). However, oral with SIL and graded TAD pre-treatments, there were significant increases and reversal ($p<0.001$ and $p<0.0001$) in the lung tissue SOD, GST, and GPx activities, restoring their activities to the control values but caused no significant ($p<0.05$) reversal in the CAT activities of the DOX-intoxicated lung tissues (Table 4). Similarly, oral SIL and TAD pre-treatments also significantly ($p<0.0001$) increased the lung tissue GSH levels when compared to the untreated DOX-intoxicated (Group II) values (Table 4). On the lung tissue MDA activities, oral SIL and TAD pre-treatments also significantly ($p<0.001$, $p<0.0001$) decreased the MDA activities in the DOX-intoxicated lung tissues (Table 4).

Table 4.
Effects of DOX intoxication, and graded oral TAD pre-treatment on the lung antioxidant profile in treated rats

Groups	SOD	CAT	MD	GST	GPx	GSH
I	3.7±0.3	21.3±0.6	1.6±0.0	25.9±2.0	53.8±1.3	39.3±1.0
II	$1.7±0.1^{c-}$	$18.2±0.3^{c-}$	$4.5±0.2^{c+}$	$20.6±0.5^{a-}$	$31.4±0.6^{c-}$	$26.3±1.1^{c-}$
III	$4.6±0.3^{c\#}$	$25.4±1.8^{c\#}$	$1.7±0.2^{c-}$	$36.2±1.7^{b\#}$	$55.4±2.1^{c\#}$	$40.9±1.4^{c\#}$
IV	$5.7±0.3^{c\#}$	19.5±0.5	$2.4±0.1^{b*}$	$46.6±1.8^{c\#}$	$67.7±0.6^{c\#}$	$50.4±0.5^{c\#}$
V	$5.4±0.3^{c\#}$	19.1±0.3	$2.4±0.0^{b*}$	$49.6±1.6^{b\#}$	$70.4±1.0^{c\#}$	$54.2±0.7^{c\#}$
VI	$4.8±0.2^{c\#}$	18.7±0.2	$2.6±0.0^{b*}$	$48.4±1.7^{b\#}$	$61.5±11.0^{c\#}$	$55.0±1.0^{c\#}$
VII	$4.4±0.3^{c\#}$	18.4±0.2	$2.7±0.0^{b*}$	$46.9±2.2^{c\#}$	$71.2±1.7^{c\#}$	$54.0±0.9^{c\#}$
VIII	$5.0±0.1^{c\#}$	17.1±0.5	$2.6±0.0^{c*}$	$50.2±1.0^{c\#}$	$69.7±1.2^{c\#}$	$55.4±1.0^{c\#}$

[c+] represents a significant increase at p<0.0001 while [a-] and [c-] represent significant decreases at p<0.05 and p<0.0001, respectively, when compared to untreated normal (Group I) rats. [b] and [c*] represent significant decreases at p<0.001 and* p<0.0001, respectively, while [b#] and [c#] represent significant increases at p<0.001 and p<0.0001, respectively, when compared to untreated DOX intoxicated (Group II) rats.

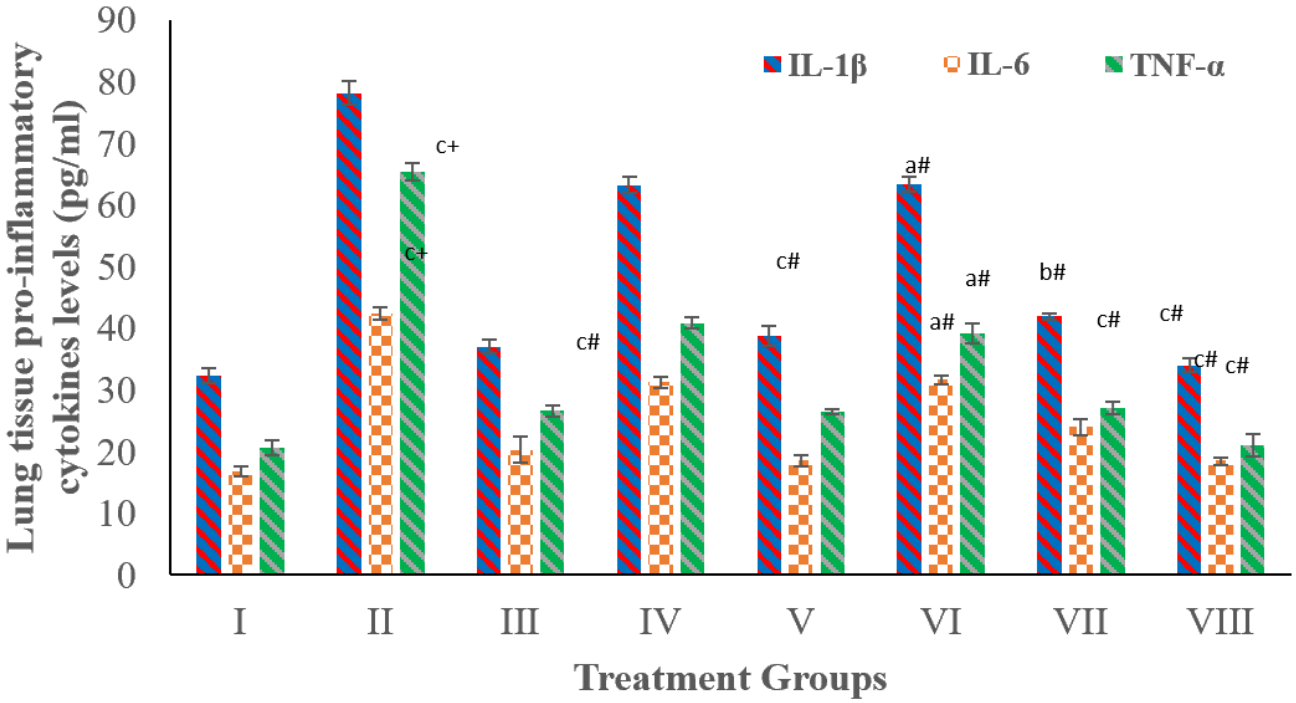

Figure 2.
Effect of graded tadalafil (TAD) and silymarin (SIL) on the lung tissue pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) levels of doxorubicin(DOX)-intoxicated control (Group II) values
$^{c+}$represents a significant increase at $p<0.0001$ when compared to the untreated normal control (Group I) values while $^{a\#, b\#}$ and $^{c\#}$ represent significant decreases at $p<0.05$, $p<0.001$ and $p<0.0001$, respectively, when compared to untreated DOX intoxicated (Group II) values.

Pro-inflammatory imprint (IL-1, IL-6 and TNF-α) of DOX and the mitigating artistry of TAD on the treated rat lung tissues: In the untreated DOX intoxicated group, there were significant ($p<0.001$ and $p<0.0001$) increases in the lung tissue levels of the measured pro-inflammatory cytokines: IL-1β, IL-6, and TNF-α levels were recorded compared to the untreated normal rats (Figure 2). However, TAD and SIL pre-treatments caused significant ($p<0.05$, $p<0.001$, and $p<0.0001$) decreases in lung tissue concentrations of IL-1β, IL-6, and TNF-α and reverting the values to almost untreated control values when compared to untreated DOX intoxicated group (Figure 2).

Hematological effect of DOX intoxication and ameliorating effects of TAD pre-treatments in rats: Table 5a shows the effects of DOX intoxication and the oral SIL and TAD pre-treatments on the hemogram of the treated rats consisting of the red blood cell counts (RBC), hemoglobin concentration (Hb), packed cell volume or hematocrit (PCV), mean corpuscular hemoglobin concentration (MCHC), total white blood cell counts (TWBC) and platelets count (PLT). Repeated i.p. injections of 2.5 mg/kg body weight DOX on alternate days for 12 days resulted in significant ($p<0.001$) decreases in TWBC and PLT without any significant ($p>0.05$) effect on RBC, Hb, PCV, MCV, and MCHC indices (Table 5a). With SIL and graded TAD pre-treatments, there were significant ($p<0.05$, $p<0.001$, $p<0.0001$) improvements in the TWBC and PLT indices while causing non-significant ($p>0.05$) alterations in the RBC, Hb, PCV, and MCHC values (Table 5a).

DOX intoxication also resulted in a significant ($p<0.0001$) decrease and increase in %LYMP and %NEUT, respectively, when compared to the untreated control (Group I) values but caused non-significant ($p>0.05$) alterations in the %MXD value (Table 5b). However, SIL and graded TAD pre-treatments significantly ($p<0.001$ and $p<0.0001$) reversed these changes and restored these hematological indices to about control values (Table 5b).

Histopathological effect of TAD pre-treatments on DOX-intoxicated rat lung tissues: Repeated DOX injection to the treated rat liver was associated with severe pulmonary interstitial congestion with cellular infiltration, diffuse inter-alveolar septa thickening, and diffuse alveolar collapse (Figure 3b) when compared to the untreated normal pulmonary histoarchitecture (Figure 3a).
Oral pre-treatment with SIL in normal rats was not associated with any remarkable pulmonary lesions (Figure 3c). SIL pre-treatment in DOX-intoxicated rats was associated with moderate pulmonary interstitial congestion and focal bronchiolar congestion with cellular infiltration (Figure 3d). In normal rats pretreated with 5 mg/kg/day TAD, there were no remarkable histological changes in the bronchiolo-alveolar architecture and pulmonary interstitium of treated rats (Figure 3e). However, oral pretreatment with graded TAD doses at 2.5 mg/kg/day, 5 mg/kg/day, and 10 mg/kg/day showed mild-to-moderate pulmonary interstitial congestion and focal alveolar collapse (Figure 3f); mild pulmonary interstitial congestion with widening of alveoli (Figure 3g), and very mild pulmonary interstitial congestion and normal alveolar distribution (Figure 3h), respectively.

Table 5a.
Effects of DOX intoxication, and graded oral TAD pre-treatment on the hematological parameters (RBC, Hb, PCV, MCHC, TWBC and PLT) in treated rats

Groups	RBC ($x10^6$/L)	Hb (g/dL)	PCV (%)	MCHC (g/dL)	TWBC ($x10^3$/L)	PLT ($x10^3$/µL)
I	7.7±0.4	12.9±0.5	46.1±2.1	31.7±1.0	11.8±1.2	653.8±99.9
II	7.6±0.7	13.2±1.1	45.2±3.5	31.5±0.1	$1.6±0.2^{c-}$	$190.0±14.8^{c-}$
III	7.7±0.2	13.8±0.4	48.2±3.5	30.6±0.2	13.1±1.7	$979.5±164.0^{c\#}$
IV	7.3±0.3	12.9±0.2	41.7±1.0	31.0±0.5	$4.1±1.6a^{\#}$	$678.7±124.2^{b\#}$
V	7.8±0.2	14.0±0.3	44.9±1.1	31.2±0.3	$13.3±1.6^{a\#}$	$856.7±225.7^{c\#}$
VI	7.4±0.5	13.1±0.8	42.0±2.9	31.3±0.5	$3.1±0.6^{a\#}$	$186.2±138.2^{c*}$
VII	7.8±0.8	12.8±1.1	39.7±1.0	37.2±3.2	$5.0±1.2^{a\#}$	$414.5±98.0^{b\#}$
VIII	7.4±0.5	13.2±0.7	41.6±2.3	31.8±0.5	$6.7±1.2^{b\#}$	$627.3±133.4^{c\#}$

$^{c-}$ represents a significant decrease at $p<0.0001$ when compared to untreated normal control (Group I) values. ^{c*} represents a significant decrease at $p<0.0001$ while $^{a\#, b\#}$ and $^{c\#}$ represent significant increases at $p<0.05$, $p<0.001$ and $p<0.0001$, respectively, when compared to untreated DOX intoxicated control (Group II) values

Table 5b.
Effects of DOX intoxication, oral SIL and graded oral TAD pre-treatment on the white blood cell differentials {percentage lymphocytes count (%LYMP), percentage neutrophil counts (%NEUT) and percentage myelocyte counts (%MXD) in treated rats

Groups	%LYMP	%NEUT	%MXD
I	81.4 ± 3.8	11.2 ± 2.1	4.0 ± 0.7
II	$37.9 ± 0.70^{c-}$	$59.4 ± 1.0^{c+}$	3.3 ± 0.3
III	$76.3 ± 6.4^{c\#}$	$19.6 ± 5.6^{c*}$	4.0 ± 0.8
IV	$59.1 ± 11.8^{b\#}$	$38.1 ± 11.4^{b*}$	2.8 ± 0.9
V	$74.4 ± 4.0^{c\#}$	$21.4 ± 3.5^{b*}$	4.2 ± 0.6
VI	$55.4±10.6^{b\#}$	$31.6±7.6^{b*}$	$13.1±9.8^{b+,\#}$
VII	$27.7±11.8^{c*}$	$68.9.3±11.7^{c\#}$	$13.5±0.6^{b+,\#}$
VIII	$47.0±5.5^{a\#}$	$39.4±6.2^{b*}$	$19.1±10.1^{c+,\#}$

$^{a+}$ represents a significant increase at $p<0.05$ while $^{a-}$ and $^{b-}$ represent significant decreases at $p<0.05$ and $p<0.001$, respectively, when compared to untreated normal control (Group I) values. ^{a*} and ^{b*} represent significant decreases at $p<0.05$ and $p<0.001$, respectively, while $^{a\#}$ and $^{b\#}$ represent significant increases at $p<0.05$ and $p<0.001$, respectively, when compared to untreated DOX-intoxicated control (Group II) values

DISCUSSION

DOX is commonly used to treat various types of cancers, including breast cancer, leukemia, and lymphoma. One of the side effects of this drug is weight loss, which can be attributed to anorexia and muscle wasting (Hiensch *et al.*, 2020; Pedrosa *et al.*, 2023). Also, DOX is known to cause gastrointestinal side effects such as nausea, vomiting, and diarrhea, which can lead to decreased food intake and subsequent weight loss. Additionally, DOX can cause taste changes that affect appetite and cause anorexia. In the present study, treatment with 15 mg/kg DOX (2.5 mg/kg given on alternate days for 12 days) caused diarrhea from day 8 of the drug treatment and lent support to the previous reports of DOX-induced diarrhea resulting from either enterocolitis or intestinal mucositis (Dahlgren *et al.*, 2021; Kawasaki *et al.*, 2023). However, the diarrhea observed in this study appears to be more like neutropenic enterocolitis (also referred to as "typhlitis" or "necrotizing enteropathy") based on the fact that this is the type of enterocolitis that is often associated with patients with DOX-induced neutropenia (Cherri *et al.*, 2020). Neutropenic enterocolitis (NEC) is a necrotizing inflammatory condition majorly affecting the terminal portion of the small intestine (often the terminal ileum) and the upper large intestine (cecum and ascending colon) in the face of neutropenia (Nesher and Rolston, 2013; Sirakaya and Inanç, 2020; Babakhanlou *et al.*, 2023). The fact that the differential leukocyte counts in the study revealed the presence of neutropenia and diarrhea affirms the induction of neutropenic enterocolitis associated with anorexia from day 10 of the DOX treatment. However, the fact that daily oral pretreatments with SIL and graded TAD did not improve the DOX-associated weight loss suggested that these drugs were not effective attenuators of this side-effect of prolonged DOX therapy. Conversely, the fact that oral SIL and TAD pretreatments reversed the profound decrease in the relative lung weight was also significant and still indicated the protective effect of these drugs on the DOX-treated rat lungs.

Pulmonary toxicity and hematotoxicity are life-threatening off-target side effects of long-term DOX therapy that are well documented in the literature (Minchin *et al.*, 1988; Testart-Pailler *et al.*, 2007; Kameo *et al.*, 2012; Kanno and Hara, 2021; Allan *et al.*, 2021; Lustberg *et al.*, 2023). DOX-induced hematotoxicity may manifest as myelosuppression, anemia, leukopenia, neutropenia, and thrombocytopenia (Thorn *et al.*, 2011; Kameo *et al.*, 2012; Madeddu *et al.*, 2021). In this study, repeated DOX administration for 12 days was associated with leukopenia, thrombocytopenia, lymphocytopenia, and neutrophilia, and these are considered predictors of DOX-induced hematological toxicity (Sleijfer *et al.*, 2018). Thus, the presence of the predictors in the hematological results obtained in this study suggests the establishment of doxorubicin toxicity. Neutrophilia is often considered a strong indicator of inflammation and cancers (Rosales, 2018; Zahorec, 2021). However, the former appears to be the more likely case, as healthy and non-cancerous rats were studied. The fact that neutrophilia was in the clinical blood picture of the DOX-treated rats suggests that DOX could be mediating its hematotoxicity via inflammation, a well-documented off-target toxicity mechanism for DOX (Supriya et al., 2016; Reis-Mendes *et al.*, 2021; 2023). This hypothesis was, perhaps, corroborated by the remarkable increases in the levels of pulmonary tissue pro-inflammatory cytokines observed in this study. However, oral TAD pretreatment not only reversed the DOX-induced hematological dysfunction and brought the values to normal but also caused a profound increase in the percentage myelocyte (MXD) count, which indicated a corresponding increase in eosinophils, monocytes, and basophils counts, and suggested activation of a general immunological response to the DOX intoxication by TAD pretreatment.

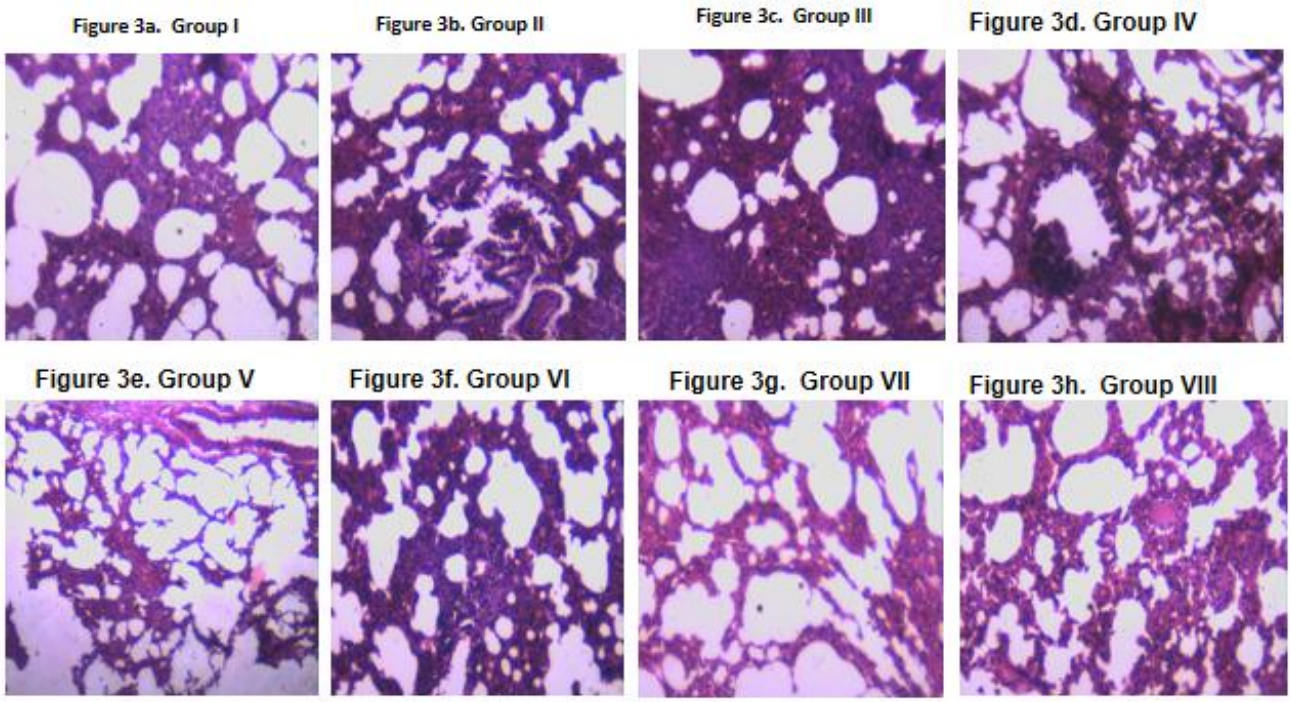

Figure 3.
A representative photographic section of (i). untreated normal control rat lung tissue showing normal bronchiole-alveolar architecture (x100 magnification, Hematoxylin & Eosin stains) **(3a)**; (ii). untreated DOX intoxicated rat lung tissue showing diffuse interstitial congestion with cellular infiltration; diffuse inter-alveolar septa thickening; and diffuse alveolar collapse (x100 magnification, Hematoxylin & Eosin stains) **(3b)**; (iii). 20 mg/kg/day SIL-only pretreated lung tissue showing normal pneumocytes, pulmonary interstitium, and inter-alveolar septa (x100 magnification, Hematoxylin & Eosin stains) **(3c)**; (iv). 20 mg/kg/day SIL pretreated + DOX-treated rat lung tissue showing moderate pulmonary interstitial congestion and focal bronchiolar congestion with cellular infiltration (x100 magnification, Hematoxylin & Eosin stains) **(3d)**; (v). 5 mg/kg/day TAD-only pretreated rat lung tissue showing normal bronchiolo-alveolar architecture and pulmonary interstitium (x100 magnification, Hematoxylin & Eosin stain) **(3e)**; (vi). 2.5 mg/kg/day TAD + 2.5 mg/kg DOX-treated rat lung tissue showing mild-to-moderate pulmonary interstitial congestion and focal alveolar collapse (x100 magnification, Hematoxylin & Eosin stain) **(3f)**; (vii). 5 mg/kg/day TAD + 2.5 mg/kg DOX-treated lung tissue showing mild pulmonary interstitial congestion with alveolar widening (x100 magnification, Hematoxylin & Eosin stains) **(3g)**; (viii). 10 mg/kg/day TAD + 2.5 mg/kg DOX-treated lung tissue showing mild pulmonary interstitial congestion and normal alveoli distribution (x100 magnification, Hematoxylin & Eosin stains) **(3h)**.

The blood HCO3- (reflective of arterial PCO2) and pH are often considered the most informative laboratory indicators of overall pulmonary function, especially in patients with pulmonary edema (Brinkman and Sharma, 2024). The primary disturbance of elevated arterial PCO2 is a decline in the ratio of arterial bicarbonate to arterial PCO2 with attendant decreased pH (Hirai *et al.*, 2019; Patel and Sharma, 2024). Thus, an arterial blood gas (ABG) and serum bicarbonate level are necessary to evaluate patients with suspected respiratory acidosis, a metabolic state marked by an elevated PCO_2, elevated HCO_3^-, and decreased pH (≤7.4) (Patel and Sharma, 2024). The fact that DOX-intoxication was associated with elevated serum HCO_3^- and reduced serum pH (7.4) suggested the presence of respiratory acidosis in the treated rats. The blood pH is known to have an inverse relationship with serum-ionized Ca^{2+} (Hamroun *et al.*, 2020). A rise in serum pH is known to promote the formation of calcium-albumin complexes and cause a decrease in ionized calcium levels; thus, a pH decrease may cause an increase in the free fraction of blood calcium. Therefore, an increase in ionized calcium levels (Hamroun *et al.*, 2020). In this study, repeated DOX treatment was associated with increased serum HCO_3^- and ionized Ca^{2+} and reduced serum pH, indicating the possible establishment of respiratory acidosis. Again, the fact that there were reversals in these measured pulmonary function parameters by TAD pretreatment suggests a potential protective effect of TAD against DOX-induced pulmonary dysfunction.

Another notable finding of this study is the effect of DOX toxicity on pulmonary tissue antioxidant activities. DOX intoxication was associated with significant reductions in the antioxidant enzyme (SOD, CAT, GPx, and GST) activities and significant increases and decreases in the pulmonary tissue MAD and GSH levels, respectively which are similar to those of other studies (Kuzu *et al.*, 2018; Kizir *et al.*, 2023). However, oral TAD pretreatment profoundly reverses the DOX-induced changes in the antioxidant profile.

The histopathological findings showed DOX-induced histological lesions such as diffuse interstitial congestion with cellular infiltration, diffuse inter-alveolar septa thickening and diffuse alveolar collapse. These observed histopathological changes remarkably improved with TAD pretreatment, highlighting the protective effect of TAD pretreatment in DOX-induced pulmonary toxicity.

Cancer chemotherapeutic agent acts on many proliferating cells or tissues, including the bone marrow. The bone marrow cells are responsible for red blood production, an important blood component. A complete

blood count assay is one of the most effective ways of monitoring drug toxicity (Sahota *et al.*, 2016; Erhabor *et al.*, 2020). Literature search shows that one of the DOX-induced hematotoxicities is bone marrow suppression that could manifest as anemia, thrombocytopenia, leukopenia, neutropenia, etc. (Isirima and Okoroafor, 2016; Ngatali *et al.*, 2022; Perpenia *et al.*, 2022). In this study, DOX intoxication did not cause leukopenia, thrombocytopenia, lymphocytopenia, myelocytosis, and neutrophila that could predispose the intoxicated rats to infections and spontaneous bleeding, respectively, from bone marrow suppression. However, oral TAD pretreatment reversed the DOX-induced hematological changes, restoring their values to about normal ranges, indicating the protective potential of graded oral TAD in preventing DOX-induced hematotoxicity.

In conclusion, this study highlights the possible chemopreventive potential of graded oral TAD against DOX-induced pulmonary and hematological toxicities, possibly mediated via anti-inflammatory and antioxidant mechanisms.

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Author contributions

AAA designed the experimental protocol, supervised the research, analyzed data and wrote the manuscript; FMO is an undergraduate student in AAA's Laboratory and carried out the experiment under AAA's supervision; OEO also contributed to the design of the experimental protocol and was involved in the manuscript editing; AOO read the slides, took the photomicrographs of the lung sections and was also involved in data analysis; IIO prepared the lung tissue slides for histopathological studies

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Full-length Research Article

Modulatory Effects of Ethanol Extract of *Dissotis rotundifolia* Whole Plant on Metabolic Syndrome-Induced Hepato-Renal Dysfunctions in Rats

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Summary: Metabolic syndrome has been associated with increased incidence of liver and chronic kidney damage. This study aimed at exploring the mitigatory action of *Dissotis rotundifolia* whole plant extract on metabolic syndrome-induced hepato-renal dysfunctions. Fifty (50) adult male Wistar rats weighing between 150-180 g were randomly distributed into five equal groups as follows: Group 1 (Control), Group 2 (MetS control), Group 3 (MetS + 100 mg/kg ethanol extract of *Dissotis rotundifolia*), Group 4 (MetS + 200 mg/kg ethanol extract of *Dissotis rotundifolia*), and Group 5 (MetS + 20 mg/kg Rosuvastatin). Metabolic syndrome was induced using a 40% high-fat diet and 20% fructose in drinking water for 8 weeks. Biomarkers of hepatic [alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP)] and renal [blood urea nitrogen (BUN) and creatinine] damage, oxidative stress [malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), antioxidant parameters [glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST)], lipid profiles [Low Density Lipoprotein cholesterol (LDL-c), High Density Lipoprotein cholesterol (HDL-c), Triglycerides (TG), and Total Cholesterol (TC)], and immunohistochemistry of the liver [Neutrophil Gelatinase-Associated Lipocalin (NGAL) and kidney [Angiotensin Converting Enzyme (ACE)] tissues of rats were determined. The results showed that metabolic syndrome caused a significant ($P<0.05$) increase in biomarkers of oxidative stress, MDA, H_2O_2 generation, LDL-c, HDL-c, TG, TC, but significantly reduced hepatic and renal GSH, SOD, GPx, and GST in comparison with the control. Furthermore, biomarkers of renal and hepatic damage were significantly ($P<0.05$) elevated in MetS untreated rats. Higher renal immune reactivity of NGAL but lower expression of ACE was recorded for MetS untreated rats. *Dissotis rotundifolia* extract mitigated biomarkers of oxidative stress, hepato-renal dysfunctions, and improved the antioxidant defense system. The observed protective effects of *Dissotis rotundifolia* on metabolic syndrome-induced hepatic and renal damage could be due to the amelioration of lipid peroxidation and increased antioxidant defense system.

Keywords*: Dissotis rotundifolia*, metabolic syndrome, Antioxidant, oxidative stress, hepato-renal dysfunction, rats.

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INTRODUCTION

Metabolic syndrome (MetS) is a complex medical condition comprising the occurrence of obesity, diabetes, and hypertension as comorbidities (Yang *et al*., 2022). The disease with a global prevalence of 25% is usually characterized by increased triglycerides, decreased high-density lipoprotein (HDL) cholesterol, insulin resistance, and hyperglycaemia (Saklayen, 2018; Kwitek, 2019). Currently, MetS is a principal contributor to disease burden and deaths in both developed and underdeveloped countries of the world (Rodríguez-Correa *et al*., 2020). Unfortunately,

effective management and treatment continue to pose great challenges to the medical community due to the multifactorial etiologies of the syndrome. Generally, the pathogenesis of MetS involves the dysregulation of the physiological mechanisms concerned with the metabolism of energy substrates, resulting in excessive fat storage in various tissues and organs (Grabner *et al*., 2021). Pathogenic mechanisms contributing significantly to the development of MetS include reduced total antioxidant capacity, impaired glucose uptake and utilization to altered insulin sensitivity by cells, and disturbed fatty acid metabolism (James *et al*., 2012; Monserrat-Mesquida *et al*., 2020). Increased oxidative stress associated with MetS results in accelerated generalized inflammatory responses, decreased vascular compliance, atherosclerosis, and hypertension (Grandl and Wolfrum, 2018).

The pathophysiology of MetS has been closely linked with abnormalities in the liver and the kidneys. As a result, these organs have been suggested to hold great potential as therapeutic targets in the management of MetS (Kotronen and Yki-Järvinen, 2007). In most cases, MetS is associated with Non-Alcoholic Fatty Liver Disease (NAFLD), characterized by the accumulation of fat in more than 5% of hepatocytes in the absence of excessive alcohol consumption (Wang *et al*., 2022). The strong association between NAFLD and MetS is highlighted by the presence of at least one component of MetS in 90% of NAFLD patients and at least three components of MetS in 33% of NAFLD patients (Almeda-Valdés *et al*., 2009). Moreover, hepatic lipid deposition positively correlates with several features of insulin resistance, a central factor in the pathophysiology of MetS, and low hepatic insulin sensitivity is associated with increased liver adipose tissue content (Rector *et al*., 2008). Likewise, chronic kidney disease is commonly associated with MetS, with severe histopathological lesions including glomerulonecrosis and loss of renal function reported in many patients (Alexander *et al*., 2009; Thomas *et al*., 2011; Carbone *et al*., 2013; Grupper *et al*., 2022).

In both renal and hepatic tissues, MetS-induced oxidative stress resulting from excessive production of reactive oxygen species has been implicated in the toxic mechanisms leading to tissue damage (Fortuno *et al*., 2006). For instance, the NADPH oxidase (NOX) family of enzymes, including NOX1, 2, and 4, which are highly expressed in the kidney, stimulate the production of reactive oxygen species in renal tissues, thereby aggravating oxidative stress with consequent induction and exacerbation of renal disease progression (Panday *et al*., 2015). Also, oxidative stress is considered one of the causative factors of liver damage, and MetS associated excessive lipid deposition in the liver may predispose to steatosis and NAFLD, generally recognized as the manifestation of MetS in the liver (Perdomo *et al*., 2019; Palladini *et al*., 2019). As a result, recent therapeutic strategies involved in the management of MetS are often geared towards the reduction of exaggerated oxidative stress in various tissues and organ using synthetic and natural antioxidants, as well as medicinal plants with high phytochemical constituents and validated antioxidant efficacies.

Dissotis rotundifolia is a medicinal plant used traditionally for the treatment of many medical conditions in several African countries including Nigeria (Gill, 1992). The plant belongs to the family Melastomataceae and is commonly referred to as pink lady (Baba and Onanuga, 2011). In different parts of Nigeria, *D. rotundifolia* is locally called Nkpisi-nku in Igbo, Ebafo in Benin and Awede in Yoruba (Friday *et al*., 2009). *D. rotundifolia* has potent antioxidant activity and has been reported to upregulate enzymatic and non-enzymatic antioxidants in *in vivo* studies (Adinortey *et al*., 2020; Djehoue *et al*., 2020). This study was designed to evaluate the probable ameliorative effects of *D. rotundifolia* whole plant on hepatic and renal toxicities associated with MetS in male Wistar rats.

MATERIALS AND METHODS

Collection and identification and preparation of *Dissotis rotundifolia* extract*:* *Dissotis rotundifolia* whole plants were collected from a farm at Erunmu, Oyo state. The plant was identified and deposited at the University of Ibadan Herbarium (UIH 22906). The plant sample was washed, cut into smaller pieces and air dried at room temperature (27°C–30°C) for four weeks. The dried plant was thereafter pulverised into its powdery form, and 1000g of this powder was extracted in 10 L of 95% ethanol (analytical grade) for two weeks using the cold extraction method (Gbadamosi *et al*., 2022). The extract was concentrated at 40 °C using a rotary evaporator, and the concentrated extract was defatted using n-hexane. The defatted ethanol extract was then stored in the refrigerator (4^{o}C) before experimental use.

Chemicals: The biochemical analyses in this study involved the use of several chemicals such as 1, 2-dichloro-4-nitrobenzene (CDNB), 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB), trichloroacetic acid (TCA), thiobarbituric acid (TBA), glutathione, hydrogen peroxide, sodium hydroxide, epinephrine, and xylenol orange from Sigma (St. Louis, MO). Also, normal goat serum, biotinylated antibody, and horse radish peroxidase (HRP) System were purchased from Kirkegaard & Perry Lab Inc (Gaithersburg, MD). NF-kB anti-body was purchased from Bioss Inc. (Woburn, MA), while diaminobenzidine (DAB) tablets were purchased from AMRESCO (LLC. OH). All other chemicals used were of analytical grade - British Drug Houses (Poole, Dorset, UK).

Antibodies: Biotinylated secondary antibodies: 2-step plus Poly-HRP Anti Mouse/Rabbit IgG Detection System with DAB solution and primary antibodies against Angiotensin Converting Enzyme1 Polyclonal Antibody (E-AB-16159: 1:500 Dilution) and Neutrophil Gelatinase-Associated Lipocalin (NGAL), Polyclonal Antibody (E-AB-16061: 1:200 Dilution) were purchased from Elabscience Biotechnology®, China).

Ethical Approval

All experiments and protocols were carried out in accordance with the guidelines of the Faculty of Veterinary Medicine, and University of Ibadan Animal Care and Use Research Ethical Committee (UI-AUREC/19/0119).

Experimental Animals Procurement and Acclimatization: Fifty (50) male Wistar rats (150 -180 g) were obtained from the Experimental Animal Unit of the Faculty of Veterinary Medicine and transported to the

Animal house of the Department of Veterinary Biochemistry and Physiology, University of Ibadan. The animals were kept in pathogen-free cages at room temperature (25-27 ºC) in a well-ventilated house under natural environmental light conditions for the period of acclimatization and throughout the experiment. They were acclimatized for two (2) weeks, fed with standard animal diet (commercial pelletized rat finisher) and allowed access to drinking water *ad libitum.*

Induction of metabolic syndrome and Grouping of animals: Metabolic syndrome was induced by feeding the rats with high carbohydrate (20%) fructose in drinking water and high fat diet (40%) for 8 weeks as earlier described by previous authors (Kohli *et al*., 2010; Mahmoud and Elshazly, 2014; Barrios-Ramos *et al*, 2014). The defatted ethanol extract of *Dissotis rotundifolia* and the standard drug for metabolic syndrome (Rosuvastatin) were administered by oral gavage daily for eight weeks, respectively. Rosuvastatin (Ros) is a member of the satin family with higher efficacy in reducing bad cholesterol (LDL cholesterol) than other statins due to a higher number of binding interactions with 3-hydroxy-3methyl-glutaryl-coenzyme A reductase (HMG-CoA), a rate limiting enzyme in cholesterol synthesis (Li *et al*., 2023).
Fifty adult male Wistar rats weighing between 150-180g were divided into five groups of ten rats each for the experiments as follows: Group 1 (Control), Group 2 (MetS control), Group 3 (MetS + 100 mg/kg ethanol extract of *Dissotis rotundifolia*) (Adinortey et al., 2020), Group 4 (MetS + 200 mg/kg ethanol extract of *Dissotis rotundifolia*) (Adinortey et al., 2020), *and* Group 5 (MetS + 20 mg/kg Rosuvastatin).

Serum Preparation and Isolation of Post-Mitochondrial Fraction: Approximately 3 mL of blood was collected from the retro-orbital venous plexus of the animals into plain sample bottles before they were sacrificed by cervical dislocation. The blood was centrifuged at 4000 rpm for 15 min to obtain the serum, which was preserved at 4ºC, and used for biochemical analysis. The liver and kidney were harvested on ice, rinsed, and homogenized in aqueous potassium buffer (0.1 M, pH 7.4) and the homogenate centrifuged at 10,000 rpm (48C) for 10 min to obtain the supernatant fraction.

Biochemical Assays: The post-mitochondrial fractions of the liver and kidney were used for the estimation of reduced glutathione (GSH) as described by Beutler *et al*. (1993). Glutathione S-transferase (GST) was measured by the method of Habig *et al*. (1974) and Glutathione peroxidase (GPx) activity was determined as described by Rotruck *et al*. (1973). Superoxide dismutase (SOD) was determined by measuring the inhibition of auto-oxidation of epinephrine at pH 10.2 as described by Misra and Fridovich (1972) and with modification from our laboratory (Oyagbemi *et al*., 2015). The MDA level was calculated as described by Varshney and Kale (1970). Hydrogen peroxide (H_2O_2) generation was estimated as described (Wolff, 1994). Protein concentration was determined by Biuret method as described by Gornall *et al*. (1949).

Immunohistochemistry

Immunohistochemical staining and imaging: Immunohistochemistry was performed as described by Oyagbemi *et al.* (2023). Antibodies against renal angiotensin-converting enzyme (ACE) and Neutrophil gelatinase-associated lipocalin (NGAL) were probed in the kidney with slight modification using 2-step plus Poly-HRP Anti Mouse/Rabbit IgG Detection System with DAB solution (Catalog number: E-IR-R217 from Elabscience Biotechnology®, China). The kidney samples were fixed with 10% neutral buffered formalin, embedded in paraffin wax, and sectioned at a thickness of 5 µm. The slides were subsequently dewaxed in xylene (100%) solution for 2 minutes and afterward, hydration was carried out in different concentrations of ethanol (100%, 90%, and 80%) for 2 minutes each. The hydrated slides were rinsed and put in a PBS buffer tank for 5 mins. The antigen retrieval was performed with citrate buffer solution containing 2.1 g of citric acid monohydrate and 14.75 g of trisodium citrate dehydrate adjusted to pH 6.0 in microwave oven. Endogenous peroxide (H_2O_2 block) was carried out following the manufacturer's instructions as directed on the kit (E-IR-217C). Drops of H_2O_2 were added to cover the sections and incubated in a humidifying chamber at room temperature for 10 min. The slides were rinsed afterwards and put back in the PBS tank for 5 min. Goat serum (E-1R-R217A) was added onto the slides to prevent nonspecific binding and incubated in humidifying chamber at room temperature (35ºC) for 30 mins. After 30 mins of incubation, the tissues were probed with primary antibodies viz-a-viz Angiotensin Converting Enzyme1 Polyclonal Antibody (E-AB-16159: 1:500 Dilution) and Neutrophil gelatinase-associated lipocalin (NGAL) Polyclonal Antibody (E-AB-16061: 1:150 Dilution) for kidney and were incubated for 2 hours at room temperature. Following incubation, the slides were rinsed with PBS and secondary antibody labelled (E-1R-R217B) was added, and the slides were incubated in humidifying chamber at room temperature for 20 min. Thereafter, the slides were rinsed and immersed in PBS tank for 5 min. Finally, a few drops of the substrate diaminobenzidine (DAB) was added at room temperature for 10 s; 50 µL of DAB concentrate (E-1R-R217D) + 1 mL DAB solution (E-1R-R217E) in the dark. The reaction was terminated with deionized water and slides were immersed in haematoxylin for 3 s before rinsing with PBS. The slides were placed in 80%, 90%, and 100% of ethanol, and then xylene (100%) for 2 minutes each. Slides were removed, allowed to dry and a DPX mountant was applied. Sections were examined using a digital camera and a Leica software application package version 3.4 light microscope (Leica LAS-EZ®).

Statistical analysis

All values are expressed as mean ± standard deviation (SD). The test of significance between two groups was estimated by Student's t-test. One way Analysis of Variance (ANOVA) with Tukey's post-hoc test with p-values < 0.05 considered statistically significant.

RESULTS

Effect of *D. rotundifolia* on Kidney and Liver function Tests: The result of the effect of *D. rotundifolia* extract on markers of kidney and liver function in MetS induced rats is

presented in Figure 1. The blood urea nitrogen (BUN) and creatinine increased significanly ($p<0.05$) in the MetS group. *D. rotundifolia*at 100 mg/kg caused significant ($p<0.05$) decrease in the urea compared with the MetS rats without *D. rotundifolia* treatment. However, *D. rotundifolia* extract at 200 mg/kg did not confer significant protection against renal damage caused by MetS. From the experiment, significant ($p<0.05$) increase was obtained in serum ALT and AST in MetS untreated rats compared ~~to~~ with the control. The extract of *D. rotundifolia* at 100 mg/kg and 200 mg/kg, and Ros caused a significant ($p<0.05$) decrease in ALT activity, while there were no significant change~~s~~ in the activity of AST following treatment with *D. rotundifolia* at 100 mg/kg and 200 mg/kg and Ros in comparison ~~to~~ with the control, respectively..

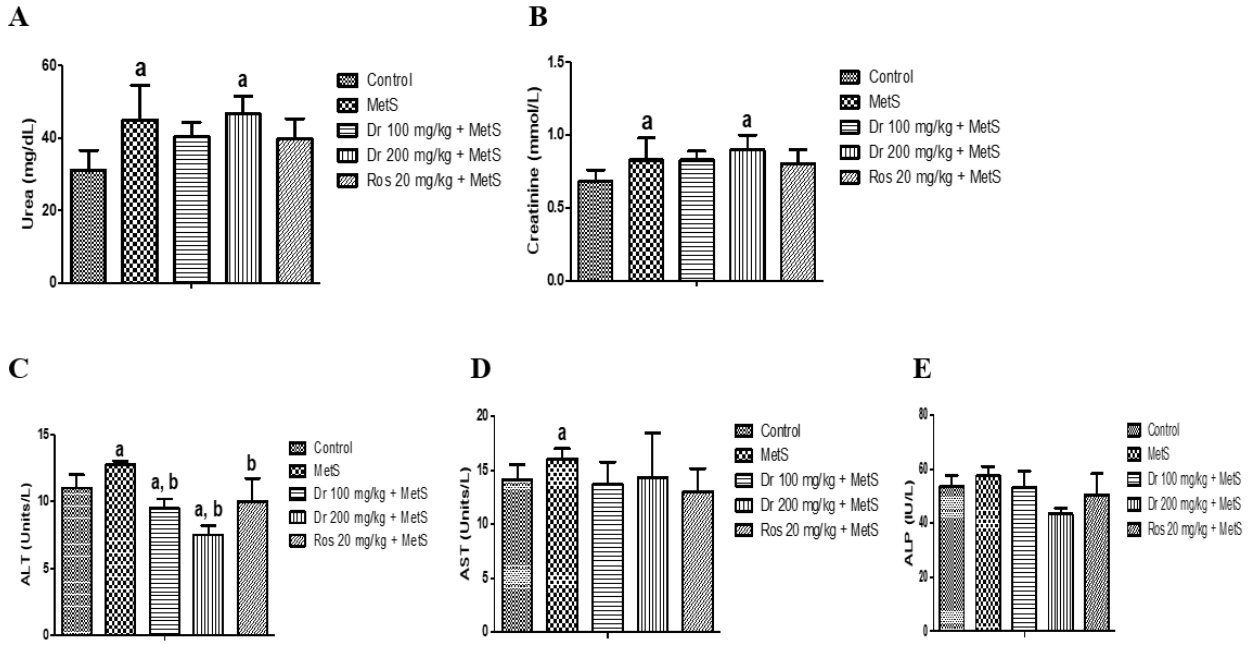

Figure 1:
The ameliorative effects of *Dissotis rotundifolia* on serum kidney and liver function tests.
Superscript (a) indicates significant difference when compared to the control and Superscript (b) indicates significant difference when compared to metabolic syndrome. $P < 0.05$ was taken as statistically significant difference. Mean ± SD (n= 5).
Abbreviations: MetS (Metabolic syndrome), Dr (*Dissotis rotundifolia*), Ros (Rosuvastatin).

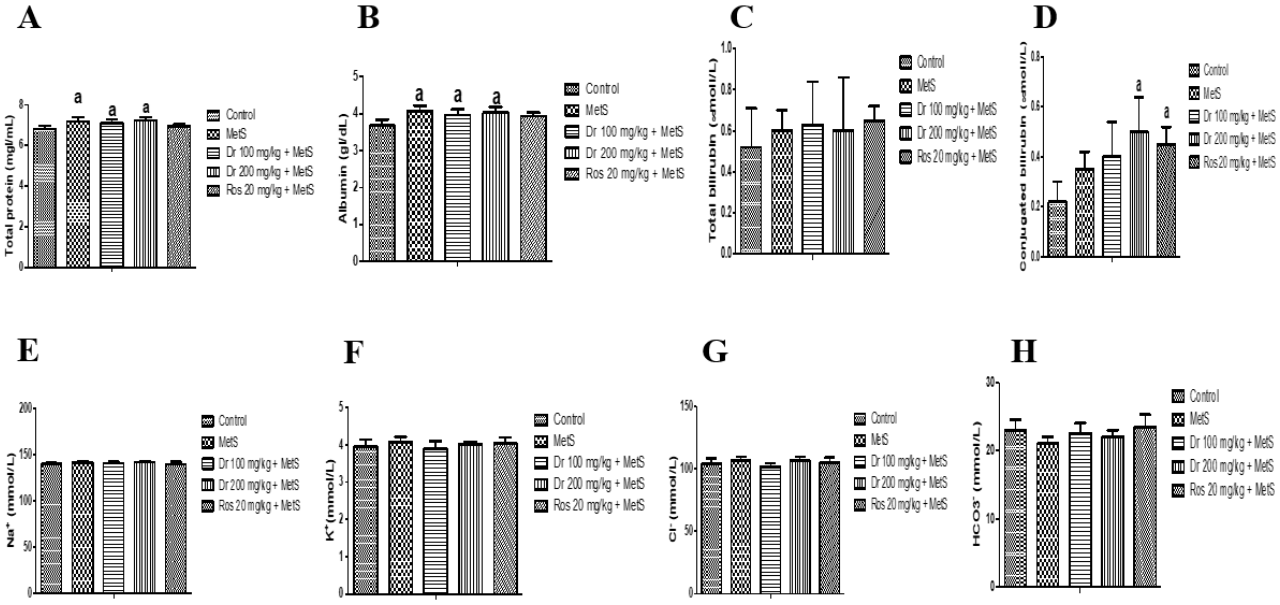

Figure 2:
The ameliorative effects of *Dissotis rotundifolia* on serum proteins and electrolytes. Superscript (a) indicates significant difference when compared to the control and Superscript (b) indicates significant difference when compared to metabolic syndrome. $P < 0.05$ was taken as statistically significant difference. Mean ± SD (n= 5). **Abbreviations:** MetS (Metabolic syndrome), Dr (*Dissotis rotundifolia*), Ros (Rosuvastatin).

Effect of *D. rotundifolia* on Serum Proteins and Electrolytes: The findings from serum proteins show a significant increase in the values of total proteins and albumin recorded in MetS rats and *D. rotundifolia*-treated

rats at 100 mg/kg and 200 mg/kg, relative to the control, respectively (Figure 2). For total bilirubin, there was no statistically significant difference in the values recorded for serum total bilirubin across treatment groups and the MetS untreated rats. However, treatment of MetS rats with *D. rotundifolia* 200 mg/kg and Ros (20 mg/kg) significantly increased the values of conjugated bilirubin compared with the control. There was no statistically significant difference in the serum sodium, potassium, chloride, and bicarbonates across all groups . However, the value of serum bicarbonates was significantly lower in MetS untreated group compared with the control.

Effect of *D. rotundifolia* on lipid profile: The results of *D. rotundifolia* extract on lipid profiles of MetS-induced rats is presented in Figure 3. MetS caused significant ($P<0.05$) increase in the total cholesterol (TC), triglyceride (TAG), low density lipoprotein-cholesterol (LDL-c), and high-density lipoprotein-cholesterol (HDL-c) when compared to the control and other treatment groups. Also,*D. rotundifolia* caused significant reduction in serum TC, TAG, and LDL-c. The HDL-c values of MetS rats were significantly higher that of the control and MetS rats treated with the extract of *D. rotundifolia* and Ros. The values of HDL-c for *D. rotundifolia* and Ros treatment groups were significantly lower than that of the MetS untreated rats.

