

Review Article

# Gestational Nutrition as a Predisposing Factor to Obesity Onset in Offspring: Role for Involvement of Epigenetic Mechanism

**\*Jeje, S.O., Adenawoola M. and Abosede C.**

*Department of Physiology, School of Health and Health Technology,  
Federal University of Technology, Akure, Nigeria*

**Summary:** Maternal lifestyle has been implicated as a predisposing factor in the development of metabolic disorders in adulthood. This lifestyle includes the immediate environment, physical activity and nutrition. Maternal nutrition has a direct influence on developmental programming through biochemical alterations in maternal metabolism and can lead to modifications in the fetal genome through epigenetic mechanisms. Imbalance in basic micro or macro nutrients due to famine or food deficiency during critical gestational periods can lead to onset of metabolic syndrome including obesity. A major example is the Dutch famine which led to a serious metabolic disorder in adulthood of affected infants. Notably due to gene variants, individualized responses to nutritional deficiencies are unconventional, therefore intensifying the need to study nutritional genomics during fetal programming. Epigenetic mechanisms can cause hereditary changes without changing the DNA sequence. The major mechanisms include small non-coding RNAs, histone modifications and most stable of all is DNA methylation. The significance association between obesity and DNA methylation is through regulation of genes implicated in lipid and glucose metabolism either directly or indirectly by hypomethylation or hypermethylation. Examples include CPT1A, APOA2, ADRB3 and POMC. Any maternal exposure to malnutrition or overnutrition that can affect genes regulating major metabolic pathways in the fetus, will eventually cause underlying changes that can predispose or cause the onset of metabolic disorder in adulthood. In this review, we examined the interaction between nutrition during gestation and epigenetic programming of obesity.

**Keywords:** Epigenetic, Fetal programming, Gestation, Nutrition, DNA Methylation

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\*Address for correspondence: [sojeje@futa.edu.ng](mailto:sojeje@futa.edu.ng); +23408023860829

Manuscript received- June 2021; Accepted- December 2021

DOI: <https://doi.org/10.54548/njps.v37i1.1>

## INTRODUCTION

Diets eaten by women before, after, and most especially during pregnancy have been known over time to influence the body system of their offspring such as metabolism and even reproductive abilities (Taylor *et al.*, 2014). Extensive epigenetic programming and reprogramming take place during embryogenesis (Muktabhant *et al.*, 2012) with maternal nutrition playing a key role (Malgorzata *et al.*, 2010) (Figure1).

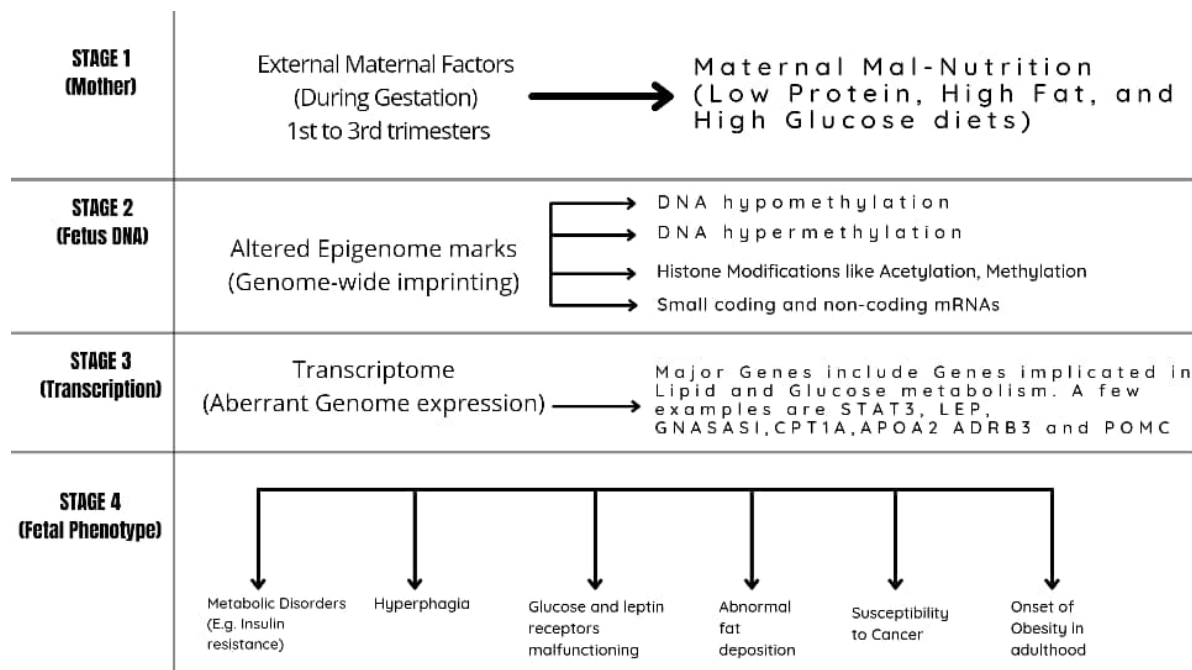
Epigenetics is the study that explains the modification of gene expressions without changes in DNA sequence (Marsal 2002). These expressions can be brought about by different modifying reactions within the genome that is largely attributed to the diet of an individual although can be caused by other factors (Gallou-Kabani and Junien 2005; Dolinoy *et al.*, 2007; Liyanage *et al.*, 2014). Epigenetic changes include short-term histone methylation, acetylation, phosphorylation, ubiquitination and longer-term DNA silencing as a result of DNA methylation (Palou *et al.*, 2010; Martinez *et al.*, 2014). These epigenetic changes are known to be transferable, and in response to in-utero conditions can induce permanent changes to the metabolic systems and functions (Palou *et al.*, 2010; Devlin and Buxseine 2012),

consequently leading to increased susceptibility to corresponding non-communicable diseases (Warner *et al.*, 2010; Innis 2011).

Developmental programming and changes that occur during the process in its entirety is a major and important scientific interest which has been able to give some explanations to the early adult onset of non-communicable diseases (Figure 1). This has especially helped prenatal nutritional prescriptions and regulations for a healthy community. This review aims to summarize contemporary studies of the epigenetic changes brought about by gestational nutrition that can predispose to metabolic abnormalities such as obesity in offspring..

## Nutritional Status and Developmental Programming

It was believed that fetal growth and development were driven by inherited traits from its parent's genome (Marsal 2002; Myatt 2006), our current understanding of epigenetics mechanisms has shown the need for better understanding of the science of fetal programming. For example, maternal malnutrition, or increased maternal nutritional demand can lead to fetal undernourishment (Marsal 2002; Myatt 2006).

**Figure 1:**

Schematic presentation of interaction between maternal environment and epigenetic programming of non-communicable diseases

Excessive supply of nutrients to the fetus is also detrimental. Fetal overgrowth (macrosomia) can occur due to excessive placental transport of glucose and other nutrients from the mother (Freinkel 1980). During the critical periods of fetal development especially the first and second trimester of gestation as shown by studies from the Dutch famine (Selassie and Sinha 2011), any stimulus such as undernutrition or overnutrition may cause fetal response and adaptation, leading to long-term or permanent changes in the structure or function of the offspring's body. These changes in later life of the offspring often predispose such offspring to adult onset Non-Communicable Diseases (NCDs) such as obesity (Lukas 1991; Ozanne *et al.*, 2004). These changes do not distort the normal DNA sequence but they alter the epigenetic state of the fetal genome (Gallou-Kabani *et al.*, 2005).

### Maternal Malnutrition and Adult-Onset of Obesity in offspring

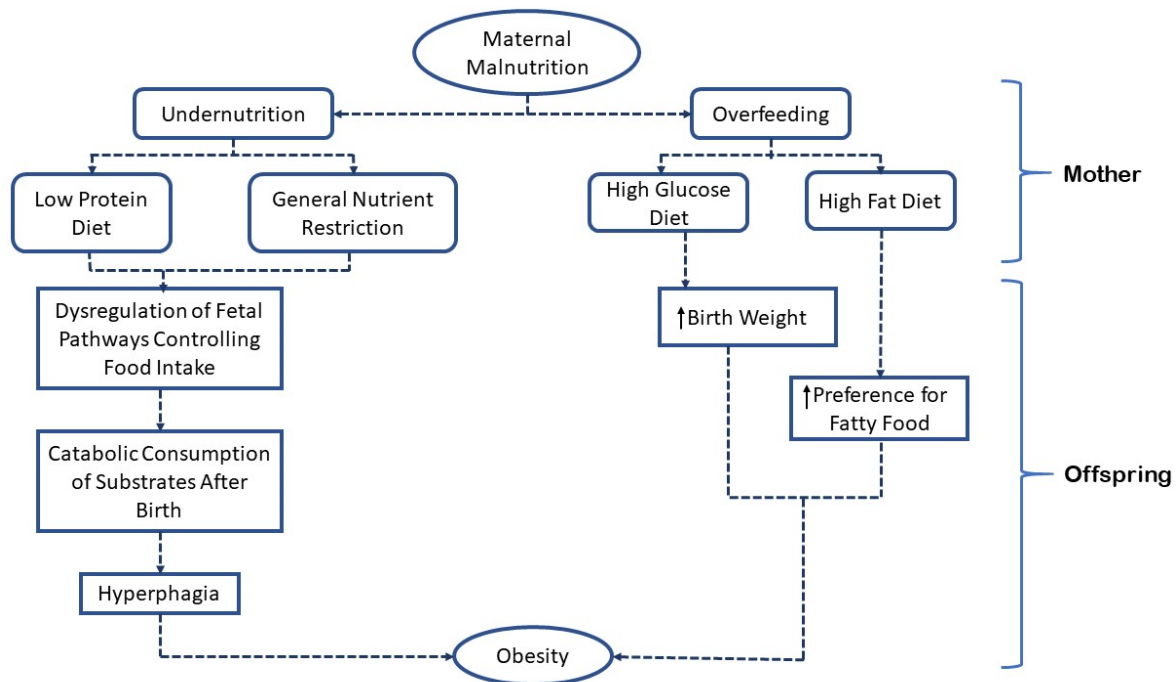
Maternal malnutrition which includes undernutrition and overnutrition can epigenetically and evidently make significant changes in the physiology of the offspring's metabolism. Although this is still controversial in the scientific space, research has shown that factors such as low-protein diet or high-fat diet during gestation can cause adult onset obesity in the offspring (Zhao *et al.*, 2012). A meta-analysis of fifteen epidemiological and clinical studies involving a total of ~22,000 individuals found that higher, but not lower, birth weight which is usually as a result of the mother's gestational overnutrition is associated with an increased risk of being overweight or obese (Zhao *et al.*, 2012). Considering that nutritional deficiency has varying effects on adult body weight, depending on its timing within gestation (Hyttén & Chamberlain 1980; Gernand *et al.*, 2016). Discrepancies in the epidemiological literature may be associated with differences in onset, duration and type of nutrient-deprivation.

### Effect of Low Protein Diet or Protein Deficiency

During pregnancy, it is important to consume the required amount of protein, which is the basic building block of maternal and fetal tissues. The required amount of protein needed during gestation is about 0.8-1.5 g/kg per day. As discussed above, human growth restriction early in gestation increases the probability of developing obesity in later life, particularly if combined with rapid compensatory growth after birth (Tian *et al.*, 2006; Dolan *et al.*, 2007; Chen *et al.*, 2012). Studies in experimental animals such as rodents and sheep reproduce these findings (Jimenez-Chillaron *et al.*, 2006; Desai *et al.*, 2007). If protein deficiency is prolonged, the fetus changes its metabolic rate and alter the production of hormones and the sensitivity of tissues to them (Figure 2).

### Gestational High-Fat Diet

Weight gain and adiposity as a result of fetal exposure to a maternal obesogenic diet can be attributed to hyperphagia and change in food preference (Wardle *et al.*, 2001). The child might tend to eat more and enjoy more of such diet because Children from obese/overweight families also have a higher preference for fatty foods (Wardle *et al.*, 2001). As stated above, offspring's resistance to hormones such as leptin which is responsible for metabolic regulations might predispose offspring to obesity through hyperphagia. It has been reported that altered maternal nutrition can programmed leptin resistance in the offspring (Vickers and Sloboda, 2012). In contrast to nutrient deficiency, global nutrient excess and obesity is emerging as one of the greatest health problems in the industrialized world (Selassie & Sinha 2011). More than half of all pregnant women in the US are considered obese, with 8% considered extremely obese (Stüber *et al.*, 2015).

**Figure 2:**

Schematic presentation of interaction between maternal Nutrition and obesity in the offspring.

## INHERITANCE OF METABOLIC COMPLICATIONS LEADING TO OBESITY

**Hyperphagia:** This is an abnormal desire or appetite for food that often leads to overeating. When the fetus is exposed to nutrient restriction during gestation, this might lead to abnormal hunger sensation and desire for food later after birth (Parlee *et al.*, 2013). This can be caused through the dysregulation of central pathways controlling food intake (Yura *et al.*, 2005; Desai *et al.*, 2007; Ikenasio-Thorpe *et al.*, 2007). The system basically programs the baby for an environment with limited nutrients and requires the need to always store enough when available. This is obviously not done by the formation of bigger gastrointestinal tract or bigger storage organs from birth but an alteration in the workings of cells responsible for the control of metabolic pathways (Desai *et al.*, 2007). For example, there may be resistance to anorexigenic hormones when the child is later born into an environment with enough and regular food source. The child could have lesser feeding habit control because of a possible epigenetic modification during programming. Hyperphagia will usually later lead to obesity (Parlee *et al.*, 2013). A particular research shows that at birth, pups exposed to maternal global nutrient-deficiency have reduced hypothalamic phosphorylation of STAT3 in response to leptin (Desai *et al.*, 2007). Following compensatory growth to three weeks of age, phosphorylation of STAT3 and reduction in food intake in response to leptin are both impaired (Desai *et al.*, 2007). During postnatal development, a surge in circulating leptin is associated with hypothalamic growth. Leptin resistance increases density of the hypothalamic neurotransmitter's neuropeptide Y, and cocaine and amphetamine regulated transcript, and causes hyperphagia (Yura *et al.*, 2005; Ikenasio-Thorpe *et al.*, 2007).

**Epigenetic mechanisms:** The main epigenetic mechanisms are DNA methylation, histone modifications and small non-coding RNAs. These types of modifications play an important role in gene expression regulation across vast biological processes at the level of chromatin structure and organization (Liyanage *et al.*, 2014). Epigenetic changes can give rise to transgenerational inheritance, which can be carried through both male and female germline.

DNA methylation is the most studied mechanism due to its stable epigenetic system. It occurs by addition of a methyl group at the 5- position of cytosine residues, mainly within CpGs, 60–80% of which are methylated within the promoter regions of genes. In most instances, hypermethylated DNA regions act to reduce gene expression and vice versa (Dolinoy *et al.*, 2007). Most DNA methylation states are stably maintained and inherited during cell division by the maintenance methyltransferases (DNMT1). These marks are critical for maintaining the physiological differentiated states of tissues and organs. Furthermore, DNMT3A, DNMT3B and co-factor DNMT3L are *de novo* DNA methyltransferases (DNMTs) which methylate DNA during embryogenesis and in differentiated cells. Other mechanisms able to affect DNA methylation exist. In fact, the methyl group on the fifth carbon of the cytosine residue within the CpG can be oxidized by the ten-eleven translocation (TET) dioxygenase family, creating the 'sixth base' defined as 5-hydroxymethylcytosine (5hmC) (Dolinoy *et al.*, 2007; Liyanage *et al.*, 2014) which are essential in regulatory functions (Auclair & Weber 2012). Histone modifications is a dynamic and unstable process, it can be easily induced and removed by many enzymes (Kouzarides, 2007). These changes can determine the DNA exposure to transcription factors due to winding and unwinding ability of the histones. This plays a major role in the gene expression regulation (Lawrence *et al.*, 2016).

Lastly, microRNAs (miRNAs) are endogenous small non-coding RNAs, playing an important role that affects gene expression in many biological processes like development, differentiation and cell cycle (Esguerra *et al.*, 2014) and immune system homeostasis (Wilczynska & Bushell 2015). This mechanism has been implicated in insulin secretion regulation, beta-cell differentiation (Kaspi *et al.*, 2014) and a new biomarker of several diseases (Ibarra *et al.*, 2018; Guarino *et al.*, 2018).

Some of these epigenetic modulations occur in vital non-imprinted genes that are involved in energy metabolism (Weinstein *et al.*, 2010). Genes that regulate adipogenesis, glucose homeostasis, inflammation, and/or insulin signaling are regulated by epigenetic mechanisms (Yang *et al.*, 2011), including genes encoding hormones (e.g., leptin) (Milagro *et al.*, 2009), nuclear receptors (adipogenic and lipogenic transcription factors PPAR $\gamma$  and PPAR $\alpha$ , respectively (Fujiki *et al.*, 2009) gluconeogenic enzymes (e.g., phosphoenolpyruvate carboxykinase, PEPCK) and transmembrane proteins (e.g., uncoupling protein 1) (Stepanow *et al.*, 2011).

### Maternal Nutrition and Offspring's Epigenomics

There are critical periods during development such as periods of pre-conception, oocyte insemination, gestation and first few infant years, during which tissues are overly sensitive to environmental exposures and lifestyle. Exposure to such environmental alterations impart on the tissues susceptibility to disease in the future (Blackmore & Ozanne 2013; Heindel *et al.*, 2015). Several studies have shown how epigenetic changes induce lifetime effects on offspring exposed to different aberrant maternal nutrition and lifestyle (Gagné-Ouellet *et al.*, 2017). This has made analysis of epigenetic modifications occurring during pregnancy an important topic in the study of environmental influence on fetal metabolic programming (Nolan *et al.*, 2011).

### Epigenetics and Programmed Obesity

The effects of perinatal malnutrition on babies were studied through the Dutch famine in 1994/95. Mothers exposed to the famine during the first two trimesters of pregnancy had offspring with lower birth weights. These low-birth weight infants rapidly grew in the first several years of life and had higher risk of adult obesity and metabolic syndrome (Monteiro & Victora 2005). The fact that maternal overnutrition induced fetal programming is beginning to gain momentum, specifically the association of maternal pre-pregnancy obesity and increased weight gain with higher birth weight in newborns (Oken *et al.*, 2008). This has been reported to leads to an increased risk of diabetes and obesity in the later life (Armitage *et al.*, 2008). Low birth weight in animal models can be induced using varieties of methods such as maternal nutrient restriction, placental uterine ligation or glucocorticoid exposure, leading to an increased risk of offspring adiposity (McMillen & Robinson 2005). Also, recent animal models of maternal over nutrition, including maternal obesity and high fat diets, similarly replicate human experience in the offspring and predispose to adult obesity (Howie *et al.*, 2009). Programmed changes occur during developmental period of growing fetuses and neonates, making them vulnerable to perturbations of the maternal nutritional and non-nutritional

milieu. These programmed changes affect organ structure, cellular responses and gene expression that impact metabolism and physiology of the offspring (Desai *et al.*, 2015).

As the association was specific for periconceptional exposure it was concluded that the periconceptual period is crucial for establishing and maintaining epigenetic marks (Heijmans *et al.*, 2008). Several genes including *INSIGF2*, *GNASAS1*, *MEG3*, *IL-10*, and *LEP*, some of which have a known role in metabolic disorders, were identified to exhibit an altered DNA methylation in blood cells of the offspring of mothers exposed to famine (Tobi *et al.*, 2009). However, nowadays most populations are confronted with an obesogenic environment and prenatal and neonatal overfeeding programs disposition to obesity.

### DNA Methylation Markers in Obesity

Numerous cross-sectional studies have reported a significant association between adiposity status and DNA methylation. For example, the offspring of obese mothers presented several CpG sites differentially methylated in cord blood in comparison with offspring from normal weight mothers (Sharp *et al.*, 2015). Other regions studied, were mainly hypomethylated in obese children located in the gene body region, and revealed a unique cluster of obese individuals that was differentiated from the normal weight children (Rhee *et al.*, 2017). In addition, some of these genes are implicated in lipid metabolism, glucose metabolism, differential body size and body composition in children (Rzehak *et al.*, 2017).

Another analysis in adult population found methylation changes in carnitine palmitoyl-transferase 1A (CPT1A) gene, associated with obesity and T2D (Aslibekyan *et al.*, 2015). CPT1A is also implicated in the control of fasting triglycerides (TG) and very low-density lipoprotein (VLDL) levels (Irvin *et al.*, 2014). These studies suggest an important role for this gene in obesity and metabolic complications. A study in three populations of diverse ancestries found that the methylation levels of the apolipoprotein A2 (APOA2) regulatory region was associated with the consumption of saturated fatty acids (Pauwels *et al.*, 2017), a nutritional factor that has been associated with an increase in the risk of obesity in previous studies. Not only is the methylation profile of blood cells associated with obesity, but also the methylation of other tissues and body fluids.

An Epigenome Wide Association Study (EWAS) identified predominantly DNA hypermethylation in White Adipose Tissue (WAT) from obese subjects, which was related to gene expression of proinflammatory pathways. These findings suggest that DNA methylation may link dysfunctional adipocytes to WAT inflammation and Insulin Resistance (IR) in obesity (Petrus *et al.*, 2017). Hypermethylation of the beta-3 adrenoceptor (ADRB3) gene was also reported in WAT of obese subjects, which was linked with an increased susceptibility to visceral obesity and altered body fat distribution (Guay *et al.*, 2014). Similar results were previously described in blood cells (Guay *et al.*, 2014), revealing that the methylation changes of ADRB3 gene in blood may reflect obesity-related DNA methylation changes of this gene in WAT.

In addition, a case-control study demonstrated an association between hypomethylation of leptin (LEP) gene

in obese individuals, measured in saliva, and obesity-related parameters (Dunstan *et al.*, 2017). Likewise, an EWAS performed in 92 children's saliva samples described 17 CpGs associated with maternal BMI (Oelsner *et al.*, 2017). The saliva sample analysis in 50 girls with and without obesity found two interesting genes, neuron navigator 3 (NAV3) and melanocortin 2 receptor (MC2R), whose methylation levels were associated with BMI (Rounge *et al.*, 2016). These studies evidenced that saliva may be a viable probe for epigenetic testing in obesity. However, further studies would have to include both, saliva and blood samples, to demonstrate that saliva is consistent with whole blood findings. Prospective studies during the early life stage described DNA methylation of several weight linked loci in newborns that continued to show a longitudinal association with adiposity, fat mass, body size and other obesity parameters in childhood (Van Dijk *et al.*, 2018; Lin *et al.*, 2017; Perng *et al.*, 2013). Interestingly, several prospective studies including adult life stage evidenced that these DNA methylation pattern changes were associated with the later risk of developing metabolic and other diseases (Dayeh *et al.*, 2016; Dayeh *et al.*, 2018). These examples described an association between early cues (environmental stimulation in utero, and in early infancy) and the later development of disease. Actually, this is the basis of the 'epigenotype model' of Developmental Origin of Health and Diseases (DOHaD), in which the environment can modulate the epigenetic signature during human life (Sambas *et al.*, 2019).

### Pomc Gene

Epigenetic alterations in the *Pomc* gene, known for its important role in the regulation of food intake, were first observed in rats and later confirmed in human. Neonatal overfeeding of rats led to rapid early weight gain and the metabolic syndrome, which was associated with a hypermethylation of the *Pomc* promoter and the inability of a *Pomc* upregulation in response to elevated leptin and insulin concentrations (Plagemann *et al.*, 2009). Accordingly, in postmortem human melanocyte-stimulating hormone (MSH)-positive neurons' alterations in DNA methylation of the *POMC* gene were strongly associated with individual body mass index (BMI). Epigenetic alterations of *POMC* appeared to be established in the early embryo and offspring methylation correlated with the paternal somatic methylation pattern (Kuhnen *et al.*, 2016).

### CONCLUSION

In conclusion, understanding the concept of developmental programming and the epigenetic basis of metabolic syndrome (such as obesity) could provide an exciting window of opportunity to prevent its development at origin or treat postnatally to reduce and control the progression of the disease. According to reports from the various studies, maternal nutrition during gestation could epigenetically modify the DNA and as a result alter nutrient metabolism in the offspring.

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Full-Length Research Article

# A Novel Gedunin-2-hydroxypropyl- $\beta$ -cyclodextrin Inclusion Complex Improves Anti-nociceptive and Anti-inflammatory Activities of Gedunin in Rodents

\*Ologe, M.O.<sup>1,2</sup>, Ajayi, A.M.<sup>2</sup>, Ben-Azu, B.<sup>2,3</sup>, Iwalewa, E.O.<sup>2</sup>, Ademowo, O.G.<sup>2,4</sup>

<sup>1</sup>Department of Pharmacology and Therapeutics, University of Ilorin, Ilorin, Nigeria.

<sup>2</sup>Department of Pharmacology and Therapeutics, University of Ibadan, Ibadan, Nigeria.

<sup>3</sup>Department of Pharmacology, Delta State University, Abraka, Nigeria.

<sup>4</sup>Institute for Advanced Medical Research and Training, University of Ibadan, Ibadan, Nigeria.

**Summary:** Gedunin is a bioactive compound, obtained from *Entandrophragma angolense* (EA), which has limited therapeutic usefulness due to poor aqueous solubility and first-pass effects. Cyclodextrins are cyclic oligosaccharides that form complexes with poorly soluble compounds, thus enhancing their pharmacological activity. In this article, we evaluated the pharmacological activities of gedunin-2-hydroxypropyl- $\beta$ -cyclodextrin complex (GCD) in rodents. The antinociceptive activity of GCD (50, 100, 200 mg/kg) and Gedunin (50mg/kg) was tested in acetic acid-induced writhing and formalin-induced paw licking in mice. The anti-inflammatory activity was investigated in carrageenan-induced paw oedema and air pouch inflammation models in rats. Leucocyte counts, Tumour Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) level, nitric oxide, malondialdehyde, reduced glutathione, and myeloperoxidase enzyme activities were assessed in the air pouch exudate. The GCD (200mg/kg) significantly decreased writhing response, reduced licking duration and decreased oedema compared with gedunin and control. Exudate volume and leucocyte count were significantly reduced by GCD (200 mg/kg), it decreased myeloperoxidase activity and inhibited TNF- $\alpha$  release. The carrageenan-induced GSH depletion, increased malondialdehyde and nitrite levels were significantly reversed by GCD (200 mg/kg) relative to gedunin and control. The GCD complex demonstrated significant antinociceptive and anti-inflammatory activities relative to gedunin alone via mechanisms associated with inhibition of oxidative stress and inflammation in rodents.

**Keywords:** anti-inflammatory, anti-nociceptive, gedunin, gedunin-2-hydroxypropyl- $\beta$ -cyclodextrin

©Physiological Society of Nigeria

\*Address for correspondence: [funmiologe@yahoo.com](mailto:funmiologe@yahoo.com); +2348033979931

Manuscript received- February 2022; Accepted- April 2022

DOI: <https://doi.org/10.54548/njps.v37i1.2>

## INTRODUCTION

Cyclodextrins are structurally related natural compounds produced when cellulose is digested by bacteria. Chemically, they are non-toxic macrocyclic biodegradable oligosaccharides with at least 6 D-(+) glucopyranose connected by  $\alpha$ -(1, 4) glucosidic linkages. They are shaped like a truncated cone rather than ideal cylinders and have a lipophilic center cavity and a hydrophilic outside surface (Brewster and Loftsson, 2007; Cusola *et al.*, 2013; Gidwani and Vyas, 2015). Cyclodextrins are capable of interacting with a vast variety of guest molecules to produce non-covalent inclusion complexes (Wankar *et al.*, 2020). The synthesis of inclusion complexes improves the physical, chemical, and biological characteristics of guest compounds in the cyclodextrin cavity's hydrophobic environment (Carneiro *et al.*, 2019). The most common use of cyclodextrins in pharmaceuticals is to improve solubility of drugs in aqueous solutions (Ueda *et al.*, 2021). A reduction in variability of oral drug absorption can then be achieved through enhancement of drug dissolution rate as a result of improved aqueous drug solubility by cyclodextrin complexation (Wankar *et al.*, 2020). This leads to an

improvement in drug bioavailability. Furthermore, complexation can increase the stability of the therapeutic substance preventing degradation or bioconversion at the absorption site (Otero-Espinar *et al.*, 2010). Other benefits of cyclodextrin complexation include the ability to mask unpleasant taste or color, fewer side effects, and the possibility of a drug release system. These have been thoroughly researched and documented in literature. Several investigations have shown that improved bioavailability associated with cyclodextrin complexation usually leads to an increase in the guest compound's pharmacological activity.

The *in vitro* physico-chemical characterization and *in vivo* analgesic and anti-inflammatory studies performed showed that the complexation process enhanced the aqueous solubility and dissolution of meloxicam. In addition, the meloxicam-cyclodextrin complex had greater gastrointestinal tolerability, much faster onset of action, and better efficacy (Bandarkar *et al.*, 2013). The evaluation of the antinociceptive effect of tetracaine complexes, using the intraorbital nerve blockade test in rats, revealed that complexation with  $\beta$ -cyclodextrin ( $\beta$ -CD) and 2-hydroxypropyl- $\beta$ -cyclodextrin (HBD) increased the

analgesic duration and intensity induced by tetracaine (Franco de Lima *et al.*, 2012). Indomethacin, etoricoxib and etodolac cyclodextrin complexes exhibited improved activity in the tail-flick and hot plate analgesic tests (Sinha and Amita Goel, 2010; Singh *et al.*, 2011; El-Feky *et al.*, 2013). The onset of action and hypoglycaemic effect was greater for  $\beta$ -CD and HBD complexes of gliquidone than gliquidone alone due to improved biopharmaceutics (Miro *et al.*, 2004). The inclusion of bupivacaine and ropivacaine in HBD lowered myotoxicity and improved the anesthetic effect following injection in the subarachnoid space or after sciatic nerve blockade, compared to free uncomplexed anaesthetic solutions at the same concentration (Dollo *et al.*, 1998; Dollo *et al.*, 2000; Araujo *et al.*, 2005; Araujo *et al.*, 2006; Araujo *et al.*, 2008; Cereda *et al.*, 2012).

Gedunin is a limonoid found in the Meliaceae family of plants (Braga *et al.*, 2020). These plants have historically been utilized as fever remedies in Africa, India, and South America. Gedunin is highly effective against *Plasmodium falciparum* *in vitro* (Khalid *et al.*, 1989; Bray *et al.*, 1990; Bickii *et al.*, 2000). It has shown anti-inflammatory and antiproliferative effects *in vitro* (Brandt *et al.*, 2008; Ravangpai, *et al.*, 2011). Poor solubility in water and short half-life have hampered gedunin's pharmacological efficacy following oral administration. Spectrophotometric studies of a novel gedunin-2-hydroxypropyl- $\beta$ -cyclodextrin binary system revealed the formation of a stable inclusion complex between gedunin and HBD at room and body temperatures (Ologe *et al.*, 2016). Inclusion complex formation was supported by the physico-chemical analysis of this binary system (Ologe *et al.*, 2021). The formation of an inclusion complex between gedunin and HBD is expected to improve the solubility of gedunin in aqueous solutions as well as increase its bioavailability. The projected increase in bioavailability should improve gedunin's pharmacological activity *in vivo*. Thus, the present study was aimed at evaluating the antinociceptive and anti-inflammatory activities of gedunin (Ged) and gedunin-2-hydroxypropyl- $\beta$ -cyclodextrin (GCD) in rodents.

## MATERIALS AND METHODS

**Drugs and Reagents:** Gedunin (Ged) was isolated from the heartwood of *Entandrophragma angolense* Welwitsch C.D.C (Meliaceae) (Okhale *et al.*, 2012). Sigma-Aldrich Chemical Company (Japan) supplied 2-hydroxypropyl- $\beta$ -cyclodextrin. Sodium nitrite, carrageenan (type 1), sodium nitroprusside, O-dianisidine, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, indomethacin and 5,5'-dithio-bis-(2-nitrobenzoic acid) were manufactured by Sigma Aldrich (Steinheim, Germany). Ethanol analytical grade and sodium nitrite were produced by BDH Chemicals Ltd, Poole, England. Mouse TNF- $\alpha$  ELISA MAX<sup>TM</sup> Deluxe set from Biolegend (San Diego, USA). Other reagents used were analytical-grade.

**Preparation of solid inclusion complex:** Ged and HBD solid complex was produced by freeze drying at a molar ratio of 1:1 based on the stoichiometric ratio determination results (Ologe *et al.*, 2016). The HBD was dissolved in distilled water and an equimolar amount of Ged, dissolved in 95% ethanol, was added. The suspension was shaken at 37°C for 6 h. The resulting solution was kept in a -20°C

freezer and lyophilized in a freeze-dryer (LTE Lyotrap Plus, UK) for 24 h (Ologe *et al.*, 2021). The freeze-dried powder was kept in a dessicator in a sealed glass vial.

**Experimental animals:** Male and female Swiss albino mice (Vom strain, 25–30 g), as well as male and female Wistar rats (150–200 g), were obtained from the Animal House at the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Nigeria. The animals were allowed to acclimatize for one week at room temperature of  $28 \pm 2$  °C, relative humidity of 60%–70%, 12:12 h light: dark cycle. All animals were given unlimited access to water and commercial rat food pellets (Ladokun Feeds Ltd., Ibadan, Nigeria). All experiments were done in line with the principles of laboratory animal care” (NIH publication No. 85-23) guidelines and protocols, which were approved by the Animal Care and Use Research Ethics Committee of the University of Ibadan (UI-ACUREC/App/2015/074).

## Antinociceptive and anti-inflammatory tests

**Acetic acid-induced writhing test:** Acetic acid-induced writhing in mice was done as previously reported (Koster *et al.*, 1959). Mice (25–30 g) were assigned randomly into six groups of five mice each. Group 1 received 10% propylene glycol (10 ml/kg). The gedunin-2-hydroxypropyl- $\beta$ -cyclodextrin inclusion complex (GCD) were administered to Groups 2, 3 and 4 at 50, 100, and 200 mg/kg respectively and group 5 received Ged alone (50 mg/kg). Group 6 received indomethacin (10 mg/kg). The vehicle, standard and test drugs were administered orally 1 h before nociception was induced with intraperitoneal injection of 10 ml/kg of 0.6% acetic acid. The number of writhes were counted for 15 min five minutes after the administration of acetic acid.

**Formalin-induced paw licking test:** Formalin test was conducted using recognized methods (Hunskar and Hole, 1987). Mice (25–30 g) were randomly assigned to groups and treated orally as described for the acetic acid-induced writhing test above. Nociception was induced 1 h later by injecting 20  $\mu$ l of 2.5% formaldehyde solution in the sub-plantar region of the left posterior paw. The duration of paw licking and/or biting the injected paw was a measure of the painful response and it was determined at 0–5 min (early phase, neurogenic) and 20–30 min (late phase, inflammatory) after injecting the noxious agent.

**Tail immersion test:** Hot water-induced tail withdrawal reflex was also used as a model of nociception (D'Amour and Smith, 1941; Barrot, 2012). Mice (25–30 g) were assigned into groups and treated orally as described in acetic acid-induced writhing test above. The tail of each animal (up to 5 cm) was immersed in water at  $55.0 \pm 0.2$ °C. The latency time (in seconds) for removal of the animal's tail from the stimulus was taken as the reaction time to pain. A cut-off time of 20 seconds was applied to avoid tissue damage. The tail withdrawal latencies were determined at 0, 1, 1.5 and 2 h after the drug administration.

**Hot plate test:** A thermostatically controlled heated metal plate (Ugo Basile, Italy) within a restraining perspex

cylinder was used for the hot plate test (Eddy and Leimbach, 1953; Franzotti *et al.*, 2000). The hot plate apparatus temperature was kept at  $55 \pm 0.5^\circ\text{C}$ . Mice (25-30 g) were randomly allotted into six groups of five mice each and treated orally as described earlier. The reaction time of each mouse was assessed at 0, 60, 90 and 120 minutes after drug administration using a 15-second cut-off period time. The differences in reaction time between treated and control groups were compared.

**Carrageenan-induced rat paw edema inflammation model:** Rats (weighing 150 – 200g) were randomly allotted into six groups and pre-treated orally, with vehicle (10 ml/kg, 10% propylene glycol), GCD (50, 100 and 200 mg/kg), Ged 50 mg/kg and indomethacin (5 mg/kg) for three days. After 1 h of last treatment, 100  $\mu\text{l}$  of 1% w/v carrageenan (Sigma, Type 1) was injected into the rat hind paw under the subplantar aponeurosis, to induce acute pedal inflammation (Winter *et al.*, 1962). The volumes of the injected and contralateral paws were measured using a Ugo Basile (7150 model) digital plethysmometer (Comerio, VA, Italy) at 1, 3, and 5 hours after inducing edema. For each animal, the edema volume was calculated as the difference between the contralateral and carrageenan-injected paws.

**Carrageenan-induced air pouch inflammation model:** The formation of air pouches was induced in rats with minor modifications (Sedgwick and Lees, 1986; Martin *et al.*, 1994). The air pouches were formed by injecting 20 ml of sterile air subcutaneously into the intra-scapular area of each rat's shaved back after anaesthesia with intraperitoneal ketamine (100 mg/kg). The created air pouches were preserved by re-injection of 10 ml of sterile air into the cavity 72 h later, followed by oral administration of vehicle (10 ml/kg) to Groups 1 and 2, GCD (50, 100 and 200 mg/kg) to groups 3, 4 and 5, Ged 50 mg/kg to group 6 and indomethacin (5 mg/kg) to group 7. The vehicle, GCD, Ged, and indomethacin were administered to the rats orally (like described) for three consecutive days before inflammation was induced. On day 6, 1 h after the oral administration of the test agents, standard drug and vehicle, 2 ml of sterile normal saline was injected into the air pouch of the saline group (group 1) while 2 ml of 1% carrageenan (Sigma, Type I) was injected into the pouch of groups 2–7 to generate an inflammatory response.

The rats were subjected to ether inhalation anaesthesia 24 h after saline or carrageenan injection. Normal saline (2 ml) was injected into the pouch, of each animal, before a small incision was gently made in the pouch wall, and the contents of the air pouch was carefully collected into a sterile tube. The total volume of collected exudate was calculated. The exudate cells were separated in a centrifuge at 10,000 rpm for 10 min at  $4^\circ\text{C}$ . The leukocytes were counted after staining with Turk solution in a Neubauer chamber under a light microscope with a  $\times 40$  objective lens (Nikkon Eclipse E200, USA). Analyses for tumor necrosis factor (TNF), reduced glutathione (GSH), nitrites, thiobarbituric acid reactant substance (TBARS/MDA) and nitrite were done on the cell-free exudate supernatant that had been aliquoted and stored at  $-80^\circ\text{C}$ . A part of the pouch lining was fixed in 10% neutral buffered formalin, processed into 4  $\mu\text{m}$  section and stained with hematoxylin and eosin (H & E) for light microscopic examination.

Another portion of the pouch lining was assayed for myeloperoxidase (MPO) activity.

### Biochemical assays

**Myeloperoxidase assay:** The MPO activity in pouch lining tissue was assessed using established procedure (Bradley *et al.*, 1982). The tissue was homogenized in 50 mM potassium phosphate buffer (pH 6.0) and centrifuged at 10,000 rpm for 15 min at  $4^\circ\text{C}$ . The pellet was suspended in extraction buffer (0.5% hexadecyltrimethyl ammonium bromide in 50 mM potassium phosphate buffer, pH 6.0) and frozen at  $20^\circ\text{C}$ . The frozen suspension was thawed and sonicated (for 10 seconds). The freezing, thawing and sonicating process was repeated thrice before centrifuging it at 15,000 rpm at  $4^\circ\text{C}$  for 15 min. The supernatant (0.2 ml) was added to 2.8 ml of mixed solution (containing 0.167 mg/ml O-dianisidine in 50 mM potassium phosphate buffer and 0.15 mM  $\text{H}_2\text{O}_2$ ). The change in absorbance at 450 nm was monitored for 3 min using a UV/VIS spectrophotometer (752N INESA, China). A unit of MPO was defined as a change in absorbance of 0.001 per minute with the specific activity represented as MPO/mg protein.

**Reduced glutathione (GSH) in pouch exudate:** The amount of reduced glutathione (GSH) amount in pouch exudates was quantified using Ellman's reagent, with minor modifications (Moron *et al.*, 1979; Sin *et al.*, 1997). Cell free exudate supernatant (0.1 ml) was diluted ten times and mixed with 1 ml Trichloroacetic acid (20%) for de-proteinization; the mixture was centrifuged at 10,000 rpm at  $4^\circ\text{C}$  for 10 mins. The supernatant (0.25 ml) was mixed with 0.75 ml sodium phosphate buffer (0.1 M, pH 7.4) and 2 ml of 0.0006 M DTNB. The absorbance was read within 5 min at 412 nm in a UV/VIS spectrophotometer (INESA 752N, China). The glutathione concentration which was calculated using a standard curve generated with standard glutathione (0-200  $\mu\text{M}$ ) was expressed as a function of the volume of pouch exudates ( $\mu\text{M}$  GSH/ml of pouch exudates).

**Thiobarbituric acid-reacting substances (TBARS) in pouch exudates:** The extent of lipid peroxidation was determined using the TBARS assay (Ohkawa *et al.*, 1979). The sample (0.1 ml) was diluted twenty times in 0.15 M Tris-KCl buffer, and mixed with 0.5 ml TCA (30%), and 0.5 ml thiobarbituric acid (0.75%). The mixture was incubated in a water bath at  $80^\circ\text{C}$  for 45 min. The test tubes were dipped in ice-cold water for 10 min to halt the reaction, and thereafter the reaction mixture was centrifuged at 4000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm in a UV/VIS spectrophotometer. The majority of TBARS are malondialdehydes (MDA), thus the result was calculated using an index of absorption for MDA (molar extinction coefficient  $1.56 \times 10^5/\text{M}/\text{cm}$ ). The concentration of TBARS in pouch exudate was expressed as nmol MDA/mg protein. Total protein content in pouch exudates for TBARS and MPO analyses was estimated by the biuret method (Gornall *et al.*, 1949). Protein concentration was determined using a standard curve generated with Bovine serum albumin (0–100  $\mu\text{M}$ ).

**Nitrite assay:** Nitric oxide production in pouch exudates was assessed by Nitrite assay using Griess reagent. Griess

reagent was freshly prepared from a mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% of N-1-naphthyl ethylenediamine dihydrochloride) at a ratio of 1:1. The samples were incubated with Griess reagent in the dark for 30 min at room temperature, absorbance was read with a spectrophotometer at 540 nm. The nitrite concentration was determined from a standard curve of sodium nitrite (0 – 100  $\mu$ M).

**TNF- $\alpha$  assay:** The amount of TNF- $\alpha$  generated in the carrageenan-induced air pouch exudate was estimated with a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit (Biolegend, USA), according to the manufacturer's instruction.

**Histopathological analysis of air pouch lining:** A portion of the pouch tissue linings was fixed in 10% neutral buffered formalin, dissected longitudinally, placed in embedding cassettes, embedded in paraffin, and then cut into 4- $\mu$ m sections. Tissue sections were stained with hematoxylin and eosin (H & E) for light microscopic examination.

**Statistical Analysis:** Results were presented as Mean  $\pm$  SEM. Statistical analysis between groups was performed by one-way analysis of variance and Student's-Newman-Keuls multiple comparison tests using Graph Pad Prism 5.0 software (Graph Pad Prism Software Inc., San Diego, CA, US). The level of significance for all tests was set at  $p < 0.05$ .

## RESULTS

**GCD reduced nociceptive reaction in mice:** GCD decreased ( $p < 0.05$ ) the number of writhes in the acetic acid-induced writhing tests compared to control although the decrease was not dose-dependent. The effect of GCD was highest at 100 mg/kg (72.6% inhibition) compared to 46% inhibition at 50 mg/kg and 65.7% inhibition at 200 mg/kg. Ged 50 mg/kg, equivalent to GCD 200 mg/kg, inhibited the writhing response by 58%. The writhing response was reduced by 58% with Ged 50 mg/kg. Ged 50 mg/kg is equivalent to GCD 200 mg/kg. Thus, GCD (200mg/kg) showed a significantly ( $p < 0.05$ ) higher response than Ged (50 mg/kg). Indomethacin decreased the writhing response by 89% (Figure 1).

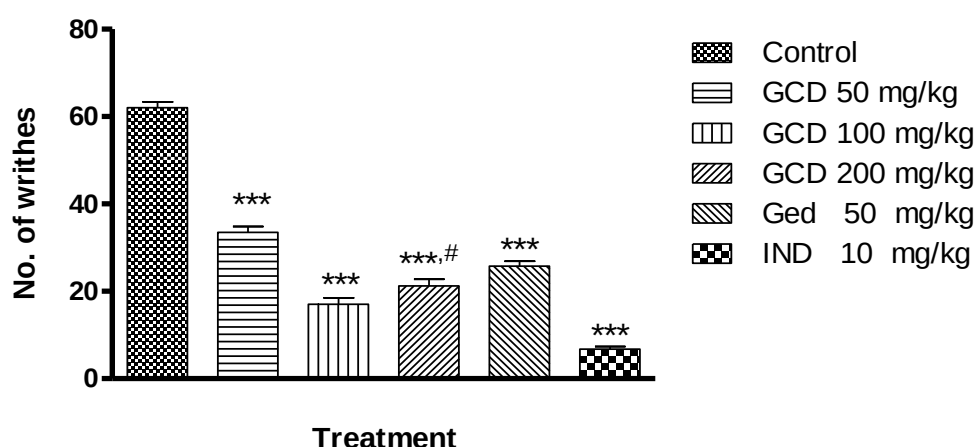


**Figure 1:**

Gedunin-cyclodextrin inclusion complex attenuates acetic acid-induced writhing in mice. The results are expressed as Mean  $\pm$  SEM (n=5). \*\*\* $p < 0.001$  vs control, # $p < 0.05$  vs Ged 50 mg/kg. GCD: gedunin – cyclodextrin; Ged: gedunin; IND: Indomethacin.

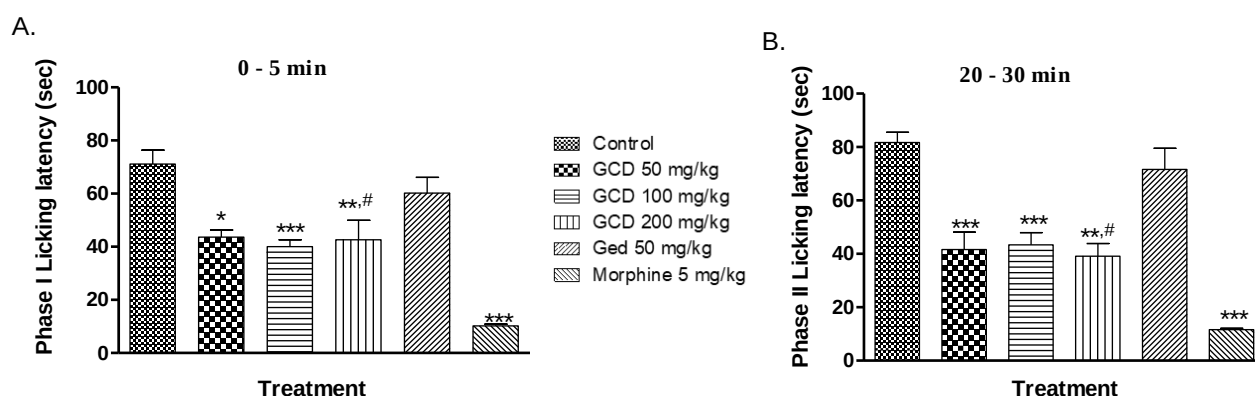


**Figure 2:**

Gedunin-cyclodextrin inclusion complex inhibits formalin-induced nociception in mice (A) Phase I and (B) Phase II. The results are expressed as Mean  $\pm$  SEM (n=5). \* $p < 0.05$  vs control, # $p < 0.05$  vs GCD 200 mg/kg. GCD: gedunin – cyclodextrin; Ged: gedunin.

GCD inhibited nociceptive reaction significantly ( $p < 0.05$ ) compared to control in both phases of the formalin test while the effect of gedunin (50 mg/kg) was comparable to control. The inhibition of nociceptive reaction due to GCD at 200 mg/kg was significantly ( $p < 0.05$ ) higher than that of gedunin at 50 mg/kg in both phases. In the first phase (neurogenic pain), GCD produced 38.7%, 43.7% and 40.1% inhibition at 50, 100 and 200 mg/kg respectively. However, these values are significantly ( $p < 0.05$ ) less than 85.7% pain inhibition produced by morphine (Figure 2a). In the second phase (inflammatory pain), GCD 200 mg/kg decreased biting and licking responses by 52.1%; this percentage reduction was highest when compared with Ged 50 mg/kg and GCD 50 and 100 mg/kg but it was lower than 85.8% inhibition of pain by morphine (Figure 2b).

**Table 1:**

Effect of gedunin-cyclodextrin inclusion complex, gedunin and morphine on reaction time in tail immersion test in mice

| Reaction time (secs) |              |                |                |                |                |
|----------------------|--------------|----------------|----------------|----------------|----------------|
| Group                | Dose (mg/kg) | 0 min          | 60 min         | 90 min         | 120 min        |
| Control              | 10 ml/kg     | 0.60<br>±0.04  | 0.61<br>±0.06  | 0.61<br>±0.06  | 0.63<br>±0.04  |
| GCD                  | 50           | 0.61<br>±0.08  | 0.86<br>±0.15* | 0.83<br>±0.05  | 0.71<br>±0.04  |
|                      | 100          | 0.61<br>±0.04  | 0.72<br>±0.04* | 0.66<br>±0.08  | 0.93<br>±0.26  |
|                      | 200          | 0.67<br>±0.04  | 0.71<br>±0.10* | 0.72<br>±0.05  | 0.74<br>±0.04  |
| Ged                  | 50           | 0.57<br>±0.03  | 0.67<br>±0.05  | 1.06<br>±0.13  | 0.77<br>±0.09  |
| Morphine             | 5            | 0.71<br>±0.07* | 3.00<br>±0.22* | 3.01<br>±0.12* | 2.37<br>±0.15* |

The results are expressed as Mean ± SEM (n=5), \* $p < 0.05$  vs control.

GCD: gedunin – cyclodextrin; Ged: gedunin

**Table 2:**

Effect of gedunin-cyclodextrin inclusion complex, gedunin and morphine on reaction time in hot plate test in mice

| Reaction time (secs) |              |                |                 |                 |                 |
|----------------------|--------------|----------------|-----------------|-----------------|-----------------|
| Group                | Dose (mg/kg) | 0 min          | 60 min          | 90 min          | 120 min         |
| Control              | 10 ml/kg     | 4.40<br>±0.61  | 4.43<br>±0.35   | 4.37<br>±0.85   | 3.47<br>±0.33   |
| GCD                  | 50           | 5.24<br>±0.85  | 4.52<br>±0.57   | 4.86<br>±0.58   | 4.06<br>±0.37   |
|                      | 100          | 5.64<br>±0.46  | 4.52<br>±0.92   | 3.72<br>±0.33   | 4.08<br>±0.33   |
|                      | 200          | 3.92<br>±0.39  | 5.04<br>±0.34   | 5.58<br>±0.84   | 6.20<br>±0.79   |
| Ged                  | 50           | 5.45<br>±0.24  | 5.53<br>±1.01   | 4.78<br>±0.46   | 5.80<br>±0.59   |
| Morphine             | 5            | 5.33<br>±0.73* | 13.23<br>±0.97* | 12.60<br>±0.93* | 10.90<br>±1.15* |

The results are expressed as Mean ± SEM (n=5). \* $p < 0.05$  vs control  
GCD: gedunin – cyclodextrin; Ged: gedunin

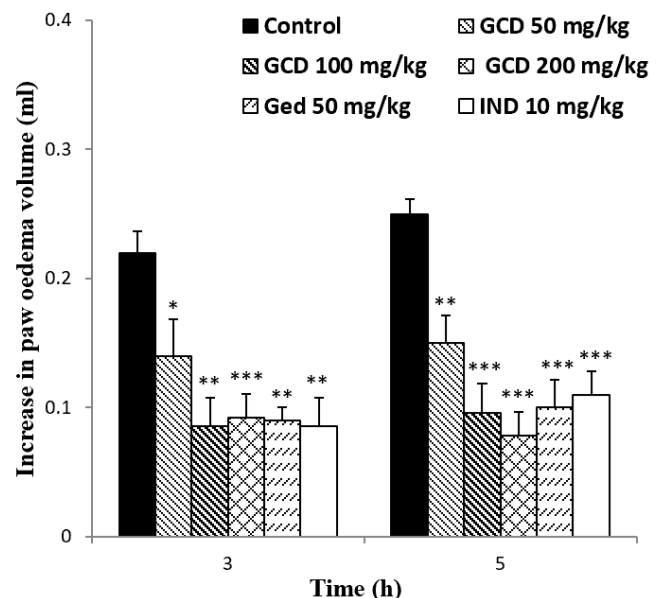
The oral administration of GCD (50, 100 and 200 mg/kg) to mice, in the tail immersion test, caused analgesia by significant ( $p < 0.05$ ) increase in reaction time to thermal stimulus of hot water relative to the control 60 min post-administration (Table 1). However, Morphine (5 mg/kg)

induced significant ( $p < 0.05$ ) analgesia relative to the control at 60, 90 and 120 min post-drug administration.

In the hot plate test, GCD 200 mg/kg and Ged 50 mg/kg increased the reaction time to pain while the lower doses of GCD showed no analgesic effect compared to controls. Morphine showed an increase in reaction time that was significant ( $p < 0.05$ ) with respect to the controls (Table 2).

### Effect of Ged and GCD on inflammation in carrageenan-induced rat paw edema:

The administration of GCD (50 - 200 mg/kg) led to significant ( $p < 0.05$ ) suppression of oedema three hours post carrageenan injection (Figure 3). A significant dose-dependent increase in GCD effect; 40.0%, 61.6% and 68.8% at 50, 100 and 200 mg/kg respectively, was observed five hours post carrageenan injection. Gedunin (50 mg/kg) produced a lower oedema suppression of 60.0% than GCD (200 mg/kg), although these values were not significantly different. Indomethacin exhibited a significant ( $p < 0.05$ ) suppression of oedema by 61.0% and 64.0% at the third and fifth-hour post carrageenan injection respectively.

**Figure 3:**

Gedunin-cyclodextrin inclusion complex reduces carrageenan-induced rat paw oedema. The results are expressed as Mean ± SEM (n=5). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs carrageenan control group.

### GCD decreased cellular migration and exudate formation in carrageenan induced air pouch:

The volume of the air pouch exudate was significantly ( $p < 0.05$ ) reduced in GCD and gedunin-treated animals compared to the carrageenan control group (Figure 4). The effect of GCD (200 mg/kg) on exudate volume was significantly ( $p < 0.05$ ) higher compared to that of Ged (50 mg/kg). Indomethacin caused a significant ( $p < 0.05$ ) decrease exudate formation.

The total number of leucocytes in the carrageenan control air pouch exudates was significantly ( $p < 0.05$ ) increased by about 80% of that of the saline control (Figure 5). Pre-treatment with GCD (50 mg/kg) reduced the leucocyte count by 55% while 100 mg/kg and 200 mg/kg lowered it by 51% and 43% respectively ( $p < 0.05$ ). Gedunin (50 mg/kg) reduced the migration of leucocytes by 16%.

The activity of GCD at the three doses was comparable to Indomethacin reduction of 41%.

#### Suppression of myeloperoxidase activity by GCD:

Infiltration of neutrophil to acute inflammatory sites is induced by carrageenan thus neutrophil activation was evaluated by the myeloperoxidase (MPO) enzyme activity in the pouch tissue lining. There was a significant ( $p < 0.05$ ) reduction of 25.3%, 55.8%, 57.4% and 49.5% in MPO activity in the pouch lining of animals treated with GCD 50, 100 and 200 mg/kg and indomethacin respectively. The activity of MPO activity was not reduced by gedunin compared to carrageenan control. GCD 200 mg/kg caused a significant ( $p < 0.05$ ) reduction in MPO activity compared to Ged 50 mg/kg (Figure 6).

**Nitrite and tumor necrosis factor- $\alpha$  levels reduction in pouch exudate by GCD:** The effect of GCD, Ged and Indomethacin on release of markers of inflammation is

shown in Figures 7A and B. The concentration of TNF- $\alpha$  was lower in pouch exudates of Ged and GCD pre-treated animals. GCD (50, 100 and 200 mg/kg) showed a significant ( $p < 0.05$ ) TNF- $\alpha$  inhibition by 33.7%, 27.3% and 49.9% respectively compared to carrageenan control (Figure 7A); Ged 50 mg/kg inhibited TNF- $\alpha$  by 25.4%. There was a significant difference ( $p < 0.05$ ) between the effect of GCD 200 mg/kg and Ged 50 mg/kg on TNF- $\alpha$  concentration. Pre-treatment with GCD reduced Nitrite level (Figure 8B). GCD 50, 100, 200 mg/kg and Ged 50 mg/kg produced a significant ( $p < 0.05$ ) reduction in nitrite values by 67.4%, 71.3%, 70% and 86.1% respectively compared to carrageenan control. However, the higher reduction in nitrite brought about by Ged was not statistically significant with respect to GCD 200 mg/kg. Indomethacin (5 mg/kg) decreased nitrite (64%) and TNF- $\alpha$  (24.5%) levels in the exudates.

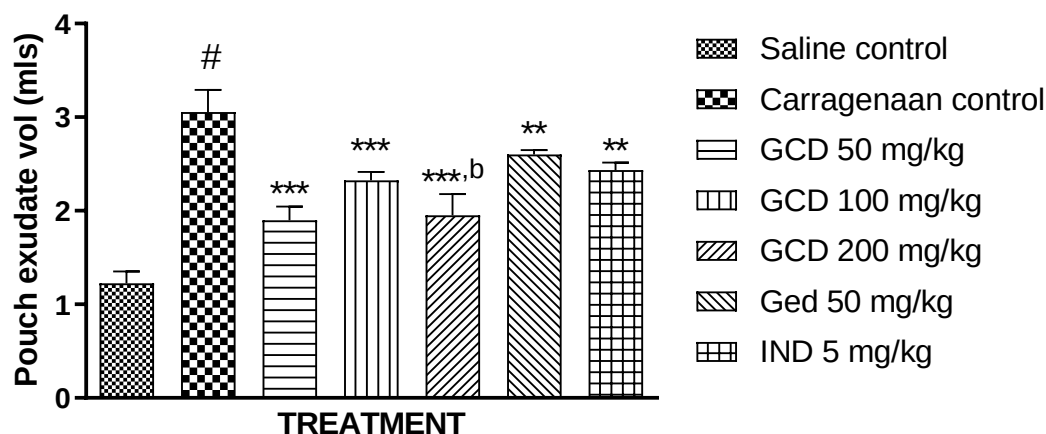


**Figure 4:**

Gedunin-cyclodextrin inclusion complex reduces exudate volume in carrageenan-induced air pouch model in rats. The results are expressed as Mean  $\pm$  SEM (n=5). # $p < 0.001$  vs saline control, \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs carrageenan control group, <sup>b</sup> $p < 0.05$  vs Ged 50 mg/kg.

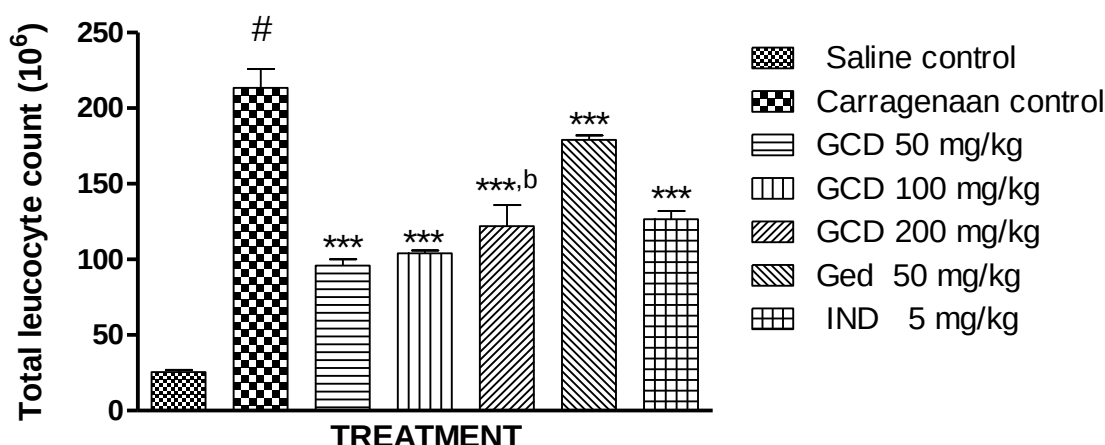
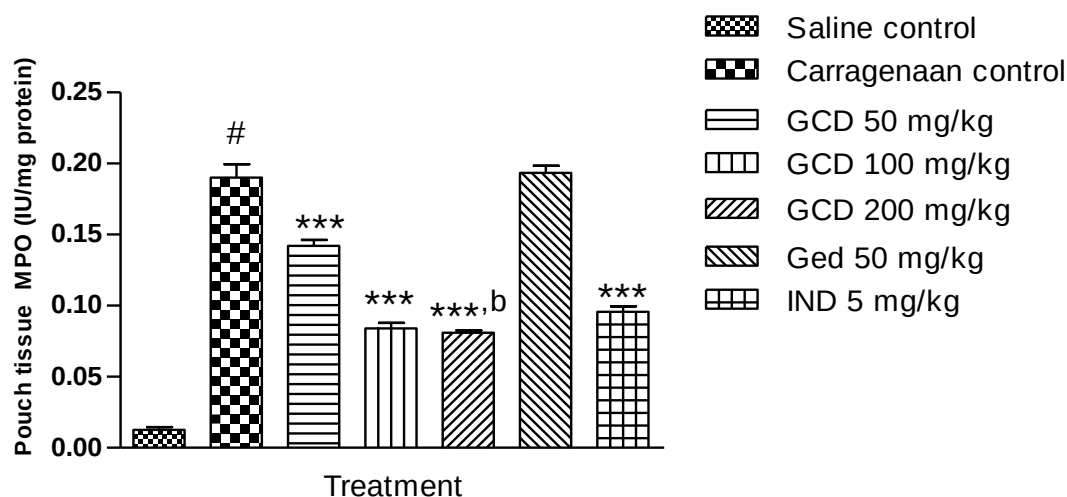
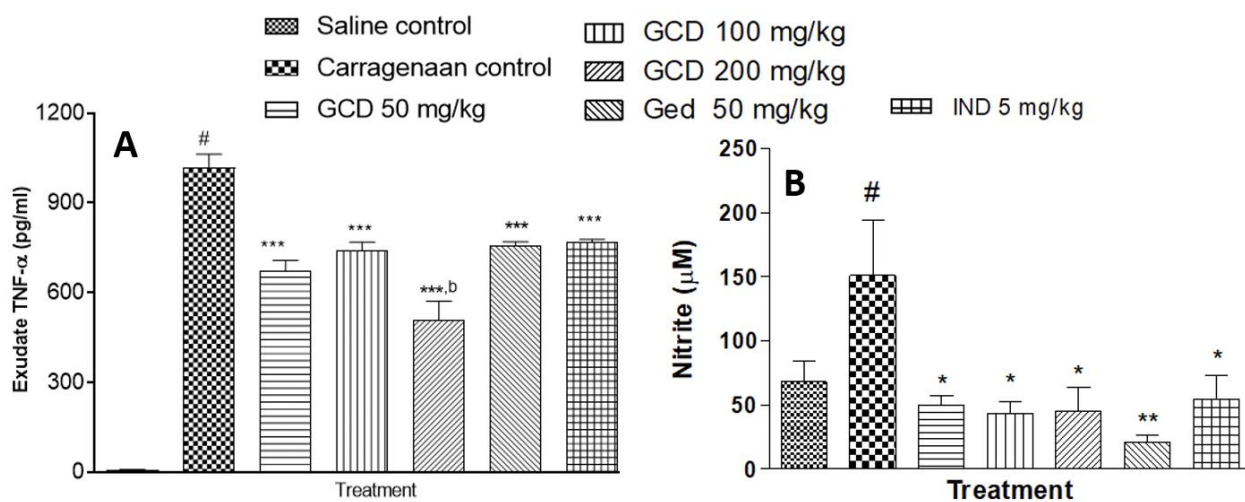


**Figure 5:**

Gedunin-cyclodextrin inclusion complex reduces total leucocyte count in carrageenan-induced air pouch cellular migration in rats. The results are expressed as Mean  $\pm$  SEM (n=5). # $p < 0.001$  vs normal saline control, \*\*\* $p < 0.001$  vs carrageenan control, <sup>b</sup> $p < 0.001$  vs Ged 200 mg/kg.