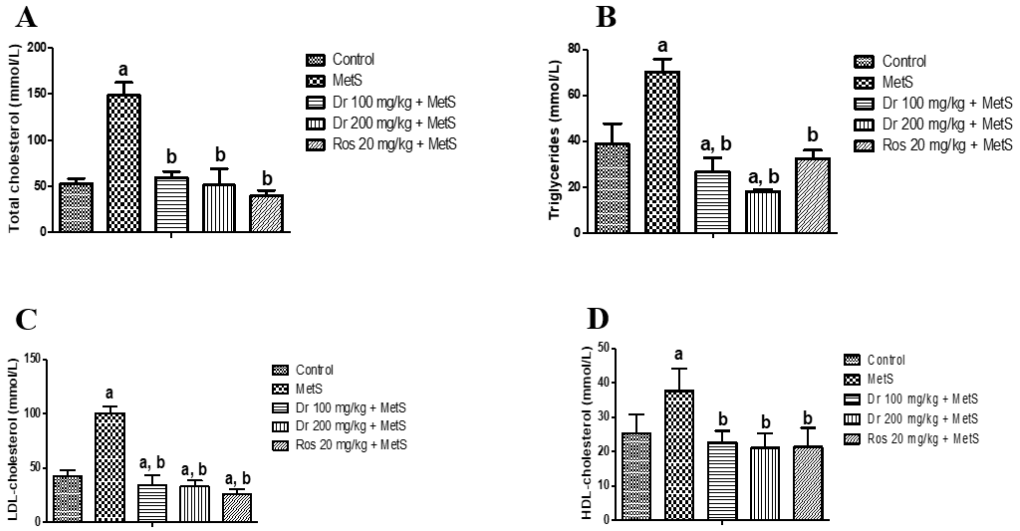

Figure 3:
The ameliorative effects of *Dissotis rotundifolia* on serum lipid profile. Superscript (a) indicates significant difference when compared to the control and Superscript (b) indicates significant difference when compared to metabolic syndrome. $P < 0.05$ was taken as statistically significant difference. Mean ± SD (n= 5). **Abbreviations:** MetS (Metabolic syndrome), Dr (*Dissotis rotundifolia*), Ros (Rosuvastatin).

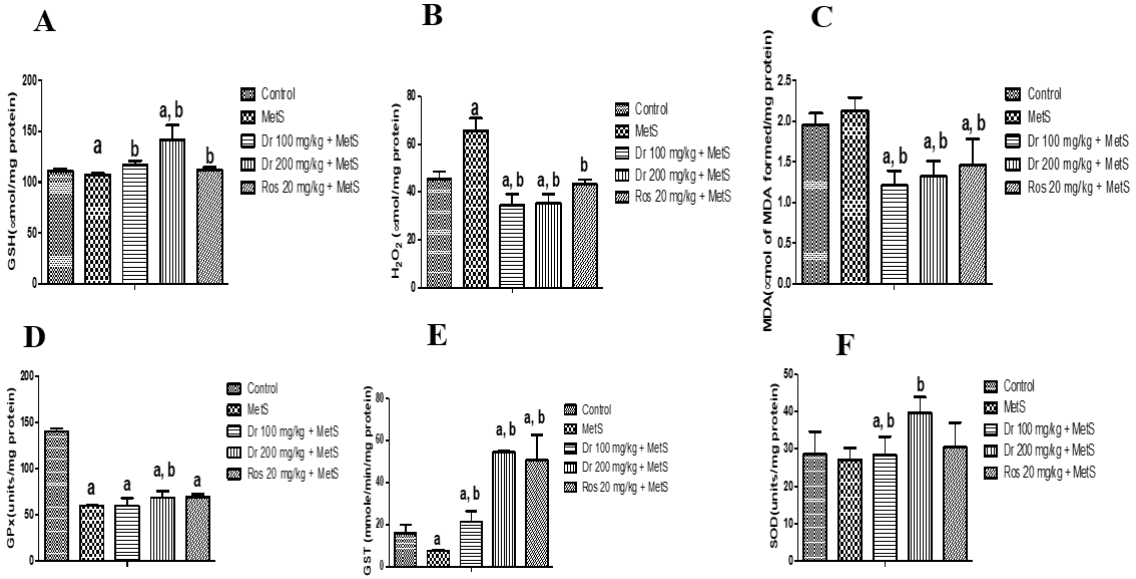

Figure 4:
The ameliorative effects of *Dissotis rotundifolia* on renal oxidative stress biomarkers. Superscript (a) indicates significant difference when compared to the control and Superscript (b) indicates significant difference when compared to metabolic syndrome. $P < 0.05$ was taken as statistically significant difference. Mean ± SD (n= 5). **Abbreviations:** MetS (Metabolic syndrome), Dr (*Dissotis rotundifolia*), Ros (Rosuvastatin).

Effect of *D. rotundifolia* on biomarkers of renal oxidative stress: The renal reduced glutathione (GSH) content in MetS untreated was significantly lower ($p<0.05$) than that in the control (Figure 4). Furthermore, hepatic GSH of rats treated with *D. rotundifolia* extract and Ros was significantly higher relative to the MetS rats. A statistically

significant increase in the values of renal hydrogen peroxide (H_2O_2) generation was recorded for MetS rats when compared to the control. *D. rotundifolia* extract and Ros significantly reduced renal biomarkers of oxidative stress in MetS treated rats. The antioxidant activity of superoxide dismutase (SOD) was enhanced by *D. rotundifolia* extract (100 mg/kg and 200 mg/kg) when compared to the MetS untreated rats. Also, MetS rats had a significant reduction in the antioxidant activities of glutathione peroxidase (GPx) and glutathione S-tranferase (GST) in comparison to the control. On the other hand, treatment of MetS rats with *D. rotundifolia* extract (100 mg/kg and 200 mg/kg) caused a significant increase in the GPx and GST activities in a dose-dependent manner.

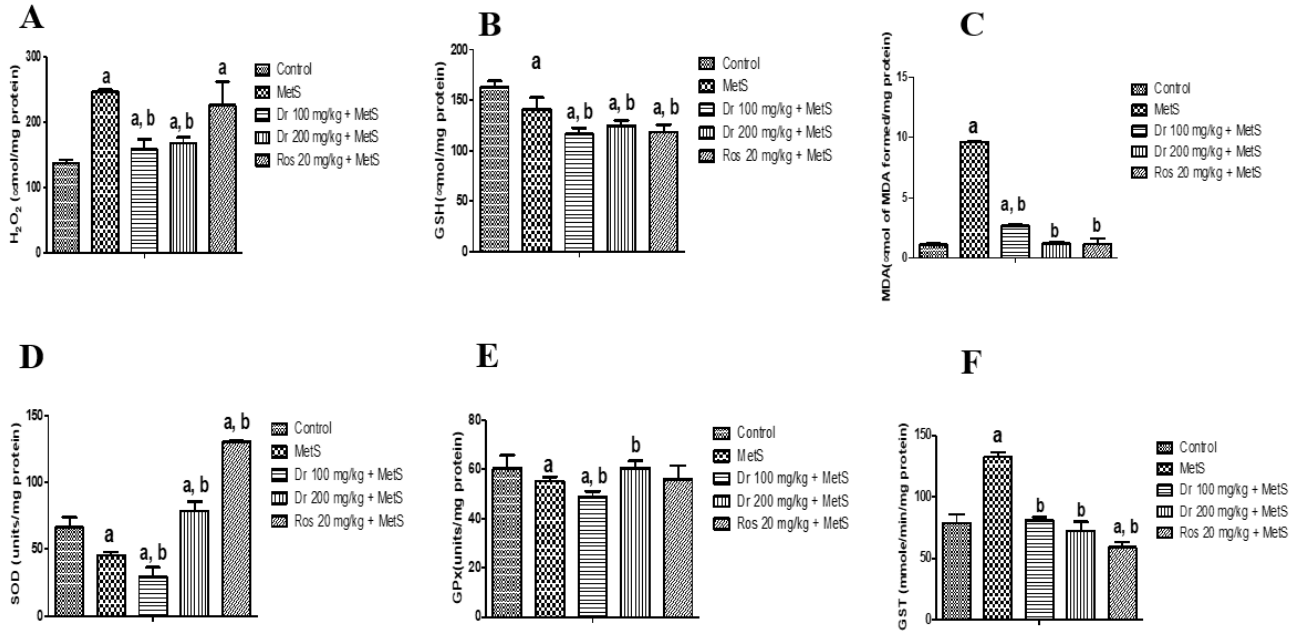

Figure 5:
The ameliorative effects of *Dissotis rotundifolia* on hepatic biomarkers of oxidative stress. Superscript (a) indicates significant difference when compared to the control and Superscript (b) indicates significant difference when compared to metabolic syndrome. $P < 0.05$ was taken as statistically significant difference. Mean ± SD (n= 5). **Abbreviations:** MetS (Metabolic syndrome), Dr (*Dissotis rotundifolia*), Ros (Rosuvastatin).

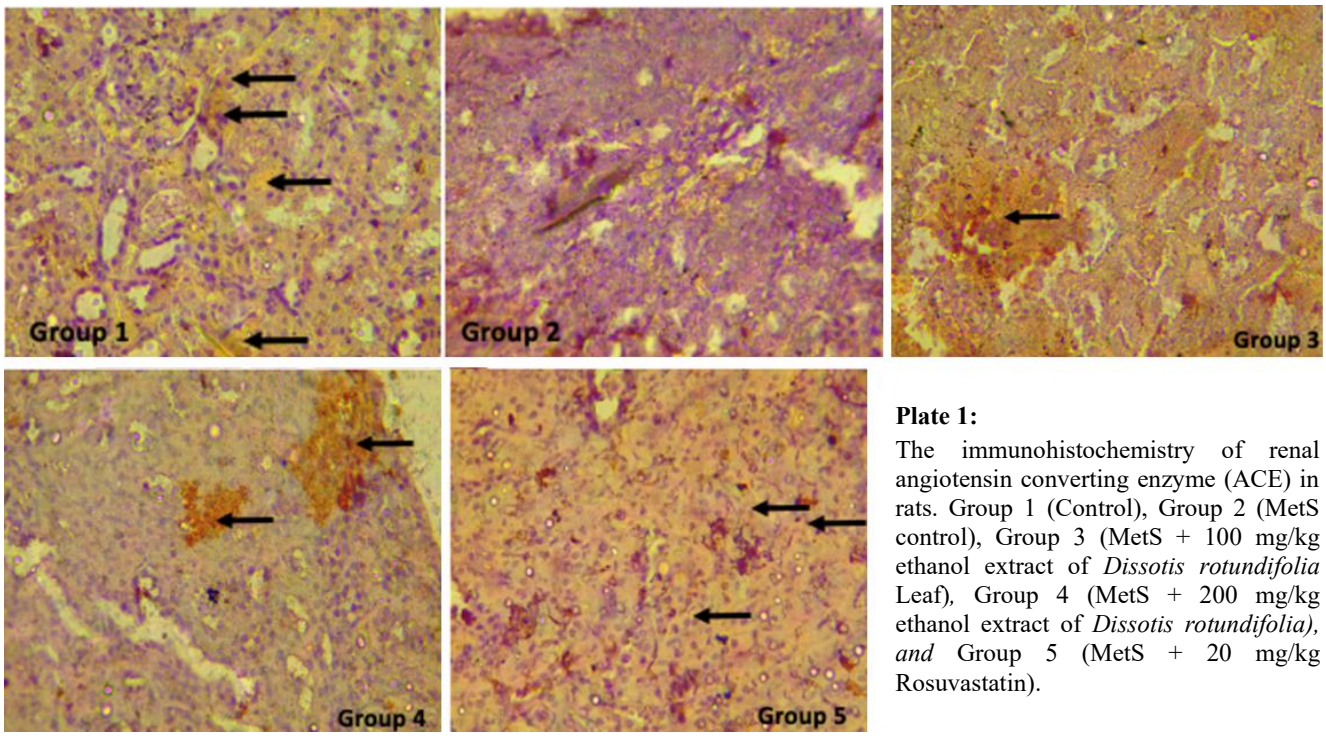

Plate 1:
The immunohistochemistry of renal angiotensin converting enzyme (ACE) in rats. Group 1 (Control), Group 2 (MetS control), Group 3 (MetS + 100 mg/kg ethanol extract of *Dissotis rotundifolia* Leaf), Group 4 (MetS + 200 mg/kg ethanol extract of *Dissotis rotundifolia), and* Group 5 (MetS + 20 mg/kg Rosuvastatin).

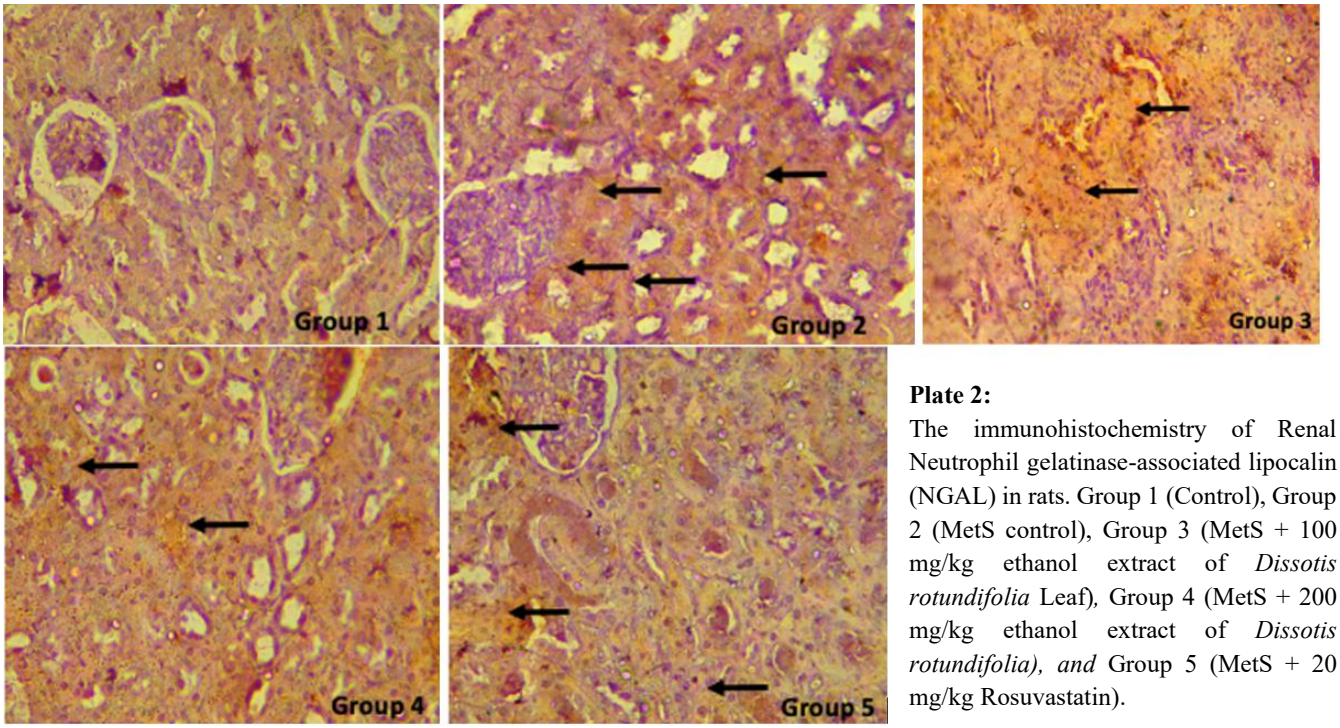

Plate 2:
The immunohistochemistry of Renal Neutrophil gelatinase-associated lipocalin (NGAL) in rats. Group 1 (Control), Group 2 (MetS control), Group 3 (MetS + 100 mg/kg ethanol extract of *Dissotis rotundifolia* Leaf), Group 4 (MetS + 200 mg/kg ethanol extract of *Dissotis rotundifolia), and* Group 5 (MetS + 20 mg/kg Rosuvastatin).

Effect of *D. rotundifolia* on biomarkers of hepatic oxidative stress: In this study, a significant reduction in hepatic GSH content, with a concomitant significant increase in hepatic H_2O_2 generation and MDA were recorded in the MetS group compared with the control and *D. rotundifolia* extract (100 mg/kg and 200 mg/kg) treated groups (Figure 5). Furthermore, *D. rotundifolia* extract caused a significant reduction in hepatic H_2O_2 generation and MDA in comparison to the MetS untreated rats. However, MetS caused a significant reduction in hepatic SOD and GPx activities when compared to the control. Also, *D. rotundifolia* at 200 mg/kg dose significantly increased hepatic SOD and GPx activities relative to the control and MetS rats. MetS significantly caused a significant increase in hepatic GST activity when compared to the control and other treatment groups. However, the extract of *D. rotundifolia* (100 mg/kg) gave a higher value of hepatic GST activity in comparison to the control.

Immunohistochemistry of renal angiotensin-converting enzyme (ACE) and neutrophil gelatinase-associated lipocalin (NGAL): In this study, immunohistochemistry revealed lower expression of angiotensin-converting enzyme (ACE) in MetS-untreated rats relative to the control and *D. rotundifolia-treated* rats at 100 and 200 mg/kg doses. However, *D. rotundifolia* at 100 and 200 mg/kg and Ros showed higher immune-positive reactions of ACE relative to the control and MetS untreated rats, respectively (Plate 1). The immunoreactivity of neutrophil gelatinase-associated lipocalin (NGAL) was higher in MetS untreated rats in comparison to the control rats (Plate 2).

DISCUSSION

Oxidative stress has been severally reported in the pathogenesis and pathophysiology of MetS and the individual components of the syndrome (Vona *et al*, 2019). As a result, in-depth investigations on oxidative-mediated organ-specific damage in disease conditions such as obesity, diabetes, and hypertension, which are clustered in MetS are currently ongoing globally (Carrier, 2017). Specifically, MetS-associated abdominal adiposity promotes inflammation and oxidative stress and other complications such as insulin resistance, hypertension, and hyperlipidemia (Rani *et al*., 2016; Francisqueti *et al*., 2017). In this study, the establishment of metabolic syndrome was manifested as elevated levels of total cholesterol, triglyceride, and low-density lipoprotein cholesterol (LDL-C) in the plasma, with decreased levels of high-density lipoprotein cholesterol (HDL-C), as earlier reported (He *et al*., 2022). Increased biomarkers of oxidative stress (OS) and decreased antioxidant defenses have been measured in the blood of patients with MetS, suggesting an *in vivo* overproduction of oxidizing species (Spahis *et al*., 2017). Increased oxidative stress can result from several variables, such as high-fat and high-carbohydrate diets, and persistent undernutrition, by activating intracellular pathways such as nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase (NOX), oxidative phosphorylation in mitochondria, glycoxidation, protein kinase C (PKC), and the polyol pathway (Korac *et al*., 2021). Furthermore, changing dietary intake from organic healthy foods to highly processed foods may lead to increased exposure to advanced glycated end products aged garlic extracts (AGEs) by a non-enzymatic chemical reaction called glycation (Fallavena *et al*., 2022).

Diets with high antioxidant content have been reported to provide beneficial effect in the management of MetS-associated oxidative stress and inflammation (Castro-Quezada *et al*., 2014; Casas *et al*., 2014). Similarly, medicinal plants and plants derived phytochemicals have been reported to positively modulate the pathophysiology and disease progression in metabolic syndrome patients (Rochlani *et al*., 2017). The observation of increased hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) levels in the MetS group of rats, in this study, suggests the induction of oxidative stress, whereas, the significantly

increased level of the antioxidants reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and glutathione transferase (GST) in rats treated with the defatted ethanol extract of *D. rotundifolia* suggests a potent antioxidant effect of the plant. . Observation in this study corroborates an earlier report of Adinortey *et al.* (2020), who reported a conservation of GSH levels, reduced MDA levels, and enhanced SOD activity in rats administered flavonoid-rich extract of *D. rotundifolia* (Adinortey *et al*., 2020). Medicinal plants, some of which have been used for thousands of years, serve as an excellent source of bioactive compounds for the treatment of MetS because they contain a wide range of phytochemicals with diverse metabolic effects (Graf *et al*., 2010). A wide array of bioactive phytochemicals from medicinal plants, such as turmeric, garlic, cinnamon, ginger, grapes, onions, and broccoli have demonstrated a positive modulatory role in the management of MetS (Rochlani *et al*., 2017). *Dissotis rotundifolia* has potent antioxidant activity that may be attributable to the high levels of flavonoids, phenols and saponins (Djehoue *et al*., 2020; Ezeabara *et al*., 2022; Gbadamosi *et al*., 2022). Flavonoids are distributed in foods and have been severally reported to provide beneficial effects in the management of many diseases by significantly modulating several metabolic parameters, such as lipid profile, blood pressure, and blood glucose (Gouveia *et al*., 2022). Moreover, natural polyphenols generally have antioxidant and anti-inflammatory effects, and have been reported to aid vascular functioning, promote gastrointestinal digestion, lower blood lipids, prevent atherosclerosis, and lower blood pressure (Cai *et al*., 2015; Bruno and Ghiadoni, 2018 *l*., 2018; Zhang *et al*., 2021). Furthermore, saponin containing medicinal plant products such as ginseng has been shown to increase fasting insulin sensitivity index and exert anti-insulin resistance as well as anti-obesity activity (Luo *et al*., 2020). Therefore, the normalisation of the lipid profile in rats treated with the defatted ethanol extract of *D. rotundifolia* compared with the control group, in this study, may be attributable to the high polyphenolic antioxidant effects of this plant, as earlier reported (Darkwah *et al*., 2018).

The liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) are routinely used clinically to assess the functional status of the liver. In this study, the induction of MetS in rats caused elevated serum levels of AST, ALT, and ALP, compared with the control, and the MetS rats that were treated with the defatted ethanol extract of *D. rotundifolia*, thus suggesting a hepatoprotective effect of the plant in MetS. Elevated ALT in the serum often suggests specific damage to hepatocytes and has been reported to be linked with various risk factors for metabolic syndrome, diabetes, and cardiovascular diseases such as obesity, hyperglycemia, dyslipidemia and increased blood pressure (Sanyal *et al*., 2015; Kathak *et al*., 2022, Gbadamosi *et al*., 2020). Thus, the observed reduced levels of the hepatic enzymes in rats administered *D. rotundifolia* extract strongly suggest hepatoprotective effects, and a desirable modulatory effect of the whole plant extract in MetS.

The elevation of creatinine and blood urea nitrogen observed in MetS-induced rats without *D. rotundifolia* treatment strongly suggests an induction of kidney damage. Blood urea nitrogen often increases due to inadequacies in its removal by the kidney, but may also be associated with several physiological conditions, such as high protein intake, intestinal bleeding, infection, fever, dehydration, medications, burns, and poisoning (Weiner et al., 2014). However, an increase in blood urea nitrogen in the presence of concomitant increased creatinine levels often signifies renal damage resulting from MetS-associated cardiovascular dysfunctions or impaired renal blood flow (Rivadeneyra-Domínguez *et al*., 2018). Likewise, increased serum creatinine levels, often associated with kidney damage, have been reported to increase in metabolic syndrome patients (Wang *et al*., 2015). Several reports have suggested significant increased likelihood of the development of chronic kidney disease characterized by changes in renal structure, decreased glomerular filtration rate (GFR), and increased urinary microalbumin in patients with MetS (Zhang and Lerman, 2017; Kawamoto *et al*., 2019).

The involvement of chronic kidney disease recently, has become one of the major risk factors in MetS (Kazancioğlu, 2023; Lin *et al*., 2023; Zohara *et al*., 2023; Scurt *et al*., 2024). The neutrophil gelatinase-associated lipocalin (NGAL) is a 25-kDa protein that is secreted mostly by immune cells such as neutrophils, macrophages, and dendritic cells. Its production is stimulated in response to inflammation (Romejko *et al*., 2023). The NGAL is known mainly as a biomarker of acute kidney injury, chronic kidney disease, and is released after tubular damage and during renal regeneration processes (Voth *et al*., 2023). Also, NGAL is useful in the diagnostic processes of cardiovascular diseases because it is highly expressed in injured heart tissue and atherosclerostic plaque (Yewale *et al*., 2023; Voth *et al*., 2023). The data from the immunohistochemistry revealed renal damage associated with MetS as indicated with highly immune-positive reactions of NGAL. The protection of the renal tissues from MetS could be associated with reduction in the NGAL immune reactivity of MetS rat treated with *D. rotundifolia* extract. Another major risk factor for MetS is hypertension. Previous research findings have reported positive association between hypertension and MetS (Das *et al*., 2023; Stanciu *et al*., 2023; Soleimani *et al*., 2023). For this reason, the immunoreactivity of angiotensin converting enzyme (ACE) was assessed. Also in this study, the immune reactivity of MetS rat treated with defatted *D. rotundifolia* extract ACE was higher than the Mets untreated rats. The angiotensin-converting enzymes (ACE and ACE2) are highly expressed in renal tubules and play an important role in the regulation of renal function by the intrarenal renin-angiotensin system (Larrinaga *et al*., 2010). ACE 2 degrades angiotensin (Ang) II to Ang (1–7) and Ang I to Ang (1–9) (Ye et al., 2004) and may reduce the incidence of hypertension since angiotensin II is a potent vasoconstrictor. Therefore, the mechanism of nephroprotection and the antihypertensive effects of *D. rotundifolia* could be through the inhibition of NGAL and upregulation of ACE signaling pathway.

In conclusion, the defatted ethanol extract of *D. rotundifolia* whole plant offered protection against metabolic syndrome-induced liver and kidney damage and hepatorenal oxidative stress, improved antioxidant defence status, and mitigated hyperlipidemia.

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Full-Length Research Article

Impact of *Zingiber officinale* on Testicular Morphometry, Sperm Quality, and Hormonal Profiles in Alcohol-Induced Toxicity

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Summary: Alcohol consumption is known to induce reproductive toxicity, leading to adverse effects on testicular morphology, sperm quality, and DNA integrity in males. *Zingiber officinale* (ginger), known for its antioxidant and anti-inflammatory properties, may counteract these effects. This study aimed to investigate the impact of ginger on testicular morphometry, sperm quality, and hormonal profiles in alcohol-induced toxicity. 30 male Wistar rats were divided into six groups (n=5/group). Group A (control) received normal saline. Group B was exposed to 40% alcohol (3.50 g/kg body weight) from Days 15–28. Group C received ginger (750 mg/kg) during the same period. Groups D, E, and F were treated with alcohol for 14 days, followed by low (250 mg/kg), medium (500 mg/kg), and high (750 mg/kg) doses of ginger, respectively, from Days 15–28. The study evaluated changes in body and testicular morphometry, antioxidant enzyme and hormonal changes. Semen analysis included sperm motility, count, and morphology, while sperm chromatin/DNA integrity was assessed using aniline blue and toluidine blue staining. Alcohol reduced body weight gain (16.5 g vs. 38.5 g in Control), testicular volume (1.11±0.03 mm³ vs. 2.16±0.27 mm³), GSI, sperm quality, hormonal levels, and GSH, reflecting oxidative damage. Ginger treatment, particularly at 250 mg/kg, restored body weight gain (29.7 g), testicular volume (1.75±0.24 mm³), GSI (1.25±0.12%), sperm parameters, LH (5.08 mIU/mL), FSH, and GSH levels, and reduced sperm DNA damage. Higher ginger doses (500–750 mg/kg) showed diminished efficacy, suggesting dose optimisation. Ginger's antioxidant properties, likely mediated by gingerols and shogaols, counteract alcohol-induced reproductive toxicity. Ginger improves reproductive health and mitigates alcohol-induced toxicity in a dose-dependent manner. Moderate doses show optimal benefits, while high doses may be detrimental. These findings support ginger's potential as a natural therapeutic agent for reproductive health.

Keywords: *Alcohol-induced toxicity, antioxidant, ginger, semen quality, testicular morphometry, Zingiber officinale.*

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INTRODUCTION

Infertility, known as the inability to conceive after months of regular, unprotected sexual intercourse (at least for 12 months), is a significant health matter (Kyrgiafini and Mamuris, 2023). Globally, infertility affects about 15% of couples of reproductive age (Assidi, 2022). Males account for about 50% of total infertility cases worldwide. The prevalence is said to increase at 0.3% annually, leading to an uneven geographical distribution ranging from 20 to 70% (Sun *et al*., 2019). Nonetheless, the rates of male infertility are underreported due to different factors such as cultural, societal, religious and patriarchal influences that hinder precise sampling and analysis (Mehra *et al*., 2018). Male infertility includes any health condition that hinders the chances of conception. It can result from abnormal sperm function or obstructions preventing ejaculation (Agarwal *et al*., 2021).

Numerous factors are reported to influence male infertility, including anatomo-pathophysiological factors, environmental factors, the process of ageing and lifestyle factors (Skoracka *et al*., 2020). Lifestyle-related factors play a significant role in causing male infertility globally (Balawender and Orkisz, 2020). An example of these lifestyle-related factors is alcohol consumption, which has gained considerable attention due to its global prevalence and modifiable nature (Anwara *et al*., 2025). Alcohol is considered one of the most prevalent dietary factors that people are exposed to and is very prevalent in many societies, with almost 60% of the global population aged 15 years and over reported to have consumed alcoholic drinks in a single year (Finelli *et al*., 2021). The consumption of alcoholic beverages has been a part of the socio-cultural

heritage of most populations since ancient times (Duca *et al*., 2019). Indeed, alcohol has been viewed as an integral part of a meal and, in some cases, as a remedy for infectious diseases or even as a cleaning agent (Neufeld *et al*., 2020). Alcohol consumption poses a significant threat to male reproductive health, affecting millions worldwide and impacting testicular structure and function through oxidative stress mechanisms (Assidi, 2022). Chronic alcohol exposure leads to histological, hormonal, and biochemical alterations, often culminating in reduced semen quality, including impaired sperm concentration and motility, which are key markers of male infertility (Neufeld *et al*., 2020).

Since alcohol causes these distortions via the increase of reactive oxygen species (ROS), many therapeutic approaches focus on synthetic or natural antioxidants derived from natural sources to mitigate the damage caused by an elevation in ROS (Ovie *et al*., 2023). An example of these natural antioxidants is *Zingiber officinale*, commonly known as ginger. The ginger rhizome is utilised for its aromatic smell and strong taste (Mahomoodally *et al*., 2021). Moreover, ginger is extensively employed in folk medicine for its numerous health benefits in treating various diseases, including chronic conditions such as cancer, diabetes, Alzheimer's, ulcers, cardiovascular disease, as well as depression (Kukula-Koch *et al*., 2018). The positive impact of ginger on these diseases primarily stems from its antioxidant, antimicrobial, and anti-inflammatory properties (Ballester *et al*., 2022). In different protective studies, ginger has been shown to overcome the reproductive toxicity of cyclophosphamide (Mohammadi *et al*., 2014), gentamicin (Zahedi and Khaki, 2014), sodium arsenite (Seif *et al*., 2021), and ethanol (Li *et al*., 2021) and increase sperm counts, viability, motility, and hormones and improve testicular architecture. Despite these promising findings, the curative potential of testicular morphometry, sperm quality, and hormonal profiles in alcohol-induced toxicity remains underexplored; hence, this study.

MATERIALS AND METHODS

Study Location: This study was carried out in the animal unit of the Anatomy department at Alex Ekwueme Federal University Ndufu Alike Ikwo (AE-FUNAI), Ebonyi State, Nigeria. Ethical approval was sought from the Research and Ethical Committee of the Faculty of Basic Medical Sciences with the code AE-FUNAI/FBMS/EAHC/24/006.

Samples Collection, Identification and Preparations: Fresh rhizomes of *Zingiber officinale* were purchased at "Ogbe Hausa" in Abakaliki Local Government Area, Ebonyi State, Nigeria. The samples were identified in the Applied Biology Department at Ebonyi State University, Abakaliki, Nigeria. The rhizomes were washed, dried at room temperature, and mechanically milled into a fine powder. The aqueous extract was prepared by soaking 100 g of powdered rhizome in 1 L of distilled water for 24 hours with intermittent stirring. The mixture was then filtered using Whatman No. 1 filter paper, and the filtrate was evaporated using a water bath at 40°C to obtain a concentrated extract. The doses of 250 mg/kg, 500 mg/kg, and 750 mg/kg of *Zingiber officinale* extract were classified as low, medium, and high, respectively, based on prior studies that reported no toxicity at doses up to 5000 mg/kg in Wistar rats (Otunola and Afolayan, 2017) and aligned with previous reproductive studies (Khaki *et al*., 2008; Morakinyo *et al*., 2008) to investigate dose-dependent effects.

Animal Care and Treatment: The alcohol (99.7- 100% vv GPR absolute ethanol) produced by NAFCO Scientific Supplies Limited, Surulere, Lagos, Nigeria, with Product Number 28304 7k, was purchased which was used as the toxicant for this experiment. According to the study by Biney *et al*. (2020) 42% had no mortality in rats.
Thirty (30) male Wistar rats randomly divided into six (6) groups (Groups A-F), each comprising five (5) randomised rats, were obtained from the animal house at AE-FUNAI and were kept at room temperature (20–22°C). The animals were housed in well-ventilated cages with suitable environmental conditions. During their acclimatisation process, which lasted for two weeks at the animal house, the rats were provided with standard feed only. The experimental groups and treatment protocols are shown in Table 1.

Animal Sacrifice and Sample Collection: On the 28th day of the experiment, animals were weighed and sacrificed. Blood samples were collected by heart puncture and centrifuged (3000 rpm for 15 min) to obtain sera, and they were then separated and stored at -80 ºC for later hormonal assays. After blood collection, testicular parameters (weight, volume and gonadosomatic/testes index) were performed. The left testis was manually homogenised and centrifuged at 3000 rpm for 10 min in a cold phosphate buffer (pH 7.4, 0.1M). The obtained supernatant was used to evaluate the level of antioxidant enzyme activity.

Testis Morphometry

Testis Weight: The weight of each testis was recorded immediately after extraction using an electronic scale.

Table 1:
Experimental Groups and Treatment Protocols

Groups	Description	Days	
		1 – 14	15 – 28
A	Control	Sterile water	Sterile water
B	Alcohol-Only	Sterile water	40% Alcohol (3.50 g/kg)
C	Ginger-Only (High Dose)	Sterile water	Ginger extract (750 mg/kg)
D	Alcohol + Low-Dose Ginger	40% Alcohol (3.50 g/kg)	Ginger extract (250 mg/kg)
E	Alcohol + Medium-Dose Ginger	40% Alcohol (3.50 g/kg)	Ginger extract (500 mg/kg)
F	Alcohol + High-Dose Ginger	40% Alcohol (3.50 g/kg)	Ginger extract (750 mg/kg)

Testis Volume: A sliding digital Vernier calliper was used to measure the width and length of each testis. The testis volume was then calculated using the spheroid formula:
Testis volume = width^2 × length × 0.523 (mm^3)

Gonadosomatic/Testes Index: The final body and testis weights were used to calculate the gonadosomatic index using the formula previously reported by Ukoha *et al.* (2014).

$$\text{Gonadosomatic index} = \frac{\text{Testis weight}}{\text{Body weight}} X\ 100(\%)$$

Biochemical Assays

Hormonal Assay: The serum levels of reproductive hormones—luteinizing hormone (LH), follicle-stimulating hormone (FSH), and inhibin B (INB)—were measured using enzyme-linked immunosorbent assay (ELISA) kits. The ELISA kits for LH and FSH were obtained from NIADDK, NIH (USA), while the kit for INB was sourced from Diagnostic Systems Laboratories (DSL-10-84100i; Webster, TX, USA), following the manufacturer's protocols, as reported by Famurewa *et al*. (2023).

Glutathione (GSH) Level: Testicular activities of antioxidant enzyme GSH were analysed in homogenate supernatant at 4°C using commercial rat ELISA kits according to the manufacturer's instructions.

Semen Analysis: Spermatozoa were collected by making a small incision (1 ml) in the caudal epididymis, followed by evaluation of sperm count, motility, and morphology. The sperm count was determined using an improved Neubauer hemocytometer. Epididymal sperm motility was calculated by measuring the number of motile spermatozoa per unit area and was expressed as a percentage of motility. Sperm morphology was analysed using the Wall and Ewas stain, with examination conducted under a microscope as previously detailed (Igwe *et al*., 2024).

Sperm Chromatin Evaluation: Standard cytochemical methods, incorporating aniline blue (AB) and toluidine blue (TB), were employed to evaluate chromatin condensation and DNA integrity. AB was selectively utilised to stain lysine-rich histones. Air-dried smears obtained from washed semen samples were positioned in 0.2 M phosphate buffer (pH 7.2) containing 3% buffered glutaraldehyde for 30 minutes at room temperature. Subsequently, each smear underwent staining in 4% acetic acid (pH 3.5) with a 5% aqueous solution of AB for 7 minutes. During the light microscopic evaluation, a meticulous count of 200 spermatozoa was conducted across various sections of each slide, utilizing a ×100 eyepiece magnification (Pourmasumi *et al*., 2019). Sperm heads stained pale blue/colourless and dark blue were considered normal (AB-) and abnormal sperm (AB+), respectively.

On the other hand, TB served as a metachromatic dye, offering insight into nuclear chromatin condensation and the quality and quantity of DNA fragmentation in sperm. Air-dried sperm smears were fixed using a mixture of 96% ethanol and acetone (1:1) for 30 minutes at a temperature of 4°C. Subsequently, the slides underwent a 5-minute incubation in 0.1 N HCl at 4°C, followed by thorough washing with distilled water three times for 2 minutes each. Finally, staining took place for 10 minutes at room temperature using 0.05% TB in 50% citrate phosphate. In the evaluation process, a minimum of 200 spermatozoa were counted in each sample using light microscopy with a ×100 eyepiece magnification (Pourmasumi *et al*., 2019). Normal sperm is pale blue, and abnormal sperm is dark blue or violet purple. For each sample, the normal (TB-) and abnormal (TB+) spermatozoa were reported as percentages.

Data Analysis: Data were subjected to analysis of variance using GraphPad Prism version 8 and presented as Means ± SD. Group means of parametric data were compared using a one-way analysis of variance, followed by Turkey's post hoc test. $p < 0.05$ was considered statistically significant.

RESULTS

Body Weight Analysis: The body weight analysis demonstrates weight changes among the groups as presented in Table 2. Group A exhibited a steady increase, resulting in a total weight change of 38.50 g. Group B showed a minimal weight gain of only 16.50 g when compared to Group A, indicating the detrimental effects of alcohol on weight. Group C displayed a weight increase of 20.50 g, whilst Groups D, E, and F were exposed to alcohol and treated with varying doses of ginger. D gained 29.00 g, while Group E had a slight weight loss of 0.75 g, and Group F gained 13.25 g.

Table 2:
Body weight analysis among the experimental groups.

Groups	Initial Weight (g)	Final Weight (g)	Weight change (g)
A	113.20±7.46	152.00±9.06	38.50±11.68
B	131±25.86	147.50±22.34	16.50±11.39
C	152.25±15.44	172.75±31.82	20.50±33.41
D	131±12.88	160.00±19.55	29.00±31.35
E	150±4.47	149.75±23.47	-0.75±24.51
F	158.40±20.74	171.00±1.16	13.25±24.73

Values represent Mean ± SD

Table 3:
Testes Morphometry

Groups	Testes Weight (g)	Testes volume (mm^3)	Gonadosomatic index (%)
A	1.57±0.39	2.16±1.76	1.04±0.28
B	1.57±0.32	1.10±0.29	1.10±0.27
C	1.81±0.13	2.61±1.31	1.10±0.31
D	1.75±0.24	1.08±0.28	1.20±0.12
E	1.66±0.28	1.18±0.08	1.11±0.12
F	1.65±0.21	1.31±0.19	0.96±0.13

Values represent Mean ± SD.

Testicular Morphometry: The evaluation of testes morphometry (Table 3) revealed no statistically significant differences ($p > 0.05$) in testes weight, testes volume, and gonadosomatic index among the experimental groups. The control group showed a mean testis weight of 1.57 ± 0.39 g, while the highest value was observed in Group C (1.81 ± 0.13 g), but this increase was not significant ($p > 0.05$). Testis volume was also highest in Group C (2.61 ± 1.31 mm^3), with the lowest in Group D

(1.08 ± 0.28 mm³); however, these differences did not reach statistical significance. The gonadosomatic index ranged from $0.96 \pm 0.13\%$ in Group F to $1.20 \pm 0.12\%$ in Group D, but no significant variation was observed across the groups ($p > 0.05$).

Semen Analysis: As shown in Table 4, the semen analysis revealed significant differences in motility, count and morphology across the groups. Group B exhibited significantly reduced progressive motile sperms when compared to Group A indicating significant impairment. Group C demonstrated notable improvement when compared to A. Groups D, E and F recorded motility levels significantly different from Group B. Regarding sperm count, Group B had a lower count compared to Group A. Groups C, D, E, and F showed counts higher than Group B. In terms of morphology, the results revealed that Groups A, C, D, and E maintained high percentages of normal sperm (around 95%), while Group B had a slightly lower percentage of 95.33±0.58. Notably, Group B displayed higher instances of head defects, with pinhead defects reported at 3.33±0.58 ($p<0.05$) compared to Group A. Additionally, in terms of midpiece defects, Group B had a bent midpiece at 0.33±0.58, while all other groups reported 0.00±0.00. Concerning tail defects, Group B exhibited a higher incidence of headless tails at 1.33±0.58 compared to Group A, which had 1.00±0.00.

Table 4:
Showing the semen analysis after exposure to alcohol and ginger

	Groups	A	B	C	D	E	F
Motility (%)	Progressive motile	71.67±2.89	36.67±2.89*	75.00±0.00	51.67±2.89#	51.67±10.41#	40±13.23#
	Sluggish motile	23.33±2.89	30.00±5.00	20.00±0.00	26.67±2.89	28.33±2.89	36.67±10.40#
	Non-motile	5.00±0.00	33.33±2.89*	5.00±0.00	21.67±2.89	20.00±10.00#	23.33±2.89
Count (10^6/ml)		37.33±6.43	20.00±7.00	38.33±8.39	34.00±5.00	27.33±11.02	33.00±12.12
Morphology (%)	Normal sperm	97.33±0.58	95.33±0.58	95.67±0.58	96.67±1.53	96.00±1.00	95.67±0.58
Head defects	Round Head	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Pinhead	1.00±1.00	3.33±0.58*	1.67±0.58	0.67±0.58	1.67±1.14	1.33±0.58#
Midpiece defects	Bent midpiece	0	0.33±0.58	0	1.00±1.00	1.33±1.15	1.33±0.58
	Coiled midpiece	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Tail defects	Headless tail	1.00±0.00	1.33±0.58	1.33±0.58	1.33±0.58	1.00±1.00	0.33±0.58
	Coiled tail	0.67±0.58	1.33±0.58	0	0.33±0.58	0	1.33±0.00
	Absence of tail	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Loop tail	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Values represent Mean ± SD. * represents a significant difference when compared to A; # represents a significant difference when compared to B.

Table 4:
Effects of alcohol and ginger on sperm chromatin

Groups	Aniline Blue		Toluidine Blue	
	AB-	AB+	TB-	TB+
A	96.83±1.61	3.17±1.60	99.33±0.76	0.67±0.76
B	92.17±3.62	7.83±3.62*	89.00±1.73*	12.33±4.04*
C	98.33±1.04	2.00±0.50	99.50±0.50	0.50±0.50
D	97.37±0.78#	2.67±0.76#	95.17±1.89#	4.83±1.89#
E	97.83±0.29#	2.67±0.58#	97.33±1.26#	2.67±1.26#
F	97.33±0.58#	2.17±0.29#	99.00±0.87#	1.00±0.87#

Values represent Mean ± SD. * represents a significant difference when compared to A; # represents a significant difference when compared to B. KEYS: normal chromatin (AB-); abnormal chromatin (AB+); normal DNA (TB-); abnormal DNA (TB+).

Sperm Chromatin: The evaluation of sperm chromatin integrity (Table 4) showed significant differences ($p < 0.05$) among the experimental groups. Group B had a significantly higher percentage of abnormal chromatin (AB+) and abnormal DNA (TB+) compared to the Control group ($p < 0.05$). Group C exhibited normal chromatin (AB-) and DNA integrity (TB-) levels comparable to the Control group ($p > 0.05$). Ginger supplementation in alcohol-treated groups (D, E and F) significantly reduced the percentage of spermatozoa with abnormal chromatin (AB+) and DNA damage (TB+) compared to Group B ($p < 0.05$).

Hormonal Assay and Antioxidant Enzyme Levels: Serum hormone levels in Table 5 showed significant differences ($p < 0.05$) among the experimental groups. Group B exhibited a significant reduction in FSH and INB levels compared to the Control group ($p < 0.05$), while LH levels were also lower but not statistically significant ($p > 0.05$). Group C showed LH, FSH, and INB levels comparable to the Control group ($p > 0.05$). In the alcohol-treated groups supplemented with ginger (groups D, E and F), FSH and INB levels were significantly higher than in Group B ($p < 0.05$), approaching values similar to the Control group, while LH levels remained statistically comparable across all groups ($p > 0.05$).

Furthermore, the testicular GSH levels showed significant differences ($p < 0.05$) among the experimental groups. Group B had a significantly lower GSH level (12.93 ± 2.41 mmol/g tissue) compared to the Control group (23.07 ± 1.76 mmol/g tissue) ($p < 0.05$). Group C showed a GSH level (25.47 ± 3.83 mmol/g tissue) comparable to the Control group ($p > 0.05$). Ginger supplementation in alcohol-treated groups (D, E and F) significantly increased GSH levels compared to the alcohol-only group ($p < 0.05$), although these values remained slightly lower than those of the Control group ($p > 0.05$), as shown in Table 5.

DISCUSSION

This study investigated the effects of alcohol-induced toxicity and aqueous *Zingiber officinale* (ginger) extract on testicular morphometry, sperm quality, hormonal profiles, and antioxidant levels in male Wistar rats. Our findings demonstrate that alcohol impairs testicular function, reduces body weight gain, and disrupts hormonal and antioxidant defenses, while ginger treatment, particularly at a low dose (250 mg/kg), mitigates these effects, improving testicular

volume, gonadosomatic index (GSI), sperm parameters, hormonal levels, and glutathione (GSH) activity. These results align with previous studies on alcohol's reproductive toxicity and ginger's ameliorative effects, while contributing novel insights into ginger's role in preserving sperm DNA integrity.

Table 5:
Effects of ginger and alcohol on levels of LH, FSH, INB and GSH

Groups	LH (mIU/mL)	FSH (mIU/mL)	IHB (pg/mL)	GSH (mmol/g tissue)
A	3.27±0.40	6.57±0.32	31.33±1.00	23.07±1.76
B	2.23±0.47	2.63±1.51 *	22.03±3.04 *	12.93±2.41 *
C	3.00±0.26	6.03±0.40	33.00±1.35	25.47±3.83
D	2.70±0.10	5.07±0.32 *	30.83±2.18 *	21.33±1.53 #
E	2.47±1.19	4.30±0.36	27.40±0.95	19.67±0.58 #
F	2.23±0.70	4.07±0.55	24.67±2.53	18.53±1.37

Values represent Mean ± SD. * represents a significant difference when compared to A; # represents a significant difference when compared to B.

Alcohol exposure was associated with an increase in body weight, which is consistent with the findings of Kołota *et al*. (2019), who observed weight gain in Wistar rats after four weeks of 10% ethanol consumption. However, other studies have reported weight loss or stagnation following prolonged alcohol exposure (Milat *et al*., 2017), as seen in this study. This reduction in weight gain likely reflects alcohol's effects on appetite, nutrient absorption, and metabolic function. Ginger treatment partially restored body weight gain, suggesting ginger's potential to counteract alcohol's metabolic disruptions, aligning with findings from Mahamoud and Elnour (2013) and Misawa *et al*. (2015), possibly via its antioxidant properties (Esomchi *et al*., 2025). Testicular morphometry was also affected where alcohol exposure in the alcohol-only group reduced testicular volume and GSI, consistent with Oremosu and Akang (2015), who reported testicular atrophy due to ethanol-induced oxidative stress and seminiferous tubule damage. Testicular weight remained similar between the alcohol-only and control groups, likely due to variations in body weight, as noted by Kołota *et al*. (2019). However, the GSI, which normalises testicular weight to body weight, was a more reliable metric, showing improvements in ginger-treated groups, particularly the alcohol + low-dose ginger group, suggesting ginger's ameliorating effect against alcohol-induced testicular damage (Esomchi *et al*., 2025). Ginger treatment, especially at 250 mg/kg, increased testicular volume and weight, aligning with Morakinyo *et al*. (2008) and Khaki *et al*. (2008), who attributed ginger's curative effects to its antioxidant properties, likely mediated by gingerols and shogaols.

Sperm morphology and seminal fluid parameters are considered primary morphological and physicochemical diagnostic markers of male infertility (Assidi, 2022). Sperm parameters were significantly impaired in the alcohol-only group, with reduced motility, count, and morphology, corroborating studies that link alcohol to oxidative stress and lipid peroxidation in sperm membranes (Oremosu and Akang, 2015). Ginger treatment markedly improved these parameters, with Group D showing the highest recovery in motility and count, consistent with Gholami-Ahangaran *et al*. (2021). A novel finding of this study is ginger's curative effect on sperm DNA integrity, assessed via TB and AB staining. Alcohol increased abnormal DNA percentages in the alcohol-only group, reflecting compromised chromatin condensation and DNA damage, as reported by Rahimipour *et al*. (2013) and Bai *et al*. (2020). Ginger treatment, particularly at 250 mg/kg, reduced DNA abnormalities, suggesting a potential role in stabilising sperm chromatin, an area previously underexplored in alcohol toxicity models.