**Figure 6:**

Gedunin-cyclodextrin inclusion complex attenuates myeloperoxidase activity in carrageenan-induced air pouch in rats. The results are expressed as Mean  $\pm$  SEM (n=5). # $p < 0.001$  vs normal saline control. \* $p < 0.001$  vs carrageenan control, <sup>b</sup> $p < 0.05$  vs Ged 200 mg/kg

**Figure 7:**

Gedunin-cyclodextrin inclusion complex inhibits carrageenan-induced release of on the release of (A) tumor necrosis factor- $\alpha$  and (B) Nitrite concentration in rats. The results are expressed as Mean  $\pm$  SEM (n=5). # $p < 0.001$  vs saline control, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs carrageenan control group, <sup>b</sup> $p < 0.05$  vs Ged 50 mg/kg.

**Table 3:**

Effect of GCD on the level of reduced glutathione (GSH) and malondialdehyde levels in pouch exudate

| Experimental groups | GSH ( $\mu$ M)                   | TBARS ( $\mu$ M of MDA/mg protein)  |
|---------------------|----------------------------------|-------------------------------------|
| Saline control      | 4.39 $\pm$ 0.17                  | 229.60 $\pm$ 37.14                  |
| Carrageenan control | 3.45 $\pm$ 0.21 <sup>#</sup>     | 412.00 $\pm$ 9.15 <sup>#</sup>      |
| GCD 50 (mg/kg)      | 5.62 $\pm$ 0.21***               | 288.90 $\pm$ 13.90**                |
| GCD 100 (mg/kg)     | 6.35 $\pm$ 0.17***               | 205.70 $\pm$ 14.18***               |
| GCD 200 (mg/kg)     | 5.48 $\pm$ 0.31***, <sup>b</sup> | 246.00 $\pm$ 11.18***, <sup>b</sup> |
| Ged 50 (mg/kg)      | 4.24 $\pm$ 0.35*                 | 346.10 $\pm$ 6.58*                  |
| IND 5 (mg/kg)       | 6.17 $\pm$ 0.16***               | 349.70 $\pm$ 13.18*                 |

The results are expressed as Mean  $\pm$  SEM (n=5). # $p < 0.05$  vs normal saline control. \* $p < 0.05$ , \*\*\* $p < 0.001$  vs carrageenan control, <sup>b</sup> $p < 0.01$  vs Ged 50 mg/kg. GCD: gedunin – cyclodextrin; Ged: gedunin, IND: Indomethacin

#### GCD altered carrageenan-induced glutathione depletion and lipid peroxidation:

Pre-treatment with GCD (50 – 200 mg/kg) and indomethacin reversed the depletion of GSH observed in the carrageenan-injected pouch (Table 3). The reversal effect of Ged 50 mg/kg on GSH depletion was less ( $p < 0.05$ ) compared to GCD 200 mg/kg.

The level of thiobarbituric acid reacting species (TBARS) was raised in carrageenan-injected pouches compared to saline group (Table 3) but administration of GCD, Ged and indomethacin reduced lipid peroxidation ( $p < 0.05$ ) compared to controls but reduction was higher in GCD treated groups. GCD 200 mg/kg achieved a higher and significant ( $p < 0.05$ ) reduction compared to Ged 50 mg/kg.

**Histological changes in the air pouch tissue lining :** The infiltration (severe, diffuse) of inflammatory cells was

observed in the pouch wall, and the tissue oedema contributed to the dispersed connective tissue fibers observed in the vehicle-treated carrageenan animal (Figure 8). The GCD-treated air pouch, on the other hand, presented signs of very mild to moderate acute inflammatory response. Ged (50 mg/kg) air pouch revealed considerable cellular infiltration. These findings imply that pre-treatment with GCD suppressed the acute inflammatory response in the pouch wall better than Ged alone.

## DISCUSSION

The antinociceptive and anti-inflammatory properties of gedunin were improved by complexation with 2-hydroxypropyl- $\beta$ -cyclodextrin (HBD) in this study.

Acetic acid-induced writhing test is a common inflammatory pain model which has high sensitivity and low specificity (Braggio *et al.*, 2002). The intraperitoneal administration of acetic acid causes local irritation which indirectly triggers the release of nociceptive endogenous mediators (e.g. bradykinin, serotonin, and prostaglandin) and proinflammatory cytokines (e.g. TNF- $\alpha$ , IL-1 $\beta$  and IL-8); chemosensitive nociceptors which contribute to the development of inflammatory pain are activated by these mediators (Manjavachi *et al.*, 2010). Acetic acid-induced writhing can be inhibited by peripherally acting drugs like indomethacin, a non-steroidal anti-inflammatory drug because the test identifies peripheral analgesics. The antinociceptive action of GCD (50–200 mg/kg), Ged 50 mg/kg and indomethacin in this test was probably due to inhibition of release and/or activity of inflammatory mediators. No particular mediator was targeted, however,

the test agents act more on the peripheral pain induced in the peritoneal cavity.

The formalin test was performed to determine whether GCD and/or Ged suppressed pain centrally or peripherally. The formalin test is more precise and has a biphasic response. The first phase is the neurogenic nociceptive response which occurs during the first five minutes after formalin injection, while the second phase is the inflammatory nociceptive response which occurs between twenty and thirty minutes post formalin injection (Ribeiro *et al.*, 2010). Centrally acting analgesics can inhibit both phases, peripherally acting analgesics inhibit the second phase only (Liao *et al.*, 2012). GCD (50 - 200 mg/kg) and morphine (5 mg/kg) decreased the nociceptive response significantly ( $p < 0.05$ ) in both phases induced by formalin but Ged (50 mg/kg) did not diminish the response in any of the phases. GCD may have central and peripheral antinociceptive action because it inhibited nociception in both phases. This confirms the possibility of GCD's anti-inflammatory property as observed in the acetic acid-induced writhing. The greater nociceptive inhibition by GCD in the acetic acid-induced writhing and formalin tests is possibly due to the improved bioavailability of the orally administered GCD compared to Ged alone (free, uncomplexed gedunin). This enhanced effect may be attributed to the high aqueous solubility of HBD resulting in an improved availability of the hydrophobic gedunin molecules aiding them in reaching and/or remaining at their site of action. In a model of articular inflammation, gedunin suppressed and diminished hyperalgesia (Conte *et al.*, 2015). This supports the effect of GCD in reducing inflammation-associated pain as seen in the antinociceptive tests results.

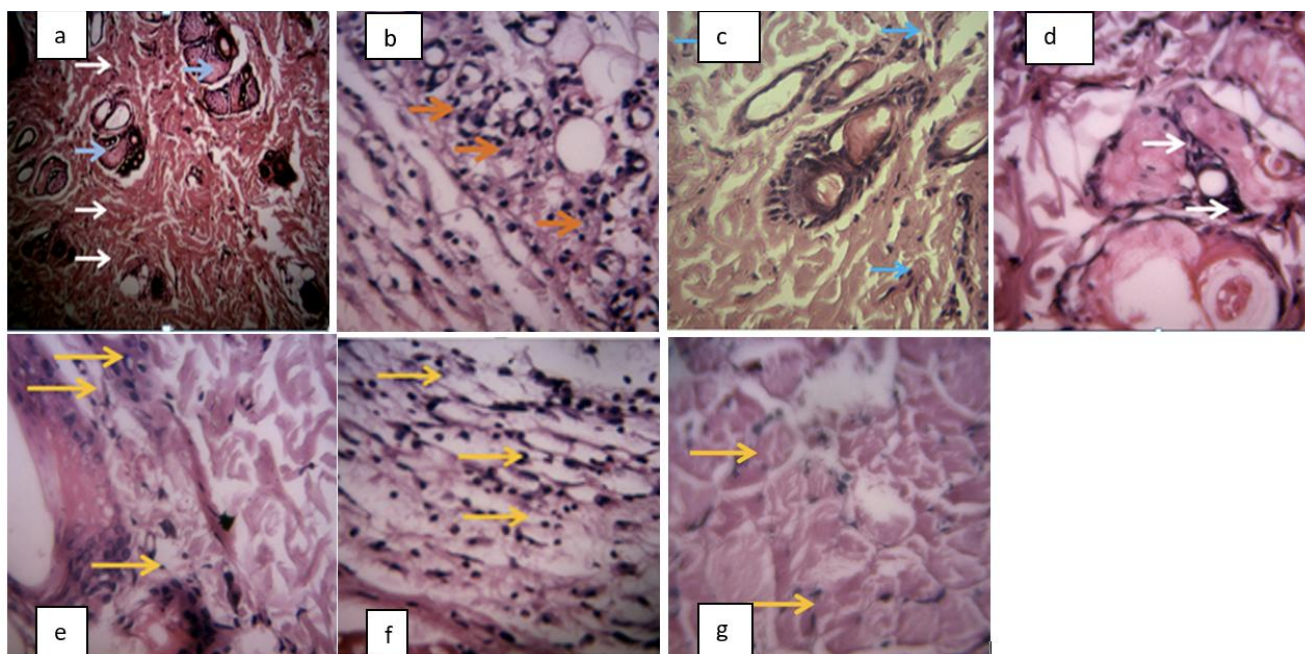


**Plate 1**

Gedunin-cyclodextrin inclusion complex attenuates carrageenan-induced pathohistological changes in the pouch lining of rats. **A.** saline control showing dermal connective tissue (white arrows) surrounding sebaceous glands (blue arrows). **B.** carrageenan control showing severe diffuse neutrophilic cellular infiltration of the dermis. The connective tissue fibers are dispersed. **C.** GCD 50 mg/kg group; mild/moderate cellular infiltration of the dermis is seen, the connective tissues are slightly dispersed. **D.** GCD 100 mg/kg; a very mild (periglandular) cellular infiltration with normal dense dermal connective tissue fibers. **E.** GCD 200 mg/kg; very mild dermal cellular infiltration, dermal connective tissues are slightly dispersed. **F.** Ged 50 mg/kg group with severe diffuse cellular infiltration by neutrophils. **G.** IND 5 mg/kg, very low infiltrative cell population. *H&E: Hematoxylin-Eosin stain (magnification x400)*

*Pharmacological activities of gedunin-2-hydroxypropyl- $\beta$ -cyclodextrin complex*

Hot plate and tail immersion tests were performed for a further assessment of GCD and Ged central antinociceptive activity. The hot-plate method is a common and selective test for evaluating centrally-mediated analgesic action. Morphine increased the reaction time to pain in the hot plate and tail immersion tests, when compared with the control, while GCD 200 mg/kg and the equivalent dose of Ged 50 mg/kg prolonged the reaction time in hot plate test although the lower doses of GCD showed no analgesic effect. This implies that GCD and Ged may have central analgesic action that is not opioid mediated. The complexation of analgesic compounds with cyclodextrins can improve their efficacy (Brito *et al.*, 2015). Cyclodextrin complexation enhanced the central analgesic property and half-life of (*R*)-(-)-linalool, a monoterpene alcohol (Quintans-Junior *et al.*, 2013). pCM (p-cymene) and pCM- $\beta$ -CD (the cyclodextrin complexed p-cymene) inhibited nociceptive behavior for 2 h and 8 h respectively in the acetic acid-writhing test (Quintans *et al.*, 2013). The findings of this study corroborate these reports.

Carrageenan-induced paw edema model is frequently used to evaluate the anti-edematogenic effect of natural products. The induced paw edema is a biphasic event; bradykinin, histamine, and 5-hydroxytryptamine (5-HT) are released in the first phase of edema (0-1 h) while cyclooxygenase-2 (COX-2), prostaglandins (PGs), TNF- $\alpha$ , IL-1 $\beta$  are produced in the second phase (1-6 h) (Ribeiro *et al.*, 2010). The increased production of PGs in the latter phase is through the activation of COX-2 and release of nitric oxide (NO). The inflammatory edema is highest at the third hour before it starts to reduce (Kirkova *et al.*, 1992). In the carrageenan-induced rat paw edema test, GCD at all doses and Ged exerted significant edema inhibition comparable to indomethacin 3 hours post-carrageenan injection. There was a significant improvement in the anti-inflammatory activity of the complexed gedunin. This implies that anti-inflammatory activity of gedunin was still significantly preserved in the complexes and complexation with cyclodextrin can improve the edema inhibitory activity of gedunin. This observation correlates well with carrageenan rat paw edema test results of cyclodextrin inclusion complexes of some drugs with anti-inflammatory action. Free celecoxib and celecoxib- $\beta$ -cyclodextrin achieved significantly higher inhibition of the inflammation induced by carrageenan compared to control group but the inclusion complex was more effective than celecoxib alone in edema inhibition (Sensoy *et al.*, 2009). In another study, dexamethasone- $\beta$  cyclodextrin complexation did not decrease the edema inhibitory and anti-inflammatory action of dexamethasone (Rodrigues *et al.*, 2014).

The anti-inflammatory activity of GCD was examined further with the carrageenan air pouch model of inflammation. Carrageenan injection into an air pouch triggers an inflammatory reaction which is associated with cellular infiltration, increased exudate, and pronounced release of pro-inflammatory mediators like prostaglandins, leukotrienes, and cytokines. These markers can be quantified and used to determine degree of inflammation, resolution of inflammation or anti-inflammatory activity of drugs (Kim *et al.*, 2006). The oral pre-treatment with GCD reduced exudate volume and number of leucocytes in the air pouch. The reduction achieved by GCD 200 mg/kg was significantly higher than the equivalent dose of Ged 50

mg/kg. This is an indication that the formation of the inclusion complex between gedunin and HBD improved the bioavailability of gedunin from the oral route.

The margination of neutrophils, an early cellular event in inflammation, can be measured using the neutrophil-specific enzyme myeloperoxidase (MPO) (Esmat *et al.*, 2012). Increased tissue and plasma MPO levels are a marker of neutrophil proliferation and degranulation (Kothari *et al.*, 2011) and intracellular MPO activity measurement can be used as a surrogate marker for the number of neutrophils contained in a biological sample (Pulli *et al.*, 2013). Thus, the decreased MPO activity due to the administration of GCD was an indication that GCD inhibited neutrophil activation and infiltration induced by carrageenan. This was confirmed by histological examination of the pouch tissue. GCD (50 - 200 mg/kg) mitigated the inflammatory histopathological modifications.

The TBARS (an index of lipid peroxidation) was increased in carrageenan injected pouches, as compared to the normal saline injected pouches, as a result of released neutrophilic reactive oxygen species triggered by carrageenan. This agrees with previous reports of increased exudates and tissue LPO that was partly mediated via increase in ROS/RNS radical production in carrageenan injected animals (Tanas *et al.*, 2010; Jain and Parmar, 2011). The end product of cell membrane decomposition, malondialdehyde (MDA), is an indicator of the inflammatory process (Chou *et al.*, 2012). Also, lipid peroxidation is an oxidative injury marker in disease conditions. This was the basis for assessing glutathione (GSH), TBARS (measured as MDA), TNF- $\alpha$  and NO levels in the pouch exudate. Pretreatment with GCD 50, 100, 200 mg/kg and Ged 50 mg/kg inhibited the increased TBARS levels significantly. However, GCD 200 mg/kg brought about a significantly higher inhibition of TBARS increase than the equivalent free Ged 50 mg/kg. Injection of carrageenan triggers an increase in glutathione level. Glutathione reacts with free radicals to generate thiol radicals. This reaction depletes GSH level over time in animals exposed to inflammatory stress (Conner and Grisham, 1996). Administration of GCD and Ged 50 mg/kg blocked carrageenan-induced GSH depletion in the air pouch. However, GCD 200 mg/kg exerted a significantly higher effect than Ged 50 mg/kg.

In conclusion, Gedunin-2-hydroxypropyl- $\beta$ -cyclodextrin complex demonstrated significant antinociceptive and anti-inflammatory activities relative to gedunin by inhibition of oxidative stress and inflammation in rodents. The improved pharmacological activities of the complex might partially be due to reduced first pass effect and increased bioavailability.

#### Acknowledgment

The authors appreciate the Staff Development Award from the University of Ilorin, Ilorin, Nigeria which led to the execution of part of the study.

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Full-Length Research Article

# Angiotensin-converting Enzyme Inhibitor Captopril Prevents Neuronal Overexpression of Amyloid-beta and alpha-synuclein in *Drosophila melanogaster* Genetic Models of Neurodegenerative Diseases

Ishola I.O.<sup>a</sup>, Afolayan O.O.<sup>b</sup>, Badru W.A.<sup>c</sup>, John N.<sup>a</sup>, Adeyemi O.O.<sup>a</sup>

<sup>a</sup>Department of Pharmacology, Therapeutics and Toxicology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Lagos, Nigeria

<sup>b</sup>Department of Anatomy, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Lagos, Nigeria

<sup>c</sup>Department of Pharmacology, Lagos State University, College of Medicine, Ikeja, Lagos State, Nigeria

<sup>d</sup>Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Lagos, Lagos, Nigeria

**Summary:** Parkinson disease (PD) and Alzheimer's disease (AD) are progressive neurodegenerative disorders characterized by loss of selective neurons in discrete part of the brain. The peptide angiotensin II (Ang II) plays significant role in hippocampal and striatal neurons degeneration through the generation of reactive oxygen species. Blockade of the angiotensin converting enzyme or ATI receptors provides protection in animal models of neurodegenerative diseases. In the present study, the neuroprotective effect of captopril was investigated in *Drosophila melanogaster* model using the UAS-GAL4 system to express the synuclein and A $\beta$ 42 peptide in the flies' neurons. The disease causing human A $\beta$ 42 peptide or  $\alpha$ -syn was expressed pan-neuronally (elav-GAL4) or dopamine neuron (DDC-GAL4) using the UAS-GAL4 system. Flies were either grown in food media with or without captopril (1, 5, or 10 $\mu$ M). This was followed by fecundity, larva motility, negative geotaxis assay (climbing) and lifespan as a measure of neurodegeneration. Elav-Gal4<A $\beta$  or DDC-GAL4< $\alpha$ -syn flies displayed a significant decrease in larva motility when compared with normal control (w<sup>1118</sup>) which was reversed by the supplementation of the media with captopril (5 or 10 mM) indicative of neuroprotection. Interestingly, supplementation of flies' media with captopril improved climbing activity in Elav-Gal4<A $\beta$  or DDC-GAL4< $\alpha$ -syn flies when compared with vehicle-treated only. Moreover, flies grown on captopril caused no significant change in lifespan. Findings from this study confirmed the neuroprotective action of captopril in genetic or familial forms of neurodegeneration.

**Keywords:** Alzheimer's disease; captopril; Parkinson's disease; neurodegeneration; *Drosophila melanogaster*; UAS-GAL4 system

©Physiological Society of Nigeria

\*Address for correspondence: oishola@cmul.edu.ng; +2348033018908

Manuscript received- August 2021; Accepted- January 2022

DOI: <https://doi.org/10.54548/njps.v37i1.3>

## INTRODUCTION

Neurodegenerative diseases represent a major threat to human health. These age-dependent disorders are becoming increasingly prevalent, in part because the elderly population has increased in recent years (Wyss-Coray, 2016). Extracellular deposits of A $\beta$  and intraneuronal tau inclusions denote AD while intracellular inclusions of  $\alpha$ -synuclein make up the Lewy pathology of PD (Jaiswal et al., 2012). Overexpression of these mutant proteins can give rise to disease-associated phenotypes (Jaiswal et al., 2012). Better elucidation of the cellular mechanisms for clearance of abnormal/misfolded proteins and of the effects of toxic protein accumulation on neuronal survival may allow the development of effective treatment for these disorders (Abeliovich and Gitler, 2016).

Various animal and cell culture models of PD and AD have been developed and have substantially contributed to

the understanding of disease pathogenesis, however, none of the existing models fully recapitulates all pathological features of PD and AD, and each model provides opportunities to investigate certain aspects of the disease. Albeit, the UAS-Gal4 system allows for the definition of which cells are expressing a particular gene or its product and is a useful tool in investigating PD and AD. *Drosophila melanogaster* which have been genetically engineered to express the Gal4 gene in a particular section of tissue can be used in the study of PD and AD (Duffy, 2002; Bier, 2005). The fly genome is smaller with substantially less gene redundancy than that of mammals, while it contains homologs of approximately 70% of human disease-related genes. In this study, we characterized the physiological effect of overexpression of amyloid- $\beta$  or  $\alpha$ -synuclein in the nervous system of *Drosophila melanogaster* using the upstream activation sequence (UAS)/yeast transcriptional activator binding to UAS (GAL4) system (Duffy, 2002;

Ishola et al., 2021). The transgenic flies over-expressing amyloid- $\beta$  or  $\alpha$ -synuclein in the nervous system showed a moderate decrease in locomotor activity and median lifespan of adult flies. Amyloid- $\beta$  protein precursor (A $\beta$ PP) and the microtubule-associated protein tau (MAPT) are the two key players involved in Alzheimer's disease (AD) and are associated with amyloid plaques and neurofibrillary tangles respectively, two key hallmarks of the disease (Jucker and Walker, 2013). *Drosophila* models have been widely used to understand the complex events leading to AD in relation to aging (Ishola et al., 2021). Similarly, genetic and environmental risk factors are associated with PD. The genetic factors includes about 20 genes, such as SNCA, parkin, PTEN-induced kinase1 (pink1), leucine-rich repeat kinase 2 (LRRK2), ATP13A2, MAPT, VPS35, and DJ-1, whereas the environmental factors consist of oxidative stress-induced toxins (Adams et al., 2000; Abeliovich and Gitler, 2016). *Drosophila melanogaster* has emerged as an excellent model organism to study both environmental and genetic factors associated with PD. Thus, provide insights to the pathways relevant for AD and PD pathogenesis, facilitating development of therapeutic strategies.

The brain RAS is implicated in neurodegenerative disorders including PD and AD (Liao et al., 2014). In humans, Angiotensin converting enzyme activity is increased in the cerebrospinal fluid of PD and AD patients which is thought to reflect a response to increased brain inflammation (Schulz and Heusch, 2006). A genetic polymorphism in the ACE gene is associated with increased risk of PD (Huo et al., 2017). Moreover, inhibition of the angiotensin-converting enzyme (ACE) by a centrally acting ACE inhibitor retards symptoms of neurodegeneration, Abeta plaque formation and tau hyperphosphorylation in experimental models of AD (Quitterer and Abdalla, 2020). Blockade of ACE, using captopril has been shown to exert neuroprotective effects in the striatum (Sonsalla et al., 2013). The purpose of the present study was to examine the neuroprotective effects of the ACE inhibitor captopril in an acute as well as a progressive neurodegeneration in *Drosophila melanogaster* model of PD and AD.

## MATERIALS AND METHODS

**Drugs and chemicals:** Captopril (Alpa Laboratories Ltd. India), diethyl-ether (Guangdong Guanghua sci. Tech CO. Ltd. China), sugar, corn flour (Benchmark foods and Spices Ltd, Lagos state, Nigeria), yeast (STK industries Ltd, China), agar (Himedia Laboratoies Pvt. Ltd, Mumbai, India), malt (Sigma Aldrich, Germany), propanoic acid (LOBA Chemie Laboratory Reagents & fine chemicals, Mumbai, India), methyl-p-hydroxy benzoate (LOBA Chemic Laboratory Reagents & fine chemicals, Mumbai, India), orthophosphoric acid (Thermo Fischer Scientific India pvt, Ltd, Mumbai, India), phosphate buffered saline (Gibco Technologies, USA).

**Fly Stocks and Culture:** Wild type W118, *ELAV-gal4/FM*, *UAS-A $\beta$ 42/TM3*, *Ddc-Gal4/TM3*, *UAS- $\alpha$ -synuclein/CyO*, and Caxton S (Cs) flies were obtained from Dr. Rakesh Mishra *Drosophila* Laboratory, Centre for Cellular and Molecular Biology, Hyderabad, India. Flies strains were maintained on standard cornflour, malt, yeast, sugar,

orthophosphoric acid, propionic acid and agar at 26 $\pm$ 2°C with 60-75% relative humidity and a 12h light/dark cycle

**Construction of A $\beta$ 42 and  $\alpha$ -synuclein transgenic *Drosophila* models:** To construct the *UAS-A $\beta$ 42/TM3* or *UAS- $\alpha$ -synuclein/CyO* transgenic *Drosophila*, respectively, with *Elav-GAL4/FM* or *Ddc-Gal4/TM3* genetic background. Virgin females of *Elav-GAL4/FM* or *Ddc-Gal4/TM3* were mated with *UAS-A $\beta$ 42* or *UAS- $\alpha$ -synuclein* transgenic *Drosophila*, respectively. *Elav-A $\beta$ 42* and *Ddc- $\alpha$ -synuclein* *Drosophila* were collected within 48 hours for lifespan or behavioural assays (Ishola et al., 2021).

**Fecundity assay:** Fecundity, also known as *reproductive rate* is a measure of the number of offspring produced by an organism over time. Assessment of fecundity allows monitoring of the effect of captopril on reproductive activity. Parameters recorded include, the number of eggs laid, number of dark pupa and the number of flies that emerged from the dark pupa through the course of the study. Cs fly strain was used; male and female mated at a ratio of 1:2 (5 males to 10 females) n=3 vials per group. The canton-special (CS) strain was used for fecundity studies. The fecundity assay began at 24 hours after mating. The number of eggs laid was counted every 24/48h by viewing under the stereomicroscope until the dark pupa as well as the number of flies that emerged from each vial were recorded.

**Larval Motility:** Third instar larvae were removed from their individual medium, after a quick wash in distilled water, twenty larvae from each group, comprising media only, media supplemented with captopril (1, 5, or 10 $\mu$ M) (n=3 vials) were gently placed on 2% w/v agar slabs in 245mm $\times$ 245mm square petri-dishes using a small paint brush; all on the surface of a 2B sheet with partitioned square boxes measuring 0.5cm by 0.5cm. The larvae was allowed to acclimatize for one minute and the number on line crosses in one minute was recorded.

**Collection of female virgins:** Collection of female virgins is easy. Females remain virgins for only 8-10 hours after eclosure and must be collected within this time frame. To obtain virgin flies, flies were anaesthetized using diethyl ether, after which they were placed under the stereomicroscope and the female virgins separated and collected.

**Larval Motility Assay:** The desired number of individual larva were collected, washed in phosphate buffered saline and transferred to a petri dish containing freshly prepared 2 % Agar (previously poured and allowed to solidify) and placed over a 2B paper with a 0.1cm<sup>2</sup> grid. Flies were left to acclimatize for a minute, until the desired number of larva has been counted. Female virgin flies of the DDC-Gal4/+ strain were crossed with male flies of the UAS-syn strain in the ratio 2:1, also female virgins of the *Elav-Gal4/+* strain were crossed with *UAS-A $\beta$*  in the ratio 2:1. On the 4<sup>th</sup> or 5<sup>th</sup> day of crossing, larva assessment was taken. The fly crossings used to larva motility are *Elav<A $\beta$*  and *DDC<syn*, with *Elav<W1118*, *DDC<W1118* as negative control (Ishola et al., 2021).

**Negative Geotaxis Assay:** To assess climbing behavior, 20 adult flies were placed at the bottom of a clean vial and second identical vial placed above. The bottom vial was labeled per centimeter. After 8 seconds, the number of flies that passed the 8cm mark was compared over time with the proportion of the control flies. The climbing behavior for a cohort of flies was followed periodically for 28days. The crossings used to assess this stringent assay were the *Elav<Aβ* and *DDC<syn*, with *Elav<W1118*, *DDC<W1118* as negative control (Ishola et al., 2021).

**Longevity Assay:** The longevity of a population of flies provides a robust estimate of their general health. It allows monitoring of the effect of genotype, environment or drug as *Drosophila melanogaster* survival throughout its lifespan. Twenty (20) male/ female flies expressing the gene of interest were placed on standard fly meal at regulated temperature (18 -23°C). The flies were counted every 2 days and transferred to new vials. At each time point, the number of flies that were observed to die and those that were lost to follow-up (for example, flies that escaped or were accidentally killed) were counted until the last fly in the vial died (Ishola et al., 2021)s.

#### Statistical Analysis

Data analyses were performed using GraphPad Prism software (GraphPad Software, Inc.). Results were expressed as mean and standard deviation values. One-way ANOVA was adopted for column analysis, while the grouped data

were analyzed with the Two-way ANOVA. The results were analyzed by Tukey's *Post hoc* multiple comparison test.

## RESULTS

### Fecundity Assay

A ratio of 1:2 of Canton S strain of flies (5 females: 10 males) were grown on normal feed and captopril supplemented feed media (1, 5 and 10 mM). *Post hoc* analysis revealed significant decrease in the number of eggs laid [ $F(3,20)=16.79, P<0.001$ ] (Fig. 1a), dark pupa [ $F(3,20)=5.15, P<0.01$ ] (Fig. 1b) and eclosion [ $F(3,20)=4.34, P<0.05$ ] (Fig. 1c) by captopril 10mM.

### Larva Motility Assay

**Effect of captopril on larva motility in synuclein expressing flies.:** One way ANOVA revealed significant effect of treatment [ $F(4,42)=3.84; P < 0.01$ ]. moreover, *Post hoc* analysis showed that *DDC-GAL4 > α-syn* flies grown on normal food media showed significant deficit in locomotor activity. However, supplementation of media with captopril (5 or 10 mM) produced significant increase in larva locomotion activity in comparison with food media only (figure 2).

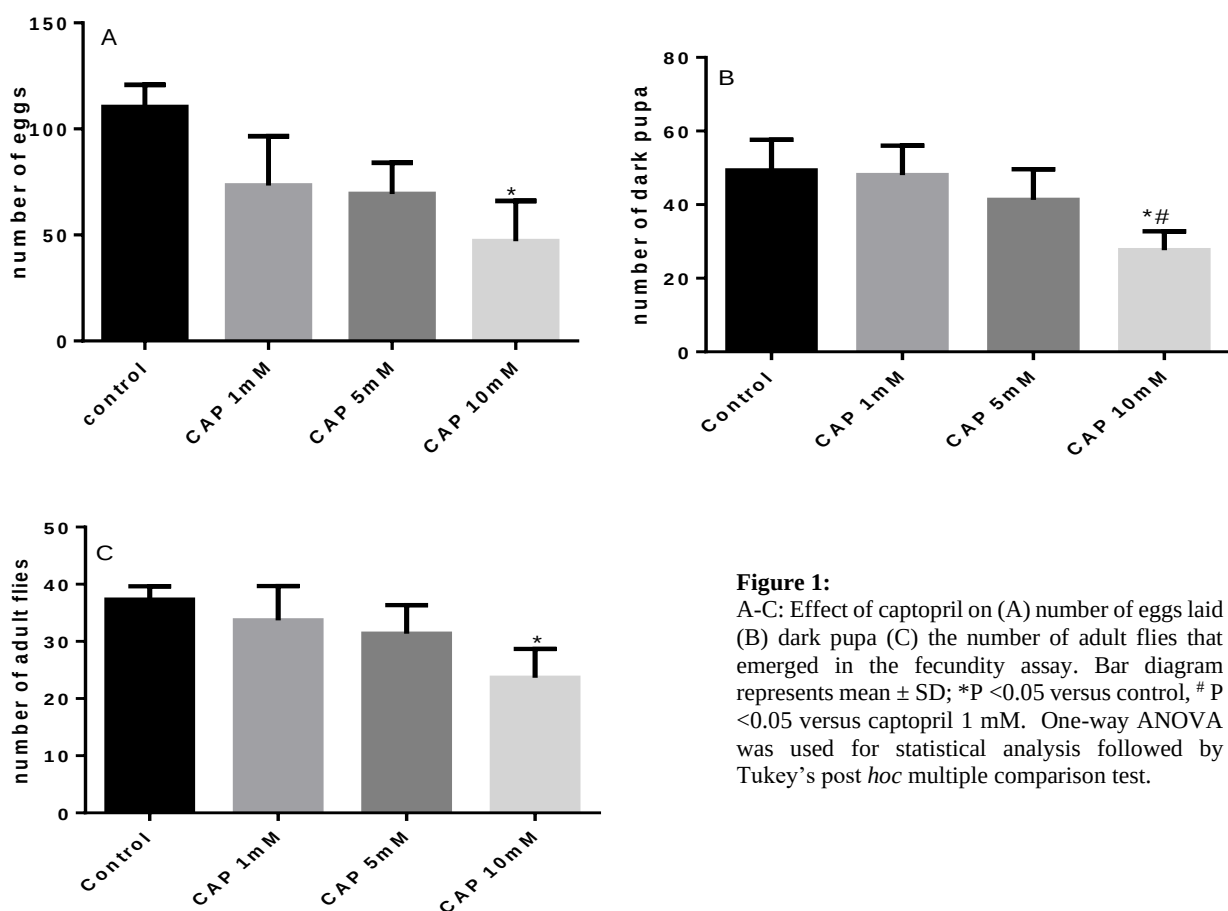


**Figure 1:**

A-C: Effect of captopril on (A) number of eggs laid (B) dark pupa (C) the number of adult flies that emerged in the fecundity assay. Bar diagram represents mean  $\pm$  SD; \*P < 0.05 versus control, # P < 0.05 versus captopril 1 mM. One-way ANOVA was used for statistical analysis followed by Tukey's *post hoc* multiple comparison test.

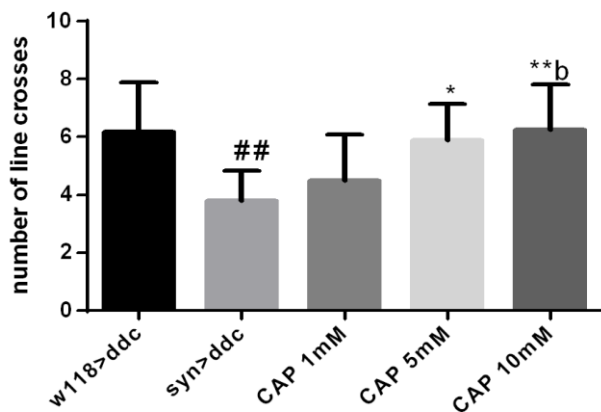


**Figure 2:**

Effect of captopril on the number of line crosses in UAS-syn < DDC-Gal4 larva. Values are expressed as mean ± SD. ##P<0.01 versus w1118; \*P<0.01 versus SYN×DDC control; \*\*P<0.01 versus SYN×DDC; <sup>b</sup>P<0.01 versus 1mM captopril. Statistical level of significant analysis by one-way ANOVA, followed by Tukey's multiple comparison test.

**Effect of captopril on larva motility of Aβ expressing flies:** Post hoc analysis showed that there was no significant change in the number of line crosses in UAS-Aβ < elav-Gal4 larva compared to control. Moreover, there was no significant difference in the number of line crossings in larva cultured on captopril supplemented feed media (1mM and 5mM) in comparison with the control. However, captopril (10mM) caused significant increase [F(4,45)=3.11; P<0.01] in number of line crosses in UAS-Aβ < elav-Gal4 larva (AD model) compared to those cultured in vehicle media (Fig. 3).

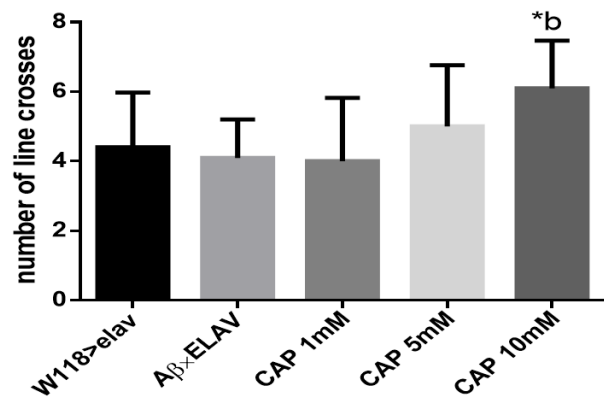


**Fig 3:** Effect of captopril on number of line crosses in UAS-Aβ < elav-Gal4 larva. values are expressed as mean ± SD. \*p<0.05 versus Aβ×Elav control, <sup>b</sup>p<0.05 versus captopril 1mM. Analysis was done by one-way ANOVA followed by Tukey's multiple comparison test.

### Climbing Assay

#### Effect of captopril on climbing in Aβ expressing flies:

Two-way ANOVA revealed significant effect of treatments on climbing activity [F(3,40)=30.56, p<0.001] (Fig.4). Post hoc analysis showed time course decrease in climbing activity with peak effect at day 28. The decline in climbing activity induced by overexpression of Aβ pan-neuronally was ameliorated in Aβ×Elav flies grown in media supplemented with captopril (5 and 10mM) (Fig. 4).

#### Effect of captopril on climbing in synuclein expressing flies:

Two-way ANOVA revealed significant effect of treatments on climbing activity [F(3,40)=31.16, p<0.001] (Fig.5). Post hoc analysis showed time course decrease in climbing activity with peak effect at day 28. The decline in climbing activity induced by overexpression of synuclein in dopamine neuron was ameliorated in syn×DDC flies grown in captopril (5 and 10mM) supplemented media (Fig. 5).

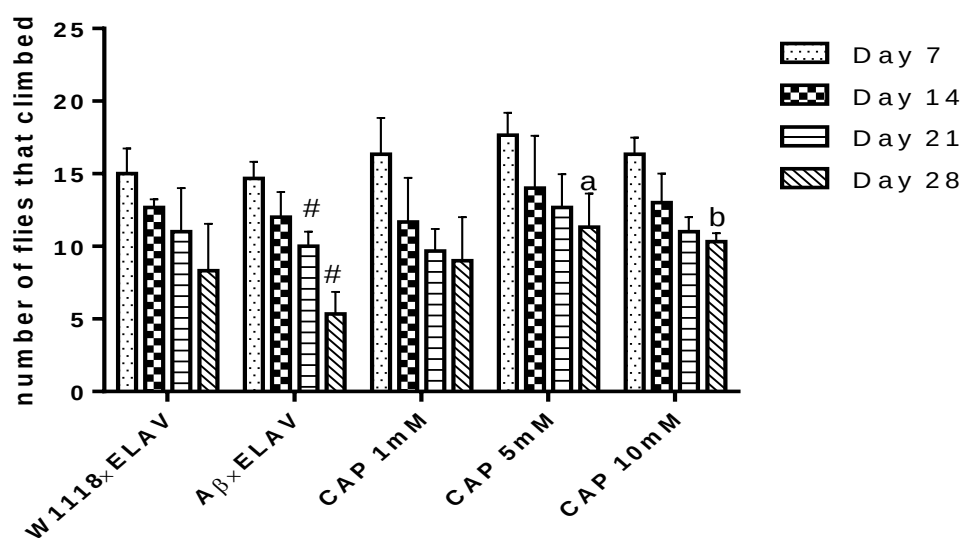


**Figure 4:**

Effect of captopril on the climbing ability of Aβ expressing flies. Bar chart represents mean ± SD; (<sup>a</sup>P<0.05, <sup>b</sup>P<0.05) versus Aβ × elav control. Data were analysed by two-way ANOVA followed by Tukey's post hoc multiple comparison test.

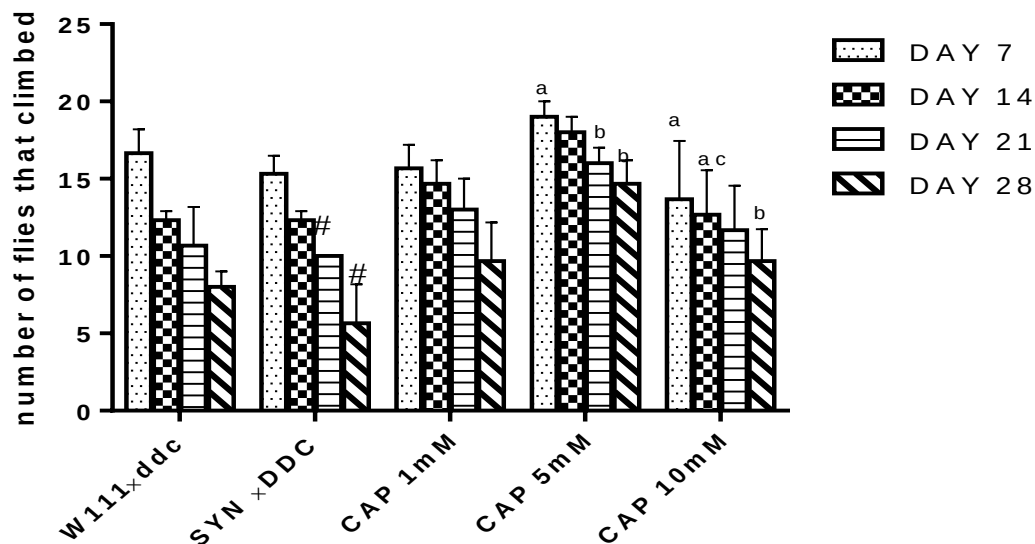
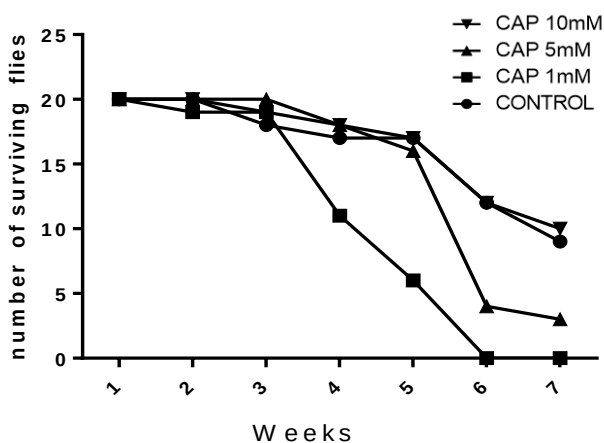


**Figure 5:**

Effect of captopril on the climbing ability following synuclein overexpression in dopamine neuron in flies. Bar chart represents mean  $\pm$  SD; #P < 0.05, ##P < 0.01 versus day 7 in syn $\times$ ddc control; <sup>a</sup>P < 0.05; <sup>b</sup>P < 0.01, <sup>c</sup>P < 0.001 versus respective days in Syn $\times$  ddc control. Data was analysed by two-way ANOVA followed by Tukey's multiple comparison test.

### Longevity assay

Two-way ANOVA revealed significant effect of treatments on lifespan [F(3,18)=5.95, P<0.0] (Fig. 6) in Cs flies. CS flies grown on feed media supplemented with captopril 5mM concentration showed a slight increase in the number of surviving flies while flies cultured on 10mM feed media showed a significant improvement in the number of surviving flies when compared to CS flies cultured on normal feed (Fig. 6).



### DISCUSSION

In this study, synuclein expressing flies replicated several features of human PD, including locomotor dysfunction, lewy body like formation and age-dependent loss of dopaminergic neurons. Treatment of synuclein expressing flies with captopril (1mM, 5 mM and 10 mM) for a period of 28 days showed a dose- dependent increase in the number

of line crosses in the larva motility assay when compared to the pathologic model (synuclein expressing flies), and thus, indicating the potential neuroprotective role of captopril in PD. The mechanism by which ACE inhibitors provide neuroprotection in PD may involve an attenuation of AngII effect on ATI receptors. Considerable evidence suggests that reducing AngII actions on ATI receptors reduces oxidative stress, neuronal loss and neuroinflammation by inhibition of ATI receptors.

One of the distinguishing characteristic features in Parkinson's disease is the loss of dopaminergic neuron and the aggregation of lewy bodies (Spillantini *et al.*, 1998) in the substantia nigra while that of Alzheimer's disease is the aggregation of A $\beta$  peptides at specific brain regions which have been implicated in the pathogenesis of AD (Selkoe, 2011). These proteins are neurotoxic proteins and can result in the loss of brain neurons which are involved in motor coordination. Several studies have shown association between ACE-I and a decrease in the incidence of PD (Gao *et al.*, 2017; Perez-Lloret *et al.*, 2017). Moreso, in vitro studies revealed that Angiotensin II (Ang II) induced apoptosis of dopaminergic neurons through its type 1 receptor (AT1R) (Gao *et al.*, 2017). Similarly, captopril ameliorate MPTP-induced PD in mice (Yazdani *et al.*, 2006; Sonsalla *et al.*, 2012), thus confirms the neuroprotective effect of captopril provides neuroprotection.

An increase or decrease in *drosophila* fertility could be due to several reasons, including temperature variations or other regulating factors which could result in increase or decrease in; number of eggs laid by mated female flies, hatchability and/ or mortality of laid eggs during development (Ram and Wolfner, 2007). Fecundity assay is a test that provides a knowledge of flies' potential to reproduce. Examination of fecundity in CS strain cultured on normal and those on feed supplemented with captopril showed that there was a decrease in the number of eggs as the concentration increased from 1mM to 10mM, though

this decrease was not significantly different from that of control. However, at the highest concentration (10mM), there was a significant decrease in the number of eggs laid, dark pupa and eclosion suggestive of possible impact of captopril on flies' fertility.

Several studies have also demonstrated an association between ACE-I and a decrease in the incidence of AD (Zou et al., 2007; Abdalla et al., 2013; Bernstein et al., 2014). However, despite the beneficial effects of ACE-I on AD, studies on the effect of ACE-I remain inconsistent and unclear. Since flies do not endogenously produce A $\beta$  peptides, A $\beta$  is produced by transgene expression thus allowing the exploration of the amyloidogenic pathway through which captopril acts. In this present study, A $\beta$  expressing flies showed significantly impaired motor function. Treatment of A $\beta$  expressing flies with various concentrations of captopril showed more improvement in the larva motility compared to control. This result suggests that captopril may play an important therapeutic role.

Aging is a phenomenon that results in steady physiological deterioration in nearly all organisms in which it has been examined. Individual aging is manifest at the population level as an increase in age-dependent mortality, which is often measured by observing life span in age-matched flies. Life span assay in *Drosophila* provides the link between insect ACE and longevity (Liao et al., 2014; Kumar et al., 2016) and depends on strict regulation of nutritional and environmental conditions. Measurement of lifespan in Canton S (CS) fly strain cultured on normal feed and those on feed supplemented with captopril showed that there was a slight increase in the lifespan at the 10mM concentration of captopril but there was no significant change in lifespan when compared to control.

In conclusion, the present study showed that captopril improves locomotor activity and confers a neuroprotective effect in *Drosophila* possibly through inhibition of amyloid beta or alpha synuclein protein aggregation. Thus, could be a potential adjunct in the management of neurodegenerative diseases.

#### Acknowledgment

Authors are very grateful to the Department of Science and Technology (DST) and Ministry of External Affairs (MEA), Government of India (GoI), through the Federation of Indian Chambers of Commerce & Industry (FICCI) for the C.V. Raman Fellowship for African Researchers award to Ishola IO. We also appreciate Centre for Cellular and Molecular Biology, Hyderabad, Andra Pradesh, India for various strains of *Drosophila melanogaster*.

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Full-Length Research Article

# Does HAART Dysregulate Angiogenesis in HIV-infected Pre-eclampsia?

Padayachee S.<sup>a</sup>, Govender N.<sup>b</sup> and \*Naickera T.<sup>a</sup>

<sup>a</sup>Optics and Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences  
University of KwaZulu-Natal, Durban, South Africa

<sup>b</sup>Department of Basic Medical Sciences, Faculty of Health Sciences, Durban University of Technology, Durban, South Africa

**Summary:** A dysregulation of angiogenic mediators has been implicated in HIV infection. Inconsistent data exists on highly active antiretroviral therapy (HAART) usage in pregnancy and its association with PE development. In view of the high prevalence of HIV infection and PE in SA, this study was aimed at determining PlGF and sFlt-1 levels in HIV-infected normotensive and preeclamptic pregnancies treated with HAART. Both PlGF and sFlt-1 were quantified in serum from HIV positive [normotensive (N+) and preeclamptic (P+)] and HIV negative [normotensive (N-) and preeclamptic (P-)] pregnancies, using a Milliplex Multiplex immunoassay. sFlt-1 was significantly upregulated in P+ vs the N+ groups. PlGF was significantly downregulated in PE vs normotensive groups, regardless of HIV status. sFlt-1/PlGF ratio was significantly increased in PE- vs the N- groups. We report an amplification of sFlt-1 in lieu of PlGF down-regulation in HIV-infected pregnancies receiving HAART.

**Keywords:** sFlt-1, PlGF, angiogenic dysregulation, HIV-1, HAART

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\*Address for correspondence: [naickera@ukzn.ac.za](mailto:naickera@ukzn.ac.za); Tel: +27 313732796

Manuscript received- February 2022; Accepted- May, 2022

DOI: <https://doi.org/10.54548/njps.v37i1.4>

## INTRODUCTION

In low-to-middle-income countries, maternal mortality is a public health challenge due to late initiation of and irregular visits for antenatal care. Most of these deaths are associated with hypertensive disorders of pregnancy (HDP), more specifically preeclampsia (PE) (Peres *et al.*, 2018). In some parts of Africa, 18% of pregnancies are complicated by PE (Robertson, 2019). In South Africa (SA), the overall HIV prevalence rate is 13.7% (8.2 million), of which 25% are women in their reproductive age (Stats SA, 2021). It is disturbing that antenatal surveillance in the province of KwaZulu-Natal (KZN), SA revealed that 40% of pregnant women are HIV positive (Woldesenbet *et al.*, 2018). Whilst the administration of antiretroviral therapy (ART) prevents mother-to-child transmission of HIV (Clouse *et al.*, 2020) it promotes PE development due to immune reconstitution (Phoswa *et al.*, 2019, Landi *et al.*, 2014).

The multifactorial disease of PE presents itself in the form of hypertension in the latter half of pregnancy (>20 gestational weeks) with/without proteinuria and multi-organ dysfunction (Brown, 2018). In KZN, the prevalence of PE is 12% (Moodley *et al.*, 2016). Currently, the only effective cure for PE is emergency delivery of the fetus and placenta. The underlying pathogenesis of PE is associated with inadequate placentation due to deficient trophoblast invasion and absence of myometrial spiral artery remodelling (Fisher, 2015). Consequently, this state of under-perfusion creates placental hypoxia with localized oxidative stress (Valenzuela *et al.*, 2012).

PE is an anti-angiogenic state as a result of increased release of anti-angiogenic factors into the maternal circulation. In PE, soluble fms-like tyrosine kinase-1 (sFlt-1) antagonizes the biological availability of both vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) by binding to their receptors (Maynard and Karumanchi, 2011). This angiogenic imbalance contributes to widespread maternal endothelial injury and heightened inflammatory response with clinical manifestation of PE (Chaiworapongsa *et al.*, 2009). Moreover, sFlt-1 via VEGF inhibition stimulates complement-mediated placental injury in PE (Yonekura Collier *et al.*, 2019). A dysregulation of angiogenic mediators have also been implicated in HIV infection (Chaiworapongsa *et al.*, 2009). Conflicting data exists on ART (HAART) usage in pregnancy and its association with PE development (Powis *et al.*, 2013).

Investigation of angiogenic factors in PE has been evaluated for its use in the early prediction and assessment of disease severity and progression (Verlohren *et al.*, 2010, Zeisler *et al.*, 2016). Given the high prevalence of HIV infection and PE in SA, this study aimed to determine PlGF and sFlt-1 levels in HIV infected normotensive and preeclamptic pregnancies treated with HAART.

## MATERIALS AND METHODS

**Study population:** Following receipt of ethical approval (BE313/18), regulatory health permissions and written informed consent, venous blood samples were collected from 80 pregnant women (n=80) attending an antenatal

clinic at a large regional hospital in eThekweni, KZN. Serum samples were stored at -80°C until immunoassay. The study population (n=80) consisted of normotensive pregnant (n=40) and preeclamptic (n=40) groups, which were further stratified by HIV status [i.e., HIV positive normotensive (N<sup>+</sup>; n=20); HIV negative normotensive (N<sup>-</sup>; n=20); HIV positive preeclamptic (P<sup>+</sup>, n=20) and HIV negative preeclamptic (P<sup>-</sup>, n=20)]. Preeclampsia was defined as systolic blood pressure  $\geq$  140 mmHg and diastolic blood pressure  $\geq$  90 mmHg with at least a two plus of proteinuria on dipstick testing after 20 weeks of pregnancy (Brown, 2018).

The relevant data of all research participants were obtained from their maternity case records. HIV testing was done after counselling using a rapid point-of-care test kit initially, as is the standard of care in South Africa. All patients recruited were of African ancestry and resident in the same geographical location in order to maintain ethnographic and anthropometric consistency. All newly diagnosed HIV-positive pregnant women were initiated on combined anti-retroviral therapy (cART) consisting of tenofovir, emtricitabine, and efavirenz, as per South African national HIV guidelines at the time of the study (South African National Department Of Health, 2020). Exclusion criteria was based on patients that declined participation and those with polycystic ovarian syndrome, abruption placentae, intrauterine death, sickle cell disease, chronic renal disease, cardiac disease, unknown HIV status, pre-existing seizure disorders and asthma.

**Milliplex Multiplex assays:** Serum was diluted for quantifying PlGF (1:3) and sFlt-1 (1:5) prior to quantification using MILLIPLEX®MAP Human Angiogenesis/Growth Factor Magnetic Bead Panel 1 (Cat.# HAGP1MAG-12K) and MILLIPLEX®MAP Human Angiogenesis Magnetic Bead Panel 2 (Cat.# HANG2MAG-12K) kits respectively. The immunoassays were performed according to the manufacturer's instructions with an overnight incubation (16-20hrs) at 2-8°C for both PlGF and sFlt-1. The plates were then analysed with the Bio-Plex MAGPIX Multiplex reader (Bio-Rad Laboratories, Pleasanton, CA, USA) with xPONENT v.3.2 software.

### Statistical analysis

All data was analysed using STATA (version 12, STATAcorp) and Graph Pad Prism version 8 (California, USA). Data were tested for normality using the D'Agostino test and histograms are presented as median and interquartile range (IQR). The Kruskal Wallis test and the Dunn's *post hoc* test were used to determine if there was a significant difference between groups. The Spearman's correlation coefficients were used to assess the relationship between sFlt-1 and PlGF between groups and between clinical factors. A  $p$  value  $\leq$  0.05 was considered statistically significant at a 95% confidence level.

## RESULTS

**Clinical demographics:** The clinical characteristics are shown in Table 1. As expected, the systolic (SBP) and diastolic (DBP) blood pressures were significantly higher in PE compared to normotensive pregnancies ( $p < 0.0001$ ). Gestational age at delivery were significantly lower in PE relative to normal pregnancies ( $p < 0.0001$ ). There was a lack of statistically significant difference for maternal age and maternal weight between normotensive pregnant and PE women, regardless of pregnancy type or HIV status.

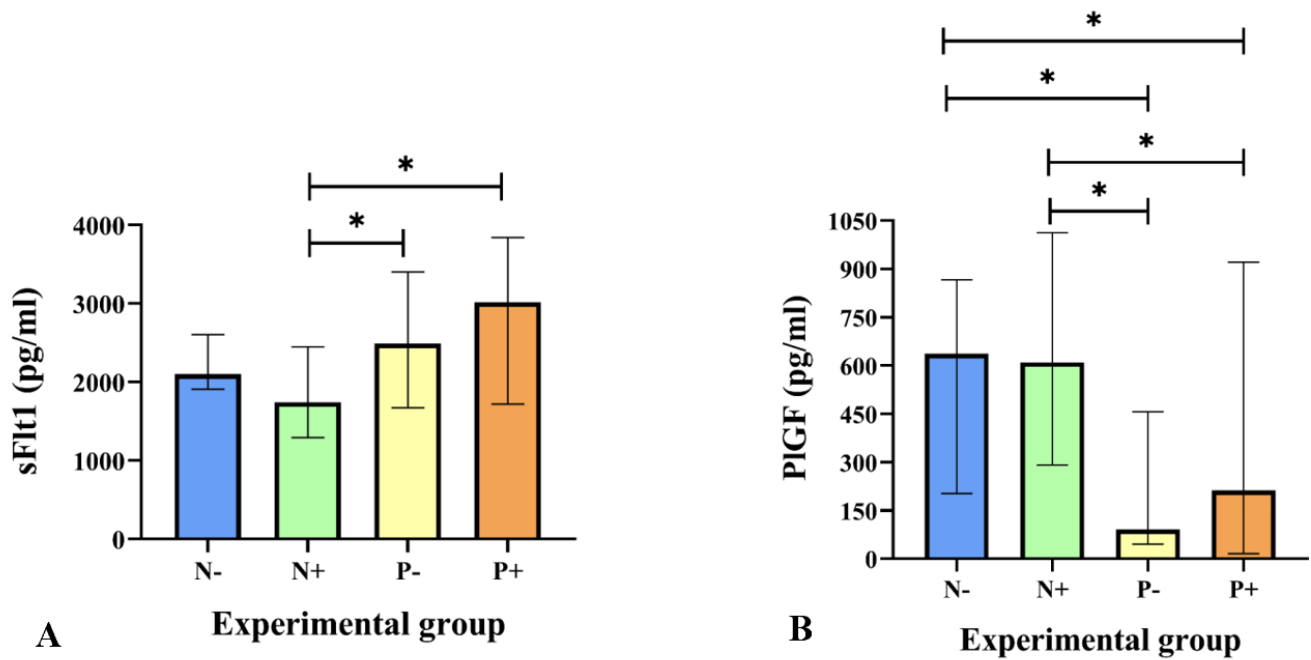
**Serum levels of sFlt-1 and PlGF:** The serum levels of sFlt-1 and PlGF are shown (Figure 1A-B). The Kruskal Wallis and Dun's *post hoc* test revealed a statistically significant difference for circulating levels of sFlt-1 between the P- vs the N+ groups, as well as between the P+ vs the N+ groups (Figure 1A). Likewise, a statistically significant difference was noted for PlGF levels between the P- versus N-; and P- vs N+ groups ( $p = 0.0001$ ). Moreover, we noted a significant difference between P+ vs the N-, and P+ vs the N+ groups ( $p = 0.0001$ ) respectively (Figure 1B). For the sFlt-1/PlGF ratio, a statistically significant difference was noted between the P- vs N-, and P- vs the N+ groups ( $p = 0.04$ ), respectively.

**Table 1.**

Clinical characteristics across all study groups [N=80; Median (25th – 75th percentile)]

|                          | Normotensive (n=40)         |                             | Pre-eclamptic (n=40)        |                             | <i>p</i> value |
|--------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|----------------|
|                          | HIV negative (N-)<br>(n=20) | HIV positive (N+)<br>(n=20) | HIV negative<br>(P-) (n=20) | HIV positive<br>(P+) (n=20) |                |
| Maternal age<br>(years)  | 26.00<br>(19.00-38.00)      | 28.00<br>(23.00-33.00)      | 25.00<br>(21.00-32.00)      | 29.00<br>(26.00-30.00)      | 0.962          |
| Maternal weight<br>(kg)  | 75.00<br>(67.00-85.00)      | 70.35<br>(65.10-75.50)      | 75.50<br>(68.00-103.00)     | 70.00<br>(67.00-81.00)      | 0.281          |
| Gestational age<br>(wks) | 39.00<br>(39.00-39.00)*     | 38.00<br>(38.00-40.00)*     | 35.00<br>(34.00-38.00)*     | 34.00<br>(34.00-36.00)*     | <b>0.0001*</b> |
| Systolic bp<br>(mmHg)    | 120.00<br>(110.00-125.00) * | 118.50<br>(114.50-126.50) * | 168.00<br>(160.00- 177.00)* | 170.00<br>(156.00-178.00) * | <b>0.0001*</b> |
| Diastolic bp<br>(mmHg)   | 69.00<br>(57-73)*           | 73.00<br>(65.00-81.00)*     | 105.50<br>(98.00-112.00)*   | 106.00<br>(97.00-114.00)*   | <b>0.0001*</b> |

\* $p < 0.05$  considered statistically significant

**Figure 1:**

Serum concentrations (A–B, median and interquartile range) of (A) anti-angiogenic sFlt1 (pg/ml) and (B) angiogenic PlGF (pg/ml) in HIV negative normotensive pregnant (N-); HIV positive pregnant (N+); HIV negative preeclamptic (P-) and HIV positive preeclamptic (P+) women.

**Spearman's correlation matrix between circulating levels of sFlt-1 and PlGF:** A bivariate Spearman's correlation analysis showed a relationship between circulating sFlt-1 and PlGF levels with clinical factors, in the N+ and P+ groups. A negative association between SBP and gestational age ( $r=-0.68$ ;  $p=0.003$ ); and a positive association between DBP and SBP ( $r=0.65$ ;  $p=0.006$ ) was noted.