Alcohol significantly reduced LH, FSH and INB levels in the alcohol-only group, disrupting the hypothalamic-pituitary-gonadal (HPG) axis, as noted by Akbari and Jelodar (2013) and Emokpae and Osabuohien (2020). In contrast, some studies report elevated gonadotropins due to compensatory feedback mechanisms (Muthusami and Chinnaswamy, 2005) highlighting variability in alcohol's effects based on dose and duration. Ginger treatment restored LH, FSH, and INB levels, aligning with Morakinyo *et al*. (2008), likely due to ginger's modulation of oxidative stress and androgen synthesis. Similarly, GSH levels, a key antioxidant, were reduced in the alcohol-only group, consistent with Basaki *et al*. (2012), but increased in ginger-treated groups, particularly ginger-only and alcohol + low-dose ginger, supporting Li *et al*. (2021).

The dose-dependent effects of ginger were evident, with the alcohol + low-dose ginger group (250 mg/kg) showing optimal recovery across most parameters, while higher doses (500 and 750 mg/kg) yielded diminishing benefits, possibly due to potential toxicity, as suggested by Khwanes *et al*. (2022). While the current study demonstrates the curative function against alcohol-induced reproductive toxicity, Biney *et al*. (2020) reports that the extent of recovery from alcohol-induced toxicity is influenced by the severity and duration of alcohol exposure.

CONCLUSION

This study demonstrates that alcohol exposure in male Wistar rats impairs body weight gain, testicular morphometry, sperm quality (motility, count, morphology, DNA integrity), hormonal profiles (LH, FSH, INB), and antioxidant defenses (GSH). Aqueous *Zingiber officinale* extract, particularly at a low dose (250 mg/kg), significantly mitigates these effects, restoring body weight gain, testicular volume, GSI, sperm parameters, hormonal levels, and GSH activity. A novel finding is ginger's effect on sperm DNA integrity, reducing alcohol-induced chromatin damage, which warrants further investigation. These results highlight ginger's therapeutic potential in ameliorating alcohol-induced reproductive toxicity, with low-dose treatment offering optimal benefits.

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Full-length Research Article

Bioassay and Histopathological Effects of Water-Soluble Fractions of Crude Oil on *Coptodon guineensis*

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Summary: Acute and subacute effects of crude oil's water-soluble fraction (WSF) and associated histopathological changes in the gills and liver of juvenile *Coptodon guineensis* were investigated. An acute toxicity test (96 hrs) was performed by a static non-renewal method at various concentrations of 0.25, 1, 5, 15, and 30%. Similarly, test organisms were tested in 2.5% sublethal concentration for 28 days. Water quality parameters, heavy metals, and total polycyclic aromatic hydrocarbons (PAHs) were determined using standard methods. After fish exposure to WSF of crude oil, gills and liver were harvested, stained with hematoxylin/eosin, and later prepared for photomicrography. Results revealed the LC50 as 26.73% (26.73 g/L), while total PAHs and heavy metal (Ni, Cd, and Pb) levels varied significantly ($p < 0.05$) in *Coptodon guineensis* throughout exposure. Histopathological alterations were evident in gills and further revealed the presence of deformed secondary lamellae, with corresponding congestion of the passive central vein and cytoplasmic vacuolation in the liver. Apart from toxicity values to unravel the adverse impacts of the bioavailable water-soluble fraction of crude oil on juvenile *Coptodon guineensis*, the histopathological assay revealed underlying sub-lethal responses that can serve as early warning signals.

Keywords: *Bioassay, water-soluble fraction, crude oil, histopathological, Coptodon guineensis.*

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INTRODUCTION

Crude oil is a naturally occurring hydrocarbon with a relatively low viscosity and specific gravity, thus allowing it to float on water. When crude oil spills in a marine environment, there is a tendency to spread over a wide surface area while the slick may remain to drift via wave actions, ultimately extending to open seas, inland waters, and terrestrial habitats (Adofo *et al*., 2022; Centers for Disease Control and Prevention, 2010). As a complex mixture, crude oil consists primarily of aliphatic and aromatic constituent hydrocarbons as well as nitrogen, oxygen, sulfur and trace amounts of cadmium, iron, nickel, vanadium, and lead (Primerano *et al*., 2024; Laws, 2017; Varjani *et al*., 2015 Olaifa, 2012; Edema, 2006). As a raw material and energy source for production of organic compounds, crude oil and its derivatives play important roles in socio-economic development of several nations (Varjani, 2017). In fact, petroleum hydrocarbons constitute one of the most vital organic pollutants in land-dwelling and marine ecosystems, thus have a tendency to contaminate natural waters and ultimately lead to emergence of other fractions including the water-soluble fractions (Ukpaka *et al*., 2020). In actual sense, continuous discharge of crude oil and its derivatives due to tanker accidents or conventional spills may lead to potentially adverse consequences in the ambient environment (George *et al*., 2025; 2014). As crude oil weathers in natural environment, its more readily bioavailable water-soluble fraction will emerge, thus leading to increased contaminant uptake in exposed organisms. Over time, accumulated oil residues in planktonic organisms and sediments may be taken up in higher organisms, resulting in bio-magnification across the aquatic food chain (Ogbeide and Eriyamremu, 2023; Chouksey *et al*., 2004). Thus, the water-soluble fraction of crude oil is assumed to be the freely dissolved component of the petroleum hydrocarbon that constitutes various toxic components, including PAHs, benzene, toluene, ethylbenzene, xylene (BTEX), phenols, heterocyclic compounds and heavy metals. Consequently, these constituents of crude oil can elicit toxic actions in exposed organisms (George, *et al*., 2025; Ambaye *et al*., 2022; Rodrigues *et al*., 2010). Typically, adverse effects due to biota exposure to crude oil residues and their derivatives can range from underlying biochemical responses to visible effects in whole organisms, particularly in early life juvenile stages (Banaee *et al*., 2025; Esteban-Sánchez, *et al*., 2021; Lee *et al*., 2017; Sadani *et al*., 2011). In particular, fish have been reported to more readily absorb dissolved petroleum hydrocarbons from contaminated water (Olaifa, 2012). The

resulting water-soluble fraction of crude oil can potentially induce adverse biological reactions, including irregular movement and ultimately death of exposed organisms. Considering that uptake of crude oil residues adversely impacts gills and other internal organs, leading to substantial damage, histopathological assays can establish contaminants' interactions and sensitivity of relevant organs as well as unravel underlying adverse consequences in exposed organisms (Agbogidi, *et al*., 2024; Pathan *et al*., 2010). Although many previous studies have assessed the potential toxicity of crude oil residues in fish (Esteban-Sánchez, *et al*., 2021; Hagerty and Ramseur, 2010), research information is scarce on the potential health consequences of its water-soluble fraction on juvenile Guinea Tilapia, *Coptodon guineensis*. Therefore, this study assessed the uptake of water-soluble fraction of crude oil, its constituent PAHs and heavy metals load by using histopathological biomarkers of toxicity in juvenile *Coptodon guineensis*.

MATERIALS AND METHODS

Test organism (*Coptodon guineensis*): Guinean Tilapia (*Coptodon guineensis*) (Gunther, 1862) is a brackish water euryhaline fish species found along the west coast of Africa. The Juvenile stage of the brackish fish was sourced from the Department of Aquaculture, Nigerian Institute for Oceanography and Marine Research (NIOMR), Lagos, Nigeria. The fish were collected as fries on day 14 after hatchery and allowed to acclimatize prior to utilization for an acute toxicity test that lasted for 21 days. The body weights of the fish fries were 0.07 - 0.10 g, while the length ranged from 1.4 to 1.6 cm. The fish were relocated to the toxicity test laboratory in a 56 x 41 x 35 cm plastic tank at a density of 50 fries per litre. Subsequently, fish were transferred to similar acclimation glass tanks at the same density.

Extraction of water-soluble fraction: Water-soluble fraction of crude oil was prepared according to procedures described by Bamidele and Eshagberi (2015) and Faksness *et al*. (2020), but with slight modifications. One part of oil was added to nine parts of filtered seawater at 10 ppt (1:9, v/v) in a 1L glass volumetric flask. The flask was capped with a stopper and covered with aluminum foil to minimize the evaporation of volatile components of the crude oil. Subsequently, the mixture was placed on a magnetic stirrer and stirred continuously for 24 hr at 250 rpm. The solution was later transferred into a separating funnel and allowed to stand for 8 hrs.. Afterwards, the aqueous phase was drained out and designated as 100% water-soluble fractions of crude oil. The stock solution was stored in a refrigerator for 24 hr and thereafter used for further bioassay experiments.

PAHs and heavy metal analysis: PAHs were analyzed using a previously developed method (Maskaoui and Hu, 2009). A 5g of the harvested fish was weighed into a mortar and homogenized in anhydrous sodium sulphate (previously baked at 160 oC for 24 hr). The homogenized sample was transferred to an amber bottle for a cold extraction in 50 mL dichloromethane for 30 mins in a sonication bath (USEPA, method 3550c; Sun *et al*., 1998; Nwaichi and Ntorgbo, 2016; Sogbanmu *et al*., 2019). The resulting extract was dried to 1 mL, prior to application to a silica gel column (4 mm i.d. × 90mm) as a further clean-up procedure. Extracts for PAH analysis were further evaporated under a gentle stream of nitrogen until 100 µL. A clean extract was reconstituted in 2 mL 2, 2, 4-trimethylpentane and later transferred into glass vials for gas chromatography analysis. The samples were assessed for all 16 US EPA priority PAHs (naphthalene, acenaphthylene, acenapthene, fluorene, phenanthrene, anthracene, fluoranthenes, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranathene, benzo[k]fluoranathene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a.h] anthracene, and benzo[g,h, i]perylene) using a GC-FID Agilent 7890 device. Prior to analysis, PAH analytical standards were used to calibrate the gas chromatograph.

Heavy metal analysis in fish tissues: The APHA-AWWA-WEF (2012) protocols according to Bello *et al*. (2019), with slight modifications were used for heavy metal analysis. A 5 g of fish muscle was weighed into a 250 mL conical flask. A further 25 mL of digestion reagent (HNO3:H2O2) in a 1:1 v/v ratio was added and allowed to stand for 30 mins in order to cool the initial reaction with the samples. Later, samples in flasks were heated for 1hr on the hot plate in a fume cupboard until 110 oC, till the volume of the contents was 5 mL. Afterwards, the sample was removed and allowed to cool for 30 mins prior to a filtration step using Whatman paper. Filtrates were collected in a standard flask and filled with deionized water to a volume of 50 mL prior to storage in a plastic dispensing bottle. Metal levels in all sample extracts were determined in an Atomic Absorption Spectrophotometer (AAS) (air-acetylene flame) (model PG AA500). A deuterium background correction was used for the metals, while blank corrections were applied for each set of the analyses. Accuracy was assessed by analyzing three replicates of samples.

Acute toxicity study: Acute test to assess the potential effects of a water-soluble fraction of crude oil on *Coptodon guineensis* was conducted according to the OECD guidelines (1993), in a static non-renewal regime. Exposures were performed in triplicates in 2L glass aquarium tanks (28cm x 13cm x 15cm). Various exposure levels tested include 0.25, 1, 5, 15, and 30% of the WSF of crude oil after each was constituted in the same habitat water as the source of fish.

Sub-lethal toxicity study: Twenty juvenile fish were exposed to 30L of the test solution in a glass aquarium tank (56 x 41 x 35cm). The weights of the fish were in the range of 9.5 - 12 g, while the standard and total lengths were 7 - 7.5 cm and 8.8 - 9.5 cm, respectively. Exposed organisms were depurated for 48 hr in clean water prior to commencement of the bioassay, in order to remove residual contaminants attached to exposed organisms. In order to assess bioaccumulation in exposed fish, individual organisms were subjected to a sub-lethal concentration of 2.5% WSF of crude oil.

Fish maintenance, exposure duration, and water quality analysis: Fish were exposed to 2.5% of WSF of crude oil for 28 days and fed compounded tilapia feed daily at 5% body weight. Exposure water was renewed every 4 days while individual fish was sacrificed every 7 days to harvest

the gills, liver and muscles for histopathological analysis, PAH, and heavy metal levels.

Water quality parameters including temperature, pH, conductivity, dissolved oxygen (DO), total dissolved solids (TDS), salinity, and turbidity of the experimental setup were monitored periodically using standard methods (APHA-AWWA-WEF, 2012).

Bioaccumulation factor (BAF): The bioaccumulation factor was determined after fish exposure to WSF of crude oil according to Kalfakakour and Akrida-Demertzi (2000) and Rashed (2001) as follows:

$$BAF = \frac{Ca}{Cw}$$

Where Ca = concentration accumulated in tissue of the juvenile fish; Cw = concentration in exposure water

Histopathological assay of *Coptodon guineensis* gills and liver tissues: At the end of 28-day exposure, fish gills and liver harvested from control as well as exposed individuals were preserved in 10% buffered normal saline. Fixed tissues were dehydrated at various levels in graded ethanol at 70 - 95%, cleared in xylene and subsequently embedded in paraffin blocks. Later, the samples were cut using a rotary microtome at a thickness of 4 - 5 µm. The resulting tissue sections were stained with hematoxylin and eosin, tested by light microscopy and photographed (Avwioro, 2014; Suvarna *et al*., 2013).

Data analysis: At the end of fish exposure in WSF of crude oil, lethal concentration (LC50) values were determined by Probit according to Finney (1971) and Akçay (2013) using the IBM SPSS Statistics Package (version 26). A one-way ANOVA was used to analyse concentration and time of exposure independently. Tukey HSD multiple posthoc tests were used to assess the significance of the differences across mean values, while a Bray-Curtis cluster analysis was applied to physico-chemical parameters of the water-soluble fraction of crude oil in relation to the control test.

RESULTS

Physico-chemical parameter values for WSF of crude oil: Mean values and Standard deviation for physico-chemical parameters determined throughout the duration of fish exposure in WSF of crude oil are shown in Table 1. Application of the Bray-Curtis index analysis (Figure 1) shows similarities in test water properties at category I (temperature, DO, conductivity, and pH), category II (TDS), and category III (turbidity and salinity).

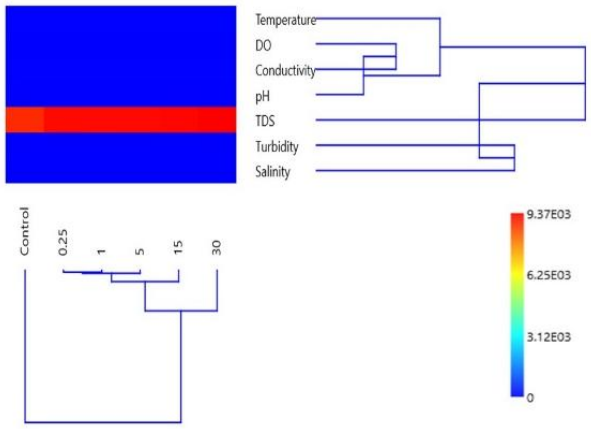

Figure 1:
Hierarchical clustering (Bray-Curtis similarities index) of physico-chemical parameters of WSF of crude oil.

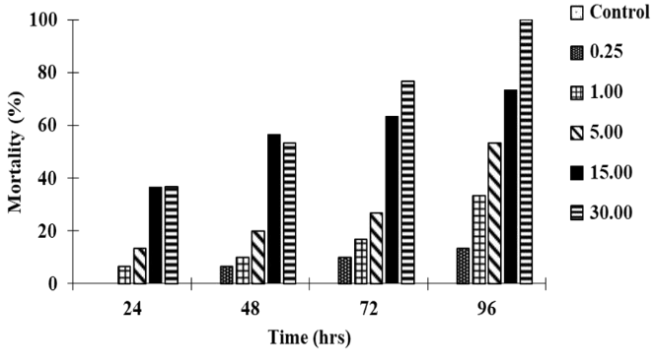

Figure 2:
Mortality of *C. guineensis* after 96hr exposure to various concentrations of WSF of crude oil

Acute toxicity: Lethal concentration (LC50) values obtained from *Coptodon guineensis* exposure to various concentrations of water-soluble fraction of crude oil until 96 hr are shown in Table 2. Mortality increased with the duration of exposure in fish treated in WSF of crude oil (Figure 2). At the end of 96 hr, a cumulative mortality of 13.3% was recorded at the lowest exposure concentration. Meanwhile, 100% mortality of fish was recorded at the highest exposure concentration.

Table 1:
Physico-chemical parameter values of WSF of crude oil in a 96 hr fish test

Conc.	Temperature (ºC)	pH	Conductivity (mS/cm)	DO (mg/L)	TDS (mg/L)	Salinity (‰)	Turbidity (mg/L)
0.0	26.6±0.1	7.6±0.0	14.4±0.1	8.8±0.1	8857.3±6.4*	8.7±0.0	0.0±0.0
0.25	27.0±0.0	7.6±0.0	14.9±0.1	8.2±0.2	9250.0±2.0*	8.7±0.0	0.0±0.0
1.0	27.1±0.2	7.7±0.0	15.0±0.1	8.3±0.1	9254.7±4.2*	8.7±0.0	0.1±0.0
5.0	27.1±0.0	7.8±0.0	15.1±0.1	8.0±0.0	9259.3±1.2*	8.6±0.0	0.8±0.0
15.0	27.0±0.0	7.8±0.0	15.6±0.1	7.9±0.0	9285.3±5.0*	8.7±0.0	1.2±0.0
30.0	27.1±0.0	7.8±0.0	15.2±0.1	7.7±0.1	9374.0±4.0*	8.8±0.0	5.9±0.0

DO: Dissolved oxygen, TDS: Total dissolved solids, * indicates significant level at $p<0.05$

Table 2:
Lethal concentration (LC_{50}) values for WSF of crude oil in a fish test

Time (hr)	LC_{50} (g/L)	lower / upper limits (95% conf.) (g/L)	
24	499.9	238.2	2,671.3
48	200.4	108.0	552.4
72	85.5	51.2	160.7
96	26.7	16.4	42.0

Accumulation of PAHs in C. guineensis exposed to WSF of crude oil: Fish body burden and the corresponding bioaccumulation factors (BAFs) increased with exposure duration until 28 days (Figure 3). Meanwhile, the cumulative uptake of PAHs peaked at 50.0 mg/kg with a BAF value of 276.7 at the end of exposure (Table 3).

Accumulation of heavy metals in C. guineensis exposed to WSF of crude oil: The selected heavy metals (Nickel, Cadmium and Lead), were determined in C. guineensis exposed to WSF of crude oil and the corresponding BAF values are shown in tables 3 and 4.

Histopathology of the gills in C. guineensis exposed to WSF of crude oil: Figure 4 (A, B, C, D and E) shows the outcome of histopathological examination of gills from *Coptodon guineensis*, which suggests there was no noticeable effects due to treatment in WSFs of crude oil upon 7day exposure. At day 14, however, there were observable toxicity effects due to the WSFs of crude oil, which was evident in the gills of *Coptodon guineensis* and showed deformed secondary lamellae but appeared mild at day 21. On day 28 (the final day of exposure), the secondary lamellae were severely deformed and damaged.

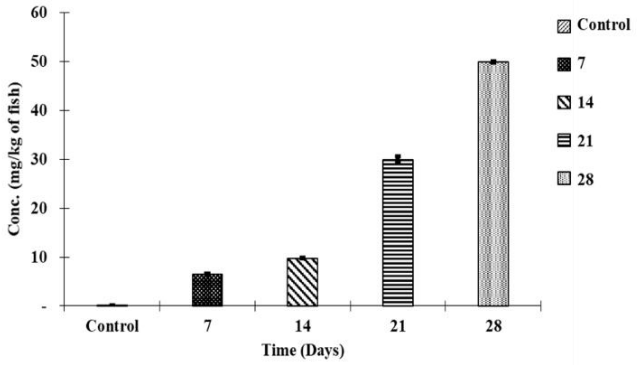

Figure 3:
Accumulation of PAHs in *C. guineensis* exposed to WSF of crude oil. Error bars represent the standard deviation of mean values

Table 3:
Heavy metals in crude oil water-soluble fractions on *Coptodon guineensis*. The data represents the mean of the triplicate samples and the standard deviation

Days	Cadmium (Cd)	Nickel (Ni)	Lead (Pb)
Control	BDL	0.35±0.03	BDL
7	BDL	3.56±0.12	1.88±0.13
14	0.07±0.01	3.25±0.15	1.50±0.10
21	0.02±0.00	3.25±0.18	1.70±0.02
28	0.02±0.00	3.56±0.13	1.88±0.11

BDL= Bellow Detection Limit

Table 4:
Bioaccumulation factors (BAF) of PAHs and selected heavy metals in *C. guineensis* exposed to water-soluble fractions of crude oil

Days	PAHs	Cadmium (Cd)	Nickel (Ni)	Lead (Pb)
7	36.3	BDL	334.0	360.9
14	54.2	25.3	305.4	288.7
21	165.9	7.3	305.4	327.2
28	276.7	7.3	333.9	360.3

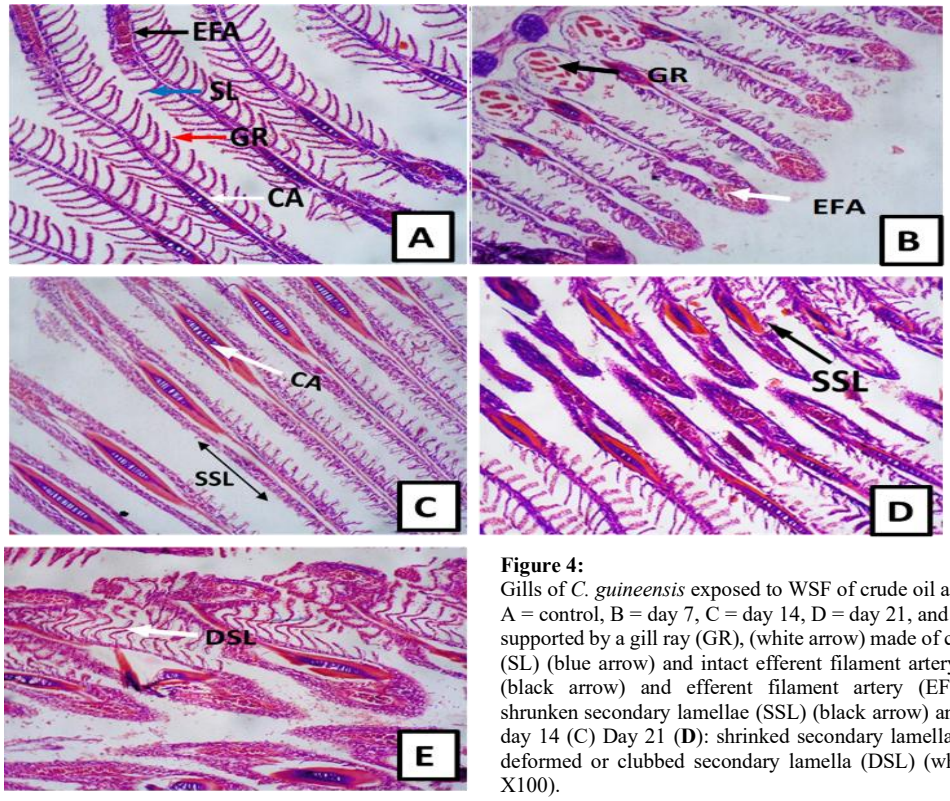

Figure 4:
Gills of *C. guineensis* exposed to WSF of crude oil after histopathological examinations. A = control, B = day 7, C = day 14, D = day 21, and E = day 28. (**A**) Gill filament (red arrow) supported by a gill ray (GR), (white arrow) made of central cartilage (CA). Secondary lamellae (SL) (blue arrow) and intact efferent filament artery (EFA) (black arrow), the gill ray (GR) (black arrow) and efferent filament artery (EFA) (white arrow) at day 7 (**B**) and shrunken secondary lamellae (SSL) (black arrow) and central cartilage (CA) (white arrow) at day 14 (C) Day 21 (**D**): shrinked secondary lamellae (SSL) (black arrow). On day 28 (E), a deformed or clubbed secondary lamella (DSL) (white arrow) was observed (H & E. mag. X100).

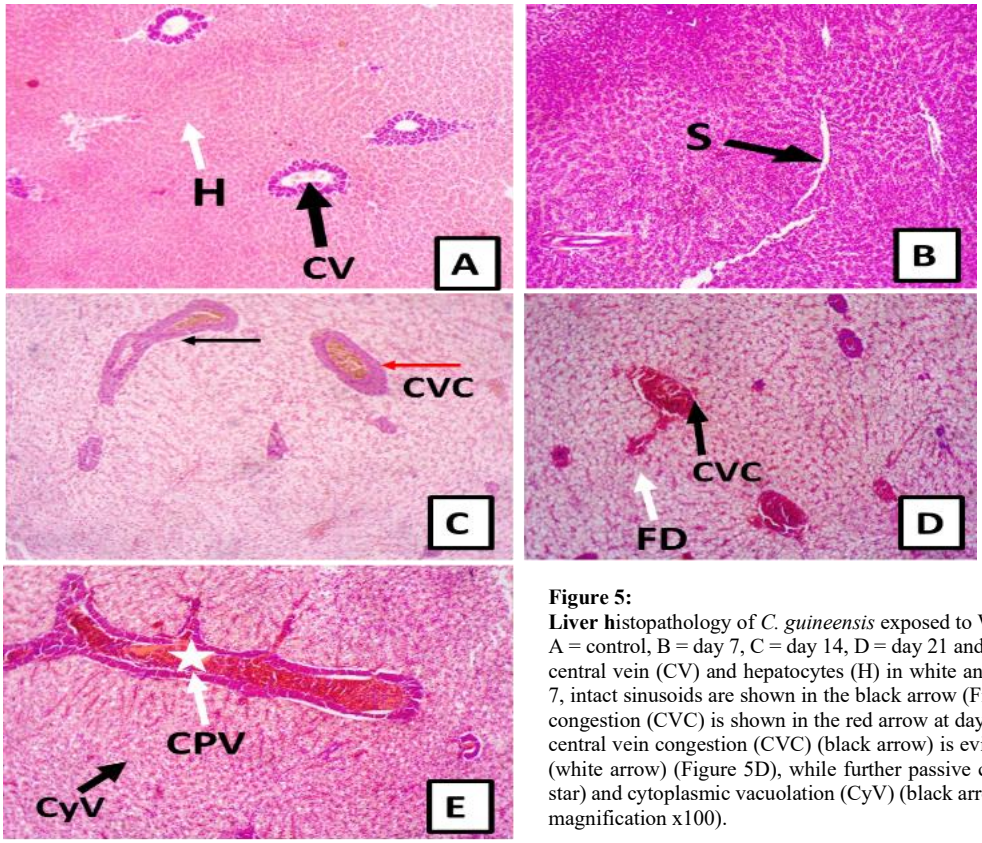

Figure 5:
Liver histopathology of *C. guineensis* exposed to WSF of crude oil.
A = control, B = day 7, C = day 14, D = day 21 and E = day 28**. Figure 5A shows** intact central vein (CV) and hepatocytes (H) in white and black arrows, respectively. At day 7, intact sinusoids are shown in the black arrow (Figure 5B), while passive central vein congestion (CVC) is shown in the red arrow at day 14 (**Figure 5C). At** day 21, passive central vein congestion (CVC) (black arrow) is evident, coupled with fat droplets (FD) (white arrow) (Figure 5D), while further passive congestion portal vein (CPV) (white star) and cytoplasmic vacuolation (CyV) (black arrow) are shown in Figure 5E. (H & E. magnification x100).

Histopathology of liver in C. guineensis exposed to WSF of crude oil: The outcome of histopathological analysis of liver from the control population showed a normal structure of the liver with undamaged central vein and hepatocytes (Figure 5 (A, B, C, D and E)). The first seven days of exposure revealed that there were no noticeable effects of wate soluble fractions of crude oil on liver tissue of *Coptodon guineensis*. However, at day 14, there were noticeable toxicity of the WSFs of crude oil, which was evident in congestion of the passive central vein of the liver. Also, damage to the central vein, congestion, dilated sinusoids, and the fat droplet at day 21 were observed. On day 28, the final day of exposure, cytoplasmic vacuolation was noticed.

DISCUSSION

The water quality characteristics were relatively steady at various tested concentrations of 0.1, 0.25 and 0.5%. The observed consistency in values is in agreement with Anwar *et al*. (2022), who reported that DO and pH did not vary significantly (P≥0.05) in the exposure test with WSF of crude oil when compared with the control vessels. The overall health of aquatic species can be negatively impacted by WSF from crude oil, according to Santos *et al*. (2016). Consistency in values of water quality parameters indicates that the observed changes were due to fish exposure to WSF of crude oil, not as a result of variations in water quality characteristics. For most water quality characteristics, values were within acceptable ranges for the sustenance of aquatic life according to the FEPA/FMEnv (1991) and not likely to contribute to the toxicity of the water-soluble fraction.

A cumulative mortality of 13.3% recorded at the lowest exposure concentration and 100% mortality of fish at the highest exposure concentration, suggest that crude oil residues may have accumulated over the period of the fish test to exert adverse consequences in exposed individuals. According to Vroumsia *et al*. (2014), toxicants can exert adverse impacts on fish and other aquatic organisms by impairment of transmission of nerve impulses, impacting vital body organs including the liver and gills, or altering haematological parameters (Friday *et al*., 1996; Chindah *et al*., 2001 and 2004). When one considers the variation in LC50 values from this test compared to previous studies with Tilapia (Akaishi *et al*., 2004; Dighiesh *et al*., 2019), it becomes pertinent to establish standard protocols for preparation of WSF of crude oil for bioassay studies. More so, the discrepancy in toxicity values compared to previous research can be attributed to the difference in source of the crude oil used for the test.

The BAF values varied greatly over the period of exposure, this implies that PAHs were taken up and accumulated in exposed fish, considering that uptake may have been greater than contaminant metabolism and subsequent elimination. Other previous research suggests that soluble hydrocarbons tend to partition more actively into animal tissues compared to other components of the environment; thus, this may explain the observed greater accumulation in fish tissues (Anyakora *et al*., 2006; Gravato and Santos, 2002). Therefore, fish is a good bio-indicator to assess contaminant uptake in biota.

Body burden for selected heavy metals, Nickel, Cadmium and Lead While Cadmium showed substantial

accumulation at day 14, further extension of exposure duration did not reveal any significant toxicant accumulation in fish tissues. The fact that these metals tend to dissolve sparingly in water-soluble fractions of crude oil, may suggest that uptake and accumulation of bioavailable fractions were slow within the duration of exposure. Also, According to a prior study by Sobhanardakani *et al*. (2011), heavy metals are rapidly absorbed by living things and are highly soluble in aquatic environments. Also, in contaminated aquatic ecosystems, Barakat (2011) stated that heavy metals have been discovered in the gills, livers, and muscles of many fish species, which is in agreement with the results of the current study as the investigated metals were found to accumulate in the muscles of the fish species used as the day of exposure progressed. Farombi *et al*. (2007) attributed the uptake, concentration, and accumulation of heavy metals in the tissues of animals as recorded in this study to the possession of metal-binding proteins in animal tissues. Due to their extreme toxicity to both humans and aquatic life, heavy metals have been utilized as markers of pollution (Omoigberale and Ogbeibu, 2007). According to Edema (2012), the WSF of crude oil can alter the physical, ionic, and heavy metal composition of the aquatic environment.

Gills of C. guineensis are essential respiratory features that allow frequent water flow over them to facilitate the absorption of dissolved oxygen from surrounding water. Considering the volume of water flowing over the gills, there is a tendency for both the dissolved and suspended contaminants to partition over the relatively large surface area of the respiratory organ. Thus, gills of fish exposed over a long period in a contaminated water system can bioaccumulate contaminants, which ultimately can elicit negative physiological responses in whole organisms. The challenge, however, is that contaminants in trace levels over short exposure durations may not substantially elicit visible deleterious effects in a whole fish if assessed by conventional toxicity endpoints. It is therefore necessary to assess underlying subtle toxicity outcomes via histopathological examination of fish tissues. This approach can serve as early warning signs to visualize potential structural changes in tissues prior to actual manifestation of severe life-threatening toxic outcome in whole organisms. For the control tests in this study, histopathological examination of gills did not result in any visible structural defects in the first 7 days (Figures 4A, 4B). At day 14, however, gills from C. guineensis treated in WSF of crude oil showed structural alterations that include deformation of the secondary lamellae (Figure 4C). A further extension of exposure duration to 21 - 28 days revealed more severe deformity in the secondary lamellae and consequently damage to the structure of the gills (Figure 4D, 4E). Overall, structural deformities were apparent in the gills of C. guineensis, which can constitute enormous health threat to exposed individuals and other fish species. These observations are consistent with the findings by Dighiesh *et al*. (2019), which reported localized hyperplasia and adhesion of secondary gill lamellae, coupled with congestion of blood vessels, in red tilapia exposed to WSF of crude oil over a 96 hr period. Similarly, the results from this study are in agreement with the outcome of research by Brand *et al*. (2001), who reported hyperplasia of secondary lamellae in the pink salamander, Oncorhynchus gorbusha exposed to crude oil. Also, Khan (2003) in a previous study of three marine fish species exposed to petroleum hydrocarbons reported substantial pathological changes, including hyperplasia of the epithelium lining the lamellae, mild necrosis and distortion, which ultimately will hamper the performance of gill filaments in diffusion of oxygen across the gill lamellae. A consequence of the physiological alterations includes reduced surface area for gaseous exchange, which will lead to development of hypoxic condition in exposed fish (Elahee and Bhagwant, 2007).

Apart from its role in maintenance of homeostasis, glucose and lipid metabolism, liver is an essential organ in contaminant detoxification (Mohamed, 2009). Liver histopathology in control population of C. guineensis showed normal structures of healthy central vein, which is consistent with the observations at day 7 of fish exposure in WSF of crude oil (Figure 5A and 5B). At day 14, however, congestions of the passive central vein were apparent in liver upon fish exposure to WSF of crude oil (Figure 5C). Further extension of exposure duration to day 21 revealed more severe damage of the central vein, congested and dilated sinusoids, coupled with the presence of fat droplets (Figure 5D). Subsequently, cytoplasmic vacuolation was observed in exposed population at day 28 (Figure 5E). Apart from operational accidents at oil rigs, crude oil contamination of marine ecosystems can also result from sabotage and activities of pirates at sea, thus leading to weathering of crude oil residues. Ecological implications of various existing routes of fish exposure to water soluble fractions from contaminating crude oil is huge, considering that oil spills can reoccur in natural marine environment. Therefore, histopathological evidence from this study suggests that WSF of crude oil can elicit various sub-lethal toxic responses in C. guineensis in a manner that increased with the duration of exposure. The results presented in this study are consistent with a previous research, which reported liver as one of the most adversely affected organs in fish exposed to WSF of hydrocarbons. It further suggests increased tendency and role of the liver to detoxify and eventually eliminate contaminants, which may have led to the observed necrosis in liver. The observed effect is expected to be more pronounced given that necrotic fish liver may not regenerate new cells. These findings are in agreement with the reports by Akaishi *et al*. (2004) and Kakkar *et al*. (2011), who observed corresponding changes in liver tissues of Astyanax sp. and Channa punctatus after exposure to WSF of crude oil, respectively. Also, the results reported by Brand *et al*. (2001) and Khan (2003) after histopathological examination of liver in freshwater and marine water fish species exposed to petroleum hydrocarbons, revealed necrosis and cellular inflammation. If one considers the frequency of threats due to petroleum hydrocarbons, it is possible that complex constituents of the WSF of crude oil can elicit toxicity in exposed fish and possibly in other marine organisms, even in trace levels. The challenge however is that toxicity of several contaminating substances are mainly assessed by using visible toxicity endpoints, which in some cases are not able to detect subtle underlying responses in exposed population. Thus, histopathological evidence can provide clues on ecological health and serve as early warning signs in event of pollution.

Various bioassay-based indices to assess water quality rely on visible parameters that often require a substantial

level of contamination in order for toxicity signatures to manifest in exposed organisms. Research evidence suggests that petroleum hydrocarbon contamination of marine water, even in low concentrations, can elicit deleterious consequences in a chronic fish test. Alternatively, histopathological examination of fish tissues as shown in this study, unravelled toxicity evidence at realistic treatment levels and short exposure time, which can serve as an early warning signal in events of crude oil contamination of marine water bodies. The results further suggest that apart from other complex constituents of crude oil, heavy metals and PAHs were detected in test water. Also, the WSF of crude oil elicited histopathological changes in gills and liver tissues even at environmentally realistic treatment levels. Utility of the WSF in this study provides a basis to assess the uptake and toxicity potential of the bioavailable portion of crude oil in the exposed population.

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Full-length Research Article

Ameliorative Effects of Methanol Extract of *Citrullus lanatus* Seed in Hyperlipidaemic Wistar Rats

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Summary: *Citrullus lanatus* (watermelon) is a commonly consumed fruit whose phytochemical constituents have known advantages; however, the seeds of this plant are usually discarded. Hyperlipidaemia is a medical condition characterized by an elevation of any or all lipid profiles and/or lipoproteins in the blood. This study aimed to investigate the haematological effects and lipid-lowering potentials of methanol extract of *C. lanatus* seed (MECLS) in egg yolk-induced hyperlipidaemic male Wistar rats. A total of 40 rats were randomly divided into four groups of ten rats each: Normal control group (NCG), Hyperlipidaemic control group (HCG), and Hyperlipidaemic treated groups (HTG1 and HTG2), which were treated with MECLS at 800mg/kg and 1600mg/kg body weight for 7 days, respectively. Lipid profiles (TC, TG, LDL and HDL) and Haematological parameters (packed cell volume, white blood cell count, red blood cell count, haemoglobin concentration, platelets) of all the animals in each group were determined. The results showed a significant decrease in Total Cholesterol, PCV, WBC, RBC, haemoglobin, and Platelets, with a corresponding significant increase in Triglycerides and HDL following treatment with MECLS in hyperlipidaemic Wistar rats. Treatment of hyperlipidaemia with the methanol extract of *Citrullus lanatus* seeds reduces total cholesterol and causes a reversal of negatively altered haematological parameters resulting from hyperlipidaemia.

Keywords*: Hyperlipidaemia, lipoproteins, Citrillus lanatus, Total Cholesterol, Triglyceride, LDL, HDL.*

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INTRODUCTION

Hyperlipidaemia has been considered the most influential risk factor for coronary heart disease (CHD), a cause of mortality in a high percentage of people around the world (Adegoke *et al.,* 2018; Roth *et al.,* 2020). One of the most common risk factors in patients with cardiovascular disease is dyslipidaemia. Hyperlipidaemia is a common pathological condition distinguished by a selective increase in plasma levels of low density lipoprotein (LDL), total cholesterol (TC) and/or triglycerides (TG) (Jellinger, 2012), which increases the risk of endothelial dysfunction and atherosclerosis (Amit *et al*., 2011), which can manifest clinically as hypertension, coronary heart disease (CHD), myocardial infarction, arrhythmias, stroke or peripheral arterial disease (Shamir and Fisher, 2000; Castilla-Guerra *et al.,* 2009).

Interest in the possible health benefits of various phytochemical constituents such as flavonoids, anthocyanin, ascorbic acid, tocopherol, phenolic compounds, dietary fibre, and carotenoids present in fruits and vegetables, has increased in recent years owing to their potent antioxidant and free-radical scavenging activities, haven been recognized as natural sources of various bioactive compounds (Pennington and Fisher, 2010; Rahman *et al.,* 2013). *Citrullus lanatus* (Watermelon), which is a vine-like flowering plant and belongs to the family *Cucurbitaceae,* is an important vegetable crop in Africa, originally from Southern Africa and can adapt to different environmental conditions (Mandel *et al.*, 2005; Adetutu *et al.,* 2015). While it is commonly consumed, its consumption is usually limited to the fruit. The seeds of *C. lanatus* have been shown to contain phytochemical constituents like alkaloids, flavonoids, tannins, amino acids, carbohydrates, cardiac glycosides, terpenoids, steroids, carotenoids, oils and fats, essential amino acid, vitamins A, B and C, and minerals (calcium, iron, magnesium, potassium, phosphorus and zinc) (Olamide *et al.,* 2011;

Omoboyowa *et al.,* 2015). Atlaş *et al.,* (2011) reported that the juice from watermelon exerts protective effects in the liver, kidney and brain against experimental Carbon tetrachloride (CCL_4) toxicity in rats, which can be attributed to the presence of antioxidants in the juice extract. The bioactive compounds observed to be present in methanol extract of *Citrullus lanatus* seed (MECLS) are known to exhibit medicinal as well as physiological activity (Sofowora, 1993; Adeniyi *et al.,* 2012). This study was designed to analyse the possible haematological and anti-hyperlipidaemic effects of methanol extract of *Citrullus lanatus* seed in hyperlipidaemic conditions.

MATERIALS AND METHODS

Plant Identification and Extract Preparation: *Citrullus lanatus* was purchased from a market in Abakaliki, Ebonyi State, identified and authenticated by a botanist in the Department of Botany, Ebonyi State University, as *C. lanatus* (Identification number: EBSU-H-1120). The seeds of *Citrullus lanatus* were air-dried and pulverized. The crushed Citullus lanatus seeds were subjected to maceration extraction using absolute methanol for total period of 24 hours. The extract was evaporated with a rotary vacuum evaporator to obtain methanol extract of *Citrullus lanatus* seed (Obonga *et al*., 2019).

Experimental Animals: Forty (40) male Wistar rats, weighing between 150g and 180g were bought from FUNAI Animal house, Nigeria. The animals were housed in animal cages with suitable temperature and humidity in the FUNAI Animal House, Ebonyi state, Nigeria. They had free access to food and water throughout the period of the experiment and they were kept under standard laboratory conditions. Handling and use of animals in this study were in accordance with the guiding principles for research involving animals as recommended by the declaration of Helsinki and the Guiding principles in the care and use of animals (Ashal *et al*., 2023).

Experimental Design: Forty (40) male Wistar rats were divided into four (4) groups of 10 rats per group, as follows: Group One: Normal Control Group (NCG), Group Two: Hyperlipidaemic Control Group (HCG), Group Three: Hyperlipidaemic Treatment group 1 (HTG1), were made hyperlipidaemic and treated with methanol extract of *C. lanatus* seed (800mg/kg body weight), for 7 days. Group Four: Hyperlipidaemic Treatment group 2 (HTG2): were also made hyperlipidaemic and treated with (1600mg/kg body weight) methanol extract of *C. lanatus* seed for 7 days.

Induction of hyperlipidaemia in Experimental Rats: Hyperlipidaemia was induced by intraperitoneal administration of egg yolk (0.2ml/10g body weight) for a day (Song *et al*., 2013; Anuwat *et al*., 2017), after which they were sacrificed after 12 hours fasting period to ascertain the attainment of hyperlipidaemia.

Haematological Analysis: At the end of the experiments, the animals were sacrificed by cervical dislocation and blood samples were collected via cardiac puncture, into Ethylene Diamine Tetra Acetic acid (EDTA) bottles. Full blood count was done using Diatron Automated A38-1 Abacus Hematology Analyzer as described by Okeke *et al*. (2023). Results obtained for packed cell volume, red blood cell count, white blood cell count, haemoglobin concentration and blood platelets were recorded.

Lipid Profile Analyses: Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were assayed using Centronic LDL and HDL kits. Ttiglyceride (TG) levels were assayed using Agappe TG kit using enzyme-linked immunosorbent assay method as described by Danboyi *et al*. (2020). Total cholesterol level was determined spectrophotometrically, using enzymatic colorimetric assay kits (Danboyi *et al*., 2020).

Statistical Analysis: Data are expressed as Mean ± SEM. Results were analysed using a standard statistical software. Comparison of results between the various groups was carried out using ANOVA with statistically significance taken at $p < 0.05$.

RESULTS

The results for lipid profile (Table 1), shows a significant increase in Total Cholesterol in the HCG compared to the control (NCG), followed by a decrease in the HTG1. Triglyceride was significantly increased after induction of hyperlipidaemia (HCG), followed by an increase in other groups. HDL was significantly reduced in HCG compared to NCG, and significantly increased in the treatment groups (HTG1 and HTG2) compared to the NCG. The results also show a significant increase in LDL in all groups compared to the NCG.

The results for haematological parameters (Table 2) shows a significant increase in Packed Cell Volume (PCV) after induction of hyperlipidaemia (HCG), followed by a decrease after treatment (HTG2). Haemoglobin concentration (Hb) was significantly increased in HCG, followed by significant reductions in both HTG1 and HTG2, compared to NCG. White blood cell count (WBC) was significantly increased in HCG, and significantly decreased in the treatment groups (HTG1 and HTG2), compared to NCG. Red blood cell (RBC) and Platelet counts (PLT) were also increased in HCG compared to NCG, and significantly decreased in HTG1 and HTG2 compared to HCG.

Table 1:
Effects of Methanol Extract of *Citullus Lanatus* Seed on the Lipid Profile of various groups.

GROUPS	Total Cholesterol (mmol/l)	Triglyceride (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
Group I (NCG)	3.82±0.01	1.11±0.02	1.34±0.01	0.55±0.02
Group II (HCG)	4.28±0.08#	1.51±0.02#	1.07±0.02#	0.84±0.04#
Group III (HTG 1)	3.67±0.06*	1.69±0.01*#	1.30±0.15	0.80±0.05#
Group IV (HTG 2)	3.91±0.05	1.73±0.07*#	1.33±0.04*	0.97±0.05#

*Values expressed as mean ± SEM. # denotes statistical significance ($p<0.05$) when compared to NCG. * denotes statistical significance ($p<0.05$) when compared to HCG*

Table 2
Effects of Methanolic *Citrullus lanatus* Seeds Extract on Haematological Parameters.

GROUPS	PCV (%)	Hb (g/dl)	WBC ($\times10^9$/mm^3)	RBC ($\times10^6$/L)	PLATELET ($\times10^9$/µL)
Group I (NCG)	38.75±0.48	17.17±0.59	6.23±0.18	2.39±0.03	122.00±1.71
Group II (HCG)	42.00±0.82$^{\#}$	19.76±0.30$^{\#}$	6.93±0.21$^{\#}$	2.82±0.05$^{\#}$	158.50±1.29$^{\#}$
Group III (HTG 1)	40.50±1.19	18.75±0.13$^{\#*}$	6.35±0.07*	2.51±0.03$^{\#*}$	138.25±0.85$^{\#*}$
Group IV (HTG 2)	37.00±0.91*	17.64±0.97*	6.33±0.09*	2.43±0.05*	120.75±0.85*

Values expressed as mean ± SEM. $^{\#}$ denotes statistical significance ($p<0.05$) when compared to NCG. * denotes statistical significance ($p<0.05$) when compared to HCG.

DISCUSSION

Hyperlipidaemia is a disorder that describes elevated lipid levels within the body (Hill and Bordoni, 2023). It can also be described as an increase in LDL, total cholesterol or triglycerides, and or a decrease in HDL (Su *et al.,* 2021). Hyperlipidaemia has been implicated in cases of hypertension (Wyszynska *et al.,* 2023), coronary heart disease (Jian-zhai *et al.,* 2004), myocardial infarction (Moertensen and Nordestgaard, 2020), arrhythmias (Ivan *et al.,* 2019) and stroke (Shigematsu *et al.,* 2015) amongst others, conditions which might be ameliorated or totally avoided if food crops with medicinal potentials are consumed as a whole, with no parts discarded (Gaire, 2018; Rathore *et al.,* 2024). This study aimed to analyse the possible haematological and anti-hyperlipidaemic effects of methanolic extract of *Citrullus lanatus* Seed (MECLS) in hyperlipidaemic conditions.

Animals in the HCG group exhibited significant hyperlipidaemia compared to the NCG, following infusion with egg yolk. Following treatment with 800mg/kg body weight of MECLS (HTG1) and 1600mg/kg body weight of MECLS (HTG2), the results showed a significant decrease in total cholesterol compared to the HCG, with results similar to that obtained in the NCG. This is in line with the finding of Danboyi *et al.* (2021), that citrulline contained in the seed of *Citrullus lanatus* has anti-dyslipidemic effect. This result is contrary to that of Uto-kando *et al.* (2021), that citrulline does not affect TC and TG.

Increased TC has been linked with increased risk of atherosclerosis (Gaggini *et al.,* 2022), potentially leading to several other conditions such as coronary artery disease, stroke and peripheral arterial disease (Jian-zhai *et al.,* 2004; Shigematsu *et al.,* 2015; Zemaitis *et al.,* 2024). Specifically, an increase in TG has been linked to increased risk of development of pancreatitis (Karalis, 2017).

This study also observed a persistent increase in TG following the induction of hyperlipidemia despite treatment with MECLS. The reason for this increase in TG remains unclear, and this observation counters that of Uto-kando *et al*. (2021), that citrulline has no effect on TG, except proven that this effect is brought about by another active compound contained in the seeds of *Citrullus lanatus.*

The dose-dependent increase in high-density lipoprotein after treatment with MECLS, suggests a positive effect, as increased HDL absorbs cholesterol in the blood, carrying it back to the liver for excretion, thus lowering the TC (Jomard and Osto, 2020). HDL has also been reported to induce endothelial nitric oxide synthase activation, leading to increased production of nitric oxide (Mineo *et al.,* 2003), a known antiatherogenic molecule (Sukhovershin *et al.,* 2015).

This study showed a significant increase in haematocrit, red blood cell count, white blood cell count and blood platelets following induction of hyperlipidaemia (Table 2). This is consistent with previous reports (Alizamir *et al.,* 2018; *Gebrie et al*., 2018; Hashemi *et al*., 2020). Dyslipidemia has also been associated with leucocytosis (Desai *et al.,* 2006; Tsai *et al.,* 2007). Though elevation of blood cell indices has been extensively reported following hyperlipidaemia (Desai *et al.,* 2006; Fessler *et al.,* 2013; Hashemi *et al.,* 2020; Tsai *et al.,* 2007), the mechanism behind remains unknown.

An interesting observation from this study is that the hematological results obtained in the HCG were corrected and brought down to baseline level as seen in the NCG, indicating a correctional effect of MECLS on the effects brought about by hyperlipidaemia. Further studies need to be carried out to ascertain the exact components of MECLS responsible for each effect observed, and to elucidate possible mechanisms.

In conclusion, treatment of hyperlipidemia with MECLS improves some lipid factors, but its effects are more visible in reversal of negatively altered haematological parameters resulting from hyperlipidemia

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Full-Length Research Article

Microscopic Assessment of the Effects of *Cannabis Sativa* Leaf Extract on the Cerebellum in Male Wistar Rats

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Summary: Cannabis remains the most abused illicit substance in the world. The growing inclination towards decriminalisation in several countries creates the need to determine how consumption affects the organs of the body, especially the brain. This research aims to determine the effect of *cannabis sativa* on the histoarchitecture of the cerebellum and motor coordination in Wistar rats. Twenty-five male rats were divided into 5 groups (I-V): Group I received food and water, Group II received 10 mg/kg, and Group III received 20mg/kg of *cannabis sativa* aqueous leaf extract for 28 days. Groups IV & V received 10 mg/kg and 20 mg/kg of *cannabis sativa* for 28 days and were allowed for another 28 days recovery period. At the end, animals were weighed and sacrificed by cervical dislocation. Results from Transmission Electron Microscope (TEM) sections of rat cerebellum in group I show normal neurons and cellular architecture. Some degrees of cytoarchitectural and neuronal distortions were observed in animals exposed to cannabis. The extent of these distortions, however, was significantly reduced following a 28-day recovery period for both doses of cannabis administered. It can be deduced, therefore, that *cannabis sativa* exposure had significant adverse ultrastructural effects on the cerebellum of adult male Wistar rats.

Keywords: *Cerebellum, transmission electron microscope (TEM), cannabis sativa, ultrastructure, histoarchitecture*

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INTRODUCTION

Cannabis sativa is a commonly abused plant due to its high content of the psychoactive compound, THC (Lucas et al., 2018; Freels et al., 2020). Though cannabis has been used for medical purposes due to its antioxidant, anticonvulsant, anti-inflammatory, and neuroprotective properties, its adverse consequences should not be underestimated (Ford et al., 2017; Bloomfield et al., 2019). Despite reported claims of cannabinoids (CBD), a non-psychoactive constituent of *Cannabis sativa* having some neuroprotective ability, inhibiting neurodegeneration, and as a promising agent in several neurodegenerative diseases (Cassano et al., 2020), there is need to clearly define its benefits and its harmful effects.

Cannabis sativa is an annual herbaceous flowering plant indigenous to Eastern Asia, but now of cosmopolitan distribution due to widespread cultivation. It has been cultivated throughout recorded history, used as a source of industrial fiber, seed oil, food, recreation, religious and spiritual moods and medicine. Each part of the plant is harvested differently, depending on the purpose of its use. The species was first classified by Carl Linnaeus in (1739).

Cannabis also produces numerous volatile sulfur compounds that contribute to the plant's skunk like aroma, with Prenylthiol (3-methyl-2-butene-1-thiol) identified as the primary odorant (Oswald et al., 2021). The active component of the marijuana plant *Cannabis sativa* was first identified as Δ9-tetrahydrocannabinol (THC) (Malabadi et al., 2023). THC, also known as dronabinol, produces numerous beneficial effects, including analgesia, appetite stimulation, nausea reduction and reduction of intraocular pressure (IOP). THC also affects bone remodeling, fertility, short term memory, tumor growth and motor coordination (Smith et al., 2020; Mechoulam, 2012; Iversen, 2005).

Cannabis use is common among adolescents and young adults, but the long-term consequences of such use are a topic of debate. Cannabis use typically starts during early adolescence and peaks when users are in their mid-20s (Hall et al., 2020; Hasin 2018). Cannabis use can have adverse health effects, including increased risks for lung, cardiovascular, and periodontal diseases (Gordon et al. 2013; Jouanjus et al. 2017; Lorenzetti et al., 2019). Its effects on development of cognitive and affective dysfunction, however, have been less conclusive. An initial study reported that cannabis use, particularly during adolescence, contributes to a lasting neurocognitive decline including an 8- point drop in IQ from childhood to adulthood (Chye et al., 2020; Jackson et al. 2016). More recent studies, however, do not support this conclusion.

Receptors for THC and other cannabinoid compounds are present in the brain, especially in the frontal cortex, basal ganglia, cerebellum, and limbic regions. Cannabinoid

action in the basal ganglia and cerebellum probably account for the effect on psychomotor control (Ashton et al., 2017). The cerebellum has an important role in motor control, with cerebellar dysfunction often presenting with motor signs. It is active in the coordination, precision, and timing of movements, as well as in motor learning (Figueiredo et al., 2020; Lorenzetti et., al 2010). They reported neuronal alterations in chronic cannabis use can lead to structural changes in the cerebellum. One of the most prominent effects is the reduction in the size and complexity of Purkinje cells, which are the principal neurons in the cerebellum. These changes may be associated with impaired motor coordination and balance in chronic users. Synaptic Changes because of cannabis impact can cause synaptic plasticity in the cerebellum, thereby affecting the integrity and efficiency of neuronal connections. This is crucial for motor learning and coordination (Battistella et al. 2014).

In a study of the combined and independent effects of chronic cannabis use and HIV on brain metabolites, Chang et al. (2006) found that cannabis use was associated with a decrease in neuronal and glial metabolites, yet a normalization of glutamate levels in PWH (Chang et al., 2006; Bahji et al., 2021). Adult studies of marijuana use often find subtle decreases in performance compared to controls in cognitive domains such as attention, memory, and processing speed; such effects have been discussed as transient in literature given limited group differences after prolonged abstinence from marijuana (Grant et al, 2003; Pope et al, 2010; Krist et al., 2020).

In contemporary research, molecular studies take central stage in biomedical research (Hindocha et al., 2020). Ultrastructural studies provide the best option available to explore histological structures, hence its adoption in this study.

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MATERIALS AND METHODS

Ethical approval: Ethical approval for this research was sought and obtained from the research ethics committee (REC) of Nnamdi Azikiwe University Awka Nnewi campus.

Animals: Rats were obtained from the Animal house of the College of Medicine and Health Sciences, Nnamdi Azikiwe University Nnewi Campus. The animals were housed within the standard facilities of a well-ventilated animal house and maintained on pelletized rat chow and water ad libitum under standard laboratory conditions of lighting and moderate temperature.

Twenty-five male rats were divided into 5 groups (I-V): Group I received food and water, Group II received 10mg/kg and Group III received 20mg/kg of cannabis sativa aqueous leaf extract for 28 days. Groups IV & V received 10mg/kg and 20mg/kg of cannabis sativa for 28 days and were allowed for another 28 days recovery period. Plant material: Fresh leaves of Cannabis sativa were obtained from the Locals. The plant was authenticated by Mr. Iroka Finian, a taxonomist at the Nnamdi Azikiwe University Herbarium, Awka, Anambra state. Yadima et al., (2017) modified method of Silva & Afkinnghorn, (1988) standard method was used for aqueous extraction. The plant material was soaked in distilled water for 24 hours and vigorously shaken intermittently. Mixture was then evaporated using a water bathe until a gummy brown deposit is formed. This was labelled and refrigerated until use. Stock solution of the extract was constituted by dissolving 1g of the extract in 10ml of distilled water.

Tissue processing: For tissue processing for H&E, small portions of rat cerebellum was cut and fixed in 10% neutral buffered formalin. After 48 hours of fixation, tissues were processed using the H&E procedure according to the method of Carleton et al., (1967). Tissues were placed in ascending grades of alcohol (60%, 70%, 80%, 90%) for one hour each and in absolute (100%) twice, one hour each. Tissues were immersed in two changes of xylene for one hour each, infiltrated in four changes of molten paraffin wax at constant temperatures of 36-40ºC in an oven of paraffin bath for one hour each and embedded with Metal blocks. Thin sections were made at 5µm using a rotary microtome, and picked up with a prelabelled glass slide made stick using egg albumin. Haematoxylin and eosin staining was done according to the procedure described by Carleton et al., (1967) and mounted in distrene plasticizer xylene (DPX) using clean glass cover slide. Tissues were then focused under Leica research light microscope and photomicrographs were taken from each group and labelled using Microsoft PowerPoint.

As regards tissue processing for Electron Microscopy, small pieces of rat cerebellar tissue were fixed in 2.5% glutaraldehyde in phosphate buffer, pH 7.4 followed by a wash in phosphate buffer, pH 7.4 for 2 hours. Tissues were immersed and postfixed for 1 hour in 1% osmium tetroxide and then placed in 70% alcohol overnight in the refrigerator at 4°C. Tissues were dehydrated through 2 changes of 95% alcohol and absolute alcohol for 20 minutes, cleared, and infiltrated with propylene oxide and Epon-Araldite resin solutions of varying ratios. First, in a solution of 3 parts propylene oxide to 1 part resin, second, in equal parts propylene oxide and resin solution, and third in a solution of 1 part propylene oxide to 3 parts resin, for a length of 30 minutes per solution. Lastly, the tissues were left overnight in resin, followed by embedding in fresh Epon-Araldite resin at 60∘C for 48 hours (Goodhew, 2011).

After polymerization, 1 μm sections were cut on a Reichert-Jung Ultracut ultra microtome (Germany) and stained with Toluidine Blue-Pyronin Y for 30 seconds, dried, and mounted in Entellan. Ultrathin gold sections were cut and placed on copper grids and stained with uranyl acetate for 3 minutes. Drops of lead citrate were placed on strips of dental wax, and once stained, grids were rinsed first in dilute sodium hydroxide, followed by distilled water and then dried (Goodhew, 2011).

The work on electron microscopy was carried out at the Noguchi Memorial Institute for Medical Research, Ghana.

RESULTS

Haematoxylin & Eosin Light Microscopy: The results for light microscopy of rat cerebellum are presented in plate 1 (L1 to L5 for rat in groups I to V respectively). The control group (L1) show typical histological features of the cerebellum. There are three distinct layers; granular layer (GL), Purkinje cell layer (PCL) and molecular layer (ML). The Purkinje layers are composed of large Purkinje neurons with large round nucleus, and prominent nucleoli with glial

cells interspersed within the neurons. The micrograph of rat cerebellum from the treated groups (L2 to L5) show no signs of tissue alteration when compared to the control group.

Electron microscopy: The micrographs of rat cerebellum are presented in plates designated EM 1 to EM5 for rats in groups I to V respectively. Each group is represented by four different micrographs designated (a to d) to show various aspects of the cerebellar ultrastructure from each group.

The ultrastructural investigation of brain tissue of control group showed normal histoarchitecture of the cerebellum under the Transmission Electron Microscope (TEM). The slides of rat cerebellum from Group I (EM 1a-d) showed normal nucleus with evenly distributed chromatin materials, presence of nucleoli and dense double layered membrane. Mitochondria in the control group exhibited homogenous dense matrix, double membrane integrity with organized cristae. Myelin sheath is thick, electron dense and tightly wrapped around their axon with good synaptic complex, presenting normal thick electron dense tightly wrapped myelin sheath.

In the test Group II (EM 2 a - d) treated with 10mg/kg body weight, TEM showed degenerative chromatin material, absence of nucleus with crescent formation, degenerated cristae, myelin sheet degeneration and no synaptic complex.

In test Group III (EM 3 a - d) treated with 20mg/kg body weight, TEM showed pyknotic nucleus, degenerated chromatin material with distorted nuclear membrane, crescent formation, degenerated cristae, disconnected myelin sheet and no synapse.

The ultrastructure of group IV rat cerebellum under the TEM (EM 4 a - d) showed some degree of recovery in form of double layer nuclear membrane, presence of nucleoli and chromatin material remarkably, close to normal mitochondria cristae and myelin sheath, with good number of synaptic complexes. In the High dose recovery group (Group V) treated with 20mg/kg, TEM (EM 5 a - c) the EM showed degenerated chromatin materials, close to normal nucleoli and broken nuclear membrane. It also showed close to normal mitochondrial integrity with visible cristae, bulged and disconnected myelin sheath with remarkable synaptic complex (Plate EM 5a-c).

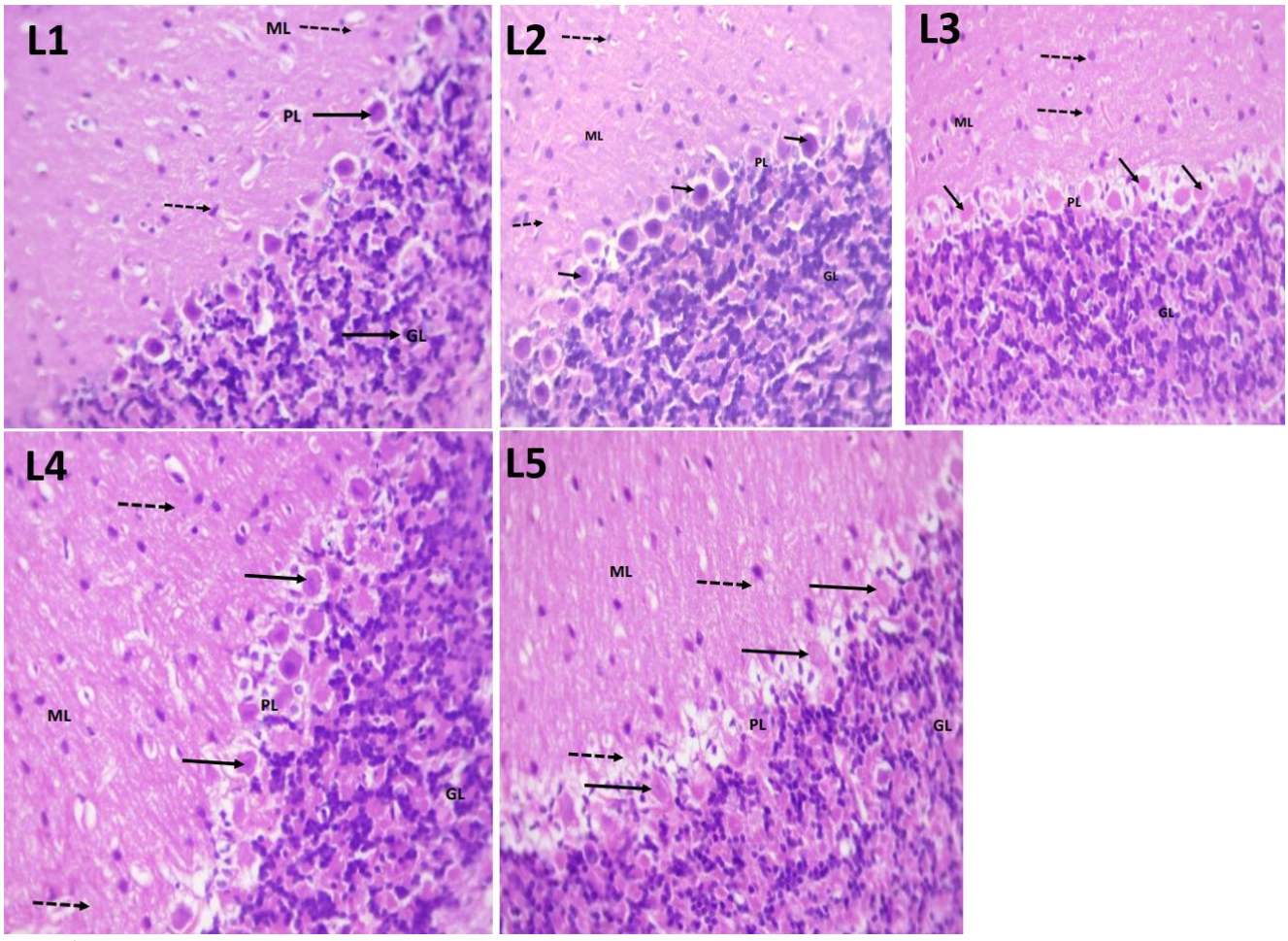

Plate 1

Photomicrograph of section of the cerebellum of rats treated with vehicle (control, L1), 10mg/kg (L2) and 20mg/kg (L3) of *cannabis sativa* for 28 days. L4 and L5 are photomicrographs of the cerebellum of 10mg/kg amd 20mg/kg of *cannabis sativa* treated rats with additional 28-day recovery period respectively..

ML – molecular layer; PL – purkinje layer; GL – granular layer; black arrows – intact purkinje neurons; dashed arrows – glial cells. H&E x400 magnification

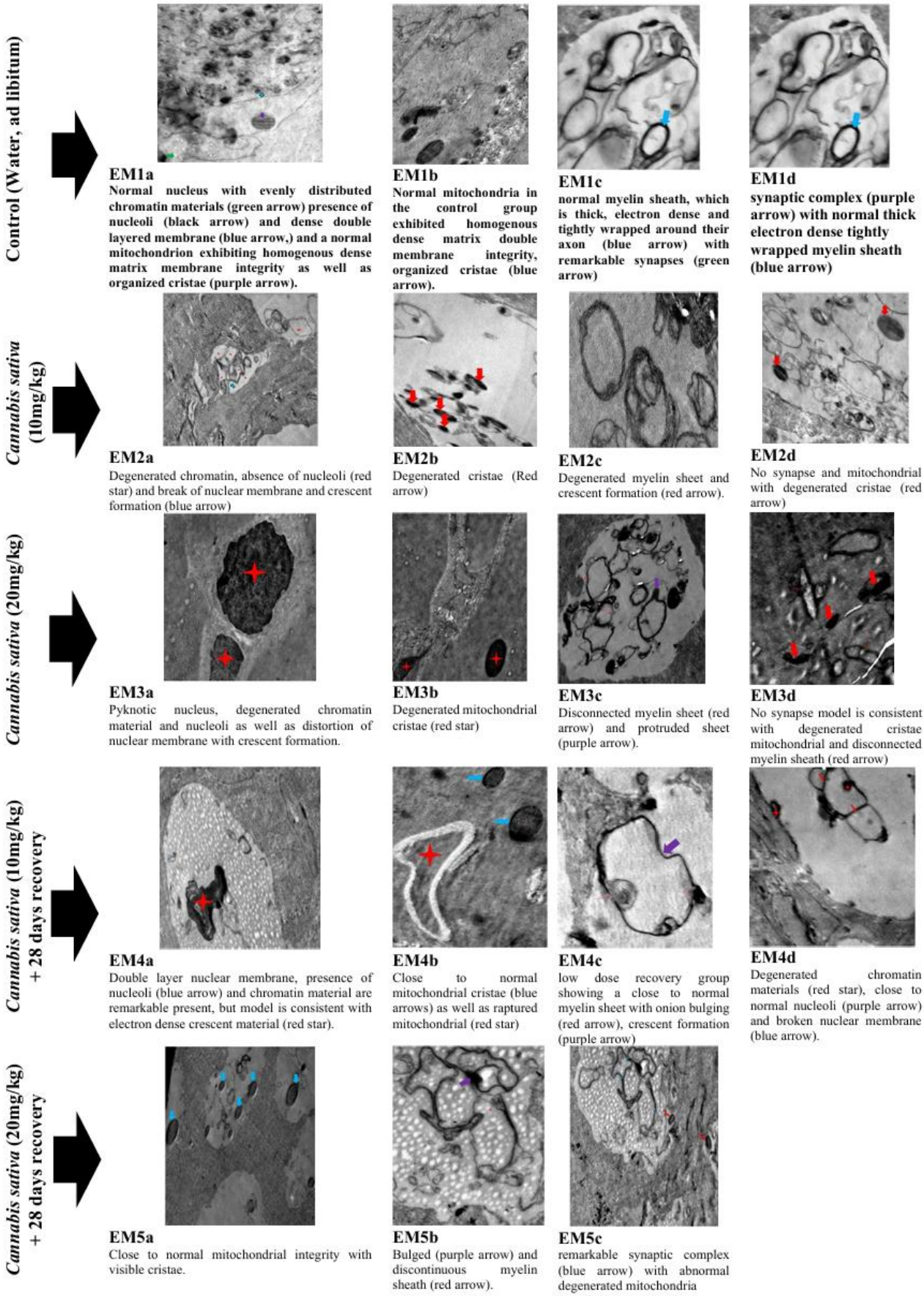

Plate 2
TEM Photomicrograph of section of the Cerebellum of Wistar rats treated with water (EM1a-d), 10mg/kg Cannnabis sativa (EM2a-d), 20mg/kg Cannnabis sativa (EM3a-d). EM4a-d and EM5a-c are TEM photomicrographs of the cerebellum of 10mg/kg and 20mg/kg of cannabis sativa treated rats with additional 28-day recovery period respectively.

DISCUSSION

The results from this study agree with the report of Battistella et al., (2014) which shows that cannabis led to synaptic changes, including synaptic plasticity in the cerebellum which affected the integrity and efficiency of neuronal connections. De Faria et al., (2021) report was consistent with this studies on myelin sheath disruption potentially affecting efficient neural communication in the cerebellum. Cannabis may disrupt myelin formation and maintenance, potentially affecting the ultrastructure of the cerebellum and reduces conduction velocity which may alters timing within neural circuits and osculation. (Pajavic et. al., 2014; Almeida and Iyons, 2017). Mild myelin abnormalities are thought to be a primary cause of behavioral alteration (Poggi et.al., 2016). This report is in line with the findings of this study.

The protocol went further to allow animals in groups IV and V treated with 10mg/kg and 20mg/kg respectively just like groups II and III another 28 days recovery period. The ultrastructure of group IV rat cerebellum under the TEM (EM IV a - d) showed some degree of recovery in form of double layer nuclear membrane, presence of nucleoli and chromatin material remarkably, close to normal mitochondria cristae and myelin sheath, with good number of synaptic complexes. Moreover, in the High dose recovery group (Group V) treated with 20mg/kg, TEM (EM V a - d) showed degenerated chromatin materials, close to normal nucleoli and broken nuclear membrane. It also showed close to normal mitochondrial integrity with visible cristae, bulged and disconnected myelin sheath with remarkable synaptic complex (Plate 5). This result throws light on the power of the natural recovery process.

A lot of cellular damage is restored naturally without treatment simply by elimination or withdrawal of the toxicant, including neurons. It therefore poses a question on the long age knowledge that neurons once damaged cannot be recovered or repaired. Removing the offending substance can lead to clinical improvement and, given the available specific treatment for some toxins, early and correct identification of the relevant toxin is important. Neurologists should consider screening for exposure to appropriate neurotoxins according to the neuropathy phenotype (Smyth et al., 2023).

Researchers have shown (Harclerode 1980) that THC could alter mitochondrial shape and induce swelling in several different tissue dependent on the dose (Whan et. al., 2006). This agrees with the findings of this research. Sarafian et.al (2003) and Whyte et.al, (2010) further suggested that both THC and CBD could modulate mitochondrial function and inhibit respiration, resulting in cell death.

Reports from Blithikioti et al., (2019) suggests that consumption of THC led to a decrease in psychomotor skills, which did not correlate with any altered brain activation. Regarding residual effects, King et al., (2011) found decreased psychomotor speed in chronic users performing a finger-tapping task but no corresponding cerebellar alteration. On the other hand, Lopez-Larson et al (2012) showed that a sample of adolescent heavy users performing a finger- tapping task had decreased cerebellar activation that was negatively correlated with lifetime exposure to marijuana, suggesting that the cerebellum might be particularly sensitive to cannabis effects during development and, therefore, associated with long-term alterations (King et al., 2011).

Several studies have suggested altered cerebellar function in chronic cannabis users (Daum et al., 1993; Safo and Regehr, 2005; Marcaggi 2015 & Koziol et al., 2014). Emerging evidence suggests that in addition to motor functions, the normal cerebellum plays a significant role in cognition in the healthy human brain (Koziol et al., 2014). It has an active role in a variety of mental activities, including facial recognition, emotion attribution, theory of mind attributions, directed attention, and many types of memory (Schmahmann, 1998). In functional imaging studies, cerebellar activations occur even when motor components of the tasks are well controlled. It is now widely accepted that many normal cognitive functions are performed by using distributed circuits that include cerebellar and thalamic components, with cortical components that vary depending on the nature of a given mental activity. Amaza et. al., (2013) in their work observed physical changes which include hyperactivity, increase in appetite as well as increase in weight. This sis due to the fact that endocannabinoids in the hypothalamus activate cannabinoids receptors that are responsible for maintaining food intake and also cannabis sativa has acute appetite enhancing effects, thereby increasing body weight in experiment model. This is consistent with the findings of this study.

From the result of this study, we can deduce that electron microscopy revealed greater details as to the nature of harm caused by *cannabis sativa* consumption on cerebellar neurons compared to light microscopy. Further aspects of this investigation are required on other parts of the brain to ascertain the extent of potential harm caused on the brain by cannabis sativa consumption.

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Full-length Research Article

Therapeutic Effects of *Persea americana* L. Phytochemicals on Key Molecular Targets in Knee Osteoarthritis: *In-silico* and *In-vivo* approach

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Summary: Knee osteoarthritis (KOA) is a prevalent degenerative joint disorder characterized by chronic inflammation, cartilage degradation, and altered bone remodelling. This study investigated the inhibitory effects of *Persea americana* L. phytochemicals on key molecular targets involved in KOA pathogenesis and compared them with clinically used drugs targeting these pathways. Specifically, Interleukin receptor-associated kinase 4 (IRAK4), Transforming Growth Factor-Beta (TGF-β), Microsomal Prostaglandin E Synthase 1 (mPGES1), Kelch-like ECH-Associated Protein 1 (Keap1), Carbonic Anhydrase 1 (CA1), and Collagen II. Molecular docking analyses were performed using Schrodinger (Maestro 12.12). All proteins' 3D X-ray crystallographic structures were screened based on the following properties (Organism, Expression system, and Resolution) using the Protein Data Bank (RCSB PDB) https://www.rcsb.org/. Various lead compounds with significant binding affinities to these targets have been identified, outperforming conventional pharmacological agents. In vivo assessments using the hot plate test were conducted on 30 male Wistar rats, which were divided into six experimental groups. Chemical induction of KOA was performed by intra-articular injection of 25uL of saline dissolved 4 mg/kg of Sodium monoiodoacetate (MIA) in four of five groups: Control, Sham, and 3 treated with Ibuprofen, Low, and High *Persea americana* L. extracts, respectively, except the KOA only group. The docking scores from all the pathways showed higher binding energy when compared to the present drug samples, except for KEAP 1, where Chlorhexidine "STD" and Quercetin had binding scores of -6.576 and -6.557, respectively. *Persea americana* L. extracts treated rats showed significantly enhanced pain thresholds in KOA models compared to pathological untreated and ibuprofen-treated groups ($p<0.006$; 0.041 respectively) by Day 21 post-induction of KOA, indicating their analgesic efficacy. These results collectively highlight the potential of *Persea americana* L. phytochemicals as novel therapeutic agents for the management of knee osteoarthritis, with strong consideration of a systemic pharmacological approach.

Keywords*: Knee Osteoarthritis, Persea americana, Pain Modality Test, Molecular Docking, Pain threshold.*

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INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis, affecting millions of individuals worldwide (Hawker 2019; Yao *et al*., 2023). The most common presentation is knee OA (KOA), which causes chronic knee pain, disability, and diminished quality of life in patients with KOA (Farrokhi *et al*., 2016; Vitaloni *et al*., 2019). Epidemiological data show that the global prevalence of knee OA is approximately 3.8% in men and 7.8% in women, of whom approximately 14 million adults are affected in the US (Chen *et al*., 2021; Allen *et al*., 2022). Knee OA pathogenesis involves an imbalance between catabolic and anabolic activity of the joint structures, including inflammation, redox imbalance, and apoptotic activities of the cartilage, bone, and synovium (Kapoor 2015; Primorac *et al*., 2020; Yunus *et al*., 2020). This has been identified as a potential target for therapeutic intervention and plays an important role in the disease process (Valenti *et al*., 2021).

IRAK4 is a vital mediator of the pathogenesis of knee osteoarthritis. Recently, it has played a central role in the Toll-like receptor (TLR) and interleukin 1 receptor (IL-1R) signaling pathways for proinflammatory responses (Li *et al*., 2021, Jrad *et al*., 2023). Activated interleukin-1 receptor-associated kinase 4 (IRAK4) promotes the production of proinflammatory cytokines, IL-1β and TNF-α, which induce cartilage matrix degradation (Jrad *et al*., 2023). Li *et al*., (2021) also reported that IRAK4 signalling disrupts chondrocyte function, inducing apoptosis and suppressing matrix synthesis. Furthermore, IRAK4 is implicated in bone remodeling, resulting in abnormal bone remodelling and osteophyte formation (Alonso-Perez *et al*., 2018). Likewise, Transforming Growth Factor-Beta (TGF-β) is a multifunctional cytokine with critical roles in the regulation of cellular processes, such as growth, differentiation, and extracellular matrix production (Tirado-Rodriguez *et al*., 2014). Elevated TGF-β levels in the subchondral bone are a major initiating factor in the pathogenesis of knee osteoarthritis (Macfarlane *et al*., 2017). However, increased TGF-β signalling is triggered by many factors, such as joint injury, mechanical stress, inflammation, genetic or epigenetic factors, metabolic disturbances, or ageing (Shen *et al*., 2017). Enhanced subchondral bone remodeling later in the process leads to the formation of new thicker sclerotic bone that is TGFβ-driven (Macfarlane *et al*., 2017). These alterations in the subchondral bone excessively change joint mechanics in the overlying articular cartilage and initiate a cascade of pathological remodeling in the cartilage, resulting in osteoarthritis.

The rate limiting step in the production pathway for bioactive PGE2 from Prostaglandin H2 (PGH2) is catalyzed by a single abundant form of microsomal Prostaglandin E Synthase 1 mPGES1 (Goodman 2018). mPGES1 increases the production of PGE2, a substance that promotes the development and progression of knee osteoarthritis. Jang *et al*., (2022) further reported that PGE2 is a potent proinflammatory mediator that leads to further cytokines and up regulation of osteoclast activity, leading to subchondral bone lesions. PGE2 also promotes synovial inflammation and angiogenesis and propagates osteoarthritis (Sanchez-Lopez *et al*., 2022). Kelch-like ECH-Associated Protein 1 (Keap1), a key regulator of the Nrf2 signaling pathway, is implicated in the pathogenesis of knee osteoarthritis (Khan *et al*., 2018). Oxidative stress and inflammation increase the induction of oxidative stress and inflammation disrupt the Keap1-Nrf2 interaction, leading Nrf2 to translocate to the nucleus and activate the expression of antioxidant and cytoprotective genes (Wen *et al*., 2019). However, dysregulation of the Keap1–Nrf2 axis is sustained and can alter chondrocyte function, leading to the progressive degeneration of articular cartilage (Riegger *et al*., 2023). However, these pathways result in collagen II depletion, breaking knee osteogenic homeostasis and causing cartilage breakdown, which is the hallmark of knee osteoarthritis (Gauci *et al*., 2017). Collagen II degradation is often initiated by an imbalance of enzymes, such as MMPs and aggrecanases, which can be upregulated by factors such as mechanical stress and joint inflammation pathogenesis (Mukherjee and Das 2024). Loss of the collagen II matrix results in knee cartilage thinning and erosion, exposing the underlying bones. This drives bone remodeling and osteophyte formation, further worsening the prognosis of knee osteoarthritis (Mukherjee and Das 2024).

With recent advancements in pharmaceutical technology, there has been an increasing awareness of the use of plant-based extracts in managing osteoarthritis (Yves *et al*., 2022), thus offering a promising alternative to conventional analgesics that are often clinically prescribed in this part of the world. This paradigm shift in the management approach can be based on the likely reduced burden of NSAID side effects and the multi-therapeutic window of the leaf extract, as they are rich in various bioactive compounds that provide additional therapeutic functions. Therefore, they can block multiple pathological pathways, such as inflammation, oxidative stress, anti-apoptotic, and chondroprotective pathways, simultaneously and not just alleviating pain alone (Silvia *et al*., 2018). *Persea americana*, commonly known as avocado, is a fruit-bearing tree native to central Mexico (Majid *et al*., 2020) that is readily found in Nigeria. Numerous studies have suggested that the fruit of *Persea americana* contains a variety of bioactive compounds, including polyphenols, flavonoids, and terpenoids, which exhibit potent anti-inflammatory, antioxidant, and chondroprotective properties (Sundararajan *et al*., 2023). These properties make *Persea americana* L. extract a promising candidate for managing knee osteoarthritis. The proposed study aims to investigate the role of *Persea americana* L. extract on experimental knee osteoarthritis using a multi-pronged approach involving in-silico molecular docking studies and in-vivo pain behavioral assessments.

MATERIALS AND METHODS

Phyto- Ligand Library creation: The chemical compounds were retrieved based on the chemical characterization of *Persea americana* L. reported by Wang *et al*. (2020) and Bonvehi *et al*. (2019). Ninety-nine (99) major compounds of *Persea americana* L. were retrieved using the search tool with the compound names, and the 2D structures of the phyto-ligands were retrieved from the PubChem Database (https://pubchem.ncbi.nlm.nih.gov) and saved in the Structure Data File (SDF) format.

Protein preparation and receptor grid generation: The 3D X-ray crystallographic structures of all proteins were retrieved from the Protein Data Bank (RCSB PDB). The protein receptor targets [TGF-B (PDB ID: 4UM8), mPGES-1 (PDB ID: 4YL3), KEAP1 (PDB ID: 5GIT), IRAK4 (PDB ID: 6EGF), Collagenase II (PDB ID: 6HG7), and CA-1 (5GMM)] were preprocessed, optimized, minimized, and refined using the Protein Preparation Wizard. During the prepossessing, bond orders and hydrogen bonds were assigned, and water molecules and other heteroatoms were removed during the pre-processing step, followed by energy minimization using the OPLS-3e force field available in Maestro 12.8. The active site x, y, and z coordinates of their respective centroid co-crystallized ligands were used to generate docking grid boxes saved in gridbox.zip files (Omoboyowa *et al*., 2021).

Protein-Ligand Docking: The Standard Precision (SP) was employed for the initial screening, and extra precision (XP) was used for the filtered ligands. Ligand docking was

performed using the Glide module of Schrodinger-Maestro v12. XP method was used to weed out false positives and provide a better correlation between good poses and good scores, estimate the theoretical interaction of the ligands with the proteins, and evaluate the interactions between the ligands and amino acids that were scored appropriately. Lower GlideScore signifies a Better Binding Affinity, which means that a more negative score means stronger predicted binding interaction (Schrödinger, 2020; Omoboyowa *et al*., 2021).

In-vivo Studies

Chemicals and Drugs: Sodium Monoiodoacetate was purchased from Santa Cruz Biotechnology (10410 Finnell Street, Dallas, USA). Ibuprofen was purchased from Aromokeye Pharmacy (Ado Ekiti, Nigeria). Phosphate buffer solution, Ethanol and Chloroform were obtained from the Department of Physiology Laboratory, College of Medicine, Ekiti State University, Ado Ekiti, Nigeria. Formalin was obtained from the Department of Anatomy, College of Medicine, Ekiti State University, Ado Ekiti, Nigeria.

SHK1 Extraction and Preparation of Sample (*Persea americana* L.): *Persea americana* leaves were obtained from a private farm in Ado Ekiti, botanically identified and authenticated at the Department of Plant Science, Ekiti State University, Ado- Ekiti with a herbarium voucher number UHAE2022071. Freshly collected leaves of *P. americana* were cleaned and dried in the shade at room temperature. After drying, the plant material was ground into smaller particles using a pestle and mortar and blended into a powder using an electric blender. The powdered sample (800 g) was then stored in airtight containers at room temperature until required. Extraction of *P. americana* L. was performed using 80% ethanol for 72 h. For extraction, the solvent was added 40 times the weight of the *P. americana* leaf powder. The extract was then filtered using a cheese cloth and freeze-dried. The supernatant from the dried extracts was then stored in airtight containers and kept at room temperature until required.

Experimental Animals: Thirty (30) wistar rats (180–220 g) were purchased from the breeding colony of the Department of Physiology, College of Medicine, Afe Babalola University, Ado Ekiti, Nigeria. The animals were maintained at 25°C on a 12hours light/dark cycle with access to food and water ad libitum. The animals were allowed to acclimatize under these conditions for two weeks before the commencement of the study and were kept under the same conditions throughout the study. This study was approved by the Ethics Review Committee of Afe Babalola University (protocol number: ABUADHREC/14/06/2024/466).

KOA induction model in Wistar rats: The infrapatellar ligament of the left knee (surgical point) was shaved after the animals were administered general anesthesia using a ketamine and xylazine combination (80/15 mg/kg body weight) administered intraperitoneally. The operation site was sterilized with chlorohexidine solution, after which a single intra-articular injection of 25uL of saline dissolved 4 mg/kg of sodium monoidoacetate (MIA), an inhibitor of aerobic glycolysis that directly injures the joint chondrocytes, was administered using a 17-gauge, 0.5 inch needle. The MIA dosage used followed that of Jiang *et al*. (2022), who reported the presentation of maximum joint discomfort at the specified dosage (Jiang *et al*., 2024).

Experimental Design: Thirty Male Wistar rats were randomly distributed into six groups (n = 5).
Group 1 (control group) received 1 ml/100 g body weight (b. w.) of water daily.
Group 2 (SHAM group) received 4 mg/kg dose of normal saline intra-articularly.
Groups 3-5 were induced with osteoarthritis (OA) by intra-articular injection of 4 mg/kg of sodium MIA in the right knee joint space. Animals in groups 3, 4, and 5 were treated for 18 days after KOA induction.
Group 3 (OA group) received intra-articular administration of 4 mg/kg of MIA.
Group 4 (OA+IBU group) received a 40 mg/kg b.w oral dose of ibuprofen.
Group 5 (OA + LDA group) received an oral dose of *P. americana* at a concentration of 50 mg/kg b.w. (Ogunmoyole *et al*., 2021)
Group 6 (OA + HDA group) received an oral dose of *P. americana* at a concentration of 100 mg/kg. (Ogunmoyole *et al*., 2021)

Measurement of Biometric Values of Pain threshold Test: All pain modality tests were performed weekly from day 0 until the last week of the experimental procedures using the hot plate test.

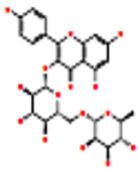
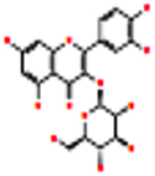
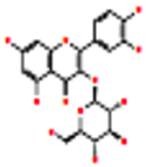
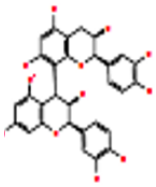
Hot Plate: This procedure was performed in accordance with the standard procedure described by Jimoh-Abdulghaffaar *et al*. (2023). Thermal hyperalgesia was assessed by placing animals on an electrical hot plate (maintained at 55°C ± 2 °C). The latency of the first sign of jumping off or paw licking by the animals from the hot plate was recorded in seconds by a blind observer as an index mark of pain threshold for that animal. A maximum time of 10 s was maintained for each procedure, after which the animal was immediately removed, regardless of whether the animal jumped. Pain response was measured at 30- and 60-minutes following extract administration. At no time was an animal allowed to stay on the hot plate for more than 10 seconds to avoid tissue damage.

Statistical Analysis: Data obtained from this study were analyzed using GraphPad Prism version 9.0 (GraphPad® Software, San Diego, CA, USA). Values are expressed as the mean ± SD. Groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons to determine statistical significance.

RESULTS

Protein-Ligand Docking: The chemical structures of all the characterized phyto-compounds of *P. americana* are shown in Table 1.

Table 1
Chemical Structures from the Phytochemical Characterization of *Persea americana*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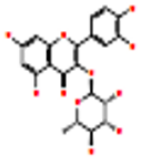	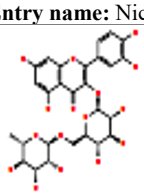	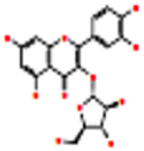	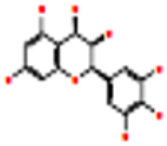	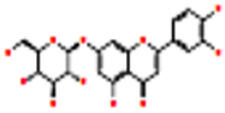
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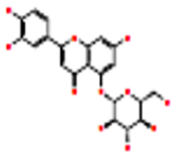	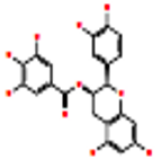	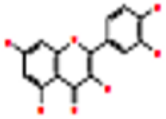	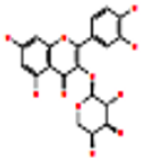	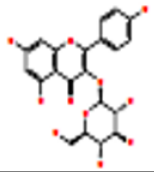
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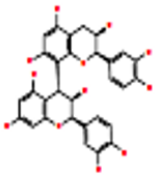	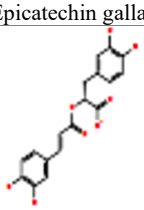	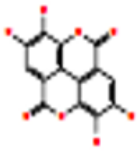	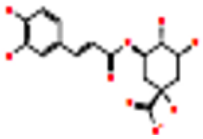	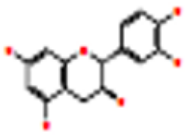
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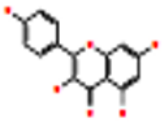	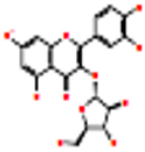	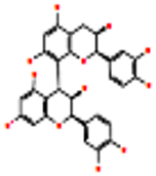	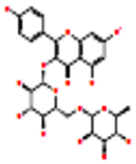	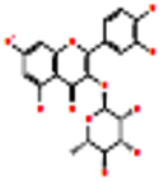
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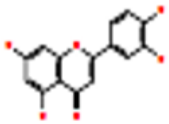	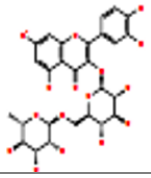	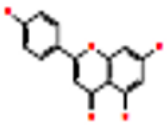	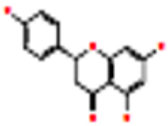	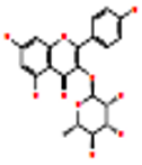
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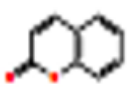	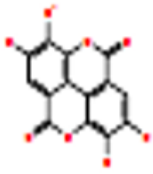	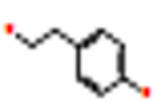	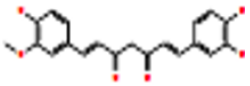	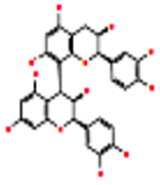
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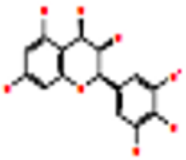	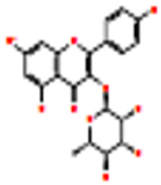	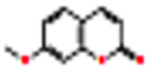	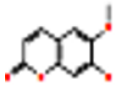	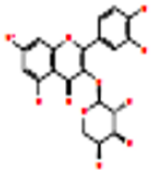
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	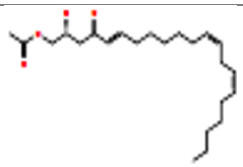		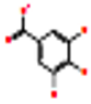	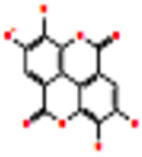
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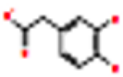	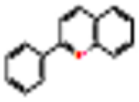	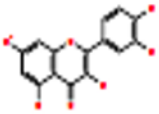	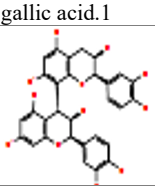	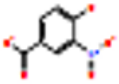
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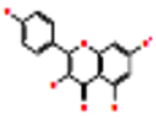		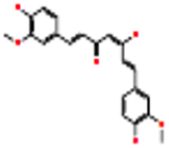	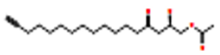	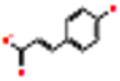
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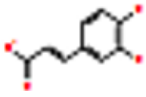	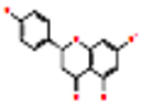		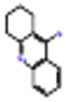	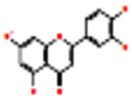
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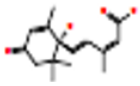
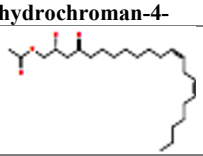
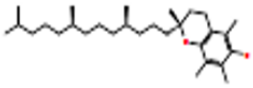
	4trihydrox 			
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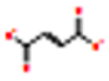	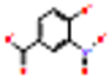	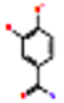	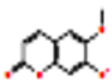	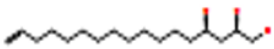
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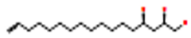	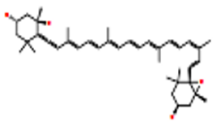	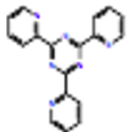	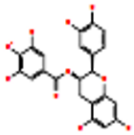	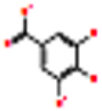
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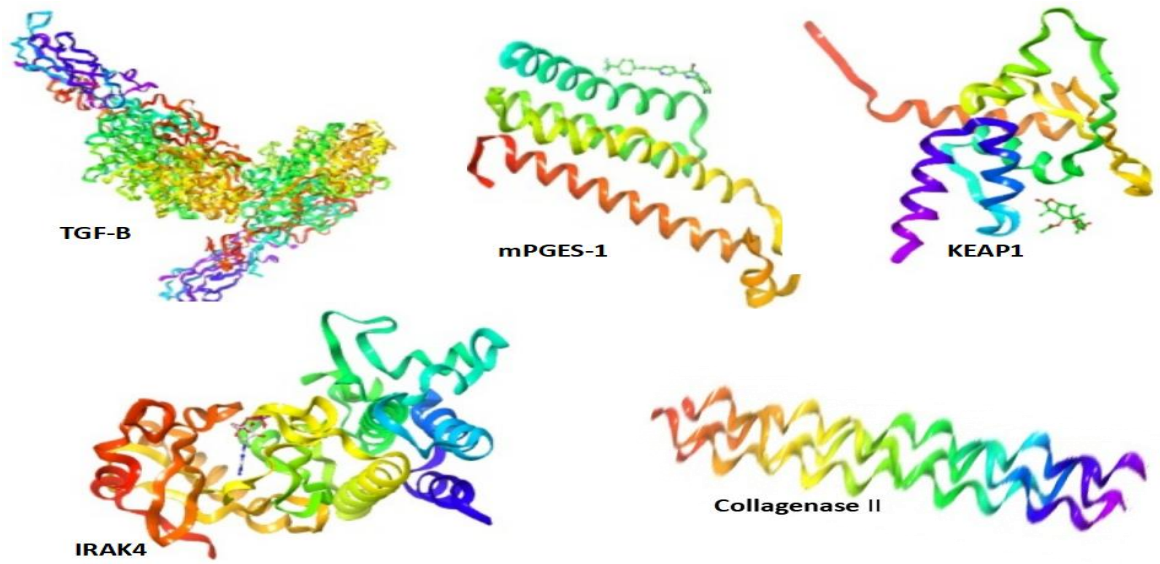

Figure 1:
X-ray Crystallography Structure of the Target Proteins: TGF-B (PDB ID: 4UM8), mPGES-1 (PDB ID: 4YL3), KEAP1 (PDB ID: 5GIT), IRAK4 (PDB ID: 6EGF), Collagenase II (PDB ID: 6HG7), CA-1 (5GMM)

The 2-dimensional structure of all the phyto-compounds present in *P. americana* in sdf format was docked against each of the active site of the target proteins: TGF-B (PDB ID: 4UM8), mPGES-1 (PDB ID: 4YL3), KEAP1 (PDB ID: 5GIT), IRAK4 (PDB ID: 6EGF), Collagenase II (PDB ID: 6HG7) and CA-1 (5GMM) (Fig. 1), along with the standard drugs Tranilast, Naproxen, Chlorhexidine, Zimlovisertib, Diclofenac, and TIMP1 (Tissue Inhibitor of Matrix Metalloproteinases 1) respectively. The docking results showed that 11 lead compounds exhibited good binding affinities with each of the target proteins (Fig. 2). Against the target protein TGF-B, all of the lead compounds have binding affinities ranging from -11.954Kcal/mol to -8.473Kcal/mol more than the standard drug Tranilast having binding affinity of -2.802Kcal/mol. Against mPGES-1, all the lead compounds exhibited good binding affinities, ranging from -6.787Kcal/mol to -5.158Kcal/mol, compared to the standard drug Naproxen, which had a binding affinity of -2.647Kcal/mol (Fig. 2). Against KEAP-1, the lead compounds exhibited good binding affinities ranging from -6.557Kcal/mol to -4.323Kcal/mol, and the standard drug Chlorhexidine exhibited a good binding affinity with a docking score of -6.576Kcal/mol. Against the target IRAK4, all the lead compounds exhibited good binding affinities ranging from -11.548Kcal/mol to -9.586 kcal/mol, better than the standard drug Zimlovisertib having binding affinity of -7.058Kcal/mol. Against the target Carbonic anhydrase 1, all the lead compounds exhibited good binding affinities with the target, with docking scores ranging from -6.385Kcal/mol to -4.749Kcal/mol, compared to the standard drug Diclofenac, which had a binding affinity of -1.967Kcal/mol. Against the target Collagenase II, nine of the lead compounds with docking scores ranging from -5.441Kcal/mol to -4.260Kcal/mol eahibited better binding affinities than the standard drug TIMP-1 with a binding affinity of -4.116Kcal/mol (Figure 2). The docking results showed that Quercetin 3-glucoside had the highest binding affinity against the target TGF-B than the standard drug Tranilast, Nicotiflorin had the highest binding affinity against the target IRAK4 compared to the standard drug Zimlovisertib, Quercetin 3-O-D-arabinopyranoside had the highest binding affinity against the target KEAP-1 and Collagenase II compared to the standard drugs Chlorhexidine and TIMP-1, respectively. Chlorogenic acid also had the highest binding affinity against the target mPGES-1 and Carbonic anhydrase 1 compared to the standard drugs Naproxen and Diclofenac, respectively (2).