Based on HIV status and pregnancy type, a statistically significant negative association was observed between sFlt-1 (P-) group and DBP ( $r=-0.57$ ,  $p=0.002$ ); and between sFlt-1 (P-) groups and sFlt-1 (P+) groups ( $r=-0.58$ ,  $p=0.01$ ). For PlGF, a statistically significant negative association was observed between P- group and SBP ( $r=-0.64$ ,  $p=0.04$ ). A statistically significant positive association was observed between sFlt-1 (P-) and PlGF (N-) groups ( $r=0.79$ ;  $p=0.02$ ). However, based only on HIV status and regardless of pregnancy type, a statistically significant negative association was shown between SBP and gestational age ( $r=-0.86$ ,  $p=0.0001$ ); DBP and gestational age ( $r=-0.74$ ;  $p=0.000$ ) and DBP and SBP ( $r=0.92$ ,  $p=0.000$ ) respectively. We also noted a positive association between sFlt-1/PlGF ratio and SBP ( $r=0.40$ ;  $p=0.03$ ); and DBP ( $r=0.4$ ;  $p=0.03$ ), in contrast to the negative association observed between the sFlt-1/PlGF ratio and gestational age ( $r=-0.50$ ;  $p=0.006$ ). A similar correlation analyses were done based on pregnancy type (normotensive vs PE); however, no significant associations were observed between the analytes and the clinical factors

## DISCUSSION

In our study, we report a statistically significant up-regulation of sFlt-1 in P+ compared to the N+ groups ( $p<0.05$ ), albeit similar levels were observed between N- vs the P- groups. It is widely accepted that sFlt-1 augmentation causes peripheral vasoconstriction with development of high blood pressure in PE, however, the altered sFlt-1 levels in our study may also be amplified from ART usage. In SA, it is a standard practice for pregnant women with previous antiretroviral exposure and documented viral load suppression, to be administered a fixed dose combinations of Tenofovir (TDF) 300mg; Lamivudine (3TC) 300mg and Dolutegravir (DTG) 50mg, loosely labelled (TLD) (Nel *et al.*, 2020). Women in our study did not switch to the revised guidelines that include DTG. Instead, these women remained on Tenofovir (TDF) + Lamivudine (3TC)/Emtricitabine (FTC) + Efavirenz (EFV) to continue their ART regimen.

In contrast to HIV-protease inhibitors (PIs), the nucleoside reverse transcriptase inhibitors (NRTIs) (*viz.*, zidovudine, efavirenz, lamivudine, emtricitabine, abacavir or tenofovir) induces endothelial dysfunction via increased reactive oxygen species production, and defective mitochondrial activity (Chen *et al.*, 2019). Notably, NRTIs interferes with angiogenic signal transduction (Weiß *et al.*, 2016, Song *et al.*, 2018, Chen *et al.*, 2019), including the mitogen-activated protein kinases (MAPK)/extracellular-signal-regulated kinases (ERK) and/or the phosphoinositide 3 kinase (PI3K)/ protein kinase B (AKT) pathways (Loizzi *et al.*, 2017). Furthermore, NRTIs suppress receptor tyrosine kinase (RTK) signalling by prohibiting VEGF-A/PlGF induced endothelial cell neovascularization, suggesting a direct effect of NRTIs on RTK activation (Song *et al.*, 2018). The dysfunctional angiogenic expression in our study

may be a consequence of NRTI suppression on VEGFR-2 activity and the inactivation of the MAPK/ERK pathway, which prevents endothelial proliferation (Liang *et al.*, 2018). Moreover, previous studies have reported that preeclamptic females receiving HAART are associated with endothelial dysfunction and angiogenic imbalance (Ajadi *et al.*, 2021). Our findings also corroborate previously reported data on HIV uninfected pregnant women (Hirashima *et al.*, 2005, Maynard *et al.*, 2003, Levine *et al.*, 2004).

An anti-angiogenic phenotype was reported between second and third trimester pregnancies (Romero *et al.*, 2010), similar to our findings albeit at term. Earlier studies establishing the reference range of sFlt-1 suggest that sFlt-1 increases with gestational age (Levine *et al.*, 2004, Thadhani *et al.*, 2004), however, we show no significant association between sFlt-1 and gestational age. Off note, an anti-angiogenic state is associated with adverse birth outcomes in HIV infected women (Conroy *et al.*, 2017).

In our study, similar levels of sFlt-1 expression were noted between the HIV infected vs uninfected groups. In a hypoxic environment, mitochondrial reactive oxygen species (mtROS) trigger the release of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ). An increased production of ROS has been previously reported in HIV-1 infection due to CD4<sup>+</sup> T cell apoptosis (Perl and Banki, 2000). HIF-1 $\alpha$  then promotes viral replication of HIV-1 in CD4<sup>+</sup> T cells and stimulates the release of extracellular vesicles (EVs) from infected cells (Duette *et al.*, 2018). HIV-1 infection thereby enhances HIF-1 $\alpha$  transcriptional activity. Duette *et al.* (2018) suggests that cART with undetected viral load may stimulate the activity of HIF-1 $\alpha$  with resultant inflammation (Duette *et al.*, 2018). It is also possible that the hyper-reactivity of HIF-1 $\alpha$  irrespective of ART promoted *Flt-1* gene activity, amplifying sFlt-1 expression in our study.

As expected, we noted a significant down-regulation in PlGF expression in the PE groups compared to the normotensive groups, regardless of HIV status. Under conditions of pathology, a permanent angiogenic imbalance exists in contrast to transient activation of physiological angiogenesis (Voron *et al.*, 2014). In turn, VEGF and PlGF exhibit immunomodulatory properties by inducing an immunosuppressive environment (Voron *et al.*, 2014, Dikov *et al.*, 2005). Therefore, the down-regulation of PlGF in PE in our study may originate from the loss of maternal tolerance induced by HIV infection together with inadequate trophoblast invasion and deficient vascular development (Albonici *et al.*, 2020). Additionally, the decreased expression of PlGF in the HIV-positive groups regardless of pregnancy type, may be attributed to PlGF/TNF- $\alpha$  stimulation. HAART is known to modify systemic TNF- $\alpha$  induced inflammation (Keating *et al.*, 2011). Notably, a shift from Th2 to Th1 immunity has been described as one of the confounding effects of HAART (Lopez *et al.*, 2012). While HAART prevents HIV disease progression, its usage is associated with adverse pregnancy outcome since normal pregnancy favours a Th2 response (Lin *et al.*, 2007). High PlGF levels produced by the placenta supports a Th2 cytokine profile in favour of normal fetal development (Lin *et al.*, 2007). In our study, the excess sFlt-1 antagonizes PlGF activity by acting as a decoy receptor; however, their levels are exacerbated by the immune restorative action of ARVs.

We report a significant up-regulation of sFlt-1/PlGF

ratio in the PE- versus the N- groups. In HIV uninfected samples, sFlt-1 binds to circulating VEGF and PlGF, inhibiting the action of these transmembrane receptors. Due to the abnormal vascular remodelling of the maternal spiral arteries, the resultant hypoxic environment promotes the excessive release of sFlt-1 into the maternal circulation (Lecarpentier and Tsatsaris, 2016). Preeclampsia arises when the functional activity of sFlt-1 surpasses that of VEGF (Rana *et al.*, 2007). Notably, a high sFlt-1/PlGF ratio is associated with an increased risk of PE development (Zeisler *et al.*, 2016).

HIV accessory proteins including *Tat*, *gp120* and *p17* contribute to endothelial vascular injury in HIV-1 infection (Jiang *et al.*, 2010, Wang *et al.*, 2014). The *Tat* protein mimics VEGF due to its arginine and lysine-rich sequence similarities to VEGF and targets its VEGFR-2/KDR receptor; hence it is a powerful angiogenic protein (Albini *et al.*, 1996, Zhou *et al.*, 2013). Furthermore, *Tat* gains access to the endothelium to facilitate HIV-1 induced oxidative stress through the arginine-glycine-aspartate motif; via the integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  and VEGFR-2/KDR of its basic domain. It negatively regulates endothelial cell morphology, gene expression, and proliferation via aberrant cell signalling and MAPK activation (Kline and Sutliff, 2008). Interestingly, ART does not inhibit *Tat* secretion (Mediouni *et al.*, 2012). Additionally, the HIV-1 glycoprotein *gp120* prompts the release of *Tat* by binding to heparin sulphate proteoglycans on endothelial cells where it induces oxidative stress (Crublet *et al.*, 2008). Consequentially, endothelin-1 is released and induces vasoconstriction (Anand *et al.*, 2018). The HIV-1 matrix protein *p17* persists in lymph nodes of patients receiving HAART (Popovic *et al.*, 2005, Fiorentini *et al.*, 2006), deregulating the biological activity of immune cells (Caccuri *et al.*, 2012), thereby affecting angiogenesis.

In conclusion, this study demonstrates an anti-angiogenic (sFlt-1 vs PlGF) milieu in PE. We report sFlt-1 amplification in lieu of PlGF downregulation in HIV infected pregnancies. Moreover, the HIV-1 *Tat*, *gp120* and *p17* proteins are responsible for endothelial injury contributing to angiogenic imbalance. The endothelial dysfunction may be a direct effect of these HIV proteins or from indirectly via HAART administration. Both PIs and NRTIs dysregulate angiogenesis via RTK cell signalling pathways. Further large-scale investigations are warranted to fully ascertain the role of HAART on angiogenesis during pregnancy.

### Acknowledgments

The authors wish to thank the UKZN College of Health Sciences for the funding received.

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Full-Length Research Article

# Luteolin Normalizes Blood Pressure Via Its Antioxidant Activity and Down-Regulation of Renal Angiotensin II Receptor and Mineralocorticoid Receptor Expressions in Rats Co-exposed to Diclofenac and Sodium Fluoride

Ajibade T.O.<sup>a</sup>, \*Akinrinde A.S.<sup>a</sup>, Adetona M.O.<sup>b</sup>, Adedapo A.D.A.<sup>c</sup>, Oyagbemi A.A.<sup>a</sup>, Larbie C.<sup>d</sup>, Omobowale T.O.<sup>e</sup>, Ola-Davies O.E.<sup>a</sup>, Saba A.B.<sup>f</sup>, Adedapo A.A.<sup>f</sup>, Oguntibeju O.O.<sup>g</sup>, Yakubu M.A.<sup>h</sup>

Departments of <sup>a</sup>Veterinary Physiology and Biochemistry, <sup>e</sup>Veterinary Medicine, <sup>f</sup>Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Ibadan, Nigeria

Departments of <sup>b</sup>Anatomy, <sup>c</sup>Pharmacology and Therapeutics, University of Ibadan, Nigeria

<sup>d</sup>Department of Biochemistry and Biotechnology, College of Science, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

<sup>g</sup>Phytomedicine and Phytochemistry Group, Oxidative Stress Research Centre, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville 7535, South Africa

<sup>h</sup>Department of Environmental & Interdisciplinary Sciences, College of Science, Engineering & Technology, Vascular Biology Unit, Center for Cardiovascular Diseases, Texas Southern University, Houston, TX, USA

**Summary:** This study was designed to investigate the modulatory role of Luteolin (Lut), a flavonoid phytochemical, on haemodynamic parameters and the potential mechanisms involving renal Angiotensin II (AT<sub>2</sub>R) and Mineralocorticoid (MCR) receptors in renal toxicity induced by co-exposure to Diclofenac (Dcf) and sodium fluoride (NaF) in rats. Male Wistar rats were administered with either vehicle (control), Dcf only (9 mg/kg orally) or concurrently with NaF (300 ppm in drinking water). Other groups were treated with LutA (100 mg/kg) or LutB (200 mg/kg) along with Dcf and NaF exposures. All treatments lasted 8 days, following which blood pressure indices were measured using tail-cuff plethysmography. Renal expressions of AT<sub>2</sub>R and MCR were studied with immunohistochemistry, while biomarkers of oxidative and antioxidant status were also measured in the kidneys. Systolic, diastolic and mean arterial pressures were significantly ( $p < 0.05$ ) reduced in Dcf-treated rats, compared to control values. However, co-treatment with NaF or Lut restored these parameters. While the expression of AT<sub>2</sub>R and MCR was high in the Dcf and Dcf+NaF groups, treatment with Lut caused obvious reduction in the renal expression of these receptors. Increased lipid peroxidation (Malondialdehyde) and protein oxidation (protein carbonyls) with a lowering of reduced glutathione levels contributed to the renal toxicity of Dcf, and these were significantly ameliorated in Lut-treated rats. In conclusion, the preservation of haemodynamic indices by Luteolin in the experimental rats was probably mediated by mechanisms involving down-regulation of renal expressions of AT<sub>2</sub>R and MCR, reduction of oxidative stress and an improvement of renal antioxidant status.

**Keywords:** Renin-Angiotensin, hypotension, Diclofenac, fluoride, oxidative stress, polyphenol antioxidant.

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\*Address for correspondence: E-mail: [as.akinrinde@gmail.com](mailto:as.akinrinde@gmail.com); Tel: +2348051659095

Manuscript received- January 2022; Accepted- April 2022

DOI: <https://doi.org/10.54548/njps.v37i1.5>

## INTRODUCTION

The toxicity of emerging anthropogenic pollutants such as residues of pharmaceuticals present in environmental compartments has become an important subject of interest in environmental toxicology not just for their individual toxicity, but also their presence in complex mixtures with other environmental stressors (Wieczerezak *et al.*, 2018). Diclofenac (Dcf) and many other Non-selective anti-inflammatory drugs (NSAIDs) are among the most widely prescribed drugs for analgesic and anti-inflammatory

purposes. Residues of these drugs are increasingly detected in environmental matrices from their discharge as effluents from drug manufacturing companies or inappropriate disposal (Freitas and Radis-Baptista, 2021). The pharmacological use of non-selective NSAIDs, including Dcf is often hampered by life-threatening adverse effects including gastrointestinal toxicity, impairment of renal function, with consequent alterations of fluid/electrolyte balance and blood pressure changes (Gwanyanya *et al.*, 2011). While several studies have focused on the effects of exposure to single chemicals or drugs, it is now increasingly

recognized that environmental chemical agents usually affect body tissues in combination, rather than alone. Thus, a novel strategy in the study of the effects of environmental pollutants on critical physiological attributes is the evaluation of possible interactions between pollutants, leading to synergism or antagonism (Nica *et al.*, 2017).

Several prescription drugs are known to affect the blood pressure of patients or individuals being treated for hypertension. Although NSAIDs are among the most common classes of medication consumed by hypertensive patients, there exist wide variations regarding their effects on blood pressure and haemodynamics of exposed humans or experimental animals (Aljadhev *et al.*, 2012). Most studies associate an increased risk of hypertension with Dcf use, occurring via its inhibition of the cyclooxygenase pathway leading to reduction in production of natriuretic prostaglandins (e.g. PGE<sub>2</sub>), salt retention, as well as reduction in the vasodilatory effects of these prostaglandins (Harris, 2002). On the other hand, there are recent suggestions that upper or lower gastrointestinal bleeding resulting from acute exposure to NSAIDs may precipitate haematemesis, melena or haematochezia, with severe cases sometimes progressing into hypovolemia, hypotension and shock (Laine *et al.*, 2021).

Exposure to fluoride salts is almost inevitable and can occur from different environmental sources such as drinking water, toothpastes and dental products (EFSA, 2013). A recent review has revealed that fluoride is often incorporated into pharmaceuticals in order to increase their biological half-lives, raising the likelihood of co-exposure with drugs, including Dcf (Yanac and Murdoch, 2019). Fluoride has been reported to cause elevation of systolic, diastolic and mean arterial blood pressures in rats (Oyagbemi *et al.*, 2017), via mechanisms involving the induction of oxidative stress. However, the effect of co-exposure of fluoride and Dcf on blood pressure parameters is not known, although, both Dcf and NaF have been reported to increase the generation of reactive oxygen species (ROS) and oxidative stress in various tissues (Islas-Flores *et al.*, 2013; Khan *et al.*, 2013).

The kidney is a major organ involved in the control of salt and water homeostasis, and hence plays vital roles in the modulation of haemodynamic changes (Wadei and Tektor, 2012). Renal control of extracellular volume is closely linked to the regulation of urinary sodium excretion which is influenced by the activity of vasoactive systems, including the renin-angiotensin-aldosterone system (Granger and Schnackenberg, 2000). Renal angiotensin II increases blood pressure either directly by enhancing tubular transport of sodium, or indirectly through mineralocorticoid (aldosterone) stimulation. Therefore, AT<sub>2</sub>R antagonists or inhibitors of the mineralocorticoid receptors (MCR) are expected to be effective in the treatment of hypertension by enhancement of natriuresis (Ivy and Bailey, 2014). The effects of co-exposures to Dcf and NaF on renal expression of AT<sub>2</sub>R and MCR have not yet been studied. Additionally, the kidney is known to often accumulate fluoride at concentrations even higher than the concentration in the plasma (Guthet *et al.*, 2020).

Luteolin (Lut), chemically 3, 4, 5, 7-tetrahydroxyflavone, is a flavonoid component of many fruits and vegetables, and is known to exhibit antioxidant, anti-inflammatory and anticancer activities (Su *et al.*, 2015).

Available evidence suggests that luteolin causes reduction in blood pressure via stimulation of nitric oxide production and arterial relaxation (Si *et al.*, 2014). Studies have shown that luteolin can influence blood pressure and cardiovascular protection by modulating proliferation of blood vessels and inhibiting hypertension-induced vascular remodelling (Qianet *et al.*, 2010). The effects of luteolin on renal regulation of vascular haemodynamics via the renin-angiotensin system are not yet fully known.

It is well known that NSAIDs, including Diclofenac (Dcf), produce nephrotoxicity with possible fluid retention and increase in blood pressure. Concurrent exposure to other agents that modulate blood pressure may either result in remission or aggravation of these undesirable effects. The present research was aimed to investigate how blood pressure indices and heart rates of Dcf-treated rats are affected during simultaneous exposure to NaF and Lut. The involvements of renal AT<sub>2</sub>R and MCR were also studied by their expression levels by immuno-histochemical staining and the redox status of the renal tissues was assessed by measuring the levels of protein and lipid oxidation, glutathione concentration, as well as the activities of some antioxidant enzymes.

## MATERIALS AND METHODS

**Chemicals:** Sodium fluoride, Luteolin, trichloroacetic acid (TCA), thiobarbituric acid (TBA), 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), 1,2-dichloro-4-nitrobenzene (CDNB), adrenaline, sodium hydroxide, xylene orange and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diclofenac sodium, sold as Voltaren<sup>®</sup> was purchased from a reputable pharmacy in Ibadan, Nigeria. Angiotensin II receptor and Mineralocorticoid receptor antibodies were purchased from Bioss Inc. (Woburn, MA, USA). Normal goat serum, Biotinylated antibody and Horse Radish Peroxidase (HRP) System were purchased from KPL, Inc. (Gaithersburg, MD) and Diaminobenzidine (DAB) was purchased from AMRESCO LLC. (OH, USA). All other chemicals used were of analytical grade and obtained from British Drug Houses (Poole, Dorset, UK).

**Animals and experimental design:** A total of forty-five male Wistar rats weighing 120-150 g were obtained from the Experimental Animal Unit of the Faculty of Veterinary Medicine, University of Ibadan for this experiment. They were housed in plastic cages in a well-ventilated animal house facility and randomly grouped into five groups (A-E) consisting of nine animals each. The environmental conditions of the animal house included a 12 h light and 12 h dark photoperiod and ambient temperature between 23-25°C. The rats were maintained on a commercial rat feed and clean tap water ad libitum throughout the duration of the experiment, which included one week of acclimatization. All animals received humane treatment as outlined in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health (PHS, 1996). This study was also conducted according to local guidelines approved by the University of Ibadan's Animal Care and Use Research Ethics Committee (ACUREC) under the approval number: UIACUREC/ 19/124.

This study employed a previously applied model of Dcf-induced toxicity (Singh et al., 2017). The treatments administered to the rats were as follows: Group A (control) received normal tap water; Group B rats were orally exposed to Dcf (9 mg/kg) twice daily for three days; Group C was exposed to NaF (300 ppm) in drinking water for 8 days and concurrently with Dcf administration on the final three days; Group D was treated with Luteolin at 100 mg/kg (LutA) along with Dcf and NaF exposures, while Group E was treated with Luteolin at 200 mg/kg (LutB) concurrently with Dcf and NaF administration.

**Measurement of Blood Pressure and Heart rate:** Blood pressure parameters (systolic, diastolic and mean arterial pressures) and heart rates of rats were measured indirectly by tail plethysmography without anaesthesia using an electro-sphygmomanometer (CODA, Kent Scientific, USA). Following acclimatization of the rats to the sphygmomanometer conditions, rats were restrained carefully on lateral recumbency and placed on a well-padded platform with a tail cuff attached and an average of at least nine readings per animal were taken in the quiescent state.

**Animal euthanasia and preparation of homogenates:** All the rats were euthanized by cervical dislocation upon termination of the experiments, approximately twenty four hours after the last administration of the different chemicals. The kidneys were harvested immediately following euthanasia, rinsed and homogenized in phosphate buffer (0.1 M, pH 7.4). The homogenate was then centrifuged at 10,000 rpm for 10 min in a refrigerated centrifuge (4°C). The supernatant was thereafter collected in separate bottles as the post-mitochondrial fraction and was used to assay for biochemical markers of oxidative stress.

**Assessment of biochemical markers of oxidative stress and antioxidant status:** The protein content of the kidney tissues was evaluated using the Biuret test as described by Gornal et al. (Gornal et al., 1949). The concentration of hydrogen peroxide in the kidney tissues was determined according to the Wolff (Wolff, 1994). The content of malondialdehyde (MDA) was used as an index of lipid peroxidation and was measured according to methods described by Varshney and Kale (Varshney and Kale, 1990), where a molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$  was used to compute the values. The renal activity of Glutathione peroxidase (GPx) was evaluated by the Rotruck et al. (Rotruck et al., 1973), while Glutathione S-transferase (GST) activity was measured as described by Habig et al. (Habig et al., 1974). The activity of Superoxide dismutase (SOD) was determined according to the methods described by Misra and Fridovich (Misra and Fridovich, 1972) by evaluating its inhibition of the autoxidation of adrenaline in an alkaline medium (pH 10.2), with slight modifications in our laboratory (Oyagbemi et al., 2015). The renal content of reduced glutathione was evaluated according to the method of Beutler et al. (Beutler et al., 1963), while the contents of total thiols and non-protein thiols were determined by the method of Ellman (Ellman, 1959).

**Immunohistochemistry of Renal AT<sub>2</sub>R and MCR:** Kidney tissues were fixed immediately in 10% formalin

after their harvest from the euthanized rats. The tissues were embedded and sectioned in paraffin and were processed for immunohistochemistry according to methods described by Todorich et al. (2011). Briefly, the paraffin sections were first melted at about 60°C in an oven and dewaxing was done using xylene followed by passage of the tissues through ethanol solutions of decreasing concentrations (i.e. 100-80%). Thereafter, peroxidase quenching was carried out by applying 1% H<sub>2</sub>O<sub>2</sub>/methanol solution (v/v) and this was followed by antigen retrieval by microwave heating in citrate buffer (0.01 M; pH 6.0). The sections were blocked in normal goat serum (10%, HistoMark® Gaithersburg MD) and then probed overnight with Angiotensin II receptor antibody (Bioss, San Diego, CA, USA), while other sections were probed with Mineralocorticoid receptor antibody (Bioss, San Diego, CA, USA), all at room temperature. Bound antibody detection was carried out by using biotinylated (goat anti-rabbit, 2.0 mg/mL) secondary antibody and then Streptavidin peroxidase (HorseRadish Peroxidase-Streptavidin), according to the manufacturer's protocol (HistoMark® Gaithersburg MD). The product of the reaction was enhanced with diaminobenzidine (DAB, Amresco®, USA) for 2-3 min with counter-staining using high definition Haematoxylin (Enzo®, NY, USA), while the slides were subsequently dehydrated in ethanol, sealed with coverslips and resinous solution and the immunoreactive regions indicating positive expression of AT<sub>2</sub>R and MCR were viewed with a light microscope (Olympus) and digital camera (Toupcam®, Touptek Photonics, Zhejiang, China).

#### Statistical Analysis:

Data were expressed as mean ± standard deviation and analyzed using One-way Analysis of Variance (ANOVA), followed by the Tukey's post hoc test for multiple comparisons. Statistical analysis was performed using the GraphPad Prism software (Version 7.00). P-values < 0.05 were considered statistically significant.

## RESULTS

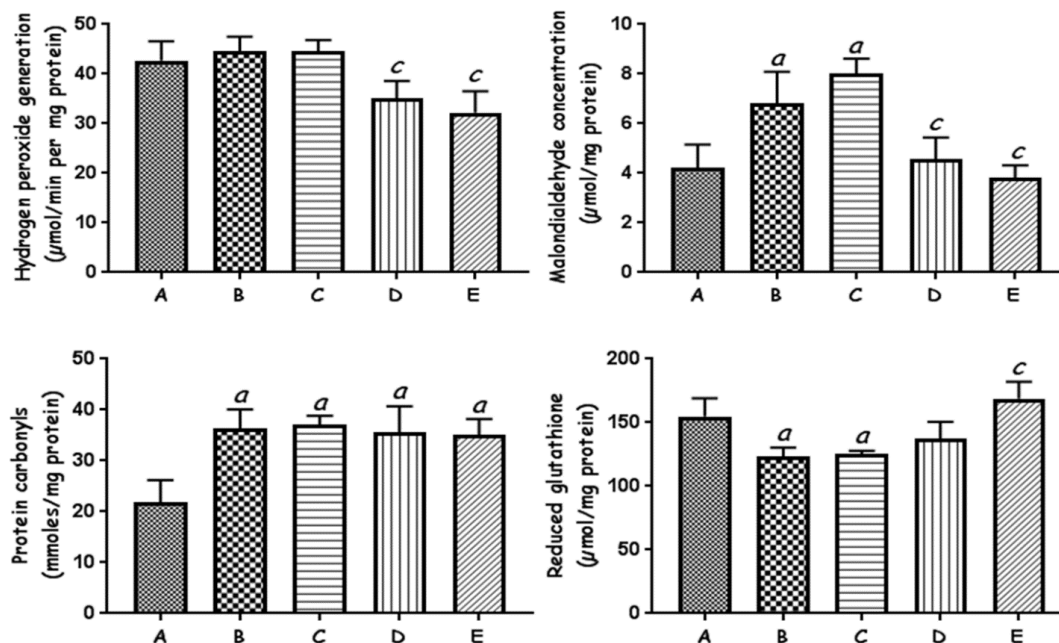
The effects of Dcf (Voltaren®), NaF and Lut exposures on blood pressure parameters and heart rates of the experimental rats are presented in Table 1. Rats exposed to Dcf alone at 9 mg/kg for three days had significant (p < 0.05) reduction in systolic (SBP), diastolic (DBP) and mean arterial (MAP) pressures, but significant (p < 0.05) increase in heart rates compared to the control rats. However, relative to exposure to Dcf alone, co-exposure to Dcf and NaF caused significant (p < 0.05) increase in SBP, DBP and MAP, but reduced heart rates. Interestingly, rats treated with Lut along with Dcf and NaF showed significant (p < 0.05) reduction in SBP, DBP and MAP compared to the group exposed simultaneously to the Dcf + NaF, with the values largely similar to those of control rats. It is important to note that SBP, DBP and MAP readings in the control and Lut-treated groups were similar to values recorded elsewhere in apparently healthy rats (Bunag and Butterfield, 1982), which reported 130 ± 5 mm Hg systolic, 100 ± 5 mm Hg mean, and 85 ± 5 mm Hg diastolic.

**Table 1:**

Blood pressure indices in rats exposed to Diclofenac, Sodium fluoride and Luteolin

| Blood pressure parameters | Control      | Dcf only                 | Dcf+NaF                    | Dcf+NaF +LutA            | Dcf+NaF+LutB              |
|---------------------------|--------------|--------------------------|----------------------------|--------------------------|---------------------------|
| SBP                       | 127.91±5.82  | 103.13±0.84 <sup>a</sup> | 137.20±2.53 <sup>a,b</sup> | 122.40±4.16 <sup>c</sup> | 117.18±10.13 <sup>c</sup> |
| DBP                       | 99.27±5.29   | 66.38±2.67 <sup>a</sup>  | 105.60±2.55 <sup>b</sup>   | 106.00±3.32              | 99.00±8.78 <sup>c</sup>   |
| MAP                       | 108.55±4.82  | 78.25±1.75 <sup>a</sup>  | 115.90±2.38 <sup>b</sup>   | 111.20±3.63              | 104.71±9.04 <sup>c</sup>  |
| Heart rate                | 350.09±42.91 | 388.13±8.37 <sup>a</sup> | 379.20±2.53 <sup>b</sup>   | 336.00±28.39             | 355.53±30.79 <sup>c</sup> |

The presented values are the means±SD. Dcf, Diclofenac; NaF, Sodium fluoride; Lut, Luteolin. Superscript (a) indicates significant differences at  $p<0.05$  when values in other groups are compared with group A; Superscript (b) indicates significant differences at  $p<0.05$  when values in other groups are compared with group B; Superscript (c) indicates significant differences at  $p<0.05$  when values in other groups are compared with group C.

**Figure 1:**

Renal markers of oxidative stress following exposure to Diclofenac (Dcf), sodium fluoride (NaF) and Luteolin (Lut). Group A (control); Group B (9 mg/kg Dcf); Group C (9 mg/kg Dcf + 300 ppm NaF); Group D (9 mg/kg Dcf + 300 ppm NaF + 100 mg/kg Lut); Group E (9 mg/kg Dcf + 300 ppm NaF + 200 mg/kg Lut). Superscript (a) indicates significant differences at  $p<0.05$  when values in other groups are compared with group A; Superscript (b) indicates significant differences at  $p<0.05$  when values in other groups are compared with group B; Superscript (c) indicates significant differences at  $p<0.05$  when values in other groups are compared with group C.

**Table 2:**

Levels of enzymic and non-enzymic antioxidants in rats exposed to Diclofenac, Sodium fluoride and Luteolin

| Parameters         | Control     | Dcf only                | Dcf+NaF                 | Dcf+NaF +LutA           | Dcf+NaF+LutB            |
|--------------------|-------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Protein thiols     | 118.77±7.43 | 118.35±5.66             | 125.49±10.98            | 122.40±6.68             | 125.38±12.54            |
| Non-protein thiols | 85.14±2.09  | 80.70±3.42 <sup>a</sup> | 80.62±2.54 <sup>a</sup> | 78.02±2.49 <sup>a</sup> | 79.39±4.40 <sup>a</sup> |
| GST                | 0.26±0.05   | 0.38±0.05 <sup>a</sup>  | 0.39±0.07 <sup>a</sup>  | 0.40±0.12 <sup>a</sup>  | 0.41±0.08 <sup>a</sup>  |
| SOD                | 18.26±2.65  | 17.58±2.31              | 15.79±2.31              | 17.59±3.71              | 17.11±5.19              |
| Vit. C             | 1.51±0.12   | 1.55±0.06               | 1.57±0.09               | 1.68±0.25               | 1.72±0.12               |

The presented values are the means±SD. Dcf, Diclofenac; NaF, Sodium fluoride; Lut, Luteolin. Superscript (a) indicates significant differences at  $p<0.05$  when values in other groups are compared with group A; Superscript (b) indicates significant differences at  $p<0.05$  when values in other groups are compared with group B; Superscript (c) indicates significant differences at  $p<0.05$  when values in other groups are compared with group C.

The effects of Dcf, NaF and Lut exposure on renal markers of oxidative stress are depicted in Fig. 1. Although, the renal levels of hydrogen peroxide in the Dcf or Dcf + NaF groups remained unchanged, the same groups of rats showed significant ( $p<0.05$ ) increase in the renal MDA content, when compared to the control group. The protein carbonyl levels in Dcf and Dcf + NaF groups were significantly ( $p<0.05$ ) elevated, while the level of GSH in these groups was significantly ( $p<0.05$ ) reduced compared to the control group. In the groups concurrently treated with LutA (100

mg/kg) or LutB (200 mg/kg), there was significant ( $p<0.05$ ) lowering of hydrogen peroxide generation and MDA content relative to the Dcf + NaF group. Furthermore, treatment of rats with Lut resulted in significant ( $p<0.05$ ) increase in GSH concentration compared to the Dcf + NaF combination, although, protein carbonyl level remained unaltered despite Lut treatment.

The results in Table 2 represent the activities and levels of enzymic and non-enzymic antioxidants, respectively. The concentrations of protein thiols and Vitamin C, as well as

the activity of SOD did not differ significantly among the various study groups. However, relative to the control group, all other groups had significantly ( $p < 0.05$ ) elevated levels of non-protein thiols and GST activity.

The immuno-histochemical staining of renal tissues revealed positive immuno-reactivity with higher expression of AT<sub>2</sub>R in the groups exposed to Dcf alone and the group

receiving a combination of Dcf and NaF, compared to the control group which showed lower expression of AT<sub>2</sub>R (Plate 1). The rats in the control group had high expression of MCR which was also similar to the expression levels in the Dcf and Dcf+NaF groups (Plate 2). On the other hand, treatment of the rats with Lut resulted in down-regulation of AT<sub>2</sub>R and MCR expression in the kidneys (Plates 2 and 3).

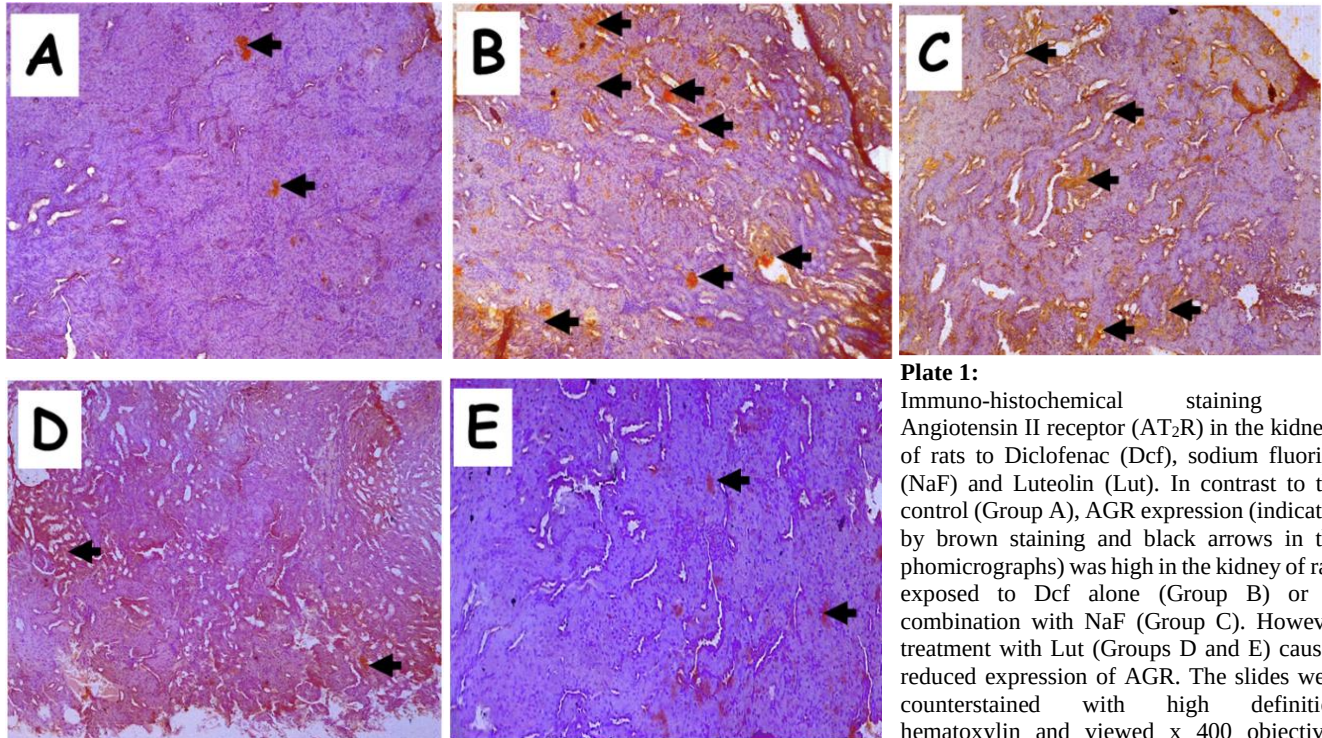


**Plate 1:** Immuno-histochemical staining of Angiotensin II receptor (AT<sub>2</sub>R) in the kidneys of rats to Diclofenac (Dcf), sodium fluoride (NaF) and Luteolin (Lut). In contrast to the control (Group A), AGR expression (indicated by brown staining and black arrows in the photomicrographs) was high in the kidney of rats exposed to Dcf alone (Group B) or in combination with NaF (Group C). However treatment with Lut (Groups D and E) caused reduced expression of AGR. The slides were counterstained with high definition hematoxylin and viewed x 400 objectives (magnification X100).

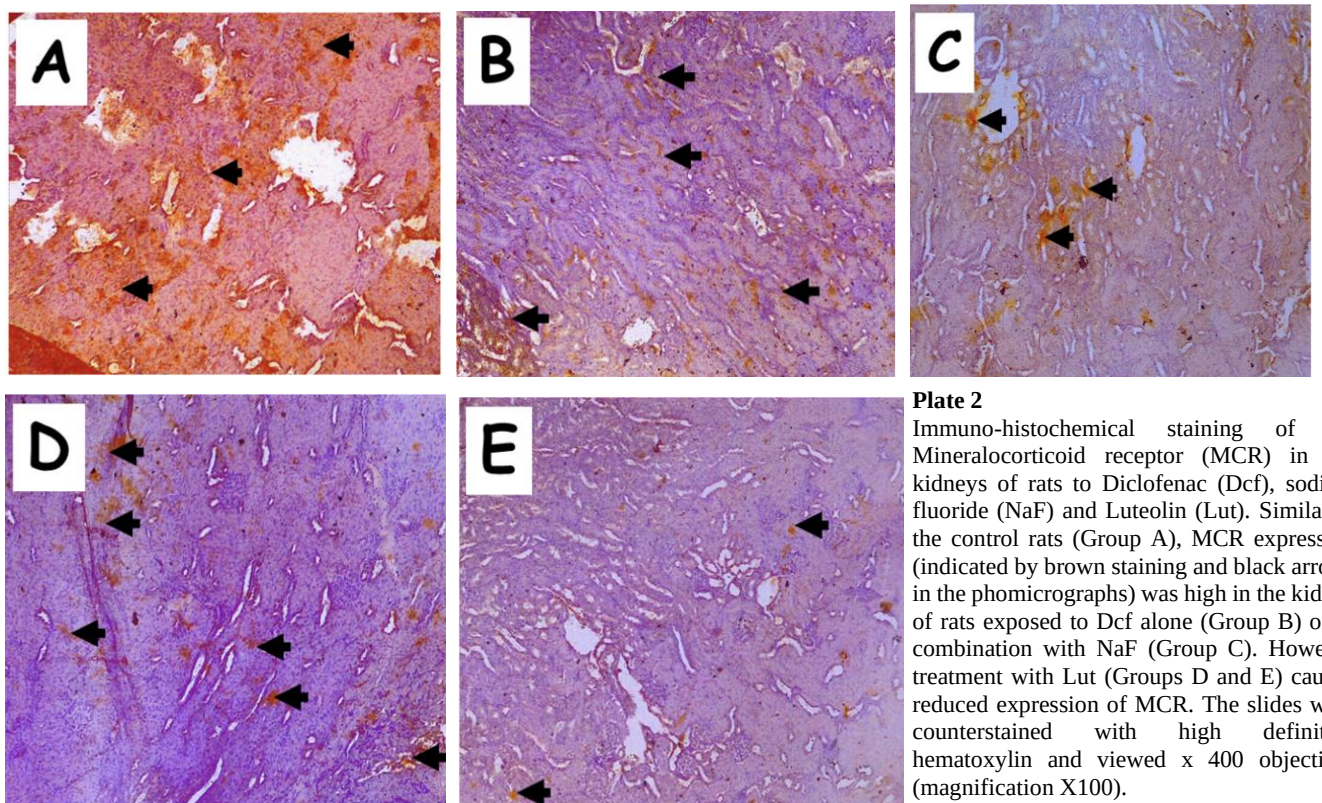


**Plate 2** Immuno-histochemical staining of the Mineralocorticoid receptor (MCR) in the kidneys of rats to Diclofenac (Dcf), sodium fluoride (NaF) and Luteolin (Lut). Similar to the control rats (Group A), MCR expression (indicated by brown staining and black arrows in the photomicrographs) was high in the kidney of rats exposed to Dcf alone (Group B) or in combination with NaF (Group C). However treatment with Lut (Groups D and E) caused reduced expression of MCR. The slides were counterstained with high definition hematoxylin and viewed x 400 objectives (magnification X100).

## DISCUSSION

NSAIDs, such as Dcf and Ibuprofen, have been associated with an increased risk of hypertension due to their inhibition of Cyclooxygenase (COX)-derived vasodilatory prostaglandins (Harris, 2002; Izhar *et al.*, 2004), although, other contrary reports have also indicated that certain NSAIDs such as Dcf do not necessarily increase the risk of hypertension (Sherve *et al.*, 2014). The present study hypothesized that combined exposure to Dcf and sodium fluoride, a compound known to modulate blood pressure (Oyagbemi *et al.*, 2017), could lead to interactions that affect blood pressure dynamics. The study, therefore, sought to investigate the effect of treatment with Luteolin, a food-derived anti-oxidative and anti-inflammatory flavonoid (Nakayama *et al.*, 2015) on relevant biomarkers involved in blood pressure regulation in Dcf- and NaF co-exposed rats. Our findings indicate a reduction in the systolic, diastolic and mean arterial pressures in rats that were exposed orally to Dcf twice daily for 3 days. In addition, there was an increase in the heart rates of this group of rats. However, combined exposure of rats to Dcf and NaF, resulted in an increase in the blood pressure parameters, representing a reversal of the effects obtained with administration of Dcf alone.

Despite the documented anti-natriuretic and vasoconstrictor effects that results from non-selective COX inhibition by NSAIDs, it appears that the findings of Dcf-induced reduction in blood pressure in the present study may be linked to separate mechanisms such as a likely reduction in extracellular fluid volume and/or hypovolaemia caused by severe haemorrhagic diarrhea observed in the rats treated with Dcf as reported in our earlier study (Akinrinde *et al.*, 2020). Differences in pharmacokinetics and dosage of the drug used in different studies have been suggested to be responsible for the conflicting reports on how NSAIDs affects blood pressure parameters (Stempak *et al.*, 2002). Furthermore, the observed drop in blood pressure in Dcf-treated rats was corroborated by a corresponding increase in the heart rate of these animals. Increased heart rate and vasoconstriction are normal compensatory responses directed at reversing hypotension, and this occurs via an increase in sympathetic stimulation of the heart, causing increase in cardiac output (DiBiona, 2004).

It was obvious from the current results that co-exposure of rats to Dcf and NaF resulted in a reversal of the above effects, probably due to reported blood pressure-enhancing effects of NaF (Yousefi *et al.*, 2018), suggesting an antagonistic interaction between the two compounds on blood pressure in the present study. Increased blood pressure following NaF administration in rats has been associated with increased vascular generation of reactive oxygen/nitrogen species (ROS/RNS) and oxidative stress (Oyagbemi *et al.*, 2018). Reactive oxygen species may increase blood pressure in the short-term by stimulating heart rate and inducing vasoconstriction, while in the long term, ROS contributes to hypertension by promoting inflammation, myocardial hypertrophy, vascular remodeling and endothelial dysfunction (Touyz and Brimes, 2011, Rodrigo *et al.*, 2011). In the present study, we examined the effect of Dcf or its co-administration with NaF on renal oxidative stress and antioxidant markers and found obvious indications of oxidative stress such as increased

levels of malondialdehyde and protein carbonyls, as well as reduction in GSH levels in the kidneys of rats treated with Dcf and NaF.

Treatment of Dcf- and NaF-exposed rats with Luteolin effectively reduced the renal contents of Hydrogen peroxide, MDA protein carbonyls, along with increase in the level of GSH, indicating the antioxidant effects of Lut in the renal tissues. This radical scavenging and antioxidant role of Lut might be responsible for maintaining the blood pressure of the affected rats. In this study, blood pressure values in the Lut-treated rats were not significantly different from those of the control rats. Indeed, Oyagbemi *et al.* (2018) reported similar values of systolic ( $124.3 \pm 12.64$  mmHg), diastolic ( $92.41 \pm 16.05$  mmHg) and Mean arterial ( $102.66 \pm 14.76$  mmHg) pressures as those recorded in the control rats used in the present study. In effect, the blood pressure of rats treated with Lut exhibited a tendency to be maintained at values close to that of normal or control rats.

The renin-angiotensin-aldosterone system (RAAS) is important in the control of blood pressure and fluid/electrolyte balance. Increased activation of the RAAS is a major contributor to the development of hypertension via stimulation of  $\text{Na}^+$  reabsorption and  $\text{K}^+$  excretion (Yatabe *et al.*, 2011); therefore, blockade of components of this system has been a useful strategy for the therapeutic control of blood pressure (Parichatikanond *et al.*, 2012). However, the usefulness of this strategy during concurrent exposure to chemicals and drugs that are capable of modulating blood pressure is not fully known. In this study we examined the effects of Dcf administration on major receptors of the RAAS i.e.  $\text{AT}_2\text{R}$  and MCR. Early reports have shown that certain NSAIDs actually possess intrinsic mineralocorticoid receptor agonist activity (Feldman and Couropmitree, 1976), while other reports also indicated that Dcf, like some other commonly prescribed NSAIDs may inhibit the glucuronidation of aldosterone in human liver and kidneys (Winner *et al.*, 2005). However, to the best of our knowledge, the effect of Dcf on the expression of the mineralocorticoid receptor has not been reported.

In this study, an obvious increase in the expression of  $\text{AT}_2\text{R}$  was observed in rats treated with Dcf alone, although this was inconsistent with the reduced blood pressure in the same group of rats. It appears, however, from our results that the effects of Dcf on these receptors of the RAAS may be masked, at least in the early stages of Dcf usage, by other haemodynamic factors including the drug's ability to induce severe gastrointestinal bleeding and possible hypovolaemia as previously reported (Akinrinde *et al.*, 2020). Hypovolemia resulting from acute loss of circulating blood volume after hemorrhage may result in low cardiac output and hypotension (Noori *et al.*, 2017). A potential limitation to our study was the inability to assess the independent effects of NaF on  $\text{AT}_2\text{R}$  and MCR expression, due to the study design employed. Nevertheless, the reversal of blood pressure in the rats given a combination of NaF and Dcf with corresponding increase in the expression of  $\text{AT}_2\text{R}$  appears to indicate that the enhancement of the RAAS pathway by NaF may have taken a more dominant role over the effects observed in rats given Dcf alone.

More significantly, in this study, we demonstrated a profound down-regulation of both  $\text{AT}_2\text{R}$  and MCR in Lut-treated rats, a finding that was also consistent with its ability to reduce the blood pressure of these rats, compared to those

co-exposed to Dcf and NaF. Dietary components and natural bioactive products from plants, including flavonoids such as quercetin or anthocyanins e.g. delphinidin, have been shown to reduce blood pressure (Parichatikanond *et al.*, 2012), although the molecular mechanisms of many of these compounds are not yet fully understood. Previous evidence supporting the blood pressure lowering effects of Lut have suggested the involvement of Lut-mediated regulation of hypertensive vascular remodeling via its inhibition of proliferation and migration of angiotensin II-induced vascular smooth muscle cells (Su *et al.*, 2015). In the present study, we provide new insights into the molecular mechanisms by which Lut can exert anti-hypertensive effects via inhibition of receptors involved in RAAS signaling. This is in addition to its ability to regulate the production of ROS and inhibition of lipid and protein oxidation.

Although the present study reveals for the first time the protective roles of Lut against nephrotoxicity and haemodynamic alterations in rats induced by co-exposure to Dcf and NaF, there may be a limitation. The experimental design does not include separate groups of rats treated with NaF or Luteolin alone, results from which further interpretations could otherwise be made. However, the ethical use of animals as approved by our local ethics committee considerably limits the use of larger numbers of animals than that used in the present study.

In conclusion, our data supports an antihypertensive effect of Luteolin mediated by inhibition or down-regulation of receptors of the RAAS pathway. The model of Dcf and NaF co-exposure showed potential for antagonistic effects between the two compounds, probably obtainable with short-term exposure. Although not usually associated with NSAIDs, Dcf-induced hypotension, in this study, may probably be a result of severe gastrointestinal bleeding and resultant hypovolemia. The nature of interactions during prolonged co-exposure to the two compounds may form the focus of future studies.

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## Full-Length Research Article

# Short-term Acute Constipation, Not Short-term Acute Diarrhea, Altered Cardiovascular Variables in Wistar Rats

**\*Odukanmi O.A., Ajala O.A., Olaleye S.B.**

Laboratory for Gastrointestinal Secretions and Inflammation, Department of Physiology, College of Medicine, University of Ibadan, Nigeria

**Summary:** Gastrointestinal dysmotility is a substantial public health challenge globally. Based on previous findings in developed countries, it has been observed that there is an association between diarrhea, constipation, and some cardiovascular variables. This study investigated the effects of experimentally-induced short-term acute constipation and short-term acute diarrhea on certain cardiovascular variables in rats. Thirty (30) male Wistar rats were divided into three groups; Control, Diarrhoea, and Constipation. The experiment was carried out in 2 phases, the period after induction and the recovery period, and 5 animals per group were used for each phase. The control group received an equivalent amount of distilled water while Diarrhoea and the Constipation group were induced by oral administration of 2ml Castor oil and administration of Loperamide (3mg/kg, b.d, orally x 3 days), respectively. Cardiovascular variables were assessed using the Edan Scientific® Electrocardiography and Heart Rate Variability machine. Recovery was allowed for 4 days after the onset of the procedure and cardiovascular parameters were reassessed. Post-induction Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), Mean Arterial Pressure (MAP) and Heart Rate (HR) significantly increased in constipated rats ( $153.2 \pm 2.9$  mmHg;  $109.0 \pm 3.7$  mmHg;  $123.7 \pm 3.2$  mmHg;  $123.4 \pm 5.6$  bpm) when compared with the control values ( $95.5 \pm 4.8$  mmHg;  $61.2 \pm 3.5$  mmHg;  $72.6 \pm 3.6$  mmHg;  $72.3 \pm 5.2$  bpm), respectively. The recovery SBP, DBP, MAP, and Heart Rate in the constipated group remained significantly higher compared to the control. Diarrhea had no significant effect on the parameters determined in both post-induction and recovery phases. The electrical activities did not change in both experimental groups compared to the control. This study revealed increased SBP, DBP, MAP, and HR in short-term acute constipated rats but not so with short-term acute experimental diarrhea.

**Keywords:** Blood pressure, heart rates, constipation, diarrhea, electrical activities, rats

©Physiological Society of Nigeria

\*Address for correspondence: E-mail: [odukanmi@yahoo.com](mailto:odukanmi@yahoo.com); Tel: +2348038224696

Manuscript received- February 2022; Accepted- May 2022

DOI: <https://doi.org/10.54548/njps.v37i1.6>

## INTRODUCTION

The fundamental functions of the gastrointestinal system include motility, sensation, absorption, digestion, secretion, and intestinal barrier function. The symptoms of functional gastrointestinal dysmotility are mostly triggered by meal intake and abnormalities in the physiological processes are responsible for the generation of symptoms. Irritable Bowel Syndrome (IBS) is a form of gastrointestinal disorder with symptoms subcategorized into painful sensations and altered intestinal motility patterns (Li *et al.*, 2020), with constipation and diarrhea presenting in an alternate pattern. Constipation is a major gastrointestinal disorder common at presentations at the primary health centres, with a prevalence of about 30% of the entire human race in their time (Bharucha *et al.*, 2013). However, constipation is often regarded as benign and may not be so dangerous (Dennison *et al.*, 2005), when it becomes chronic, the quality of life of patients becomes compromised socially and economic-wise (Guerin *et al.*, 2014). It is one of the commonest disorders of gastrointestinal motility and secretions which result in infrequent and difficulty in stool passage with pains and stiffness. This often is due to the very slow movement of food residues through the colon (slow transit constipation)

or difficulty moving bowels after reaching the rectum (outlet dysfunction) (Martins *et al.*, 2015). Acute constipation causes closure of the intestine in which surgery may be required. The common symptoms of constipation are hard or lumpy stools with consequential straining to defecate; less than three bowel movements per week; and blocked rectum (Mugie *et al.*, 2011).

Diarrhea, on the other hand, is categorized as a gastrointestinal dysmotility that occurs as a symptomatic secretory response of the gastrointestinal mucosa to a wide range of stimuli (Berthoud *et al.*, 2004). The increase in water content and fluidity of stool is due to the increased secretion of the gastrointestinal mucosa with a possibility of an underlying infection or disease. The underlying disease could cause inflammation or ulceration of the intestinal mucosa, especially in the large intestine or the rectum, then there is the tendency for blood and mucus to be seen in the faeces (Grover *et al.*, 2012). Diarrhoeal diseases are related to about 1.3 million annual reported deaths (Troeger *et al.*, 2017). The majority of these deaths occur in low-income countries (Kotloff *et al.*, 2013; Fiedoruk *et al.*, 2015; Troeger *et al.*, 2017). Stools with excess loss of water and electrolytes can result in dehydration, hyponatremia, hypokalemia, and loss of other essential electrolytes which

are critical for survival. All of these could predispose individuals to the barrage of complications emanating from the alterations in the epithelial barrier from inflammation, ulceration, and altered circulation (Bern *et al.*, 1992).

It is well-known that 10% of deaths in the United States have gastrointestinal disorders as the root cause (Peery *et al.*, 2015), while some patients present with cardiovascular complaints as the primary symptoms which have made the relationship between cardiovascular symptoms and gastrointestinal disorders an interesting area of focus (Martins *et al.*, 2015). Constipation is associated with the risk of cardiovascular events when treated with laxatives (Sumida *et al.*, 2019). In some cases, the associated risk of atherosclerosis is increased blood pressure, and other cardiovascular events are due to changes in altered intestinal microbiota (Attaluri *et al.*, 2010; Kim *et al.*, 2015; Ishiyama *et al.*, 2019). The prevalence of constipation increases with the increasing age of an individual (Ishiyama *et al.*, 2019). Other reports implicated arteriosclerotic cardiovascular disease in constipation (Salmoirago-Blotcher *et al.*, 2011; Sumida *et al.*, 2019), and increased risk of coronary heart disease and ischaemic stroke (Ishiyama *et al.*, 2019). In the case of diarrheal diseases, it was suggested with the associated dehydration and electrolytes derangement the possibility of deranged blood pressure in childhood, but that assertion was debunked in the cohort study in children where there was no blood pressure change, no risk of coronary heart disease (Batty *et al.*, 2007a). Other reports explored the association between diarrhea in pediatrics or childhood and its effect on blood pressure later in life and also its relationship with coronary heart disease (Batty *et al.*, 2007b; Pearce *et al.*, 2008).

Considering the enormous suffering from gastrointestinal dysmotility by patients any observed relationship with cardiovascular events or diseases would be of utmost interest to public health and in the management of the associated cardiovascular risk if it ensued. This study was designed to further characterize the relationship between short-term acute constipation and short-term acute diarrhea in rats' cardiovascular events with the hope to relate the outcome to the previous findings on these experimentally induced gastrointestinal derangements.

## MATERIALS AND METHODS

**Materials:** Plastic cages, castor oil, loperamide, gloves, 5ml, and 2ml syringe, cotton wool, methylated spirit, surgical instrument, distilled water, Edan Scientific® Electrocardiography Heart Rate Variability Machine, Kent Scientific® Blood Pressure machine, USA, were used.

**Animal Care and Grouping:** Thirty male Wistar rats with a bodyweight range of 155-178g were used. The rats were bred and acclimatized for 14 days in the Animal House of the Department of Physiology, College of Medicine, University of Ibadan, Ibadan, Nigeria. The rats were randomly distributed into cages based on the groupings, with beddings that were replaced every other day. The rats were grouped into 3 (n=10 per group). The 10 rats in diarrhea and constipation groups were induced, and 5 rats each from each group were examined for post-induction changes and the recovery phase. The rats were monitored for cardiovascular changes in both phases of the experiment.

**Group 1 (Control):** Rats in this group were allowed free access to water and given an equivalent amount of distilled water as a sham. **Group 2 (Diarrhea):** rats in this group were orally administered 2ml of castor oil per rat to induce diarrhea. **Group 3 (Constipation):** rats in this group were administered loperamide (3 mg/kg) twice per day for 3 days to induce constipation.

**Animal ethics:** Experimental protocols and procedures used in animal handling were strictly in adherence to the ethical guidelines of the University of Ibadan, Animal Care and Use for Research Ethics Committee (UI-ACUREC/046-0521/20).

**Diarrhea and Constipation Induction procedures:** The rats in group 2 were orally administered with castor oil -2ml per rat to induce diarrhea and waited for 2 hours after the onset of diarrhea before determining the cardiovascular changes. The rats were observed for soft, smooth, or mucus-coated and confirmed diarrheic clinically. Group 3 rats were orally administered loperamide (3 mg/kg) twice per day for 3 days to induce constipation (Bustos *et al.*, 1991; Wintola *et al.*, 2010). The rats were examined for constipation with evidence of a distended abdomen and were used for the experiment.

After the initial assessment, the experimental rats were kept in the animal house and total recovery was noticed after 4 days. Constipated rats were examined and discovered to no longer have distended abdomen and diarrhea was resolved evidenced by the passage of normal stool.

**Assessment of Arterial Blood Pressure (BP):** Blood pressure assessment of rats was determined non-invasively with a rat tail-cuff connected to a volume pressure sensor of the Kent Scientific® Blood Pressure Machine. Each rat was restrained in a cone restrainer and placed on a manually-controlled warming plate with its tail inserted into the V and O cuff of the machine's transducer. The systolic blood pressure (SBP) and diastolic blood pressure (DBP) were monitored through the transducer connected to a computer via a data acquisition system. The blood pressure variables were recorded in triplicate and the mean values of the assessed parameters were determined per session.

**Determination of Mean Arterial Pressure (MAP):** The MAP was determined using the formula below (Vedel *et al.*, 2016);

$$MAP = DBP + \frac{\text{Pulse Pressure}}{3}$$

Where:

Pulse pressure = SBP – DBP;

MAP = Mean Arterial Pressure;

DBP = Diastolic Blood Pressure

**Assessment of Heart Rate Variability (HRV):** The HRV of each rat was monitored through the connection to the Edan Scientific® Electrocardiography Heart Rate Variability machine for 5 minutes, and the mean of recorded HRV values was determined as previously reported by Reed *et al.*, (Reed *et al.*, 2005)

**Determination of Electrical Activities of the Heart:** Rats from each group were anesthetized intra-peritoneally with 0.1 ml/100 g of xylazine and 25 mg/100g of ketamine

hydrochloride. The ECG lead electrodes were placed in their respective positions (chest, right arms, right legs, left arms, and left legs) on the animals as previously described (Farraj et al., 2011; Ahmad et al., 2016).

**Statistical Analysis:** The results of the experiment were expressed as Mean  $\pm$  Standard Error Mean (SEM) and analyzed using Analysis of Variance (ANOVA). The Bonferroni test (GraphPad Prism Version 7.0® Software) was used to analyze all results where significant. Data were considered significant at  $P \leq 0.05$ .

## RESULTS

**Effect of Experimentally Induced Diarrhea and Constipation on Systolic Blood Pressure (SBP):** Table 1 describes the SBP (mmHg) significantly increased in constipated rats ( $153.2 \pm 2.9$ ) compared with the control rats ( $95.5 \pm 4.8$ ). The SBP significantly increased in constipated rats ( $124.5 \pm 2.3$ ) compared with the SBP of control rats ( $113.4 \pm 2.1$ ) after recovery. There was no significant change when the diarrhea group was compared to the control in both the induction and recovery phases.

**Effect of Experimentally Induced Diarrhea and Constipation on Diastolic Blood Pressure (DBP):** The DBP (mmHg) increased significantly in constipated rats ( $109.0 \pm 3.7$ ) compared with the control rats ( $61.2 \pm 3.5$ ), table 2. The significant increase in DBP was sustained during the recovery phase in constipated rats ( $89.3 \pm 2.0$ ) when compared with the control rats ( $78.7 \pm 1.4$ ). Again, there was no significant change in DBP of the diarrhea group compared to the control in both the induction and recovery phases (Table 2).

**Table 1:**

Systolic Blood Pressure of Rats After the Induction and Recovery from Diarrhoea and Constipation Compared with the Control Group

| Groups       | Systolic Blood Pressure (mmHg) |                      |
|--------------|--------------------------------|----------------------|
|              | Induction Phase                | Recovery Phase       |
| Control      | $95.5 \pm 4.8$                 | $113.4 \pm 2.1$      |
| Diarrhoea    | $100.0 \pm 5.4^{ns}$           | $108.0 \pm 3.7^{ns}$ |
| Constipation | $153.2 \pm 2.9^*$              | $124.5 \pm 2.3^*$    |

\* = Significant Increase at  $P \leq 0.05$  compared with control, ns = no significant difference compared to control

**Table 2:**

Diastolic Blood Pressure of Rats After the Induction and Recovery from Diarrhoea and Constipation Compared with the Control Group

| Groups       | Diastolic Blood Pressure (mmHg) |                     |
|--------------|---------------------------------|---------------------|
|              | Induction Phase                 | Recovery Phase      |
| Control      | $61.2 \pm 3.5$                  | $78.7 \pm 1.4$      |
| Diarrhoea    | $69.9 \pm 3.9^{ns}$             | $72.0 \pm 3.0^{ns}$ |
| Constipation | $109.0 \pm 3.7^*$               | $89.3 \pm 2.0^*$    |

\* = Significant Increase at  $P \leq 0.05$  compared with control, ns = no significant difference compared to control

**Table 3:**

Mean Arterial Pressure of Rats After the Induction and Recovery from Diarrhea and Constipation

| Phases    | Groups         |                     |                   |
|-----------|----------------|---------------------|-------------------|
|           | Control        | Diarrhea            | Constipation      |
| Induction | $72.6 \pm 3.6$ | $79.9 \pm 3.9^{ns}$ | $123.7 \pm 3.2^*$ |
| Recovery  | $90.3 \pm 0.9$ | $84.2 \pm 3.1^{ns}$ | $101.0 \pm 2.0^*$ |

\* = Significant Increase at  $P \leq 0.05$  compared with control, ns = no significant difference

**Effect of Experimentally Induced Diarrhea and Constipation on Mean Arterial Pressure (MAP):** The MAP (mmHg) significantly increased in constipated rats ( $123.7 \pm 3.2$ ) compared with the MAP of control rats ( $72.6 \pm 3.6$ ). A significant increase in MAP in constipated rats ( $101.0 \pm 2.0$ ) was sustained after the recovery phase compared to control rats ( $90.3 \pm 0.9$ ), (Table 3). The diarrhea values obtained were not significantly different from control rats in both the induction and recovery phases.

**Effect of Experimentally Induced Diarrhea and Constipation on Heart Rate Variability (HRV):** Heart rate variability (HRV) (m/s) increased significantly in constipated rats ( $123.4 \pm 3.2$ ) compared with the control rats ( $72.3 \pm 3.7$ ), Figure 1. The significant increase was persistent in the recovery phase in HRV of constipated rats ( $100.8 \pm 1.9$ ) when compared with control rats ( $89.9 \pm 1.0$ ) (Figure 1).