Molecular Interactions: The 2-dimensional molecular interactions of the lead compounds with each of the standard drugs are illustrated in Figure 3. Figure 3a shows the 2D interaction of the compound Rutin, which has the highest binding affinity against the target TGF-B and the standard drug Tranilast. Figure 3b shows the 2D interaction of chlorogenic acid, which has the highest binding affinity against the target mPGES-1 and the standard drug Naproxen. Figure 3c shows the 2D interaction of the compound Quercetin 3-O-D-arabinopyranoside, which has the highest binding affinity among the lead compounds against the target KEAP-1 and the standard drug Chlorhexidine. The 2D interaction of the compound with the highest binding affinity for docking *P. americana* and Zimlovisertib against IRAK4 is depicted in Figure 3d. The 2D interaction of the highest-binding compound of The docking of the docking of *P. americana* Chlorogenic acid and the standard drug Diclofenac against the target Carbonic anhydrase 1 is shown in Figure 3e. Figure 3f shows the 2D interaction of Quercetin 3-O-D-arabinopyranoside, which has the highest binding affinity against the target Collagenase II and the standard drug TIMP1.

In-Silico Drug likeness, Pharmacokinetics, and Toxicity Prediction: The pharmacokinetics and toxicology predictions of all the lead compounds are depicted in Figure 4, showing hepatotoxicity, carcinogenicity, mutagenicity, immunotoxicity, Lipinski's rule of five, and other drug likeness predictions.

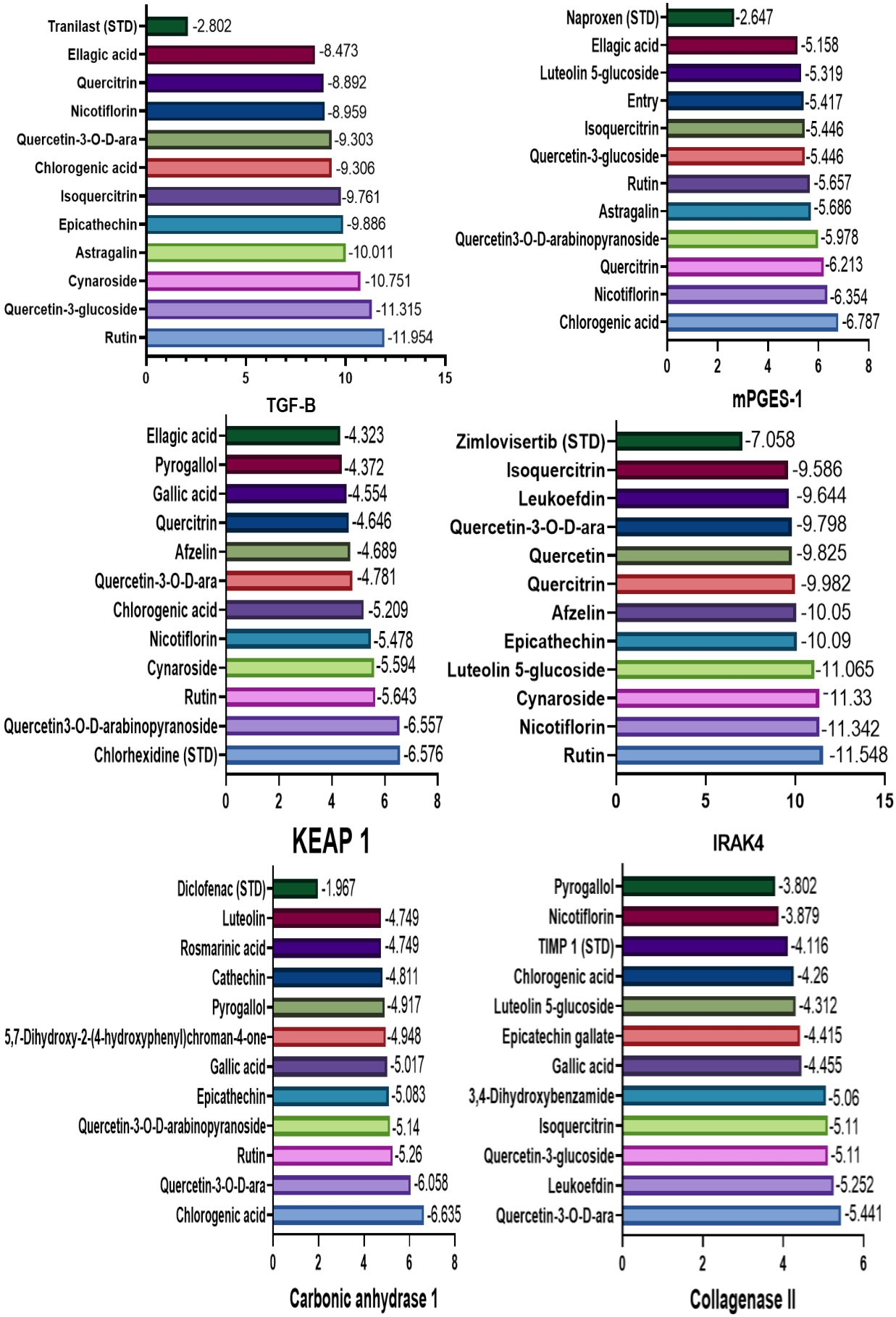

Figure 2:
Docking Scores/ Binding Affinities of the lead compounds from *Persea americana* docked against the target proteins

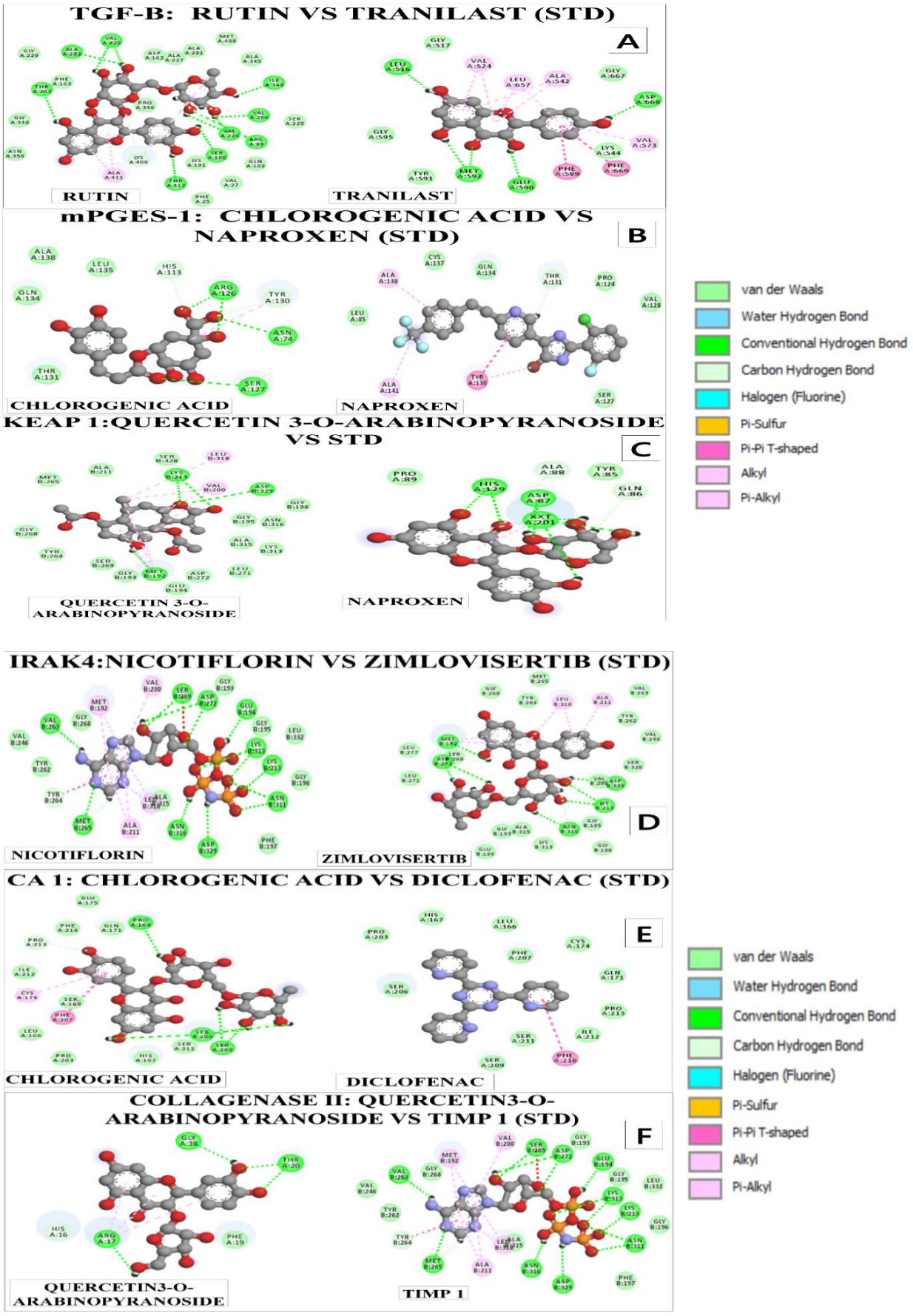

Figure 3:
2D-molecular interactions of the lead compounds from *Persea americana* docked against the target proteins.

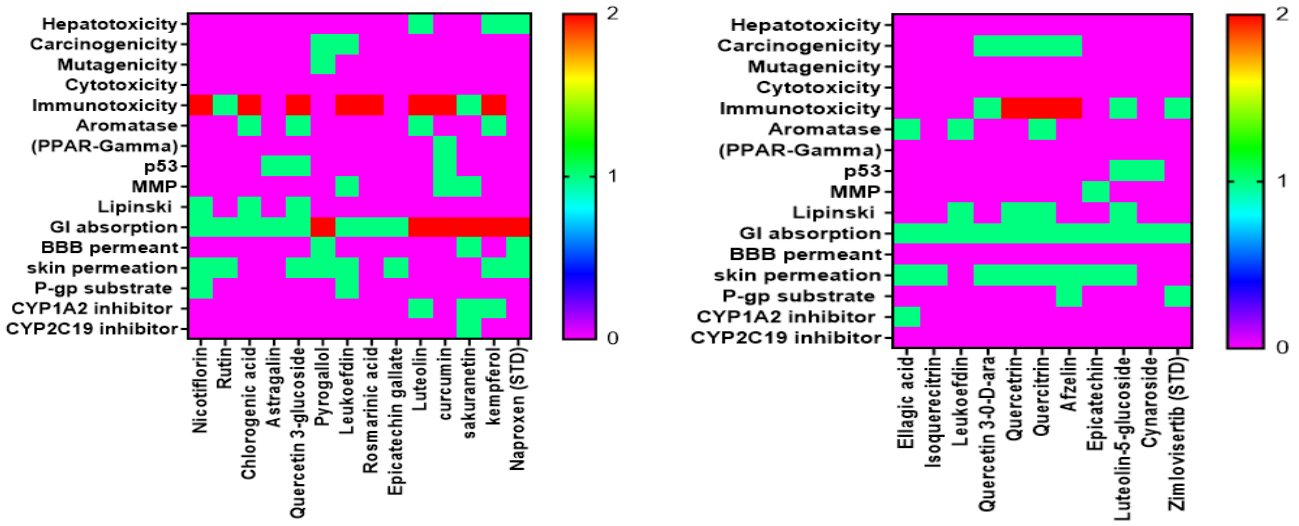

Figure 4:
Heat map showing the drug likeness and toxicity predictions of *Persea americana* lead compounds docked against the active site of all the target proteins

Table 2:
Effect of *Persea americana* L. dose-dependent treatment on the pain sensation threshold assessment of KOA-induced rats using the Hot Plate Test (s).

	CONTROL	SHAM	OA	OA+IBU	OA+LDA	OA+HDA
DAY 0	24.52±1.608	24.42±3.597	23.46±2.419	24.42±2.887	25.22±2.592	23.94±2.186
DAY 3	23.80±2.984	5.55±0.4924[a]	5.380±0.593[a]	5.320±1.370[a]	5.4±2.713[a]	5.540±3.024[a]
DAY 7	22.20±1.306	5.050±0.4528[a]	4.980±0.580[a]	6.140±1.737[a]	6±2.209[a]	6±2.014[a]
DAY 14	21.30±1.920	4.470±0.616[a]	4.1±0.741[a]	11.79±1.299[ab]	13.42±1.319[ab]	15.36±0.819[b]
DAY 21	20.36±0.650	4.170±0.426[a]	3.7±0.447[a]	15.19±0.959[ab]	16.62±1.416[b]	19.36±1.088[bc]

Control - 1 ml/100 g body weight (b. w.) of water daily; SHAM group received 4 mg/kg dose of normal saline; OA group received 4 mg/kg of MIA; OA+IBU group received 40 mg/kg b.w oral dose of ibuprofen; OA + LDA group received 50 mg/kg b.w of *P. americana* p.o; OA + HDA group received received 100 mg/kg b.w of *P. americana* p.o

Pain Modality Test

Effect of *Persea americana* L. Extract on Hot plate Test: The results of the pain threshold using the Hot Plate test across the six groups are shown in Table 2. The baseline measurement on day 0 showed no significant differences across the six groups. On day 3 (post-induction), our results showed a significantly decreased pain threshold in groups 2-6, relative to the control group. On day 7 post-treatment, all treatment groups showed statistically lower pain thresholds compared with the control group (p=0.0041; 0.0113; 0.0005; 0.0141), and by day 21 post-treatment, our results showed an improvement in the pain response sensitivity, with the OA+HDA treatment group demonstrating more potent amelioration of the pain response compared to the OA and OA+IBU groups (p=0.0012; 0.0417, respectively).

Table 2 shows the effect of *Persea americana* L. dose-dependent treatment on the pain sensation threshold assessment of a KOA induced rat by the hot plate test (secs) expressed as mean±SD, n =5. Data were analysed by one-way ANOVA followed by Tukey's multiple post hoc test. ap < 0.05 vs. Control, bp < 0.05 vs. OA cp < 0.05 vs. OA+IBU. OA (Osteoarthritis), IBU (Ibuprofen), LDA (Low dose *Persea americana* L.), HDA (High dose *Persea americana* L.).

DISCUSSION

Knee osteoarthritis is characterized by a complex interplay between inflammatory processes, cartilage degeneration, and altered bone remodeling (De Roover *et al*., 2023). The disease involves an imbalance between catabolic and anabolic activities within the joint, primarily driven by dysregulation of cytokines and other signaling molecules (Yunus *et al*., 2020). Key proteins such as Interleukin receptor-associated kinase 4 (IRAK4), Transforming Growth Factor-Beta (TGF-β), Microsomal Prostaglandin E Synthase 1 (mPGES1), Kelch-like ECH-Associated Protein 1 (Keap1), Carbonic Anhydrase 1 (CA1), and Collagen II play critical roles in these processes. Targeting these proteins may be an attractive strategy to limit KOA progression. Molecular docking studies showed that all lead compounds from *Persea americana* L. showed favorable binding affinities against the target proteins, which were significantly better than those of the standard drugs. The values were well above the binding affinity of the standard

drug, Tranilast, which was measured at -2.802 kcal/mol. [SHK1] The high binding affinities observed likely make these phyto compounds powerful inhibitors of TGF β activity, a key mediator of inflammatory responses and fibrotic pathologies in KOA (Zhang *et al*., 2021). Interactions between the lead compounds and TGF-β suggest the ability of these compounds to modulate the signaling pathways that promote cartilage degradation and joint inflammation by binding to this protein target, thus preventing its activation effect in KOA pathogenesis. The docking results for mPGES-1 showed a total binding affinity for the compounds ranging from -6.787 kcal/mol to -5.158 kcal/mol. The standard drug Naproxen bound to this site with a binding affinity of -2.647 kcal/mol. The high binding affinities of the lead compounds suggest that they may be good inhibitors of mPGES-1, an enzyme that is the chief prostaglandin E2 (a key mediator of pain and inflammation) synthesizing enzyme (Dos Santos Nascimento *et al*., 2022). The inhibition of these compounds could potentially reduce the inflammatory response of KOA, leading to therapeutic pain relief and joint function improvement. The lead compounds exhibited excellent binding affinities in the range of -6.557 to -4.323 kcal/mol in the docking study of Keap1. The comparable binding affinity observed for our standard drug, Chlorhexidine, was -6.576 kcal/mol. Thus, the results of this study indicate that phyto-compounds can efficiently fine-tune the Keap1-Nrf2 signaling module and regulate cellular antioxidant defenses as well as inflammatory states (Tu *et al*., 2019). By enhancing the activation of Nrf2, these compounds could contribute to the mitigation of oxidative stress in the joints, further supporting their potential role in the management of KOA.

The docking results for IRAK4 indicated that the lead compounds exhibited binding affinities that were significantly higher than that of Zimlovisertib, a standard drug used to block this pathway in clinical practice. The affinities for these compounds are sufficiently strong to suggest that they can effectively inhibit IRAK4, a key kinase in the inflammatory signaling cascade. Thus, the combination of these compounds with IRAK4 targeting might reduce the inflammatory responses involved in the progression of KOA and present a novel therapeutic approach (Deligiannidou *et al*., 2020). For CA1, the binding affinities of the lead compounds ranged from -6.385 to -4.749 kcal/mol. The standard drug Diclofenac showed a much lower binding affinity of -1.967 kcal/mol. The findings suggest that the phyto-compounds may inhibit CA1, an enzyme that helps regulate pH and bicarbonate levels in the tissues. Inhibiting both PDR mice and KOA, these compounds could modulate the inflammatory processes within each joint; subsequently, these compounds may also influence the metabolic environment of the cartilage and synovial fluid in KOA (Wang *et al*., 2022). The docking results for Collagenase II showed that nine of the lead compounds exhibited binding affinities ranging from -5.441 kcal/mol to -4.260 kcal/mol. The standard drug TIMP-1 had a binding affinity of -4.116 kcal/mol. These findings indicate that phyto compounds prevent collagenase activity from degrading collagen in cartilage (Mixon *et al*., 2022). These compounds would prevent (or slow) the progression of KOA by inhibiting collagenase, which is one of the critical aspects of KOA progression; thus, this could potentially help preserve cartilage integrity and function.

Drug likeness and toxicity predictions of various lead molecules (from *Persea americana* L.) were analyzed in a heat map to provide insight into their potential as therapeutic agents for knee osteoarthritis (KOA). Lipinski's Rule of Five (Chen *et al*., 2020) was applied to each compound to rank the key drug-like properties of the compounds. The profiles of these compounds, such as Nicotiflorin, Quercetin 3-glucoside, and Chlorogenic Acid, all with favorable profiles, suggest their possible oral bioavailability and systemic absorption. The heat map also shows the toxicity of the lead compounds in terms of hepatotoxicity, carcinogenicity, and mutagenicity. Of particular note are Nicotiflorin and Quercetin 3-glucoside, which demonstrated lower toxicity predictions and are therefore good candidates for further development. Conversely, compounds such as Leukoefidin and its derivatives have higher toxicity risks, which may limit their clinical usefulness. Additionally, the assessment of gastrointestinal absorption and blood-brain barrier permeability revealed that several lead compounds, including Chlorogenic Acid, may not only alleviate pain associated with KOA but also have the potential for central nervous system effects, enhancing their therapeutic efficacy. Comparing these natural compounds with established drugs, such as Naproxen, provides a benchmark for evaluating their safety and efficacy. The heat map indicates that some lead compounds align closely with conventional therapies, suggesting that they could serve as effective alternatives or adjuncts in KOA treatment.

The analgesic property of *Persea americana* L.extracts was further evaluated by using standard Hot plate rat model test for measurement of nociceptive responses (Bannon and Malmberg, 2007; Oyesanmi *et al*.,2019). According to our results at different stages of pain assessment, as shown in Table 3.5.1, the group treated with the high dose of the extract demonstrated the best analgesic control compared to all other treatment groups. It is evident that this analgesic potential of *Persea americana* L extract is due to its multiple modulating effects on inflammation pathways and its strong supportive effect on collagen matrix integrity in KOA, as shown in the *in silico* segment of the study. Therefore, the *in vivo* pain behavioral assessments underscore the substantial analgesic effects of *Persea americana* L. extracts, further affirming its potential as an alternative therapeutic option for managing pain in knee osteoarthritis. As shown in this study, the sustained improvements in pain thresholds over the study period are further supported by our *in silico* results, which underline that natural compounds of *Persea americana* L. are efficacious for the management of chronic pain, thus motivating further investigation into their mechanisms and possible clinical applications in the management of chronic pain.

Together with in silico and in vivo validation, this study highlights the promising therapeutic potential of *Persea americana* L. compounds as inhibitors of key inflammatory and cartilage-degrading proteins in knee osteoarthritis. Our molecular docking results indicate that these phyto-compounds have higher binding affinities than currently used pharmacological agents being clinically utilized in the management of chronic pain, particularly against TGF-β and IRAK4, which are important in inflammatory mechanisms pertinent to KOA.

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Full-length Research Article

Chemo-Intervention of *Paullinia pinnata* Methanol Leaf Extract on Ethylene Glycol Monomethyl Ether–Induced Toxicity in Wistar Rats

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Summary: *Paullinia pinnata* (PP) is a medicinal vine used folklorically as a result of this attribute to treat various ailments. Ethylene glycol monomethyl ether (EGME) is a solvent of wide application and is toxic. This study is designed to elucidate the potential of *P. pinnata* methanol leaf extract in preventing the deleterious effect of EGME in the liver and kidney. The leaves of *P. pinnata* were extracted after defatting by Soxhlet extraction using absolute methanol. As a sequel to our previous study, seventy adult male Wistar rats were weight-matched into seven groups (n=10). Groups I and II served as controls and received distilled water and 10% dimethyl sulfoxide, respectively. Group III received EGME (200 mg/kg) only. Groups IV-VII were co-treated with EGME (200 mg/kg) and PP at 25, 50, 75 and 100 mg/kg doses, respectively. The administration was done by oral gavage daily for 14 consecutive days. On day 15, the animals were euthanized by cervical dislocation and the liver and kidneys were excised. Sections of the liver and kidney were fixed in 10% formalin for histology. The remainder of the liver and kidney were homogenized in Tris-HCl/KCl buffer and the supernatant was used for liver function and kidney function assays using standard laboratory techniques and ion selective electrode, respectively. EGME significantly ($p<0.05$) increased the activities of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase and gamma glutamyl transferase in the liver, while the concentration of sodium ion was reduced, but that of chloride-, potassium-, calcium-, phosphate- ions, urea, uric acid and creatinine was increased in the kidney. Lesions were observed in the EGME only and EGME + PP (25 mg/kg) groups and not in the other co-administered groups. The methanol leaf extract of *Paullinia pinnata* prevented the perturbations of EGME at moderate doses in the liver and kidney.

Keywords*: Paullinia pinnata, ethylene glycol monomethyl ether, liver, kidney.*

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INTRODUCTION

Paullinia pinnata (PP) is a medicinal climber used folklorically as a result of this attribute to treat various diseases topically and via ingestion. Some of the folkloric applications include treatment of whooping cough, fever, as an emetic, rickets and management of gynaecological challenges (Burkill, 2000). Some of these uses have been supported by scientific demonstrations. These include antimicrobial, anti-cancer, anti-typhoid, and antioxidant manifestations (Adeyemo-Salami, 2020). Ethylene glycol monomethyl ether (EGME) is a solvent that is employed widely domestically and industrially and has been shown to be toxic to various organs upon exposure (Adeyemo-Salami, 2021). Aberrations in liver function as a result of exposure to EGME have been reported by Takei *et al*. (2010) and Bendjeddou and Khelili (2014). Given that investigations to ameliorate the toxic effect of EGME is of interest, as an extension to our previous observation of the effect of *P. pinnata* methanol leaf extract on the toxic effect of EGME on enzymatic and non-enzymatic antioxidant parameters in the liver and kidney (Adeyemo-Salami *et al*., 2024), we assessed the effect on the functions of these organs.

This study is therefore aimed at unraveling the result of exposure to EGME and the effect of *P. pinnata* methanol leaf extract on the damage caused by EGME on the function of the liver and kidney.

MATERIALS AND METHODS

Plant material: *P. pinnata* leaves were collected and authenticated at the Herbarium of Forestry Research Institute of Nigeria (F.R.I.N.), and the specimen identification FHI 106555 was assigned. The leaves were processed using the method of Adeyemo-Salami and Makinde (2013). Briefly, rinsed, air-dried and pulverized leaves of *P. pinnata* were defatted using n-hexane and then extracted using absolute methanol in a Soxhlet extractor. A 14% yield of the plant was realized with absolute methanol as the extract.

Ethical Approval: Ethical approval was sought and granted by the Animal Care and Use Research Ethics Committee of

the University of Ibadan, Nigeria, and the number UI-ACUREC/ APP/ 10/2016 /003 was assigned.

Experimental Animal and Care: Seventy (70) adult male Wistar rats weighing 140- 190g were obtained from the Department of Veterinary Anatomy, University of Ibadan, Oyo State, Nigeria, and were weight-matched into seven groups of ten animals each. They were acclimatized for a week in standard laboratory cages and given feed (Breedwell Feed, Nigeria) and tap water ad libitum at the Animal house of the Department of Biochemistry of the same University. The 12hour light/dark cycle was maintained.

Plate 1.
Paullinia pinnata Linn. Leaves

Experimental Design: The following treatment protocol was adopted and all administrations were done by oral gavage daily for 14 consecutive days:
Group I- distilled water
Group II- 10% dimethyl sulfoxide (DMSO) (vehicle for PP)
Group III- EGME (200 mg/kg) only constituted with distilled water
Group IV- EGME (200 mg/kg) + PP (25 mg/kg)
Group V- EGME (200 mg/kg) + PP (50 mg/kg)
Group VI- EGME (200 mg/kg) + PP (75 mg/kg)
Group VII- EGME (200 mg/kg) + PP (100 mg/kg)
The weight of the animals was monitored weekly. On day 15, the animals were euthanized by cervical dislocation and the liver and kidneys were excised and weighed. The liver and kidney were homogenized in Tris-HCl/KCl buffer and the supernatant was stored at -20^{0}C until time for biochemical analyses which were liver function (albumin, total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT)) using Randox kits (U.K.), and electrolytes (sodium, potassium, and chloride ions) were analyzed using ISE SFRI Medical Diagnostics 4000 (France) while the other kidney function parameters (urea, creatinine, calcium ion, phosphate ion, and uric acid) were analyzed using Architect plus c4000 Chemistry Analyser (Abbott, USA).Sections of the liver and kidney were fixed in 10% formalin and subjected to histology. These tissues were processed for histopathology examination using a routine paraffin-wax embedded method by dehydrating using different grades of alcohol, de-alcoholizing in xylene, embedding in paraffin wax, and then rehydrating using alcohol. Sections of 5 micrometer thickness were stained with hematoxylin and eosin. The slides were then examined using a light microscope for lesions and were evaluated by a pathologist at the Department of Veterinary Anatomy, University of Ibadan, Nigeria.

Statistical Analysis: All data are expressed as mean ± standard error of mean and analyzed using one-way analysis of variance. P-values less than 5% were taken to be significant and post-hoc test was carried out using Bonferroni's multiple comparison test.

RESULTS

Figure 1 shows that there was decrease in weight gain in the groups treated with EGME only (from 17% to 10%) and EGME+ PP (25 mg/kg) (from 20% to 15%) after the first week of administration while the weight of the animals in the other groups increased with that in the EGME+ PP (75 mg/kg) being the least.

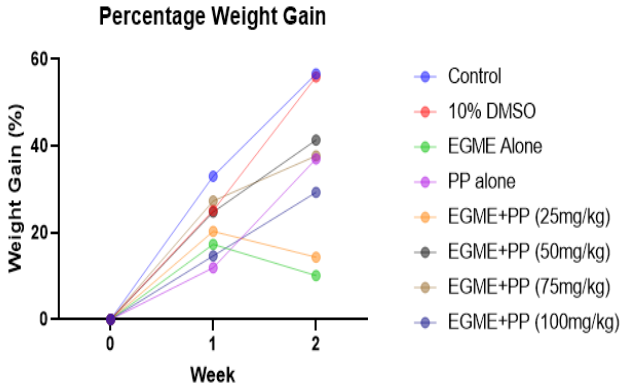

Figure 1:
Percentage weight gain over the period of the study

Table 1:
Effect on relative organ weight of animals co-treated with EGME And *P. pinnata*

Dose	Relative organ weight (%)	
	Kidney	Liver
Control	0.74±0.03	4.10±0.24
10% DMSO	0.64±0.10	3.65±0.24
EGME	0.26±0.02*	2.11±0.25*
EGME + PP (25mg/kg)	0.23±0.02*	2.02±0.44*
EGME + PP (50mg/kg)	0.72±0.11	3.64±0.42
EGME + PP (75mg/kg)	0.67±0.05	3.62±0.20
EGME + PP (100mg/kg)	0.72±0.09	3.52±0.20

Note: n=10; *- significantly differs from control at $p < 0.05$; all data are mean ± standard error of mean

Treatment with EGME only caused a significant ($p < 0.05$) reduction in the relative weight of the kidney and liver compared to the control. However, co-treatment with EGME and *P. pinnata* methanol leaf extract at 50, 75 and 100 mg/kg doses did not affect the relative weight of the organs except at the 25 mg/kg dose compared to the control (Table 1).

Table 2:
The influence of co-administration of EGME and *P. pinnata* on non-enzymatic liver function parameters

Dose	Albumin (g/dL)	Total bilirubin (mg/dL)
Control	3.94 ± 0.14	0.18 ± 0.03
10% DMSO	3.32 ± 0.14	0.26 ± 0.03
EGME (200 mg/kg)	2.40 ± 0.33[a]	0.69 ± 0.03[a]
EGME+PP (25 mg/kg)	1.82 ± 0.57[a,b]	0.57 ± 0.05[a]
EGME+PP (50 mg/kg)	4.31 ± 0.16[b]	0.25 ± 0.02[b]
EGME+PP (75 mg/kg)	3.31 ± 0.24[b]	0.16 ± 0.02[b]
EGME+PP (100 mg/kg)	3.16 ± 0.29[b]	0.22 ± 0.03[b]

Note: *n=10; all data are mean ± standard error of mean; a - significantly different from control at $p < 0.05$; b- significantly different from EGME group at $p < 0.05$*

Treatment with EGME only, significantly ($p < 0.05$) reduced the level of albumin and significantly ($p < 0.05$) increased the level of bilirubin in the liver in comparison with the control. The level of albumin in the groups co-administered with EGME and *P. pinnata* at 50, 75 and 100 mg/kg doses were significantly ($p < 0.05$) elevated when compared to the EGME only group but not with the control, except at the 25 mg/kg dose. The level of bilirubin was significantly ($p < 0.05$) reduced in the groups co-administered with EGME and *P. pinnata* methanol leaf extract at 50, 75 and 100 mg/kg doses when compared to the EGME only group but not with the control, except at the 25 mg/kg dose (Table 2).

Table 3 shows that administration of EGME only, significantly ($p < 0.05$) elevated the activities of ALT, AST, ALP and GGT in the liver compared with the control. Co-administration with EGME and *P. pinnata* at 50, 75 and 100 mg/kg doses significantly ($p < 0.05$) douced the activities of the enzymes when compared with the EGME only group and not the control, except at the 25 mg/kg dose.

Table 3:
The effect of co-administration of EGME and *P. pinnata* on certain enzymatic liver function biomarkers

DOSE	ALT (U/l)	AST (U/l)	ALP (U/l)	GGT (U/l)
Control	51.17 ±3.00	8.00 ±0.26	38.09 ±1.95	8.89 ±0.42
EGME (200 mg/kg)	74.40 ±1.29[a]	16.35 ±0.58[a]	101.00 ±1.64[a]	15.39 ±0.80[a]
EGME+PP (25 mg/kg)	66.50 ±1.50[a]	15.50 ±1.50[a]	112.10 ±10.00[a]	22.42 ±1.84[a,b]
EGME+PP (50 mg/kg)	46.50 ±2.50[b]	8.25 ±0.34[b]	48.76 ±3.54[b]	7.11 ±0.89[b]
EGME+PP (75 mg/kg)	58.00 ±1.87[b]	9.18 ±0.56[b]	37.44 ±1.18[b]	7.74 ±0.26[b]
EGME+PP (100 mg/kg)	57.80 ±1.99[b]	9.51 ±0.14[b]	39.83 ±1.10[b]	11.63 ±0.31[b]

Note: n=10; all data are mean ± standard error of mean; a - significantly different from control at $p < 0.05$; b- significantly different from EGME group at $p < 0.05$; ALT- alanine aminotransferase; AST- aspartate aminotransferase; ALP- alkaline phosphatase; GGT- gamma glutamyl transferase

Except for sodium ions, treatment with EGME only significantly ($p < 0.05$) increased the concentrations of potassium, chloride, calcium and phosphate ions and similarly, the concentrations of uric acid, urea and creatinine in the kidney in comparison with the control. Co-administration with EGME and *P. pinnata* at 50, 75 and 100 mg/kg doses resulted in significant ($p < 0.05$) increase in sodium ion level and significant ($p < 0.05$) decrease in the concentrations of potassium, chloride, calcium and phosphate ions, and that of uric acid, urea and creatinine in comparison to the EGME only group and not the control, except at the dose of 25 mg/kg (Table 4).

Table 4:
The influence of co-administration of EGME and *P. pinnata* on certain parameters for kidney function

DOSE	Na^+ (mmol/L)	K^+ (mmol/L)	Cl^- (mmol/L)	Urea (mg/dL)	Creatinine (mg/dL)	Ca^{2+} (mg/dL)	PO_4^{2-} (mg/dL)	Uric Acid (mg/dL)
Control	25.00 ±1.08	66.07 ±1.84	61.00 ±0.93	42.01 ±1.14	0.11 ±0.01	1.15 ±0.10	9.48 ±0.43	16.90 ±1.41
10% DMSO	25.67 ±0.88	75.85 ±0.65	65.33 ±1.45	51.64 ±0.98	0.18 ±0.02	1.40 ±0.06	11.54 ±0.37	19.25 ±0.75
EGME (200 mg/kg)	10.67 ±0.66[a]	92.83 ±2.82[a]	117.30 ±6.36[a]	71.21 ±1.19[a]	0.22 ±0.02[a]	1.77 ±0.09[a]	20.15 ±0.92[a]	26.75 ±1.25[a]
EGME+PP (25 mg/kg)	10.00 ±0.00[a]	102.00 ±0.00[a]	120.00 ±0.00[a]	53.50 ±0.00[a]	0.20 ±0.00[a]	1.70 ±0.00[a]	17.70 ±0.00[a]	20.00 ±0.00[a]
EGME+PP (50 mg/kg)	23.25 ±1.55[b]	66.40 ±1.20[b]	59.00 ±0.91[b]	40.93 ±2.24[b]	0.15 ±0.01[b]	1.12 ±0.06[b]	10.33 ±0.32[b]	14.48 ±0.51[b]
EGME+PP (75 mg/kg)	29.50 ±1.85[b]	69.37 ±1.27[b]	62.50 ±2.78[b]	38.52 ±2.31[b]	0.12 ±0.01[b]	1.13 ±0.09[b]	11.13 ±0.40[b]	14.43 ±0.56[b]
EGME+PP (100 mg/kg)	28.00 ±0.91[b]	70.18 ±2.70[b]	65.67 ±1.20[b]	50.42 ±1.92[b]	0.12 ±0.01[b]	1.18 ±0.09[b]	11.34 ±0.18[b]	16.80 ±0.40[b]

Note: n=10; all data are mean ± standard error of mean; a - significantly different from control at $p < 0.05$; b- significantly different from EGME group at $p < 0.05$; Na^+-sodium ion; K^+-potassium ion; Cl^--chloride ion; Ca^{2+}-calcium ion; PO_4^{2-}-phosphate ion

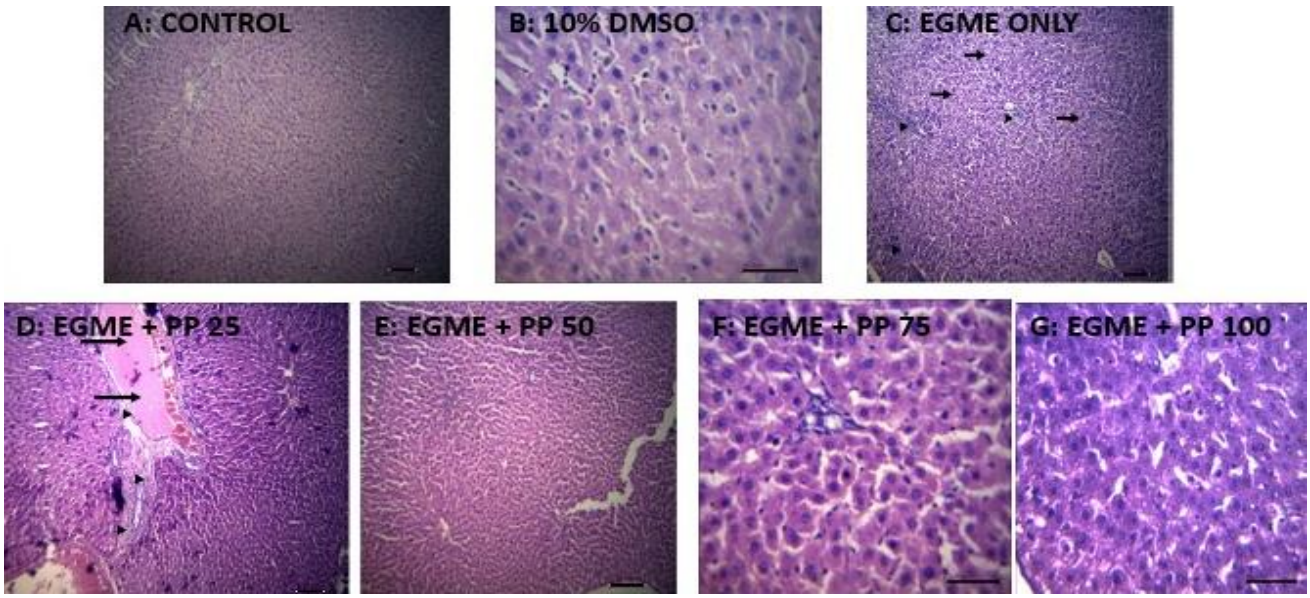

Plate 2:
Photomicrographs of the liver sections showing the effect of co-administration of EGME and *P. pinnata*: - A, B, and E - G - No lesions; **C**-There is a moderate to severe diffuse hydropic degeneration and necrosis of hepatocytes, there is also a diffuse lymphocytic cellular infiltration; **D-** Severe portal congestion with a mild to moderate periportal cellular infiltration by neutrophils and macrophages. The periportal connective tissue is also prominent.

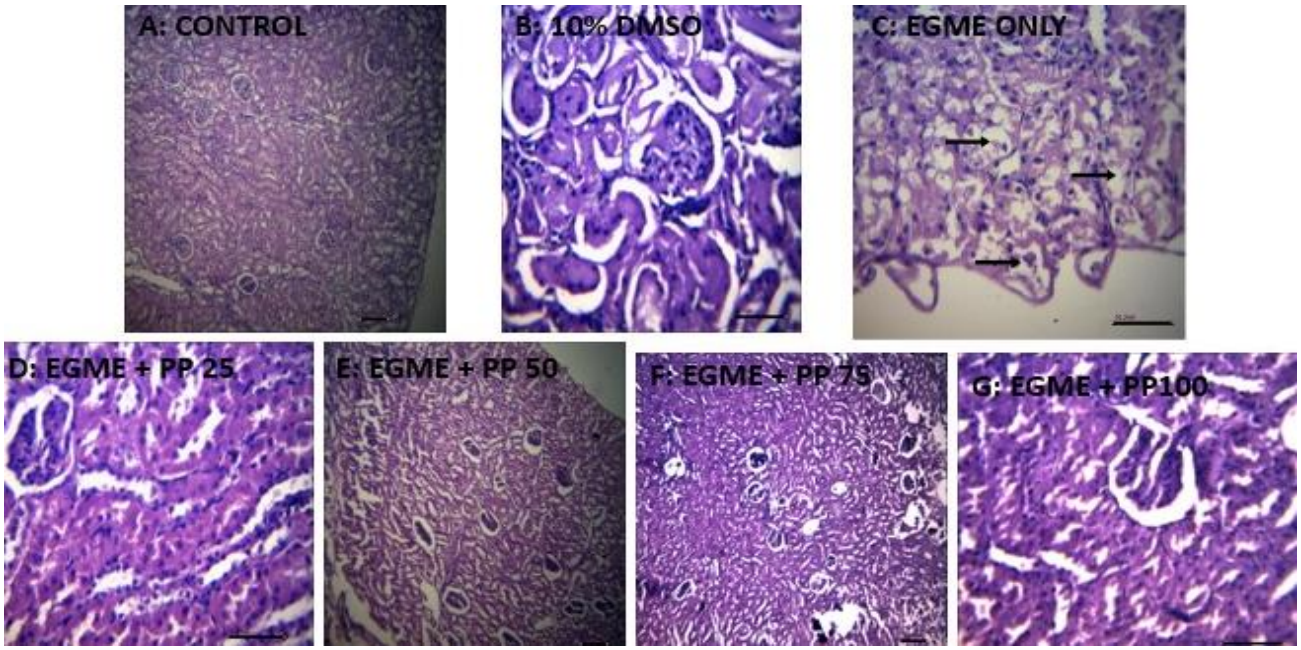

Plate 3:
Photomicrographs of the kidney sections showing the effect of co-administration of EGME and *P. pinnata*:- A, B and D - G- No lesions; **C**-There is a severe diffuse tubular degeneration and necrosis, especially below the capsule

DISCUSSION

The investigation showed that co-treatment with *P. pinnata* methanol leaf extract barred the perturbations of EGME in the liver and kidney at moderate doses.

The percentage weight gain in the EGME only treated group and that co-administered with EGME and *P. pinnata* methanol leaf extract at 25 mg/kg dose was reduced. Similarly, the relative organ weight had the same presentation. These are signs of toxicity, revealing that *P. pinnata* methanol leaf extract did not prevent the deleterious effect of EGME at that dose. In the groups co-administered with EGME and *P. pinnata* methanol leaf extract at 50, 75 and 100 mg/kg doses, the percentage weight gain and relative organ weights of the liver and kidney were not affected. This implies that at those doses the *P. pinnata* methanol leaf extract barred the adverse effect of EGME.

Albumin is a protein produced in the liver that assists with the transportation of fatty acids in the plasma including a host of other functions (Soeters and de Leeuw, 2021). In the macrophage-monocyte system, bilirubin is generated by the hydrolysis of haemoglobin to biliverdin and subsequently to bilirubin. Bilirubin is then transported via the plasma to the liver from which bilirubin diglucuronide is formed and subsequently released into the bile. Hence, bilirubin level serves as an indicator of liver and bile tract function (Washington and Hoosier, 2012). Administration of EGME lowered the albumin level and elevated the bilirubin level in

the liver. Co-administration with *P. pinnata* prevented the effect at 50, 75 and 100 mg/kg doses with the exception of the co-administration at the 25 mg/kg dose. This shows that the deleterious effect of EGME was douced at those doses, and indicates that the function of the liver is preserved and enhanced in the presence of the toxicant.

The activities of alanine aminotransferase, aspartate aminotransferase alkaline phosphatase, and gamma-glutamyl transferase are all indicators of the function of the liver. Alanine aminotransferase (ALT), formerly known as serum glutamate pyruvate transaminase (SGPT), functions in a transamination reaction by catalyzing the transfer of an α –amino group between α-ketoglutarate and alanine to generate glutamate and pyruvate, respectively. ALT is found in serum and organs with it being most abundant in the liver (Washington and Hoosier, 2012). It may be elevated in the light of biliary duct and liver damage, hepatitis, myopathy and congestive heart failure (Washington and Hoosier, 2012). Aspartate aminotransferase (AST), formerly known as serum glutamate oxalate transaminase (SGOT), is an enzyme that catalyzes the transamination reaction between α-ketoglutarate and aspartate to form glutamate and oxaloacetate respectively. With the exception of the bone, AST is found in all tissues and is highest in the skeletal muscle and liver. AST activity is elevated after neoplasia, trauma, infection, bruising and necrosis of the liver or muscle (Washington and Hoosier, 2012). Elevated activities of ALT and AST in the EGME only group tend to point to the fact that there is liver damage. Alkaline phosphatase is a metalloenzyme which is membrane bound, and is made up of three isozymes which are tissue specific. One of the isozymes is abundant in the hepatic tissue (Sharma *et al*., 2014). At high optimum pH (pH 9-10), it catalyzes the hydrolysis of monophosphate esters (in molecules such as proteins and nucleotides) with the concomitant release of inorganic phosphate (Sharma *et al*., 2012; Washington and Hoosier, 2012). Gamma-glutamyl transferase (GGT) has multiple catalytic functions including the transfer of gamma-glutamyl moiety to amino acids and short peptides and hydrolysis of reduced glutathione (GSH) to cysteinylglycine and gamma-glutamyl moiety in GSH conjugate metabolism (Anadon *et al*., 2014). Elevated GGT in the serum is an indicator of hepatobiliary insult including cholestasis and biliary effect, although it is also expressed in the bile ducts, kidney and pancreas. Also, it may be a biomarker of severe adverse drug reactions (Anadon *et al*., 2014). The EGME only treated group presented increased activities of ALP and GGT. This evidently suggests that there is insult on the function of the liver which may involve hepatobiliary dysfunction and cholestasis, characterized by reduced or paused bile flow which leads to the accumulation of bile in the liver and bloodstream, resulting in itching and jaundice (Cleveland Clinic, 2022). Co-administration with EGME and *P. pinnata* significantly reduced the toxic effect at 50, 75 and 100 mg/kg doses, except at the 25 mg/kg dose, on the activities of ALT, AST, ALP and GGT. Thus, showing that *P. pinnata* methanol leaf extract has the capacity to enhance liver function, in the light of exposure to EGME, at moderate doses. This observation is akin to the report of Bardi *et al*. (2014) where they administered thioacetamide to Sprague Dawley rats and co-treatment with *Andrographis paniculata* ethanol leaf extract reduced the effect. Moreover, this is similar to the documentation of Abdou *et al*. (2012) who showed that sesame oil barred the effect of cypermethrin on the liver function parameters and the antioxidant parameters in rats.

Sodium, potassium, chloride, calcium, and phosphate ions are all electrolytes found in the kidney. The kidney helps to maintain balance in the concentrations of these electrolytes. They help maintain water balance, regulate nerve and muscle function and acid-base balance (Lewis, 2021). With the exception of sodium ion, the concentrations of all the other electrolytes were elevated upon administration of EGME only and this was prevented upon co-treatment with *P. pinnata* at the doses of 50, 75 and 100 mg/kg with the exception of the 25 mg/kg dose. This is in tandem with Marhoume *et al*. (2021) who observed that co-administration with polyphenol-rich extracts of *Rubia tinctorum* reduced the effect of ethylene glycol and ammonium chloride on the electrolytes.