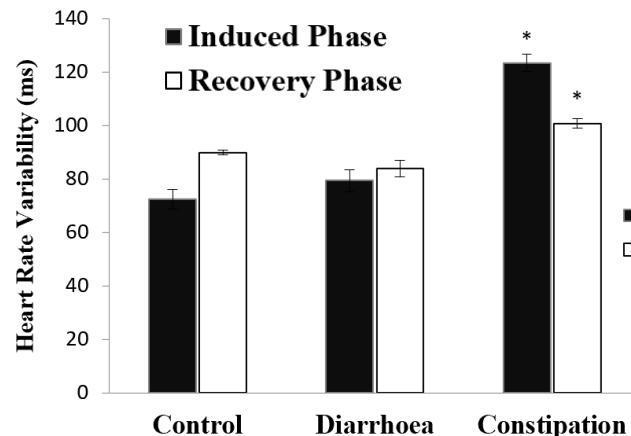


**Figure 1:**

The Heart Rate Variability After the Induction and Recovery from Diarrhea and Constipation

\*Significant increase at  $P \leq 0.05$  compared to control.

**Effect of Experimentally Induced Diarrhoea and Constipation on ECG Tracing:** Based on the statistical analysis of both the induced phase of gastrointestinal dysmotility and after recovery, there was no significant change in the ECG tracing of both diarrhea and constipated rats when compared to the control rats (Plate 1 and Table 4).

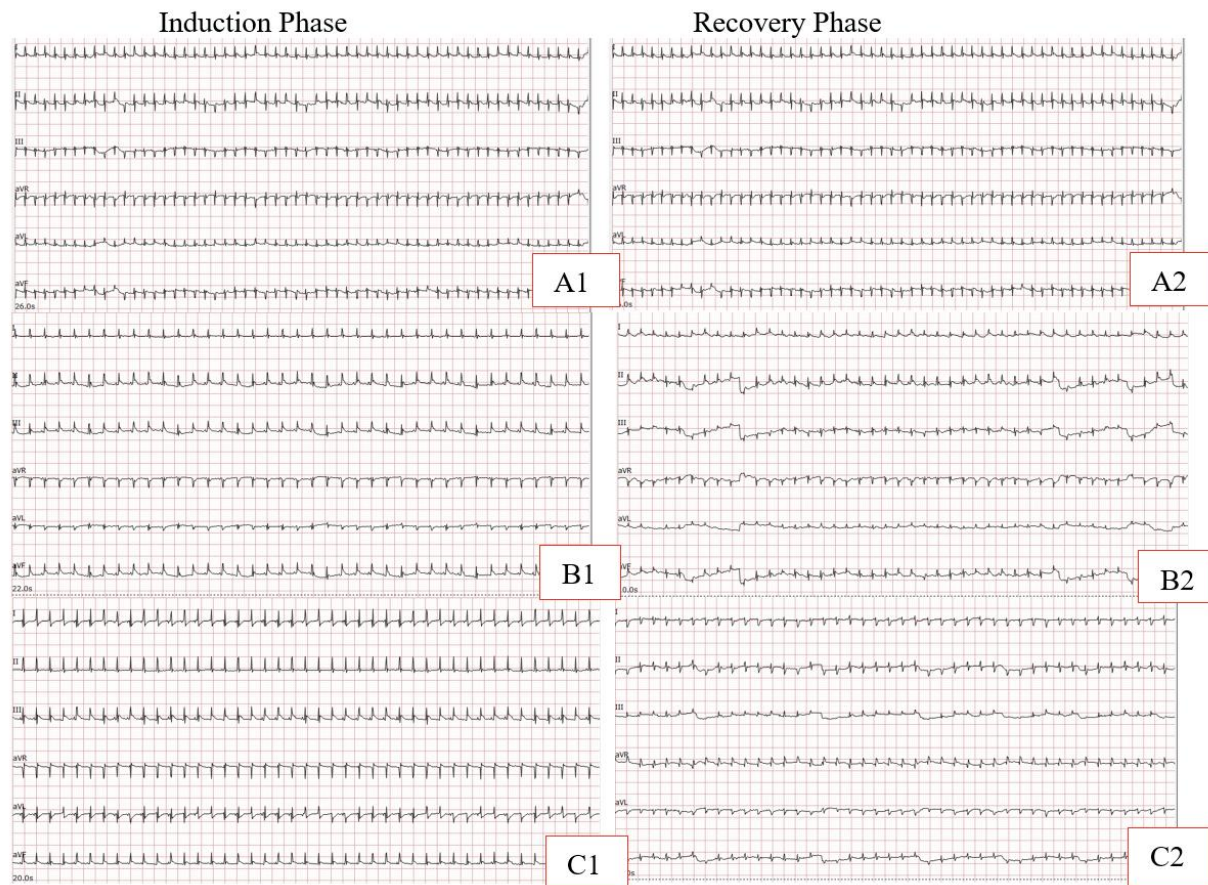


**Plate 1:** Representative ECG Tracings After the Induction and Recovery from Diarrhea and Constipation. A = Control, B= Diarrhea, C= Constipation, 1= induction Phase, 2=recovery phase

**Table 4:** ECG Tracing in Rats After the Induction and Recovery from Diarrhea and Constipation

| ECG Tracing    | Groups          |                         |                          |                |                          |                          |
|----------------|-----------------|-------------------------|--------------------------|----------------|--------------------------|--------------------------|
|                | Induction Phase |                         |                          | Recovery Phase |                          |                          |
|                | Control         | Diarrhea                | Constipation             | Control        | Diarrhea                 | Constipation             |
| <b>P (ms)</b>  | 29.5±5.0        | 32.0±6.0 <sup>ns</sup>  | 35.5±2.0 <sup>ns</sup>   | 39.0±3.7       | 26.0±5.4 <sup>ns</sup>   | 32.5±3.1 <sup>ns</sup>   |
| <b>PR (ms)</b> | 43.5±8.9        | 53.5±1.3 <sup>ns</sup>  | 46.0±1.1 <sup>ns</sup>   | 49.3±4.9       | 37.8±6.1 <sup>ns</sup>   | 42.8±1.5 <sup>ns</sup>   |
| <b>QRS(ms)</b> | 19.0±2.7        | 22.0±1.3 <sup>ns</sup>  | 21.75±2.3 <sup>ns</sup>  | 28.0±5.7       | 24.8±2.8 <sup>ns</sup>   | 24.0±3.2 <sup>ns</sup>   |
| <b>QT (ms)</b> | 76.3±5.2        | 96.3±2.6 <sup>ns</sup>  | 111.0±11.2 <sup>ns</sup> | 101.8±19.3     | 89.0±15.2 <sup>ns</sup>  | 129.3±10.9 <sup>ns</sup> |
| <b>QTC(ms)</b> | 159.8±12.7      | 189.8±8.9 <sup>ns</sup> | 238.5±21.6 <sup>ns</sup> | 207.0±40.8     | 183.3±33.1 <sup>ns</sup> | 280.3±33.4 <sup>ns</sup> |
| <b>Ramv</b>    | 0.3±0.0         | 0.5±0.1 <sup>ns</sup>   | 0.4±0.1 <sup>ns</sup>    | 0.3±0.1        | 0.2±0.1 <sup>ns</sup>    | 0.4±0.0 <sup>ns</sup>    |

ns = no significant difference compared to the control

## DISCUSSION

Gastrointestinal dysmotility is a cause of significant morbidity worldwide, and it is one of the most prevalent conditions presented to medical practitioners, specialists, and surgeons across sub-specialties (Peery *et al.*, 2015). Few studies have reported the association of gastrointestinal dysmotility, especially regarding diarrhea and constipation with increased cardiovascular risk (Hunter *et al.*, 2001; Batty *et al.*, 2007b; Ishiyama *et al.*, 2019; Salmoirago-Blotcher *et al.*, 2011; Sumida *et al.*, 2019) but none have been able to describe the relationship in the line of the basic blood pressure variables, mean arterial pressure, heart rate variableness, and the electrical activities of the events during short-term acute constipation and short-term acute diarrhea. In this study, we observed the aforementioned

changes during the induction phases of diarrhea and constipation and determined further if these changes were sustained after the recovery period.

Blood pressure (SBP and DBP) was increased in the constipated group when compared with the control rats. This increase in BP was sustained during the recovery period as well. Rats suffering from hypertension during the onset of constipation did not recuperate completely from high blood pressure after the period of recovery. It has been previously explained that the distention of the smooth muscle walls during straining to defecate and the similarity of the smooth muscle components to the blood vessels directly affect the normal functioning of the vascular system (Martins *et al.*, 2015). More so, the smooth muscles of the blood vessels and the gut walls have similar fibers. They rely on the same principle even though their numbers differ considerably.

*Short-term acute constipation affects cardiovascular variables*

These similarities aid in lower gastrointestinal dysmotility being accompanied by BP fluctuation (Martins *et al.*, 2015). Thus, the increase in BP following straining to defecate by a constipated person allows the flow of a large volume of blood to the brain, which then increases pressure in blood vessels and arteries (Salmoirago-Blotcher *et al.*, 2011).

In the current study, constipation was observed to affect MAP. The reported increase in MAP in the constipated rats during both the induction and recovery phases corroborates the findings on the SBP and DBP in the constipated group. It further gave an insight into the reported increase in blood pressure as reported from our findings. Previous studies revealed that disturbances in the microbial gut are most common in patients with constipation and have been associated with stiffness of the arteries (Mancabelli *et al.*, 2017; Menni *et al.*, 2018), and increased blood pressure (Yang *et al.*, 2015). Other effects of constipation have to include atherosclerotic cardiovascular diseases (Wang *et al.*, 2011), and heart failure (Tang *et al.*, 2019). Although the lipid profile and the microbiomes were not considered in this study, it is important to consider these as major limitations of our study. The heart rate variability was observed to be increased compared with the HRV of control rats and as well during the recovery phase. Abdominal cramps and straining can stimulate the vagus nerve thereby increasing the blood pressure via increased heart rate.

Diarrhea had no significant effect on both SBP and DBP from our study. This is in line with observations from previous studies that show no association between diarrheal disease and blood pressure or mortality in later life (Batty *et al.*, 2007a; Batty *et al.*, 2008), but infant diarrhea that results in severe dehydration in early infancy has been linked to increased blood pressure later in life (Davey Smith *et al.*, 2006; Lawlor *et al.*, 2006). Even though the rats used were adult rats, the associated prevalence of the microbiomes and arteriosclerosis as earlier discussed in constipation could not be ascertained in diarrhea disease. The physiological mechanism following the promptness and duration of diarrhea before assessment in our study could also have reduced the expected effect in terms of volume and electrolyte loss.

The MAP and HRV did not change significantly in the diarrhea group compared to the control. Further buttressing its docile effect on blood pressure and invariably on the measured cardiovascular variables in this study. Also, diarrhea and constipation had no significant effect on ECG tracing. This is particularly strange with diarrhea which is often associated with fluid and electrolyte loss. These elements are important for electrical activities and are usually deranged in diarrhea. However, the short-term acute diarrhea nature of our study might not be adequate to elicit any significant change in the ECG tracing, particularly for diarrhea.

In conclusion, constipation has been observed to have a significant aftermath effect on SBP, DBP, MAP, and HRV whereas diarrhea from our study did not show any effect on the cardiovascular variables determined. The short duration following the diarrhea induction and determination of cardiovascular variables measured was different from the usual presentation of diarrhea and constipation in terms of duration. Despite the limitation, it is however important for health practitioners to pay more attention to the

cardiovascular variables that might accompany patients who present with constipation.

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## Full-Length Research Article

# Insulin and Zinc Co-Administration Ameliorates Diabetes Mellitus-Induced Reproductive Dysfunction in Male Rats

\*Ujah G.A.<sup>1</sup>, Emmanuel I. B<sup>2</sup>, Ansa F<sup>1</sup>, Ukoh A<sup>1</sup>, Ani E. J<sup>1</sup>, Osim E.E<sup>1</sup>

<sup>1</sup> Department of Physiology, University of Calabar, Calabar, Nigeria

<sup>2</sup> Biomedical Research Centre, Forestry Research Institute of Nigeria, Ibadan, Nigeria

**Summary:** Impaired male reproductive function is a major complication associated with diabetes mellitus (DM). Whether or not insulin, when co-administered with zinc will reverse or ameliorate reproductive dysfunction in male diabetics is not known. This study thus sought to establish if co-administration of insulin and zinc reverses or ameliorates male reproductive dysfunction in DM better than either insulin or zinc. Five (5) normal and twenty (20) diabetic sexually mature rats were assigned into five groups of five animals each. Group A consisted of normal rats and had access to only food and water. Group B consisted of diabetic animals with no treatment and served as DM control. DM was induced by a single intraperitoneal injection of 60mg/kg of streptozotocin after an overnight fast. Groups C and D consisted of diabetic animals that received insulin and zinc respectively. Group E consisted of diabetic animals that received both insulin and zinc. All diabetic animals had free access to food and water. Insulin in all cases was given subcutaneously twice daily in the morning and evening at 1 unit and 4 units respectively. Zinc (10mg/kg) was given orally once daily. Treatments in all cases commenced two weeks after DM was confirmed. The treatment lasted ten days. Samples were thereafter collected for analyses. DM decreased sperm count, sperm motility, sperm viability, normal sperm cells, semen pH, Serum Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and testosterone, while increasing sperm cells with defective tails. DM also impaired testicular morphology. Insulin and zinc co-administration improved sperm viability, Sertoli cell count, Johnsen's score, serum FSH, LH and testosterone. Co-administration also improved semen pH towards normal. Insulin or zinc ameliorated several aspects of DM-induced male sexual dysfunction. However, the co-administration of insulin and zinc provided better results.

**Keywords:** Diabetes mellitus, Insulin, Zinc, Co-administration, Reproductive dysfunction, Ameliorates

©Physiological Society of Nigeria

\*Address for correspondence: E-mail: [gasukole@yahoo.com](mailto:gasukole@yahoo.com); Tel: +2348038224696

Manuscript received- March 2022; Accepted- May 2022

DOI: <https://doi.org/10.54548/njps.v37i1.7>

## INTRODUCTION

Diabetes mellitus (DM) is a chronic and complex metabolic condition that is characterized by increased blood glucose, otherwise known as hyperglycaemia. It is the result of aberrations in either insulin secretion or insulin action. DM could also result from a combination of aberrations in both insulin secretion and action (American Diabetes Association, 2011). Today, DM has become a disease of major public health concern. The 2017 International Diabetes Federation report estimates that there are 425 million diabetics worldwide. This figure will increase to 693 million people by 2045. At present, 79% of diabetic cases are in low and middle-income countries (IDF, 2017). DM is associated with a number of complications including cardiovascular disorders, neuropathy, nephropathy, retinopathy and infertility (Atkinson and Maclaren, 1994; Melendez-Ramirez *et al.*, 2010). A number of studies have shown that DM impairs male reproductive capacity at various levels. DM impairs steroidogenesis (Baccetti *et al.*, 2002; Schoeller, *et al.*, 2012), alters glucose metabolism in testes (Alves *et al.*, 2013), induces changes in testicular morphology (Ricci *et al.*, 2009), lowers sperm quality and quantity (Scarano *et al.*, 2006), reduces libido, causes

ejaculatory dysfunction (Sexton and Jarow, 1997) and impairs spermatogenesis (La Vignera *et al.*, 2012). DM impairs male reproductive capacity by increasing oxidative stress through the generation of reactive oxygen species (ROS) (Tabak *et al.*, 2011; La Vignera *et al.*, 2012). Since DM is mainly characterized by hyperglycaemia, the management of the glycaemic state is crucial to reducing the complications associated with it. Insulin is the most powerful antihyperglycaemic agent commonly used for glycaemic control in diabetics (Peyrot *et al.*, 2010). Diabetes mellitus (DM) is usually diagnosed in the advanced phase, thus allowing the progression of functional deterioration in cells that may not be reversed with insulin therapy alone (Donner and Munoz, 2012). About half of the people living with DM are unaware they have the disease (IDF, 2017). Diabetes mellitus is usually accompanied by reduced zinc levels (Kelly, 1998) and increased oxidative stress levels (La Vignera *et al.*, 2012), both of which impair male sexual function. Zinc is a trace element that is essential for testosterone production (Giugliano *et al.*, 1996). It also has antioxidant effects (Stumvoll *et al.*, 2005). Whether or not zinc, when co-administered with insulin in male diabetics, who already present with complications, will ameliorate

reproductive impairments better than insulin administration alone is what this study hopes to establish.

## MATERIALS AND METHODS

Streptozotocin (S0130), Insulin (Humulin) and Zinc gluconate were obtained from Sigma-Aldrich (St. Louis, MO, USA), Novo Nordisk A/S (Denmark) and Pharmedic (Vietnam) respectively. All other chemicals and reagents were commercially available and of analytical grade.

**Experimental animals:** Twenty-five (25) male Wistar rats aged between 8 and 10 weeks and weighing between 150 – 230g purchased from the Animal House of the Anatomy Department of the University of Benin, Benin, Nigeria were used for this experiment. These animals were housed in standard animal cages with sawdust as beddings in the Animal House of Physiology Department, University of Calabar, Calabar, Nigeria. The animals were allowed a period of two weeks to acclimatize. Animals were kept in standard conditions and had free access to rat feed and water.

**Experimental design/Drug administration:** Five (5) normal and twenty (20) diabetic rats were assigned into five (5) groups of five (5) animals each (The animals were assigned into groups such that the mean differences in weight before the start of the experiment were similar): Group 1 – Normal control, Group 2 – Diabetes mellitus (DM) control, Group 3 – Diabetes mellitus (DM) + Insulin, Group 4 – Diabetes mellitus (DM) + Zinc, Group 5 – Diabetes (DM) + Insulin + Zinc. Diabetes mellitus (DM) was induced after overnight fast by single intraperitoneal injection of 60mg/kg of streptozotocin (STZ) (De Young *et al.*, 2004). Fresh 0.1M citrate buffer (pH 4.5) was used as vehicle. Blood from tail vein prick was assayed for fasting glucose forty eight hours after DM induction. Animals with fasting blood glucose of 250 mg/dL and above were considered diabetic (Cao *et al.*, 2012). Animals were then allowed a period of two weeks, with free access to just normal rat feed and water before commencement of treatments. Insulin was subcutaneously administered twice daily to animals in group 3, at 1 and 4 units in the morning and evening respectively (Pinheiro *et al.*, 2011). Oral zinc (10mg/kg) was given once daily to animals in group 4 (Shidfar *et al.*, 2010). Animals in group 5 received both insulin and zinc in the doses and routes as above. The treatments lasted for ten (10) days after which samples were collected for analyses.

**Collection of blood samples and Serum Preparation:** Under chloroform anaesthesia, 5mls of blood was collected by means of cardiac puncture into plain sample bottles and then made to stand for thirty (30) minutes. The blood was then centrifuged for ten (10) minutes at 4000g for the collection of serum for hormonal assay.

**Preparation of testicular homogenates:** Testes (Right) were each homogenized separately in cold 0.1M phosphate buffer (pH 7.4) with Heidolph homogenizer and Teflon pestle. The homogenate thereafter was centrifuged at 3500g for ten (10) minutes at 4°C. The supernatants were then assayed for biochemical parameters.

**Measurement of body weight and testicular weight:** Total body weight of rats was measured on a weekly basis throughout the duration of the study. The testis was harvested, cleared of adjoining tissue and also weighed. Digital weighing scale was used for both measurements.

**Hormonal assay:** Testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) in serum were determined using ELISA kits following manufacturer's protocol.

**Semen analysis:** The epididymis together with the vas deferens were harvested and sperm count, viability, morphology and semen pH assessed from caudal portion of the epididymis (Ujah *et al.*, 2021). Sperm motility was determined from cells in the vas deferens. Method was as in a previous study (Nna *et al.*, 2017).

**Histopathological techniques:** Haematoxylin and eosin (H and E) technique was used for testicular histopathology. The left testis was harvested under chloroform anaesthesia and immediately placed in Bouin's fluid for fixation. It was then dehydrated and embedded in paraffin blocks. The tissue blocks were sectioned and stained with H and E on glass slides. The slides were appropriately labelled and photomicrographs taken at x40 magnification under a light microscope. Tissue sections were evaluated for Sertoli cell count, seminiferous tubular diameter, number of germ cell layers, leydig cell count, seminiferous epithelial height and Johnsen's score. Seminiferous tubular diameter and seminiferous epithelial height were measured by ocular micrometer calibrated with stage micrometer (D'Souza, 2004). The Johnsen's score was evaluated using the Johnsen scoring method as shown in table 1 (Johnsen, 1970).

**Table 1**  
Johnsen's Score

| Score | Features                                                            |
|-------|---------------------------------------------------------------------|
| 10    | Complete spermatogenesis with perfect tubules                       |
| 9     | Many spermatozoa present but disorganized spermatogenesis           |
| 8     | Few spermatozoa present                                             |
| 7     | No spermatozoa but many spermatids present                          |
| 6     | Only a few spermatids present                                       |
| 5     | No spermatozoa or spermatids present but many spermatocytes present |
| 4     | Only a few spermatocytes present                                    |
| 3     | Spermatogonia alone present                                         |
| 2     | Germ cells absent                                                   |
| 1     | No germ cells no Sertoli cells present                              |

Authors should state the source of the table

## Statistical analysis

Graph pad prism 7.0 (Graph Pad Software Inc., La Jolla, CA, USA) was used for data analysis. The One-way analysis of variance (ANOVA) followed by post-hoc test (turkey) was used to analyse data. Results were presented as mean  $\pm$  standard error of mean. A p value below 0.05 was considered significant.

## RESULTS

**Fasting blood glucose:** Fig. 1 shows a significant increase ( $P<0.001$ ) in fasting blood glucose concentration in the DM control group ( $602.80 \pm 0.73$  mg/dL), DM + insulin group ( $129.40 \pm 3.20$  mg/dL) and DM + Zinc group ( $582.20 \pm 20.05$  mg/dL) when compared to the normal control group ( $99.20 \pm 1.24$  mg/dL). Results further showed that fasting blood glucose concentration was significantly higher ( $P<0.001$ ) in the DM + Zinc group when compared to the DM + Insulin group. There was a significant decrease in fasting blood glucose concentration in the DM + Insulin + Zinc group ( $116.00 \pm 1.18$  mg/dL) when compared with the DM control ( $P<0.001$ ) and DM + Zinc ( $P<0.001$ ) groups.

**Semen analysis:** There was a significant decrease ( $P<0.001$ ) in sperm count in DM control ( $0.16 \times 10^6 \pm 0.04$ /ml), DM + insulin ( $0.16 \times 10^6 \pm 0.02$ /ml), DM + zinc ( $0.12 \times 10^6 \pm 0.02$ /ml) and DM + insulin + zinc ( $0.20 \times 10^6 \pm 0.03$ /ml) when compared with the normal control group ( $19.08 \times 10^6 \pm 0.63$ /ml) (Table 2).

There was a significant decrease ( $P<0.001$ ) in sperm motility in DM control ( $0.00 \pm 0.00\%$ ), DM + insulin ( $12.00 \pm 1.22\%$ ), DM + zinc ( $0.00 \pm 0.00\%$ ) and DM + insulin + zinc ( $10.00 \pm 0.00\%$ ) groups when compared to normal control group ( $77.00 \pm 4.36\%$ ). When compared to DM control however, sperm motility increased significantly in the DM + insulin group ( $P<0.01$ ) and DM + insulin + zinc group ( $P<0.05$ ). Sperm motility decreased significantly ( $P<0.01$ ) in the DM + zinc group when compared with the DM + insulin group. There was a significant increase ( $P<0.05$ ) in sperm motility in the DM + insulin + zinc group when compared with the DM + zinc group. (Table 2).

Sperm viability decreased significantly ( $P<0.001$ ) in DM control ( $12.00 \pm 1.22\%$ ), DM + insulin ( $24.60 \pm 0.24\%$ ), DM + zinc ( $24.00 \pm 0.55\%$ ) and DM + insulin + zinc ( $25.00 \pm 0.32\%$ ) groups when compared to normal control group ( $84.20 \pm 0.49\%$ ). Results further show a significant increase ( $P<0.001$ ) in sperm viability in DM + insulin, DM + zinc and DM + insulin + zinc groups when compared to DM control group. (Table2).

Abnormal sperm morphology increased significantly in DM control ( $15.20 \pm 0.73\%$ ,  $P<0.001$ ) and DM + zinc group ( $4.80 \pm 0.66\%$ ,  $P<0.05$ ) groups when compared to normal control group ( $1.80 \pm 0.37\%$ ). There was however a significant decrease ( $P<0.001$ ) in abnormal sperm morphology in DM + insulin group ( $3.60 \pm 0.40\%$ ), DM +

zinc group and DM + insulin + zinc group ( $3.80 \pm 0.49\%$ ) when compared to the DM control. (Table 2).

**Sperm head defect:** Table 2 shows comparison of sperm head defect between normal control ( $0.00 \pm 0.00\%$ ), DM control ( $1.00 \pm 0.77\%$ ), DM + insulin ( $0.60 \pm 0.24\%$ ), DM + zinc ( $0.60 \pm 0.24\%$ ) and DM + insulin + zinc ( $0.40 \pm 0.24\%$ ) groups. Results show no significant change ( $P>0.005$ ) in sperm head defect between the different groups.

**Sperm neck defect:** Table 2 shows results for sperm neck defect in normal control ( $0.40 \pm 0.24\%$ ), DM control ( $1.20 \pm 0.49\%$ ), DM + insulin ( $0.60 \pm 0.24\%$ ), DM + zinc ( $0.40 \pm 0.24\%$ ) and DM + insulin + zinc ( $1.40 \pm 0.24\%$ ) groups. There was no significant difference ( $P>0.05$ ) between the groups.

**Sperm tail defect:** There was a significant increase in sperm tail defect in DM control group ( $12.80 \pm 0.37\%$ ,  $P<0.001$ ) and DM + zinc group ( $3.60 \pm 0.51\%$ ,  $P<0.05$ ) when compared to normal control group ( $1.40 \pm 0.24\%$ ). There was however a significant decrease in sperm tail defect in DM + insulin ( $2.60 \pm 0.40\%$ ,  $P<0.001$ ), DM + zinc ( $P<0.001$ ) and DM + insulin + zinc ( $2.00 \pm 0.63\%$ ,  $P<0.001$ ) when compared to the DM control (Table 2)

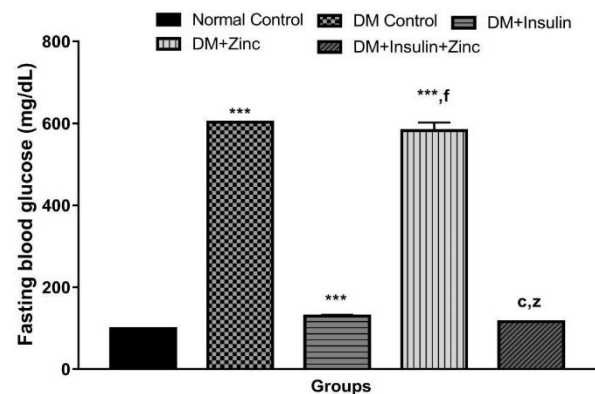


**Figure 1:**

Comparison of fasting blood glucose concentration between the different groups.

Values are mean  $\pm$  SEM,  $n = 5$ .

\*\*\* vs Normal control ( $p<0.001$ ); c vs DM control ( $p<0.001$ ); f vs DM +

Insulin ( $p<0.001$ ); z vs DM + Zinc ( $p<0.001$ )

**Table 2**  
Semen Analysis

|                                                | Normal control        | Diabetes mellitus     | Diabetes mellitus+Insulin    | Diabetes mellitus + zinc       | Diabetes mellitus+insulin + zinc |
|------------------------------------------------|-----------------------|-----------------------|------------------------------|--------------------------------|----------------------------------|
| <b>Sperm count <math>\times 10^6</math>/ml</b> | 19.08 $\pm$ 0.63      | 0.16 $\pm$ 0.04***    | 0.16 $\pm$ 0.02***           | 0.12 $\pm$ 0.02***             | 0.20 $\pm$ 0.03***               |
| <b>Sperm motility (%)</b>                      | 77.00 $\pm$ 4.36      | 0.00 $\pm$ 0.00***    | 12.00 $\pm$ 1.22***,b        | 0.00 $\pm$ 0.00***,e           | 10.00 $\pm$ 0.00***,a,x          |
| <b>Sperm viability(%)</b>                      | 84.20 $\pm$ 0.49***,c | 12.00 $\pm$ 1.22***,c | 24.60 $\pm$ 0.24***,c        | 24.00 $\pm$ 0.55***,c          | 25.00 $\pm$ 0.32***,c            |
| <b>Abnormal sperm morphology(%)</b>            | 1.80 $\pm$ 0.37       | 15.20 $\pm$ 0.73***   | 3.60 $\pm$ 0.40 <sup>c</sup> | 4.80 $\pm$ 0.66 <sup>x,c</sup> | 3.80 $\pm$ 0.49 <sup>c</sup>     |
| <b>Head defect(%)</b>                          | 0.00 $\pm$ 0.00       | 1.00 $\pm$ 0.77       | 0.60 $\pm$ 0.24              | 0.60 $\pm$ 0.24                | 0.40 $\pm$ 0.24                  |
| <b>Neck defect(%)</b>                          | 0.40 $\pm$ 0.24       | 1.20 $\pm$ 0.49       | 0.60 $\pm$ 0.24              | 0.40 $\pm$ 0.24                | 1.40 $\pm$ 0.24                  |
| <b>Tail defect(%)</b>                          | 1.40 $\pm$ 0.24       | 12.80 $\pm$ 0.37***   | 2.60 $\pm$ 0.40 <sup>c</sup> | 3.60 $\pm$ 0.51 <sup>x,c</sup> | 2.00 $\pm$ 0.63 <sup>c</sup>     |
| <b>Semen pH</b>                                | 6.12 $\pm$ 0.04       | 5.80 $\pm$ 0.04***    | 6.22 $\pm$ 0.08 <sup>c</sup> | 6.30 $\pm$ 0.00 <sup>c</sup>   | 6.06 $\pm$ 0.02 <sup>b,x</sup>   |

Values are mean  $\pm$  SEM,  $n = 5$ .

\*\*\* vs Normal control ( $p<0.001$ ); a vs DM control ( $p<0.05$ ), b vs DM control ( $p<0.01$ ); c vs DM control ( $p<0.001$ ); e vs DM + Insulin ( $p<0.01$ ); x vs DM + Zinc ( $p<0.05$ )

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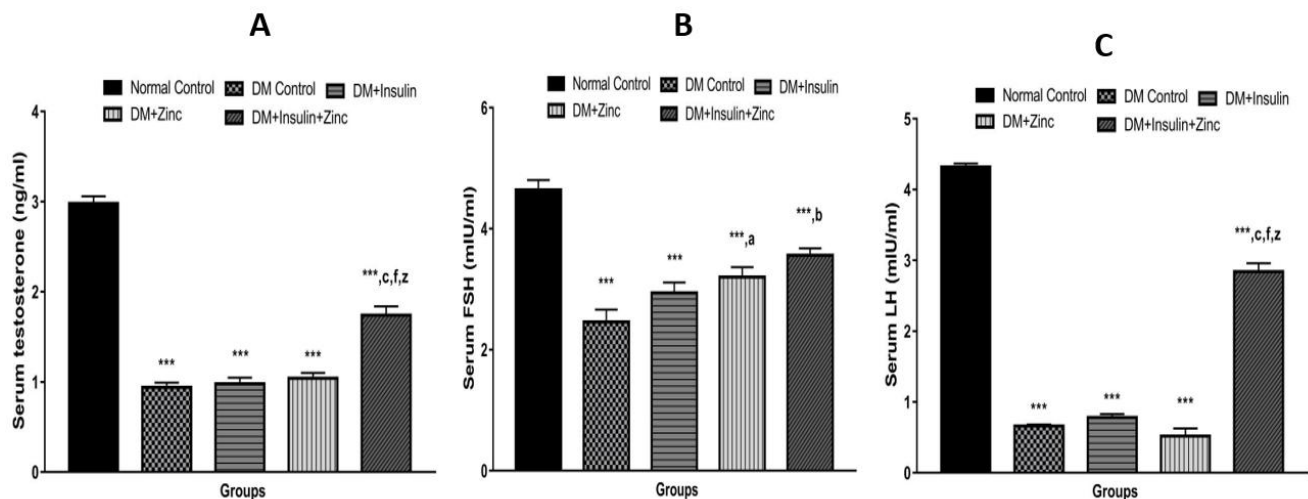


**Figure 2:**

Comparison of serum reproductive hormone concentration between the different groups.

Values are mean ± SEM, n = 5.

\*\*\* vs Normal control ( $p < 0.001$ ); a ( $p < 0.05$ ), b ( $p < 0.01$ ) vs DM control. c vs DM control ( $p < 0.001$ ); f vs DM + Insulin ( $p < 0.001$ ); z = vs DM + Zinc ( $p < 0.001$ )

**Semen pH:** Table 2 also compares the semen pH across the different groups. There was a significant decrease ( $P < 0.001$ ) in semen pH in the DM control group ( $5.80 \pm 0.04$ ) when compared to normal control group ( $6.12 \pm 0.04$ ). There was a significant increase ( $P < 0.001$ ) in semen pH in DM + insulin ( $6.22 \pm 0.08$ ) and DM + zinc ( $6.30 \pm 0.00$ ) groups when compared to DM control. There was however a significant decrease ( $P < 0.01$ ) in semen pH in the DM + insulin + zinc group ( $6.06 \pm 0.02$ ) when compared to the DM control. There was a significant decrease ( $P < 0.05$ ) in semen pH in the DM + insulin + zinc group when compared to DM + zinc group.

**Serum testosterone:** Figure 2A shows comparison of serum testosterone between the different groups. There was a significant decrease in serum testosterone ( $P < 0.001$ ) in the DM control group ( $0.94 \pm 0.05$  ng/ml), DM + Insulin group ( $0.98 \pm 0.07$  ng/ml), DM + zinc group ( $1.04 \pm 0.06$  ng/ml) and DM + insulin + zinc group ( $1.74 \pm 0.10$  ng/ml) when compared with the normal control ( $2.98 \pm 0.08$  ng/ml). Results also show a significant increase in serum testosterone in the DM + insulin + zinc group when compared with DM control ( $P < 0.001$ ), DM + insulin ( $P < 0.001$ ) and DM + zinc ( $P < 0.001$ ) groups.

**Serum follicle stimulating hormone (FSH):** Serum FSH decreased significantly ( $P < 0.001$ ) in DM control ( $2.46 \pm 0.20$  mIU/ml), DM + insulin ( $2.94 \pm 0.17$  mIU/ml), DM + zinc ( $3.20 \pm 0.16$  mIU/ml) and DM + insulin + zinc ( $3.56 \pm 0.12$  mIU/ml) groups when compared to the normal control group ( $4.64 \pm 0.16$  mIU/ml). serum FSH was significantly increased in DM + zinc ( $P < 0.05$ ) and DM + insulin + zinc ( $P < 0.01$ ) groups when compared to the DM control group (Fig. 2B)

**Serum luteinizing hormone (LH):** Figure 2C shows that serum LH was significantly decreased ( $P < 0.001$ ) in DM control ( $0.66 \pm 0.02$  mIU/ml), DM + insulin ( $0.78 \pm 0.05$  mIU/ml), DM + zinc ( $0.52 \pm 0.11$  mIU/ml) and DM + insulin + zinc ( $2.84 \pm 0.12$  mIU/ml) groups when compared

to the normal control group ( $4.32 \pm 0.05$  mIU/ml). The serum LH level increased significantly in the DM + insulin + zinc group when compared with DM control ( $P < 0.001$ ), DM + insulin ( $P < 0.001$ ) and DM + zinc ( $P < 0.001$ ) groups.

**Sertoli cell count:** Sertoli cell count significantly decreased ( $P < 0.001$ ) in DM control ( $5.02 \pm 0.25$ ), DM + insulin ( $6.34 \pm 0.06$ ), DM + zinc ( $5.65 \pm 0.21$ ) and DM + insulin + zinc ( $7.00 \pm 0.36$ ) when compared to the normal control group ( $8.60 \pm 0.14$ ). There was a significant increase in sertoli cell count in DM + insulin ( $P < 0.01$ ) and DM + insulin + zinc ( $P < 0.001$ ) groups when compared to DM control group. A significant increase ( $P < 0.01$ ) in Sertoli cell count was recorded in the DM + insulin + zinc group when compared to the DM + zinc group (Fig. 3A)

**Leydig cell count:** Results show a significant decrease ( $P < 0.001$ ) of Leydig cells in DM control ( $3.64 \pm 0.15$ ), DM + insulin ( $3.69 \pm 0.06$ ), DM + zinc ( $3.71 \pm 0.10$ ) and DM + insulin + zinc ( $4.03 \pm 0.08$ ) groups when compared to the normal control group ( $5.30 \pm 0.05$ ). (FIG. 3B).

**Germ cell layers:** Fig. 3C shows a significant decrease ( $P < 0.001$ ) in the number of germ cells in the DM control group ( $1.80 \pm 0.20$ ), DM + zinc group ( $2.60 \pm 0.24$ ), DM + insulin + zinc ( $3.00 \pm 0.32$ ) and DM + insulin group ( $4.20 \pm 0.49$ ,  $P < 0.05$ ) when compared to the normal control group ( $5.60 \pm 0.24$ ). There was a significant increase ( $P < 0.001$ ) in the number of germ cell layers in DM + insulin group when compared to the DM control group. The number of germ cell layers significantly decreased ( $P < 0.05$ ) in the DM + zinc group when compared with the DM + insulin group.

**Seminiferous tubule diameter:** There was a significant decrease ( $P < 0.001$ ) in seminiferous tubule diameter in DM control ( $63.30 \pm 0.45$   $\mu$ m), DM + insulin ( $154.64 \pm 1.42$   $\mu$ m), DM + zinc ( $68.56 \pm 0.65$   $\mu$ m) and DM + insulin + zinc ( $83.78 \pm 2.01$   $\mu$ m) groups when compared to the normal control group ( $170.44 \pm 1.01$   $\mu$ m). Results further show a significant increase ( $P < 0.001$ ) in seminiferous tubule

diameter in DM + insulin and DM + insulin + zinc groups when compared with DM control group. A significant decrease ( $P<0.05$ ) in seminiferous tubule diameter was seen in DM + insulin group when compared to DM control group. Seminiferous tubule diameter was significantly decreased ( $P<0.001$ ) in DM + zinc and DM + insulin + zinc groups when compared to DM + insulin group. Seminiferous tubule diameter significantly increased ( $P<0.001$ ) in DM + insulin + zinc group when compared to DM + zinc group. (Fig.3D).

**Seminiferous epithelial height:** Result (Fig. 3E) show a significant decrease ( $P<0.001$ ) in seminiferous epithelial height in DM control ( $19.38 \pm 0.32 \mu\text{m}$ ), DM + insulin ( $28.70 \pm 1.23 \mu\text{m}$ ), DM + zinc ( $27.36 \pm 1.62 \mu\text{m}$ ) and DM + insulin + zinc ( $27.32 \pm 0.93 \mu\text{m}$ ) groups when compared to the normal control group ( $65.14 \pm 0.38 \mu\text{m}$ ). There was a significant increase ( $P<0.001$ ) in seminiferous epithelial height in DM + insulin, DM + zinc and DM + insulin + zinc groups when compared to DM control group.

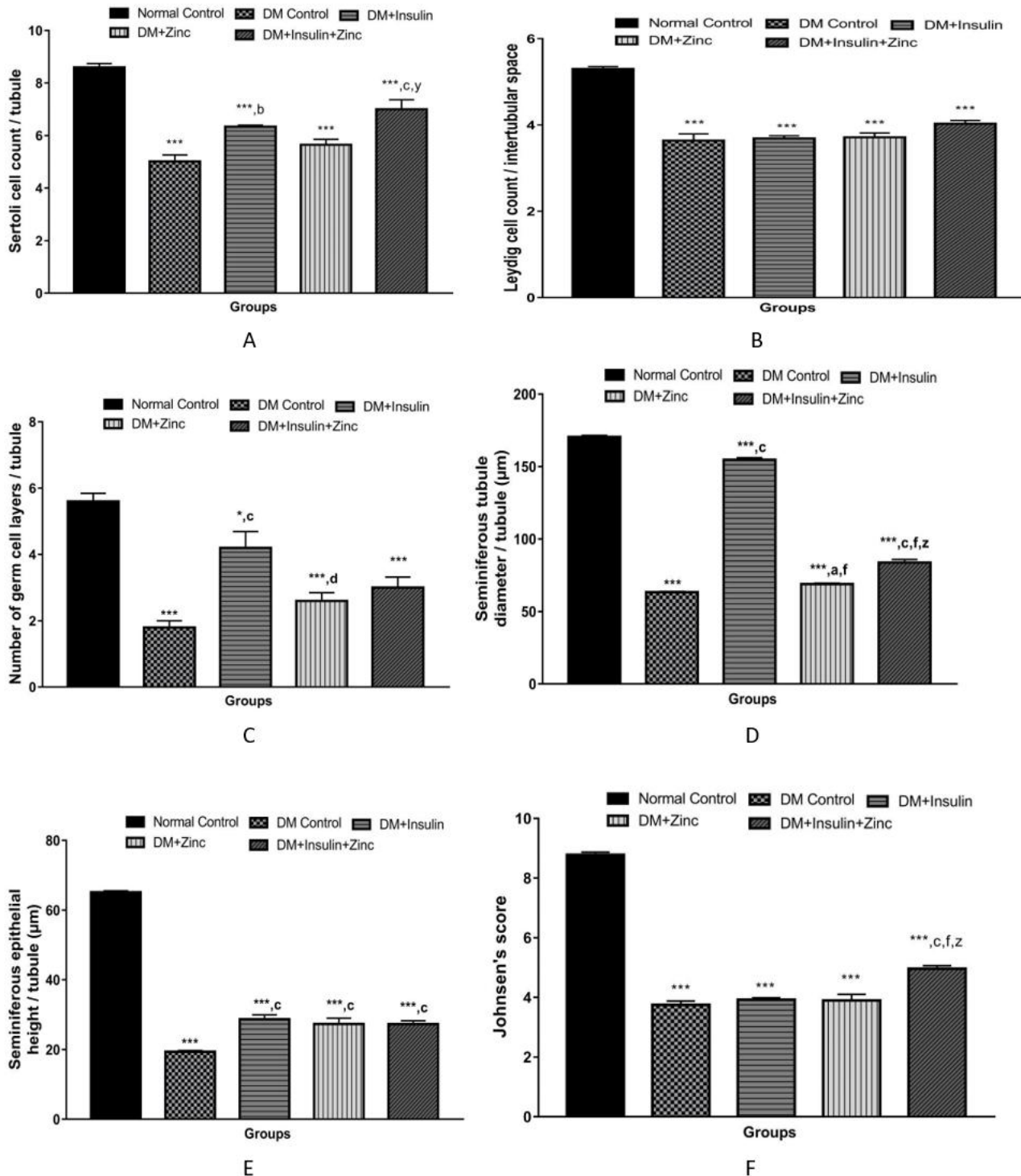


**Figure 3**

Comparison of histological indices between the different groups.

Values are mean  $\pm$  SEM,  $n = 5$ ; \*\*\* vs Normal control ( $p<0.001$ ); b ( $p<0.01$ ), c ( $p<0.001$ ) vs DM control; d vs DM + insulin ( $p<0.05$ ); f vs DM + insulin ( $p<0.001$ ); y = vs DM + Zinc ( $p<0.01$ ); z vs DM + zinc ( $p<0.001$ )

**Johnsens's score:** Johnsen's score decreased significantly ( $P < 0.001$ ) in DM control ( $3.75 \pm 0.13$ ), DM + insulin ( $3.92 \pm 0.06$ ), DM + zinc ( $3.90 \pm 0.20$ ) and DM + insulin + zinc ( $4.96 \pm 0.11$ ) groups when compared to the normal control group ( $8.78 \pm 0.09$ ). There was a significant increase in Johnsen's score in the DM + insulin + zinc group when compared to DM control ( $P < 0.001$ ), DM + insulin ( $P < 0.001$ ) and DM + zinc ( $P < 0.001$ ) groups. (Fig.3F).

#### Histopathology of testicular tissue comparing seminiferous tubules and intertubular interstitium between the groups:

Figs 4a and 5a show histopathology of seminiferous tubules and intertubular interstitium respectively in the normal control group. The seminiferous tubules contain proliferating spermatogonia cells at various stages of maturation. These cells include spermatogonia A and B that are closer to the basement membrane, the primary spermatocytes, secondary spermatocytes, spermatid and spermatozoa. The cells have regular nuclei contour and there are 10 to 12 sertoli cells per tubules and the leydig cell population within the peritubular and intertubular interstitium is 4 to 6 cells.

Figs 4b and 5b show histopathology of seminiferous tubules and intertubular interstitium respectively in the DM + insulin group. Section of the testis shows seminiferous tubules containing scanty spermatogonia with a thickened basement membrane. There is complete absence of spermatogonia in majority of the seminiferous tubules and some few scanty spermatogonia A and B. The spermatocytes, spermatids and spermatozoa are absent. The sertoli cell population is less than six per tubule and the leydig cell population is scanty and less than three per

cluster. The scanty spermatogonia are atrophic and few macrophages are seen in the lumen.

Figs 4c and 5c show histopathology of seminiferous tubules and intertubular interstitium respectively in the DM + zinc group. Section of the testis shows atrophic seminiferous tubules containing sparsely populated spermatogonia. The basement membranes are thickened and the spermatogonia are atrophic. The spermatogonia are less than two cell layer thick and the intervening interstitium is scanty and contains less than three round to oval leydig cells. The supporting Sertoli cells are also reduced in number. The other cell lineage such as the spermatocyte, spermatid and spermatozoa are absent.

Figs 4d and 5d show histopathology of seminiferous tubules and intertubular interstitium respectively in the DM + insulin + zinc group. Seminiferous tubules with thickened basement membrane are seen. The tubules contain sparsely populated spermatogonia at various stages of maturation. The cells are less than four layers thick and are atrophic. The luminal cavities are empty and the Sertoli cell population is less than seven and the Leydig cells are less than four per cluster. Some of the tubules contain only spermatogonia cells. The cells have deeply stained atrophic nuclei.

Figs 4e and 5e show histopathology of seminiferous tubules and intertubular interstitium respectively in the DM control group. The tubules contain scanty spermatogonia cells with complete absence of the late series which includes spermatocytes, spermatid and spermatozoa. There is atrophy of the spermatogonia cells and reduction in the Sertoli cell population to less than five per tubule. The separating interstitium is scanty and contains less than six Leydig cells per cluster

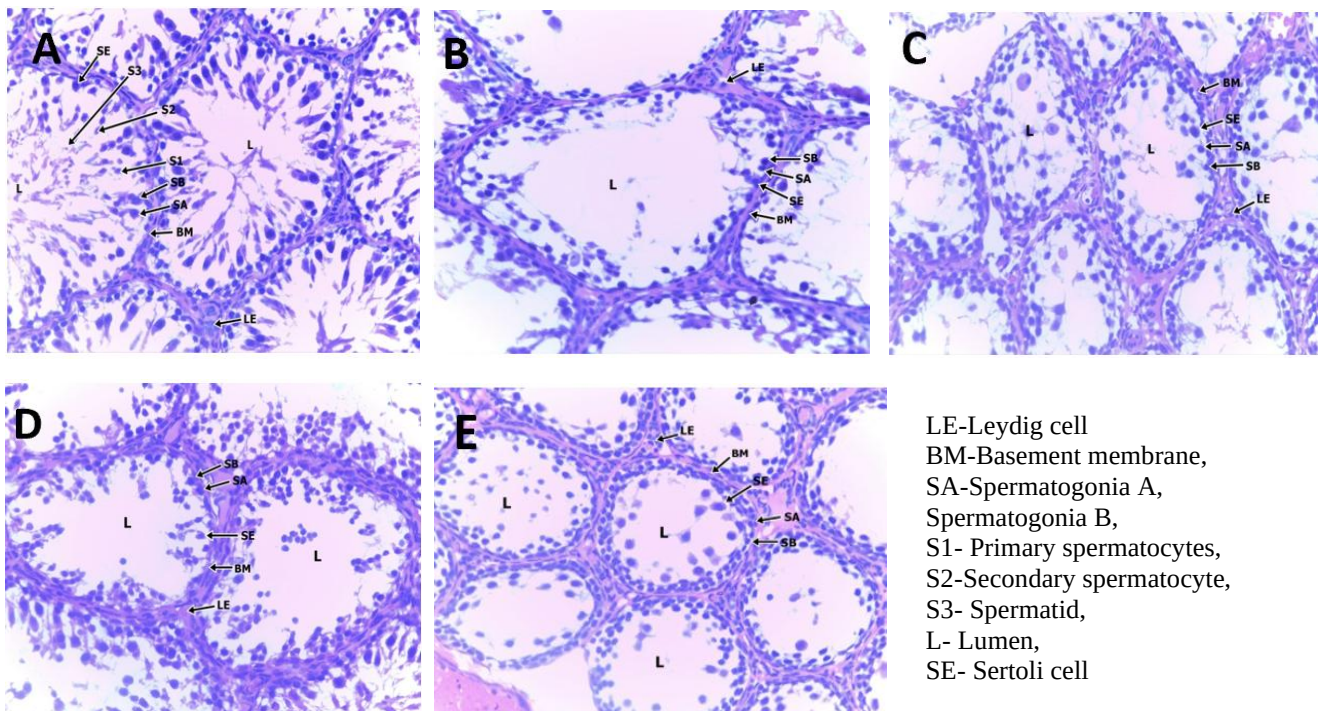


**Plate 1**

4a. Normal seminiferous tubules (ST) containing proliferating spermatogonia cells at various stages of maturation. F4b (DM + insulin group) shows ST with scanty spermatogonia. 4c (DM + Zinc group) shows atrophic ST containing sparsely populated spermatogonia. 4d (DM + insulin + zinc group) shows ST with sparsely populated spermatogonia. 4e (DM control group) shows ST containing scanty spermatogonia with complete absence of spermatocytes, spermatid and spermatozoa. All figures were viewed at x400 magnification

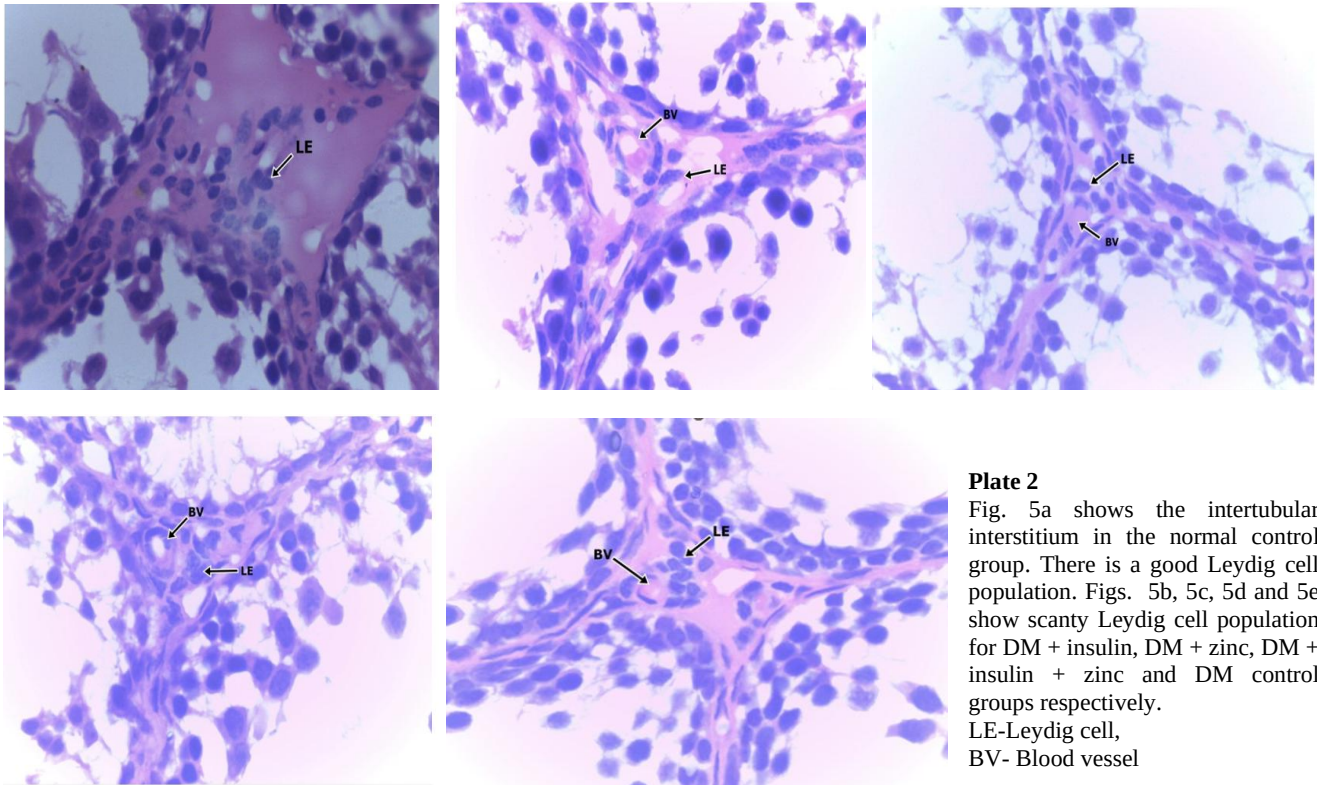
**Plate 2**

Fig. 5a shows the intertubular interstitium in the normal control group. There is a good Leydig cell population. Figs. 5b, 5c, 5d and 5e show scanty Leydig cell population for DM + insulin, DM + zinc, DM + insulin + zinc and DM control groups respectively.

LE-Leydig cell,  
BV- Blood vessel

## DISCUSSION

This study sought to establish if zinc and /or insulin could reverse or ameliorate diabetes mellitus (DM) – induced reproductive impairments in male rats.

True to its antihyperglycaemic effect, insulin decreased fasting blood glucose level in DM. the decrease was better when zinc was co-administered with insulin. It thus appears that while zinc may not have an antihyperglycaemic effect, it enhances insulin action by either prolonging its duration of action (Sharma and Divya, 2020) or increasing its tissue sensitivity (Thoen et al., 2019).

Results from the study showed that both body weight and testicular weight were decreased in diabetic conditions. This is consistent with other works (Scarano et al., 2006; Cao et al., 2012). The decrease in weight has been reported to be a consequence of metabolic alterations occasioned by insulin absence or insufficiency (Arduino, 1980). Also, testicular weight is a reflection of the size of the testis. Sertoli cells (SC) are important components of the testis, and the number of SCs is usually linked to testicular size (Sharpe et al., 2003). Insulin administration however improved body weight and its co-administration with zinc improved both general body weight and testicular weight in diabetic state. It appears that the improvement in body weight was mostly due to insulin effects. This is likely due to the improvement in metabolic activities following insulin therapy as insulin promotes cell division and growth (Wilcox, 2005). For testicular weight however, only in combination with zinc was insulin able to improve weight. This is likely due to the improvement in SC count in same group as shown by results.

Testosterone is a hormone that is critical to male fertility (Walker, 2010). Testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) are key

requirements for spermatogenesis, and together with gonadotropin-releasing hormone (GnRH) form the hypothalamus-pituitary-testicular axis (Ramaswamy and Weinbauer, 2015). In this study, the diabetic state decreased testosterone, FSH and LH. This is in line with a number of studies (Djursing et al., 1983; Scarano et al., 2006; Ricci et al., 2009; Mohasseb et al., 2011; Rato et al., 2015). The decreased levels of FSH and LH could be the result of diabetic neuropathy that may have affected the hypothalamus and/or pituitary (Dinulovic and Radonjic, 1990; Chao et al., 2022). The low level of testosterone in the diabetic state is likely the result of a decrease in Leydig cells (Rehman et al., 2001). Leydig cell count is usually directly proportional to testosterone concentration, as they are responsible for testosterone secretion (Nna et al., 2017). While insulin has been reported to have stimulatory effect on leydig cells (Ballester et al., 2004) and zinc to be essential for testosterone synthesis and release of FSH and LH (Al- Ani et al., 2015), neither the single administration of insulin nor zinc increased the level of any of these hormones in the diabetic states, except for FSH, where zinc administration increased its level in comparison to the diabetic untreated group. In combination however, insulin and zinc increased the levels of these hormones. There appears to be a synergy of actions of insulin and zinc when co-administered. Insulin binding to tissues is reported to be greater when co-administered with zinc (Thoen et al., 2019). Insulin sensitivity has been shown to be increased by zinc (Alkaladi et al., 2014). Its action and duration of action are also known to be potentiated by zinc (Dodson and Steiner, 1998; Owens, 2011). Zinc on the other hand is a co-factor in over 300 enzymes and hormones (Rink and Gabriel, 2000) and required for testosterone synthesis (Bhowmik et al., 2010). Insulin may have created an environment that enhanced zinc actions.

*Insulin and zinc ameliorate sexual dysfunction in diabetic males*

Leydig cell count decreased in all the diabetic groups and this was associated with a corresponding decrease on testosterone level in all the diabetic groups, except in the diabetic group treated with both insulin and zinc, where testosterone was increased. It is important to note that an increase (though not significant) in Leydig cell count in the insulin and zinc treated diabetic group was accompanied by a significant increase in LH in same group. LH is known to stimulate testosterone synthesis by Leydig cells (Ramaswamy and Weinbauer, 2015). This increased LH and the previous finding that zinc inhibits the conversion of testosterone to oestrogen (Al-Ani et al., 2015) may have been responsible for this phenomenon. It is also possible that the combination of insulin and zinc may have stimulatory effect on adrenal testosterone synthesis in ways that their separate administrations could not. The adrenal gland is known to synthesize testosterone (Nakamura et al., 2009) and this study assessed testosterone level in the serum.

Semen analysis is an essential tool in determining male fertility status. Sperm count, motility and viability were all decreased and abnormal sperm morphology increased in the diabetic groups. This is corroborated by several other studies (Scarano et al., 2006; Afifi et al., 2015; An et al., 2018; Laleethambika et al., 2019). Sperm quality is the end product of reproductive and other systems working together (Dinulovic and Radonjic, 1990) and hence a number of factors may have been responsible for the poor sperm quality observed in this study. Alterations in the hypothalamic-pituitary-gonadal axis, as manifested in decreased FSH, LH and testosterone is one way the sperm quality may have been compromised. These hormones are required for effective sperm production (Ramaswamy and Weinbauer, 2015). Other mechanisms may include; germ cell depletion and Sertoli cell deterioration (Bruning et al., 2000; Baccetti et al., 2002), all of which are evident in this study. Treatments in all cases seemed not to have improved sperm count compared to the untreated animals even though treatments improved a number of parameters (hormones, germ cell layer, Sertoli cell count, amongst others) influencing sperm count. It may be that the duration of study did not allow for full completion of the spermatogenic cycle which is averagely six weeks in rat (De Krester and Kerr, 1988). There was an increase in viability following treatments.

The increase in sperm motility in the diabetic untreated group following insulin and insulin and zinc co-treatment is mainly due to insulin as zinc when administered alone showed no significant change. Insulin being an antihyperglycaemic agent may have decreased oxidative stress by reducing the formation of glycation products. There is evidence that insulin interacts with sperm cells (Schoeller et al., 2012). Abnormal sperm morphology decreased to near normal level with treatments. It thus appears that zinc's antioxidant role (Sacan et al., 2016) and insulin's antihyperglycaemic effect reversed defects in sperm cells. Zinc concentration has been reported to be low in seminal fluid in DM (Dinulovic and Radonjic, 1990). Thus supplementation may have increased zinc levels to influence the observed changes. DM seems to have adverse effect on sperm morphology by mostly causing tail defects as this study shows. The head and neck of sperm cells were largely unaffected. This may also explain how DM decreases motility.

Semen pH was slightly decreased in diabetic untreated rats. DM is a metabolic disease that has been shown to be associated with high concentration of certain amino acids and their derivatives in the seminal fluid, believed to be the result of defective kreb's cycle (Kaemmerer and Mitzkat, 1985). This is likely how semen pH is decreased. This could also be implicated in the impairment of sperm motility and formation of defective sperm cells (Kaemmerer and Mitzkat, 1985). With either insulin or zinc treatment, semen pH was increased. The best result was however with insulin and zinc co-administration, as pH was brought to near normal levels. Zinc is essential for metabolism (Prasad, 2009) and it may have, especially in combination with insulin, reduced the amount of amino acids formed in the seminal fluid.

Effective male reproductive function is fundamentally dependent on a healthy testicular tissue. Testicular morphology was compromised by DM. Thickened basement membrane of the seminiferous tubules, sparsely populated spermatogonia and a scanty intertubular interstitium are some of the morphological changes that were seen from photomicrographs. Some of these changes have been similarly reported by Wankeu-Nya et al., (2013). A quantitative assessment of the testicular tissue revealed more morphometric deviations. Sertoli cell count, Leydig cells and germ cell layers were all depleted by DM. The SCs are key testicular cells that play a major role in spermatogenesis by nourishing the germ cells as has been stated earlier. Insulin and insulin co-treatment with zinc increased the SC count. The trend was similar with the number of germ cell layers except in the co-treatment group. While it could be said that the increase in the number of germ cell layers following insulin treatment is a consequence of the increase in SCs, same cannot be said for co-treatment. It may be that zinc has a yet unknown role in limiting germ cell proliferation, as even when administered alone; the number of germ cell layers was significantly lower than in insulin treatment. This wasn't the case with the SC count.

Seminiferous tubular diameter and seminiferous epithelial height are parameters that tell of testicular integrity (Nna et al., 2017). Oxidative stress causes testicular atrophy which manifests as a decrease in both tubular diameter and tubular epithelial height (Nna et al., 2017). The increased formation of glycation products is DM causes oxidative stress (Ahmed, 2005). Treatments improved epithelial height in equal measure while insulin treatment offered improvement in tubular diameter.

The Johsen's score is an index on a scale of 1-10 that reflects spermatogenesis (Johnson, 1970). DM impaired spermatogenesis. Insulin and zinc co-treatment improved spermatogenesis. This didn't however reflect in the sperm count. While the Johsen's score evaluates and scores all stages of spermatogenesis, sperm count measures only spermatozoa.

In conclusion, insulin or zinc ameliorates several aspects of DM-induced male reproductive dysfunction. However, the co-administration of insulin and zinc provides better results.

#### **Acknowledgements**

The authors thank Mr. Umoh Ededet and Dr. Nna Victor for their technical assistance in the laboratory and data analysis respectively.

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Full-Length Research Article

# Modulation of Memory and Neurochemical Changes by Resveratrol and Environmental Enrichment in Rodent Model of Alzheimer's Disease

Muhammad M.S.<sup>1,2</sup>, Ayo J.O.<sup>3</sup>, Danjuma N.M.<sup>4</sup>, AbdulWahab A.<sup>2</sup>, Isa A.S.<sup>2</sup> and Umar A.H.<sup>2</sup>

<sup>1</sup>Department of Human Physiology, College of Medical Sciences, Gombe State University, Nigeria  
<sup>2</sup>Departments of <sup>2</sup>Human Physiology, <sup>3</sup>Veterinary Physiology, Ahmadu Bello University Zaria, Nigeria  
<sup>4</sup>Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello, University Zaria, Nigeria

**Summary:** Alzheimer's disease (AD) is the most common cause of dementia that affects one patient every seven seconds, with over 35 million people currently affected worldwide. The aim of the study was to investigate the modulation of memory and neurochemical responses by resveratrol and environmental enrichment (EE) in aluminium chloride (AlCl<sub>3</sub>) model of Alzheimer's disease in mice. Male mice used for the study were divided into nine groups, of seven animals each. Group I (negative control): 0.2 ml normal saline/kg, Group II: 0.2 ml CMC/kg. Group III: resveratrol (200 mg/kg), Group IV: CMC and kept in EE, Group V: AlCl<sub>3</sub> at dose of 50 mg/kg, Group VI: resveratrol at dose of 200 mg/kg and kept in EE, Group VII: AlCl<sub>3</sub> (50 mg/kg) + resveratrol (200 mg/kg), Group VIII: AlCl<sub>3</sub> (50 mg/kg) and kept in EE, Group IX: AlCl<sub>3</sub> (50 mg/kg) + resveratrol (200 mg/kg) and kept in enriched environment. All treatments were oral and lasted for 8 weeks. Assessments of memory was carried out before treatment, and at weeks 4 and 8, after the first treatment. The mice were sacrificed and hippocampal samples collected for neurochemical analysis. The findings of the study suggest that AlCl<sub>3</sub> induced contextual fear memory deficit over time ( $p < 0.05$ ), which was improved by resveratrol. Both A $\beta$  and Nrf2 significantly ( $p < 0.05$ ) increased in AlCl<sub>3</sub> + EE + resveratrol group. In conclusion, Individual treatment with either resveratrol or EE improved memory over the combined treatment in AlCl<sub>3</sub> model of AD by decreasing A $\beta$  protein concentration.

**Keywords:** Resveratrol, Environmental enrichment, Aluminium chloride, Alzheimer's disease, Oxidative stress

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\*Address for correspondence: E-mail: [mshehu84@gmail.com](mailto:mshehu84@gmail.com), [msmuhammad@qsu.edu.ng](mailto:msmuhammad@qsu.edu.ng); Tel: +234-7032527295

Manuscript received- September- 2021; Accepted- March, 2022

DOI: <https://doi.org/10.54548/njps.v37i1.8>

## INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia that affects one patient every seven seconds, with over 35 million people currently affected worldwide (Mohandas et al., 2009; Prince et al., 2018). It has been projected that the disease will affect about 115 million people by 2050 (Prince et al., 2013), which may be caused by both genetic and environmental factors (Gatz et al., 2010). Indeed, it is the most frequent cause of dementia found in the elderly, with an estimated prevalence of 25-50% in people over the age of 85, making it one of the most important medical problem in the elderly (Hong-Qi et al., 2012). The principal histological hall-marks of the disease are the presence of aggregated amyloid-beta (A $\beta$ )-laden plaques and hyperphosphorylated tau-laden neurofibrillary tangles (NFTs) (Selkoe et al., 2001). However, the neurodegeneration that occurs in AD has been proposed to arise not only from the accumulation of A $\beta$  and/or aberrant modification of tau, but also from a number of other factors that include oxidative stress, inflammation, vascular disease and accumulation of metals, such as zinc and aluminium

(Al) (Mohandas et al., 2009; Armstrong, 2011; Craddock et al., 2012).

The negative impacts of aluminium toxicity include diseased ageing induced by alteration of cellular and molecular mechanisms through gene activation and silencing, eventually leading to various age-related disorders, such as AD. Interestingly, Al is an important component of many household materials, such as clays, glasses, and alum (Rui and Yongjian, 2010; Kawahara and Kato-Negishi, 2011). Exposure to Al has been associated with the impairment of mitochondrial functions and the antioxidant defence systems leading to oxidative stress and increased lipid peroxidation, which could eventually promote A $\beta$  peptide formation, deposition, and AD-like amyloidosis (Niu et al., 2005; Kumar, et al., 2008; Kumar and Gill, 2009).

Resveratrol (3, 4'-5- trihydroxystilbene), which is a natural polyphenolic compound found in the skin and seeds of grapes, has been reported to possess antioxidant and anti-inflammatory properties (Das and Das, 2007; Roy et al., 2011; Poulsen et al., 2015). It induces sirtuin 1-dependent histone deacetylase (HDAC) activities (Howitz et al., 2003;

Aggarwal et al., 2005). Resveratrol is more effective in inhibiting oxidative damage than the conventional antioxidants. It scavenges ROS, such as lipid hydroperoxyl, hydroxyl and superoxide anion radicals, through modulation of glutathione biosynthesis via Nrf2 antioxidant-response-element signalling (Pervaiz, 2003; Rahman, 2008). Resveratrol possesses numerous health benefits such as increase in life-span in yeast, nematode worm, fruit fly and short-lived fish, through a direct sirtuin 1-dependent mechanism, or indirectly through activation of mitogen-activated protein kinase (Bauer et al., 2004; Viswanathan et al., 2005; Valenzano et al., 2006; Hawley et al., 2010; Wang et al., 2012). However, the life-span-prolonging effect of resveratrol has been seriously debated (Pearson et al., 2008; Miller et al., 2011), and the molecular mechanisms responsible for therapeutic potential in AD need further investigation.

Environmental enrichment (EE) is a sustained and progressive increase in cognitive and sensorimotor stimuli, with cumulated voluntary physical activity and complex social interactions (Anastasia et al., 2009). It possesses many benefits such as neuroprotection both in health and disease conditions, especially in ageing and animal models, against neurodegenerative disorders (Faherty et al., 2005; Nithianantharajah and Hannan, 2006; Laviola et al., 2008). The synthesis and release of trophic factors (TFs) have been suggested to play a crucial role in the neuroprotection induced by EE. The EE has been observed to alter the expression of TFs and their receptors in several brain areas, and it induces astroglialogenesis (Spires et al., 2004; Steiner et al., 2006). It increases brain-derived neurotrophic factor (BDNF) expression in the striatum and glia cell-line-derived neurotrophic factor (GDNF) mRNA in the substantia nigra in animals (Bezard et al., 2003; Faherty et al., 2005). Furthermore, it improves spatial memory in rodents by elevating histone acetylation in the hippocampus (Levenson et al., 2004; Chwang et al., 2007; Fischer et al., 2007).

The global health burden of dementia of AD has been on the increase in an alarming rate, next to cardiovascular disease with estimated global societal economic cost of over \$604 billion per annum (Wimo and Prince, 2010). Reports indicated that unless progress is made in the management of AD, the annual national cost of managing AD will reach a projected US\$ 1.2 trillion in the US alone by 2050 (Vradsburg, 2015), which further buttress the need to carry out more researches in AD with the aim of proffering viable therapeutic approach that will ameliorate its pathophysiological progression. However, research efforts focused on elucidation of the combined role of EE and resveratrol in Al-induced AD are currently lacking in the available literature. Measures to alleviate Al-induced AD via resveratrol and EE interventions have not been investigated. This study intends to evaluate the effect of resveratrol and environmental enrichment on memory and neurochemical responses in rodent model of AD.

## MATERIALS AND METHODS

**Animals and management:** A total of 63 male mice, 8-12 weeks of age, and weighing 22-27 g were used for this study. The animals were purchased from the Animal House Facility of the Department of Pharmacology and

Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. They were given free access to standard commercial grower's mash feed and water. The mice were allowed to acclimatise to the environment for two weeks before the commencement of the experiment. All experimental protocols were carried out in accordance with the Ahmadu Bello University Research policy, ethics and regulations, governing the care and use of experimental animals (NIH Publication no. 85-23, revised 1996). Ethical clearance was received from Ahmadu Bello University Animal Use and Care committee (ABUCAUC/2018/056). The experiments were conducted in a quiet laboratory from 9: 00 h to 16: 00 h, with light-dark cycle of about 12:12 h.

**Chemicals and drugs:** Resveratrol (Candlewood Stars Incorporated, Danbury, USA, Batch Number: MR 150528), Aluminium chloride (100 g), purchased from ACROS Organics, New Jersey USA (CAS: A0352086), and carboxymethyl cellulose (CMC) (Product No: 27929, BDH Laboratory Chemicals Limited Poole, England), 70% alcohol, ELISA biochemical assay kits were used in the study.

**Preparation and Administration of Drugs:** *Trans*-resveratrol (200 mg/kg/body weight/day), was suspended in 10 g/L carboxymethyl cellulose (CMC) because it is poorly soluble in water, and administered orally (Juan et al., 2002; Roy et al., 2011). Aluminium chloride (50 mg/kg/body weight/day) was administered orally (Bihaqi et al., 2009).

**Animal Housing and management:** The enriched cage (66 cm long × 46 cm wide × 38 cm high) used in keeping the mice was constructed as described by Harburger *et al.* (2007). The cage contained tubes, ramps, stairs, and different 'toys' (hard plastic balls, cubes, cones, and sticks). The toys were changed twice a week to avoid contamination by faeces and urine, and to continuously encourage exploration of the environment. The complexity (the number of objects) of the housing facility was increased progressively every two days with two to four objects added to the environment. After housing the mice in the enriched cage for ten (10) days, the complexity of the cage was expected to be maximal, but the positions of the objects were changed continuously every 2 days to ensure maximal exploration by the mice (Anastasia et al., 2009). Seven mice were housed together to allow social interactions. The standard (control) condition consisted of cages made without objects or running wheels, housing seven mice per cage.

Mice were kept in EE housing for eight weeks, while receiving the appropriate treatment as described by Steiner *et al.* (2006). The control mice were given normal saline and kept under good housing.

**Animal groupings:** The mice were divided into nine groups of seven animals each (Table 1). All drugs were administered via the oral route. All treatments lasted for 8 weeks. Neurobehavioral studies to assess memory and learning was carried out in three phases; 7 days before treatment, and at weeks 4 and 8 after the first treatment. The animals were sacrificed 24 hours after the last

neurobehavioral assessment and the hippocampus was collected for biochemical analysis.

**Table 1:**

Animal Groupings

| S/No | Groups     | Treatment                                                                     | Duration |
|------|------------|-------------------------------------------------------------------------------|----------|
| 1    | Group I    | 0.2 ml normal saline/kg body weight (negative control)                        | 8 weeks  |
| 2    | Group II   | 0.2 ml CMC/kg body weight                                                     | 8 weeks  |
| 3    | Group III  | Resveratrol (200 mg/kg) orally                                                | 8 weeks  |
| 4    | Group IV   | CMC + Enriched housing                                                        | 8 weeks  |
| 5    | Group V    | AlCl <sub>3</sub> (50 mg/kg)                                                  | 8 weeks  |
| 6    | Group VI   | Resveratrol (200 mg/kg) + Enriched environment                                | 8 weeks  |
| 7    | Group VII  | AlCl <sub>3</sub> (50 mg/kg) + Resveratrol (200 mg/kg)                        | 8 weeks  |
| 8    | Group VIII | AlCl <sub>3</sub> (50 mg/kg) + Enriched environment.                          | 8 weeks  |
| 9    | Group IX   | AlCl <sub>3</sub> (50 mg/kg) + Resveratrol (200 mg/kg) + Enriched environment | 8 weeks  |

### Neurobehavioral assessments

**Assessment of contextual fear memory using passive avoidance test:** The passive avoidance chamber (40 cm × 25 cm × 25 cm) as described by Zhu et al. (2001) and modified by Ambali et al. (2010), was used for the evaluation of emotional memory, based on contextual fear conditioning learning (Altman et al., 1987). The floor of the chamber consisted of parallel 2-mm-calibre stainless steel bars, copper rods spaced 1 cm apart, and a well-insulated 2.5 cm × 8 cm × 25 cm wooden platform placed on the extreme left of the chamber. An electric shock was delivered through the floor bars. Each mouse was placed in the chamber for a three-minute adaptation period and then placed on the platform. The latency to step-down on the grid with all four paws was measured. Upon stepping down on the copper bars, each mouse received an immediate mild electrical shock (40-volt foot shock). To avoid the shock, the mouse demonstrated an instinctive reaction to jump back onto the platform. Each mouse was tested in this manner for 5 minutes (Shuchang et al., 2009). The number of times the mouse stepped down from the platform within 5 minutes was considered acquisition errors. This procedure was repeated 24 hours later, and the step-down latency was used as a measurement of memory retention. The number of times the mouse stepped down from the platform within the 5-minute interval was recorded as retention errors.

**Biochemical Assessments:** All the mice were humanely sacrificed. The hippocampus was removed from each brain sample using surgical incision and rinsed with ice-cold isotonic saline. The hippocampus was then ground in a cold glass mortar and homogenised with ice-cold 100 mM phosphate buffer (pH 7.4; 1 g of tissue/9 mL). The homogenates (10 % w/v) were then centrifuged at 1000g for 20 min and the supernatants were used for the following biochemical analyses.

### Quantification of mouse amyloid beta peptide 1-42

**concentration:** The Mouse Amyloid Beta Peptide 1-42 Elisa assay kit (GA-E0181MS) was used to assess the concentration of Aβ1-42 in mouse hippocampus, based on the principle of biotin double antibody sandwich technology (Song et al., 2011). Exactly 40 µL of the sample, 10 µL of mouse Aβ1-42 antibody and 50 µL streptavidin-HRP were added to wells, pre-coated with Aβ1-42 monoclonal antibody. Mouse Aβ1-42 antibody (10 µL) was added to the blank well, with no sample added to it. The standard well contained 50 µL of the standard + 50 µL streptavidin-HRP (no biotin antibody added). The plate was covered with a seal and shaken gently to mix. It was incubated at 37°C for 60 minutes. Thereafter, the plate was removed and drained off liquid. Each well was filled with washing solution and allowed to stand for 3 seconds, after which it was blot off. The washing was repeated five times. Chromogen A (50 µL) and Chromogen B (50 µL) were added to the blank, standard and sample wells, respectively. The plate was shaken to mix the solution, and incubated at 37°C for 10 minutes away from light for colour development. The plate was removed after 10 minutes and 50 µL stop solution was added to each well to stop the reaction. Colour changes were observed from blue to yellow. The absorbance of each well was measured one by one under 450 nm, 10 minutes after adding stop solution. The optical density was measured and concentration of the samples was determined using MyAssays software.

### Quantification of malondialdehyde concentration:

The Mouse malondialdehyde (MDA) Elisa assay kit (GA-E0638MS) was used to assess the concentration of MDA in mouse hippocampus on the basis of biotin double antibody sandwich technology (Janero et al., 1990), according to the manufacturer's protocol.

### Quantification of nuclear factor E -related factor 2 (Nrf2) concentration:

The Mouse Nrf2 Elisa assay kit (GA-E3955MS) was used to assess the concentration of Nrf2 in mouse hippocampus on the basis of biotin double antibody sandwich technology (Taguchi et al., 2011), according to the manufacturer's protocol.

### Statistical Analyses

The analysis of data was carried out using SPSS version 22 (NY: IBM Corp, 2013) and values obtained were expressed as Mean ± SEM. All analyses were done using one-way analysis of variance (ANOVA) for biochemical parameters and mixed analysis of variance for neurobehavioural evaluation, followed by Tukey's and Bonferroni *post-hoc* tests in order to evaluate the significance of the differences between the means, respectively. Values of  $P < 0.05$  were considered significant.

## RESULTS

### Assessment of memory and learning deficits induced by AlCl<sub>3</sub> in mice

#### Acquisition error (learning)-induced deficits in mice:

Table 1 shows the effect of resveratrol and EE on AlCl<sub>3</sub>-induced acquisition error deficits using passive avoidance test in mice. A significant ( $p < 0.05$ ) difference was

observed in acquisition error across the three phases of the study: [ $F(2, 42) = 6.47$ ;  $p = 0.004$ ]. A significant increase in acquisition error was observed in  $\text{AlCl}_3$  + EE treatment group ( $6.33 \pm 1.42$ ) in phase 2, when compared to the baseline mean score of the same treatment group ( $1.67 \pm 0.64$ ). There was no significant [ $F(8, 21) = 2.99$ ;  $p = 0.2$ ] difference between groups, and there was no interaction between groups and time in all the three phases of the study: [ $F(16, 42) = 0.67$ ;  $p = 0.80$ ].

#### Step down latency (memory-induced deficits in mice):

The effect of resveratrol and EE on  $\text{AlCl}_3$ -induced latency (memory) deficits using passive avoidance test in mice showed a significant [ $F(2, 42) = 39.65$ ;  $p = 0.001$ ] difference across the three phases of the study (Table 2). A significant ( $p < 0.05$ ) decrease in latency (s) was recorded across the three phases in: normal saline; phases 2 ( $13.00 \pm 27.54$ ) and 3 ( $27.25 \pm 46.82$ ), CMC; phases 2 ( $22.00 \pm 31.80$ ) and 3 ( $2.67 \pm 54.06$ ), EE; phases 2 ( $39.00 \pm 31.80$ ) and 3 ( $20.00 \pm 54.06$ ) treatment groups, when compared to the mean scores of their respective phase 1 (base-line). A significant decrease in latency (s) was also recorded in  $\text{AlCl}_3$ ; phase 3 ( $13.33 \pm 54.06$ ), EE + resveratrol; phase 2 ( $6.75 \pm 27.54$ );  $\text{AlCl}_3$  + resveratrol; phases 2 ( $155.67 \pm 31.80$ ) and 3 ( $124.00 \pm 54.06$ );  $\text{AlCl}_3$  + EE; phase 2 ( $5.00 \pm 31.80$ ), and  $\text{AlCl}_3$  + EE + resveratrol; phase 2 ( $67.75 \pm 27.54$ ) treatment groups, when compared to the mean scores of their respective phase 1 (base-line). However, significant decrease in latency (s)

was also observed in  $\text{AlCl}_3$ -treatment group in phase 3 ( $13.33 \pm 54.06$ ), when compared to phase 2 ( $235.33 \pm 31.80$ ), EE + resveratrol in phase 2 ( $6.75 \pm 27.54$ ), when compared to phase 3 ( $172.25 \pm 46.82$ ); and  $\text{AlCl}_3$  + EE in phase 2 ( $5.00 \pm 31.80$ ), compared to phase 3 ( $208.33 \pm 54.06$ ), respectively.

A significant difference was observed between groups: [ $F(8, 21) = 3.7$ ;  $p = 0.01$ ], in phases 1 and 2 of the study. The latency(s) in resveratrol group was lower ( $144.67 \pm 42.22$ ), when compared to that of  $\text{AlCl}_3$  + EE + resveratrol ( $251.25 \pm 36.57$ ) group at base-line (phase 1). In phase 2 of the study, a significant decrease in latency (s) was recorded in the control ( $13.00 \pm 27.54$ ), CMC ( $22.00 \pm 31.80$ ) EE ( $39.00 \pm 31.80$ ), EE + Resveratrol ( $6.75 \pm 27.54$ ),  $\text{AlCl}_3$  + EE ( $5.00 \pm 31.80$ ) and  $\text{AlCl}_3$  + EE + resveratrol ( $67.75 \pm 27.54$ ) treatment groups, compared to resveratrol ( $217.00 \pm 31.80$ ) or  $\text{AlCl}_3$  ( $235.33 \pm 31.80$ ) groups, respectively.

A significant interaction between groups and time was observed in latency (s) in the study: [ $F(16, 42) = 3.29$ ;  $p = 0.001$ ]. Using Bonferroni *post-hoc* test, a significant increase ( $p < 0.001$ ) was obtained in latency (s) of  $\text{AlCl}_3$  ( $235.33 \pm 31.80$ ) group in phase 2, when compared to normal saline ( $13.00 \pm 27.54$ ), CMC ( $22.00 \pm 31.80$ ), EE ( $39.00 \pm 31.80$ ), EE + Resveratrol ( $6.75 \pm 27.54$ ),  $\text{AlCl}_3$  + EE ( $5.00 \pm 31.80$ ) and  $\text{AlCl}_3$  + EE + resveratrol ( $67.75 \pm 27.54$ ) treatment groups, respectively. The latency (s) of  $\text{AlCl}_3$  decreased over time in phase 3 ( $13.33 \pm 54.06$ ).

**Table 1:**

Effect of resveratrol and environmental enrichment on acquisition error in aluminium chloride-induced cognitive deficits using passive avoidance test

| Groups                             | Phase 1 (Base-line) | Phase 2 (Four weeks) | Phase 3 (Eight weeks) |
|------------------------------------|---------------------|----------------------|-----------------------|
| Normal Saline                      | $2.75 \pm 0.55$     | $5.00 \pm 1.23$      | $3.25 \pm 0.85$       |
| CMC                                | $1.67 \pm 0.64$     | $3.33 \pm 1.42$      | $3.33 \pm 0.98$       |
| Resveratrol                        | $2.33 \pm 0.64$     | $4.33 \pm 1.42$      | $3.67 \pm 0.98$       |
| EE                                 | $3.67 \pm 0.64$     | $5.33 \pm 1.42$      | $2.33 \pm 0.98$       |
| $\text{AlCl}_3$                    | $1.67 \pm 0.64$     | $2.00 \pm 1.42$      | $1.33 \pm 0.98$       |
| EE + Resveratrol                   | $2.00 \pm 0.55$     | $4.75 \pm 1.23$      | $2.50 \pm 0.85$       |
| $\text{AlCl}_3$ + Resveratrol      | $1.33 \pm 0.64$     | $2.00 \pm 1.42$      | $2.00 \pm 0.98$       |
| $\text{AlCl}_3$ + EE               | $1.67 \pm 0.64$     | $6.33 \pm 1.42^*$    | $2.33 \pm 0.98$       |
| $\text{AlCl}_3$ + EE + Resveratrol | $1.75 \pm 0.55$     | $2.00 \pm 1.23$      | $2.50 \pm 0.85$       |

\* Indicate significance ( $p < 0.05$ ) when compared to phase 1 (base-line). CMC = Carboxymethyl cellulose, EE = Environmental enrichment,  $\text{AlCl}_3$  = Aluminium chloride,  $n = 7$

**Table 2:**

Effect of resveratrol and environmental enrichment on aluminium chloride-memory deficits using passive avoidance test in mice

| Groups                             | Step-down latency (s)<br>Phase 1 (Base-line) | Step-down latency (s)<br>Phase 2 (Four weeks) | Step-down latency (s)<br>Phase 3 (Eight weeks) |
|------------------------------------|----------------------------------------------|-----------------------------------------------|------------------------------------------------|
| Normal Saline                      | $247.75 \pm 36.57$                           | $13.00 \pm 27.54^{*,b,c}$                     | $27.25 \pm 46.82^*$                            |
| CMC                                | $180.00 \pm 42.22$                           | $22.00 \pm 31.80^{*,b,c}$                     | $2.67 \pm 54.06^*$                             |
| Resveratrol                        | $144.67 \pm 42.22^a$                         | $217.00 \pm 31.80$                            | $88.33 \pm 54.06$                              |
| EE                                 | $266.67 \pm 42.22$                           | $39.00 \pm 31.80^{*,b,c}$                     | $20.00 \pm 54.06^*$                            |
| $\text{AlCl}_3$                    | $297.67 \pm 42.22$                           | $235.33 \pm 31.80$                            | $13.33 \pm 54.06^{*#}$                         |
| EE + Resveratrol                   | $261.50 \pm 36.57$                           | $6.75 \pm 27.54^{*,b,c}$                      | $172.25 \pm 46.82$                             |
| $\text{AlCl}_3$ + Resveratrol      | $300.00 \pm 42.22$                           | $155.67 \pm 31.80^*$                          | $124.00 \pm 54.06^*$                           |
| $\text{AlCl}_3$ + EE               | $284.33 \pm 42.22$                           | $5.00 \pm 31.80^{*,b,c}$                      | $208.33 \pm 54.06$                             |
| $\text{AlCl}_3$ + EE + Resveratrol | $251.25 \pm 36.57$                           | $67.75 \pm 27.54^{*,b,c}$                     | $191.75 \pm 46.82$                             |

\* Indicates significant ( $p < 0.05$ ) difference when compared to phase 1 (base-line), # Indicates significant ( $p < 0.05$ ) difference compared to phase 2, + Indicates significant ( $p < 0.05$ ) difference compared to phase 3, <sup>a, b and c</sup> Indicates significant ( $p < 0.05$ ) difference compared to  $\text{AlCl}_3$  + EE + resveratrol, Resveratrol and  $\text{AlCl}_3$  treatment groups, respectively. CMC = Carboxymethyl cellulose, EE = Environmental enrichment,  $\text{AlCl}_3$  = Aluminium chloride, s = seconds,  $n = 7$

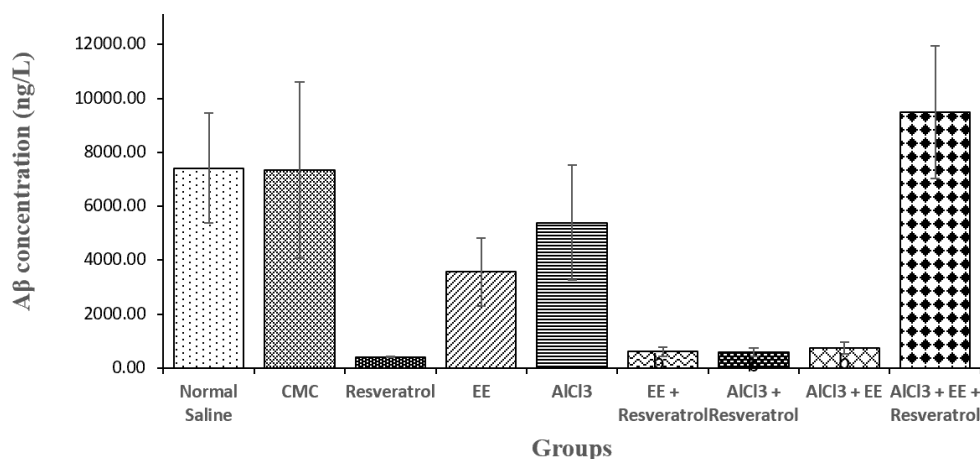


**Figure 1:** Effect of resveratrol and environmental enrichment on amyloid-beta concentration in aluminium chloride model of Alzheimer's disease.

<sup>b</sup> Indicate significant ( $p < 0.05$ ) difference, when compared to AlCl<sub>3</sub> + EE + Resveratrol. CMC = Carboxymethyl cellulose, EE = Environmental enrichment, AlCl<sub>3</sub> = Aluminium chloride,  $n = 4$

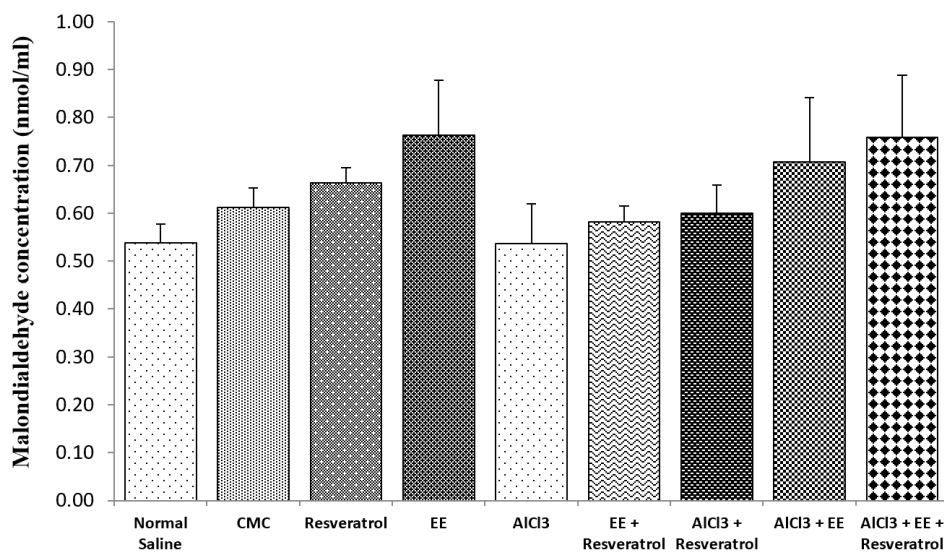


**Figure 2:** Effect of resveratrol and environmental enrichment on malondialdehyde concentration in aluminium chloride model of Alzheimer's disease in mice. AD = Alzheimer's disease, CMC = Carboxymethyl cellulose, EE = Environmental enrichment, AlCl<sub>3</sub> = Aluminium chloride, MDA = Malondialdehyde,  $n = 4$

### Determination of Amyloid beta Peptide (Aβ)

**Concentration:** Results of Aβ peptide concentration (ng/L) obtained during the study are represented in Figure 1. There was no significant difference in concentration (ng/L) of Aβ protein across all the treatment groups, when compared to the control (normal saline). However, a significant ( $p < 0.05$ ) decrease was observed in concentration (ng/L) of Aβ protein in resveratrol ( $412.35 \pm 25.84$ ), EE + resveratrol ( $617.35 \pm 171.38$ ), AlCl<sub>3</sub> + resveratrol ( $590.30 \pm 150.75$ ) and AlCl<sub>3</sub> + EE + Resveratrol ( $9495.00 \pm 2462.40$ ) groups, respectively, [ $F(8, 21) = 4.71$ ;  $p = 0.02$ ].

### Assessment of oxidative stress biomarkers in aluminium chloride model of Alzheimer's disease

#### Determination of malondialdehyde (MDA)

**concentration:** Results of MDA concentration (nmol/ml) obtained during the study are represented in Figure 2. There was no significant difference in MDA concentration (nmol/ml) in all the treatment groups, when compared to the controls, [ $F(8, 24) = 0.86$ ;  $p = 0.56$ ].

#### Determination of nuclear factor erythroid 2-related factor 2 (Nrf2) concentration:

Results of Nrf2

concentration (ng/ml) obtained during the study are represented in Figure 3. There was a significant [ $F(8, 23) = 4.59$ ;  $p = 0.002$ ] difference in concentration (ng/ml) of Nrf2 in Resveratrol ( $5.62 \pm 0.31$ ), AlCl<sub>3</sub> + Resveratrol ( $5.92 \pm 0.53$ ) and AlCl<sub>3</sub> + EE ( $6.96 \pm 0.31$ ) groups, compared to the controls ( $13.24 \pm 0.72$ ). The concentrations (ng/ml) of Nrf2 in Resveratrol ( $5.62 \pm 0.31$ ) and AlCl<sub>3</sub> + Resveratrol ( $5.92 \pm 0.53$ ) groups were lower ( $P < 0.05$ ), when compared to that of AlCl<sub>3</sub> + EE + Resveratrol ( $12.61 \pm 0.78$ ).

### DISCUSSION

The result of the study demonstrated that resveratrol and environmental enrichment modulate memory and neurochemical changes in AlCl<sub>3</sub> model of AD in mice.

The results of the current study agreed with the findings that environmental stimuli such as physical exercise, pollutants, life-style, chemicals, pesticides, nutrition, physical stress, behavioural stress and exposure to metals such as aluminium affect the normal inherited methylome throughout the life-span of an organism, leading to either healthy or diseased ageing (Nicolia et al., 2015). The negative impacts of these stressors such as Al toxicity have been shown to lead to diseased ageing by altering cellular and molecular mechanisms through gene activation and silencing.

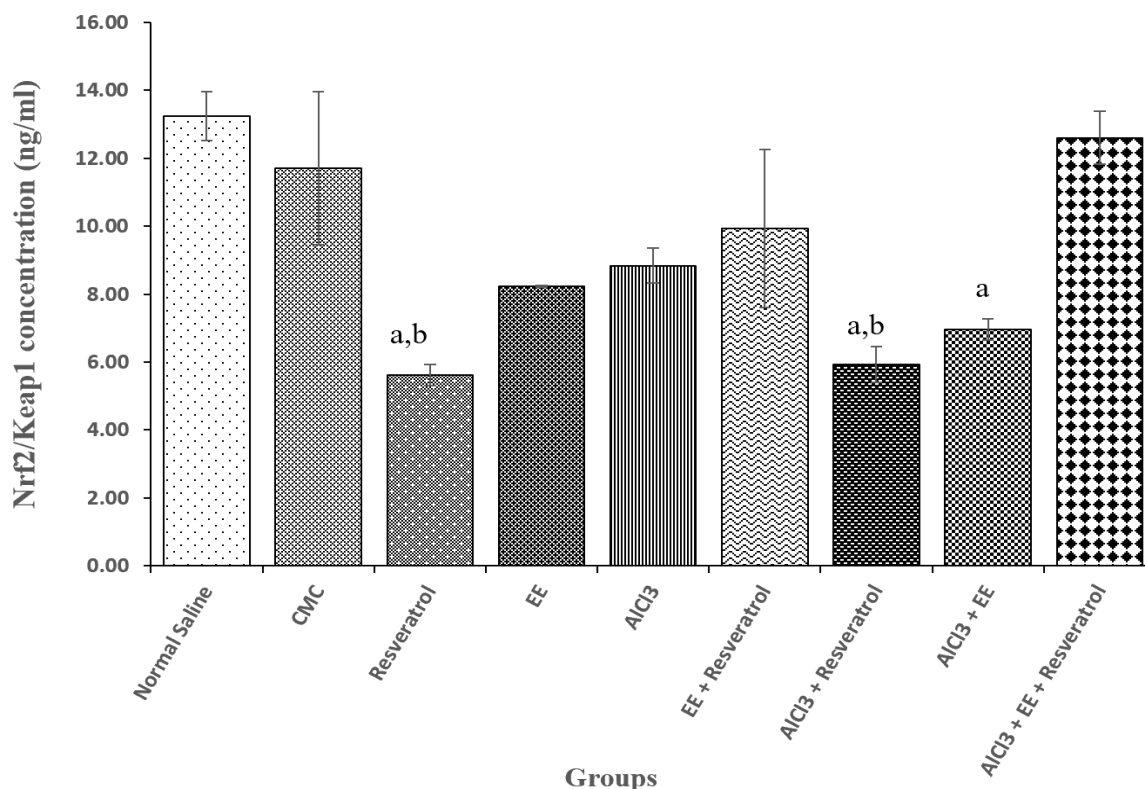


**Figure 3:**

Effect of resveratrol and environmental enrichment on nuclear factor erythroid 2-related factor 2 concentration aluminium chloride model of AD in mice

<sup>a</sup> and <sup>b</sup> = significant ( $p < 0.05$ ) difference, when compared to the control and  $\text{AlCl}_3 + \text{EE} + \text{Resveratrol}$ , respectively. AD = Alzheimer's disease, CMC = Carboxymethyl cellulose, EE = Environmental enrichment,  $\text{AlCl}_3$  = Aluminium chloride, Nrf2/keap-1 = Nuclear factor erythroid 2-related factor 2/keap-1,  $n = 4$

The impacts eventually lead to the manifestation of various aged-related disorders such as Alzheimer's disease. Environmental enrichment and dietary supplement of resveratrol have been shown to act as epigenetic factors that regulate gene activation and silencing (Kawano et al., 2015; Nicolai et al., 2015). Experimental models have unveiled numerous pathways; thus, providing novel insights into the mechanisms of their epigenetic control, especially in pathological conditions. Therefore, modulation of neurobehavioral and cellular responses obtained in the present study by resveratrol and EE may be of prophylactic and therapeutic potentials in the management of AD.

The significant increase observed in acquisition error in  $\text{AlCl}_3 + \text{EE}$  group in the fourth week of the study, when compared to the mean base-line score demonstrated the effect of time on learning process. The significant decrease in latency (memory) of the animals to stay on the safe platform over time across the various groups using the passive avoidance test indicated the effect of time and ageing on cognitive functions. However, in EE + resveratrol and  $\text{AlCl}_3 + \text{EE}$  treatment groups, the significant decrease in latency observed in at the fourth week, compared to the base-line was followed by significant increase in latency at the eighth week of the study. This finding may be attributed to improvement in cognitive function, elicited by both resveratrol and EE. This finding was further corroborated by significant decrease in  $\text{A}\beta$  concentration in the same treatment groups. Both treatments have been reported to improve cognitive functions in ageing and animal models of AD by reducing oxidative stress, neuroinflammation, and CREB pathway; thereby decreasing the levels of  $\text{A}\beta$ -

precursor protein and promoting the synthesis of brain derived neurotrophic factor, consequently leading to improved memory and learning (Kawano et al., 2015; Yazir et al., 2015; Grinan-Ferre et al., 2015; Zhang et al., 2016).

The significant decrease in latency of the animals to stay on the safe platform observed in the resveratrol group, when compared to  $\text{AlCl}_3 + \text{EE} + \text{resveratrol}$  group at base-line, could be as a result of behavioural variation due to anxiety of animals exposed to novel environment. Although the animals were well acclimatised to the passive avoidance test apparatus prior to the commencement of the experiment, anxiety may play a role in the decline in latency observed in the resveratrol group, compared to  $\text{AlCl}_3 + \text{EE} + \text{resveratrol}$  treatment group at base-line, as animals responses to novelty differ. After four weeks of the study (phase 2), a significant decrease in latency of the animals to stay on the platform was observed in the controls, CMC, EE, EE + Resveratrol,  $\text{AlCl}_3 + \text{EE}$  and  $\text{AlCl}_3 + \text{EE} + \text{resveratrol}$  groups, compared to resveratrol and  $\text{AlCl}_3$  groups, respectively. The significant increase in latency observed in the resveratrol group, compared to the various treatment groups may be due to its ability to improve cognitive functions, which was further corroborated by significant decrease in  $\text{A}\beta$  concentration in the same treatment group. Resveratrol has been reported to improve memory and learning in humans and animal models of AD by activating sirt-1-dependent mechanism, increasing hippocampal production of insulin-like growth factor-1 via sensory neurone stimulation, reducing cellular level of iNOS and lipid peroxidation by increasing haeme oxygenase-1 (HO-1) production and reducing the expressions of miR-134 and miR-124, thus in

turn up-regulating CREB levels, thereby promoting BDNF synthesis and decreasing A $\beta$  concentration (Harada et al., 2011; Huang et al., 2011; Zhao et al., 2013; Yazir et al., 2015). These are possible mechanisms via which resveratrol improves memory and learning as observed in the present study.

The significant increase in latency of the animals to stay on the platform in the AlCl<sub>3</sub> group, observed in the fourth week of the study, decreased over time after the eighth week of the study period (phase 3). This demonstrated a cognitive decline induced by AlCl<sub>3</sub> at the eighth week and further shows that, the increased latency observed over time was transient in the AlCl<sub>3</sub> treatment group at the fourth week, indicating a significant interaction between group and time in the present study.

Results of A $\beta$  peptide concentration obtained during the study showed no significant difference in concentration of A $\beta$  protein across all the groups, when compared to the control (normal saline) group. However, a significant decrease in concentration of A $\beta$  protein was observed in resveratrol, EE + resveratrol, AlCl<sub>3</sub> + resveratrol and AlCl<sub>3</sub> + EE treatment groups, compared to AlCl<sub>3</sub> + EE + Resveratrol treatment group. The increase in concentration of A $\beta$  protein observed in AlCl<sub>3</sub> + EE + Resveratrol implies that the combined treatment of resveratrol and EE with AlCl<sub>3</sub> did not decrease A $\beta$  protein concentration, rather it increased the value far beyond the value obtained in the AlCl<sub>3</sub> treatment group.

Available data suggest that both caloric restriction and EE exert similar beneficial effects on neurones in the brain by inducing a mild stress, thereby sending signals that increase the production of cell survival-promoting molecules, including growth factors (insulin like growth factors, brain derived neurotrophic factors, and nerve derived neurotrophic factor), neurotransmitters (glutamate, serotonin), protein chaperones (heat-shock proteins 70 and glucose-regulated protein 78), antioxidants (superoxide dismutase, reduced glutathione), increased calcium influx and gene expression and enhanced energy metabolism (electron-transport chain and glycolysis). These factors consequently lead to enhanced cognitive function, disease resistance, increased insulin sensitivity and improved lipid metabolism (Mattson et al., 2001; Bordone and Guarente, 2005; Mattson et al., 2005; Sinclair, 2005). Resveratrol acting as an established CR mimetic, could act via similar mechanism to elicit its beneficial role in AD (Markus and Morris, 2008). However, the combination of resveratrol and EE in AlCl<sub>3</sub> model of AD may reverse the beneficial role of the individual treatment by exaggerating the beneficial mild stress response, elicited by the individual treatments in the AD model. This may lead to oxidative stress that consequently increased A $\beta$  protein production as observed in the AlCl<sub>3</sub> + EE + Resveratrol group. Decreasing the dose of resveratrol from below 200 mg/kg/body weight may result in better response in the combined treatment.

The results of oxidative stress biomarkers obtained from the study showed that there was no significant difference in MDA concentrations in all the groups, when compared to the controls. However, the Nrf2 concentrations obtained during the study showed significant decrease in the concentration in Resveratrol, AlCl<sub>3</sub> + Resveratrol and AlCl<sub>3</sub> + EE groups, compared to the controls. This finding showed that both resveratrol and EE did not to up-regulate

the concentration of Nrf2 independently and in combination with AlCl<sub>3</sub> above those of the controls, and AlCl<sub>3</sub> group. This is in disagreement with the findings of Tamaki et al. (2014) and that of Yang et al. (2015), who reported an increase in Nrf2 protein level of rats exposed to chronic cerebral hypo-perfusion and periodontitis by EE and resveratrol, respectively, thereby improving the antioxidant status of the animals. Both resveratrol and EE may probably exert their beneficial effect via alternate pathway in the present study, improving oxidative status by either increasing the synthesis of high molecular weight endogenous antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase enzymes or constitutive nitric oxide which has unpaired electron in its outermost shell thereby making it more attractive to ROS and sparing Nrf2 for other scavenging roles (Das and Maulik, 2006).

The increase in Nrf2 concentration observed in AlCl<sub>3</sub> + EE + Resveratrol compared to Resveratrol and AlCl<sub>3</sub> + Resveratrol could be adaptive in order to contain the oxidative stress-induced neuropathology. The increase, apparently, resulted from increased MDA concentration observed in the same group, with a trend towards significance, which consequently led to increase in A $\beta$  concentration. This result demonstrated the synergistic role of resveratrol and EE in up-regulating Nrf2 concentration to convert the increased oxidative stress level and A $\beta$  protein concentration, thereby suggesting the antioxidant role of the combined treatments in AlCl<sub>3</sub> model of AD. This cascade could be via activation of SIRT1-dependent mechanism as both resveratrol and treadmill exercise, which is an important component of EE, were reported to increase the level of Nrf2 via SIRT1-dependent mechanism (Tamaki et al., 2014; Koo et al., 2017).

In conclusion and based on the results of this study, it was concluded that time significantly influenced memory in the various groups at the fourth week of the study. Resveratrol administration improved contextual fear memory. Resveratrol and EE treatments alone significantly lowered A $\beta$  concentration over the combined treatment in AlCl<sub>3</sub> model of AD. Combined treatment of resveratrol and EE enhanced the antioxidant status by upregulating Nrf2 concentration in AlCl<sub>3</sub> model of AD.

### Acknowledgments

The authors would like to acknowledge Mr. Tahir Mohammed of the DNA laboratory, Kaduna State, Nigeria for his contribution in the biochemical analyses.

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*Full-Length Research Article*

# Effect of Caffeine and Adrenaline on Memory and Anxiety in Male Wistar Rats

**Imam-Fulani A.O. and Owoyele B.V.**

*Department of Physiology, College of Health Sciences, University of Ilorin, Ilorin, Nigeria*

**Summary:** The present study was designed to investigate the effects of caffeine and adrenaline administration on memory and anxiety in male rats. Rats weighing about 140-200g were used for the study. They were divided into three groups (4 animals per group). Study groups 1; a,b,c,d were healthy rats administered normal saline, 5,10 and 15mg/kg bw caffeine intraperitoneally (i.p.), respectively for 6 weeks. Study groups 2; a,b,c,d administered normal saline, 0.1, 0.2 and 0.31mg/kg bw adrenaline (i.p.), respectively for 6 weeks. Study groups 3; a,b,c,d administered normal saline, 5mg/kg caffeine (i.p.) + 0.1mg/kg adrenaline (i.p.), 10mg/kg Caffeine (i.p.) + 0.2mg/kg Adrenaline (i.p.) and 15mg/kg Caffeine (i.p.) + 0.3mg/kg Adrenaline (i.p.) respectively for 6 weeks. The result showed no significant difference in spatial memory across all animals in study groups 1: b,c,d when compared to control (a). Study groups 2: (b, c) showed increase in spatial memory when compared to control (a). 2(d) showed a significant ( $p<0.05$ ) decrease. Study groups 3: b, c, d showed no significant difference in spatial memory when compared to control (a). Study groups 1: b, c showed significant ( $p<0.05$ ) reduction in duration for the short and long-term memory test when compared to control. Study groups 2 showed reduction in duration for both the long- and short-term memory test when compared to control. Study groups 3 no significant ( $p<0.05$ ) difference in short and long term memory test across all animals in the group. It was also observed that adrenaline enhanced short and long term memory and only high dose of adrenaline distorted spatial memory. Study groups 1; (b,c) showed significant ( $p<0.05$ ) increase in the number of entries to the open arm of the elevated plus maze when compared to control (a). Study groups 2; (b), showed significant ( $p<0.05$ ) increase in the frequencies of entries to the closed arm of the elevated plus maze when compared to control (a). Study groups 2; (d), showed a significant ( $p<0.05$ ) decrease in the frequency of entries to the open and closed arm when compared to control (a). The study revealed that co- administration of caffeine and adrenaline led to elevation of mood, increased activity and reduction of anxiety in Wistar rats. In addition, it was observed that only high dose of adrenaline increased anxiety. It was also observed that caffeine and adrenaline enhanced short and long term memory and only high dose of adrenaline distorted spatial memory.

**Keywords:** *adrenaline, anxiety, behavioural tests, caffeine, memory, Wistar rats.*

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\*Address for correspondence: E-mail: [faminat2@gmail.com](mailto:faminat2@gmail.com); Tel: +234-8032146355

Manuscript received- September- 2021; Accepted- March, 2022

DOI: <https://doi.org/10.54548/njps.v37i1.9>

## INTRODUCTION

Memory is a process which involves storage of information or experiences in the brain. Encoding information is one of the processes involved in remembering episodes (McDermott and Roediger, 2022) and allows information that is from the outside to get into the senses in the forms of chemical and physical stimuli. The process of memory consolidation is in stages: Sensory memory, which is the ability to see an item and remember what it looked like within few seconds of observation. Short term memory is a type of memory that allows recall for a period of several seconds to about a minute without rehearsal as seen in recalling an eleven-digit telephone number. Long-term memory can store much larger information for potentially unlimited time or duration, sometimes it takes a whole life span. The hippocampus is the major organ responsible for acquisition of new information and also for memory consolidation of information from short term to long-term memory. If there is a damage or problem with the hippocampus, the ability to make new information and

memories are not possible to be stored into long-term memory (Anand and Dhikav, 2012). Long-term memory can be categorised into declarative and non-declarative memories (Camina and Guell, 2017). Declarative memory requires conscious recall and it can be sub-divided into semantic memory and episodic memory. Semantic memory allows the encoding of abstract knowledge about the continents (Binder and Desai, 2011). Episodic memory is more for personal memories, such as the sensations, emotions, and personal associations with a particular place or time. Some factors e.g. odour can affect memory and can also be used to re-activate new memories in the brains of people when sleeping and the individuals would remember better when they are awake (Shanahan et al., 2018). Emotion is also a major link to recall. Interference can hamper memorisation and retrieval. Sometimes, retroactive interference can occur where learning new information leads to hard recall of old information and proactive interference, where previous learning interferes with the recall of new information (Susic-Vasic et al., 2018).

Caffeine (1,3, 7 trimethylxanthine), a bitter, white crystalline purine has been discovered as a CNS stimulant and also classified as an organic molecule called methylxanthine (Fiani et al., 2021). Caffeine counteracts adenosine that normally circulates at high levels throughout the body and especially in the nervous system. Adenosine acts as an inhibitory neurotransmitter that suppresses activity in the central nervous system. It produces a certain alertness of short duration (Camargo and Camargo, 2019), increases wakefulness, focus, and better general body coordination. Low doses (20±200 mg) produce positive feelings (e.g. heightened energy, ability to concentrate, liking (Lieberman et al., 1987), while higher doses have been associated with feelings of severe anxiety, nervousness and agitations which can increase in severity with dose (Walter, 2022). Herz (1999) reported that psychoactive dose of caffeine does not impair or enhance memory, even though it produced a significant mood elevation in arousal. It has been proposed that arousal elevation sufficient to alter memory encoding must impact on  $\beta$ -adrenergic systems (Cahill and McGaugh, 1998) and 5 mg/kg caffeine may not have produced this effect.

Adrenaline, also called epinephrine, is a stress hormone produced from the adrenal glands of the kidneys and in some neurons of the central nervous system. It helps in preparing the body for a fight-or-flight reaction in threatening environments. Evidence suggests that adrenergic hormones modulate the consolidation of long-term memory for experiences that induce their release. For example, post-training injection of adrenergic drugs or hormones enhances memory consolidation in many species (Segal et al., 2012; Villain et al., 2016; Schramm et al., 2016). Adrenergic hormones, released by emotional arousal modulate memory consolidation of the events (McGaugh, 2013). The relationship between the effects of adrenaline and the anxiety state is further emphasised by the emotive action of adrenaline (Richter, 1940).

This study was aimed at investigating the effect of caffeine and adrenaline on memory and anxiety in male Wistar rats. The objective of this study is to administer caffeine and adrenaline singly and in combination to evaluate its synergistic effects on spatial memory, long and short-term memory, and anxiety.

## MATERIALS AND METHODS

**Experimental Animals:** Male albino rats of Wistar strain weighing between 140-200g were obtained from the animal holding of the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria. The animals were fed on rats pellet (Premier Feed Limited, Apapa, Lagos, Nigeria) and water *ad libitum*. All animals were maintained under standard laboratory conditions of temperature (22±2°C) and natural photoperiod of about 12h light: dark cycle with relative humidity of 40-50%. Ethical approval was obtained from the University of Ilorin Ethical Review Committee with ethical approval number (UERC/ASN/2016/487).

**Study design:** The rats were grouped into study groups A, B and C. Study groups 1; a,b,c,d were healthy rats administered normal saline, 5,10,15mg/kg bw caffeine intraperitoneally (i.p.), respectively for 6 weeks. Study

groups 2; a,b,c,d administered normal saline, 0.1, 0.2, 0.31mg/kg bw adrenaline (i.p.), respectively for 6 weeks. Study groups 3; a,b,c,d administered normal saline, 5mg/kg caffeine (i.p.) + 0.1mg/kg adrenaline (i.p.), 10mg/kg Caffeine (i.p.) + 0.2mg/kg Adrenaline (i.p.), 15mg/kg Caffeine (i.p.) + 0.3mg/kg Adrenaline (i.p.) respectively for 6 weeks. At the end of six weeks of administration, the animals were subjected to series of behavioural tests which include spatial learning and memory and anxiety.

**Spatial learning and memory test using morris water maze:** The methods of Morris (1984) and D'Hooge, and De Deyn (2001) were used for this study. The animals were subjected to 5days training in the Morris water maze prior to the learning test and memory test. During this period, 10cm platform was visible i.e. 1cm above the water level in the maze. This was followed by the learning and memory test at the end of the drug administration where the hidden platform was 1cm below the water.

Each rat was placed in the Morris water maze at a designated start point of a quadrant and allowed to swim freely to locate the hidden platform within a maximum duration of 1minute.

**Anxiety test using elevated plus maze:** Following the drug administration, animals were placed in the central platform facing the closed arm and their behaviour recorded for 5 min. based upon the study by Montgomery, (1958). The duration of time spent in the arms was calculated as time in open arms or closed arm, the number of entries into the arms was calculated as number of entries into open or closed arms/total number of entries.

**Spatial memory test using y-maze:** Each rat was placed in the centre of the arm compartments and was allowed to move freely until its tail completely enters a particular arm. The sequence of arm entries was manually recorded, the arms being labelled A, B, or C. An alternation is defined as entry into all three arms consecutively, for instance if the animal makes the following arm entries; A,C,B,C,A,B,C,A,C,A,B,C,A, in this example, the animal made 13 arm entries 8 of which are correct alternations. The number of maximum spontaneous alternations is the total number of arms entered minus two, and the percentage alternation is calculated as {(actual alternations /maximum alternations) x 100}. For each animal the Y-maze testing was carried out for 5 minutes. The apparatus was cleaned with 5% alcohol and allowed to dry between sessions (Onaolapo et al., 2012).

**Open field test:** The procedure followed the method of Danenberg, (1969). Each rat was placed in the center of the open field maze and allowed to move freely for the duration of 5minutes. Each line crossing frequency, centre square frequency and center square duration was recorded. Rearing frequency was also recorded. Time spent moving, cleaning/grooming was also noted.

## Statistical analysis

Results obtained are expressed as the mean ± standard error of mean (SEM). The test for significance was done using one-way ANOVA, Student's t-test and Duncan *post-hoc* test. Differences were considered statistically significant at

$p < 0.05$ . Data analysis was performed using statistical packages for social sciences (SPSS) version 16.

## RESULTS

### Memory test

#### Y-maze test

**Effect of caffeine administration on spatial memory in male Wistar rats:** There was no significant difference across all animals administered caffeine when compared to control group in the spatial memory test using Y-Maze as indicated in Figure 1

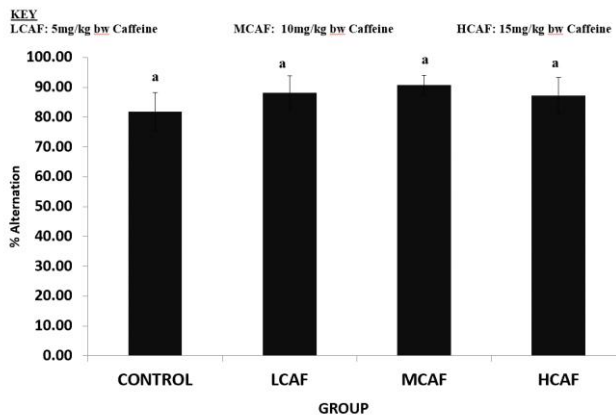


**Figure 1:** Effect of caffeine administration on spatial memory in male Wistar rats. Bars with different letters are significantly ( $p < 0.05$ ) different

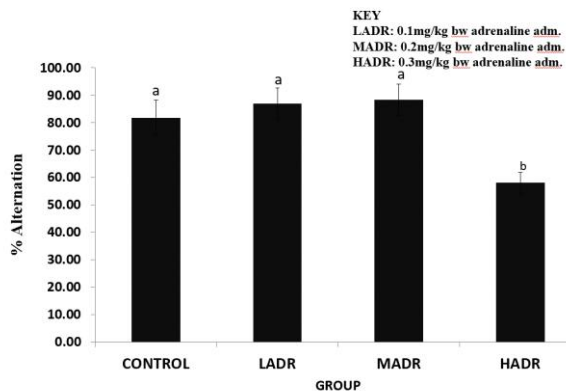


**Figure 2:**

Effect of adrenaline administration on spatial memory in male Wistar rats. Bars with different letters are significantly ( $p < 0.05$ ) different.

**Effect of adrenaline administration on spatial memory in male Wistar rats:** Only high dose of caffeine showed significant decrease in the measure of spatial memory (correct alternation (%)) in Y-Maze) when compared to control, other animals showed no significant difference as shown in figure 2.

**Effect of combined administration of caffeine and adrenaline on spatial memory in male Wistar rats:** There was no significant difference across all the caffeine and adrenaline co-administered animals when compared to control (Figure 3).

#### Morris water maze

**Effect of caffeine administration on short and long-term memory of male Wistar rats:** Caffeine animals (10 and 15mg/kg bw) showed significant ( $p < 0.05$ ) reduction in duration for the short and long term memory test when compared to control. Low dose of caffeine (5mg/kg bw) only showed significant ( $p < 0.05$ ) reduction in duration for long term memory test when compared to control as shown in Figure 4.

**Effect of adrenaline administration on short and long-term memory of male Wistar rats:** All doses of adrenaline group showed reduction in duration for both the long and short term memory test when compared to control as shown in Figure 5.

**Effect of combined administration of caffeine and adrenaline on short and long-term memory of male Wistar rats:** No significant ( $p < 0.05$ ) difference in short and long term memory test across the animals as shown in Figure 6.

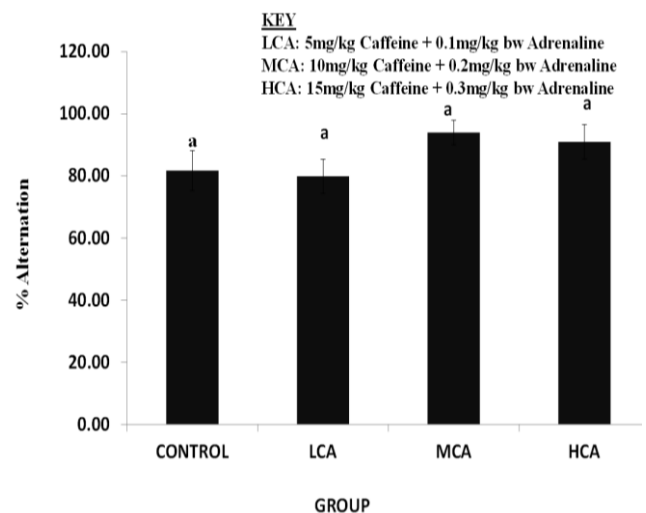


**Figure 3:** Effect of combined administration of caffeine and adrenaline on spatial memory in male Wistar rats. Bars with different letters are significantly ( $p < 0.05$ ) different.

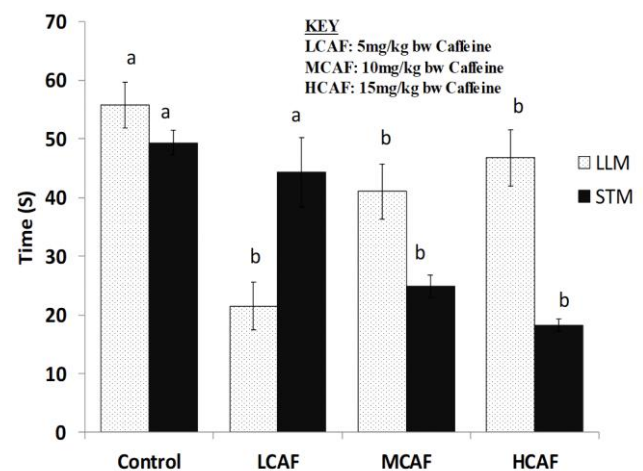


**Figure 4:** Effect of caffeine administration on short and long-term memory of male Wistar rats. Bars with different letters are significantly ( $p < 0.05$ ) different.

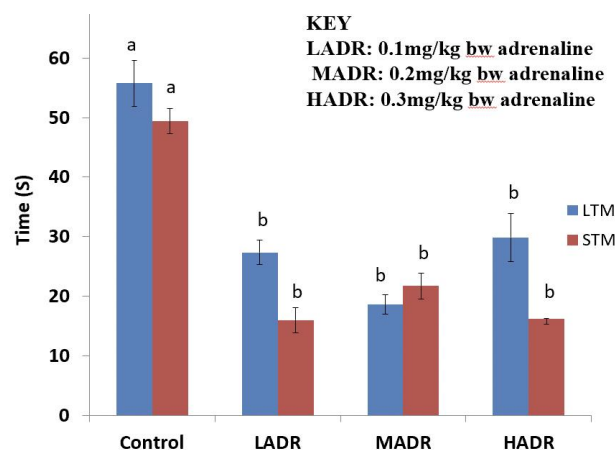


**Figure 5:** Effect of adrenaline administration on short and long-term memory of male Wistar rats. Bars with different letters are significantly ( $p < 0.05$ ) different.

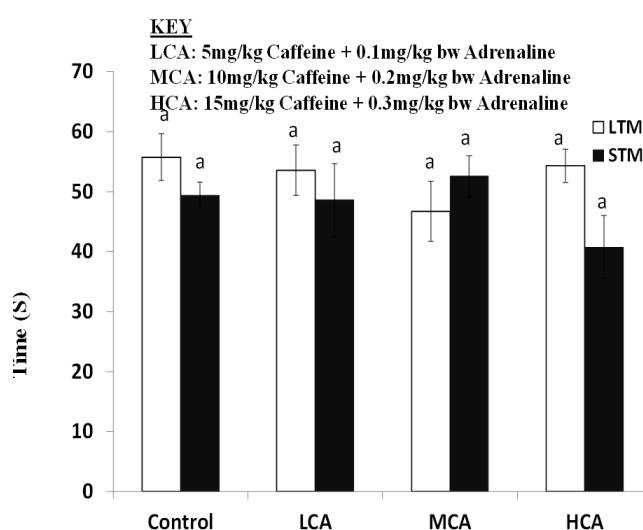


**Figure 6:** Effect of combined administration of caffeine and adrenaline on short and long-term memory of male Wistar rats. Bars with different letters are significantly ( $p < 0.05$ ) different.

### Anxiety Tests Using Elevated Plus Maze

#### Open and closed arm entry frequencies

**Effect of caffeine administration on anxiety of male Wistar rats:** Animals administered caffeine 5mg/kg bw and 10mg/kg bw showed significant ( $p < 0.05$ ) increase in the number of entries to the open arm of the elevated plus maze when compared to control as shown in Figure 7.

**Effect of adrenaline administration on anxiety of male Wistar rats:** Adrenaline group administered 0.1mg/kg bw showed significant ( $p < 0.05$ ) increase (LADR,  $3.88 \pm 0.611$ ) in the frequencies of entries to the closed arm of the elevated plus maze when compared to control with no significant ( $p < 0.05$ ) difference in the open arm entry frequency. Group administered 0.2mg/kg bw adrenaline showed no significant ( $p < 0.05$ ) difference in frequencies of entries to the open and closed arm when compared to control. However administered 0.3mg/kg bw adrenaline showed a significant ( $p < 0.05$ ) decrease in the frequency of entries to the open and

closed arm when compared to control, group as shown in Figure 8.

**Effect of combined administration of caffeine and adrenaline on anxiety of male rats:** Only the moderate dose of caffeine+adrenaline showed a significant ( $p < 0.05$ ) increase in the number of entries to the open arm and closed arm of the elevated plus maze when compared to control. Moderate and high dose caffeine + adrenaline showed no significant difference in number of entries to the open arm when compared to control as shown in Figure 9.

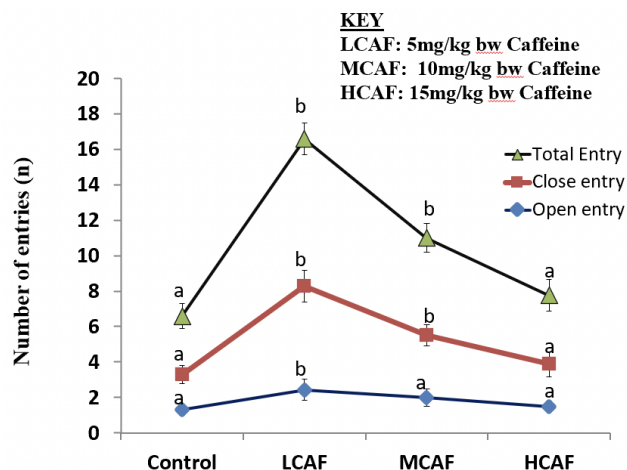


**Figure 7:** Effect of caffeine administration on anxiety of male Wistar rats. Groups with different letters are significantly ( $p < 0.05$ ) different.