Urea, also called blood urea nitrogen (BUN) when measured in the blood, is also a non-protein nitrogenous waste product that is a byproduct of protein metabolism and an index for renal function because it is excreted from the body via the kidney (Salazar, 2014). Creatine supplies the muscles with energy and creatinine is the non-protein nitrogenous waste product of creatine. Creatinine is removed from the body by the kidney. Therefore it is a measure of the function of the kidney (Salazar, 2014). Uric acid is the final product of oxidation in purine metabolism which is excreted by the kidney (Giordano *et al*., 2015). It has been shown that elevated levels of urea, creatinine, and uric acid are associated or may be associated with impaired kidney function which plays a key role in the excretion of these waste products (Salazar, 2014; Giordano *et al*., 2015; Gounden *et al*., 2021). The most common renal vascular disease or damage is nephrosclerosis which is characterized by damage to blood vessels, glomeruli and tubulointerstitium (Vaidya and Aeddula, 2024). Therefore, the elevated levels of these products which was caused by the administration of EGME only and was barred by co-administration with *P. pinnata* at 50, 75 and 100 mg/kg doses but not at the 25 mg/kg dose, again shows that *P. pinnata* methanol leaf extract has the capacity to protect the kidney against damage from EGME, at moderate doses. These observations were similar to that of Romi *et al*. (2017) who reported an increase in the plasma levels of urate and creatinine upon administration of urate which was prevented by treatment with allopurinol, and that of Ogundipe *et al*. (2016) where aqueous leaf extract of *Ocimum gratissimum* was used to reverse the effect of gentamicin on the urine and plasma levels of urea and creatinine. Also, it is akin to the demonstrations of Abdou *et al*. (2012) who showed that sesame oil barred the perturbations in the kidney upon exposure to cypermethrin in rats. Therefore, co-administration with EGME and *P. pinnata* methanol leaf extract at 50, 75 and 100 mg/kg doses circumvented the reduced concentration of Na+ and increased concentrations of K+, Cl-, Ca2+, PO42-, urea, creatinine and uric acid caused by exposure to EGME, except at the dose of 25 mg/kg. Thus showing that *P. pinnata* methanol leaf extract protect the kidney from damage as a result of exposure to EGME. All of these results were supported by the histopathology report where varying lesions were observed in the liver and kidney for the EGME only treated group and

the group co-administered with EGME and *P. pinnata* methanol leaf extract at 25 mg/kg. This shows that *P. pinnata* methanol leaf extract had the capacity to protect the organs at 50, 75 and 100 mg/kg doses. The limitation of the study is that it was conducted using animals. Primate and human studies may be necessary to support the findings.

In conclusion, *Paullinia pinnata* methanol leaf extract protects the liver and kidney from the deleterious effect as a result of exposure to EGME, at moderate doses

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Full-length Research Article

Methanolic Extract of Kola Nut (*Cola acuminata*) Decreased Body Weight and Elevated Total Plasma Cholesterol Level in Rats (Rattus norvegicus)

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Summary: Kola and kola-containing beverages are among the most consumed products globally for their stimulatory and energy-boosting potentials. The precise impact of kola nut consumption on various biological indices of consumers remains debatable, necessitated by the fact that the phytochemical compositions of kola nuts are dependent on a range of factors. This study investigated the impacts of a 28-day administration of a methanolic extract of *C. acuminata* on the body weight and serum cholesterol indices of adult male albino rats (Wistar strain). The quantitative photochemical compositions and lethal dose (LD50) of the extract were determined using standard bioassay procedures. Rats were randomized into four experimental groups that received various concentrations of the extract: 0, 100, 150, and 200 mg/kg. Results show an LD_{50} of 3101.37 mg/kg for the extract, whereas flavonoids and tannins were the most abundant phytochemicals. In addition, extract administration caused a dose-dependent reduction in body weight, while significant elevations in total serum cholesterol levels were recorded in rats that received 100 and 150 mg/kg of the extract. These suggest that kola nut consumption may negatively impact body weight and cholesterol metabolism.

Keywords*: Kola nut, Cola acuminata, body weight, cholesterol, lethal dose*

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INTRODUCTION

Kola and kola-containing beverages are one of the heavily consumed nervous system stimulants and energy boosters globally. Common sources of kola include various species of kola nuts, *C. nitida* and *C. acuminata*, and many coca-cola beverages (Adesida *et al*., 2021). Nigeria accounts for about 70 % of world kola nut production (Quarco, 1969; Jacob, 2023), 90% of which are consumed locally (Quarco, 1973). Quantitatively, kola nuts are rich in water, carbohydrates, ash, alkaloids, flavonoids, tannins, and cellulose (Purseglove, 1968, Igbinovia *et al*., 2009). The stimulatory and energy-boosting potentials of kola nuts are due to its caffeine and other methylxanthine alkaloid contents (Jacob *et al*., 2023). Although there is no extant data on the precise rate or dynamics of consumption. Kola nut, especially *C. nitida* and *C. acuminata* are greatly employed as a symbol of hospitality (Purseglove, 1977), and during cultural, religious, and social gatherings (Jacob *et al*., 2023). Kola nuts are also used in the production of various beverages and wines (Beattie, 1970; Ogutuga, 1975; Ajiboye & Afolayan, 2009).

As stimulants and energy boosters, there are undocumented claims of local farmers, especially in Africa, subsisting on water and kola for hours without food. Again, it is common in Nigeria, including among students, to chew kola nut seeds to stay awake for various reasons. For instance, Erinfolami *et al*. (2011) found 11.2%, 29.1%, and 74.8% for 30-day, one-year and lifetime prevalence rate of kola nut consumption among secondary school students most of who started chewing kola nut from the age of 14 in Osogbo, Osun State, Nigeria. That study also found that several factors, including poor school attendance, polygamy, low maternal and high paternal educational attainment, and over-permissiveness on the part of the mother. Consequently, the rate of kola nut consumption appears to be on the increase even in these modern times, strengthening the growing concern about potential negative impacts of kola consumption on crucial biological indices of consumers of different ages, socio-economic status, and health. However, most extant studies on the impact of kola nut consumption focused on *C. nitida* (Ikegwuonu *et al*., 1981; Obidike *et al*., 2011; Nku *et al*., 2014; Ewenighi *et al*., 2016), making us wonder if *C. acuminata* consumption would elicit harmful or beneficial consequences. For instance, Asogwa *et al*. (2014) reported dose and time-dependent changes in the stress response and inflammatory biomarkers of male Albino rats associated with an in vivo exposure to methanolic extract of *C. acuminata*. These changes were typified by elevated serum cortisol levels and leukocytosis. We reasoned that *C. acuminata*, which is most

consumed of all kolas in Eastern Nigeria, may have a wide-ranging effect on different biological systems apart from the stress hormonal and leukopoietic impacts earlier reported (Asogwa *et al*., 2014). So, we extended our investigation to evaluate the potential effects of *C. acuminata* extract on the body weight and total plasma cholesterol levels of adult male Albino rats (Rattus norvegicus) for 28 days. We first determined the quantitative phytochemical composition and lethal dose of *C. acuminata* used in this study. Our findings are consistent with the hypothesis that *C. acuminata*¸ like C. nitida, has weight-reducing potentials and an obvious tendency to spike total plasma cholesterol levels. The extent to which *C. acuminata* impacts on the body weight and lipid biochemical indices of human consumers remain to be ascertained.

MATERIALS AND METHODS

Procurement of kola nut seeds: Seeds of *C. acuminata* were purchased from Ogige Market, Nsukka, Enugu State, Nigeria. Identity of the seeds was authenticated at the Taxonomy Unit, Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.

Experimental animals: Adult male albino rats (*Rattus norvegicus*, Wistar strain, 180-200 g) used for this study were purchased from the Genetics and Animal Breeding House, Department of Zoology and Environmental Biology, University of Nigeria, Nsukka. They had *ad libitum* access to commercially available rat chow and water for the duration of the investigation.

Preparation of *C. acuminata* extract: The methanolic extract of *C. acuminata* was prepared as described earlier (Asogwa *et al*., 2014). Briefly, 2 kg of the seeds of *C. acuminata* was floor-dried and pulverized with an electric blender. 50 g of the powder was put into a conical flask to which 200 ml of absolute methanol was added. The mixture was allowed to stand for 24 hr. It was then filtered using a clean muslin cloth. The extract was concentrated to dryness using a rotary evaporator. The concentrated extract was used to prepare various concentrations administered to the experimental animals.

Quantitative phytochemical analysis: Detailed quantitative phytochemical determinations were previously described: alkaloids (Henry, 1973), tannins (Dawra *et al*., 1988), saponins (Brunner, 1984), flavonoids (Zhishen *et al*., 1999), terpenoids (Łukowski *et al*., 2022), steroids (Birner, 1969), and total cyanide (Haque & Bradbury, 2001).

Determination of lethal dose (LD_{50}): The lethal dose (LD_{50}) of the extract was determined according to the method of Lorke (1983). Briefly, a preliminary test was done using 10, 100, and 1000 mg/kg body weight of the extract (n=5 mice/group). When no mortality was recorded after 24 hr, a new set of mice administered 1600 mg/kg, 2900 mg/kg, and 5000 mg/kg of the extract (n=5 mice/dose). The number of dead mice were recorded for 24 hrs, while a probit curve was plotted to deduce the LD_{50} of the extract.

Experimental design: A total of 60 adult male albino rats were randomly divided into four groups (0, 100, 150, and 200 mg/kg, 3 replicates/group, n=5/replicate), each group with 3 replicates (n=5/replicate). The rats were maintained at optimum laboratory conditions (25^{o}C, 12L/12D photoperiod) with *ad libitum* access to food and water. Rats were weighed using a sensitive balance before extract administration and after every 7 days following administration.

Collection of blood sample: Blood samples were collected from the orbital sinus before the commencement of extract administration and every other 7 days. Blood was allowed to clot and centrifuged at 1200 rpm to separate the serum. Serum samples were stored frozen if not analysed immediately (Machado *et al*., 2009). All Biochemical analyses were performed at Shalom Diagnostic Laboratories, Nsukka Local Government Area, Enugu State, Nigeria.

Determination of total cholesterol concentration: Total plasma cholesterol levels were determined as previously described (Kishi *et al*., 2002) using a commercially available diagnostic kit (Randox, Germany). This method was based on the principle that cholesterol esters are hydrolysed by cholesterol esterase to free cholesterol and fatty acids, while free cholesterol is oxidized to cholest-4-ene-3-one and hydrogen peroxide, which in the presence of phenol and amino-4-antipyrin forms a red complex whose optical absorbance is measured at 550 nm.

Statistical Analysis

The data obtained were subjected to a Two-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS ver. 20 for Windows, IBM Statistics, USA). Post hoc tests (Duncan) were utilized to identify significant changes in the mean body weight and total cholesterol of both the control and treated groups. Statistical significance was set at $p \leq 0.05$, and the results were presented as mean ± standard error of the mean (SEM).

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RESULTS

Quantitative phytochemical composition of the methanolic extract of *C. acuminata:* Our phytochemical analyses revealed in qualitative and quantitative terms, the presence of saponins, alkaloids, flavonoids, tannins, terpenoids, and cyanides (Table 1). Additionally, the concentration of alkaloid was only lower than those of flavonoids and tannins, and with a negligible amount of cyanide.

Table 1:
Phytochemical compositions of the methanolic extract of *C. acuminata*

Phytochemicals	Qualitative	Quantitative (mg/100 g)
Saponins	+	0.31±0.004
Alkaloids	++	3.06±0.0032
Flavonoids	++	3.90±0.0027
Tannins	++	3.64±0.0036
Terpinoids	+	1.32±0.002
Steroids	+	1.51±0.003
Cyanide	+	0.31±0.004

Notes. +: present, ++: moderately present. Each determination was performed in triplicates (n = 3). Table shows a higher amount (mg/100 g) of flavonoids, tannins, and alkaloids than saponins, terpinoids, and steroids. Quantitative values are Mean±SEM.

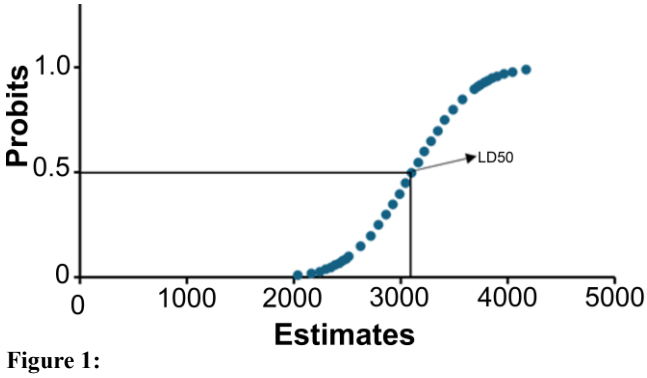

Figure 1:
Dose-response curve of the methanolic extract of *C. acuminata*. Adult male mice (n = 3 for each of the 10, 100, 1000, 1600, 2900, and 5000 mg/kg doses) were used for the LD_{50} determination. Rectangular tracing shows the estimated dosage that would cause a 50% mortality of the test mice.

Lethal dose (LD_{50}) of the methanolic extract of *C. acuminata*: The acute toxicity test of the methanolic extract of *C. acuminata* revealed an LD_{50} of 3082.81 mg/kg of body weight (Figure 1).

Effects of the methanolic extract of *C. acuminata* on body weight: The administration of graded doses of *C. acuminata* caused a time-dependent increase in body weights of rats in the 100 mg/kg group on day 28, while rats that received 200 mg/kg experienced a time-dependent reduction in body weight starting from day 7 until day 28 (Figure 2, replicates/group, n = 5/replicate, Two-way ANOVA, $p \leq 0.05$). Similarly, there was a dose-dependent reduction in body weight of rats given 200 mg/kg of extract on day 21, and rats that received 150 and 200 mg/kg on day 28 (Figure 2, replicates/group, n = 5/replicate, Two-way ANOVA, $p \leq 0.05$).

Effects of the methanolic extract of *C. acuminata* on serum total cholesterol concentration: The study recorded a significant time-dependent elevations in serum total cholesterol concentrations in the 100 and 150 mg/kg treatments on Days 14 and 21 compared with baseline values (Figure 3, replicates/group, n = 5/replicate, Two-way ANOVA, $p \geq 0.05$). However, no significant (Figure 3, replicates/group, n = 5/replicate, Two-way ANOVA, $p \leq 0.05$) elevations in serum cholesterol concentration were recorded in the 200 mg/kg group except on day 7 of extract administration (Figure 3, replicates/group, n = 5/replicate, Two-way ANOVA, $p \leq 0.05$). On the other hand, it was only on day 7 that a significant dose-dependent elevations in total serum cholesterol was recorded (Figure 3, replicates/group, n = 5/replicate, Two-way ANOVA, $p \leq 0.05$).

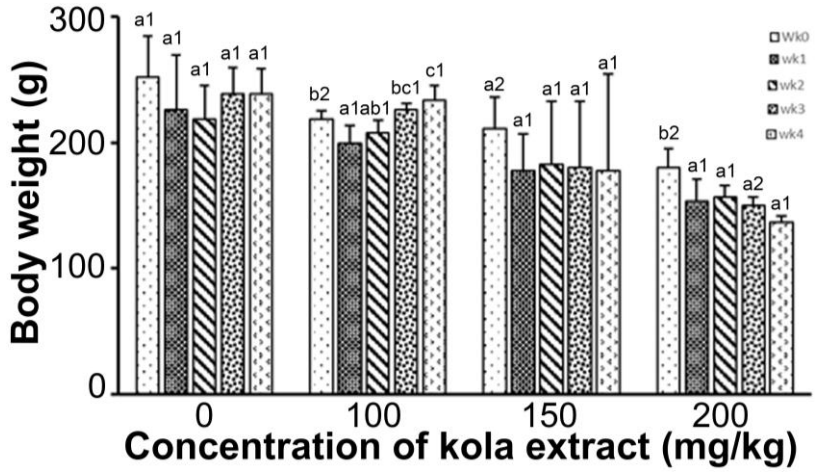

Figure 2:
Effects of a 28-day administration of the methanolic extract of *C. acuminata* on the body weight of adult male albino rats. Different bars with different alphabets within a treatment group are significantly different (3 replicates/group, n = 5/replicate, One-way ANOVA, $p \leq 0.05$). The same bars with different numbers across the treatment groups are significantly different (3 replicates/group, n = 5/replicate, One-way ANOVA, $p \leq 0.05$). Plot shows clear dose-dependent reductions in the body because of extract administration. Plotted values are mean±SEM. Error bars represent SEM.

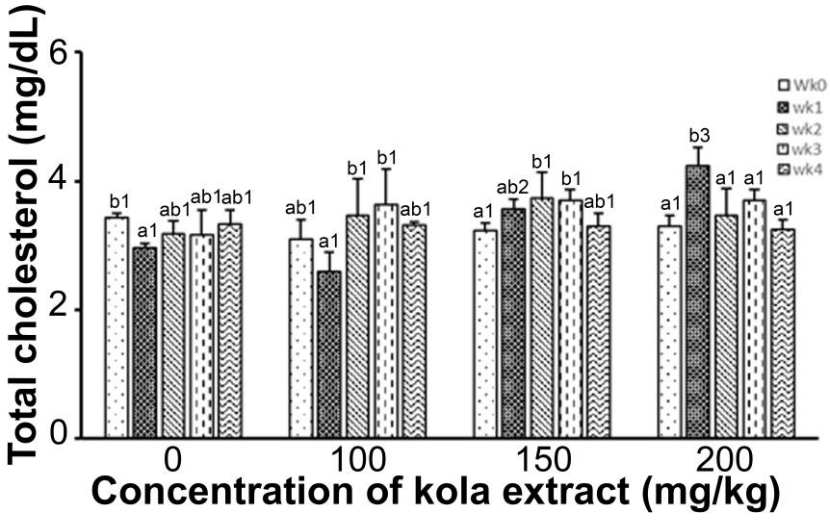

Figure 2:
Changes in total plasma cholesterol levels of adult male albino rats administered graded doses of the methanolic extract of C. acuminata for 28 days. Plotted values are mean±SEM. Error bars represent SEM. Top right: legends. Different bars with different alphabets within a treatment group are significantly different (3 replicates/group, n = 5/replicate, One-way ANOVA, $p \leq 0.05$). The same bars with different numbers across the treatment groups are significantly different (3 replicates/group, n = 5/replicate, One-way ANOVA, $p \leq 0.05$). Plot depicts a gradual dose-dependent elevation in total plasma cholesterol levels in the treated groups compared with the control (0 mg/kg).

DISCUSSION

This study investigated the proximate composition and impacts of chronic sublethal doses of the methanolic extract of *C. acuminata* on the body weight and some lipid profile biomarker of adult male albino rats for 28 days. Our findings reveal that the extract contains varying amounts of alkaloids, tannins, flavonoids, terpenoids, steroids, and cyanide. In addition, at the highest dose administered, methanolic extract of *C. acuminata* strongly reduced the body wight of albino rats. Additionally, the two lowest doses of the extract caused a significant time-dependent increase in the serum cholesterol concentration relative to baseline values.

Although we found similar phytochemical constituents in the kola seeds used in this study, there are obvious variabilities in the specific amount of each phytochemical present. For example, whereas we found that flavonoids and tannins were the most abundant phytochemicals followed by alkaloids and tannins, Osaro *et al*. (2024) reported that tannins and alkaloids followed by saponins and flavonoids. On the other hand, Uwabunkeonye *et al*. (2015) reported higher amounts of tannins and phenols compared with alkaloids and flavonoids. These findings demonstrate that although *C. acuminata* contains a lot of beneficial phytochemicals, the presence of methylxanthine alkaloids points to the fact that potentially unrecorded harmful effects may be associated with *C. acuminata* consumption. The discrepancies between our findings and previous studies could be due to differences in farming, soil, humidity, post-harvest handling, and method and length of storage (Touati *et al*., 2014; Kapcum & Uriyapongson, 2018; DeBenedictis *et al*., 2023; Tedeschi *et al*., 2023).

The effects of methanolic extract of *C. acuminata* on body weight and lipid profile indices recorded in this study compares largely with previous investigations in humans and rodents. For instance, intraperitoneal injections of 20 and 30 mg/kg caffeine extract of Cola nitida into adult male albino rats resulted in a significant reduction of body weight compared with the control and 10 mg/kg injected group. Similarly, Umoren *et al*. (2009) and Salahdeed *et al*. (2009) reported that supplementing rat diet with different concentrations of *C. nitida* extract, or the caffeine extracted thereof, resulted to a significant weight reduction in the exposed rats. Even though these studies employed C. nitida, another species of *C. acuminata*, and that the routes of administration differed, the observation that kola or its extracts have weight-reduction potential is consistent with our current findings. It is possible that *C. acuminata* extract may have modulated the activity of the feeding/satiety center that could have led to changes in feeding habit, ultimately resulting to apparent changes in body weight. Although the likely extract-related changes in feed consumption were not recorded in this study, previous studies showed that kola or caffeine ingestion caused a significant change in dietary intake in both humans and rats. The possible impacts of *C. acuminata* on food and feeding habits remain to be ascertained.

Earlier investigations found that diet supplementation with *C. nitida rubra* (15 g/ 100 g of rat feed) significantly elevated total cholesterol concentration, whereas a higher supplementation (30 g/100 g of rat feed) significantly reduced total cholesterol levels compared with control rats (Nku *et al*., 2014) These findings are consistent with the results of the current study, which show that lower concentrations of the methanolic extracts of *C. acuminata* (100 and 150 mg/kg) let to a significant increase in total plasma cholesterol compared to control rats. In contrast to the significant reduction in total cholesterol levels recorded in rats fed diets supplemented with a higher amount of *C. nitida* (Nku *et al*., 2014), the highest concentration of *C. acuminata* (200 mg/kg) used in our study did not result in significant changes in total plasma cholesterol levels. This suggests that there may be a concentration threshold required to produce significant alterations in cholesterol metabolism. In a human-based study, chronic kola nut consumers (aged 55.3±9.15 yrs) were found to have significantly higher serum total cholesterol levels compared with age-matched non-consumers (Ewenighi *et al*., 2016). Since other studies suggested weight-reducing potentials for flavonoids and terpenoids, we predict that the observed impact of the methanolic extract of *C. acuminata* could be the result of synergistic interactions among essential phytochemicals. Future investigations should explore this.

Although the lipotropic potentials of caffeine were proposed over a century ago (Heppel *et al*., 1947), the consumption of caffeine and caffeine-containing products remain unabated. As we progress through a fast-paced and increasingly complex world, there is projected increase in stress and stress-related complications owing to rising job demands, anxiety, depression, and social isolation. To counteract the attendant adverse consequence, people would usually seek over-the-counter energy boosters and sleep-influencing drugs. We believe that kola nut and other kola-containing products would also be highly sort after. Therefore, we recommend a cautious use of *C. acuminata* in its various formulations, especially when stress, age, physical activities, and existing health conditions may be of major concerns. Since the proximate and phytochemical compositions of kola nut depend on a variety of factors, including cultivars, season, soil type, farming system, method and length of storage (Touati *et al*., 2014; Kapcum & Uryapngson, 2017; DeBenedictis *et al*., 2023; Tedeschi *et al*., 2023), a periodic assessment of the potential impacts of commercially available kola nuts on various biological indices has become crucial.

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Full-length Research Article

Persea americana bark extract Modulates N^{w}–nitro-L-arginine methyl ester (L-NAME)-induced hypertension through NF-ƙβ/NRF2/KIM-1/CTnI signaling pathways in Wistar rats

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Summary: Hypertension is one of the major risk factors for cardiovascular diseases. It has become a significant public health concern in both developed and developing countries. In this study we evaluated the ameliorative effect of methanol bark extract of *Persea americana* (PA) on L-NAME-induced hypertension and its attendant cardiac and renal complications. Sixty rats were divided into six groups. Group A was the negative control and received distilled water throughout the study. Group B received a daily repetitive dose of L-NAME alone at 40 mg/kg for 21 days. Groups C, D, and E received a daily repetitive dose of L-NAME at 40 mg/kg and extract at 100 mg/kg, 200 mg/kg and 400 mg/kg, respectively, for 21 days. Group F received a daily repetitive dose of L-NAME at 40 mg/kg and lisinopril at 10 mg/kg for 21 days. The results showed that L-NAME significantly elevated blood pressure, markers of renal damage, oxidative stress, and expression of KIM-1, NRF2, NF-KB and CTnI, while it decreased both enzymatic and non-enzymatic antioxidant parameters. The extract and lisinopril, however, ameliorated these effects in the rat model. These findings showed that the bark extract of PA may play a role in reducing oxidative stress, inflammation, cardio-renal organ damage and blood pressure levels in hypertension, possibly through free radical scavenging, antioxidant system potentiation, NF-ƙβ/NRF2/KIM-1/CTnI signaling pathways.

Keywords: *Persea americana* bark extract, hypertension, oxidative stress, antioxidant, L-NAME, lisinopril

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INTRODUCTION

Hypertension accounts for about 15 percent of health loss in adults and little decreases in the prevalence of hypertension can facilitate great health gains in blood pressure values (Abreu *et al*., 2018). It is characterized by elevations in blood pressure with attendant organ damage in the heart, kidneys, and the eye (Carasin *et al*., 2007). The pathogenesis of hypertension is multifactorial, and beyond reducing blood pressure values, an ideal antihypertensive should also ameliorate the complications of hypertension seen in several other target organs such as the heart, brain and the kidneys (Dais *et al*., 2018). This, however, is not targeted in current drug strategies.

It has been shown that a prerequisite of hypertension is constriction of renal blood vessels and this is facilitated by discrepancies in the levels of renal vasoconstrictors and vasodilators favoring vasoconstrictors (Johnson et al., 2018). The kidney plays a huge role in the development of hypertension. Ultimately, hypertension leads to cardiac damage and elevated blood pressure, particularly systolic blood pressure, is a significant factor contributing to myocardial infarction (Messerli *et al*., 2017).

Persia americana (avocado pear) is widely found in America, Africa, and the tropics. The leaf is simple, finely toothed, glossy, and green in colour. The fruit has a bell shape with colour mostly green or brown. Apart from the nutritional value of *Persia americana,* extracts from the leaf

and seed of the plant have been found to be of good medicinal value (Ojewole *et al*., 2007).

Lisinopril, an angiotensin converting enzyme (ACE) inhibitor acts by reducing the synthesis of angiotensin-2 by inhibiting the action of ACE. ACE Inhibitors have been shown to prevent the onset of nephropathy in hypertension (NHFA, 2016).

In this study we evaluated the ability of *Persea americana* to reduce blood pressure and ameliorate the consequent renal and cardiac damage and probable mechanisms of action.

MATERIALS AND METHODS

Animals: Sixty male Wistar rats were used in this study. The rats were randomly selected and acclimatized to the feed and the environment for about three weeks. They were provided with standard diet rat feed and water *ad libitum*. They were kept in spacious cages in a well-ventilated house under natural light conditions; 12 hours light, 12 hours dark daily for the period of acclimatisation. Animals used for this experiment were handle in accordance to the guidelines, rules and regulations of handling experimental animals and ethical approval was given by the University of Ibadan Animal Care and Research Ethic Committee with the approval number UI-ACUREC/17/0118.

Experimental design: The rats were divided into six groups of ten rats each. A daily repetitive dose of L-NAME (at a dosage of 40 mg/kg), lisinopril (at a dosage of 10 mg/kg) and extract was given for 21days to each rat according to the group/experimental demand. A (Control), B (L- NAME alone), C (L-NAME + 100 mg of extract), D (L-NAME + 200 mg of extract), E (L-NAME + 400 mg of extract), F (L-NAME + Lisinopril at 10 mg/kg).

Blood pressure measurements: Blood pressure parameters, including systolic, diastolic, and mean arterial blood pressures, were determined non-invasively in conscious animals by tail plethysmography using an automated blood pressure monitor (CODA 4.1, Kent Scientific Corporation, Connecticut, USA). The average of at least nine readings, taken in the quiescent state, following acclimatization, was recorded per animal (Omobowale *et al*., 2019)

Serum preparation: Approximately three milliliters of blood were collected by retro–orbital venous puncture using plain capillary tubes into plain bottles and allowed to clot. The clotted blood was then centrifuged at 4, 000 revolutions per minute (rpm) for 10 minutes. Clear serum was separated with Pasteur pipette into another plain tube and then stored at 4^{o}C until they were analysed.

Renal and cardiac homogenate preparation: The organs excised were rinsed and homogenized using 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% KCl. The homogenates were subjected to cold centrifugation at 4^{o}C using a speed of 10, 000 for 15 minutes. The post mitochondrial fractions (PMFs) obtained from cardiac and renal homogenates were used for biochemical assays.

Biochemical analysis

Renal and Cardiac Makers of Oxidative Stress: Hydrogen peroxide generation was determined according to the method of Wolff 1994. The reaction mixture was subsequently incubated at room temperature for 30 minutes. The mixtures were read at absorbance at 560 nm and H_2O_2 generated was extrapolated from H_2O_2 standard curve. The Malondialdehyde (MDA) content as an index of lipid peroxidation was quantified in the PMFs of cardiac and renal tissue according to the method Varshney and Kale, 1990. The absorbance was measured against a blank of distilled water at 532 nm. Lipid peroxidation was calculated with a molar extinction coefficient of 1.56×10^5/M/cm. Protein carbonyl (PCO) contents in the renal and cardiac tissues were measured using the method of Reznick and Packer, 1994. The absorbance of the sample was measured at 370 nm. The carbonyl content was calculated based on the molar extinction coefficient of $4 \quad 1 \quad 1$ DNPH ($2.2\ 10$ cm M) and expressed as nmoles/mg protein while vitamin C contents were measured as earlier described by Jacques-Silva *et al.* (2001).

Renal and Cardiac Antioxidants: The Superoxide dismutase (SOD) assay was carried out by the method of Misra and Fridovich, (1972), with slight modification by Omobowale *et al.* (2014). The increase in absorbance at 480 nm was monitored every 30 s for 150 s. The one unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome. Reduced glutathione (GSH) was estimated by the method of Jollow and Mitchell, (1974). Catalase (CAT) activity was determined according to the method of Sinha, 1972. One unit of CAT activity represents the amount of enzyme required to decompose 1 µmol of H_2O_2/min. Glutathione peroxidase (GPx) activity was also measured according to Beutler *et al*. 1963. Glutathione-S-transferase (GST) was estimated by the method of Habig *et al.* (1974) using 1-chloro-2, 4-dinitrobenzene as substrate. The protein and non-protein thiol contents were determined as described by Ellman, (1954). Protein concentration was determined by the Biuret method of Gornal *et al.*, 1949 , using bovine serum albumin (BSA) as standard.

Determination of Serum Markers of Oxidative Stress.: The serum nitric oxide concentrations were measured spectrophotometrically at 548 nm according to the method of Olaleye *et al*. (2007). The serum myeloperoxidase (MPO) activity was determined according to the method of Xia and Zweier, 1997. The advanced oxidation protein product (AOPP) contents were determined as described by Kayali *et al.* (2006). Briefly, 0.4 ml of cardiac and renal PMFs were treated with 0.8 ml phosphate buffer (0.1 M; pH 7.4). The absorbance of the reaction mixture was immediately recorded at 340 nm wavelength. The content of AOPP for each sample was calculated using the extinction coefficient of 261 cm^{-1} mM^{-1} and the results were expressed as µmoles/mg protein. The activity of xanthine oxidase was determined according to method of Akaike *et al.* (1990).

Serum Markers of Renal and Liver Damage: The blood urea nitrogen (BUN), creatinine, ALT, AST and ALT were

determined using Randox kits following the manufacturer's instructions.

Histopathology: Small pieces of kidney and heart were fixed in 10% formalin, embedded in paraffin wax, and sections of 5-6 mm in thickness were made and thereafter stained with hematoxylin and eosin for histopathological examination according to the methods as previously described by Drury *et al*. (1996). Thereafter, the sections were examined with light microscopy.

Immunohistochemical staining for kidney injury molecule I (Kim-1), nuclear factor kappa beta (NF-κB), nuclear factor erythroid 2-related factor 2 (Nrf2) and **cardiac troponin I (CTnI) expressions :** Immunohistochemistry procedures were conducted as previously documented (Oyagbemi *et al*., 2017). For the assessment of kidney injury molecule I (Kim-1), nuclear factor kappa beta (NF-κB), cardiac troponin I (CTnI), and the suppression of nuclear factor erythroid 2-related factor 2 (Nrf2) expression in both kidney and heart tissues, fixed specimens were embedded in paraffin and sliced into 5 µm thick sections. The regions displaying positive immunoreactivity for Kim-1, NF-κB, Nrf2, and CTnI in anti-rabbit staining were observed, starting from a low magnification on each section and subsequently at 400× magnification using a photomicroscope (Olympus) and a digital camera (Toupcam®, Touptek Photonics, Zhejiang, China).

Statistical Analysis : Data obtained were analyzed with One-way ANOVA with Dunnett's post-test at a 95% confidence limit. All values are expressed as mean ±S.D. The test of significance between two groups was estimated by Student's t test.

RESULTS

Percentage differences in the body weight of hypertensive rats: The percentage (%) weight changes across the groups were as follows: Groups A, C, and D experienced weight gains of 1.4%, 0.57%, and 21.3%, respectively. In contrast, groups B, E, and F incurred weight losses of 9.12%, 12.1%, and 11.1%, respectively.

Organ weight: There are no significant differences in the weights of the heart, kidney, and liver across the groups (Table 2).

Blood pressure: Rats treated with L-NAME only had a significant ($P<0.05$) increase in systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial blood pressure (MAP) values when compared with the control, extract and lisinopril treated groups (Table 3).

Electrocardiograph of hypertensive rats treated with *Persea americana* bark extract: Heart rate of group C was significantly higher when compared with Groups B, E and F. The PR interval of group B rats was significantly ($p<0.05$) higher than that of group A. There was no significant difference ($P>0.05$) in the QRS complex across all the group. Group B showed a statistically significant ($P<0.05$) increase in the QT interval compared to groups A, C, D, E, and F. Similarly, the QTc value in group B rats was markedly greater than that reported in group A (Table 4).

Haematological and Serum Biochemical Parameters: Treatment with L-NAME only caused significant ($P<0.05$) decreases in packed cell volume, haemoglobin, red blood cell values when compared with the control, extract and lisinopril-treated groups. These parameters were, however, restored in extract-treated groups (Table 5).

Table 1:
Effect of *Persea americana* bark extract on body weight of Wistar rats

Groups	Initial weight (g)	Final weight (g)	(%) weight gain/loss
(A) Control	198.82 ± 4.11^{b}	201.67 ± 6.07^{a}	1.4%
(B) L-NAME	188.90 ± 4.39^{bc}	171.67 ± 5.89^{b}	-9.12%
(C) L-NAME + PABE A (100 mg/kg)	169.75 ± 3.68^{de}	170.71 ± 4.55^{bc}	0.57%
(D) L-NAME + PABE B (200 mg/kg)	167.17 ± 3.10^{e}	202.78 ± 5.66^{a}	21.3%
(E) L-NAME + PABE C (400 mg/kg)	179.54 ± 2.36^{cd}	157.78 ± 5.90^{c}	-12.1%
(F) L-NAME + Lisinopril (10 mg/kg)	212.43 ± 4.81^{a}	188.89 ± 7.44^{ab}	-11.1%

PABE- Persea americana bark extract. L-NAME- N^{w}–nitro-L-arginine methyl ester at 40 mg/kg body weight
Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A (100 mg/kg), Group D (L-NAME+ PABE B (200 mg/kg), Group E (L-NAME + PABE C (400 mg/kg). F (L-NAME + Lisinopril (10 mg/kg). The alphabet superscripts indicate significant differences across groups at P<0.05.

Table 2:
Effect of *Persia americana* bark extract on organ weight of Wistar rats

Treatment	Mean ± Standard error				
	Body weight (mg)	Heart (mg)	Kidney (mg)	Liver (mg)	
Control	201.67 ± 6.07^{a}	0.68 ± 0.10^{a}	0.80 ± 0.10a	6.07	±0.74a
L-NAME	171.67 ± 5.89^{b}	0.60 ± 0.04^{a}	0.86 ± 0.02a	5.61	± 0.23a
L-NAME + PABE A (100 mg/kg)	170.71 ± 4.55^{bc}	0.59 ± 0.11^{a}	0.74 ± 0.09^{a}	4.41	$\pm 0.63^{a}$
L-NAME + PABE B (200 mg/kg)	202.78 ± 5.66^{a}	0.57 ± 0.07^{a}	0.89 ± 0.09^{a}	5.12	$\pm 0.63^{a}$
L-NAME + PABE C (400 mg/kg)	157.78 ± 5.90^{c}	0.53 ± 0.03^{a}	0.81 ± 0.08^{a}	5.17	$\pm 0.44^{a}$
L-NAME + Lisinopril 10 mg/kg	188.89 ± 7.44^{ab}	0.61 ± 0.07^{a}	0.88 ± 0.06^{a}	5.57 ± 0.45^{a}	

PABE- *Persea americana* bark extract. L-NAME- N^{w}–nitro-L-arginine methyl ester at 40 mg/kg body weight
Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A (100 mg/kg), Group D (L-NAME+ PABE B (200 mg/kg), Group E (L-NAME + PABE C (400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The alphabet superscripts indicate significant differences across groups at P<0.05.

Table 3:
Effect of *Persea americana* bark extract on Systolic, Diastolic, Mean Arterial Blood Pressure of Wistar rats

Treatment	Mean ± Standard error		
	Systolic Blood Pressure (mmHg)	Diastolic Blood Pressure (mmHg)	Mean Arterial Pressure (MAP) (mmHg)
Control	124.67 ± 6.89	79.67 ± 9.33	94.50 ± 6.25
L-NAME	230.00 ± 3.51^{a}	181.67 ± 13.28^{a}	197.33 ± 9.84^{a}
L-NAME + PABE A (100 mg/kg)	160.33 ± 17.95^{b}	131.33 ± 14.25^{b}	140.67 ± 15.25^{b}
L-NAME + PABE B (200 mg/kg)	130.33 ± 10.91^{b}	91.33 ± 17.84^{c}	104.67 ± 15.17^{c}
L-NAME + PABE C (400 mg/kg)	149.67 ± 10.68^{b}	113.67 ± 9.49^{bc}	125.67 ± 9.62^{bc}
L-NAME + Lisinopril 10 mg/kg	126.33 ± 9.82^{b}	82.33 ± 6.64^{c}	96.67 ± 7.79^{c}

PABE- Persea americana bark extract. L-NAME- N^{w}–nitro-L-arginine methyl ester at 40 mg/kg body weight
Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE 100 mg/kg), Group D (L-NAME+ PABE 200 mg/kg), Group E (L-NAME + PABE 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The alphabet superscripts indicate significant differences across groups at P<0.05.

Table 4:
Effect of *Persea americana* bark extract on ECG parameters of Wistar rats.

Groups	HEART RATE	P (ms)	PR (ms)	QRS (ms)	QT (ms)	QTc (ms)	Ra (mV)
A	198.67±27.94	19.33±5.03	48.33±8.96	10.00±3.46	29.33±3.79	52.33±9.50	0.28±0.11
B	181.00± 19.98	24.67±3.51	51.00±3.61^{a}	15.67±2.08	88.33±3.73^{a}	141.33±15.59^{a}	0.40±0.09
C	247.67±2.08^{b}	19.00±1.00	27.33±1.53^{ab}	15.67±4.16^{b}	76.33±1.53^{a}	138.00±2.65^{a}	0.33±0.09
D	197.00±1.00	31.00±2.65	48.00±1.00^{ac}	13.00±1.00	67.67±11.06^{abc}	115.33±24.17^{a}	0.27±0.04
E	177.33±40.28^{c}	23.00±9.85	43.33±1.53^{ac}	13.33±1.52	47.67±2.52^{abcd}	89.33±2.08^{bc}	0.26±0.06
F	182.33±3.06^{c}	21.33±1.53	36.00±1.00^{abd}	8.33±1.33^{b}	53.00±2.65^{bcd}	87.67±2.31^{bc}	0.33±0.16

PABE- Persea americana bark extract. L-NAME- N^{w}–nitro-L-arginine methyl ester at 40 mg/kg body weight
Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME+ PABE B 200 mg/kg), and Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The alphabet superscripts indicate significant differences across groups at P<0.05.

Table 5:
Effect of *Persea americana* bark extract on red blood cell indices of Wistar rats

Groups	Mean ± Standard error			
	PCV (%)	Hb	RBC (10^{6}cells/µL)	Platelet (10^{3}cells/µL)
A	40.40 ± 2.60^{a}	13.78 ± 1.23^{a}	6.69 ± 0.57^{a}	139400.00 ± 13151.43^{a}
B	36.75 ± 3.12^{a}	12.13 ± 1.07^{a}	5.94 ± 0.62^{a}	132250.00 ± 9294.94^{a}
C	41.25 ± 2.53^{a}	13.60 ± 0.84^{a}	6.90 ± 0.65^{a}	127250.00 ± 16779.82^{a}
D	41.20 ± 3.07^{a}	13.74 ± 0.99^{a}	6.92 ± 0.62^{a}	188800.00 ± 41921.83^{a}
E	37.00 ± 4.25^{a}	12.76 ± 1.68^{a}	6.37 ± 0.71^{a}	131200.00 ± 9774.46^{a}
F	36.86 ± 2.04^{a}	12.17± 0.73^{a}	6.17 ± 0.36^{a}	132600.00 ± 22174.80^{a}

PABE- Persea americana bark extract. L-NAME- N^{w}–nitro-L-arginine methyl ester at 40 mg/kg body weight
Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME+ PABE B 200 mg/kg), Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The alphabet superscripts indicate significant differences across groups at P<0.05.

Table 6:
Effect of *Persea americana* bark extract on White Blood Cell (WBC) Indices of Wistar rats in murine model of hypertension

Groups	WBC (10^{3}cells/µL)	Lymphocyte (10^{3}cells/µL)	Neutrophil (10^{3}cells/µL)	Monocyte (10^{3}cells/µL)	Eosinophil (10^{3}cells/µL)
A	4800.00 ± 1028.47^{a}	66.00 ± 1.76^{a}	31.00 ± 1.92^{a}	2.00 ± 0.45^{a}	1.00 ± 0.32^{a}
B	4437.50 ± 798.80^{a}	68.00 ± 1.78^{a}	27.75 ± 2.84^{a}	2.25 ± 0.48^{a}	2.00 ± 0.71^{a}
C	4675.00 ± 1478.53^{a}	67.00 ± 1.96^{a}	29.75 ± 2.14^{a}	1.50 ± 0.29^{a}	1.75 ± 0.75^{a}
D	3960.00 ± 341.47^{a}	65.20 ± 2.40^{a}	28.80 ± 2.04^{a}	2.00 ± 0.32^{a}	2.00 ± 0.32^{a}
E	4580.00 ± 363.52^{a}	69.00 ± 1.82^{a}	27.20 ± 1.59^{a}	1.80 ± 0.37^{a}	2.00 ± 0.32^{a}
F	4342.86 ± 330.84^{a}	66.57 ± 2.44^{a}	29.00 ± 1.88^{a}	1.86 ± 0.34^{a}	1.71 ± 0.29^{a}

PABE- Persea americana back extract. L-NAME- N^{w}–nitro-L-arginine methyl ester at 40 mg/kg body weight
Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME+ PABE B 200 mg/kg), Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The alphabet superscripts indicate significant differences across groups at P<0.05

Across the treatment groups, the leucocytes parameters including; White blood cell count, Lymphocyte, Neutrophils, Monocyte and Eosinophil had no statistically significant changes (Table 6).

Furthermore, a significant ($p<0.05$) increases in hepatocellular leakage enzymes (AST and ALP) and also, markers of renal damage such as BUN, creatinine were recorded in the group treated with L-NAME alone when compared with the controls (Table 7).

Oxidants and antioxidant status of hypertensive rats treated with *Persea americana* bark extract: Non-enzymatic antioxidant indicators for the heart and kidneys, non-protein thiol, protein thiol, and reduced glutathione were all significantly decreased by L-NAME, whereas these markers were significantly increased in the extract and lisinopril-treated groups (Table 8).

Superoxide dismutase, glutathione peroxidase, and glutathione-s-transferase levels in the heart and kidneys were significantly decreased in the hypertensive group, whereas they were significantly improved in the extract and lisinopril-treated groups (Table 9).

Markers of oxidative stress such as malondialdehyde, hydrogen peroxide, and protein carbonyl levels were significantly elevated in both the heart and the kidneys after L-NAME administration. Groups given extract and lisinopril demonstrated noticeably lower levels (Table 10).

When compared to the control, extract, and lisinopril-treated groups, L-NAME significantly reduced serum nitric oxide while significantly raising serum myeloperoxidase and advanced oxidative protein products (Table 11).

Immunohistochemistry: When compared to the control, extract, and lisinopril treatment groups, the L-NAME alone group displayed the highest levels of CTnI and NF-kB in heart tissue (Figures 1 and 2), as well as the highest expression of NRF-2 in the heart and kidney tissue (Figures 3). In the kidney and cardiac tissues, L-NAME alone also reveals the highest expression of KIM-1 and NF- kB (Figure 4).

Table 7:
Effect of *Persea americana* bark extract on serum biochemical parameters of Wistar rats in murine model of hypertension.

Groups	ALP (U/L)	ALT (U/L)	AST (U/L)	BUN mg/dL	CREATININE (mg/dL)	HDL (mmol/L)
A	93.5±9.15	27.50±1.00	38.00±2.26	15.48±0.88	0.65±0.06	20.58±2.41
B	112.67±11.75^{a}	30.00±1.00^{a}	48.00±1.00^{a}	16.73±0.61^{a}	0.73±0.06	25.40±0.92^{a}
C	95.00±6.56^{b}	31.00±1.00^{a}	47.33±1.53^{a}	18.13±0.38^{ab}	0.83±0.15^{a}	24.73±1.07^{a}
D	110.25±5.32^{ac}	32.00±1.41^{ab}	46.25±2.36^{a}	17.17±0.77	1.00±0.16^{ab}	23.13±1.91
E	117.75±4.03^{ac}	32.75±1.26^{ab}	46.75±1.71^{a}	16.98±0.39^{a}	0.90±0.18^{a}	22.33±1.66^{b}
F	95.67±9.45^{de}	29.00±1.00	40.67±1.15^{b}	17.23±0.93^{a}	0.70±0.10	21.93±1.33^{b}

PABE- Persea americana back extract. L-NAME- N^{w}–nitro-L-arginine methyl ester at 40 mg/kg body weight
Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME+ PABE B 200 mg/kg), Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The alphabet superscripts indicate significant differences across groups at P<0.05.

Table 8:
Effect of *Persea americana* bark extract on cardiac and renal non-enzymatic antioxidant system of Wistar rats in murine model of hypertension.

Groups (mg/kg)	PT heart	PT kidney	NPT heart	NPT kidney	GSH heart	GSH kidney
A	35.81±7.0	53.6±17.27	20.94± 2.06	20.69 ± 2.49	64.13±24.66	93.12±17.82
B	30.11±5.0^{a}	35.52±2.93^{a}	13.32±5.89^{a}	13.46±2.714^{a}	51.82±13.2^{a}	59.35±20.20^{a}
C	34.59±10.62	46.69±6.98	15.32±4.91	18.50±8.37	54.21±13.2	94.64±14.81^{b}
D	35.58±13.83	41.90±9.06^{c}	15.45±4.80	16.49±4.54	73.72±11.53^{bc}	94.29±19.99^{b}
E	39.4±11.19^{b}	45.34±16.72	16.65±3.74	16.03±4.34	57.88±13.35	76.82±22.71
F	36.99±9.57^{b}	41.88±8.88^{ab}	16.07±3.16	16.74±6.14	74.21±15.09^{bc}	87.14±13.06^{b}

PABE- Persea americana back extract. L-NAME- N^{w}–nitro-L-arginine methyl ester at 40 mg/kg body weight
Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME+ PABE B 200 mg/kg), Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The alphabet superscripts indicate significant differences across groups at P<0.05.GSH (reduced glutathione; µmol /mg protein), non-protein thiol (µmol /mg protein), protein thiol (µmol /mg protein).

Table 9:
Effect of *Persea americana* bark extract on cardiac and renal enzymatic antioxidant system of rats in murine model of hypertension

GROUPS	SOD heart	SOD kidney	GPX heart	GPX kidney	GST heart	GST kidney
A	16.92±4.20	21.57±4.5	93.53±15.8	116.4±35.03	0.11±0.05	0.09±0.05
B	12.7±3.27^{a*}	13.09±4.47	72.13±10.3^{a}	93.95±22.97^{a}	0.03±0.01^{a}	0.03±0.02^{a}
C	16.03±5.44	20.06±8.40	96.69±21.57^{b}	112±52.97	0.09±0.01^{b}	0.08±0.06^{b}
D	15.12±6.01	16.19±0.85	95.77±25.40^{b}	101.5±5.51	0.10±0.02^{b}	0.08±0.04^{b}
E	17.96±3.72^{b}	16.17±5.92	109.8±30.84^{b}	111.5±27.46^{b}	0.28±0.^{09a,b,c,d}	0.06±0.04
F	16.47±3.60^{b}	18.26±7.24	89.49±17.71^{b}	104.9±7.625	0.12±0.02^{be}	0.09±0.03^{b}

PABE- Persea americana back extract. L-NAME- N^{w}–nitro-L-arginine methyl ester at 40 mg/kg body weight
Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME+ PABE B 200 mg/kg), Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The alphabet superscripts indicate significant differences across groups at P<0.05 .SOD (superoxide dismutase; units/mg protein), GST (glutathione-S-transferase; mmole1-chloro-2,4-dinitrobenzene-GSH complex formed/min/mg protein), GPx (glutathione peroxidase; units/mg protein)

Table 10:
Effect of *Persea americana* bark extract on cardiac and renal markers of oxidative stress of rats in murine model of hypertension

Groups	MDA heart	MDA kidney	H_2O_2 heart	H_2O_2 kidney	PC heart	PC kidney
A	0.57±0.22	0.49±0.22	82.39±19.36	121.2±28.9[a]	960±187.5	918.6±478.7
B	0.87±0.25[a]	1.20±0.12	110.1±13.9[a]	153.30± 17.66[a]	1252±475.4[a]	1709±559.3[a]
C	0.54±0.27[b]	0.47±0.19	100.5.6±10. `91[a]	109.3±13.68[b]	1027±418.1	1363±356.7
D	0.53±0.14[b]	0.60±0.19	97.30±15.22	117.5±10.51[b]	901.5±356.50	832.8±137.70[ab]
E	0.6±0.11[b]	0.34±0.12	99.08±10.99	114.2±13.79[b]	1029±122[b]	1043±381.40[b]
F	0.42±0.103[b]	0.50±0.07	91.04±15.73[b]	108.4 ±9.77[b]	940.1±185.6[b]	821.9±308.9[bc]

PABE- *Persea americana* back extract. L-NAME- N^w–nitro-L-arginine methyl ester at 40 mg/kg body weight
Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME+ PABE B 200 mg/kg), Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The alphabet superscripts indicate significant differences across groups at P<0.05.MDA (malondialdehyde; µmol /mg protein), H_2O_2 (hydrogen peroxide; µmol /mg protein), PC (protein carbonyl: µmol /mg protein).