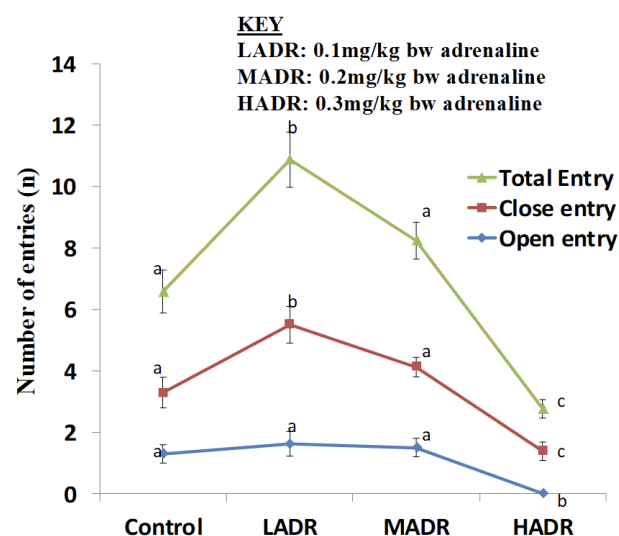
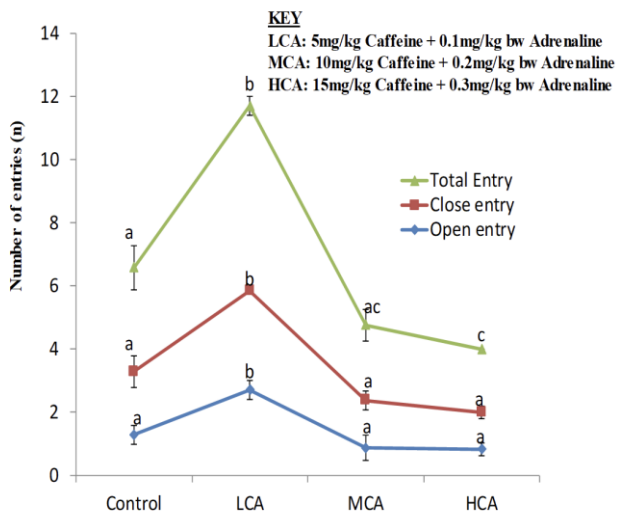


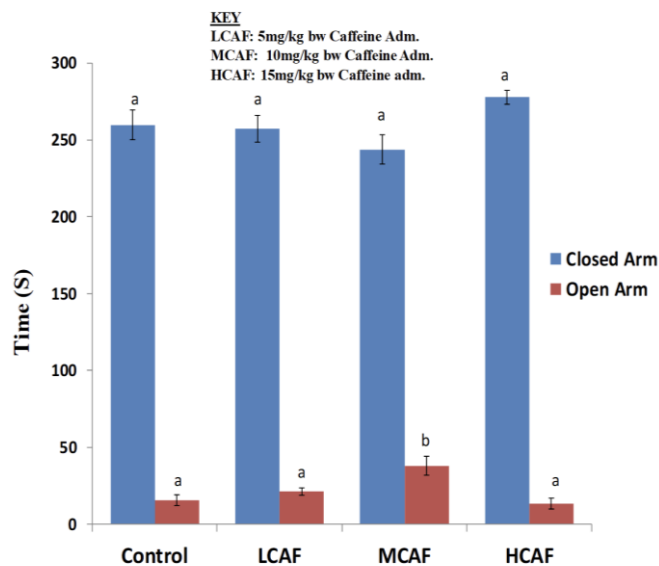
**Figure 8:** Effect of adrenaline administration on anxiety in male Wistar rats. Groups with different letters are significantly ( $p < 0.05$ ) different.

#### Opened and closed arm duration (seconds)

**Effect of caffeine administration on anxiety of male Wistar rats:** There was no significant ( $p < 0.05$ ) difference in the closed arm duration in all animals administered caffeine when compared to control. Moderate dose of caffeine showed significant ( $p < 0.05$ ) increase in open duration when compared control as shown in Figure 10.

**Figure 9:**

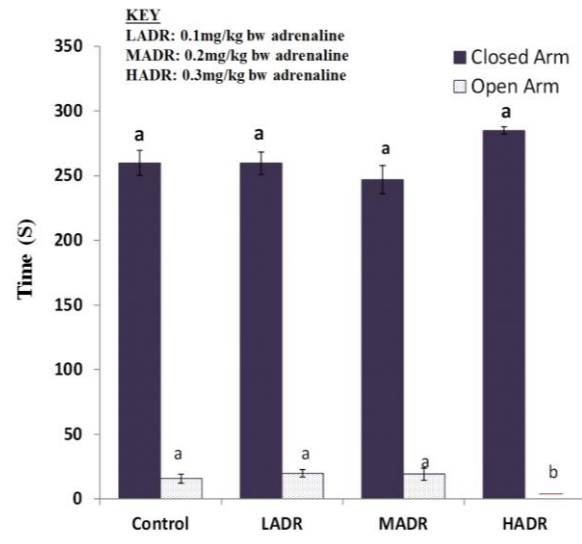
Effect of combined administration of caffeine and adrenaline on anxiety in male Wistar rats. Groups with different letters are significantly ( $p < 0.05$ ) different.

**Figure 10:**

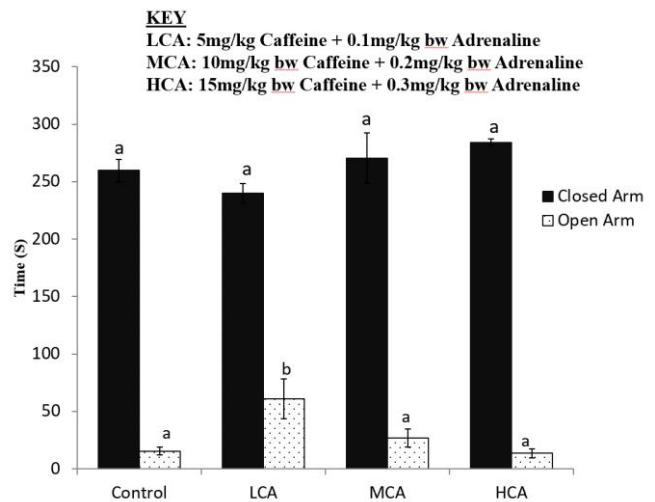
Effect of caffeine administration on anxiety of male Wistar rats. Bars with different letters are significantly ( $p < 0.05$ ) different.

**Effect of adrenaline administration on anxiety of male Wistar rats:** There was no significant ( $p < 0.05$ ) difference in the closed arm duration in animals administered adrenaline (0.1 and 0.2 mg/kg bw) when compared to control. Only group administered 0.3mg/kg bw adrenaline showed significant ( $p < 0.05$ ) reduction in open arm when compared to control as shown in Figure 11.

**Effect of combined administration of caffeine and adrenaline on anxiety of male Wistar rats:** There was no significant ( $p < 0.05$ ) difference in the closed arm duration in all animals co-administered caffeine and adrenaline when compared to control. Only low dose of caffeine and adrenaline showed significant ( $p < 0.05$ ) increase in open arm duration when compared to control as shown in Figure 12.

**Figure 11:**

Effect of adrenaline administration on anxiety of male Wistar rats. Bars with different letters are significantly ( $p < 0.05$ ) different.

**Figure 12:**

Effect of combined administration of caffeine and adrenaline on anxiety of male Wistar rats. Bars with different letters are significantly ( $p < 0.05$ ) different.

### Anxiety test using open field maze

#### Effect of caffeine, adrenaline and their combination on rearing frequency in the open field of male Wistar rats:

There was no significant ( $p < 0.05$ ) difference in rearing frequency of animals administered 15mg/kg bw caffeine and 0.3mg/kg bw adrenaline when compared to control, all other animals showed significant ( $p < 0.05$ ) increase in rearing frequency when compared to control as indicated in Table 1.

#### Effect of caffeine, adrenaline and their combination on grooming duration (S) in the open field of male Wistar rats:

There was significant ( $p < 0.05$ ) reduction in grooming time of animals administered 0.1 and 0.2mg/kg bw adrenaline when compared to the control. Other animals showed no significant ( $p < 0.05$ ) difference when compared to control (Table 1).

**Table 1:**

Effect of caffeine, adrenaline and their combination on rearing, grooming, center duration and movement time in the open field maze

| Animals | Rearing Frequency        | Grooming Duration (s)     | Center duration (s)     | Edge duration (s)         | Active movement time (s)  |
|---------|--------------------------|---------------------------|-------------------------|---------------------------|---------------------------|
| CONTROL | 7.29±1.36 <sup>a</sup>   | 30.71±2.12 <sup>ac</sup>  | 5.14±1.10 <sup>a</sup>  | 293.57±0.81 <sup>a</sup>  | 105.57±5.34 <sup>a</sup>  |
| LCAF    | 14.86±1.55 <sup>c</sup>  | 24.57±2.43 <sup>ab</sup>  | 9.14±1.05 <sup>b</sup>  | 291.14±1.18 <sup>bc</sup> | 118.29±3.08 <sup>b</sup>  |
| MCAF    | 15.43±1.11 <sup>c</sup>  | 26.43±1.84 <sup>abc</sup> | 10.42±0.48 <sup>b</sup> | 289.85±0.67 <sup>c</sup>  | 155.00±2.79 <sup>e</sup>  |
| HCAF    | 9.57±1.04 <sup>ab</sup>  | 24.71±1.98 <sup>ab</sup>  | 6.71±0.57 <sup>a</sup>  | 293.29±0.57 <sup>ab</sup> | 143.14±2.69 <sup>cd</sup> |
| LADR    | 12.57±0.84 <sup>bc</sup> | 18.00±1.73 <sup>d</sup>   | 10.14±0.51 <sup>b</sup> | 289.85±0.51 <sup>c</sup>  | 151.86±2.37 <sup>d</sup>  |
| MADR    | 13.86±0.94 <sup>c</sup>  | 22.00±2.31 <sup>bd</sup>  | 10.14±0.46 <sup>b</sup> | 289.86±0.46 <sup>c</sup>  | 157.85±4.21 <sup>e</sup>  |
| HADR    | 9.86±0.51 <sup>ab</sup>  | 24.57±2.30 <sup>ab</sup>  | 6.57±0.65 <sup>a</sup>  | 293.43±0.65 <sup>a</sup>  | 113.57±2.11 <sup>ab</sup> |
| LCA     | 12.14±0.60 <sup>bc</sup> | 30.86±2.05 <sup>ac</sup>  | 5.71±0.87 <sup>a</sup>  | 294.89±0.87 <sup>a</sup>  | 135.86±4.15 <sup>c</sup>  |
| MCA     | 20.42±2.34 <sup>d</sup>  | 31.43±1.60 <sup>c</sup>   | 11.00±0.87 <sup>b</sup> | 289.00±0.87 <sup>c</sup>  | 138.57±2.31 <sup>c</sup>  |
| HCA     | 11.57±0.75 <sup>bc</sup> | 26.14±2.20 <sup>abc</sup> | 5.86±0.80 <sup>a</sup>  | 294.14±0.33 <sup>a</sup>  | 109.29±4.49 <sup>ab</sup> |

Animals with different letters are significantly different. LCAF=5mg/kg caffeine, MCAF=10mg/kg caffeine, HCAF=15mg/kg caffeine, LADR=0.1mg/kg adrenaline, MADR=0.2mg/kg adrenaline, HADR=0.3mg/kg adrenaline, LCA=5mg/kg caffeine + 0.1mg/kg adrenaline, MCA=10mg/kg caffeine + 0.2mg/kg adrenaline, HCA=15mg/kg caffeine + 0.3mg/kg adrenaline

### Effect of caffeine, adrenaline and their combination on center area duration (S) in the open field of male Wistar rats:

There was no significant ( $p < 0.05$ ) difference in center time duration of animals administered 15mg/kg bw caffeine, 0.3mg/kg bw adrenaline when compared to control. Also no significant ( $p < 0.05$ ) difference in center time duration of animals administered 5mg/kg bw caffeine + 0.1mg/kg bw adrenaline and 15mg/kg bw caffeine + 0.3mg/kg bw adrenaline (Table 1).

### Effect of caffeine, adrenaline and their combination on edge duration (S) in the open field of male Wistar rats:

Animals administered 15mg/kg bw caffeine, 0.3mg/kg bw adrenaline, 5mg/kg bw caffeine + 0.1mg/kg bw adrenaline and 15mg/kg bw caffeine+0.3mg/kg bw adrenaline showed no significant ( $p < 0.05$ ) different in the edge duration when compared to control while other animals showed significant ( $p < 0.05$ ) decrease in edge duration when compared to control as shown in Table 1.

### Effect of caffeine, adrenaline and their combination on active movement time (S) in the open field of male Wistar rats:

Animals administered 0.3mg/kg bw adrenaline and 15mg/kg bw caffeine + 0.3mg/kg bw adrenaline showed no significant ( $p < 0.05$ ) different in the active movement time duration when compared to control while other animals showed significant ( $p < 0.05$ ) increase in movement duration when compared to control as shown in Table 1.

## DISCUSSION

The aim of the present study was to evaluate the cognitive effects of caffeine, adrenaline and their combination. Caffeine has been widely studied, some reports states that in moderate doses, caffeine typically does not affect learning and memory. Other studies reported that low dose caffeine has a greater effect on activation of brain and improves cognitive functions (Zhang et al., 2020).

All doses of caffeine, and adrenaline used in this study enhanced both short and long term memory although adrenaline showed more profound memory enhancement. The CACA however, did not have any effect on memory of the animals. Spatial memory tested using Y-maze was not altered following caffeine and adrenaline administration, except for the high dose of adrenaline that seems to impair spatial memory.

Studies suggest that caffeine improves memory consolidation of long-term memory in humans and that it can also improve memory retrieval in a task specific for the spatial/relational memory system that models the human hippocampal memory system (Angelucci et al., 2002; Borota et al., 2014).

While many studies suggest that caffeine produces improvement in memory either in animal (Munawar, 2022; Villanueva-Garcia et al., 2020) or in human (Borota et al., 2014; Sherman et al., 2016; Hartney, 2020) models of learning and memory, other studies report that caffeine does not affect memory (Nehlig, 2010) or even its craving impairs memory (Palmer et al., 2017). Some studies reported that long-term consumption of caffeine could inhibit hippocampus-dependent learning and memory partially through inhibition of hippocampal neurogenesis (Han et al., 2007).

According to Gold, low-to-moderate doses of adrenaline facilitate memory, while high doses impair memory (Gold 2014; Roozendaal and Hermans 2017). Considerable evidence suggests that adrenergic hormones modulate the consolidation of long-term memory for experiences that induce their release. For example, post-training injection of adrenergic drugs or hormones has been reported to enhance memory consolidation in many species (Segal et al., 2012; Villain et al., 2016; Schramm et al., 2016).

The brain's response to stress, or any kind of emotional arousal, is in part mediated by the release of the hormone adrenaline. This type of stress memory, or adrenaline-mediated memory formation, is important because it could explain the pathological memory formation that is commonly seen in phenomena such as Post Traumatic Stress Disorder (Samuelson, 2011).

The results of this study showed that low to moderate dose of caffeine reduce anxiety and elevated mood with an increase in general activity. Low dose of adrenaline produced elevated mood and increase motor activities. Anxiety is increased with high dose of adrenaline, which also led to moody state in the animals with no to minimal general activities.

CACA in this study eradicated the observed anxiety following high dose of adrenaline. Low dose of CACA reduced anxiety to a greater extent, it also showed an elevated mood with increased general activity, although activity is reduced following high dose of caffeine and adrenaline. Therefore, caffeine and adrenaline has dose-

dependent anxiolytic effect and could be used to lighten mood. It was reported that caffeine increases anxiety in some individuals and patients with panic disorder (Richards and Smith, 2015; Klevebrant and Frick, 2022). Caffeine has also been shown to have anxiogenic-like behaviour in animals (Rogers et al., 2010). On the other hand, caffeine has also been reported to have anxiolytic effect in rats (Pedraza et al., 2018; Hughes et al., 2014). Increase alertness by caffeine is associated with a high core body temperature (McHill et al., 2014). Also adrenaline has been reported to induce somatic symptoms as well as mental anxiety (Argyropoulos and Nutt, 2003).

The part played by adrenaline in the major emotions of anger and fear has been generally recognized since the classical work of Cannon, who obtained evidence of a liberation of adrenaline from the adrenals and showed that the main somatic accompaniments of anger and fear could be attributed to the action of adrenaline (Richter, 1940). Anxiety has also been reported to increase the body's metabolism, and being anxious increases body temperature (Folk and Folk, 2016). This study reported increased memory in rats administered caffeine and adrenaline.

In conclusion, the study revealed that CACA led to elevation of mood, increased activity and reduction of anxiety in Wistar rats. In addition, it was observed that only high dose of adrenaline increased anxiety. It was also observed that caffeine and adrenaline enhanced short- and long-term memory and only high dose of adrenaline distorted spatial memory. This finding most likely revealed the underlying mechanism to enhance memory, elevate mood and increase anxiety and increased activity in the Wistar rats.

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Full-Length Research Article

# Physiological Changes in Serum Calcium, Phosphate, Vitamin D, Parathyroid Hormone and Calcitonin During Pregnancy and Lactation in Randomised Population of Zaria Women

Avidime O.M.<sup>1</sup>, Avidime S.<sup>2</sup>, Randawa A.J.<sup>2</sup>, Kawu M.U.<sup>3</sup>, Mohammed A.<sup>4</sup>,  
Oweh O.T.<sup>5</sup>

<sup>1</sup>Department of Human Physiology, Faculty of Basic Medical Sciences, Kaduna State University

<sup>2</sup>Department of Obstetrics and Gynaecology, Ahmadu Bello University Teaching Hospital, Zaria

<sup>3</sup>Department of Veterinary Physiology, Ahmadu Bello University, Zaria.

<sup>4</sup>Department of Human Physiology, Ahmadu Bello University

<sup>5</sup>Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Kaduna State University.

**Summary:** Pregnancy and lactation are usual but stressful physiological conditions accompanied by changes in calcium and phosphate metabolism and their regulatory hormones which may lead to calcium-related disorders in pregnant women. The aim of this study was to evaluate the changes in serum levels of calcium, phosphate, vitamin D and their regulatory hormones in pregnant and lactating women in Zaria, Nigeria. A cross-sectional descriptive study was conducted at Ahmadu Bello University Teaching Hospital, Zaria for a period of three (3) months. Blood samples were collected, anthropometric measurements (weight, height and body mass index) of 179 women were taken. Serum calcium, phosphate, vitamin D, parathyroid hormone and calcitonin were determined using standard methods. Data were presented as mean  $\pm$  SD, analysis was performed using one-way ANOVA and Pearson's correlation analysis. Values were considered significant at  $p \leq 0.05$ . There was a significant decrease in serum calcium concentration ( $p < 0.01$ ) during the third trimester of pregnancy and lactation. An increase in serum concentration of vitamin D, parathyroid hormone and calcitonin in the 2<sup>nd</sup> trimester and a decreased during the third trimester and lactation although not statistically significant when compared with the control. There was a negative correlation between serum calcium concentration and gestational age ( $r = 0.255$ ) while no correlation between gestational age and serum phosphate concentration. Changes in serum calcium, vitamin D, parathyroid hormone and calcitonin during pregnancy and lactation have been demonstrated suggesting a relationship between calcium metabolism and these hormones at some stages of pregnancy.

**Keywords:** *Tocopherol, arsenic, stomach, ileum, colon*

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\*Address for correspondence: E-mail: [nenemakaju@yahoo.com](mailto:nenemakaju@yahoo.com); Tel: 08037053285

Manuscript received- December- 2021; Accepted- March 2022

DOI: <https://doi.org/10.54548/njps.v37i1.10>

## INTRODUCTION

Women during certain physiological stages such as pregnancy and lactation experience an alteration in vitamin D and calcium metabolism as the growing fetus obtains calcium from maternal stores to aid skeletal development (Kovacs, 2021). The demand for calcium during pregnancy varies from about 2-3mg/day in the 1st trimester to about 3-fold in the 3rd trimester (WHO, 2016) which is accompanied by an increase in intestinal absorption of calcium under the influence of vitamin D. This shows the importance of calcium and phosphate during pregnancy hence serum levels need to be maintained in a certain range with a complex interplay of hormones involved to maintain a balance (Fukumoto, 2014). The most important factors involved in the balance are vitamin D and parathyroid hormone (Hysaj et al., 2021). Vitamin D regulates absorption of calcium from the gastrointestinal tract as well as its excretion from kidney (Fleet, 2017). Serum phosphate

level is however regulated by the absorption of phosphate in the intestine, renal phosphate management and a balance of extracellular phosphate with that in bone or intracellular fluid (Yee et al., 2021). Parathyroid hormone mainly function through conversion of vitamin D to its active metabolite (1,25-hydroxycholecalciferol) in the kidneys, which regulates intestinal absorption, renal excretion, and exchange between the extracellular fluid and bone (Levine et al., 2014).

Studies have revealed that there is variation in the levels of serum calcium, inorganic phosphate, vitamin D and parathyroid hormone at different stages of pregnancy however there is paucity of information on the variation of these biochemical markers among pregnant women in Zaria, a major town in Northern Nigeria. Therefore, by measuring the levels of calcium, inorganic phosphate, vitamin D and their regulatory hormones at various stages of pregnancy, local reference values may be established that will contribute to the growing body of knowledge concerning

calcium and phosphorus disorders during pregnancy in Nigerian women. The result of this study would establish the relationship between calcium and its regulatory hormones in pregnant women in Zaria, and its environs. This could also be beneficial in the management of pregnant women presenting with calcium associated disorders.

## MATERIALS AND METHODS

**Demography of Study Area:** Zaria is a heterogeneous city in Kaduna State. It is inhabited by about 1,018,827 people (National Bureau of Statistics, 2015). Kaduna is located in the North-west geopolitical zone of Nigeria, Kaduna State is one of the 36 states in Nigeria with its capital in Kaduna city. Zaria occupies a portion of the high plains of Northern Nigeria, 652.6 meters above sea level and some 950 kilometers from the coast at 11°03'N, 7°42'E.

**Study Design and Study Population:** This study is a cross-sectional descriptive study that was conducted between within a period of three (3) months. A total of one hundred and seventy nine (179) women attending antenatal, family planning and immunization clinics at the Ahmadu Bello University Teaching Hospital (ABUTH) Shika, Zaria, Maternal and Child Health Centre, ABUTH, Ban Zazzau, Zaria and Comprehensive Health Centre, ABUTH, Sabon Gari, Zaria were recruited for the study. A structured questionnaire about pregnancy history was administered. Blood samples were collected by a competent Laboratory Technologist at the Departments of Obstetrics and Gynaecology, ABUTH, Shika and measurements (body weight and height) were also recorded from the subjects. Body mass Index (BMI) was calculated from data collected from the anthropometric indices.

**Experimental Grouping:** A total of one hundred and seventy nine (179) women involved in the study were grouped as follows: Group 1 non-pregnant women (n=15), group 2 were pregnant women in the first trimester (n=40), group three involved pregnant women in their second trimester (n=41), group four involved pregnant women in their third trimester (n=53) while group 5 involved lactating women (n=30).

**Inclusion and exclusion Criteria:** Healthy consenting women attending antenatal, family planning and immunization clinics at Ahmadu Bello University Teaching Hospital (ABUTH) Shika; Women and Child Centre, ABUTH, Ban Zazzau; Women Centre, ABUTH Sabon Gari, Antenatal Clinic, Sickbay, Ahmadu Bello University. Pregnant women with any disorder that affects metabolism of calcium or bone, history of endocrine, renal or liver illnesses, hypertension of pregnancy, gestational diabetes, thyroid disorders, or treatment with anti-tubercular or antiepileptic drugs in the previous 3 months were excluded from the study.

**Ethical Approval Statement:** Approval was obtained from the Scientific and Ethical Committee on Human Research, ABUTH, Shika, Zaria (Reference number: ABUTHZ/HREC/K30/2014) and verbal consent was obtained from all subjects.

**Collection and processing of samples:** Exactly 10 ml of blood sample was collected by venipuncture, transferred to clean plain serum bottles immediately and allowed to stand at room temperature (27°C) for two hours and centrifuged at 3000 g for 5 minutes to obtain the serum. The separated serum was stored at -20°C in a deep freezer at the department of Chemical pathology until needed for assay.

**Determination of serum calcium and inorganic phosphate level:** The assay for serum calcium was done using O-cresolphthaline complexone method as described by Morin (Morin, 1974) while serum inorganic phosphate was analyzed using Fiske-Subbarow method as described by Fiske and Subbarow (Fiske and Subbarow, 1925)

**Quantification of serum 1, 25 (OH) hydroxyvitamin D:** Serum 1, 25(OH) hydroxyvitamin D was assayed using an enzyme-linked immunosorbent assay (ELISA) technique. The absorbance was read at 450 nm using Mindray MR-96A microplate reader (Shenzhen, China). The concentration of serum 25(OH) D in the test samples was calculated using the standard curve plotted with the different standards and their known antigen concentration.

**Determination of serum parathyroid hormone and Serum Calcitonin:** Serum PHT and calcitonin were assayed for using an enzyme-linked immunosorbent assay (ELISA) technique as described by Essley *et al.*, 2011.

**Data Analysis:** Data was collected, curated, cleaned up and was presented as mean  $\pm$  SD. Analysis of data was done using One Way ANOVA followed by Holm-Sidak post hoc test and level of significance was tested at  $P < 0.05$ . Pearson's correlation analysis was done to determine the inter-relationship between the variables

## RESULTS

The anthropometric indices of the subjects are as shown in Table 1. The study showed that the mean age of the control group was  $29.79 \pm 5.56$  years; while those in the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> trimesters were  $27.00 \pm 6.41$ ,  $28.17 \pm 6.11$ ,  $26.62 \pm 5.58$  years, respectively. Mean age of the lactating group was  $28.37 \pm 5.13$  years. The mean weight of the control women was  $67.40 \pm 9.49$  Kg; mean weight of 1st, 2<sup>nd</sup> and 3<sup>rd</sup> trimester women were  $63.39 \pm 16.91$ ,  $63.39 \pm 16.91$ ,  $68.39 \pm 13.04$  Kg; while the mean weight of the lactating group was  $63.13 \pm 12.82$ kg. Mean height of control, 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> trimesters and lactating groups were  $159.27 \pm 9.13$ cm,  $157.10 \pm 8.29$ cm,  $161.78 \pm 7.12$ cm,  $159.82 \pm 6.52$ cm and  $160.90 \pm 7.38$ cm, respectively. Mean BMI was  $26.56 \pm 3.12$ Kg/m<sup>2</sup>,  $25.32 \pm 6.98$ Kg/m<sup>2</sup>,  $24.58 \pm 7.06$ Kg/m<sup>2</sup>,  $24.00 \pm 8.35$ Kg/m<sup>2</sup> and  $24.32 \pm 4.18$ Kg/m<sup>2</sup> for the control, 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> trimesters and lactating groups, respectively. Mean gestational ages in months were  $2.71 \pm 0.49$  months,  $5.42 \pm 0.81$  months and  $7.74 \pm 0.79$  months for the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> trimesters, respectively.

**Serum Levels of Calcium, Vitamin D, Parathyroid Hormone and Calcitonin:** Table 2 shows the profile of mean calcium, phosphate (PO<sub>4</sub>), vitamin D, parathyroid hormone (PTH) and calcitonin in the control, pregnant and lactating women. The mean values for serum calcium was

highest in the 1<sup>st</sup> trimester, significantly reduced in the 3<sup>rd</sup> trimester and lowest in the lactating group with the highest and lowest value at 2.52 mmol/L and 2.25 mmol/L respectively. Mean serum phosphate concentration was highest in the control group 1.13±0.15 mmol/L and lowest in the 1<sup>st</sup> trimester; 1.01±0.13mmol/L. Furthermore, the mean serum vitamin D level was highest in the 2<sup>nd</sup> trimester;

46.57±20.90 µg/L and lowest in the control group; 39.29±4.51 µg/L. Mean PTH level was highest in the 2<sup>nd</sup> trimester. 45.60±17.62 pg/ml and lowest in the 1<sup>st</sup> trimester; 40.66±6.64 pg/ml. Mean calcitonin concentration was highest in the 2<sup>nd</sup> trimester 3.95±4.10 ng/L and also lowest in the 1<sup>st</sup> trimester 3.05±0 ng/L.

**Table 1:**

Table of anthropometric indices of control, pregnant and lactating women

| Parameters                         | Age (yrs)  | Weight (kg) | Height (cm) | BMI (Kg/m <sup>2</sup> ) | Gest. Age(mths) |
|------------------------------------|------------|-------------|-------------|--------------------------|-----------------|
| Control (n = 15)<br>non-pregnant   | 29.79±5.56 | 67.40±9.49  | 159.27±9.13 | 26.56±3.12               | -               |
| 1 <sup>st</sup> trimester (n = 40) | 27.00±6.41 | 63.39±16.91 | 157.10±8.29 | 25.32±6.98               | 2.71±0.49       |
| 2 <sup>nd</sup> trimester (n = 41) | 28.17±6.11 | 66.42±14.53 | 161.78±7.12 | 24.58±7.06               | 5.42±0.81       |
| 3 <sup>rd</sup> trimester (n = 53) | 26.62±5.58 | 68.39±13.04 | 159.82±6.52 | 24.00±8.35               | 7.74±0.79       |
| Lactating gp (n = 30)              | 28.37±5.13 | 63.13±12.82 | 160.90±7.38 | 24.32±4.18               | -               |

Key: n – number of women per group, Gest. Age – Mean gestational age, BMI – Body Mass Index

**Table 2:**

Mean (±SD) Serum concentration of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin in control, pregnant and lactating women

| Parameters               | Control (n = 15) | 1 <sup>st</sup> Trim (n = 40) | 2 <sup>nd</sup> Trim (n = 41) | 3 <sup>rd</sup> Trim (n = 53) | Lactating Gp (n = 30) |
|--------------------------|------------------|-------------------------------|-------------------------------|-------------------------------|-----------------------|
| Calcium (mmol/L)         | 2.30±0.10        | 2.32±0.11                     | 2.31±0.15                     | 2.24±0.14**                   | 2.21±0.17**           |
| PO <sub>4</sub> (mmol/L) | 1.13±0.15        | 1.01±0.13                     | 1.08±0.14                     | 1.08±0.15                     | 1.09±0.14             |
| Vitamin D (µg/L)         | 39.29±4.51       | 41.21±5.68                    | 46.57±20.90                   | 42.44±8.50                    | 43.33±20.08           |
| PTH (pg/ml)              | 40.79±8.43       | 40.66±6.64                    | 45.60±17.62                   | 41.84±10.40                   | 44.81±12.63           |
| Calcitonin (ng/L)        | 3.47±0.91        | 3.05±0.93                     | 3.95±4.10                     | 3.20±0.92                     | 3.10±0.77             |

Key: \*\* Represents  $P < 0.01$  when compared to the control, Trim - Trimester, Gp - Group, PTH - parathyroid hormone

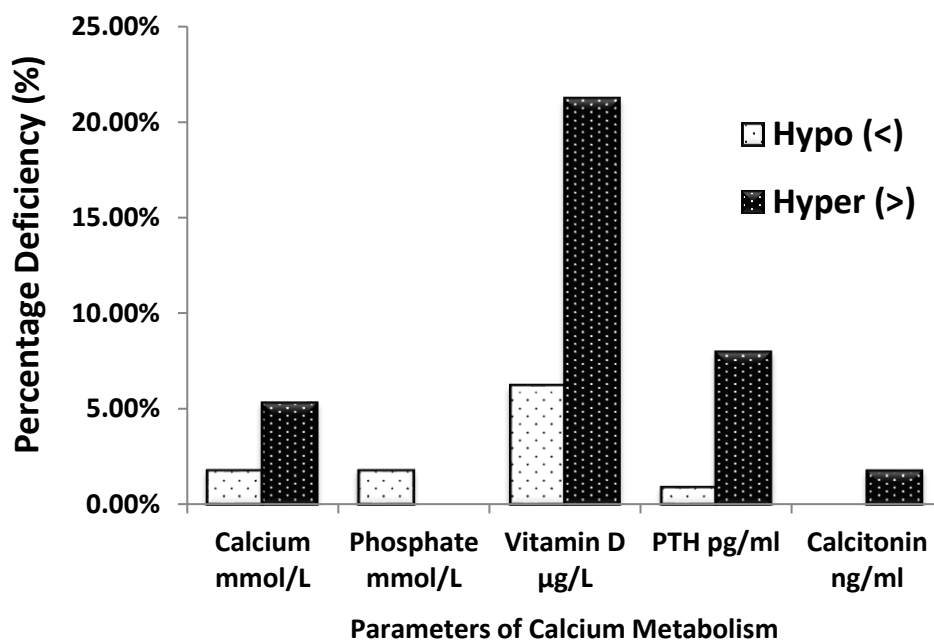


**Figure 1:**

Prevalence of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin deficiency among pregnant women in Zaria, Nigeria

*Calcium, phosphate, vitamin D in pregnant and lactating women in Zaria*

**Table 3:**

Relationship between age, gestational age, BMI and serum concentration of calcium, phosphate, parathyroid hormone, vitamin D and calcitonin

| Parameters                        | R       | P value |
|-----------------------------------|---------|---------|
| Body mass index and Age           | 0.290   | <0.001  |
| Calcium and Gestational Age       | - 0.255 | <0.01   |
| Phosphate and Calcium             | - 0.270 | <0.001  |
| Vitamin D and Calcium             | - 0.422 | <0.001  |
| Parathyroid hormone and Calcium   | - 0.448 | <0.001  |
| Parathyroid hormone and Vitamin D | 0.756   | <0.001  |
| Calcitonin and Calcium            | 0.279   | <0.001  |
| Calcitonin and Phosphate          | - 0.239 | <0.001  |

**Prevalence of Calcium, Phosphate, Vitamin D, Parathyroid Hormone and Calcitonin Deficiency:** Figure 1 shows 1.76% of pregnant women with calcium below the normal range and 5.31% above the normal range in this study. Also, 1.76% of the women had phosphate levels below the normal range and none of them was hyperphosphataemic. The vitamin D concentration was low in 6.19% and high in 21.24% of the pregnant women. Furthermore, parathyroid hormone level was low in less than 1% (one per cent) of the study population with 7.97% above the normal range. Calcitonin was not below the normal range in any of the groups, but there was a 1.76% prevalence above the normal range.

Table 3 shows the correlation between age, gestational age, body mass index (BMI), and serum concentrations of calcium, phosphate, parathyroid hormone (PTH), vitamin D and calcitonin. About 20% (twenty percent) of the variables correlated significantly. Positive correlation was observed between age and BMI and parathyroid hormone and vitamin D; while negative correlation was observed between phosphate and calcium, vitamin D and calcium, parathyroid hormone and calcium and calcitonin and phosphate. These correlations were statistically significant ( $P < 0.001$ ).

## DISCUSSION

This study was conducted to evaluate the biochemical changes in serum concentration of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin in pregnant and lactating women in Zaria.

Serum calcium has been reported to decrease during late pregnancy in humans (Ajong *et al.*, 2019). In this study, ionized calcium was measured, since it is the physiologically significant fraction of extracellular calcium. The normalcy in the level of serum calcium during the first trimester as compared to the control may probably be due to less need for calcium during the period. The drop in serum calcium by the third trimester and during lactation correlates with gestational age. This is because pregnancy and lactation cause a substantial increase in demand for calcium (Kovacs, 2021). Thus, increased intestinal calcium absorption, which more than doubles during pregnancy

(Kovacs, 2021). Maternal skeleton reabsorption also helps to provide most of the calcium content of breast milk during lactation (Kovacs, 2008). This report does not correspond with Almaghamsi *et al.*, 2018 which showed that ionized calcium does not change during human pregnancy. The decreased in serum calcium concentration during the third trimester and lactation agrees with the report of Almaghamsi *et al.*, 2018 who also found low calcium levels in pregnant women with preeclampsia.

The lowest value of serum calcium concentration among the pregnant women in the present study was 1.76%. This very low value could be as a result of low calcium intake of less than 500 mg/day or as a result of decreased calcium metabolism during periods of low intake. This fact is supported by the report of Madukosiri *et al* 2011 on the food intake of pregnant and lactating women in Plateau state, Nigeria. Furthermore, more women were hypercalcaemic (5.31%) and this could be as a result of calcium rich diets, such as nuts and soya bean cake supplements (tofu, wara) (common in Zaria and environs) which the pregnant women may have been taking. Intake of calcium supplements could cause raised concentrations of serum calcium (Institute of Medicine, 2011). This result corresponds with the report of Benali and Demouche, 2014 who observed a high prevalence (70.55%) of hypocalcaemia among pregnant women in Algeria. However, Kumar *et al.*, 2010 reported that approximately two thirds of pregnant women in India were hypocalcaemic though; none of the women had any manifest symptoms.

This study also revealed an increase in serum phosphate concentration from the 1<sup>st</sup> to the 3<sup>rd</sup> trimester; though this increase was not statistically significant. The observed increase in serum phosphate level could possibly be due to an increase in macromolecular metabolism during pregnancy (Correia-Branco *et al.*, 2020) since phosphorus plays a vital role in intermediary metabolism of carbohydrates and is also an important component of physiological substances such as organic phosphate esters, phospholipids, nucleic acids and nucleotides. The increase in serum phosphate concentration in this study agrees with the report of Kametas *et al.*, 2003 which showed that serum phosphate levels are within the non-pregnant range possibly. However, Møller *et al.*, 2013 reported no significant changes in the serum phosphate of pregnant women during the various trimester whereas, Ikekpeazu *et al*, 2010 reported a significant decrease in serum inorganic phosphate levels in the second and third trimesters as compared to the non-pregnant, non-lactating group. The population of pregnant women in this study showed a prevalence of 1.76% hypophosphataemia and none was observed to have hyperphosphataemia.

In the present study, vitamin D concentration was highest in the 2<sup>nd</sup> trimester. But the 3<sup>rd</sup> trimester showed a lower vitamin D concentration than the 1<sup>st</sup> trimester. The increase in Vitamin D concentration in the second trimester could be due to negative feedback stimulation from decreased calcium levels which was also reported during the second trimester and probably due to an increase in the synthesis of vitamin D which occurs in the maternal kidneys, maternal decidua, placenta, and fetus (Kovacs, 2016). The higher vitamin D levels observed in this study during the 2<sup>nd</sup> trimester of pregnancy corresponds with the finding of Møller *et al*, 2013. However, the hypovitaminosis

D observed as compared to the control in this study was 6.19% while the hypervitaminosis D was 21%. This is in contrast to the 29% vitamin D deficiency reported by Gbadegesin *et al.*, 2017 in a study of pregnant women in Lagos, Nigeria.

In this study, parathyroid hormone (PTH) levels were higher in the 1<sup>st</sup> trimester and 2<sup>nd</sup> trimester, and lower in the 3<sup>rd</sup> trimester. This decrease in PHT in the 3<sup>rd</sup> trimester could be due to direct inhibition of PTH production either by previously high concentrations of 1,25-dihydroxyvitamin D or by augmented intestinal absorption of calcium Holt, 2013. This finding agrees with earlier studies (Madar *et al.* 2009, Marwaha *et al.*, 2011, Roth *et al.*, 2013 and Schoenmakers *et al.*, 2013). Furthermore, there was a prevalence of 0.88% hypoparathyroidism and 7.97% hyperparathyroidism.

In this study, serum calcitonin levels were increased in the 2<sup>nd</sup> trimester, but decreased by the 3<sup>rd</sup> trimester. There was also a slight decrease in serum calcitonin concentration in the lactating group. The increase in calcitonin level during the 2<sup>nd</sup> trimester may be attributed to increased production by the C-cells of the thyroid gland and additional sources of expression that may contribute to the circulating level of calcitonin, such as mammary tissue, placenta, and pituitary tissue (Kovacs, 2016). The slight increase in serum calcitonin concentration observed in the lactating group could be attributed to its possible physiological role in protecting the maternal skeleton during pregnancy and lactation (Kovacs, 2016). There was no hypocalcitoninaemia observed in this study, but there was a 1.76% prevalence of hypercalcitoninaemia. This finding contradicts the findings of Møller *et al.*, 2013 who found no change in the level of calcitonin; during the three trimesters of pregnancy.

There was strong negative correlation between vitamin D and calcium as well as vitamin D and phosphate. This suggests that as vitamin D level increases, calcium and phosphate concentrations were decreasing. Also, the proliferation of the lactiferous ducts, lobules of the breasts and foetal calcium accretion. This negative correlation between vitamin D and calcium could be as a result of the diversion of essential elements such as sodium, potassium and calcium to the site of growth and synthesis for growth to take place (especially during the 3<sup>rd</sup> trimester of pregnancy and the lactation period), causes an increase in calcium demands during this phase. As a result of this increase in demand, there is a negative feedback triggered causing a decrease in the blood serum concentration of calcium and the subsequent increase in vitamin D synthesis, intestinal absorption and reduced renal excretion. This corresponded with the report of Kirby *et al.*, 2013 who demonstrated that serum vitamin D concentrations could increase up to seven times during the course of a normal pregnancy in rats and mice, and this corresponds to the time of rapid foetal mineral accretion (in the third trimester of pregnancy) which could lead to reduced serum calcium levels.

Parathyroid hormone showed a significant negative correlation with calcium. This finding corroborates with the report of Gillies *et al.*, 2015 who reported that high calcium in the diet caused a lower serum PTH concentration. Some animal models have shown that pregnant mice have very low PTH levels (Kirby *et al.*, 2013), these maybe as a result of negative feedback mechanisms.

In conclusion, changes in serum calcium, vitamin D, parathyroid hormone as well as calcitonin during pregnancy and lactation have been demonstrated suggesting a relationship between calcium metabolism and these regulatory hormones at some stages of pregnancy. These variations observed may be critical to the developmental processes during pregnancy.

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Full-Length Research Article

# Tocopherol Enhances the Antioxidant Defense System and Histomorphometric Parameters in the Gastrointestinal Tract of Rats Treated with Sodium Arsenite

\*Oladokun O.O.<sup>1</sup>, Olaleye T.C.<sup>1</sup>, Moses N.M.<sup>1</sup>, Oladosu O.A.<sup>1</sup>, Babatunde A.A.<sup>1</sup>,  
Adedokun K.I.<sup>1</sup>, Owonikoko M.W.<sup>2</sup>, and Ajeigbe K.O.<sup>3</sup>

<sup>1</sup>Department of Physiology, Faculty of Basic Medical Sciences, Osun State University, Osogbo, Nigeria

<sup>2</sup>Department of Physiology, Faculty of Basic Medical Sciences, Igbinedion University, Okada. Okada, Nigeria

<sup>3</sup>Department of Physiology, Faculty of Basic Medical Sciences, Federal University, Oye-Ekiti, Nigeria

**Summary:** Arsenic compromises the gastrointestinal integrity and function via the body's anti-oxidative system breakdown. Hence, this study aimed to investigate the effects of tocopherol on redox imbalance and histoarchitectural alterations in rats' gastrointestinal tract exposed to sodium arsenite. Sodium arsenite (10mg/Kg) and graded doses of tocopherol were administered for four weeks concurrently (per oral) to experimental rats assigned to different groups as follows i: Control normal saline (1 mg/Kg); ii: Rats treated with Sodium arsenite (10 mg/Kg); iii: Rats treated with 100 mg/kg tocopherol + Sodium arsenite; iv: Rats treated with 300 mg/kg tocopherol + Sodium arsenite; v: Rats treated with Olive oil (0.5ml/Kg) only; vi: Rats treated with Olive oil and 100 mg/kg tocopherol; vii: Rats treated with Olive oil and 300 mg/kg tocopherol. Redox status assay was done in homogenized samples by spectrophotometry. Parietal cell mass and mucous cell density (stomach), villus height and crypt depth (ileum), goblet cells count, and crypt depth (colon) were evaluated by histomorphometry. Inflammatory cells infiltration was also assessed using a semi-quantitative procedure. Sodium arsenite caused a significant increase in Malondialdehyde and Myeloperoxidase but, decreased Superoxide dismutase, Catalase, Nitric oxide, Glutathione peroxidase, Glutathione, and Glutathione-S-Transferase. Tocopherol treatment reversed the changes ( $p < 0.05$ ) though not largely dose-dependent. Furthermore, tocopherol annulled sodium arsenite-induced increase in parietal cell mass and decrease in mucous cell density in the stomach, decrease in villus height and villus height/crypt depth ratio in the ileum, and decrease in goblets cells and increase in crypt depth in the colon. Moreover, activated inflammatory cell infiltration by sodium arsenite was mitigated by tocopherol. Sodium arsenite provokes not only marked inflammatory cellular infiltration but a focal loss of glands, hyperplasia of crypts, atrophic villi, and hypertrophy of Peyer's patches in the intestines, which are all lessened with tocopherol treatment. These findings underscore the anti-oxidative properties of tocopherol as a potent dietary factor against sodium arsenite toxicity in the gastrointestinal tract.

**Keywords:** Tocopherol, arsenic, stomach, ileum, colon

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\*Address for correspondence: E-mail: [olayemi.oladokun@uniosun.edu.ng](mailto:olayemi.oladokun@uniosun.edu.ng); Tel: +2348037282899

Manuscript received- February- 2022; Accepted- April 2022

DOI: <https://doi.org/10.54548/njps.v37i1.11>

## INTRODUCTION

Arsenic (AS) is one of the most abundant toxic metals to which approximately 200 million humans are exposed particularly in developing countries (WHO, 2012). More people are being increasingly predisposed due to the periodic societal adjustments in the processing and quality of foodstuff. For example, plants, an intermediate energy level in the food chain pathway preceding human exposure have been reported to bioconcentrate AS in the form of fruits, vegetables, and beverages (Chen et al., 2011). There is evidence of AS water contamination in many countries of the world such as India (Pradhan and Kumar 2014), Australia (Kiddee et al., 2014), China (Chau et al., 2015), Vietnam (Smith et al., 2000), Burkina Faso (Somé et al., 2012), and Ghana (Asante et al., 2012) particularly through electronic wastes. The recent incorporation of rice in baby diets may add the latter to the growing list of AS exposed

subjects. Roxarsone, a dietary supplement used to stimulate growth in the poultry industry contains organic arsenic (Nachman et al., 2013). In Nigeria, AS has been reported to contaminate the soil (Anselm et al., 2021), water (Karkarna and Matazu 2021), plants (Olafisoye et al., 2013) dust (Ibe et al., 2018), and even blood/serum (Popoola et al., 2019) owing to poor environmental management.

Most heavy metals are impervious to digestive activities. In the case of AS, intestinal absorption follows the ingestion of inorganic AS polluted water and is thereafter sequestered into bio accumulative tissues via circulation (Kitchin and Kirk 2001). This process is accompanied by AS-induced toxicity such as ocular burning sensation, body weakness, lower limbs swelling, pulmonary distress, hepatic fibrosis, neurological disorder, and non-specific organ cancer (Singh et al., 2011) as the toxicosis traverses several areas of the body (Szymanska-Chabowska et al., 2002). One of the

notable pathophysiological disease mechanisms for which AS is identified including carcinogenesis, is induction of oxidative stress Chih-Hung et al., 2010) through the generation of Reactive Oxygen Species (ROS) which in turn disrupt the structure and function of important biomolecules such as protein, lipid, and DNA (Mo et al., 2006) thereby altering biochemical status (Yousef et al., 2008). AS and its intermediates in the process of their systemic clearance in the body are bio-transformed into methylated products that are not only bioavailable but are also cytotoxic (Petrick et al., 2000), genotoxic (Mass et al., 2001) and are biomolecular inhibitor of several enzymes (Drobná et al., 2003) and signaling pathway disruptors (Stýblo et al., 2002). Due to the ingestive nature of human exposure to AS (Chiocchetti et al., 2019), the gastrointestinal system is the prime target for its toxicity with such symptoms as abdominal discomfort, vomiting, and most commonly diarrhea. AS exposure has been reported to affect intestinal homeostasis, disrupt the mucosal layer (Chiocchetti et al., 2018), and predispose the gastrointestinal tract to ulceration (Adebayo-Gege et al., 2018).

Tocopherol commonly referred to as Vitamin E is a fat-soluble diet-derived natural antioxidant known to be well absorbed in the gastrointestinal tract (Traber and Arai 1999) as it mixes well with micelles and ferries through the enterocytes into extra-gastrointestinal organs (Reboul 2017). It has been well established that vitamin E is a major antioxidant in cellular membranes preventing peroxidation of cell membrane (Jiang 2014) by scavenging peroxy, oxygen, and superoxide anion radicals. Though its potency as an exogenous gastroprotective and therapeutic factor has been mentioned (Kamisah et al., 2014), the level of knowledge on the mitigating effect of tocopherol on AS exposed gastrointestinal system is still incompletely understood. But it is established that the etiology of AS-induced gastrointestinal injury is biochemically mediated. Hence, this study investigates arsenite-induced oxidative stress and histoarchitectural changes in stomach, ileum, liver, and colon tissues, and the possible amelioration by tocopherol using laboratory rats.

## MATERIALS AND METHODS

**Animals and Experimental Design:** Thirty-five (35) male Wistar rats weighing 100–120 g were obtained from the College Central Animal Facility of College of Health Sciences, Osun State University, Osogbo, Nigeria. The animals were kept in plastic cages in a well-ventilated animal house. They were fed with commercial rat chow and water *ad libitum*. Handling and care of the experimental animals were in strict accordance with the criteria outlined in the Current Animal Care Regulations and Standards approved by the Institute for Laboratory Animal Research (Guide for Care and Use of Laboratory Animals in Biomedical and Behavioral Research). The institution's Animal Ethics and Research Committee approved the experimental protocols (Protocol I.D: UNIOSUNHREC/2021B/003).

Animals were randomly divided into seven groups of five animals each as follows- i: Control rats receiving normal saline (1 mg/Kg); ii: Rats treated with Sodium arsenite (10 mg/Kg); iii: Rats treated with tocopherol (100 mg/kg; peroral), followed by administration Sodium

arsenite; iv: Rats treated with tocopherol (300 mg/kg; per oral), followed administration of Sodium arsenite; v: Rats treated with Olive oil (0.5ml/Kg) only; vi: Rats treated with Olive oil and tocopherol (100 mg/Kg); vii: Rats treated with Olive oil and tocopherol (300 mg/Kg).

Administration of sodium arsenite and tocopherol was done for four weeks concurrently. Rats were euthanized 24 h after the last administration, then, the stomach, ileum, and colon were removed. The stomach was cut open along the greater curvature while the intestines were opened along their entire lengths to wash off the debris. Small portions of the different segments of the gastrointestinal tract were cut for histological processing and analysis. The remaining portions were rinsed and homogenized using 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% KCl. The homogenate was centrifuged at 4°C for 10 minutes at a speed of 10,000x g, and the supernatant thereof was kept at -8°C and used for the estimation of the biochemical parameters.

**Drugs and Chemicals:** Tocopherol capsules (Vitamin E) were purchased from a local Pharmacy duly registered by the Pharmacists' Council of Nigeria (PCN). Sodium arsenite, adrenaline, glutathione, 5,5'-dithiobis-2-nitrobenzoic acid, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), thiobarbituric acid, and trichloroacetic acid, were purchased from Sigma Chemical (St. Louis, MO). All other reagents used were of analytical grade and were obtained from British Drug houses.

**Biochemical Assays:** MDA concentration as an index of lipid peroxidation was quantified according to the method described by Varshney and Kale (1990). Myeloperoxidase (MPO) activity was determined by the method of Xia and Zweier (1997). Superoxide dismutase (SOD) assay was carried out by the method of Misra and Fridovich (1972), Catalase (CAT) activity using H<sub>2</sub>O<sub>2</sub> as substrate was measured by the method of Claiborne (1995) while Nitric Oxide (NO) content was quantified as previously described by Olaleye et al. (2007). Glutathione (GSH) concentration was determined using the method of Jollow et al. (1974). Glutathione peroxidase (GPX) activity was measured by the method of Rotruck et al. (1973). Glutathione S-transferase (GST) was estimated by the method of Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene as substrate.

**Histopathological Studies:** Samples of the stomach, ileum, and colon were collected from each animal and fixed in 10% neutral formalin for 48–72 h. The tissue samples were processed in an automatic tissue processor – dehydrated and embedded in paraffin wax. Sections were cut at a thickness of 4 µm and stained with hematoxylin and eosin for histopathology. Standard glass microscope slides were used for mounting.

**Histomorphometry:** Inflammatory cells infiltration assessment was done subjectively using the scale: 0 = no infiltration; 1 = very mild infiltration; 2 = mild infiltration; 3 = moderate infiltration; 4 = marked infiltration. Parietal cell mass and mucous cell density were calculated as described by Ajeigbe et al., (2014) as the number of cells per mm<sup>2</sup> multiplied by the thickness of the glandular layer.

In the ileum and colon, villous height, villous width, and crypt depth were measured and assessed. At light microscopy (10 × objective lens), intact and 10 well-oriented villi and crypts from each intestinal section of each animal were randomly selected for measurement. The villus length was measured from the villus-crypt junction to the villus tip and the crypt depth from the crypt base to the villus-crypt junction. Using the same villus and crypt columns, the number of goblet cells was determined, expressed per 100 enterocytes. The villus/crypt ratio was calculated by dividing the villus height by crypt depth (Van Zuidewijn *et al.*, 1992). The samples were evaluated, and measurements were performed with Olympus CX43 microscope with a color digital camera connected to a computerized image analysis system (Image Pro, USA).

### Statistical Analysis

Data are presented as mean ± SEM and subjected to one-way analysis of variance (ANOVA) and Newman-Keul

posthoc test using the Graphpad Prism version 6.0 for Windows from GraphPad Software, San Diego, CA, USA. Values of  $p < 0.05$  were regarded as significant

### RESULTS

#### Effect of tocopherol on lipid peroxidation induced by sodium arsenite in the gastrointestinal tissues of rats:

The ameliorative effect of tocopherol on sodium arsenite-induced lipid peroxidation is shown in Figures 1a, 2a, 3a and 1b, 2b, 3b. Sodium arsenite significantly increased malondialdehyde (MDA), an important marker of lipid peroxidation, when compared with the normal saline (control) group ( $p < 0.05$ ). However, treatment with 100 mg/Kg and 300 mg/Kg tocopherol reduced the elevation observed in the sodium arsenite alone group ( $p < 0.05$ ). Likewise, myeloperoxidase (MPO) activity was reduced in the stomach, ileum, and colon tissues by tocopherol in the sodium arsenite rats ( $p < 0.05$ ).

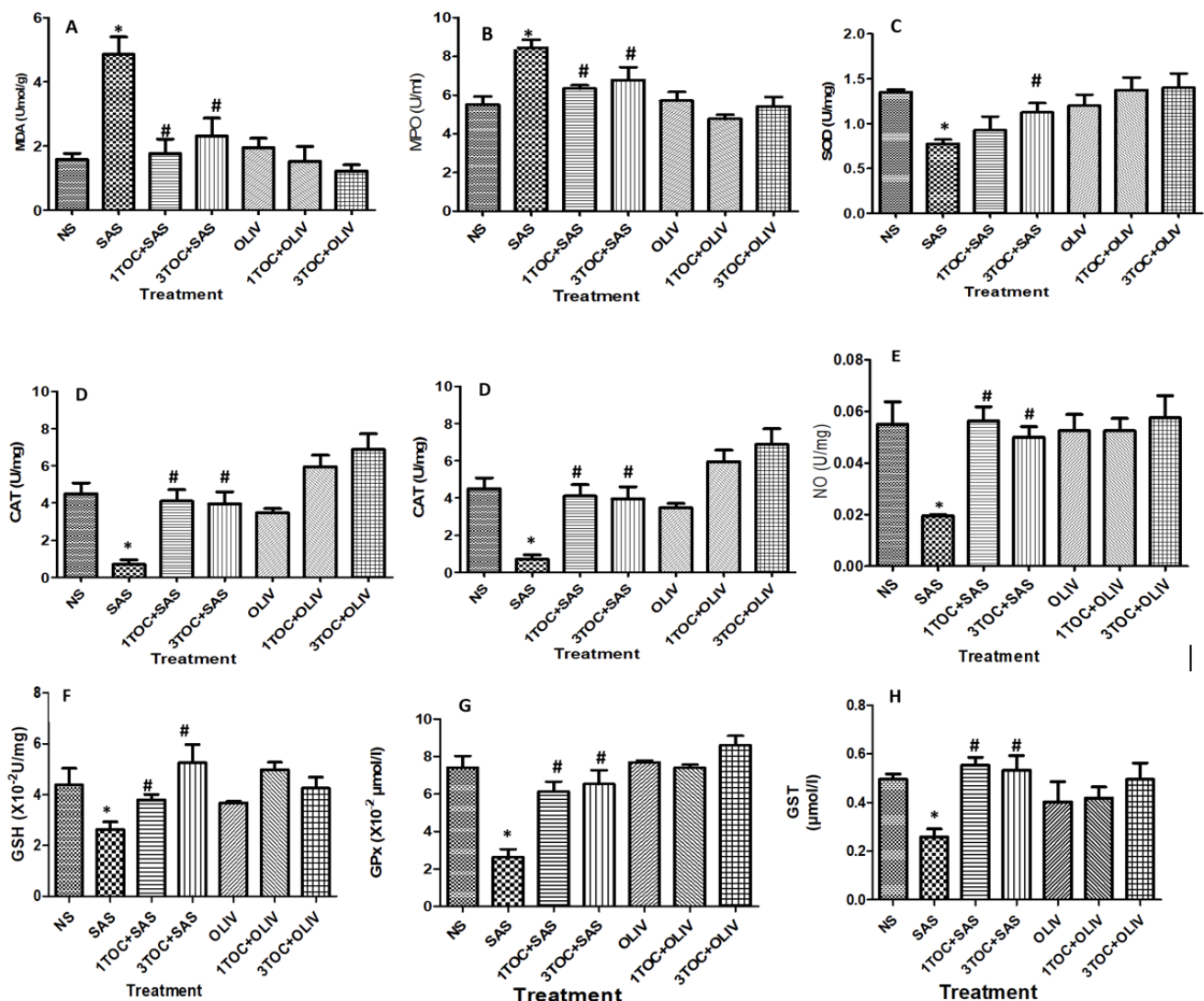


**Figure 1:**

**Redox status in normal and sodium-arsenite exposed stomach tissue upon treatment with tocopherol.** (A); MDA=Malondialdehyde, (B); MPO= Myeloperoxidase, (C); SOD= Superoxide dismutase, (D); CAT= Catalase, (E); NO= Nitric Oxide, (F); GSH= Glutathione (G); GPx= Glutathione Peroxidase, (H); GST= Glutathione S-Transferase. NS= Normal saline, SAS= Sodium-arsenite, 1TOC+SAS= 100 mg/kg Tocopherol + Sodium arsenite, 3TOC+SAS= 300 mg/kg Tocopherol + Sodium-arsenite, OLIV= Olive oil, 1TOC+OLIV= 100 mg/kg Tocopherol + Olive oil, 3TOC+OLIV= 300 mg/kg Tocopherol + Olive oil. Each bar represents means value and standard error,  $n=5$ . Significant difference is shown as \* $p < 0.05$  when compared with the control (NS), and # $p < 0.05$  when compared with the sodium-arsenite treated (SAS) only.

In the sodium arsenite alone group, lipid peroxidation was significantly enhanced by increased myeloperoxidase activity when compared with the normal saline (control) group ( $p < 0.05$ ). The groups treated with tocopherol alone showed no significance when compared with the normal saline group ( $p > 0.05$ ) either in MDA or MPO activity.

#### Effect of tocopherol on antioxidant systems of the gastrointestinal tissues exposed to sodium arsenite:

Sodium arsenite exposure reduced all the antioxidant enzymes activities viz: Superoxide dismutase (SOD), Catalase (CAT), Glutathione (GSH), Glutathione peroxidase (GPx) and Glutathione S-transferase (GST) in

the stomach (Figure 1c, d, f, g, h), ileum (Figure 2c, d, f, g, h) and colon (Figure 3c, d, f, g, h) tissues when compared with the normal saline (control) group ( $p < 0.05$ ). However, 100 mg/Kg and 300 mg/Kg tocopherol increased the activities of all the antioxidants in the gastrointestinal tissues when compared with the sodium arsenite alone treated group. Similarly, an erstwhile reduction in gastric, ileal, and colonic nitric oxide by sodium arsenite was reversed with tocopherol treatment ( $p < 0.05$ ) (Figure 1e, 2e, 3e). The groups treated with tocopherol alone showed no significance when compared with the normal saline group ( $p > 0.05$ ) either in the nitric oxide or antioxidant enzymes activities.

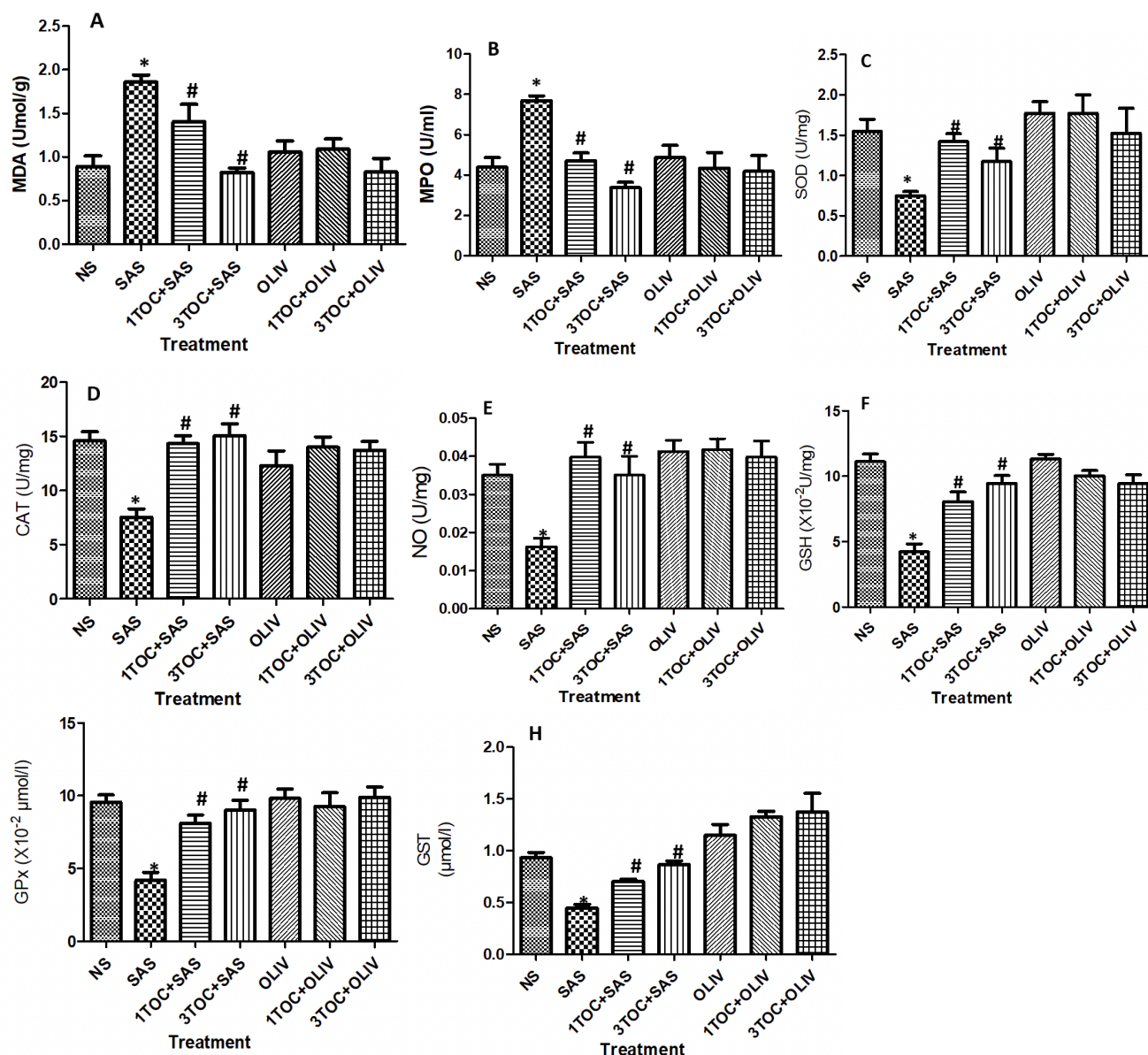
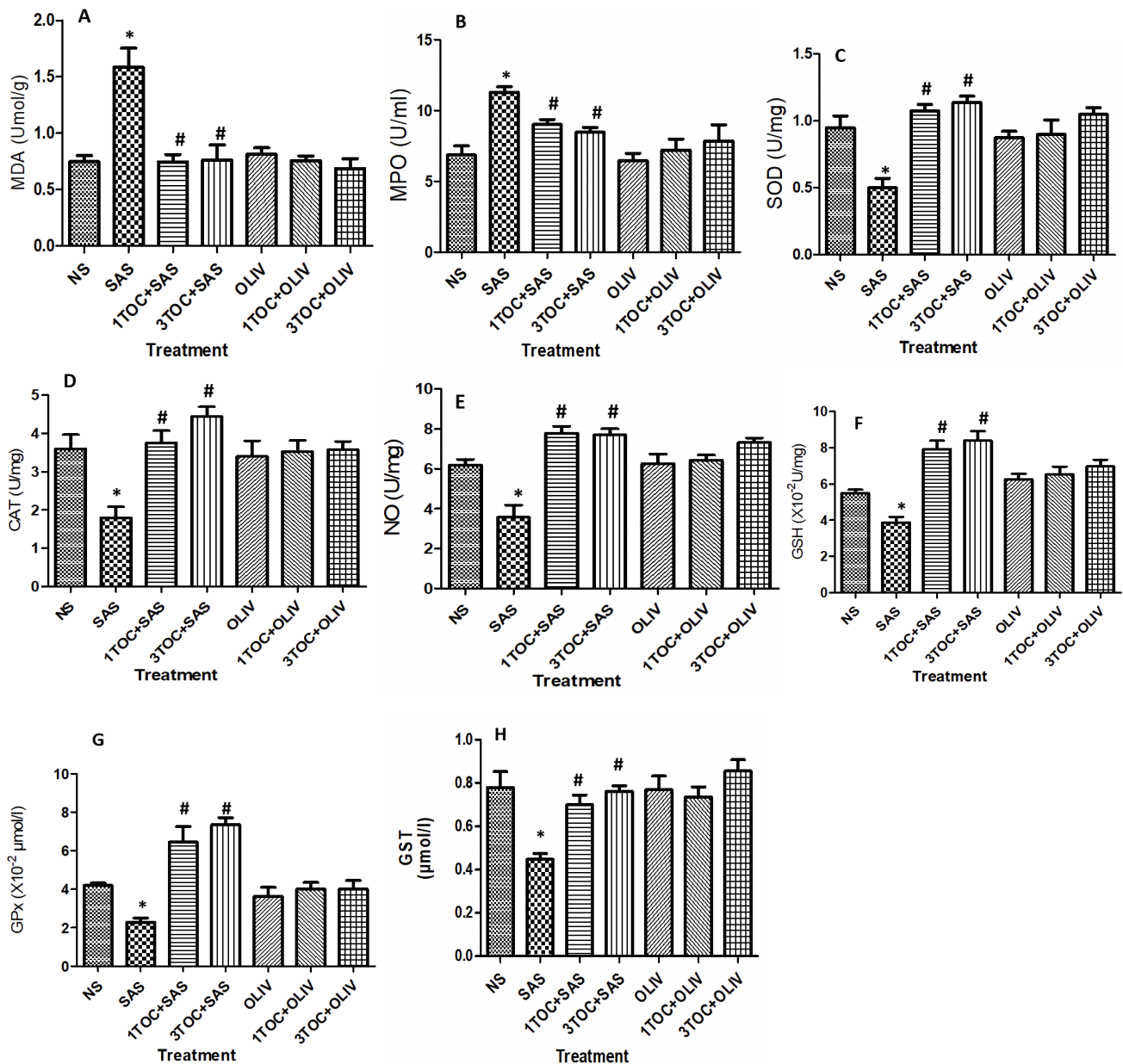


**Figure 2:**

**Redox status in normal and sodium-arsenite exposed ileum tissue upon treatment with tocopherol.** (A); MDA=Malondialdehyde, (B); MPO= Myeloperoxidase, (C); SOD= Superoxide dismutase, (D); CAT= Catalase, (E); NO=Nitric Oxide, (F); GSH=Glutathione (G); GPx=Glutathione Peroxidase, (H); GST=Glutathione S-Transferase. NS=Normal saline, SAS=Sodium-arsenite, 1TOC+SAS= 100 mg/kg Tocopherol + Sodium arsenite, 3TOC+SAS= 300 mg/kg Tocopherol + Sodium-arsenite, OLIV=Olive oil, 1TOC+OLIV= 100 mg/kg Tocopherol + Olive oil, 3TOC+OLIV= 300 mg/kg Tocopherol + Olive oil. Each bar represents means value and standard error,  $n=5$ . Significant difference is shown as \* $p < 0.05$  when compared with the control (NS), and # $p < 0.05$  when compared with the sodium-arsenite treated (SAS) only

**Figure 3:**

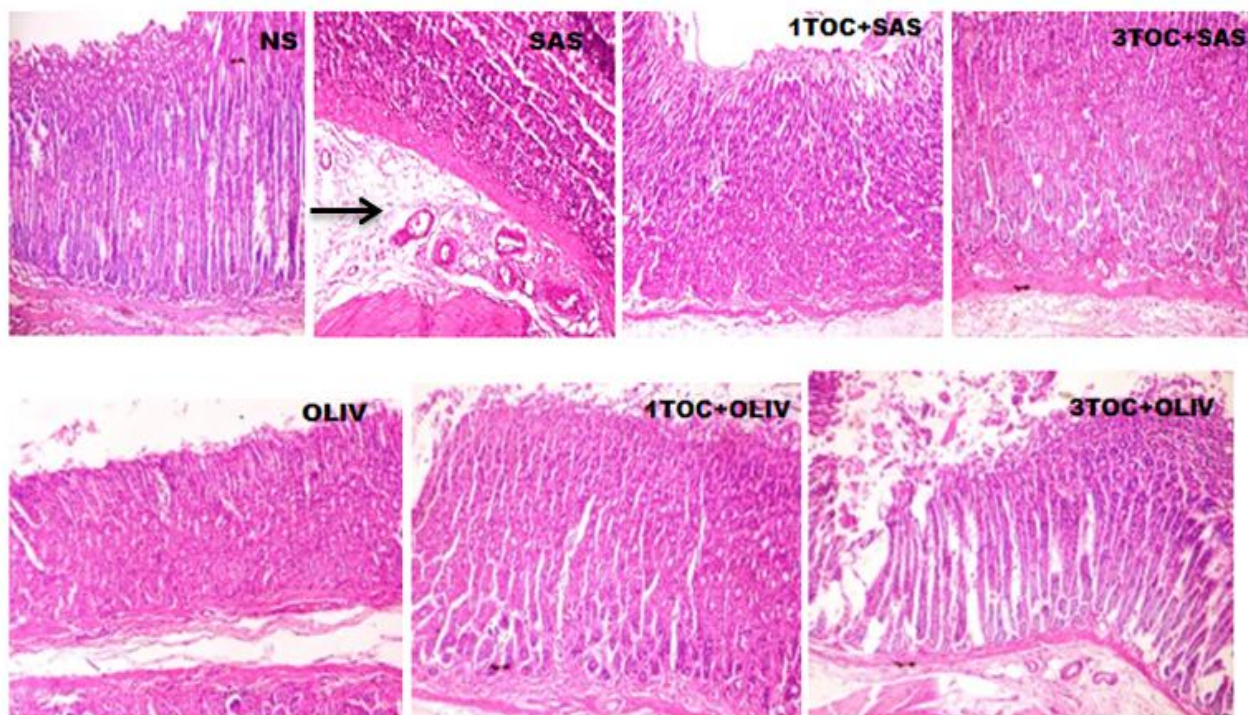
**Redox status in normal and sodium-arsenite exposed colon tissue upon treatment with tocopherol.** (A); MDA=Malondialdehyde, (B); MPO= Myeloperoxidase, (C); SOD= Superoxide dismutase, (D); CAT= Catalase, (E); NO=Nitric Oxide, (F); GSH=Glutathione (G); GPx=Glutathione Peroxidase, (H); GST=Glutathione S-Transferase. NS=Normal saline, SAS=Sodium-arsenite, 1TOC+SAS= 100 mg/kg Tocopherol + Sodium arsenite, 3TOC+SAS= 300 mg/kg Tocopherol + Sodium-arsenite, OLIV=Olive oil, 1TOC+OLIV= 100 mg/kg Tocopherol + Olive oil, 3TOC+OLIV= 300 mg/kg Tocopherol + Olive oil. Each bar represents means value and standard error, n=5. Significant difference is shown as \*p<0.05 when compared with the control (NS), and #p<0.05 when compared with the sodium-arsenite treated (SAS) only.

**Table 1:**

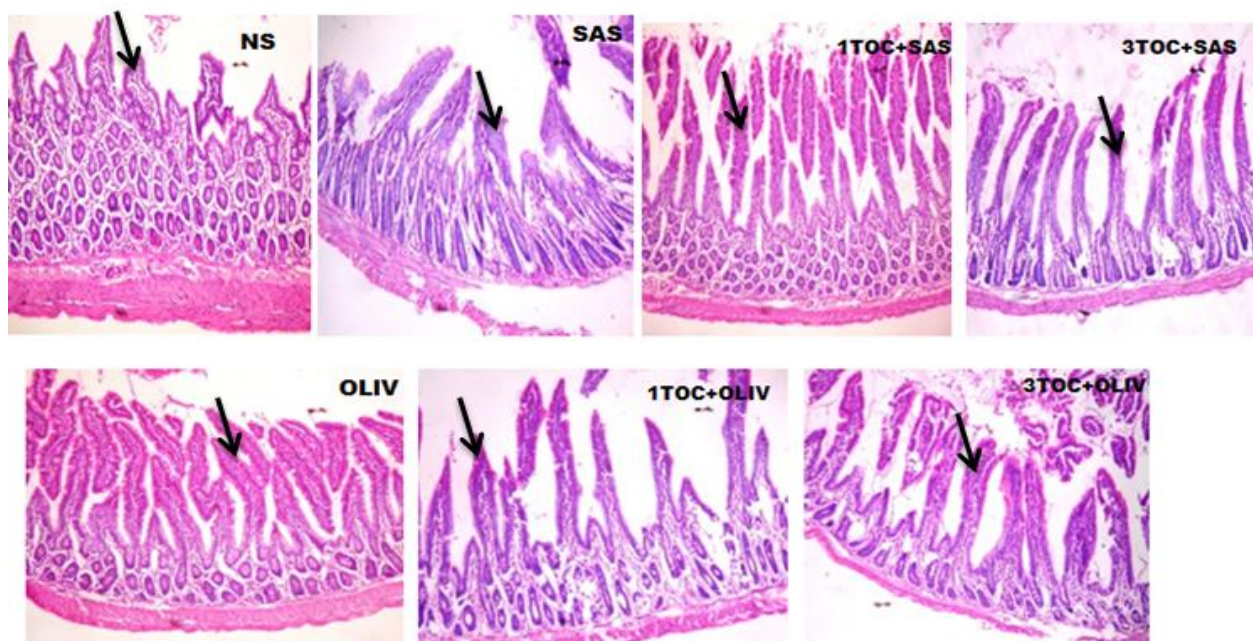
Effects of graded doses of tocopherol on sodium arsenite-induced toxicity in the rat's stomach: Parietal cell mass, mucous cell count, and Inflammatory cell infiltration

|           | Parietal Cell Mass | Mucous Cell Count | Inflammatory Cell Infiltration score |
|-----------|--------------------|-------------------|--------------------------------------|
| NS        | 8.60±0.40          | 13.20±0.37        | 0.60±0.20                            |
| SAS       | 15.00±0.30*        | 6.20±0.40*        | 2.20±0.20*                           |
| 1TOC+SAS  | 10.20±0.60#        | 8.80±0.30#        | 1.20±0.20#                           |
| 3TOC+SAS  | 10.40±0.50#        | 10.60±0.40#       | 1.20±0.20#                           |
| OLIV      | 8.30±0.37          | 12.00±0.30        | 0.60±0.20                            |
| 1TOC+OLIV | 9.20±0.58          | 14.80±0.35        | 0.60±0.20                            |
| 3TOC+OLIV | 8.80±0.37          | 13.40±0.50        | 0.60±0.20                            |

The value represents means value and standard error, n=5. A significant difference is shown as \*p<0.05 when compared with the control (NS), and #p<0.05 when compared with the sodium-arsenite treated (SAS) only.

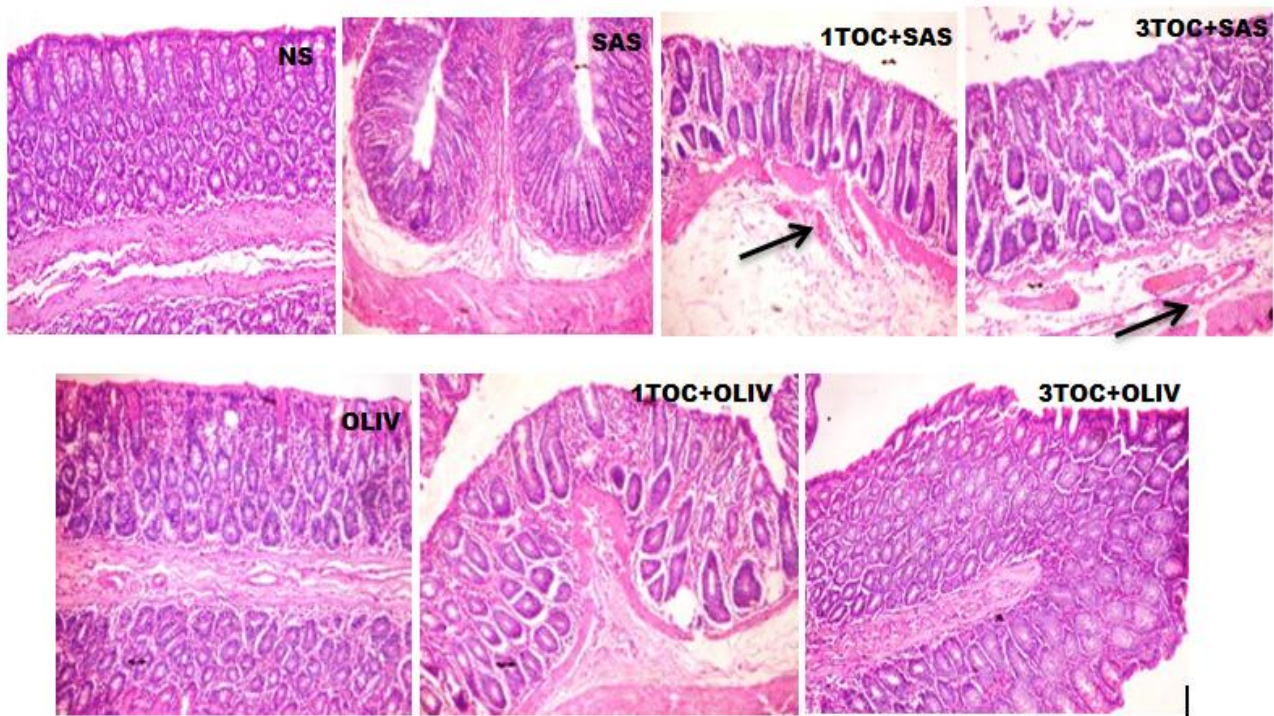
**Plate 1:****Histological presentation of normal and sodium arsenite exposed stomach treated with tocopherol. (X100)**

NS=Normal mucosa, submucosa and muscularis. The surface epithelial is well preserved. SAS= Mild edema (black arrow) and infiltration by acute inflammatory cells. 1TOC+SAS= Mild inflammation of the mucosa and submucosa. 3TOC+SAS= Normal mucosa, submucosa and muscularis. The surface epithelial is well preserved. No significant lesion. OLIV= Normal mucosa, submucosa and muscularis. The surface epithelial is well preserved. 1TOC+OLIV=Normal mucosa, submucosa and muscularis. 3TOC+OLIV=Normal mucosa, submucosa and muscularis.

**Plate 2:****Histological presentation of normal and sodium arsenite exposed ileum treated with tocopherol. (X100)**

NS= Normal villi structure, normal mucosa, submucosa, and muscularis, and normal crypt-villous architecture (black arrow) is well preserved. SAS= Focal area of loss of intestinal glands and gross distortion of the villous-crypt structure. 1TOC+SAS= Normal villi structure, normal mucosa, submucosa and muscularis, and the normal crypt-villous architecture. 3TOC+SAS= Normal villi structure, normal mucosa, submucosa, and muscularis; normal crypt-villous architecture. OLIV= Normal villi structure, normal mucosa, submucosa, and muscularis with normal crypt-villous architecture. 1TOC+OLIV=Normal villi structure, normal mucosa, submucosa, and muscularis, and normal crypt-villous architecture is well preserved. 3TOC+OLIV= Normal villi structure, normal mucosa, submucosa, and muscularis and normal crypt-villous architecture are well preserved.

*Sodium arsenite toxicity in GIT is ameliorated by tocopherol*

**Plate 3:****Histological presentation of normal and sodium arsenite exposed colon treated with tocopherol**

NS= normal mucosa, submucosa and muscularis. No significant lesion was seen. SAS= Focal area of loss of tubular glands and replacement by chronic inflammatory cells and fibroblasts. 1TOC+SAS= mild vascular congestion, 3TOC+SAS= mild vascular congestion (black arrow) and inflammation of the submucosa. OLIV=Normal mucosa, submucosa and muscularis. 1TOC+OLIV= normal mucosa, submucosa and muscularis. 3TOC+OLIV= normal mucosa, submucosa and muscularis.

**Table 2:**

Effects of graded doses of tocopherol on sodium arsenite-induced toxicity in the rat's ileum: Villus Height, Crypt Depth, and Inflammatory cell infiltration

|           | Villus Height (HD, $\mu\text{m}$ ) | Crypt Depth (CD, $\mu\text{m}$ ) | VH/CD                        | Inflammatory Cell Infiltration score |
|-----------|------------------------------------|----------------------------------|------------------------------|--------------------------------------|
| NS        | 120 $\pm$ 2.5                      | 51 $\pm$ 1.8                     | 2.35 $\pm$ 0.05              | 0.4 $\pm$ 0.2                        |
| SAS       | 55 $\pm$ 1.0*                      | 52 $\pm$ 2.0                     | 1.05 $\pm$ 0.01*             | 2.4 $\pm$ 0.2*                       |
| 1TOC+SAS  | 98 $\pm$ 2.0 <sup>#</sup>          | 55 $\pm$ 2.5                     | 1.78 $\pm$ 0.02 <sup>#</sup> | 1.0 $\pm$ 0.3 <sup>#</sup>           |
| 3TOC+SAS  | 100 $\pm$ 1.4 <sup>#</sup>         | 50 $\pm$ 1.5                     | 2.00 $\pm$ 0.01 <sup>#</sup> | 0.8 $\pm$ 0.2 <sup>#</sup>           |
| OLIV      | 110 $\pm$ 3.0                      | 51 $\pm$ 3.5                     | 2.15 $\pm$ 0.01              | 0.8 $\pm$ 0.2                        |
| 1TOC+OLIV | 125 $\pm$ 5.0                      | 54 $\pm$ 1.0                     | 2.31 $\pm$ 0.02              | 0.6 $\pm$ 0.2                        |
| 3TOC+OLIV | 120 $\pm$ 1.0                      | 50 $\pm$ 2.0                     | 2.40 $\pm$ 0.01              | 0.6 $\pm$ 0.2                        |

The value represents means value and standard error, n=5. A significant difference is shown as \*p<0.05 when compared with the control (NS), and <sup>#</sup>p<0.05 when compared with the sodium-arsenite treated (SAS) only.

**Table 3:**

Effects of graded doses of tocopherol on sodium arsenite-induced toxicity in the rat's colon: Goblet cell count, Crypt Depth, and Inflammatory cell infiltration

|           | Goblet cell count (Cells/field) | Crypt Depth ( $\mu\text{m}$ ) | Inflammatory Cell Infiltration score |
|-----------|---------------------------------|-------------------------------|--------------------------------------|
| NS        | 11.4 $\pm$ 0.4                  | 85 $\pm$ 1.5                  | 0.6 $\pm$ 0.2                        |
| SAS       | 5.8 $\pm$ 0.3*                  | 140 $\pm$ 2.0*                | 2.4 $\pm$ 0.2*                       |
| 1TOC+SAS  | 8.8 $\pm$ 0.3 <sup>#</sup>      | 100 $\pm$ 1.7 <sup>#</sup>    | 1.0 $\pm$ 0.3 <sup>#</sup>           |
| 3TOC+SAS  | 9.6 $\pm$ 0.4 <sup>#</sup>      | 95 $\pm$ 1.5 <sup>#</sup>     | 0.6 $\pm$ 0.2 <sup>#</sup>           |
| OLIV      | 10.4 $\pm$ 0.3                  | 82 $\pm$ 1.0                  | 0.6 $\pm$ 0.2                        |
| 1TOC+OLIV | 11.0 $\pm$ 1.0                  | 87 $\pm$ 2.2                  | 0.6 $\pm$ 0.2                        |
| 3TOC+OLIV | 10.8 $\pm$ 0.3                  | 80 $\pm$ 2.5                  | 0.6 $\pm$ 0.2                        |

The value represents means value and standard error, n=5. A significant difference is shown as \*p<0.05 when compared with

the control (NS), and <sup>#</sup>p<0.05 when compared with the sodium-arsenite treated (SAS) only

**Histology:** Upon histopathological examination of the gastrointestinal cytoarchitectural integrity, clear alterations and distortions were observed in the epithelium of the sodium arsenite alone group. Sodium arsenite provokes marked inflammatory cellular infiltration in the stomach (Plate 1), focal loss of glands (Plate 2 and 3), hyperplasia of crypts (Plate 3), atrophic villi, and hypertrophy of Peyer's patches in the intestines (Plate 2). All these were mitigated in the tocopherol treated group. With histomorphometry, it was observed that tocopherol annulled sodium arsenite-induced increase in parietal cell mass and decrease in mucous cell density in the stomach (Table 1), decrease in villus height and villus height/crypt depth ratio in the ileum (Table 2), and decrease in goblets cells and increase in crypt depth in the colon (Table 3).

## DISCUSSION

In the present study, we presented findings of sodium arsenite toxicity on the gastrointestinal tract, and subsequent improvement by tocopherol intervention. Sodium arsenite is an inorganic compound whose exposure causes damage to the gastrointestinal tract with clinical manifestations such as nausea, vomiting, and diarrhea (Jomova *et al.*, 2011). Previous workers have also reported cytoarchitectural alterations and lesions in the stomach and intestinal epithelium in laboratory animals treated with sodium arsenite (Hemalatha *et al.*, 2013).

Reactive Oxygen Species (ROS) plays an important role in lipid peroxidation implicated in the pathogenesis of many diseases. In this study, Arsenite (AS) increased the level of lipid peroxidation (MDA) and Myeloperoxidase activity (MPO) which are indicators of oxidative stress. The increased lipid peroxidation results in oxidative stress which occurs when the dynamic balance between pro-oxidant and antioxidant mechanisms is impaired (Flora *et al.*, 2008). This underscores the fact that AS exerts its effects through the production of free radicals that cause cellular damage as previous findings have shown increased lipid peroxidation even at low doses (Petrick *et al.*, 2000). Nevertheless, tocopherol showed a distinct reduction in MDA contents and MPO activity in the stomach, ileum, and colon of the rats, though not clearly in a dose-dependent fashion. The lessening effects of tocopherol might easily be linked to its antioxidant property.

Upon histological examination, a clear correlation was established between increased MPO activity and inflammatory cell infiltration in the GI segments which is a common phenomenon with arsenic toxicity and evidenced in this study. The activated inflammatory cell infiltration observed in the sodium arsenite-treated stomach, ileum, and colon was mitigated by tocopherol. This suggests that the anti-inflammatory effect of tocopherol seen in this study is via lowering the activated and infiltrated inflammatory cells in the tissues and associated MDA and MPO. Several lines of evidence have demonstrated how infiltrated neutrophils and inflammatory cells release reactive oxygen species upon which altered MDA content and MPO activity is predicated (Ajeigbe *et al.*, 2014). In the stomach, parietal cells increased while the mucous cells decreased in the arsenite group which was upturned with tocopherol. Parietal cells secrete hydrochloric acid hugely needed for protein digestion, and there exists a direct relationship between parietal cell count and the acid output. Hence, this lends credence to the findings of Adebayo-Gege *et al.*, (2018), that reported parietal cell hyperplasia and elaborated acid secretion in sodium arsenite-treated stomachs. Focal loss of glands, hyperplasia of crypts, atrophic villi, and hypertrophy of Peyer's patches in the intestines of the arsenite treated were all lessened with tocopherol treatment. Khan *et al.*, (2013) had equally reported crypt hyperplasia and sloughing of villi in broiler chicks exposed to sodium arsenite. Alteration in the colonic crypt architecture and depletion of goblet cells are other histological ways of grading inflammation in the gastrointestinal system (Kim *et al.*, 2012). Interestingly, tocopherol treatment reversed all these.

Further, data from this study revealed enhancement of Superoxide Dismutase and Catalase activities by tocopherol

in all the GI segments examined. As the first line of defense against oxidative stress, SOD and CAT are essential in maintaining cellular health and protecting the body from free radicals and other harmful agents. While there was a deficiency of these important enzymes in the gut samples of the group exposed to arsenite, an upgrade in the level of these enzymes was evident in all the groups treated with tocopherol. SOD is known for its ability to convert superoxide radicals into  $H_2O_2$  while CAT breaks  $H_2O_2$  into water molecules. An elevation of superoxide radicals is associated with reduced SOD and CAT (Ighodaro and Akinloye 2018), to a large extent as it is seen in the arsenite-only treated group.

Glutathione serves as a second line of defense in the body and plays an important role in preventing free radicals with its -SH group. Glutathione-related enzymes like GPx, GST function in the detoxification process to regulate cellular homeostasis (Yamanaka *et al.*, 1991). The results from the study showed that there was a significant alteration in the GSH level. For recycling of this enzyme, GPx catalyzes the conversion of GSH into GSSG and regeneration of GSH from GSSG. Hence, activities of GPx and GR are strictly regulated with the changes in the GSH level. In the current study, the decrease observed in both GPx and GST activities is an easily attributable reduction in GSH level in the stomach, ileum, and colon of the arsenite-only treated rats when compared to the control. It can thus be hypothesized that altered glutathione-linked enzymes lead to an oxidative imbalance in all the GI segments examined and precipitated oxidative stress. Meanwhile, tocopherol reversed the depletion of GSH, GST, and GPx. Alterations of nitric oxide (NO) in the periphery have been detected after arsenic exposure. The nitroergic dysfunction can also be linked to oxidative stress since inadequate levels of tetrahydrobiopterin, one of the cofactors for NO synthesis are thought to contribute to superoxide formation by nitric oxide synthase (NOS) (Kamada *et al.*, 2005). In this study, the activity of NO decreased in the arsenite group, which also presented the characteristic effects of lipid peroxidation and increase of ROS production associated with arsenic exposure.

In conclusion, tocopherol is a great dietary supplement that has the potential to mitigate arsenic toxicity on the gastrointestinal system via its anti-oxidative properties.

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## Full-Length Research Article

# Age-Dependent Effects of Copper Toxicity on Connective Tissue Structural Stability in Wistar Rats Skin

El-ta'alu A.<sup>1</sup> and Ahmad M.M.<sup>2</sup><sup>1</sup>Department of Human Physiology, Faculty of Basic Medical Sciences, Bayero University, Kano.<sup>2</sup>Department of Physiology, Khon Kaen University, Khon Kaen 40002, Thailand

**Summary:** Over the last three decades, there has been increasing global concern over the public health impacts attributed to direct and indirect environmental pollution, in particular, the global burden of disease. The World Health Organization estimates that, about a quarter of the diseases facing mankind today occur due to prolonged exposure to environmental pollution; the health of 200 million people in lower-income countries is at risk from toxins such as lead, copper, mercury, and that, nearly a quarter of deaths in developing countries including Nigeria and Ghana, are linked to pollution. The purpose of study was to investigate the effects of ingestion of large dose of copper on the structural stability of collagen molecules, as well as reveal age-dependent differences in the phenomena. The content of de novo synthesized collagen was determined by hydroxyproline concentration using Stegmann-Staeder's method as modified by Utevskaia and Persky; the nature of intra- and inter-molecular covalent cross-links in collagen matrix was estimated by electrophoretic separation of products of partial thermal denaturation of collagen in polyacrylamide gel. There was intensification of synthesis over degradation in young rats, and that administration of copper led to a decrease in collagen solubility. This study concluded that, effects of copper toxicity on the structural stability of collagen appeared mostly in young rats.

**Keywords:** Age, Collagen, Copper, Hydroxyproline, Thermo-Stability, Structural Stability.

©Physiological Society of Nigeria

\*Address for correspondence: E-mail: [nenemakoju@yahoo.com](mailto:nenemakoju@yahoo.com); Tel: 08037053285

Manuscript received- December- 2021; Accepted- March, 2022

DOI: <https://doi.org/10.54548/njps.v37i1.12>

## INTRODUCTION

Most environment-related diseases are not easily detected but may be acquired during childhood and manifested in later ontogenesis (UN Report, 2017). One type of pollution that bears a pronounced technological character is that of salts of heavy metals: lead, mercury, copper, arsenic, to mention a few, the level of pollution of these compounds is increasing every year.

Copper (Cu) is an essential trace element for humans and animals because it is a key constituent of the respiratory enzyme cytochrome oxidase (MIC, 2019, Muriel *et al.*, 2016). In the human body, copper shifts between the cuprous ( $\text{Cu}^{1+}$ ) and cupric ( $\text{Cu}^{2+}$ ) forms, though the majority of the body's copper is in the latter form. The ability of copper to easily accept and donate electrons explains its significance in oxidation-reduction (redox) reactions, involving copper-containing oxidases; and in scavenging free radicals (Peter, 2018). Copper is a critical functional component of several essential enzymes known as cupro-enzymes (Ludmila *et al.*, 2018, Peter, 2018, Prohaska, 2011). A cupro-enzyme, lysyl oxidase, is required for the cross-linking of collagen and elastin, which are essential for the formation of strong and flexible connective tissue. The action of lysyl oxidase helps maintain the integrity of

connective tissue in the heart and blood vessels and also plays a role in bone formation (Prohaska *et al.*, 2012).

The oxidative potential of copper may be responsible for some of its toxicity in excess ingestion cases (Turnlund, 2006). At high concentrations, copper is known to produce oxidative damage to biological systems, including peroxidation of lipids or other macromolecules. It is therefore important to study the impact of this toxic metal on living organisms. The commonest type of tissue in the human body is the connective tissue and therefore, the effect of copper pollution on its various components must be the commonest in the body as a whole.

One of the most important functional and structural components of the connective tissue is collagen (Elena *et al.*, 2013). Unlike most other proteins, the turnover of collagen is extremely slow and can therefore, reflect age-related changes (Bailey and Paul, 1999). There is no doubt that, a change in collagen matrix under the influence of various toxic substances will have a significant impact on both the connective tissue and organs containing it. The stabilisation of collagen fibers during development through growth to maturation is now fairly documented; it is a carefully controlled enzymatic process, which produces inter-molecular cross-links at specific locations (Muriel *et al.*, 2016, NPIC, 2014).

Collagen concentration in tissues; the degree and types of protein's cross covalent bonds; as well as content of hydroxyproline in individual collagen molecules, are the basic parameters of structural stability of matrix sub-molecular collagen complexes.

The biomedical potential of natural collagen is limited by its poor mechanical strength, thermal stability, and enzyme resistance, but exogenous physico-chemical or biological cross-links have been used to modify the molecular structure of the protein to minimize degradation and enhance mechanical stability. By way of thermal stability of collagen in sub-molecular complexes of the extracellular matrix, structural stability of the skin could be assessed (Altug and Bumsoo, 2016, Selestina and Vanja, 2011, Tsereteli *et al.*, 1996).

Changes in collagen physical properties that occur towards old age are stochastic and involve oxidative reactions that result in the formation of glucose-mediated cross-links. This excessive and random cross-linking leads to deterioration in the functional properties of the connective tissue at old age, i.e. leads to a devastating loss of tissue functionality of vital organs (Chan-Sik *et al.*, 2017, Jess and Alfonso, 2014, NPIC, 2014, Elena *et al.*, 2013, Yamauchi and Sricholpech, 2012 Turnlund, 2006, Waller and Maibach, 2006). In addition, specific residues involved in cell-matrix interactions may become chemically modified; and this can affect the expression of cells, as well as lead to the formation of inappropriate collagen matrix during its slower turnover in ontogenesis (UN Report, 2017). This is exemplified in the ubiquitous disorders, osteoporosis and osteoarthritis, as well as age-related diseases in which gene-regulated changes in the collagen are deposited. At the same time, post-translational changes such as over-hydration of lysine residues were noted (Chan-Sik *et al.*, 2017, Jess and Alfonso, 2014, NPIC, 2014, David *et al.*, 2019); both effects can have a profound deleterious impact on the functioning of the matrix tissue. In view of this, the study of the influence of copper heavy metal on collagen structural stability is therefore important.

Hydroxyproline (4-OHPr), is a non-proteinogenic amino acid formed by the post-translational hydroxylation of proline, and is a major component of collagen, where it serves to stabilize the helical structure; it accounts for 13-14% of collagen's total amino acid content (Ivan *et al.*, 2018, Paietta, *et al.*, 2013, Richard and Rosemary, 2007). Endogenous hydroxyproline reflects rate of degradation of collagen, which in turn, is related at least in part, to the rate of synthesis of collagen; due to its highly restricted distribution in collagen, the measurement of OHPr levels can be used as an indicator of both the presence and metabolism of collagen (Carmen, *et al.*, 2016, Bishnu. and Eric, 2014, Kondo, *et al.*, 2014, Huije, 2013, Corrine *et al.*, 2011, El-ta'alu *et al.*, 2010, Deyl and Miksik, 2000). We estimated this de novo synthesized collagen content using oxidation and condensation methods earlier proposed by Stegmann and Staeder (1967) as modified by Utevskaia and Persky (1982). The method is based on condensation reaction of products obtained from the interaction of hydroxyproline oxidation (pyrole) with para-di-methyl-amine-benzo-aldehyde (DABA). Separation of products of partial thermal collagen denaturation was carried out by electrophoresis in polyacrylamide gel (Carmen, *et al.*, 2016, Deyl and Miksik, 2000, Corrine *et al.*, 1979).

The purpose of this study was to investigate copper toxicity on the structural stability of collagen molecules, as well as reveal age-dependent differences in the phenomena. At present however, there are practically no reports that focus on age-related effects of copper toxicity on integrated structural stability of collagen structures. It is therefore, expedient to deeply study the aforementioned phenomenon, as well as the consequential impact on the structural stability of collagenous structures. To achieve this aim, the following tasks were set as objectives: 1. Determine the concentration of hydroxyproline in the skin and based on this, the content of de novo synthesized collagen in the organ. 2. Estimate the nature of intra- and inter-molecular covalent cross-links in collagen matrix based on analysis of electrophoretic separation of products of partial thermal denaturation of collagen in polyacrylamide gel. 3. Evaluate the stability of collagen cross-links through determination of the degree of collagen solubility in neutral salt solution and determination of the degree of thermal stability of collagen samples.

## MATERIALS AND METHODS

**Subjects/Materials and Methods:** The study was partly carried out in A.M Gorky Kharkov State University, Ukraine, and partly in the Physiology Research Laboratory of the Department of Human Physiology, Faculty of Basic Medical Sciences, Bayero University, Kano-Nigeria. Experimental animals were handled in accordance with international principles of the European Convention «On protection of vertebrate animals used for experimental and other scientific purposes» (European Convention, 1985), as well as norms of biomedical ethics in accordance with the Law of Ukraine «On protecting animals from wicked handling».

A total of 40 male Wistar rats comprising of eleven 3-months old (young) and nine 20-months old (adults) animals weighing between 150-250 grams were studied. Rats that corresponded with the same age-groups were used as control. Before decapitation, animals received large doses of copper, three (3) times after every 48 hours by intravenous injection of 1mg of CuSO<sub>4</sub>/100g of body weight (NPIC, 2014).

In order to study the trend of the effect of copper on collagen protein, loose connective tissue of dorsal skin (skin of the spine region) of young and adult Wistar rats was chosen (Zaki, 2015). This allowed the tracing of not only the general pattern of copper pollution, but also revealed the age-related characteristic features of its effects. Briefly after obtaining informed consent, Wistar rats were lulled to sleep by intravenous injection of 0.5cm<sup>3</sup> of 0.4% solution of sodium thiopental (Stephen, 2002), and then decapitated. Dorsal skin samples (20 × 5) mm were cleaned from subcutaneous fat and hair.

Data were expressed as Mean±SEM. Chi-square method and Student's Test were used to determine differences in hydroxyproline concentration between normal and psoriatic patients. All analyses were carried out using IBM-SPSS version 20.0 statistical software. P < 0.05 was set as level of significance.

**Skin Incubation:** Samples of skin were incubated in Ringer-Kreb's medium for warm-blooded animals, which contained 9.075g of tris HCl; 8.2998g NaCl; 0.5206g KC1;

2222g  $\text{MgCl}_2$ ; 0.4333g  $\text{K}_2\text{HSO}_4$  and 5.4g of  $\text{C}_6\text{H}_2\text{O}_6$  in 1 litre of distilled water (El-ta'alu *et al.*, 2010). Incubation was carried out for 6 hours, at  $37^\circ\text{C}$ .

**Isolation of Type I Collagen:** Incubated samples were defatted using acetone and diethyl ether for 24 hours in each case, weighed, and ground to powder form in liquid nitrogen. Type I collagen was extracted from the powder using 1M solution of NaCl; and freshly synthesized collagen isolated from the extract was obtained by dialysis against phosphate buffer solution (pH 8.0) for 10 days with daily change of buffer (El-ta'alu *et al.*, 2010).

**Determination of the Content of *de novo* Synthesized Collagen:** This was carried out by hydroxyproline concentration using Stegmann-Staeder's method as modified by Utevskaia and Persky;

**Determination of the Nature of Intra- and Inter-molecular Covalent Cross-links in Collagen Matrix:** This was estimated by electrophoretic separation of products of partial thermal denaturation of collagen in polyacrylamide gel (Laurent and Marianne, 2011, Corrine *et al.*, 1979).

**Evaluation of Stability of Collagen Cross-links:** To determine the extent and nature of cross-links in collagen of studied samples, the degree of protein's solubility in neutral salt solution, before and after heating, was studied (Laurent and Marianne, 2011, Marta and Krystyna, 2012).

**Determination of Degree of Collagen Solubility in Neutral Salt Solution :** About  $5\text{cm}^3$  of 1M solution of NaCl were added to 100mg of fresh skin samples. Extraction was carried out for 3 days in the cold, and collagen released into solution was precipitated by adding dry NaCl up to a concentration of 3.5M. Solubility of collagen was evaluated by the ratio of hydroxyproline released into solution during collagen extraction to the total content of hydroxyproline in the skin before extraction, and the resulting value was expressed in percentage.

**Determination of the Degree of Collagen Thermal Stability:** 100mg of fresh skin samples were heated in  $3\text{cm}^3$  of distilled water for 20 minutes at  $50^\circ\text{C}$ . The degree of thermal stability of collagen was evaluated by the relative ratio of hydroxyproline concentration released from collagen into solution to its total content before heating. Obtained result was expressed in percentage.

## RESULTS

Results on age-related effect of copper on hydroxyproline concentration in the skin of rats, as well as changes in total collagen degradation in age groups (Table 1; Figure 1) showed an intensification of synthesis over degradation in young rats.

Tables 2 and 3; Figures 2 and 3 showed that, administration of copper led to a decrease in collagen solubility in 1M solution of NaCl before and after heating in both age groups; these significant changes in solubility were observed only in young rats.

**Table 1:** Age-dependent Effect of Copper Administration on Hydroxyproline Concentration in the Skin of Rats

| Age of Rats (Months) | Condition of Investigation | Hydroxyproline Concentration (mg/100mg of Fresh Skin) |
|----------------------|----------------------------|-------------------------------------------------------|
| 3                    | Control                    | $1.81 \pm 0.09$                                       |
|                      | Experiment                 | $1.94 \pm 0.08$                                       |
| 20                   | Control                    | $2.80 \pm 0.11$                                       |
|                      | Experiment                 | $2.63 \pm 0.09$                                       |

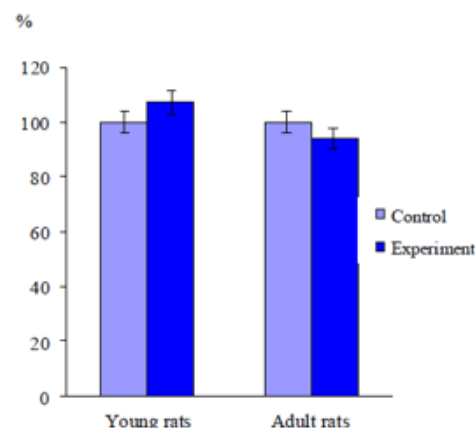


**Figure 1:** Age-dependent Effect of Copper Over-dose on Hydroxyproline Concentration in the Skin of Wistar Rats (% of Control)

**Table 2:** Age-dependent Effect of Copper Administration on Collagen Solubility before Heating in 1M Solution of NaCl

| Age of Rats (Months) | Condition of Investigation | Content of Collagen in Solution (% of Total Collagen Concentration) |
|----------------------|----------------------------|---------------------------------------------------------------------|
| 3                    | Control                    | $2.31 \pm 0.08$                                                     |
|                      | Experiment                 | $2.05 \pm 0.11^*$                                                   |
| 20                   | Control                    | $1.50 \pm 0.08$                                                     |
|                      | Experiment                 | $1.41 \pm 0.08$                                                     |

\* Significant compared to control ( $P < 0.05$ )

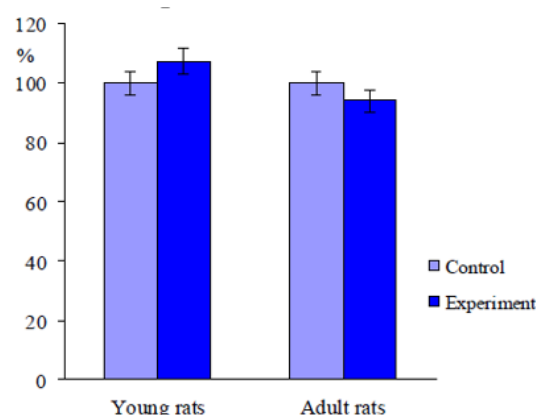


**Figure 2:** Age-dependent Effect of Copper on Skin Collagen Solubility in 1M NaCl Solution (% of Control)

**Table 3:**

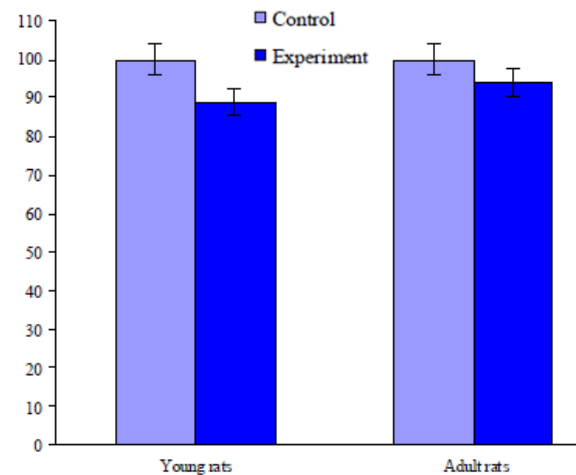
Age-dependent Effect of Copper Administration on Collagen Solubility after Heating in 1M Solution of NaCl

| Age of Rats (Months) | Condition of Investigation | Content of Collagen in Solution (% of Total Collagen Concentration) |
|----------------------|----------------------------|---------------------------------------------------------------------|
| 3                    | Control                    | 22.1±1.0                                                            |
|                      | Experiment                 | 19.6±0.9*                                                           |
| 20                   | Control                    | 9.0±0.3                                                             |
|                      | Experiment                 | 8.1±0.4                                                             |

\* Significant compared to control ( $P < 0.05$ )

Analyses of cross-links in collagen of control and experimental rats by electrophoretic separation of collagen that was released into solution after heating (thermo-labile collagen - Table 4; figures 4 and 5) showed a marked increase in collagen structures in both study groups. This effect was manifested in the decrease of relative content of  $\alpha$ -particles, corresponding to free unbound cross-linked  $\alpha$ -chains. At the same time, the relative content of  $\alpha$ -linked chains increased. It should be noted that, such an increase was expressed in both young and adult rats (3- and 20-months old, respectively). An increase in the stiffness in the linking of  $\alpha$ -chains in both young and adult rats were different: intensification of cross-linking in 3-months old rats was accompanied not only by a rise in the number of  $\beta$ -

particles but also by an increase in the content of  $\gamma$ -particles; in 20-months old rats, an increase was observed only in the content of  $\beta$ -particles (Table 5).

**Figure 3:**

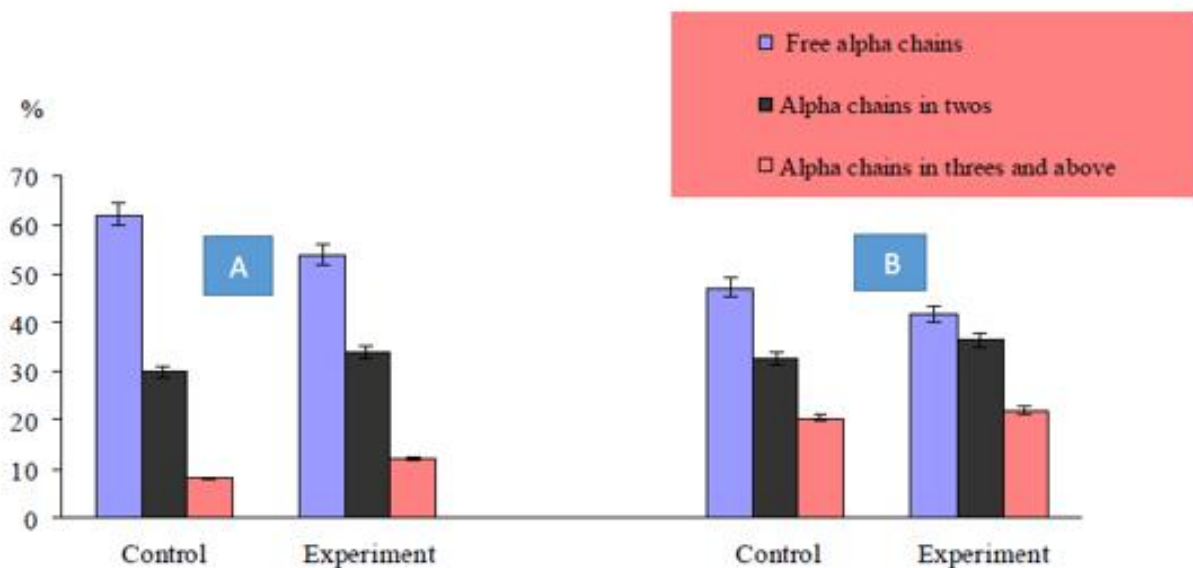
Age-dependent Effect of Copper Over-dose on Collagen Solubility After Heating (% of Control)

**Table 4:**

Age-dependent Effect of Copper Administration on Relative Contents of  $\alpha$ -chains of Varying Degrees of Polymerization in Skim Collagen after heating (% of Heat-labile Collagen)

| Age of Rats (Months) | Condition of Investigation | Content of $\alpha$ -chains of Different Degrees of Bonding (%) |                                  |                                              |
|----------------------|----------------------------|-----------------------------------------------------------------|----------------------------------|----------------------------------------------|
|                      |                            | Free $\alpha$ -chains                                           | $\alpha$ -chains Bound (in twos) | $\alpha$ -chains Bound (in threes and above) |
| 3                    | Control                    | 62.1±0.7                                                        | 29.9±0.3                         | 8.0±0.1                                      |
|                      | Experiment                 | 53.9±0.6*                                                       | 34.0±0.3*                        | 12.1±0.1*                                    |
| 20                   | Control                    | 47.1±0.5                                                        | 32.5±0.2                         | 20.4±0.1                                     |
|                      | Experiment                 | 41.7±0.6*                                                       | 36.4±0.3*                        | 21.9±0.2                                     |

\* Significant compared to control ( $P < 0.05$ )

**Figure 4:**

Effect of Copper Over-dose on the Degree of Polymerization of  $\alpha$ -chains of Thermo-labile Collagen in 3-months (A) and 20-months old (B) Wistar Rats (%).

**Table 5:**Age-dependent Effect of Copper Administration on the Ratio  $\beta$ -particles: $\gamma$ -particles in Heat-labile Collagen of the Skin

| Age of Animals<br>(Months) | Control | Experiment |
|----------------------------|---------|------------|
| 3                          | 3.74    | 2.80       |
| 20                         | 1.59    | 1.66       |

## DISCUSSION

Intensification of synthesis over degradation of collagen in young rats. These changes subsequently became balanced. Thus, changes in collagen concentration due to copper toxicity are reflections of different sensitivities of synthetic and catabolic processes to the toxic effects of copper in the skin of both young and adult Wistar rats. In the skin, where damaging effects are less; the degree of cross-linking, assessed based on the solubility of collagen in neutral salt solutions before and after heating varied depending on the age of rats. This agrees with findings by El-ta'alu *et al* (2010).

It should be noted that, thermal destruction of the collagen matrix in the skin was less pronounced than in neutral salt solution; the degree of the decrease in solubility for 1M solution of NaCl and temperature during heating were about the same (about 10%). In the skin of experimental rats, exit of collagen into solution after heating, and after treatment with salt, was also about the same as in young rats but remained below the reference value (in controls - about 5-7%). As reported by Laurent and Marianne, 2011, as a result of this, the difference in reliability from the control, for solubility in salt solution was only 90%.

It has been well documented by Marta and Krystyna (2012) that the different types of cross-covalent bonds in collagen have different sensitivities to thermal treatment. Cross-links formed in the initial stages of the process of collagen cross-linking through Aldole condensation, were more readily destroyed by heat than aldimine bonds that were formed later (Laurent and Marianne, 2011). Modified as a result of further post-synthetic modifications of cross-links, as a result of for example (1) changes in Schiff's bases to form ketoamines, and (2) formation of poly-functional cross-links, with the formation of pyridine cross-links, etc., cross-links were less destroyed by heat (Laurent and Marianne, 2011). This led to the fact that, heat initially destroyed labile cross-links, and then thermo-stable ones and as a result, aggregates of  $\alpha$ -chains of varying degrees of bonding found their way into solution depending on the number of the bonds themselves and their chemical nature.

Analyses of cross-links in collagen of control and experimental rats by electrophoretic separation of collagen that was released into solution after heating (thermo-labile collagen) showed a marked increase in collagen structures in both study groups was manifested in the decrease of relative content of  $\alpha$ -particles, corresponding to free unbound cross-linked  $\alpha$ -chains. At the same time, the relative content of  $\alpha$ -linked chains increased. It should be noted that, such an increase was expressed in both young and adult rats (3- and 20-months old, respectively). It is interesting to note that, an increase in the stiffness in the linking of  $\alpha$ -chains in both young and adult rats were

different: intensification of cross-linking in 3-months old rats was accompanied not only by a rise in the number of  $\beta$ -particles but also by an increase in the content of  $\gamma$ -particles; in 20-months old rats, an increase was observed only in the content of  $\beta$ -particles. This also agrees with the findings of El-ta'alu *et al* (2010).

This study concluded that effects of copper appeared mostly in young rats; copper toxicity led to stiffness and hardening of collagen structures of the skin in 3- and 20-months old Wistar rats and this was a result of increased intra- and inter-molecular bonding; and in adult rats mainly due to only intra-molecular bonding; the toxicity also led to a decrease in the concentration, and an increase in the degradation of collagen; there was a decrease in the solubility of collagen after heating in 1M solution of NaCl; cross-linking of collagen increased in 3- and 20-months old Wistar rats.

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Full-Length Research Article

# A Comparative Study of Skull Shape Variations in *Eidolon helvum* (African Fruit Bat) from Two Geographical Locations in Nigeria

**\*Igado, O. O. and Joannis, J. S**

Department of Veterinary Anatomy, University of Ibadan, Nigeria

**Summary:** The shape and size of a skull provides insight into the age, breed and gender of the animal. Skull shape variations have been reported in different animals, with some theories linking these variations to evolution and/or migration. This study assessed the variations observed in the skull shape, size and gross morphometrics of two groups of the *Eidolon helvum* obtained from two geographical regions in Nigeria (south and north). All skulls were rostro-caudally elongated, having a dolichocephalic appearance. The skulls from the north had a distinct dome shape, with a more prominent zygomatic process, absence of a 'diastema' and an extra upper molar, while the southern skulls showed a more dorsally flattened skull and a less prominent zygomatic process. The shape of the sagittal crest was different in the two groups, while there was the presence of an accessory infraorbital foramen in some of the southern skulls. The southern skulls lacked the palatine foramen. The lacrimal foramen was observed to be more caudally placed in the southern skulls. Values for most linear measurements were higher in the northern skulls, although statistically significant difference was not present in all. The value for the neurocranial volume was considerably higher in the northern skulls ( $4.41 \pm 0.28$  mls) relative to the southern skulls ( $2.0 \pm 0.27$  mls). Statistically significant differences were not observed between males and females (within regions). Data obtained from this study may find application in evolution and migration studies, wildlife medicine and surgery and comparative and forensic anatomy.

**Keywords:** Anthropometry; clinical implications; craniofacial osteometrics; megachiroptera; wildlife and comparative anatomy

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\*Address for correspondence: E-mail: [mayowaigado@yahoo.com](mailto:mayowaigado@yahoo.com); [oo.igado@mail1.ui.edu.ng](mailto:oo.igado@mail1.ui.edu.ng); Tel: +234-8035790102

Manuscript received- November- 2021; Accepted- April, 2022

DOI: <https://doi.org/10.54548/njps.v37i1.13>

## INTRODUCTION

Craniometry is an anthropometric parameter that involves the measurement of cranial features to categorise them according to race, intelligence, etc (Durbar, 2014). By extension, cranial osteometrics (measurement of bones of the skull) has proven to be invaluable in identifying species, breeds and gender, providing baseline anatomical data and finding usefulness in comparative anatomy and forensics. In recent years, there has been an increasing report in animals, for example, goats (Olopade and Onwuka, 2004), bats (Bullen and McKenzie, 2008), and dogs (Igado, 2017; Janeczek *et al.*, 2008; Onar and Günes, 2003).

This study focuses on the variation of the skull shape, size, general arrangement of some sutures and gross morphometrics of the *Eidolon helvum* (the straw-coloured fruit bat), obtained from two different geographic locations (south and north) in Nigeria.

In Africa, the *E. helvum* is reputed to be the most conspicuous megachiroptera (large bats), it is widely distributed throughout sub-Saharan Africa, and is reputed to be the most hunted for bush meat among all bat species in West Africa (Gibson *et al.*, 2021; Richter and Cumming, 2006). It is the second largest, is fruit-eating and also bears unique morphological and ecological characteristics. It is however a poorly diversified genus (Juste *et al.*, 2000),

having only the species *E. helvum*. It belongs to the Order Chiroptera, Suborder Megachiroptera, Family Pteropodidae, Genus *Eidolon* (DeFrees and Wilson, 1988). It is the only known flying mammal, with an average body weight of  $225 \pm 33$  g and average wing span of  $76 \pm 7$  cm. They are reputed to be of economic importance (pollination), economic waste (destruction of fruits) and of public health importance (transmission of the rabies virus) (Balthazary *et al.*, 2007).

Being highly diversified, having over 1,400 species, bats have been reported to have significant variation in ecology and morphology, and so offer myriads of opportunities for studies (Brokaw and Smotherman, 2020) involving understanding the correlations between morphology, ecology, evolution and adaptability.

This study of the variation in the skull shape and characteristics of the *E. helvum* may play a role in better understanding the evolution of the frugivorous bats, while also finding application in the field of wildlife craniofacial/maxillofacial applied anatomy.

## MATERIALS AND METHODS

**Ethical considerations:** Ethical approval for this study was obtained from the Ethical Committee of the Faculty of Veterinary Medicine, University of Ibadan, Nigeria, Ethical

code number 'ethic/12/13/03'. All procedures followed the Guide for the care and use of experimental animals, according to guidelines by the National Institute of Health (NIH), USA and the Animal Care Use and Research Ethics Committee (ACUREC), University of Ibadan, Nigeria. All bats were handled humanely, to avoid undue stress or pain to them.

**Trapping of bats and sacrifice:** A total of nineteen adults (10 males and 9 females) were used for this study. Bats were obtained from two geographical locations in Nigeria – 4 males and 3 females from Pandam Game Reserve, Qua'an Pan Local Government Area, Plateau State (Northern Nigeria) and 3 males and 3 females from forests and roosting sites in Ibadan, Oyo State (Southern Nigeria). An additional 6 skulls (3 males and 3 females) obtained previously from *E. helvum* from the same roosting sites in Ibadan were added to the specimens, bringing the total number of the southern skulls to 12 (6 males, 6 females). A larger number of this wildlife could not be used for this study for ethical reasons (especially those collected from the Game Reserve), even though the *E. helvum* are currently not on the list of threatened animals. Using Google map, the geographical coordinates of Qua'an Pan Local Government Area was 8°48'N 9°09'E, while that of Ibadan was 7°23'47"N 3°55'0"E. The bats were captured humanely using mist nets and transported to the Research Laboratory in cages. Bats were certified to be adults using the weight according to previous reports (Richter and Cumming, 2006), and weights were determined using a digital kitchen weighing scale (Electronic Kitchen Scale, Camry® EK5350, China). All subjects used for this study had the typical orange/yellowish coloured ventral aspect peculiar to the *E. helvum*.

They were anaesthetized with ketamine HCl at 50 mg/kg and xylazine 10 mg/kg, administered intramuscularly in the pectoral muscle. This dose was a modification from a previous report in bats (Sohayati *et al.*, 2008). After ascertaining that the bats were deceased (lack of response to strong tactile stimuli and cessation of respiration and heart-beat), they were eviscerated, skinned and all muscles were removed as much as possible. All bones were left intact. Animals were tagged individually for easy identification.

**Cold water maceration:** The heads were separated from the bodies at the atlanto-occipital junction, and macerated individually in labelled plastic containers. The skulls were macerated using the cold-water maceration method previously described (Igado and Ade-Julius, 2018). The skulls were placed in individual plastic containers with lids, in water solution containing 5% sodium hydroxide (NaOH) pellets for 5 – 9 days at room temperature. This was done to remove the remaining muscles and ligaments still attached to the bone. The water solution was changed during the duration of time of maceration. Then the skulls were rinsed to remove any soft tissue and then transferred to a water solution containing 4.2 % sodium hypochlorite overnight. The skulls were again rinsed in clean water and then left in the sun to air-dry.

**Linear morphometry:** A total of fifty-two (52) parameters were measured on each skull (Figures 1 – 14). Some of the parameters measured were adapted from previous methods and definitions (Igado, 2017; Igado and Ekeolu, 2014; Onar,

1999). Nomenclatures were derived from veterinary anatomy texts (Getty, 1975; Singh, 2017). Measurements were determined with the aid of a digital vernier calliper (Neiko®, sensitivity of 0.01mm, Germany), centimetre rule, mathematical dividers and compass to the nearest 0.01mm. Pictures were obtained using a digital camera (Sony® Cyber-shot, DSC-HX400V, 50x optical zoom, China).

The parameters measured and their landmarks are described below. All measurements were recorded in millimeters (mm), all paired measurements were obtained consistently from the left side e.g. measurements of orbit.