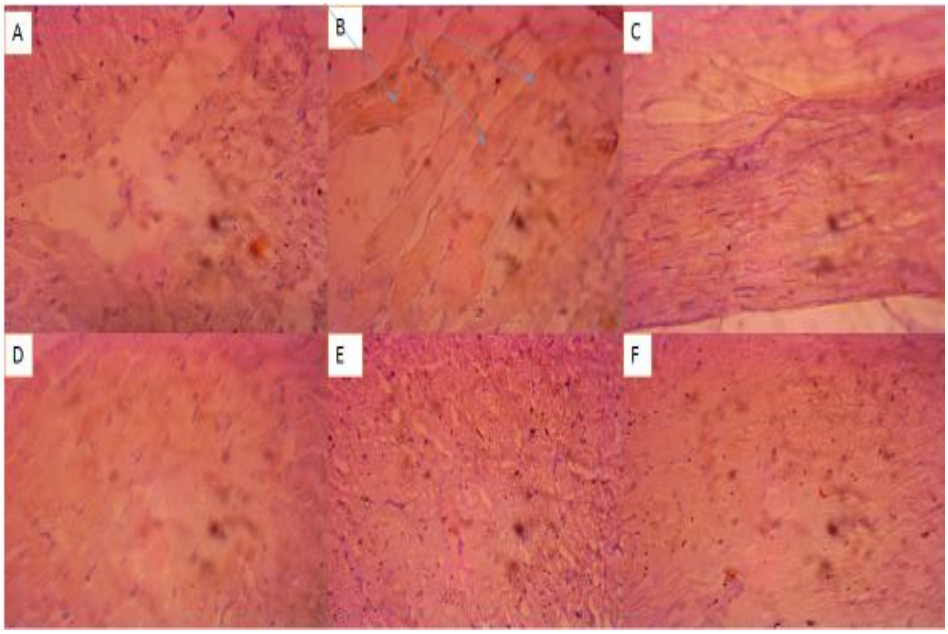

Plate 1
Immunohistochemistry of NF-kB in heart tissue in rats. PABE- Persea americana back extract. L-NAME- Nw–nitro-L-arginine methyl ester at 40 mg/kg body weight. Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME+ PABE B 200 mg/kg), Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The slides were counterstained with high-definition hematoxylin and viewed with ×100 objectives.

Plate 1
Immunohistochemistry of NF-kB in heart tissue in rats. PABE- *Persea americana* back extract. L-NAME- Nw–nitro-L-arginine methyl ester at 40 mg/kg body weight. Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME + PABE B 200 mg/kg), Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The slides were counterstained with high-definition hematoxylin and viewed with ×100 objectives.

Table 11:
Effect of *Persea americana* bark extract on serum markers of inflammation and oxidative stress of Wistar rats in a murine model of hypertension

Treatment groups	NO	MPO	AOPP
A	0.44±0.51	17.73±4.05	44.37±14.57
B	0.32±0.08	26.54±4.19[a]	69.88±29.60[a]
C	0.46±0.093[b]	25.7±2.90[a]	53.43±15.24
D	0.39±0.04[b]	24.29±7.67[a]	47.97±14.28[b]
E	0.42±0.15	19.73±3.99[b]	65.36±18.54[a]
F	0.46±0.15[b]	22.53±6.31[a]	49.3±8.17[b]

PABE- *Persea americana* back extract. L-NAME- N^w–nitro-L-arginine methyl ester at 40 mg/kg body weight
Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME+ PABE B 200 mg/kg), Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The alphabet superscripts indicate significant differences across groups at P<0.05. NO (nitric oxide; units/mg protein), MPO (myeloperoxidase; µmole/L), AOPP (advanced oxidative protein product; units/mg protein).

DISCUSSION

Hypertension is a major risk factor for renal and cardiac disease and a decrease in blood pressure significantly lowers the risk of attending cardiovascular events (Hermann *et al*., 2006). *Persea americana* significantly lowered SBP, DBP and MAP values and corrected the resultant cardiac arrhythmia in this study to values comparable to lisinopril, an ACE inhibitor.

Several studies have elucidated the importance of nitric oxide in the pathogenesis of hypertension as it plays a major role in the regulation of blood pressure. The impairment of nitric oxide bioavailability is an important part of hypertension (Hermann *et al*., 2006). Some antihypertensives act by exploiting the effect of nitric oxide on vascular smooth muscle, they elicit the release of nitric oxide and thus facilitate vasodilation (Adefegha and Oboh, 2016). In this study, we reported significant decrease in nitric oxide bioavailability in the L-NAME group whereas the groups treated with *Persea americana* and lisinopril exhibited significant increases in nitric oxide bioavailability. Thus, suggesting that *Persea americana* acts by facilitating nitric oxide bioavailability.

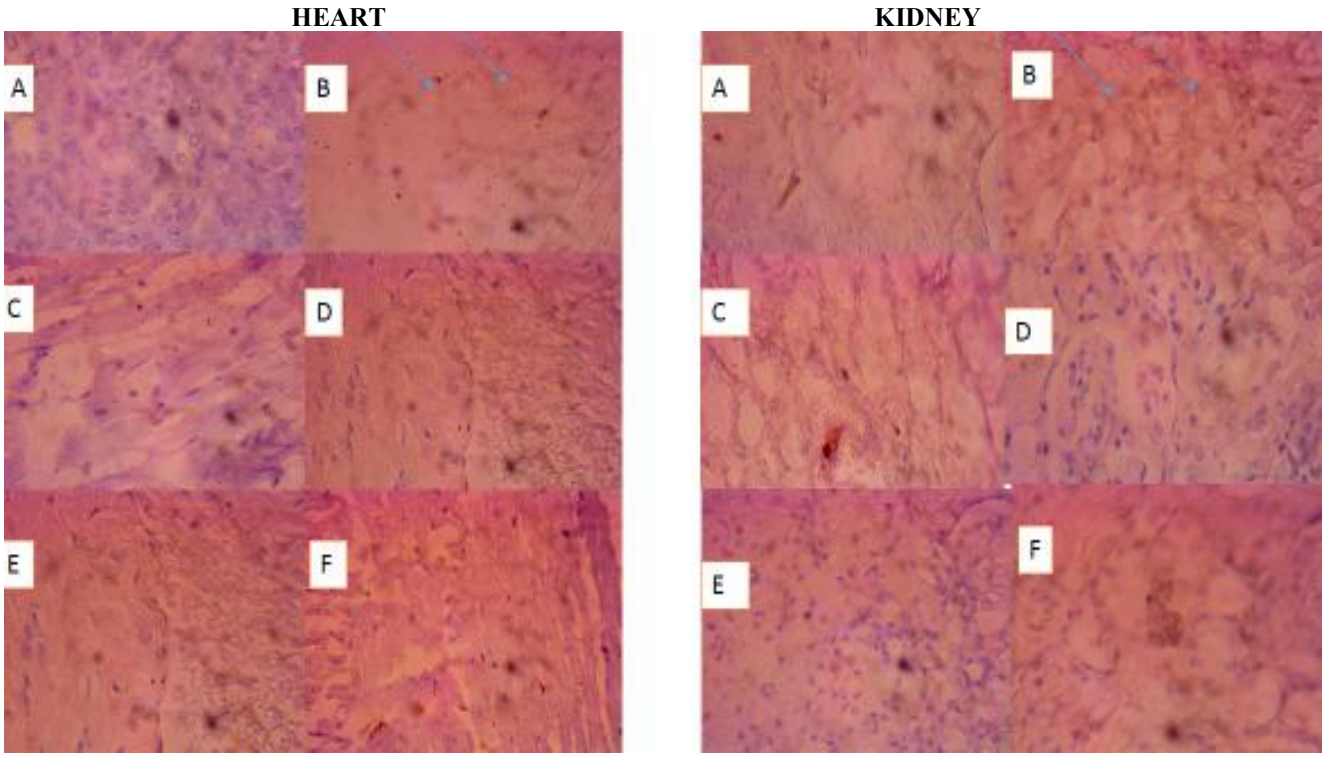

Plate 2:
Immunohistochemistry of Nrf-2 in heart and kidney tissues of rats.
PABE- *Persea americana* bark extract. L-NAME- N^{w}–nitro-L-arginine methyl ester at 40 mg/kg body weight. Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME+ PABE B 200 mg/kg), Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The slides were counterstained with high definition hematoxylin and viewed ×100 objectives.

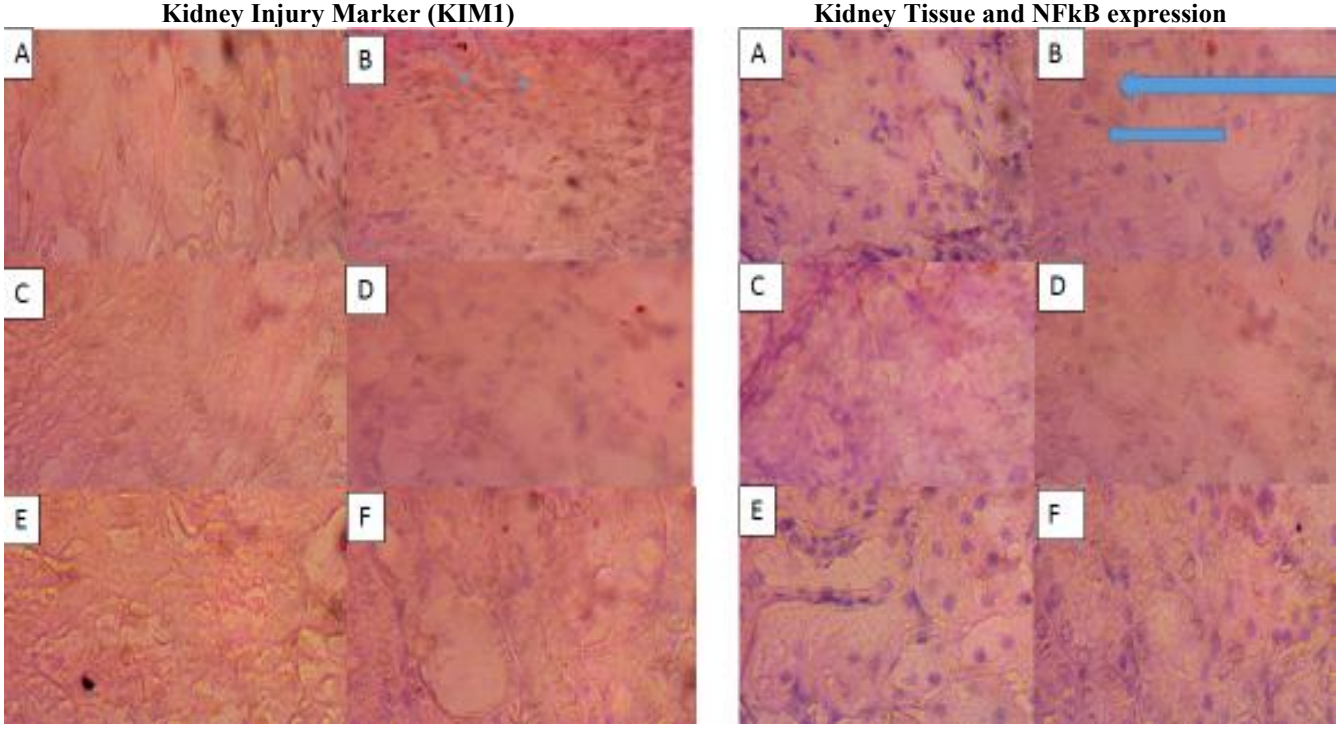

Plate 3:
Immunohistochemistry of Kim-1 and NFkB in kidney tissue of rats.
PABE- *Persea americana* back extract. L-NAME- N^{w}–nitro-L-arginine methyl ester at 40 mg/kg body weight. Values presented as mean ± SEM. Group A (Control), Group B (L-NAME), Group C (L-NAME + PABE A 100 mg/kg), Group D (L-NAME+ PABE B 200 mg/kg), Group E (L-NAME + PABE C 400 mg/kg). F (L-NAME + Lisinopril 10 mg/kg). The slides were counterstained with high-definition hematoxylin and viewed ×100 objectives.

Hepatocellular leakage enzymes such as AST and ALP were significantly increased in the L-NAME alone group, these enzymes are mostly associated with the liver parenchyma, and elevate serum levels are usually seen in acute hepatic injury (Kannan *et al*., 2013). The increase seen in the L-NAME alone group suggests hepatic damage as a complication of hypertension.

BUN and creatinine are traditional markers of renal damage, with creatinine showing more specificity than BUN (Onuigbo *et al*., 2017). These markers were significantly increased in the L-NAME alone group confirming that kidney damage is a complication of persistent elevations in arterial blood pressure. *Persea americana* however, caused a reduction in the levels of both BUN and creatinine to levels comparable with Lisinopril, a known antihypertensive.

Oxidative stress, which is defined as an imbalance in the levels of oxidants and antioxidants favoring oxidants, has been implicated in the advent of hypertension, where it causes damage to macromolecules such as DNA, protein and lipids and it is also exacerbated by insufficient antioxidants in the body (Wu and David, 2015). Renal and cardiac dysfunction in hypertension is associated with oxidative damage.

In the kidney, oxidative stress promotes vasoconstriction, increases sodium retention and increases vascular resistance, therefore worsening hypertension (Duni *et al*., 2018). Cardiac diseases are also associated with oxidative stress (Jin *et al*., 2017)

In this present study, we reported significant elevations in various markers of oxidative stress, such as advanced oxidative protein products, malondialdehyde, protein carbonyl, hydrogen peroxide in the renal and cardiac homogenate of the L-NAME alone group. Treatment with *Persea americana* however brought their values to levels comparable with the control and lisinopril treated groups.

Several antioxidants such as superoxide dismutase, catalase, reduced glutathione, glutathione peroxidase acts as the first line of defense against oxidative stress-induced damage (Nguyen *et al*., 2007). It is thus widely accepted that consuming food with high concentrations of these antioxidants can be useful in the prevention and treatment of various cardiovascular and renal diseases (Smith *et al*. 2016). SOD acts by removing superoxide radical thus preventing the deleterious effects of superoxide radical (Kabel, 2014), the glutathione system including reduced glutathione, glutathione peroxidase, glutathione transferase functions in detoxification metabolism. Reduced glutathione functions in maintaining the redox system of the cell (Kabel, 2014). In this study, L-NAME significantly elicited a depletion in levels of these enzymatic and non-enzymatic antioxidants in the renal and cardiac homogenates, however, treatment with *Persea americana* and lisinopril brought on significant increases in their levels, confirming the antioxidant effect of *Persea americana* .

Inflammation goes hand in hand with oxidative stress, and both act in an interrelated way in hypertension (Duni *et al*., 2018). In this study, myeloperoxidase, a marker of inflammation was significantly elevated in the L-NAME alone group confirming the role of inflammation in the pathogenesis of hypertension, *Persea americana* however significantly reduced its levels in the treated groups.

Nuclear erythroid-2 like factor-2 (Nrf2) is a transcription factor that controls the expression of antioxidants in the body by activating the Antioxidant Response Elements (ARE), this activity of Nrf2 is only turned on when the body is assailed with oxidants (Smith *et al*. 2016). Therefore, enhancing the activity of Nrf2 will help to downregulate the effects of reactive oxygen species (Rajappa *et al*., 2017). In this study, we reported decreased expression of Nrf2 in the heart and kidney tissue of the L-NAME alone group, this finding is supported by the decreased levels of both enzymatic and non-enzymatic antioxidants in the renal and cardiac homogenate of the L-NAME alone group. *Persea americana* and Lisinopril however brought on an increased in Nrf2 expression in the treated groups, this is also supported by the increased levels of antioxidants in the renal and cardiac homogenate of the treated groups.

NF-kB modulates cell growth, cell survival, development processes, immune and inflammatory responses as well as apoptosis and its activation has been linked to a number of cancers as it has been shown to trigger the activation of reactive oxygen species (Kusano and Bucalen, 2011). Inflammatory signals from the tissue also serves as an activator of NF-kB and it has been implicated in the pathogenesis of several diseases including hypertension, cardiomyopathy and diabetes (Kusano and Bucalen, 2011). Its downregulation in the kidney and heart tissue of the extract treated groups thus suggests that *Persea americana* possesses anti-inflammatory and anti-apoptotic effects.

Kidney Injury Molecule 1 (Kim1) is a specific biomarker for tubular injury, it is usually undetectable in normal kidneys but markedly increased in kidney injury thus making it a sensitive and specific biomarker for renal damage (Huo *et al*., 2010). In this study, KIM-1 was highly expressed in the L-NAME alone group whereas the extract and lisinopril treated groups showed decreased expression, this thus confirms the ability of *Persea americana* to protect against hypertension induced renal damage.

Cardiac troponins are sensitive and specific markers of myocardial injury although they do not give any information regarding the mechanism of injury. They have redefined how acute myocardial infarction is diagnosed in humans (Weito and May, 2008). Troponins are regulatory proteins that are part of the contractile apparatus of skeletal and cardiac muscle tissue. They are not present in smooth muscle tissue; Elevated cardiac troponin levels therefore indicate myocardial damage (Wells and Meg, 2008). We reported increased expression of CTnI levels in the heart tissue of the L-NAME alone group whereas *Persea americana* and Lisinopril treated groups showed lesser expressions.

Our findings support the assertion that oxidative stress is involved in the pathogenesis of hypertension and the target organ complications. It also justifies the use of *Persea americana* bark extract in folkloric medicine. A desirable effect of an antihypertensive is that in addition to reducing blood pressure, it should also ameliorate hypertensive cardiac and kidney damage, this makes *Persea americana* an excellent antihypertensive drug candidate.

The findings of this study suggest an important role for *Persea americana* bark extract in the management of hypertension and its complications.

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Review Article

Educational and Family Factors in Child Autism: A Systematic Literature Review

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Summary: The early detection of autism spectrum disorders is considered the best strategy for managing, controlling, and intervening with the patients before they reach adulthood. There is a consensus among medical professionals and research communities about the mismatching criteria, confusing symptoms, and inexhaustive nature of symptoms. Also, there are no established diagnostic factors to date due to many more non-deterministic factors like gender, race, age and region. In this study, a systematic literature review (SLR) was done on educational and family factors affecting child autism spectrum disorder at early stages. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were adopted for selecting the forty-eight (48) items for the study. The results of this study revealed the educational and family factors for children with autism. This, in turn, will assist medical practitioners during the screening process, as well as help schools and specialised centres for autism plan effectively to meet the needs of integrated educational services for children with autism. This study also included a set of educational tools related to managing children with autism. This reveals some requirements in the field of assistive software, especially in the commencement of AI assistive tools for helping better and effective education in ASD kids. In addition to future research directions and areas of additional studies related to autism.

Keywords*: Autism, Diagnosis, Children. Educational Factors, Family Factors, , Control, Management.*

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INTRODUCTION

Autism spectrum disorder (ASD) is a combined phrase for a family of complex developmental disabilities, inclusive of "Autistic Disorder, Pervasive Developmental Disorder not Otherwise Specified (PDD-NOS), and Asperger's Disorder". "ASD is characterized not only by persistent impairments in reciprocal social communication and social interactions, but is also manifested by restricted, repetitive patterns of behaviour, interests, or activities" (Joon, Kumar and Parle, 2021; Edwards *et al*., 2024). At the inception of the Century, the autism spectrum disorder ailment continues to spread unabated among children around the world. Consequently, there are more autism spectrum patients under 4 years of age seeking help with Pediatric health providers (James and Smith, 2020).

Autism is considered a serious health challenge in children. It is caused by heterogeneous etiologies, disrupted growths, poor signal flow among system parts, and distinct phenotypes in genetic makeups (complex interactions with genes and external factors). The condition negatively affects children's linguistic, neurological, adaptive, biological, social, and cognitive functions. Its symptoms and needs are complex and vary throughout their lives. There is understanding of hereditary predisposition, where a particular autism spectrum disorder gene may be absent without a detectable medical marker when it is present (Hus and Segal, 2021).

Neurodevelopmental disorder sufferers show intellectual disability, speech impairment, motor deficits, sleep disturbances, gait alterations, and impairments in behaviour and psychological functioning. The symptoms of attention deficit hyperactivity disorder (ADHD), poor social skills, and communication difficulties are common in ASD, alongside hyperactivity and inattention. However, there are indications that seizures could decrease after 10 years. Still, the cognitive, gait, behavioural, and motor comorbidities tend to increase and persist throughout adulthood, requiring lifelong care (Strzelczyk *et al*., 2023).

The adult population is increasingly being diagnosed with autism spectrum in the past decades, owing to factors like awareness, improvement in diagnostic procedures, and better clinicians. The key factors impacting the speedy and timely diagnosis of autism spectrum disorder include: co-occurring disability, autism subtype, and demography (that is, ethnic background and gender), developmental regression, and mental health. There are several inconsistencies in the literature about what factors predict the existence of autism spectrum disorders in children or younger adults. In particular, there are no interrelationships between high socioeconomic status (income, maternal

education, and urban residency), gender, ethnicity or nationality, and family history with screening of the autism spectrum disorder in people (Huang *et al*., 2021; Hus and Segal, 2021).

Several determinants or factors have been recognized to impact the timing of autism spectrum disorder diagnosis. Studies have mentioned socioeconomic (ethnicity/race) and birth cohort variables in many cases. Children in order birth cohorts have better diagnoses much later against those in much early cohorts, which implies autism spectrum disorder age is diminishing as time passed. The children of non-colour at age 1 to 2 have lower chances of being positively diagnosed of autism spectrum than children of colour (Wiggins *et al*., 2020). Several parents have explained their children's behavioural concern or medical issue to likely linked to autism spectrum disorder diagnosis, and not from the standpoint of developmental defects (Wiggins *et al*., 2020).

Majority of research in autism in recent years were conducted in western countries having better income and living standard. Over 80% world's population are low-income countries, which informed the sparingly slow focus and extensive study in autism related subjects. There are quite wide gap and inequality on the knowledge and understanding of symptom and manifestation of the autism spectrum disorder, and the diagnosis and screening instruments. Moreso, there are seemingly a neglect of the population severally impacted by autism especially in the low-and-middle income nations, which implies shortages in access to diagnosis and evidence-motivated education and support (de Leeuw, Happé and Hoekstra, 2020).

The main problem in dealing with autism spectrum disorder in children is largely related to the relatively complex process of diagnosis. This requires that children be observed over a long period of time their behaviour and history of growth to determine whether the conditions are discovered. The similarity in the symptoms can be confusing as to the existence of autism spectrum disorder in children, including: maturational changes, co-occurring conditions, and many developing criteria. This has led to misdiagnosis at early stages of autism spectrum disorder in certain children, but much later than in others, regardless of possessing a comparable behavioural profile (Huang *et al*., 2020).

On the part of clinicians, the standard methods [contained in the Diagnostic and Statistical Manual of Mental Disorders: Fifth Edition (DSM-5)] for screening autism spectrum cases are related to following: intellectual functioning, behavioural characteristics, co-occurring conditions, history of ailment in genealogy, and probability of suffering (de Leeuw, Happé and Hoekstra, 2020; Wiggins *et al*., 2020). There are no effective diagnostic and biomarkers approaches are available for detecting autism in children at the earliest possible time (Hodges, Fealko and Soares, 2020). This renewed the calls for the most suitable autism diagnostic and screening tools matching the diverse cross-cultural and contextual inclinations (de Leeuw, Happé and Hoekstra, 2020). Studies have highlighted that the occurrence of comorbidities raises phenotype heterogeneity, though may alter or mask autism spectrum disorder symptoms (Elsabbagh, 2020). Therefore, children are highly probable to be misdiagnosed, delaying diagnosis. Also, children having medical or developmental or comorbidities like anxiety, hearing impairment, and epilepsy could risk losing altogether their preliminary autism spectrum disorder diagnosis (Hus and Segal, 2021).

Joon, Kumar and Parle (2021) described autism or autism spectrum disorder as multi-factorial, heterogeneous, developmental disability that gives rise to abnormal pattern of growth at infancy and toddler periods. Considering the developmental disability, it refers to the chronic and severe condition of a person due to physical and mental impairments at the stage of growing up and beyond causing huge restriction in occupational, social, and everyday life activities. Autism is chief among developmental disorders in addition to others like learning difficulties, cerebral palsy, attention-deficit hyperactivity disorder, hearing/vision impairment, Asperger's disorder, Rett syndrome, and motor disorders. While the mental disorders cover the conditions limiting the process of thinking, behaviour, mood and feelings, which could be momentary or permanent. Specific cases of mental disorders have been identified as anxiety, personal disorder, depression, bipolar disorder, schizophrenia, dementia, and the lists are inexhaustible.
The standard procedure of diagnosing psychological and physical conditions of autism spectrum disorder relied largely on the documents that compared symptoms and diagnostic criteria for detecting availability or otherwise of the ailment. Two major standards autism diagnosis are available presently: (a) Diagnostic and Statistical Manual of Mental Disorders authored by the American Psychiatric Association as most common among clinicians in the Americas; (b) International Classification of Diseases distributed by the World Health Organization (WHO) and mostly accepted in the practice of clinicians in the European countries.

This paper conducted a systematic literature review on the educational factors and family factors affecting autism in children. The key contributions of the paper include:

- To understand the trends in children autism research.
- To identify the educational and family factors for the autistic child literacy.
- To identify the educational tools for the autistic children management.
- To present future research directions.

MATERIALS AND METHODS

The systematic study is to extract the factors that affect autism in children from the literature. The process of conducting the systematic study utilizes Kitchenham and Charters protocol (Kitchenham & Charters, 2007), the preferred reporting items for systematic review and meta-analysis, for developing the search mechanism as shown in Figure 1.

Research Questions: The mapping studies are required as input data realized from the following research questions including:
RQ1.What are trends in children autism research?
RQ2.What are the educational and family factors for the autistic children?
RQ3.What are educational tools for the autistic children management?
RQ4.What are the future research directions?

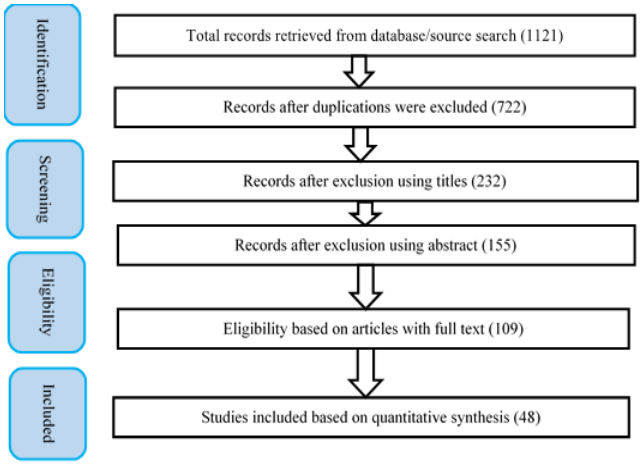

Figure 1.
The SLR process using the PRISMA standard protocol

Search Mechanism: The following sets of strings and keywords were formulated matching the research questions to facilitate academic records retrieval including: "Autism, Child autism, Autism in children, Diagnosis of autism, Factors for the early detection of autism spectrum disorder, interventions of the child autism, educational tools for managing autism spectrum disorder problems in children, future research directions of child autism educational interventions".

Data Extraction: The data was initially extracted from Google Scholar records (www.scholar.google.com), that is, the selected reputable and peer-reviewed studies on 27th June 2024 to 27th November 2024 for the period of 2018-2024 using these customised exclusion and inclusion criteria shown in Table 1.

From Table 1, the articles were screened using the title followed by abstract and conclusion. The selected articles as not conforming to the screening criteria were excluded. The selected articles as conforming to the screening criteria were accepted and included in this study.

Table 1.
The study inclusion and exclusion criteria

S/N	Study inclusion	Study exclusion
1.	Article focuses on educational and family factors and educational tools for the autistic children.	Article does not focus on educational and family factors and educational tools for the autistic children.
2.	Article mode of communication is English.	Article mode of communication is not English.
3.	Article is published in reputable research society, and peer-reviewed.	Article is not published in reputable research society, and not peer-reviewed.
4.	Article is gotten from conferences or journals with high reputation.	Article is not gotten from conferences or journals without high reputation.
5.	Article discusses autism spectrum disorder current trends, educational factors, family factors for children.	Article does not discuss autism spectrum disorder current trends, educational factors, family factors for children.

Research System Dynamic Diagram: The taxonomy of the research in area of autistic children educational development is represented in Figure 2.

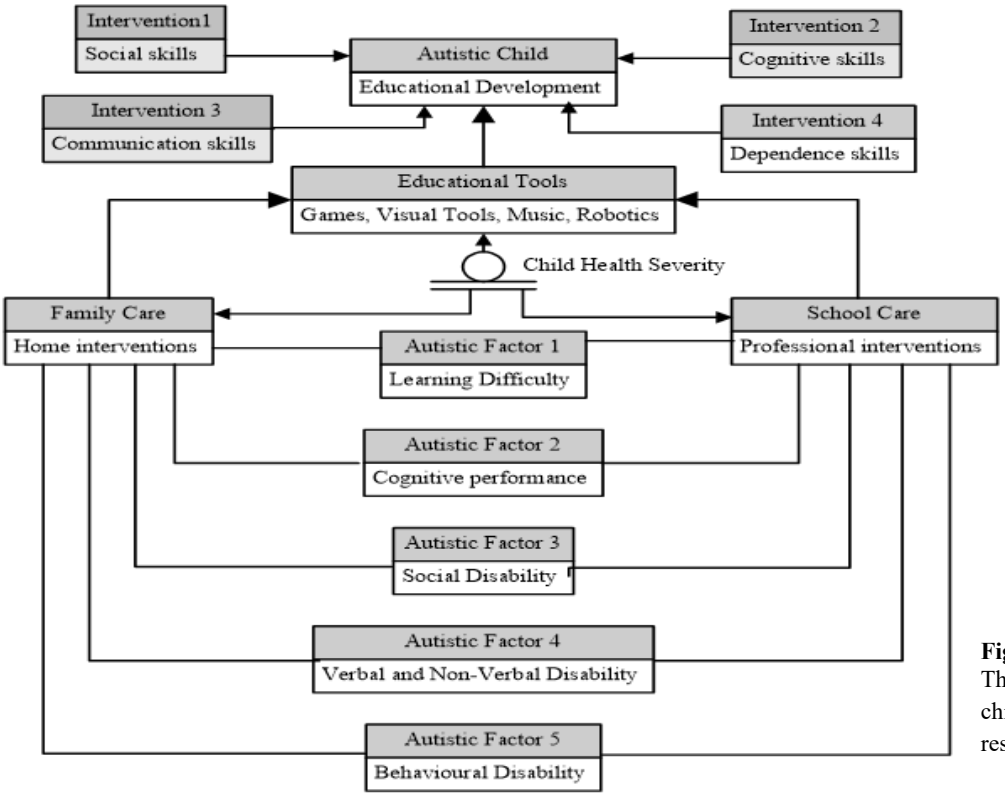

Figure 2.
The taxonomy of the autistic children educational development research

From Figure 2, the literature reviewed from the included studies, child autism research is still an evolving field of endeavour, especially directed at improving educational development and appreciation. Several interventions are being planned for autistic children in areas of social skills, communication skills, cognitive skills and dependence skills. The concept of educational tools such as computer games, visual tools, music, robotics, and many other technologies to speed skills transfer and acquisition on the part of facilitators (family members and professionals) and the children suffering from autism spectrum disorders. More so, the child health severity is the main consideration at the root of all planning and implementation of educational activities to stimulate cognitive performance, learning effectiveness, social development, verbal and non-verbal abilities, and behavioral stability. Most importantly, the included studies identified two environments for the effective educational development of child autism, which are the family homecare and school care.

Synthesis and Analysis of the Studies

Current Advances in Child Autism Research: The reviewers' analyses of each included study, focusing on the author(s), region of study, year of publication, publisher, and scope of the study, are shown in Table 2. These explain the recent and current advances in child autism research globally, stated as RQ1

Table 2.
The initial data extraction form

S/N	Author(S)	Authors' country	Year	Publisher	Scope
1.	(Zanuttini, 2023)	Australia	2023	Elsevier	Child autism
2.	(Purnama *et al.*, 2021)	Indonesia	2021	Elsevier	Child autism
3.	(Xinogalos and Tsikinas, 2019)	Greece	2019	Springer	Child autism
4.	(Mozolic-Staunton *et al.*, 2020)	Australia	2020	Elsevier	Child autism
5.	(Maddox *et al.*, 2020)	USA	2020	Routledge Taylor & Francis Group.	Child autism
6.	(Saleh, Hanapiah and Hashim, 2021)	Malaysia	2021	Taylor & Francis Group.	Autism
7.	(Syriopoulou-Delli and Gkiolnta, 2022)	Greece	2022	Taylor & Francis Group	Child autism.
8.	(Zilli, Parsons and Kovshoff, 2020)	UK	2020	The British Psychological Society	Child autism
9.	(Ke, Moon and Sokolikj, 2022)	USA	2022	SAGE	Child autism
10.	(Syrdal *et al.*, 2020)	UK	2020	DE GRUYTER	Child autism
11.	(Taheri *et al.*, 2021)	UK	2021	DE GRUYTER	Child autism
12.	(Hyman, Levy and Myers, 2020)	USA	2020	The American Academy of Pediatrics	Child autism
13.	(Chaidi *et al.*, 2021)	Greece	2021	Unknown	Child autism
14.	(Hussain, Mkpojiogu and Okoroafor, 2021)	Malaysia	2021	Unknown	Child autism
15.	(Fachantidis, Syriopoulou-Delli and Zygopoulou, 2020)	Greece	2020	Talyor & Francis Group	Child autism
16.	(Bamicha and Drigas, 2022)	Greece	2022	Unknown.	Child autism
17.	(Aloizou *et al.*, 2021)	Greece	2021	Routledge Taylor & Francis Group	Child autism
18.	(Galitskaya and Drigas, 2020)	Greece	2020	Unknown	Child autism
19.	(Chaidi and Drigas, 2020)	Greece	2020	Unknown	Child autism
20.	(Kirby *et al.*, 2022)	USA	2022	WILEY	Child autism
21.	(Baldassarri *et al.*, 2021)	Spain	2021	Springer	Child autism
22.	(Zhang *et al.*, 2022)	China	2022	MDPI	Child autism
23.	(Taresh *et al.*, 2020)	Malaysia.	2020	MDPI	Child autism
24.	(Carmona-Serrano *et al.*, 2020)	Spain	2020	MDPI	Child autism
25.	(Daulay, 2021)	Indonesia.	2021	Elsevier	Child autism
26.	(Bravou, Oikonomidou and Drigas, 2022)	Greece	2022	Unknown	Child autism
27.	(Singh *et al.*, 2023)	USA	2023	MDPI	Child autism
28.	(Elshahawy, Aboelnaga and Sharaf, 2020)	Egypt	2020	IEEE	Child autism
29.	(Davis, Fletcher-Watson and Digard, 2021)	UK	2021	Frontiers	Child autism
30.	(Blasco-Magraner *et al.*, 2021)	Spain	2021	MDPI	Child autism
31.	(Jackson and Hanline, 2020)	USA	2020	SAGE	Child autism
32.	(Sanromà-Giménez *et al.*, 2021)	Spain	2021	Unknown	Child autism
33.	(Harris *et al.*, 2021)	USA	2021	Unknown	Child autism
34.	(Pillay, Duncan and de Vries, 2021)	South Africa	2021	SAGE	Child autism
35.	(Gallardo-Montes, Caurcel Cara and Rodríguez Fuentes, 2022)	Spain	2022	Springer	Child autism
36.	(Bolourian *et al.*, 2021)	USA	2021	Springer	Child autism
37.	(Barbaro and Yaari, 2020)	Australia	2020	BMC	Child autism
38.	(O'Keeffe and McNally, 2023)	Ireland	2023	Springer	Child autism

39.	(Khalil *et al.*, 2020)	Egypt	2020	Scholars Middle East Publishers	Child autism
40.	(Sweidan *et al.*, 2022)	Jordan	2022	Routledge Taylor & Francis Group	Child autism
41.	(Chinchay *et al.*, 2024)	Spain	2024	Taylor & Francis Group	Child autism
42.	(Fernández Cerero, Montenegro Rueda and López Meneses, 2024)	Spain	2024	MDPI	Child autism
43.	(Đorđević *et al.*, 2022)	Bosnia and Herzegovina	2022	The British Society of Developmental Disabilities	Child autism
44.	(McDevitt, 2021)	USA	2021	Elsevier	Child autism
45.	(Nisa, Zain and Rahmah, 2024)	Indonesia	2024	Faculty of Education and Teacher Training State Institute for Islamic Studies Batusangkar.	Child autism
46.	(Hermanto and Pamungkas, 2023)	Indonesia	2023	Unknown	Child autism
47.	(Yahya *et al.*, 2023)	Malaysia	2023	Unknown	Child autism
48.	(Kurniastuti, Evanjeli and Sari, 2023)	Indonesia	2023	Unknown	Child autism

RESULTS AND DISCUSSION

Table 2 presents the frequency of publications. The year 2021 had the highest turnover in terms of research outputs in the child autism spectrum disorder around the globe. The graphical representation of the published articles in the study for the period of seven years is shown in Figure 3.
From Figure 3, the most active period of the research is year 2021 with 15 articles, followed by year 2020 at 14 articles, and year 2018 had no articles published.

Also, the distribution of the publishers of the included studies is shown in Table 4, which revealed that MDPI provided the most research works of 6, followed by Elsevier Inc. and Springer Inc., with 5 articles each on the child autism spectrum disorders and the relative factors from the educators' and parents' standpoints. Furthermore, the distribution of the regions of the included studies on the autism spectrum disorder in children, educational tools and interventions, educational and family factors are presented in Figure 4

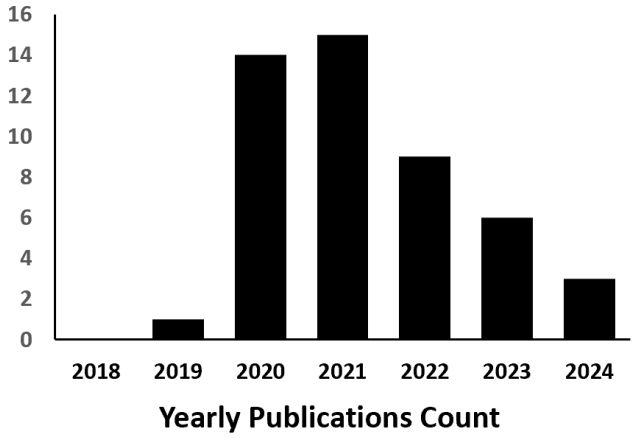

Figure 3:
The distribution of published articles included in the study

From Figure 4, the most productive research regions on child autism and related investigations are the USA and Greece, at 9 authors each, closely followed by Spain at 7 authors, then, Bosnia and Herzegovina, China, Ireland, Jordan, South Africa produced 1 author each to be placed in the least productive regions. The reason can be attributed to early knowledge and understanding of the ailment and high autistic populations with large clinical and academic studies towards its management and controls.

Educational and family factors for the autistic children: The answers to the research question two (RQ2), which are the most important educational and family factors of the child autism covered in included studies are presented in Table 4.

Table 3.
The distribution of the publishers of the included studies

Article Publisher	Count
BMC	1
DE GRUYTER	2
Elsevier Inc	5
Faculty of Education and Teacher Training State Institute for Islamic Studies Batusangkar	1
Frontiers	1
IEEE	1
MDPI	6
Routledge Taylor & Francis Group	3
SAGE	3
Scholars Middle East Publishers	1
Springer	5
Taylor & Francis Group	4
The American Academy of Pediatrics	1
The British Psychological Society	1
The British Society of Developmental Disabilities	1
Unknown	11
WILEY	1
Elsevier Inc	5

From Table 4, the general factors adopted by educators, clinicians, and parents for identifying child autism spectrum disorder cases include:

- Socioeconomic factors: Race, ethnicity, socio-cultural, literacy levels of parents, living standards, environmental, social class, cultures and beliefs, demography.
- Biological factors: Birth cohorts, age, gender, co-occurring, facial dysmorphic traits, neurological signs, developmental disorders, genetic disorders, repetitive behaviours, comorbid concerns, neuropsychiatric disorders.
- Psychological and behavioural factors: Seizures, sensory impairment, hearing and vision difficulties, social functioning, intellectual disorders, communication changes, hyperactivity, intelligence quotient, cognitive ability, relationships, social, learning difficulty, mental health.
- Medical and healthcare factors: Expertise, synaptic dysfunction, medical co-occurring conditions, language skills, parental care, epilepsy, medical caregivers' experiences, and speech delays, attention deficit, nutrition, heavy metal exposure, perinatal, and risky lifestyles, presence of regression.
- Observatory factors: Physical activities, adaptive behaviours, non-verbal reasoning, social functioning, Asperger's syndrome, perspective development disorders, contextual and cultural factors, diagnostic criteria, behavioural analysis, knowledge and awareness of illness.

Educational factors matching different children autism during the early development stages including: Intellectual disability, learning difficulty, social, practical and intellectual functioning skills (Xinogalos and Tsikinas, 2019), verbal communication problems, cognitive performance (Mozolic-Staunton *et al*., 2020), child cognitive disability (Maddox *et al*., 2020; Saleh, Hanapiah and Hashim, 2021; Ke, Moon and Sokolikj, 2022), mutual attention, imitation skills, verbal communication skills (Syriopoulou-Delli and Gkiolnta, 2022), Repetitive behaviour, severe and irritable behaviours, hyperactivity, inattention, distractibility (Hyman, Levy and Myers, 2020), Learning and communication disabilities (Yahya *et al*., 2023), deficiencies in interaction skills, daily skills, domestic skills, learning skills (Hermanto and Pamungkas, 2023; Kurniastuti, Evanjeli and Sari, 2023), Academic performance, emotional disorders, lack of cultural and linguistic identity (Fernández Cerero, Montenegro Rueda and López Meneses, 2024), attention deficit, hyperactivity disorder, physical, mental and neurological challenges, communication deficiency, personality (Nisa, Zain and Rahmah, 2024).

Similarly, the family factors related to child autism detection including: persistent restricted, repetitive pattern interests or behaviours (Purnama *et al*., 2021), speech/language, social developmental history, adaptive behaviour (Maddox *et al*., 2020), Social and cognitive skills (Saleh, Hanapiah and Hashim, 2021; Syriopoulou-Delli and Gkiolnta, 2022), lack of social and collaborative play (Syrdal *et al*., 2020), Disruptive activities at home, discomfort, and self-injurious behaviours and other co-occurring behaviourial symptoms (Hyman, Levy and Myers, 2020), motor disorders, autism, speech delay, learning difficulties, speech impaired, slow learners, deaf, mentally retarded, blind, quadriplegic, unsociable (Kurniastuti, Evanjeli and Sari, 2023), communication, social interaction, offensive behaviours, stress (Fernández Cerero, Montenegro Rueda and López Meneses, 2024), self-development, cognition, and learning activities, behavioural and social defects (Nisa, Zain and Rahmah, 2024).

Figure 4.
The distribution of included studies by region of the authors

Table 4.
The educational and family factors of the child autism

No	Author(s)	Educational Factors	Family Factors
1	Zanuttini (2023)	Bullying, well-being, transition.	Uncovered.
2	Purnama *et al*. (2021)	Social interaction and communication. Cognition.	Persistent restricted, repetitive pattern interests or behaviours.
3	Xinogalos *et al*. (2020)	Intellectual disability, learning difficulty, social, practical and intellectual functioning skills.	Independent living skills
4	Mozolic-Staunton *et al*. (2020)	Verbal communication problems, cognitive performance.	Social and emotional developmental delays, stress.
5	Maddox *et al*. (2019)	Child cognitive disability.	Speech/ language, social developmental history, adaptive behaviour.
6	Saleh *et al*. (2020)	Severe intellectual disability, learning skills.	Social and cognitive skills.
7	Syriopoulous-Delli & Gkiolnta (2020)	Mutual attention, imitation skills, verbal communication skills.	Social skills.
8	Zilli *et al*. (2019)	Poor thinking skills, poor interest, social disconnection.	Unspecified.
9	Ke *et al*. (2022)	Social and communication skills.	Unspecified.
10	Syrdal *et al*. (2020)	Social and communication skills, narrow interests, repetitive and stereotypical behaviours.	Lack of social and collaborative play.
11	Taheri *et al*. (2021)	Stereotypical behaviours, cognitive defects.	High parenting stress levels.
12	Hyman *et al*. (2020)	Repetitive behaviour, severe and irritable behaviours, hyperactivity, inattention, distractibility.	Disruptive activities at home, discomfort, and self-injurious behaviours and other co-occurring behaviourial symptoms.
13	Chaidi *et al*. (2021)	Learning difficulties.	Lack self-esteem, social interaction deficiency.
14	Hussain *et al*. (2021)	Language and communication skills.	Social interaction with verbal and non-verbal dimensions. Unusual response to stimulus.
15	Fachantidis *et al*. (2018)	Attention deficiency, social and communication problem, stereotypical behaviours.	Lack of autonomy and low quality of life.
16	Bamicha & Athanasios (2022)	Perception, social interaction, information processing, verbal and non-verbal communication, social and cognitive behaviour.	Social communication difficulty, uncontrollable repetitive behaviours, and interests.
17	Aloizou *et al*. (2021)	Communication, behavioural and cognitive deficiencies.	Communication and beviourial disorders.
18	Galitskaya & Drigas (2020)	Concentration and learning difficulties, intellectual disabilities, movement challenges.	Object recognition, movement difficulties.
19	Chaidi & Drigas (2020)	Communication and socio-emotional skills, attention disorders.	Communication skills, quality of life, behavioural disorders, difficulty in retaining knowledge, generalization problem, sleep disturbances,
20	Kirby *et al*. (2022)	Cognitive problems, adaptive behaviours, emotional instabilities, attention, and aggression.	Adaptive behaviour disorders, difficulty with sensory parts of the environment (extreme sensitivity, stress, sensitive to noise, over-responsiveness, excessive mouthing of objects).
21	Baldassarri *et al*. (2021)	Communication and attention disorders, cognition, concentration, contextualized challenges.	Emotional challenges (happiness, fears, disgust, surprise, sadness, neutral), social interactions and communication.
22	Zhang *et al*. (2022)	Cognitive skills, social and interaction skills, social communication (language and speech, emotion recognition, social functioning).	Atypical view patterns, irregular emotions, speech and language skills, social communication skills.
23	Taresh *et al*. (2020)	Behavioural disorders, learning difficulties, mental health issues, psychological disorders, emotional and physical disability.	Behavioral disorders, social and learning difficulty.

24	Carmona-Serrano *et al*. (2020)	Narrative skills, communication, participation, retention and assimilation of information.	Behavioural challenge, stress, dependence on daily life.
25	Daulay (2021)	Repetitive emotions, and behvavioural patterns, communication, learning difficulty.	Burden of caregiving and negative emotions, socially delayed, inappropriate behaviours, poor adaptability.
26	Bravou *et al*. (2022)	Behavioural and learning difficulties, social and distracted problems.	Attention deficit, mental imagery disorders, information processing patterns, facial expressions, interpersonal skills.
27	Singh *et al*. (2023)	Non-verbal communication, seizures, learning difficulties, and verbal disorders.	Learning and social interaction problems.
28	Elshahawy *et al*. (2020)	Problem solving skills, language, social and communication skills, intellectual abilities.	Communication and behavioural deficiencies, social, restrictive, repetitive and stereotyped behaviours.
29	Davis *et al*. (2021)	Early language difficulty.	Early language and cognitive defects, low emotional processing.
30	Blasco-Magraner *et al*. (2021)	Learning and emotional difficulties. Psychological and deductive disorders.	Emotional intelligence, academic performance, and prosocial skills.
31	Jackson *et al*. (2020)	Learning disability, language and communication problem, and verbal disorders.	Social and learning disorders, verbal disorders.
32	Sanroma-Gimenez *et al*. (2021)	Learning difficulty, attention deficiency, lack of participation, interaction, commitment,	Low quality of life, learning disorders.
33	Harris *et al*. (2021)	Communication, play, and behaviour, learning difficulty, cognition and language functioning.	Social engagement, play, behviour, and speech disorders.
34	Pilay *et al*. (2020)	Co-occurring intellectual disability, hyperactivity, language and speech disorders, attention-deficit.	Speech and language disorders, visual and hearing impairment, physical and intellectual disorders, hyperactivity/ attention-deficit, bevariour and seizures.
35	Gallardo-Montes *et al*. (2022)	Language function, emotional disorders, entertainment.	Behviours, verbal/ non-verbal impairments, and thinking, language and communication, interpersonal reciprocity.
36	Bolourian *et al*. (2022)	Social difficulties, fixed/focused interests, physical impairments, emotional and behavioural difficulties, inattention.	Communication skills and intellectual functioning, social communication and repetitive behaviours, and inappropriate behaviours.
37	Barbaro & Yaari (2020)	Social attention, communication and behavioural disorders.	Social attention, communication and behavioural disorders.
38	O'Keeffe & McNally (2021)	Social interaction disorders, communication, cognition.	Communication skills and social skills disorders.
39	Khalil *et al*. (2020)	Behavioural disorders, social interactions, communication deficit, intellectual disorders.	Behavior, social interaction, verbal and non-verbal communication, disorders.
40	Sweidan *et al*. (2019)	Social skills, language skills, inattention, learning difficulty, behavioural disorders.	Intellectual disabilities, personal development, social behavior and thinking deficiencies, speaking skills.
41	Chinchay *et al*. (2023)	Emotional instability, communication and social disorders.	Isolation, quarantine, physical and executive function disorders, language, cognition, memory deficiencies.
42	Cerero *et al*. (2024)	Academic performance, emotional disorders, lack of cultural and linguistic identity.	Communication, social interaction, offensive behaviours, stress.
43	Dordevic *et al*. (2022)	Mental health play, well-being, depression, relationship and communication problem.	Child behavioral difficulties, educational and learning deficiencies, developmental disabilities.
44	McDevitt (2021)	Socio-emotional instability.	Academic performance, social and communication issues,
45	Nisa *et al*. (2024)	Attention deficit, hyperactivity disorder, physical, mental and neurological challenges, communication deficiency, personality.	Self-development, cognition, and learning activities, behavioural and social defects.
46	Hermanto & Pamungkas (2023)	Learning disabilities, physical and developmental barriers, social and mobility deficiencies, cognitive skills defects, sight and hearing impairments, emotional and behavioural issues.	Learning difficulties, visual and hearing impairments, inattention,
47	Yahya *et al*. (2023)	Learning and communication disabilities.	Uncovered.
48	Kurniastuti *et al*. (2023)	Deficiencies in interaction skills, daily skills, domestic skills, learning skills.	Motor disorders, autism, speech delay, learning difficulties, speech impaired, slow learners, deaf, mentally retarded, blind, quadriplegic, unsociable.

Table 5.
The educational and assistive tools for autistic children management

S/N	Author(S)	Education/ Assistive Tools
1.	Purnama *et al*. (2021)	Sqizzy
2.	Xinogalos *et al*. (2020)	Computer-based serious games.
3.	Maddox *et al*. (2019)	Special education services.
4.	Saleh *et al*. (2020)	Robot applications
5.	Syriopoulous-Delli & Gkiolnta (2020)	Assistive technology.
6.	Ke *et al*. (2020)	Virtual reality
7.	Syrdal *et al*. (2020)	Kaspar Humanoid robot.
8.	Taheri *et al*. (2021)	Social robot for music lessons.
9.	Chaidi *et al*. (2021)	Educational robotics.
10.	Hussain *et al*. (2021)	Mobile applications.
11.	Fachantidis *et al*. (2018)	Assistive robotics.
12.	Bamicha & Athanasios (2022)	Computer assisted technology.
13.	Aloizou *et al*. (2021)	Tele-conferencing technologies.
14.	Galitskaya & Drigas (2020)	Digital and computer technologies. Assisted learning technologies.
15.	Chaidi & Drigas (2020)	Parent education and therapy. Family care and support packages.
16.	Kirby *et al*. (2022)	Autistic screening tools with sensory feature capabilities.
17.	Baldassarri *et al*. (2020)	Video games.
18.	Zhang *et al*. (2022)	Virtual reality technology.
19.	Taresh *et al*. (2020)	Teachers' education on the means to identify autistic children.
20.	Carmona-Serrano *et al*. (2020)	Technological interventions such videos, websites and gamified environment.
21.	Daulay (2021)	Online training of parents and teachers about coping with autistic children.
22.	Bravou *et al*. (2022)	Computer assistive tools such as role-playing games, interactive games.
23.	Singh *et al*. (2023)	Robotics.
24.	Elshahawy *et al*. (2020)	Computer-based solutions such as games
25.	Davis *et al*. (2021)	Bilingualism mobile app.
26.	Blasco-Magraner *et al*. (2021)	Music
27.	Jackson *et al*. (2019)	RECALL visual technology: Shared reading as an instructional context.
28.	Sanroma-Gimenez *et al*. (2021)	Educational mobile applications.
29.	Harris *et al*. (2021)	Electronic questionnaire for child autism screening during early literacy.
30.	Pilay *et al*. (2020)	Centralized education management information system for enrollment and tracking of children in school system.
31.	Gallardo-Montes *et al*. (2022)	Specialized Mobile applications and digital technologies.
32.	Bolourian *et al*. (2022)	Visual aids, learning module.
33.	Barbaro & Yaari (2020)	ASDetect Mobile applications for early detection of ASD by parents.
34.	O'Keeffe & McNally (2021)	Play-based interventions such as JASPER, ASAP, ENGAGE, SKILLS, FRIENDS.
35.	Khali *et al*. (2020)	Visual activity, verbal reinforcement, and telling social stories as most practical behavioral strategies.
36.	Sweidan *et al*. (2019)	Autistic innovative Assistant android smart phone application.
37.	Chinchay *et al*. (2023)	Assistive technologies such as desktop and mobile settings.
38.	Cerero *et al*. (2024)	Assistive technologies. Synergy between parents, educated and health professionals.
39.	Dordevic *et al*. (2021)	Parent-teacher interactions and collaboration for tending with autistic children.
40.	McDevitt (2021)	Parent education and training program for home-based interventions.
41.	Nisa *et al*. (2024)	Special assistant teachers.
42.	Hermanto & Pamungkas (2023)	Learning media, and resources such as textbooks, journals.
43.	Yahya *et al*. (2023)	Instruction digital model such as virtual and learning technologies, mobile apps, adaptive devices, augmented reality and virtual reality, social media and communication apps, virtual classroom, teletherapy.
44.	Kurniastuti *et al*. (2023)	Learning media and resources.

Educational tools for autistic child management: Research question three (RQ3) answers detail the educational and assistive tools used by teachers and parents for advancing and managing autistic children as presented in Table 5.

In Table 5, the major educational and assistive tools adapted by teachers and parents to meet the needs of autistic children include: Virtual reality and gaming technologies (Galitskaya and Drigas, 2020; Baldassarri *et al.*, 2021; Zhang *et al.*, 2022); Music (Blasco-Magraner *et al.*, 2021); Gaming applications (Xinogalos and Tsikinas, 2019; Baldassarri *et al.*, 2021; O'Keeffe and McNally, 2023); Robot applications (Syrdal *et al.*, 2020; Chaidi *et al.*, 2021; Taheri *et al.*, 2021; Singh *et al.*, 2023); RECALL visual technology: Shared reading as an instructional context (Jackson and Hanline, 2020); Assistive technologies such as mobile and desktop applications (Galitskaya and Drigas, 2020; Bravou, Oikonomidou and Drigas, 2022; Gallardo-Montes, Caurcel Cara and

Rodríguez Fuentes, 2022; Chinchay *et al.*, 2024; Fernández Cerero, Montenegro Rueda and López Meneses, 2024); Learning media and resources (Hermanto and Pamungkas, 2023; Kurniastuti, Evanjeli and Sari, 2023)

Future research directions: The fourth research question (RQ4) answers provide the future research directions on the basis of the included studies, as shown in Table 6.

Table 6
Future research directions and area of further studies

S/N	Author(S)	Future Research Directions
1.	Zanuttini (2023)	Under-presented study areas around the globe.
2.	Purnama *et al*. (2021)	Design of assistive technologies could be more realistic.
3.	Xinogalos *et al*. (2020)	No user validation.
4.	Mozolic-Staunton *et al*. (2020)	No universal developmental surveillance for child autism.
5.	Maddox *et al*. (2019)	Educational autism classification utilizes ADOS-2 criteria in most advanced economies.
6.	Saleh *et al*. (2020)	To use robots to assess educational factors like rating of subjects' attention rate could be explored subsequently.
7.	Syriopoulous-Delli & Gkiolnta (2020)	Situating interventions to age, intellectual abilities of autistic children.
8.	Zilli *et al*. (2019)	Assistive communication solutions are probable.
9.	Ke *et al*. (2020)	Adapting non-natural solutions to users.
10.	Syrdal *et al*. (2020)	Evolving and complex research area.
11.	Taheri *et al*. (2021)	The use of educational tools had no significant improvements on the autistic child.
12.	Hyman *et al*. (2020)	Traditional screening approaches are less-effective.
13.	Chaidi *et al*. (2021)	Adaptive solutions to keep pace with learners' abilities.
14.	Hussain *et al*. (2021)	Effective usage of mobile apps to support ease of teaching and learning.
15.	Fachantidis *et al*. (2018)	Enhancing functionalities on the robotics solution.
16.	Bamicha & Athanasios (2022)	To adaptive digital interventions to specific educational needs of autist children.
17.	Aloizou *et al*. (2021)	Ineffectiveness of digital interventions for severe and mobility difficult children.
18.	Galitskaya & Drigas (2020)	Limited to the geometric mathematics skills.
19.	Chaidi & Drigas (2020)	To evolve effective and early intervention for autistic children.
20.	Kirby *et al*. (2022)	Sociodemographic disparities impart on sensory features recognition and treatment.
21.	Baldassarri *et al*. (2020)	Inappropriate EEG devices for capturing data. Lack of analytical tools for explaining emotions of autistic children.
22.	Zhang *et al*. (2022)	Evolving technology little immersion. More functionalities expected,
23.	Taresh *et al*. (2020)	Preparing parents for early detection of child autism.
24.	Carmona-Serrano *et al*. (2020)	Continuous improvement of pedagogical interventions and tools for autistic children.
25.	Daulay (2021)	Poor knowledge on taking care of autistic children.
26.	Bravou *et al*. (2022)	The suitability of the gadgets for effective interactions.
27.	Singh *et al*. (2023)	The majority of child autism solutions are cost-ineffective.
28.	Elshahawy *et al*. (2020)	No usability studies were performed on available assistive technologies.
29.	Davis *et al*. (2021)	More cultural and social settings inclusion.
30.	Blasco-Magraner *et al*. (2021)	Evolving field of study in socio-emotional area.
31.	Jackson *et al*. (2019)	Limited to reading and comprehension skills in sciences.
32.	Sanroma-Gimenez *et al*. (2021)	Standardizing and Personalizing learning experiences for autistic children.
33.	Harris *et al*. (2021)	Impart of demographic information could be investigated.
34.	Pilay *et al*. (2020)	Lack policy documents on enrolment of autistic children into schools. Lack of appropriate tools for special educators in low-income nations.
35.	Gallardo-Montes *et al*. (2022)	Adaptability of technologies matching children with autism.
36.	Bolourian *et al*. (2022)	Focus to be on evidence-based tools for teachers in managing autistic children.
37.	Barbaro & Yaari (2020)	Parent literacy technologies could help child autism in low-income countries.
38.	O'Keeffe & McNally (2021)	Interventions are limited by interests and levels of development. Play-based curriculum is still evolving.
39.	Khali *et al*. (2020)	Professional standpoints on behavioural strategies for managing autistic children are expected.
40.	Sweidan *et al*. (2019)	Inclusion of artificial intelligence for a more personalized and adaptive experiences.
41.	Chinchay *et al*. (2023)	Ineffective due to large digital skills gaps of teachers, caregivers, and parents.
42.	Cerero *et al*. (2024)	Low availability of appropriate educational materials.
43.	Dordevic *et al*. (2021)	Scanty literature from developing countries.
44.	McDevitt (2021)	Lack of appropriate resources and professionals for schooling and services to autistic children. Educational tools could be situated to special needs of children.
45.	Nisa *et al*. (2024)	AI could be introduced in improving learning assistance of shadow teachers.
46.	Hermanto & Pamungkas (2023)	Strengthening of teacher-parent relation on improved education of autistic children. Artificial intelligence and expert systems could improve assistive learning.
47.	Yahya *et al*. (2023)	Low access to educational resources for autistic children.
48.	Kurniastuti *et al*. (2023)	More teacher training on care and teaching processes of autistic children.

From Table 6, the included studies revealed the highly pressing need for urgent attention in the child autism research. These include:

- The relevance of educational tools usage on autistic children cannot be ascertained in terms of significant improvements (Taheri *et al.*, 2021).
- The low-income countries are still underrepresented when compared to advanced countries in child autism research (Zanuttini, 2023).
- There are still no usability assessments performed on available assistive technologies, in which the effectiveness cannot be established (Elsabbagh, 2020).
- There is a need to standardize and personalize the learning experiences for the autistic children (Sanromà-Giménez *et al.*, 2021).
- The adoption of AI and machine learning algorithms to improve on autistic children management (Hermanto and Pamungkas, 2023; Nisa, Zain and Rahmah, 2024).
- There is a seemingly absence of appropriate resources and professionals for the schooling and services to autistic children, especially in low-income countries (McDevitt, 2021).
- Educational tools could be situated to special needs of children (Zhang *et al.*, 2022).
- There is a need to focus on evidence-based tools by teachers in the management of autistic children (Bolourian *et al.*, 2021).
- There is a consensus on the ineffectiveness of educational tools due to large digital skills gaps of teachers, caregivers, and parents (Chinchay *et al.*, 2024).

CONCLUSION

This study conducted an SLR to investigate educational and family factors of child autism spectrum disorder globally. The trends of research favour the more advanced and high-income countries, with sparse research contributions across low-income countries. The majority of research endeavours are more inclined to the diagnosis and the detection of autism disorders. The study identified factors associated with early, mild and severe symptoms of autism disorder among children from their teachers in schools, and parents and family members. There is little awareness about the educational and assistive tools capable of helping in the management of child autism cases in the high-income and low-income regions of the world. Though, majority of assistive tools are incorporating expert systems and AI for a more adaptive experiences of the available educational interventions on the part of family and educator.

The key patterns and factors exhibited by the children in the early autism spectrum disorder were established from the perceptive of the parents and teachers which could help the medical practitioners during the process of screening, and schools could effectively plan for different educational service needs. The various educational services and tools for addressing certain autistic children's problems were identified. This study provides the numerous areas of research requiring urgent attention in the future works section

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Study protocol

Sugar-Sweetened Beverages Consumption among Adolescents in Nigeria (SURRENDER): Research Plan and Procedures of Mixed-Method Survey

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Summary: Sugar-sweetened beverage (SSB) consumption has been estimated to be the highest in sub-Saharan Africa, with a significant impact on the increasing burden of diet-related non-communicable diseases among adolescents. This manuscript highlights the research plan and procedures for SSB consumption among adolescents in Nigeria (SURRENDER), a survey designed to assess the magnitude and patterns of sweetened beverages (SSB) consumption with its associated factors and the potential association with cardiometabolic risk factors, including obesity, high blood pressure and elevated blood sugar, among in-school adolescents in Nigeria. The SURRENDER study surveyed in-school adolescents (males and females, aged 10–19 years) from secondary schools across three main cities: Abuja, Ikeja, and Ibadan, Nigeria. Participant enrollment, including questionnaire administration, physical examination and blood specimen collection, started in 2023, using the World Health Organization's STEPS Instrument for Chronic Disease Risk Factor Surveillance for data collection. Data were collected on sociodemographics, parent and family characteristics, lifestyle factors, food access and dietary diversity, SSB consumption, physical and sedentary activity, school environment, anthropometric measurements (including weight in kilograms, height and mid-upper arm circumference in centimetres) and blood pressure assessment (including systolic and diastolic blood pressure and pulse rate) and blood glucose measurements by trained personnel in keeping with standing protocol, procedures and tools. A total of 1,699 (females – 58.6%) were recruited (29.4% - Abuja, 29.4% - Ikeja and 41.1% - Ibadan) with a 74.9% response rate and focused group discussions are underway to understand the etiological basis for perceptions, attitudes, and environmental drivers of dietary SSB consumption among adolescents. The SURRENDER would be promising, as it would provide evidence-based and innovative data that would ultimately inform the design of public health guidelines, advisories, and policy initiatives to support strong adolescent health in Nigeria and can be extrapolated for guidance in other African countries.

Keywords: *SURRENDER; Adolescent health; Obesity; Hypertension; Diabetes; Chronic diseases; Africa.*

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INTRODUCTION

Sugar-sweetened beverages (SSB), defined as carbonated or non-carbonated drinks containing added sugars, have become the primary source of added sugar in many diets (Malik *et al*., 2006). There has been a significant increase in consumption levels, especially among teenagers and young adults, as well as notable increases in low- and middle-income countries (LMIC) (Lara-Castor *et al*., 2023). SSB consumption provides trifling nutritional benefits, promoting less satiety than healthier diets, large volume consumption, and reduced intake of balanced diet (Popkin and Hawkes, 2016). All these factors have been linked to increased energy intake and a significant contribution to weight gain (Malik *et al*., 2006) and diet-related non-communicable diseases (NCDs) (Malik *et al*., 2010), with solid evidence suggesting causal etiologies for cardiometabolic diseases worldwide (Malik and Hu, 2022).

Several reports have documented SSB consumption trends and influences on cardiometabolic risk factors from different world regions. For example, the Global Dietary Database reported a mean global SSB intake of 2.7 servings (equivalent to 8 oz, or 248 grams) per week among adults worldwide (Lara-Castor *et al*., 2023). Similarly, the Centre for Disease Control of the United States (US) revealed that SSBs are the foremost source of added sugars in the

American diet, with five in every ten adults drinking SSB daily and an average of 145 calories per day attributable to SSB between 2011 and 2014 (Rosinger *et al*., 2017a). The situation is no different in Europe, where 12% of men and 7% of women consume sugar-sweetened soft drinks daily (Eurostat, 2021). Despite the enormous stakes and the potential public health risk portended by SSB, minimal studies are available on the trend and consumption of SSB in LMICs, especially in sub-Saharan Africa (SSA).

In addition, the excessive consumption of refined sugars, especially from SSB, promotes neurobehavioral alterations (Burger, 2017; Jacques *et al*., 2019) that interrupt the encephalon reward network by impairing food signalling systems, leading to obsessive consumption (Falbe *et al*., 2019) or satiety manipulation (Shearrer *et al*., 2016), with a significant impact on obesity and NCD manifestations (Audain *et al*., 2019; Yin *et al*., 2021; Malik and Hu, 2022). Consequently, it is worthwhile to discern the pattern of SSB consumption among adolescents in SSA as a potential resource for designing appropriate interventions. Furthermore, the SSB consumption trend among adolescents has been documented to be on the increase among adolescents globally, in Europe (Eurostat, 2021; Chatelan *et al*., 2023), Australia (Clifton *et al*., 2011), and the US (Rosinger *et al*., 2017b) with little information on this phenomenon, especially among adolescents from SSA. A similar effort in Burkina Faso and Kenya recently demonstrated that half of 3759 women of reproductive age reported SSB consumption without articulating relevant information for adolescents (Semagn *et al*., 2023). Similarly, a nationally representative study of 74,055 in-school adolescents aged 12 to 15 years from eighteen predominantly LMIC fell short of evidence on the trend of SSB consumption in SSA (Smith *et al*., 2024). Nigeria, which is the most populous black nation worldwide, is not exempted from this issue, as data on SSB consumption and the associated factors are scarce, thereby making it tedious to support healthcare systems in SSA with critical information in addressing nutrition policy and public health practice to improve its preparedness in addressing the snowballing burden of NCDs (Gouda *et al*., 2019).

Furthermore, recent global estimates suggest that populations from SSA currently experience the most significant increase in SSB consumption (Lara-Castor *et al*., 2023). However, there is limited information on the proximal and distal factors associated with SSB consumption, particularly among adolescents. Studies aimed at identifying the magnitude and characteristics associated with SSB consumption among adolescents would be a worthwhile investment, providing vital information to help adolescents transition into healthy adulthood and contribute to the development of a healthy workforce. This information is critical for preventing the potential negative impact of SSB consumption not only in adolescents but also in later adulthood. The food environment is crucial in informing dietary behaviour (Kelly *et al*., 2019), especially for younger populations, including adolescents, who are vulnerable to advertising and marketing pressures that could manipulate their dietary choices (Harris and Graff, 2011) to naively inform dietary lifestyle practices that could potentially lead to adverse health outcomes that could impair functionality and promote disability in later adulthood. To this effect, the necessity of assessing the significance of SSB consumption, its associated factors and its impact on cardiometabolic health outcomes among adolescents cannot be overemphasized in strengthening stakeholders' efforts (such as government, donor agencies, and public health practitioners, among others) in improving health system quality service delivery for the struggling healthcare systems in SSA which simultaneously grapples under the dual burden of infectious and NCDs.

Additionally, the Sugar-Sweetened Beverage Consumption among Adolescents in Nigeria (SURRENDER) study is a home-grown research effort for in-school adolescents, primarily aimed at achieving three main goals. First, to examine the trends and factors associated with SSB consumption. Second, to determine the potential association of SSB with the prevalence of cardiometabolic risk factors, including obesity, high blood pressure, and high blood glucose. Third, to quantify the magnitude of cardiometabolic risk factors, offer information and promote evidence-based public health guidelines, advisories, and policy initiatives that support adolescent health.

MATERIALS AND METHODS

Project Organization: Study investigators for the SURRENDER study were professionals with diverse educational backgrounds (in medicine, medical laboratory science, pharmaceutical science, biostatistics, and nutrition) and research training in epidemiology, maternal, child, and adolescent health. The study also employed research assistants, volunteer interviewers, and data clerks who were fluent in English and specific local languages to facilitate smooth communication with the participants and minimize language barriers. Additionally, the technical staff reviewed data collection, entry, and administration for consistency and accuracy. All research staff were accountable to the study investigators across study sites, and the principal investigator coordinated the study's administration.

Study Design: The SURRENDER study was a multicenter, cross-sectional study that collected data among in-school adolescents from private and public schools across three major cities in Nigeria: Abuja, Ikeja, and Ibadan, using predefined inclusion and exclusion criteria. The Ethics Review Committee approved the study across study sites. In Abuja, the National Health Research Ethics Committee of Nigeria, located at the Federal Ministry of Health, Abuja, Nigeria, approved the study (Approval number: NHREC/01/01/2007-02/05/2023). In Ikeja, the Institutional Review Board of the National Institute of Medical Research, Lagos State, Nigeria, approved the study (Project number: IRB/23/017). In Ibadan, the Research Ethics Review Committee of the Ministry of Health, Oyo State Government, Nigeria, approved the study (NREC Assigned number: NHREC/OYOSHRIEC/10/11/22). All interviews and data sampling were conducted during school hours after permission from the school principal. Informed consent was obtained prior to participation, following receipt of a signed explanatory note and informed consent that was shared with parents or guardians to explain the study's purpose and assent from eligible in-school adolescents.

Specific Aims and Hypotheses: The SURRENDER study has three main aims and underlying hypotheses. First, the

trends and factors associated with SSB consumption among in-school adolescents will be explored. Second, to determine the potential association of SSB consumption with the prevalence of cardiometabolic risk factors, including obesity, high blood pressure and high blood glucose among in-school adolescents. Third, to quantify the magnitude of cardiometabolic risk factors and the qualitative and quantitative contributions of sociodemographic factors, lifestyle habits, and sedentary behaviors in a sample of in-school adolescents in Nigeria. The critical premise guiding these aims hypothesizes that the distribution of the risk factors, including SSB consumption, will likely modify the undercurrents and magnitude of cardiometabolic risk factors among in-school adolescents from diverse backgrounds in Nigeria. We hypothesize that differences in the SBB consumption trends and risk factors may explain changes in cardiometabolic risk factors among in-school adolescents in Nigeria. The central idea behind this objective is to obtain reliable phenotypic data, including SSB consumption, to assess cardiometabolic risk factors among in-school adolescents in Nigeria. Additionally, chronic exposure to these risk factors may alter the vasculature, influencing the assessment of the magnitude and burden of cardiometabolic risk factors among in-school adolescents.

The cross-sectional recruitment of in-school adolescents (including males and females) from diverse school settings in Abuja, Ikeja, and Ibadan, Nigeria, has been completed (Fig. 1). Furthermore, focus group discussions and key informant interviews were conducted to clarify the knowledge, perception, and beliefs of in-school adolescents about voluntary participation in research, SSB consumption, cardiometabolic risk, with potential traditional risk factors. The premise for these objectives stems from the need to promote viable research for designing evidence-based public health interventions, guidelines, advisories, and policy initiatives for promoting strong cardiometabolic health among adolescents.

Study Location: Enrollment and data collection for this study took place across three study sites (Figure 1), including Abuja, Ikeja, and Ibadan, Nigeria, due to the rich multicultural diversity and diverse demographic and socioeconomic backgrounds representative of Nigeria's multi-ethnic and multicultural populace.

Abuja – FCT: The FCT – Abuja is the eighth most populous city in Nigeria, located on longitude 6° 45" and 7° 45" East of the Greenwich Meridian and latitude 8°35′ and 9° 25′ North of the equator, with a land area of about 8,000km^2, managed across six (6) area councils with a total population of 776,298 according to the 2005 population census (Ola Balogun and Balogun, 2001; National Population Commission, 2006). The Abuja Municipal Area Council was purposively selected for this study, as it serves as the administrative and commercial hub of the FCT in Abuja. Furthermore, thirteen (four private and nine public) schools were randomly selected from the entire sample frame of three hundred and seven (307) registered secondary schools in the Abuja municipal area council for participant recruitment.

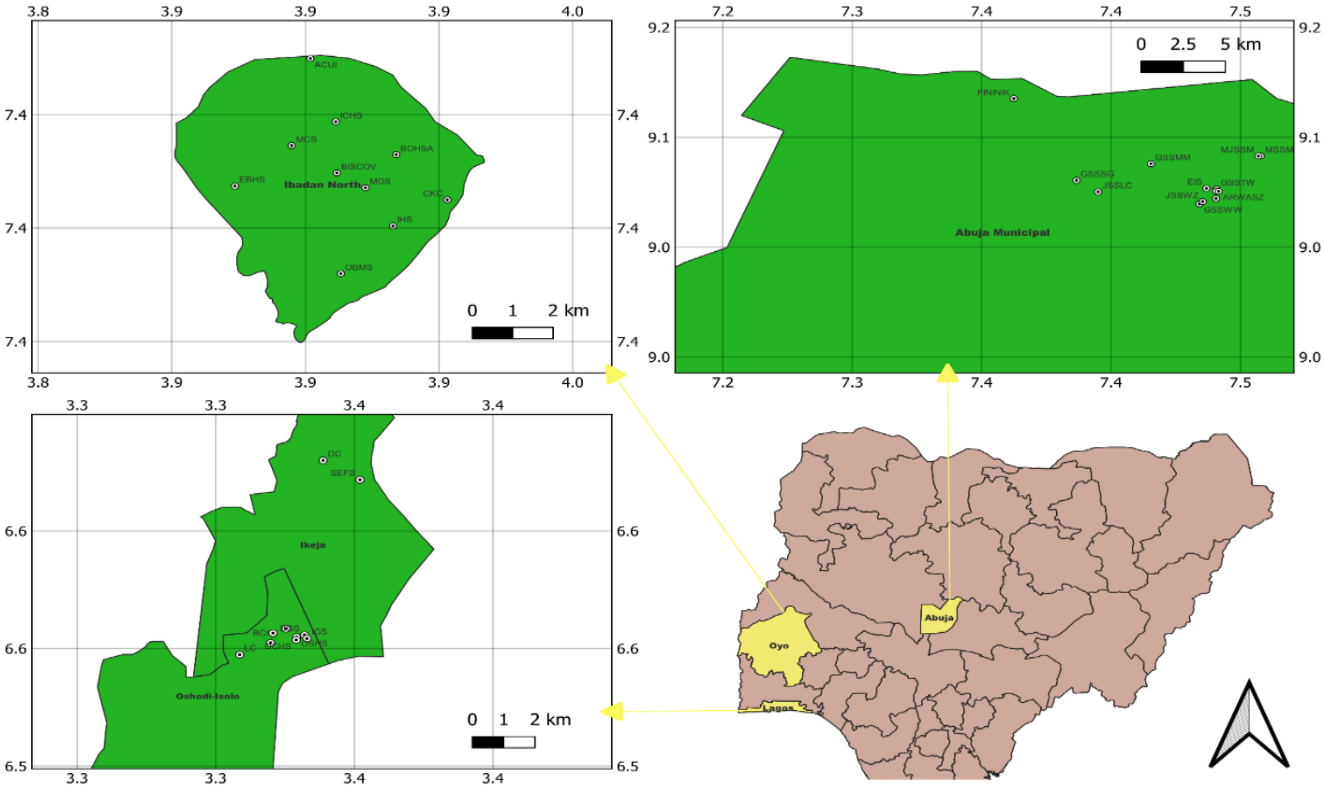

Figure 1:
Sampling locations for the SURRENDER study in Abuja, Ikeja and Ibadan, Nigeria

Ikeja – Lagos State: Lagos is the core economic hub of Nigeria, located on longitude 2° 42′–4° 20′ East of the Greenwich Meridian and latitude 6° 22′–6° 42′ North of the equator, with an enormously diverse population of about 8,048,430 according to the 2006 census and administratively governed across twenty (20) local government areas – LGA (YO Balogun *et al*., 1999; National Population Commission, 2006). Oshodi-Isolo LGA was purposively selected from the sampling frame of LGAs in Lagos, Nigeria, based on its administrative significance and the diversity of its population, which provides wide-ranging information on socioeconomic, demographic, and health outcomes. Ten (10 – five private and five public) schools were randomly selected from the entire sample frame of one hundred and six (106) registered secondary schools in Oshodi-Isolo LGA for participant recruitment. Furthermore, two schools declined to participate, and two additional schools were purposively selected from Ikeja LGA next to Oshodi-Isolo LGA.

Ibadan – Oyo State: Ibadan is one of the largest metropolitan cities in Nigeria, located at longitude 3°5′ East of the Greenwich Meridian and latitude 7°2′ North of the equator, with eleven (11) local governments (Okekunle *et al*., 2015) and a population well-known for its unique accretion of people from diverse socioeconomic and cultural backgrounds representative of the Nigerian population (Fabiyi, 2004). The Ibadan North LGA was purposively selected from the sampling frame of LGAs in Oyo State, Nigeria, given that it harbours the highest number of educational institutions and is currently the central hub of commercial activities in Ibadan, Nigeria. Also, ten (10 – five private and five public) schools were randomly selected from the entire sample frame of one hundred and eight (108) registered secondary schools in Ibadan North LGA for participant recruitment.

Sample size estimation and power validation: The sample size was estimated per study site using 80% power and a two-sided 95% confidence level. With a 24.2% prevalence of daily SSB consumption among adolescents from Sagamu, Nigeria (Sholeye *et al*., 2018), a 50% R-squared, and a 10% non-response rate, a minimum sample size of n = 463 (approximated to 480) per study site was needed to detect an odds ratio ≥1.50 for SSB association with cardiometabolic risk factors in the study being guided by a technique by the World Health Organization (Lwanga *et al*., 1991). The sample size was proportionately assigned to the selected schools based on the projected in-school adolescent population.

Sampling Technique: The eligible participants for this study were in-school adolescents selected from public and private secondary schools across study sites through multistage sampling techniques. The schools were randomly selected from a sampling frame of private and public schools in each study site, specifically from a selected LGA or area council. In-school adolescents were recruited from a total of thirty-three secondary schools, including ten (four private and nine public) in Abuja, ten (five private and five public) in Ikeja, and ten (five private and five public) in Ibadan, Nigeria.

Abuja – Federal Capital Territory: In Abuja, the Abuja Municipal Area Council was purposively selected for this study, and thirteen (four private and nine public) schools were randomly selected from the sampling frame of registered secondary schools (13/307; 4.2%) in the area council, intending to recruit nine hundred and thirty-nine (939) participants each across selected private schools and public schools. In the third step at each selected school, eligible adolescents were proportionately selected from a sampling frame of adolescents by class (excluding those in the examination class at junior and senior secondary schools). The first participant per class was randomly selected, and subsequent participants were selected using the kth term based on the sampling frame to ensure the sampling of seventy-two (72) participants per school.

Ikeja – Lagos State: Oshodi-Isolo LGA was purposively selected in Ikeja, and eight (four private and four public) and an additional two (from Ikeja LGA) schools were randomly selected from the sampling frame of registered secondary schools (10/106; 9.4%) in the LGA, intending to recruit three hundred (300) participants each across all private schools and public schools selected. In the third step at each selected school, eligible adolescents were proportionately selected from a sampling frame of adolescents by class (excluding those in the examination class at junior and senior secondary schools). The first participant per class was randomly selected, and subsequent participants were selected using the kth term based on the sampling frame to ensure that sixty (60) adolescents were sampled per school, achieving a total of five hundred eighty (580) participants.

Ibadan – Oyo State: Ibadan North LGA was purposively selected in Ibadan, and ten (five private and five public) schools were randomly selected from the sampling frame of registered secondary schools (10/108; 9.3%) in the LGA. In the third step at each selected school, eligible adolescents were proportionately selected from a sampling frame of adolescents by class (excluding those in the examination class at junior and senior secondary schools). The first participant per class was randomly selected, and subsequent participants were selected using the k^{th} term based on the sampling frame to ensure that seventy-five (75) adolescents were sampled per school, thereby achieving a sampling target of seven hundred and fifty (750) participants.

Inclusion and Exclusion Criteria: Participants were selected from in-school adolescents (aged ≥10 years) enrolled in one of the schools chosen at the time of the study will be/were recruited. To participate in the study, in-school adolescents must be between 10 and 19 years old on their last birthday, enrolled in the school for at least the last six months preceding the survey, and apparently healthy with signed informed consent from their parents or guardians before participating. In-school adolescents were excluded from the study based on the following criteria: being over 19 years old, being unhealthy, currently preparing for or taking a test or examination, lacking informed consent from a parent or guardian, and declining interest in the study.

Recruitment: Participant recruitment began with a detailed presentation of the study's aims to the school principals, who granted access to allow participants to indicate their interest

in the study. An eligible in-school adolescent who indicated interest and assented to participate was provided with informed consent, signed by the parent or guardian, approving their participation. Participants without signed informed consent from the parent or guardian were not allowed to participate in the survey.

Data Collection: Data collection consists of three parts: completing the questionnaire, taking physical measurements (including anthropometric and blood pressure assessments), and conducting biochemical assessments (Table 1). Trained research personnel guided in-school adolescents in filling out the questionnaire privately, with the opportunity to withdraw from the recruitment at any time without suffering any harm or consequences. Furthermore, physical examinations, including anthropometric measurements and blood pressure checks, were conducted following standard protocol, and blood draws were performed by a trained phlebotomist.

Table 1
Overview Variables and Measurements in the SURRENDER study

Date/Information	Variable or instrument for data collection	Study sites		
		Abuja	Ikeja	Ibadan
Survey Information	Questionnaire			
	School name	○	○	○
	Area of Residence	○	○	○
	Type of Residence	○	○	○
	Parental consent	○	○	○
Demographic Characteristics	Questionnaire			
	Sex	○	○	○
	Date of birth	○	○	○
	Age	○	○	○
	Type of school	○	○	○
	Current School Class	○	○	○
Family Characteristics	Questionnaires			
	Living with who?	○	○	○
	Parents' marital status	○	○	○
	Number of siblings	○	○	○
	Household size	○	○	○
Parents' Demographic Characteristics	Questionnaires			
	Father's characteristics	○	○	○
	Mother's characteristics	○	○	○
School Environment	Questionnaires			
	Presence of convenience stores, including snacks and soft drinks stores within school premises	○	○	○
	Presence of sporting or recreational facilities within the school premises	○	○	○
Nutrition Knowledge of SSB	Knowledge Questionnaire			
	SSBs are high in sugars	○	○	○
	High levels of SSB consumption contribute to dental caries/tooth decay	○	○	○
	High levels of SSB consumption contribute to overweight.	○	○	○
	High levels of SSB consumption contribute to diabetes.	○	○	○
SSB Beverage Consumption Pattern	Adapted Beverage Intake Questionnaire (BEVQ)	○	○	○
Physical Activity	Physical Activity Questionnaire for Older Children and Adolescents	○	○	○
Screen Time Assessment	Questionnaires	○	○	○
Dietary Diversity	Dietary Diversity Scale	○	○	○
Anthropometric measurements	Weighing Scale and Stadiometer			
	Weight (kg)	○	○	○
	Height (cm)	○	○	○
	Mid-upper arm Circumference (cm)	○	○	○
	Waist circumference (cm)	○	○	○
Blood pressure measurements	Electronic Blood Pressure			
	Systolic blood pressure (mmHg)	×	○	×
	Diastolic blood pressure (mmHg)	×	○	×
	Pulse (beats/minutes)	×	○	×
Blood Glucose measurements	Blood Glucometer			
	Blood Glucose (*m*mol/*l*)	○	×	×
	Family History of Diabetes Mellitus	○	×	×

SSB: Sugar-Sweetened Beverages

○: The information was collected at the study site.

×: The information was not collected at the study site.

Questionnaire: Standard pretested questionnaires were self-administered by participants under the guidance of trained personnel to collect information on sociodemographic, socioeconomic, family and parent characteristics. Furthermore, using standardized instruments, participants provided information on nutrition knowledge, school environment, physical activity (Kowalski *et al*., 2004), screen time, and diversity (Food and Technical Assistance (FANTA) III, 2006).

Anthropometric measurements: First, non-stretchable tape was applied to measure participants' mid-upper arm circumference (MUAC), height, and waist circumference to the nearest 0.1 cm. The MUAC was measured at the midpoint between the tip of the shoulder and the elbow tip (olecranon process and the acromium) of the right upper arm (World Health Organization, 2007). Height was measured while participants stood using a stadiometer (wall height chart). Waist circumference was measured at the midpoint, between the lower border of the rib cage and the iliac crest. Also, the weight of participants was taken while participants were in school uniform without wearing shoes using a digital body weighing scale using Gromy (model: PS-2003A, Gromy Scale Co., Ltd) (Scale, 2008) in Abuja, CAMRY (model: EB9015, Camry Electronic Ltd) (SCALES, 2023) in Ikeja and OMRON (model: HN289) (Healthcare, 2024b) in Ibadan. All anthropometric measurements were taken twice by trained personnel, following the standard protocol (WHO, 2020).

Blood pressure measurements: In keeping with standard protocol (Unger *et al*., 2020), participants' blood pressure, including systolic, diastolic, and pulse rates, were measured three times in a sitting position at a 5-minute interval while they were in a resting position using the blood pressure monitors (OMRON M1 Basic, model: HEM-7121J-AF) (Healthcare, 2024a).

Fasting Blood sample collection: A trained phlebotomist used a blood glucometer (Accu-Chek Active, Model GU, Roche Diabetes Care Middle East FZCO) (Roche, 2024) to check participants' blood glucose levels. The participant's index finger was pricked with a needle at the tip. The second or third drop of blood was carefully placed on the green field of the glucometers' strips. The blood glucose results in mmol/L, as displayed on the digital blood glucometer, were recorded according to the standard protocol (S. Karon *et al*., 2008; Singh *et al*., 2019; Roche, 2024).

Focused Group Discussion: In discerning the etiological basis for perceptions, attitudes, and environmental drivers of dietary SSB consumption among adolescents, the study plans to conduct focused group discussions (FGD) in at least two schools (preferably with the highest response rate in private and public schools) per study site using non-probability sampling methods (Hennink and Leavy, 2014). The study investigators would develop an FGD protocol to gather information from participants with sound knowledge of SSB consumption among adolescents through open discussions. Each FGD session will last approximately 60-90 minutes and will be conducted in a neutral, calm and safe environment to ensure autonomy of thought, impartiality and honesty in the discussion by trained personnel using probing questions to elicit accurate information on the aims and study questions. All conversations will be recorded using an audio recorder, transcribed and independently examined before analysis to guarantee accurate transcription of responses. The transcribed data will be analysed using both thematic and content analysis to identify central themes and patterns. All identified themes will then be interpreted and discussed in the context of the study's aims and research questions. All qualitative coding and analysis would be carried out using the Atlas—ti Web version (ATLAS.ti Scientific Software Development GmbH., 2023).

Participant feedback and referral: Generally, health education, including nutrition advisories and information on healthy lifestyles, was provided to all participants. However, parents of participants with arbitrarily high blood pressure or glucose levels were counselled and advised to check with trained physicians for appropriate check-ups, medical diagnoses and intervention.

Quality control, Data management and Statistical analysis: A detailed review of data collection, procedures and methods was conducted to ensure consistency of data collection across study sites. Additionally, thorough data cleaning techniques were employed to ensure the high-quality analysis of data and to guarantee the reliability of the data and its findings. First, all questionnaires were in English, and trained personnel were available to translate them into the most common site-specific language and to assist participants who had a limited understanding of the English language. Second, all questionnaire questions were reviewed with participants on-site to clarify any incongruity in responses before data entry using IBM SPSS Statistics for Windows, version 25 (IBM Corporation, Armonk, NY USA). All questionnaires were stored in a safe lock accessible to the site and principal investigator after data entry. Additionally, multiple imputation procedures are planned to address data missingness, taking into account the missing data apparatus, functionality, and selection of confounders in accordance with data management principles reported elsewhere (VanderWeele, 2019). Data analysis will commence using suitable statistical methods, including suitable bivariate and multivariate statistical techniques contingent on the outcome variable of the hypothesis being tested. All statistical analyses will be performed using IBM SPSS Statistics for Windows, version 25 (IBM Corporation, Armonk, NY USA) and R statistical program (version 3.6.2) at P-value < 0.05.

RESULTS

Overall, the response rate for participants in the study was 74.9%, with rates of 53.2% in Abuja, 86.2% in Ikeja, and 93.2% in Ibadan.

A total of 1,699 in-school adolescents (29.4% from Abuja, 29.4% from Ikeja and 41.1% from Ibadan) representing 58.9% females. The distribution of response rate to each variable and instrument includes ≥97.2% for demographic information, ≥98.4% for household characteristics, and ≥99.2% for family and parent characteristics. Furthermore, the response rates for other scales and instruments, such as nutrition knowledge

(100.0%), school environment (≥98.6%), physical activity (≥96.6%), and screen time (≥67.9%), were also moderately high, as detailed in Table 2.

Sugar-sweetened beverages (SSB) were self-reported by participants, with an overall response rate for SSB was ≥ 84.0%. Physical activity had an overall response rate of at least 96.8%. The overall response rate for anthropometric measures was at least 99.4%. The response rates for blood pressure and glucose measurements were 99.2% and 100.0%, respectively, as this information was primarily assessed in Ikeja and Abuja. Details of the response rate are in Table 2.

Table 2
Response rate, n (%) of critical items and variables in the SURRENDER study

Instruments/Scale /Variable	Total		Abuja		Ikeja		Ibadan	
	Frequency (n)	% Response rate	Frequency (n)	% Response rate	Frequency (n)	% Response rate	Frequency (n)	% Response rate
Participant recruitment	1699	74.9%	500	53.2%	500	86.2%	699	93.2%
Questionnaires								
Survey Information	1332 – 1699	≥ 78.4	500	100.0	268 – 500	≥ 53.6	564 – 699	≥ 80.7
Demographic Characteristics	1652 – 1699	≥ 97.2	500	100.0	465 – 500	≥ 93.0	687 – 699	≥ 98.0
Household Characteristics	1672 – 1695	98.4 – 99.8	500	100.0	483 – 494	96.6 – 98.8	685 – 697	≥ 98.0
Parents' Demographic Characteristics								
Father's characteristics	1686 – 1699	≥ 99.2	500	100.0	497 – 500	≥ 99.4	692 – 699	≥ 99.6
Mother's characteristics	1685 – 1699	≥ 99.2	500	100.0	494 – 497	≥ 98.8	691 – 699	≥ 98.4
School Environment	1675 – 1684	≥ 98.6	500	100.0	486 – 490	≥ 97.2	689 – 694	≥ 98.6
Nutrition Knowledge of SSB	1699	100.0	500	100.0	500	100.0	699	100.0
SSB Beverage Consumption	1427 – 1699	≥ 84.0	500	100.0	205 – 500	≥ 41.1	592 – 699	≥ 84.7
Physical Activity	1644 – 1675	96.8 – 98.6	500	100.0	468 – 500	≥ 93.6	676 – 699	≥ 96.7
Screen Time Assessment	1154 – 1562	67.9 – 91.9	285 – 471	57.0 – 94.2	376 – 462	75.2 – 92.4	493 – 629	70.5 – 90.0
Dietary Diversity Scale	1690 – 1699	≥ 99.5	491 – 500	≥ 98.2	500	100.0	699	100.0
Anthropometric measurements								
Weight (kg)	1690	99.5	500	100.0	500	100.0	690	98.7
Height (cm)	1689	99.4	500	100.0	499	99.8	690	98.7
Mid-upper arm Circumference (cm)	1683	99.1	500	100.0	494	98.8	689	98.6
Waist circumference (cm)	1679	98.8	500	100.0	493	98.6	686	98.1
Blood pressure measurements								
Systolic blood pressure (mmHg)	496	99.2	0	0.0	496	99.2	0	0.0
Diastolic blood pressure (mmHg)	496	99.2	0	0.0	496	99.2	0	0.0
Pulse (beats/minutes)	314	62.8	0	0.0	314	62.8	0	0.0
Blood Glucose								
Blood Glucose (*m*mol/*l*)	500	100.0	500	100.0	0	0.0	0	0.0
Family History of Diabetes Mellitus	500	100.0	500	100.0	0	0.0	0	0.0

SURRENDER: **Su**ga**r**-Sweetened Bever**a**g**e**s Co**n**sumption among A**d**ol**e**scents in Nige**r**ia; SSB: Sugar-sweetened beverages

DISCUSSION

Higher SSB consumption has been linked with poor cardiometabolic health (Lara-Castor *et al*., 2023), and the case for the impact of SSB on cardiometabolic health among adolescents in higher-income settings (Clifton *et al*., 2011; Rosinger *et al*., 2017b; Chatelan *et al*., 2023) has been well documented, with little information on adolescents from low- and middle-income countries like Nigeria. This lack of viable information is a considerable setback for understanding the magnitude and potential drivers of SSB consumption, with poor insights into the likely underlying impact on the cardiometabolic well-being and related complications to help deploy fiscal resources to support preventative interventions and clinical management of the increasingly elusive burden and implications of SSB consumption among adolescents in low- and middle-income countries.

The SURRENDER study will deliver relevant and critical information on SSB consumption and its impact on cardiometabolic risk factors, including obesity, high blood pressure and elevated blood sugar, among in-school adolescents. Similarly, it will provide unique insights into the proximal and distal drivers of cardiometabolic health among adolescents, thereby offering opportunities to draw informed conclusions about the magnitude of SSB consumption and its impact on the cardiometabolic well-being of adolescents. These findings will support healthcare providers and health policy stakeholders with information vital for guiding the distribution of resources and efforts to improve cardiometabolic health among adolescents in Nigeria and, by extension, in low and middle-income countries.

There are some limitations in the design, execution and preliminary findings. First, this is a cross-sectional study, and causal associations cannot be inferred from the results. However, the study is potentially promising, providing critical information to guide fiscal budgeting for addressing adolescent health, especially in a low and middle-income country like Nigeria, where budgetary provision for health is meagre and consideration for adolescent health is non-existent. Secondly, misclassification bias is likely, especially with self-reported information on some sections of the instruments for data collection. Still, the meticulous efforts to thoroughly review all questionnaires to resolve all incongruities minimized this bias. Although the statistical threshold for participant recruitment was met at all study sites, the non-response rate in Abuja was very low, primarily due to the inability of parents and guardians to sign consent forms for potential participants in the study.

Thirdly, the adapted BEVQ used to assess dietary exposure was limited to SSB consumption only and has yet to be validated. Validation studies are necessary to ensure the reflection of long-term diet exposure and the reliability of the information collected. However, this might not significantly impact the study's findings because adolescents typically exhibit good memory and are able to provide accurate information during their growth spurt. Similarly, study findings are promising to discern the overall consumption of dietary and lifestyle exposures in the pathophysiology of adolescent health outcomes. In addition, this study holds promise for extending the frontiers of understanding adolescent health in low- and middle-income countries, thereby complementing the global literature on adolescents with sound information and well-informed science worldwide.

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Authors' contributions

IAA and APO were the principal investigators and conceptualized the study; AMO, SIO and MHB were study investigators; SAO was responsible for training study investigators and oversight of the data collection at the study sites; SAO and APO supervised the data acquisition; DKD and APO managed the qualitative methodology for the study, IJO, SAO and OJA were the data managers and responsible for data curation; IJO, SAO, and APO drafted the manuscript; APO and IAA critically revised the manuscript for important intellectual content. All authors read, contributed to the interpretation, approved the final version to be published and agreed to be accountable for the work.

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