#### Neurocranial and viscerocranial measurements

1. **Whole skull height (WSH):** This is the total height of the skull; from the highest level of the frontal bone to the base of the mandible.
2. **Whole skull length (WSL):** This is the total length of the skull; from the most rostral aspect of the dental pad to the most caudal aspect of the occipital bone.
3. **Skull height without the mandible (SHWM 1):** Height of the skull, from the highest limit of the frontal bone to the most ventral aspect of the sphenoid bone, below the foramen magnum.
4. **Maximum width of the skull (MWS):** This is the breadth/width of the skull, measured from zygion to zygion (zygomatic bone to zygomatic bone).
5. **Total length of frontal bone (FBL):** From the rostral tip of the bone, where it articulates with the maxilla and nasal bones, to the caudal border where it forms a suture with the parietal bone.
6. **Overall length of the nasal bone (NBL):** From the rostral end of the frontal bone to the rostral tip of the nasal bone.
7. **Length of parietal bone (FNE):** From the fronto-parietal suture to the nuchal eminence.
8. **Length of palate (PAL):** This was measured along the midline, from the most rostral aspect of the incisive bone, to the most rostral aspect of the choanae.
9. **Length of the perpendicular plate of the palatine (UMP):** From the most rostral aspect of the choanae to the suture between/joining the perpendicular plate of the palatine bone and the pterygoid.
10. **Tympanic bulla length (TBL):** From the rostral aspect to the caudal aspect of the bulla.
11. **Width of the tympanic bulla (TBW):** From one lateral aspect of the bulla to the other lateral aspect.
12. **Orbital height/vertical diameter (OVD):** Height of the orbit, from the ventral aspect of the orbital rim in a vertical line to the dorsal aspect of the rim.
13. **Orbital width/horizontal diameter (OHD):** From the limit of the zygomatic process of the frontal bone to the median canthus of the orbit.
14. **Orbital index:** 
$$\left( \frac{\text{orbital height} - \text{OVD}}{\text{orbital width} - \text{OHD}} \right) \times 100$$
15. **Cephalic index:** 
$$\left( \frac{\text{skull width} - \text{MWS}}{\text{skull length} - \text{WSL}} \right) \times 100$$
16. **Occipital height (OCH):** Nuchal crest to the lower rim of the foramen magnum.
17. **Maximum width of the occipital condyles (OCW):** Maximum width or distance, from the lateral aspect of one occipital condyle to the lateral aspect of the other occipital condyle.
18. **Neurocranial volume (NCV):** Cotton wool was used to block all the foramina of the intact skull; fine grains were then poured into the neurocranium via the foramen magnum until filled up. The grains were emptied into a measuring cylinder and volume determined.
19. **Length of zygomatic process of frontal bone (LPO):** This was measured from the origin of the zygomatic process on the frontal bone, to its most lateral limit.

20. **Zygomatic process to zygomatic process (POPO):** This was measured from the most lateral limit of one zygomatic process of the frontal bone to the most lateral limit of the other process.
21. Vertical distance from the most lateral limit of zygomatic process to zygomatic bone (POZB).
22. Distance from the rostral tip of the nasal bone to the rostral tip of the incisive bone (NIL)

#### Mandibular measurements

23. **Length of the mandibular bone (MDL):** From the most rostral point of the dental bone to the caudal limit of the mandibular condyle.
24. Length of the lower jaw from the most rostral point of the dental bone to the most caudal projection of the coronoid process (MDL-1)
25. Length of the lower jaw from the most rostral point of the dental bone to the most caudal projection of the mandibular condyle (condylar process) (MDL-2)
26. Length of the lower jaw, from the most rostral point of the dental bone to the most caudal projection of the angular process (MDL-3)
27. **Height of the mandibular symphysis (HSPH):** This was measured as the vertical distance from the dorsal to the ventral limits of the mandibular symphysis
28. **Length of the mandibular symphysis (LSPH):** This was measured from the rostral to the caudal limits of the mandibular symphysis.
29. **Width of the condylar process (WCP):** This was measured from one lateral aspect to the other lateral aspect of the condylar process of the mandible.
30. Distance between the two lower (mandibular) premolars, at the level of the 2nd premolars, measured along the transverse plane (D2P)
31. Distance between the lower (mandibular) 2<sup>nd</sup> molars, measured along the transverse plane (DLM)
32. Height of the mandibular body between the mid-point of premolar 1 and 2 and the mandibular base (HMP)
33. Thickness of mandible at molar 1 (TM-1)
34. Thickness of the mandible, between the medial and lateral parts of the mandible, caudal to the last molar tooth (TR)
35. Length of the mandible between the cranial and caudal angles (RAM). This was measured as the distance from immediately caudal to the last molar tooth (cranial angle), to the ventral aspect of the angular process (caudal angle).

**Foramen diameters and foramen-related measurements:** Parameters relating to some foramina which may aid in maxillofacial anaesthesiology (rostrofacial indices). The lacrimal fossa was used as a reference point for some parameters, since the edge of the orbit may be easier to palpate and access in some live patients.

36. **Occipital height without foramen magnum (OCHW):** Nuchal crest to the upper rim of the foramen magnum.
37. **Height of the foramen magnum (FMH):** Maximum height of the foramen magnum, or distance between the dorsal and ventral limits of the foramen magnum.
38. **Width of the foramen magnum (FMW):** Maximum width, between the two lateral limits of the foramen magnum.
39. **Foramen magnum index (FMI):** calculated as  $\left(\frac{FMH}{FMW}\right) * 100$ , expressed in percentage.
40. Distance between the most medial points of the most rostral left and right mental foramina (RMF)
41. Distance between the alveolar border of the 3rd lower incisor and the most ventral point of the most rostral mental foramen (IRMF)

42. Length along a horizontal line, from the ventral limit of the mandibular foramen to the caudal border of the mandible (MFCB)
43. Length, along a vertical line, from the ventral limit of the middle mental foramen, to the ventral border of the mandibular foramen (MFMF)
44. Length, along a vertical line, from the ventral limit of the mandibular foramen, to the base of the mandible (MFMB)
45. Length along a vertical line, from the ventral limit of the mandibular foramen, to the most dorsal aspect of the coronoid process (MFCP)
46. Distance between the caudal limit/rim of the lacrimal foramen and the medial canthus of the orbit (IFMO)
47. Distance from the ventral limit of the lacrimal foramen to the alveolar border between the upper 3rd and 4<sup>th</sup> premolars (IFP).
48. Distance, along a horizontal line, between the caudal rim of the lacrimal foramen and the cranial tip of the facial crest (FCIF)
49. Distance, from the zygomatic arch to the caudal rim of the lacrimal foramen (ZPIF).
50. **Diameter of the lacrimal foramen (DIF):** This was measured vertically, in a straight line, as the distance between the dorsal and ventral limits of the lacrimal foramen.
51. **Diameter of infraorbital foramen (DIC):** This was measured vertically, in a straight line, as the distance between the dorsal and ventral limits of the infraorbital foramen.
52. Distance between the infraorbital foramen and the medial canthus of the orbit/the orbit (ICMC).

#### Statistical analysis

All data were analysed using the Graphpad prism version 5 software. Statistically significant difference between different group parameters (gender and location) were determined using the Student 't' test. Pearson's correlation was assessed by correlating the whole skull length with other parameters. Statistically significant difference was at  $\alpha_{0.05}$ .

#### RESULTS

Linear parameters were measured in millimetres (mm) and recorded as mean  $\pm$  standard deviation (S.D.). Morphometric results are presented in Tables 1 and 2, while Pearson's correlation data are presented in Table 3.

**General appearance of the skulls:** The skulls of the *E. helvum* were rostro-caudally elongated, having a dolichocephalic appearance (Figure 1). All the skulls possessed a bilaterally placed supraorbital foramen, at the cranio-rostral aspect of the beginning of the zygomatic process (Figure 2). The supraorbital foramen led ventrally into a canal (supraorbital canal) which opened into the roof of the orbit. The nasal bone protruded slightly more cranially than the incisive bone. No palatine fissure or inter-incisive canal was present (Figure 3). The paracondylar processes were short, resulting in the skull resting on the occipital condyles.

The lacrimal foramen was external in both skull types. In the northern skulls, it was placed above the 1<sup>st</sup> and 2<sup>nd</sup> molar, while in the southern skull, it was placed behind the 2<sup>nd</sup> molar (dorso-caudal) (Figure 1). The foramen magnum had the typical spherical appearance in all the skulls assessed (Figure 4).

**Table 1:** Linear morphometric values of skulls of *Eidolon helvum* from Nigeria (north and south regions)

|                                                    | S/N | Parameters<br>(unit – mm) | Mean ± S.D. (Total)<br>n = 19 | (Male) n = 10<br>Mean ± S.D. | (Female) n = 9;<br>Mean ± S.D. |
|----------------------------------------------------|-----|---------------------------|-------------------------------|------------------------------|--------------------------------|
| Neurocranial and viscerocranial measurements       | 1.  | WSH                       | 24.02 ± 2.03                  | 24.21 ± 1.29                 | 23.80 ± 2.85                   |
|                                                    | 2.  | WSL                       | 55.42 ± 2.16                  | 55.89 ± 1.30                 | 54.94 ± 2.88                   |
|                                                    | 3.  | SHWM1                     | 17.09 ± 3.29                  | 17.29 ± 3.22                 | 16.90 ± 3.73                   |
|                                                    | 4.  | MWS                       | 30.77 ± 3.15                  | 30.79 ± 2.50                 | 30.75 ± 4.12                   |
|                                                    | 5.  | FBL                       | 18.80 ± 2.42                  | 18.95 ± 3.00                 | 18.62 ± 1.81                   |
|                                                    | 6.  | NBL                       | 24.02 ± 3.35                  | 24.35 ± 4.57                 | 23.63 ± 1.29                   |
|                                                    | 7.  | FNE                       | 15.79 ± 2.43                  | 15.50 ± 2.45                 | 16.13 ± 2.65                   |
|                                                    | 8.  | PAL                       | 31.30 ± 4.05                  | 31.69 ± 4.79                 | 30.84 ± 3.45                   |
|                                                    | 9.  | UMP                       | 3.48 ± 1.18                   | 3.38 ± 1.43                  | 3.635 ± 0.86                   |
|                                                    | 10. | TBL                       | 3.60 ± 0.33                   | 3.57 ± 0.254                 | 3.63 ± 0.42                    |
|                                                    | 11. | TBW                       | 2.99 ± 0.27                   | 2.96 ± 0.07                  | 3.04 ± 0.42                    |
|                                                    | 12. | OVD                       | 11.82 ± 1.3220                | 12.04 ± 1.02                 | 11.54 ± 1.71                   |
|                                                    | 13. | OHD                       | 11.88 ± 0.79                  | 12.05 ± 0.66                 | 11.68 ± 0.97                   |
|                                                    | 14. | Orbital index (%)         | 101.10 ± 6.44                 | 100.30 ± 5.49                | 102.10 ± 8.00                  |
|                                                    | 15. | Cephalic index (%)        | 55.95 ± 5.54                  | 56.00 ± 5.44                 | 55.90 ± 6.28                   |
|                                                    | 16. | OCH                       | 10.90 ± 1.58                  | 11.04 ± 1.55                 | 10.76 ± 1.78                   |
|                                                    | 17. | OCW                       | 9.73 ± 0.90                   | 9.92 ± 1.06                  | 9.54 ± 0.77                    |
|                                                    | 18. | NCV (mls)                 | 3.53 ± 1.24                   | 3.52 ± 1.06                  | 3.55 ± 1.56                    |
|                                                    | 19. | LPO                       | 8.88 ± 2.36                   | 8.96 ± 1.90                  | 8.77 ± 3.06                    |
|                                                    | 20. | POPO                      | 22.95 ± 3.50                  | 23.17 ± 2.50                 | 22.73 ± 4.60                   |
|                                                    | 21. | POZB                      | 5.82 ± 0.46                   | 5.86 ± 0.28                  | 5.77 ± 0.64                    |
| Mandibular measurements                            | 22. | NIL                       | 6.59 ± 0.249                  | 6.64 ± 0.27                  | 6.53 ± 0.23                    |
|                                                    | 23. | MDL                       | 44.67 ± 2.57                  | 45.00 ± 2.53                 | 44.29 ± 2.86                   |
|                                                    | 24. | MDL-1                     | 41.58 ± 2.10                  | 42.20 ± 1.27                 | 40.84 ± 2.78                   |
|                                                    | 25. | MDL-2                     | 42.81 ± 3.18                  | 42.89 ± 3.53                 | 42.72 ± 3.12                   |
|                                                    | 26. | MDL-3                     | 41.28 ± 2.48                  | 41.51 ± 2.85                 | 41.01 ± 2.25                   |
|                                                    | 27. | HSPH                      | 4.55 ± 0.45                   | 4.58 ± 0.33                  | 4.52 ± 0.60                    |
|                                                    | 28. | LSPH                      | 6.20 ± 0.84                   | 6.43 ± 0.84                  | 5.92 ± 0.84                    |
|                                                    | 29. | WCP                       | 4.86 ± 0.44                   | 4.86 ± 0.23                  | 4.86 ± 0.64                    |
|                                                    | 30. | D2P                       | 6.88 ± 0.50                   | 6.89 ± 0.57                  | 6.87 ± 0.47                    |
|                                                    | 31. | DLM                       | 13.57 ± 1.72                  | 13.60 ± 1.23                 | 13.55 ± 2.35                   |
|                                                    | 32. | HMP                       | 4.48 ± 0.34                   | 4.53 ± 0.30                  | 4.42 ± 0.42                    |
|                                                    | 33. | TM-1                      | 2.212 ± 0.27                  | 2.22 ± 0.28                  | 2.20 ± 0.28                    |
|                                                    | 34. | TR                        | 1.45 ± 0.23                   | 1.46 ± 0.24                  | 1.43 ± 0.25                    |
|                                                    | 35. | RAM                       | 17.66 ± 2.05                  | 17.71 ± 2.59                 | 17.60 ± 1.44                   |
| Foramen diameters and foramen-related measurements | 36. | OCHW                      | 6.84 ± 1.33                   | 6.806 ± 1.04                 | 6.874 ± 1.71                   |
|                                                    | 37. | FMH                       | 5.23 ± 0.59                   | 5.30 ± 0.60                  | 5.16 ± 0.63                    |
|                                                    | 38. | FMW                       | 6.10 ± 0.47                   | 6.128 ± 0.59                 | 6.06 ± 0.38                    |
|                                                    | 39. | FMI                       | 85.75 ± 6.57                  | 86.59 ± 7.73                 | 84.92 ± 5.95                   |
|                                                    | 40. | RMF                       | 0.67 ± 0.21                   | 0.65 ± 0.22                  | 0.68 ± 0.23                    |
|                                                    | 41. | IRMF                      | 1.58 ± 0.47                   | 1.45 ± 0.48                  | 1.74 ± 0.46                    |
|                                                    | 42. | MFCB                      | 6.21 ± 2.10                   | 6.53 ± 2.53                  | 5.82 ± 1.65                    |
|                                                    | 43. | MFMB                      | 29.27 ± 1.96                  | 29.60 ± 1.27                 | 28.88 ± 2.68                   |
|                                                    | 44. | MFMB                      | 4.24 ± 0.69                   | 4.22 ± 0.35                  | 4.27 ± 1.02                    |
|                                                    | 45. | MFCP                      | 14.04 ± 0.99                  | 14.47 ± 0.75                 | 13.53 ± 1.07                   |
|                                                    | 46. | IFMO                      | 1.45 ± 0.41                   | 1.35 ± 0.33                  | 1.60 ± 0.51                    |
|                                                    | 47. | IFP                       | 6.19 ± 1.57                   | 6.43 ± 0.92                  | 5.84 ± 2.40                    |
|                                                    | 48. | FCIF                      | 14.70 ± 2.75                  | 14.58 ± 2.86                 | 14.90 ± 2.99                   |
|                                                    | 49. | ZPIF                      | 17.45 ± 2.17                  | 16.99 ± 2.17                 | 18.14 ± 2.30                   |
|                                                    | 50. | DIF                       | 1.01 ± 0.19                   | 1.048 ± 0.21                 | 0.96 ± 0.17                    |
|                                                    | 51. | DIC                       | 1.17 ± 0.23                   | 1.14 ± 0.25                  | 1.21 ± 0.22                    |
|                                                    | 52. | ICMC                      | 1.62 ± 0.60                   | 1.53 ± 0.66                  | 1.72 ± 0.57                    |

A mid-sagittal cut of the skull was made to reveal the neurocranium. The frontal sinus appeared to be limited only to the cranial aspect of the bone and was very small, appearing like pin-point holes, probably due to the thinness of the bones of the skull. The neurocranial cavity was relatively smooth; revealing few impressions of corresponding gyri and sulci from the brain (Figure 5).

Major morphological differences observed between the skulls of the two regions were:

1. The bat skulls from Jos (northern Nigeria) had a distinct dome-shape, formed by the parietal bone, unlike the bats from the south which had a flattened shape (Figure 1), making the southern skulls appear longer.
2. The zygomatic process emanating laterally (and projecting latero-ventrally) from the frontal bone was conspicuously more pronounced in the northern skulls, in some cases, twice as long as in the southern skulls (Figure 2).

**Table 2:**

Linear morphometric values of *Eidolon helvum* skulls from the northern region (Jos) compared to the southern region (Ibadan) of Nigeria, with gender-based values

|                                                    |                           | Jos (northern Nigeria)    |                             |                              | Ibadan (southern Nigeria) |                             |                               |
|----------------------------------------------------|---------------------------|---------------------------|-----------------------------|------------------------------|---------------------------|-----------------------------|-------------------------------|
| S/N                                                | Parameters<br>(unit – mm) | Mean ± S.D.<br>n = 7      | Mean ± S.D.<br>(Male) n = 4 | Mean ± S.D.<br>(Female) n =3 | Mean ± S.D.<br>n = 12     | Mean ± S.D.<br>(Male) n = 6 | Mean ± S.D.<br>(Female) n = 6 |
| Neurocranial and viscerocranial measurements       |                           |                           |                             |                              |                           |                             |                               |
| 1.                                                 | WSH                       | 25.32 ± 0.66              | 24.99 ± 0.42                | 25.77 ± 0.73                 | 21.75 ± 1.43              | 22.66 ± 0.74                | 20.85 ± 1.52                  |
| 2.                                                 | WSL                       | 55.65 ± 1.11*             | 55.47 ± 1.04                | 55.88 ± 1.40                 | 54.88 ± 4.09              | 57.56 ± 0.00                | 53.54 ± 4.77                  |
| 3.                                                 | SHWM1                     | 19.08 ± 0.73              | 18.71 ± 0.50                | 19.58 ± 0.75                 | 12.45 ± 0.93              | 11.59 ± 0.00                | 12.88 ± 0.80                  |
| 4.                                                 | MWS                       | 32.92 ± 0.86              | 32.35 ± 0.41                | 33.67 ± 0.67                 | 27.01 ± 1.41              | 27.66 ± 1.17                | 26.37 ± 1.70                  |
| 5.                                                 | FBL                       | 20.24 ± 1.44              | 20.72 ± 1.41                | 19.60 ± 1.48                 | 16.28 ± 1.43              | 15.42 ± 1.34                | 17.14 ± 1.19                  |
| 6.                                                 | NBL                       | 22.34 ± 1.57#             | 21.52 ± 1.63                | 23.44 ± 0.49                 | 26.97 ± 3.80              | 30.01 ± 0.67                | 23.93 ± 2.43                  |
| 7.                                                 | FNE                       | 17.39 ± 1.22              | 16.90 ± 1.42                | 18.03 ± 0.56                 | 12.99 ± 0.59              | 12.70 ± 0.67                | 13.28 ± 0.50                  |
| 8.                                                 | PAL                       | 28.74 ± 1.03 <sup>β</sup> | 28.64 ± 0.79                | 28.87 ± 1.49                 | 35.79 ± 3.24              | 37.79 ± 1.03                | 33.78 ± 3.78                  |
| 9.                                                 | UMP                       | 4.17 ± 0.37               | 4.28 ± 0.40                 | 4.040 ± 0.35                 | 1.87 ± 0.56               | 1.59 ± 0.40                 | 2.420 ± 0.00                  |
| 10.                                                | TBL                       | 3.73 ± 0.30               | 3.67 ± 0.16                 | 3.81 ± 0.46                  | 3.30 ± 0.12               | 3.19 ± 0.00                 | 3.36 ± 0.11                   |
| 11.                                                | TBW                       | 3.10 ± 0.25               | 2.97 ± 0.09                 | 3.29 ± 0.29                  | 2.80 ± 0.22               | 2.94 ± 0.00                 | 2.67 ± 0.28                   |
| 12.                                                | OVD                       | 12.67 ± 0.26              | 12.69 ± 0.22                | 12.64 ± 0.34                 | 10.33 ± 1.02              | 10.75 ± 0.14                | 9.90 ± 1.54                   |
| 13.                                                | OHD                       | 12.35 ± 0.25              | 12.37 ± 0.28                | 12.32 ± 0.26                 | 11.07 ± 0.77              | 11.41 ± 0.86                | 10.72 ± 0.74                  |
| 14.                                                | Orbital index (%)         | 97.46 ± 1.38 <sup>β</sup> | 97.46 ± 1.56                | 97.46 ± 1.44                 | 107.60 ±6.92              | 106.10 ±6.63                | 109.00 ±9.55                  |
| 15.                                                | Cephalic index (%)        | 59.18 ± 2.08              | 58.34 ± 1.66                | 60.30 ± 2.37                 | 48.40 ± 1.77              | 46.61 ± 0.00                | 49.30 ± 1.21                  |
| 16.                                                | OCH                       | 11.84 ± 0.55              | 11.71 ± 0.53                | 12.01 ± 0.64                 | 8.72 ± 0.43               | 8.39 ± 0.00                 | 8.89 ± 0.45                   |
| 17.                                                | OCW                       | 10.23 ± 0.44              | 10.36 ± 0.50                | 10.06 ± 0.37                 | 8.57 ± 0.33               | 8.20 ± 0.00                 | 8.75 ± 0.14                   |
| 18.                                                | NCV (mls)                 | 4.41 ± 0.28               | 4.20 ±0.08                  | 4.68 ± 0.13                  | 2.0 ± 0.27                | 2.15 ± 0.07                 | 1.85 ± 0.35                   |
| 19.                                                | LPO                       | 10.46 ± 0.98              | 10.14 ± 0.60                | 10.89 ± 1.36                 | 6.11 ± 0.72               | 6.62 ± 0.63                 | 5.60 ± 0.29                   |
| 20.                                                | POPO                      | 24.99 ± 1.29              | 24.23 ± 0.96                | 26.01 ± 0.95                 | 18.20 ± 1.29              | 18.96 ± 0.00                | 17.83 ± 1.56                  |
| 21.                                                | POZB                      | 5.97 ±0.33*               | 5.80 ± 0.34                 | 6.21 ± 0.07                  | 5.55 ± 0.56               | 6.00 ± 0.02                 | 5.10 ± 0.38                   |
| 22.                                                | NIL                       | 6.68 ± 0.19*              | 6.72 ± 0.22                 | 6.62 ± 0.15                  | 6.45 ± 0.31               | 6.49 ± 0.40                 | 6.40 ± 0.34                   |
| Mandibular measurements                            |                           |                           |                             |                              |                           |                             |                               |
| 23.                                                | MDL                       | 43.81 ± 1.09#             | 43.44 ± 0.81                | 44.30 ± 1.39                 | 46.19 ± 3.86              | 48.11 ± 1.02                | 44.27 ± 5.38                  |
| 24.                                                | MDL-1                     | 41.32 ± 0.87#             | 41.45 ± 0.46                | 41.14 ± 1.37                 | 42.04 ± 3.57              | 43.71 ± 0.80                | 40.38 ± 5.15                  |
| 25.                                                | MDL-2                     | 41.37 ± 1.37#             | 40.66 ± 0.71                | 42.31 ± 1.60                 | 45.33 ± 4.08              | 47.34 ± 1.15                | 43.33 ± 5.71                  |
| 26.                                                | MDL-3                     | 40.23 ± 1.01#             | 39.80 ±0.76                 | 40.80 ± 1.14                 | 43.13 ± 3.37              | 44.93 ± 1.97                | 41.33 ± 4.15                  |
| 27.                                                | HSPh                      | 4.61 ± 0.44*              | 4.56 ± 0.34                 | 4.68 ± 0.62                  | 4.45 ± 0.51               | 4.63 ± 0.44                 | 4.28 ± 0.69                   |
| 28.                                                | LSPh                      | 6.71 ± 0.52               | 6.85 ± 0.69                 | 6.53 ± 0.13                  | 5.30 ± 0.34               | 5.59 ± 0.02                 | 5.01 ± 0.12                   |
| 29.                                                | WCP                       | 5.12 ± 0.21               | 4.99 ± 0.12                 | 5.29 ± 0.16                  | 4.41 ± 0.35               | 4.61 ± 0.15                 | 4.22 ± 0.45                   |
| 30.                                                | D2P                       | 6.69 ± 0.40#              | 6.63 ± 0.51                 | 6.76 ± 0.26                  | 7.22 ± 0.53               | 7.41 ± 0.19                 | 7.03 ± 0.81                   |
| 31.                                                | DLM                       | 14.65 ± 0.82              | 14.32 ± 0.57                | 15.09 ± 1.00                 | 11.69 ± 1.07              | 12.15 ± 0.56                | 11.24 ± 1.51                  |
| 32.                                                | HMP                       | 4.41 ± 0.26#              | 4.42 ± 0.18                 | 4.40 ± 0.40                  | 4.593 ± 0.48              | 4.75 ± 0.46                 | 4.44 ± 0.62                   |
| 33.                                                | TM-1                      | 2.38 ± 0.14               | 2.39 ± 0.12                 | 2.37 ± 0.19                  | 1.91 ± 0.09               | 1.89 ± 0.04                 | 1.94 ± 0.13                   |
| 34.                                                | TR                        | 1.60 ± 0.11               | 1.61 ± 0.05                 | 1.58 ± 0.19                  | 1.18 ± 0.04               | 1.16 ± 0.04                 | 1.20 ± 0.01                   |
| 35.                                                | RAM                       | 16.53 ± 0.89 <sup>β</sup> | 16.20 ± 0.71                | 16.98 ± 1.03                 | 19.63 ± 2.06              | 20.73 ± 2.14                | 18.53 ± 1.82                  |
| Foramen diameters and foramen-related measurements |                           |                           |                             |                              |                           |                             |                               |
| 36.                                                | OCHW                      | 7.55 ± 0.82               | 7.25 ± 0.34                 | 7.94 ± 1.19                  | 5.193 ± 0.40              | 5.030 ± 0.00                | 5.275 ±0.53                   |
| 37.                                                | FMH                       | 5.54 ± 0.37               | 5.50 ± 0.46                 | 5.51 ± 0.32                  | 4.50 ± 0.06               | 4.48 ± 0.00                 | 4.52 ± 0.08                   |
| 38.                                                | FMW                       | 6.33 ± 0.31               | 6.34 ± 0.43                 | 6.31 ± 0.14                  | 5.56 ± 0.29               | 5.30 ± 0.00                 | 5.69 ± 0.26                   |
| 39.                                                | FMI (%)                   | 87.70 ± 6.55              | 87.11 ± 8.83                | 88.50 ± 3.15                 | 81.20 ± 4.58              | 84.53 ± 0.00                | 79.54 ± 5.03                  |
| 40.                                                | RMF                       | 0.73 ± 0.19*              | 0.76 ± 0.19                 | 0.70 ± 0.23                  | 0.55 ± 0.23               | 0.44 ± 0.01                 | 0.66 ± 0.33                   |
| 41.                                                | IRMF                      | 1.54 ± 0.28#              | 1.44 ± 0.31                 | 1.67 ± 0.20                  | 1.66 ± 0.76               | 1.47 ± 0.93                 | 1.86 ± 0.84                   |
| 42.                                                | MFCB                      | 4.94 ± 0.87 <sup>β</sup>  | 4.96 ± 0.95                 | 4.92 ± 0.97                  | 8.42 ± 1.73               | 9.66 ± 0.04                 | 7.18 ± 1.68                   |
| 43.                                                | MFMF                      | 29.18 ± 1.19#             | 29.09 ± 0.96                | 29.30 ± 1.67                 | 29.44 ± 3.14              | 30.63 ± 1.46                | 28.25 ± 4.67                  |
| 44.                                                | MFMB                      | 4.61 ± 0.47               | 4.40 ± 0.22                 | 4.89 ± 0.61                  | 3.60 ± 0.55               | 3.85 ± 0.26                 | 3.35 ± 0.56                   |
| 45.                                                | MFCP                      | 14.01 ± 0.92#             | 14.28 ± 0.85                | 13.65 ± 1.05                 | 14.11 ± 1.25              | 14.86 ± 0.43                | 13.37 ± 1.52                  |
| 46.                                                | IFMO                      | 1.23 ± 0.15 <sup>β</sup>  | 1.15 ± 0.15                 | 1.34 ± 0.09                  | 1.95 ±0.35                | 1.75 ± 0.01                 | 2.36 ± 0.00                   |
| 47.                                                | IFP                       | 5.48 ± 1.30 <sup>β</sup>  | 5.86 ± 0.24                 | 4.99 ± 2.08                  | 7.84 ± 0.51               | 7.58 ± 0.30                 | 8.38 ± 0.00                   |
| 48.                                                | FCIF                      | 13.11 ± 1.09 <sup>β</sup> | 12.86 ± 1.30                | 13.44 ± 0.87                 | 18.43 ± 0.83              | 18.02 ± 0.59                | 19.25 ± 0.00                  |
| 49.                                                | ZPIF                      | 18.62 ± 0.93              | 18.17 ± 0.68                | 19.22 ± 0.97                 | 14.73 ± 1.68              | 14.64 ± 2.36                | 14.91 ± 0.00                  |
| 50.                                                | DIF                       | 1.08 ± 0.17*              | 1.12 ± 0.24                 | 1.04 ± 0.04                  | 0.85 ± 0.12               | 0.92 ± 0.02                 | 0.71 ± 0.00                   |
| 51.                                                | DIC                       | 1.27 ± 0.20               | 1.25 ± 0.23                 | 1.30 ± 0.19                  | 0.99 ±0.18                | 0.90 ± 0.01                 | 1.08 ± 0.26                   |
| 52.                                                | ICMC                      | 1.20 ± 0.16 <sup>β</sup>  | 1.11 ± 0.12                 | 1.33 ± 0.14                  | 2.34 ± 0.21               | 2.38 ± 0.06                 | 2.31 ± 0.35                   |

\* Indicates values which were higher in Jos skulls but no statistically significant difference was observed when compared to Ibadan skulls ( $p > 0.05$ )

# Indicates values which were higher in Ibadan skulls although no statistically significant difference was observed ( $p > 0.05$ )

<sup>β</sup> Indicates values higher in Ibadan skulls, with statistically significant difference observed ( $p < 0.05$ )

All other values were higher in the Jos skulls with statistically significant difference observed ( $p < 0.05$ )

3. The frontal bone in the northern skulls extended more caudally, the crests formed by the two lateral aspects extended through the parietal bone to the occipital bone, forming a double sagittal crest (V-shaped); while in the southern skulls, the caudal aspect of the frontal bone formed a distinct divergent 'Y-shape' which demarcated the fronto-parietal suture, and continued caudally as a single crest. The sagittal crest in both skull types were distinct and pronounced (Figures 1 and 2).
  4. There was the presence of a diastema in the southern skulls, unlike the northern skulls (Figure 1).
  5. The nasal bone in the southern skulls had a slightly more convex appearance (Figure 2).
  6. The southern skulls had a dental formula of  $I^{2/2} C^{1/1} PM^{1/2} M^{2/3}$  (total 28), while the northern skulls had  $I^{2/2} C^{1/1} PM^{2/2} M^{3/3}$  (total 32). (Figure 3). This finding was consistent in both genders.
  7. The suture lines in the southern skulls were more distinct than the northern skulls. For example, the nasal bones showed a more distinct demarcation in the southern skulls relative to the northern skulls, where the maxillo-nasal sutures were not so apparent (Figure 2).
  8. From a dorsal view, the southern skulls appeared more streamlined and narrower relative to the northern skulls (Figure 2).
  9. Two of the southern skulls had accessory infraorbital foramen (Figure 6).
  10. The palatine bone in the southern skull was more concave than that of the northern bone which was quite flatter (Figure 3).
  11. Palatine foramen observed in northern skulls but not in southern skulls (Figure 3).
- All the differences observed and stated above were consistent in all the skull specimens examined.

**Morphometric results:** Combined results of the skulls, without geographical segregation, showed no statistically significant differences between the sexes (Table 1).

However, analysis based on geographical location showed statistical differences among the skulls. Six (6) parameters were observed to be higher in the northern skulls with no statistically significant difference observed ( $p > 0.05$ ) – WSL, RMF, NIL, DIF, HSPH, POZB; ten (10) values were higher in the southern skulls with no statistically significant difference observed ( $p > 0.05$ ) – NBL, MDL, IRMF, MFMF, MFCF, MDL-1, MDL-2, MDL-3, HMP, D2P; while nine (9) parameters were higher in the southern skulls with statistically significant differences observed ( $p < 0.05$ ) – PAL, orbital index, MFCB, RAM, IFMO, IFP, FCIF, ICMC. All other parameters were higher in the northern skulls with statistically significant differences observed ( $p < 0.05$ ) (Table 2).

The neurocranial volume of the northern skulls was about twice that of the southern skulls, while in the southern skulls, the distance between the mandibular foramen and the caudal border of the mandible (MFCB) was almost twice that observed in the northern skulls. The southern skulls generally had longer mandibles, and the 'caudal angle' of the ramus dipped more ventrally than in the northern skulls.

Pearson's correlation coefficient to WSL revealed the highest positive correlations to lengths of mandible, MDL ( $r = 0.822$ ), MDL-1 ( $r = 0.880$ ), MDL-2 ( $r = 0.674$ ), MDL-3 ( $r = 0.727$ ); FCIF ( $r = 0.718$ ) and MFCP ( $r = 0.712$ ). Highest negative correlations were observed with UMP ( $r = -0.718$ ), ZPIF ( $r = -0.676$ ) and the orbital index ( $r = -0.640$ ) (Table 3).

$r = 0.727$ ); FCIF ( $r = 0.718$ ) and MFCP ( $r = 0.712$ ). Highest negative correlations were observed with UMP ( $r = -0.718$ ), ZPIF ( $r = -0.676$ ) and the orbital index ( $r = -0.640$ ) (Table 3).

**Table 3:**

Pearson's correlation coefficient values ( $r$ ) relative to WSL

| S/N                                                 | Parameters     | r value | S/N                                                       | Parameters | r value |
|-----------------------------------------------------|----------------|---------|-----------------------------------------------------------|------------|---------|
| <b>Neurocranial and viscerocranial measurements</b> |                |         |                                                           |            |         |
| 1                                                   | WSH            | 0.541   | 27                                                        | LSPH       | 0.243   |
| 2                                                   | SHWM1          | 0.041   | 28                                                        | WCP        | 0.073   |
| 3                                                   | MWS            | 0.299   | 29                                                        | D2P        | 0.489   |
| 4                                                   | FBL            | 0.333   | 30                                                        | DLM        | 0.480   |
| 5                                                   | NBL            | 0.539   | 31                                                        | HMP        | 0.435   |
| 6                                                   | FNE            | 0.078   | 32                                                        | TM-1       | 0.293   |
| 7                                                   | PAL            | 0.329   | 33                                                        | TR         | 0.095   |
| 8                                                   | UMP            | -0.718  | 34                                                        | RAM        | 0.357   |
| 9                                                   | TBL            | 0.283   | 35                                                        | OCHW       | 0.292   |
| 10                                                  | TBW            | 0.191   | <b>Foramen diameters and foramen-related measurements</b> |            |         |
| 11                                                  | OVD            | 0.506   | 36                                                        | FMH        | 0.104   |
| 12                                                  | OHD            | 0.430   | 37                                                        | FMW        | 0.297   |
| 13                                                  | Orbital index  | -0.640  | 38                                                        | FMI (%)    | -0.136  |
| 14                                                  | Cephalic index | -0.047  | 39                                                        | RMF        | 0.259   |
| 15                                                  | OCH            | 0.245   | 40                                                        | IRMF       | 0.453   |
| 16                                                  | OCW            | 0.234   | 41                                                        | MFCB       | -0.151  |
| 17                                                  | NCV            | 0.298   | 42                                                        | MFMF       | 0.844   |
| 18                                                  | LPO            | 0.271   | 43                                                        | MFMB       | 0.553   |
| 19                                                  | POPO           | 0.526   | 44                                                        | MFCP       | 0.712   |
| 20                                                  | POZB           | 0.206   | 45                                                        | IFMO       | 0.606   |
| 21                                                  | NIL            | 0.264   | 46                                                        | IFP        | 0.082   |
| 22                                                  | MDL            | 0.822   | 47                                                        | FCIF       | 0.718   |
| <b>Mandibular measurements</b>                      |                |         | 48                                                        | ZPIF       | -0.676  |
| 23                                                  | MDL-1          | 0.880   | 49                                                        | DIF        | -0.201  |
| 24                                                  | MDL-2          | 0.674   | 50                                                        | DIC        | -0.268  |
| 25                                                  | MDL-3          | 0.727   | 51                                                        | ICMC       | 0.024   |
| 26                                                  | HSPH           | 0.422   |                                                           |            |         |

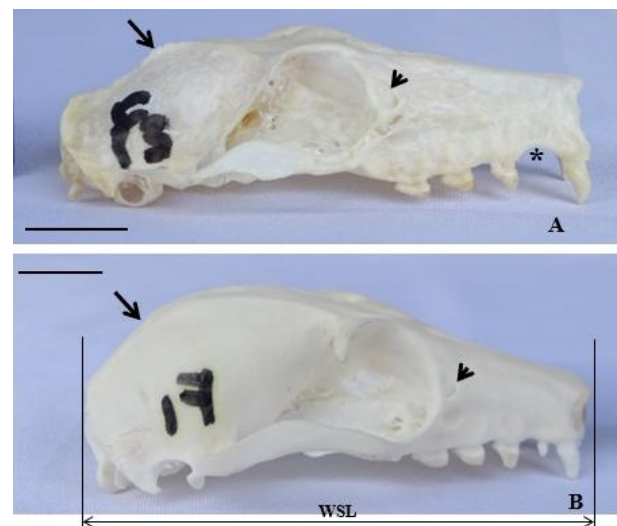
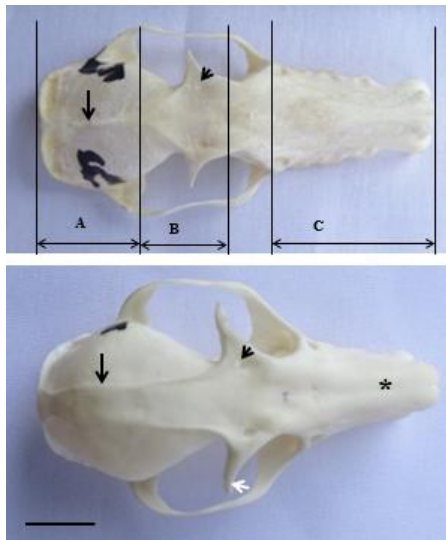
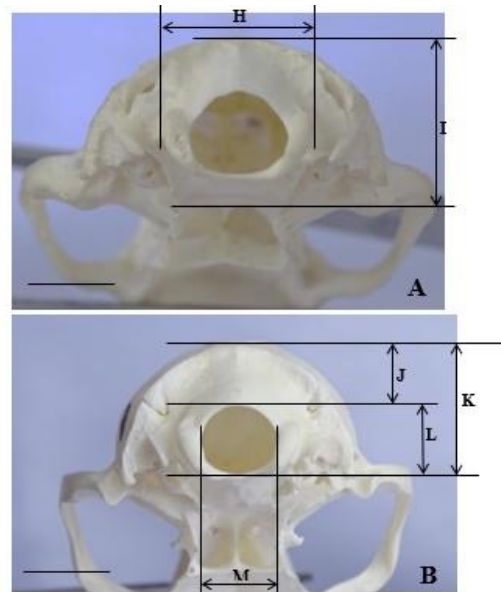


**Plate 1:**

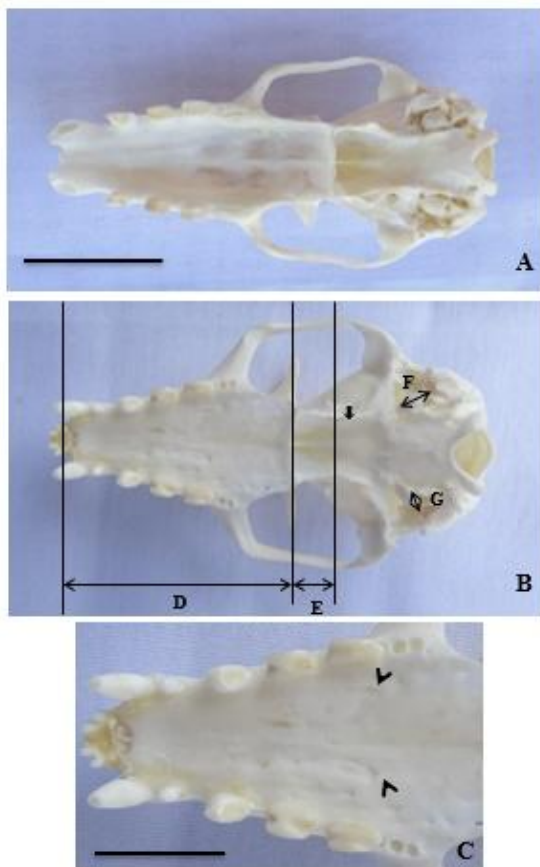
Lateral view of the female *Eidolon helvum* skulls showing the external lacrimal foramen (arrow heads). A is the southern skull while B is the northern skull. Note the more pronounced external sagittal crest (arrows) and the diastema (\*) in the southern skull, and the dome-shaped appearance of the northern skulls relative to the flattened appearance of the southern skulls. Note also the whole skull length (WSL). Scale bar – 1 cm.

**Plate 2:**

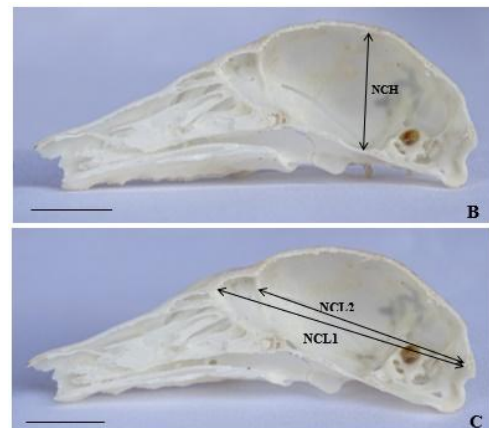
Dorsal view of the skull of the female *Eidolon helvum*, southern skull (top panel) and northern skull (lower panel), showing the length of the parietal bone – FNE (A), the total length of the frontal bone – FBL (B) and the overall length of the nasal bone – NBL (C). Note the supra-orbital foramen present in both skulls (arrow head), the more convex nasal bone in the southern skull relative to northern skull (\*), the difference in the shapes of the external sagittal crests (arrows) and the longer zygomatic process of the frontal bone (white arrow) in the northern skull. Scale bar 1 cm.

**Plate 4:**

Occipital view of the skull of the female *Eidolon helvum*, A – southern skull, B – northern skull. Pictures show the maximum width of the occipital condyles – OCW (H); skull height without mandible 1 – SHWM-1 (I); occipital height without foramen magnum – OCHW (J); occipital height – OCH (K); height of the foramen magnum – FMH (L); and width of the foramen magnum – FMW (M). Scale bar 1 cm.

**Plate 3:**

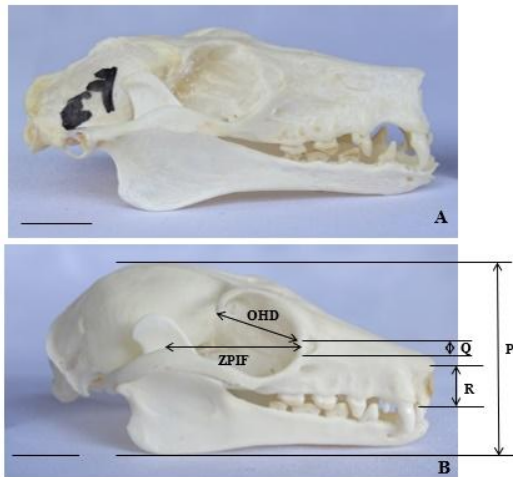
Ventral view skull of the female *Eidolon helvum*. A – southern skull, B – northern skull. Note – length of the palate, PAL (D); the length of the perpendicular plate of the palatine bone, UMP (E); length of the tympanic bulla, TBL (F); width of the tympanic bulla, TBW (G). Arrow indicates the hamulus of the pterygoid bone. Note the more pronounced concavity of the southern skull (A), the absence of palatine fissure or inter-incisive canal on the 2 skulls, the variation in dentition and the presence of the palatine foramen on the northern skull (arrow heads on panel C). C is magnification of palatine bone of B (northern skull). Scale bar – 2 cm (A & B), 1 cm (C).

**Plate 5:**

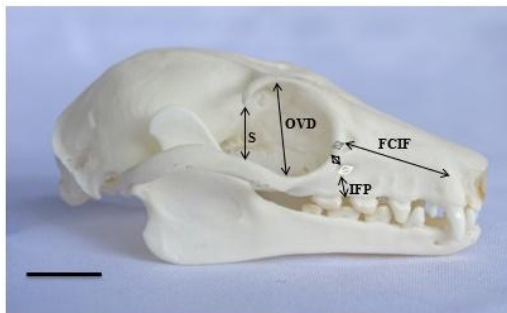
Medial view of the neurocranium of the female *Eidolon helvum*, showing the neurocranial height – NCH; neurocranial length with the ethmoid fossa – NCL1 and the neurocranial length without the ethmoid fossa – NCL2. Scale bar 1 cm

**Plate 6:**

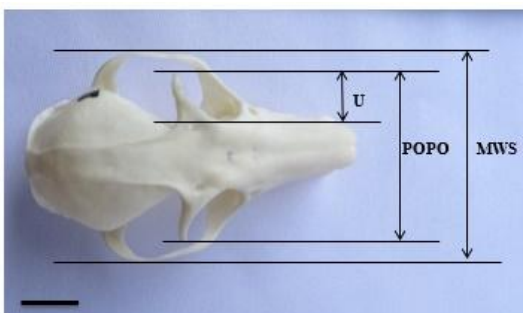
Lateral and rostral views of the *Eidolon helvum* southern skull. Note the lacrimal foramen (arrow) and the infraorbital foramen and accessory (arrow heads).

**Plate 7:**

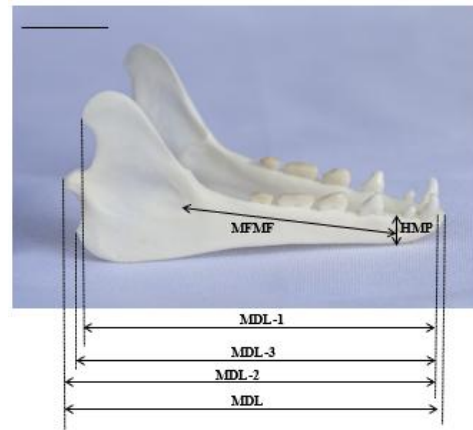
Lateral view of the skull (northern) of the female *Eidolon helvum*, showing the whole skull height – WSH (P); the horizontal diameter of the orbit – OHD; the distance from the zygomatic arch to the caudal rim of the infra-orbital foramen – ZPIF; the diameter of the lacrimal foramen – DIF (Q) and the distance from the rostral tip of the nasal bone to the rostral tip of the incisive bone – NIL (R). A – southern skull, B – northern skull; note the similarity in the dentition of the lower jaw (lower first premolar and canine missing in southern skull). Scale bar 1 cm.

**Plate 8:**

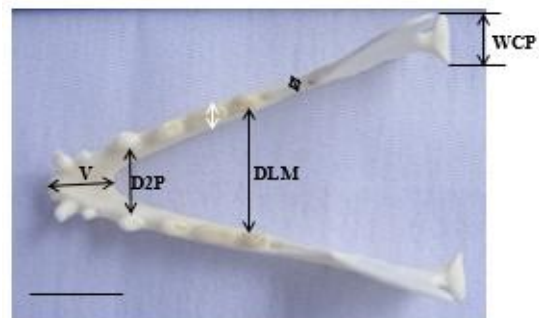
Lateral view of the skull of the female *Eidolon helvum*, showing the vertical diameter of the orbit – OVD, the vertical distance from the most lateral limit of postorbital process to zygomatic bone – POZB (S), the distance, along a horizontal line, between the caudal rim of the lacrimal foramen and the cranial tip of the facial crest – FCIF, the distance from the ventral limit of the infraorbital foramen to the alveolar border between the upper 3rd and 4<sup>th</sup> premolars – IFP, the distance between the infraorbital foramen and the median canthus of the orbit/the orbit – ICMC (black arrow), the diameter of the infraorbital foramen – DIC (white arrow), and the distance between the caudal limit/rim of the lacrimal foramen and the medial canthus of the orbit – IFMO (grey arrow). Scale bar 1 cm.

**Plate 9:**

Dorsal view of the skull of the female *Eidolon helvum*, showing the maximum width of the skull, MWS; the length of the zygomatic process of the frontal bone, LPO (U); and the distance from one limit of the zygomatic process to the other, POPO. Scale bar 1 cm.

**Figure 10:**

Lateral view of the mandible of the female *Eidolon helvum*, showing the height of the mandibular body from the mid-point of premolar 1 and 2 to the mandibular base, HMP; the length, along a vertical line, from the ventral limit of the middle mental foramen, to the ventral border of the mandibular foramen, MFMF; the length of the lower jaw from the most rostral point of the dental bone to the most caudal projection of the coronoid process, MDL-1; the length of the lower jaw from the most rostral point of the dental bone to the most caudal projection of the mandibular condyle (condylar process), MDL-2; the length of the lower jaw, from the most rostral point of the dental bone to the most caudal projection of the angular process, MDL-3; and the length of the mandibular bone, MDL. Scale bar 1 cm.

**Figure 11:**

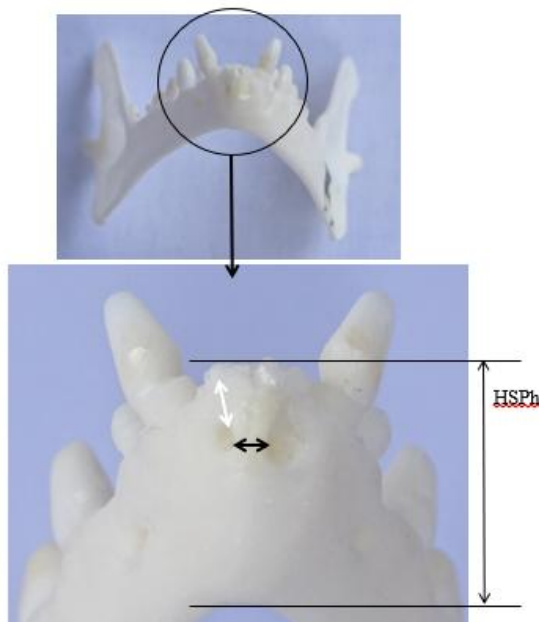
Dorsal view of the mandible of the female *Eidolon helvum*, showing the length of the mandibular symphysis, LSPH (V); the distance between the two lower (mandibular) premolars, at the level of the 2nd premolars, measured along the transverse plane, D2P; the distance between the lower (mandibular) 2nd molars, measured along the transverse plane, DLM; the thickness of mandible at molar 1, TM-1 (white arrow); the thickness of the mandible, between the medial and lateral parts of the mandible, caudal to the last molar tooth, TR (black arrow); and the width of the condylar process, WCP. Scale bar 1 cm.

## DISCUSSION

Skull variation in size and shape has been reported among different species of mammals, example, the gray wolf (Milenković *et al.*, 2010), but has been reported to occur more in dogs, with skull shapes varying from dolichocephalic, brachycephalic to mesaticephalic (Drake and Klingenberg, 2010; Janeczek *et al.*, 2008; Onar and Günes, 2003). Bat skulls have been documented to show variations in shape, differing from species to species (Brokaw and Smotherman, 2020; Camacho *et al.*, 2019; Freeman, 1998, 2000; Pedersen, 1995). But so far, no documentation on skull variations within bat species could be ascertained at the time of this write-up. The two skull types showed a distinct dolichocephalic appearance, consistent with previous pictorial reports in the *E. helvum* (DeFrees and Wilson, 1988).

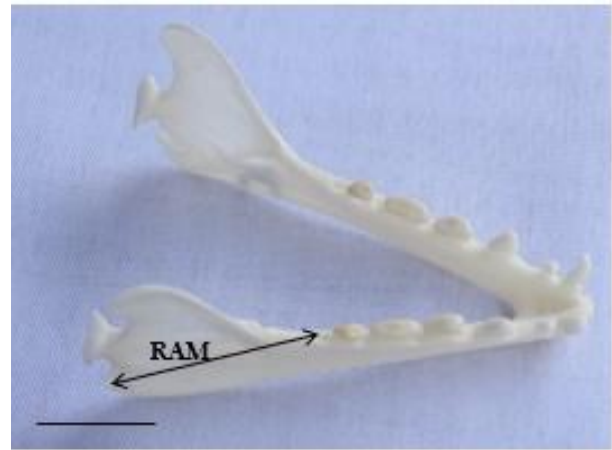
**Figure 12:**

Caudo-lateral view of the mandible of the female *Eidolon helvum*, showing the distance between the ventral limit of the mandibular foramen and the caudal border of the mandible, MFCB (X); the mandibular foramen to the base of the mandible, MFMB (W); the ventral limit of the mandibular foramen to the most dorsal aspect of the coronoid process, MFCP (Y). Scale bar 1 cm.

**Figure 13:**

Rostral view of the mandible of the female *Eidolon helvum*, showing the distance between the most medial points of the most rostral left and right mental foramina, RMF (black arrow); the distance between the alveolar border of the 2<sup>nd</sup> incisor and the rostral mental foramen, IRMF (white arrow); and the height of the mandibular symphysis – HSPh.

The zygomatic process of the frontal bone, emanating from the dorso-lateral aspect, was observed to be more pronounced than that observed in dogs. Not all animals have this process, e.g. the equine; but in some of those that possess it (like the dog, cat, porcine) the supraorbital foramen on its dorsal aspect is absent. The foramen has however been reported to be present in ruminants (Getty, 1975; Singh, 2017). Unlike the cat however, the bats lack the frontal process of the zygomatic bone, which is directed dorsally, emanating from the zygomatic arch (Getty, 1975; Singh, 2017). The frontal bone accommodates the frontalis muscle and some other muscles controlling the movements of the eyelids and the nasal region. The prominence of the zygomatic process may indicate that some of these muscles are stronger or bigger in these species. The supraorbital foramen houses the supraorbital nerve which innervates the upper eyelid and the adjacent parts of the skin of the forehead (Singh, 2017).

**Figure 14:**

Dorso-lateral view of the mandible of the female *Eidolon helvum*, showing the length of the mandible from the angular process to the last molar tooth, RAM. Scale bar 1 cm.

The sagittal crest is usually described as a ridge of bone, on the dorsal and midline aspect of the skull, running lengthwise. It has been observed in the skulls of mammals and reptiles and functions for muscle attachment (Igado, 2017; Igado *et al.*, 2015; Singh, 2017). This crest accommodates and serves as attachment for some muscles of mastication (Getty, 1975; Singh, 2017), therefore, the difference in shape of the sagittal crests observed could indicate a difference or adaptation in eating and chewing pattern, based on geographical location.

Previous pictorial reports on the skull of the *Eidolon helvum* show a more dome-shaped appearance, similar to that observed in the northern skulls (DeFrees and Wilson, 1988). The dome shape observed in the northern skulls could probably indicate that the brains of these animals were relatively bigger than the southern skulls. This dome shape was mainly due to the more convex shape of the frontal, parietal and occipital bones, which are the dorsal bones of the neurocranium (Singh, 2017). This hypothesis of the northern brains being bigger is also supported by the fact that the neurocranial volume in the northern skulls was statistically significantly higher than that of the southern skulls. The relatively smooth inner surface of the skull in both skull types is reflected in the lissencephalic nature of the brain (Igado *et al.*, 2012), as the presence of gyri results in corresponding grooves on surface of the neurocranium. Further research may reveal the differences in the brain size and also general neuro-architecture.

In the mammalian embryo, teeth development is through a number of interactions between the “odontogenic epithelium and the neural crest-derived ectomesenchyme of the early jaw primordia” (Cobourne and Sharpe, 2010). Intra-species variation in dentition has been reported in the Ipanema bat (Dick, 2002), and also in the *Eidolon helvum* (Igado *et al.*, 2018). These variations in dentition were due to differences in the number of the cheek teeth. Interestingly, the *Eidolon helvum* were obtained from the southern part of Nigeria, but in a different state from that used in the current study. The study reported differences between the genders, with the males have 30 to 36 teeth in total, and the females having 30 to 34. The reasons for the fact that no differences were observed in the dentition by the current authors could possibly be due to the number of bats used. The diastema observed in the southern skulls could

also contribute to the reduction in the number of teeth relative to the northern skulls.

As reported in domestic dogs, variation in the skull shape has been reported to have obvious functional consequences, for example on the force of bite and on breathing (Drake and Klingenberg, 2010). The shape of the external nares and its orientation are probably involved in the control of airflow into the nasal cavity for the purposes of olfaction and respiration (Clifford and Witmer, 2004; Craven *et al.*, 2007; Ranslow *et al.*, 2014). Nasal shape variation has been reported generally in bats, differing from species to species. This variation in nasal shape and other facial features has been reported to correlate with feeding ecology and echolocation (Brokaw and Smotherman, 2020), however in this study, the two groups of bats used were of the same species, having the same fruit diet. Morphological adaptations of the nose also improve the ability to detect and track odours, an essential aspect of animal survival. While odour detection may be enhanced by increased nostril separation (Brokaw and Smotherman, 2020) (an aspect which was not explored in this study), the slightly more convex appearance in the southern skulls may indicate an increased nostril separation, probably implying their possession of a keener sense of smell.

The palatine bone showed distinct differences – more concave in the southern skulls coupled with the presence of the palatine foramen. The presence of the palatine foramen may make some dental and oral anaesthesiology easier in surgical interventions and examinations. The long and narrow shape of the palate and fused mandibular symphysis are consistent with reports by Freeman (Freeman, 1998) on observations in other species of frugivorous megachiropterans. The fused mandibular symphysis helps to stabilise and strengthen the rostral aspect of the jaw (Freeman, 1998). The differences observed in the palatine bone and the distinctiveness of the suture lines may be attributed to changes in the development of the cranium resulting in modifications of the skull or the whole skeleton (Camacho *et al.*, 2019).

Some reports have been documented of variation in skull shape and size in breeds of animals obtained from different geographical locations, for example, Rodent *Ctenomys* (Fernandes *et al.*, 2009), the gray wolf (Milenković *et al.*, 2010), domestic dogs (Drake and Klingenberg, 2010) and based also on the racial variations in humans (Durbar, 2014). In spite of the fact that the bats used in this study were all phenotypically *Eidolon helvum*, the results obtained from this study indicate the possibility of an ecology-based variation. In addition, since bats are animals that migrate over long distances (DeFrees and Wilson, 1988), these skull differences may also indicate an adaptation for survival based on the distances travelled and the peculiarities of the geographical locations. In humans, craniometry has been used to determine the race and sex of the subject (Durbar, 2014). It remains to be seen if this can be applied completely to bats, although in this study, results obtained show that measurements alone may not be sufficient to distinguish males from females.

In conclusion, the *Eidolon helvum* still remains one of the most common frugivorous bats in Nigeria. These distinct variations in skull features based on location may shed more light on the effect of ecology on skeletal morphology, and may act as a guide when handling these species, most

especially in clinical and surgical manoeuvres. This report is probably the first documenting skull shape variation in the *E. helvum* obtained from different locations in the same country.

### Acknowledgements

The authors gratefully acknowledge the assistance of Dr. T. B. Joannis of the Nigerian Veterinary Research Institute (NVRI), Jos, Plateau State, in capturing the bats from the north (Jos) and also Mr. A.W. Ramoni, of Veterinary Anatomy Department, University of Ibadan for his technical assistance in the maceration.

### Author contributions

OOI: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, manuscript draft, review and editing.

JSJ: Data curation, formal analysis, investigation, methodology, manuscript draft.

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Full-Length Research Article

# Anti-Hypertensive Effects of Anthocyanins from Hibiscus sabdariffa Calyx on the Renin-Angiotensin-Aldosterone System in Wistar Rats

Ubom R.E., \*Ugwu P.I., Madueke S.P., Okorie P.O., Nwachukwu D.C.

Department of Physiology, College of Medicine, University of Nigeria, Enugu Campus

**Summary:** Hibiscus sabdariffa (HS) has gained attention as an anti-hypertensive agent. In the present study, we hypothesized that anthocyanins from HS may attenuate salt-induced hypertension in rats by suppressing the components of renin-angiotensin-aldosterone system (RAAS). Hypertension was induced in the rats by adding 8% NaCl to their constituted diet for six weeks. Wistar rats (n=5 each) were randomly divided into seven groups. Group 1 was the normotensive control group and was fed with normal rat chew and water ad libitum; groups 2 and 3 served as hypertensive control (negative untreated and positive treated with captopril 30mg/kg respectively); groups 4, 5, and 6 served as treatment groups and were administered with graded doses of anthocyanins (50, 100, 200mg/kg respectively) while group 7 received both 100mg anthocyanins and 30mg captopril per day for 4 weeks. Using HPLC, anthocyanins were isolated from HS calyx following standard protocol. Anthocyanins significantly ( $p < 0.05$ ) reduced blood pressure and heart rate in hypertensive rats in a dose-dependent manner. The blood pressure reduction by anthocyanins was associated with a reduction in serum ACE and plasma aldosterone in the hypertensive rats. The effects of anthocyanins on blood pressure and biomarkers of RAAS were similar to those of captopril, a reference anti-hypertensive drug. The results suggest that anthocyanins exerts a significant anti-hypertensive potency on rats, probably mediated by the reduction in components of the RAAS.

**Keywords:** hypertension, anthocyanins, renin, aldosterone, captopril, rats

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\*Address for correspondence: E-mail: [princewill.ugwu@unn.edu.ng](mailto:princewill.ugwu@unn.edu.ng); Tel: +2347063197745

Manuscript received- October- 2021; Accepted- April, 2022

DOI: <https://doi.org/10.54548/njps.v37i1.14>

## INTRODUCTION

Hypertension has been identified as a major risk factor for the emergence of cardiovascular disease and is associated with substantial global morbidity and mortality (Gou *et al*, 2011; Okubadejo *et al*, 2019). In Nigeria, the prevalence of hypertension, dependent on the location, is said to range between 28.9% and 46.6%, with a high prevalence of poor control observed among her population (Adeloye *et al*, 2015; Paul *et al*, 2021). In addition to conventional anti-hypertensive drugs, there are several herbal formulations with documented anti-hypertensive potency, and the use of these agents has increased among patients of all socioeconomic levels (Shinta *et al*, 2019). However, there is a general lack of evidence regarding the mechanisms of action and potential interactions of these herbal preparations with routine anti-hypertensive drug therapy (Ajay *et al*, 2007). The anti-hypertensive efficacy of HS extract has been established in various studies (Nwachukwu *et al*, 2015; Abubakar *et al*, 2015).

Anthocyanins constitute one of the components in HS which is associated with anti-hypertensive actions (Ojeda *et al*, 2010). However, there is a paucity of information on the mechanisms of action of anthocyanins in human and animal models of hypertension. The therapeutic manipulation of the RAAS is a known treatment strategy for hypertension (Crowley and Coffman, 2012; Fountain and Lipton, 2019).

There is evidence that agents that suppress RAAS have vasopressor and diuretic effects that improve the prognosis in hypertensive patients (Munoz-Durango *et al*, 2016). In available reports, anti-hypertensive activities of crude extracts of HS (Mojiminiyi *et al*, 2012; Abubakar *et al*, 2015) and total anthocyanins mixtures (Cortez *et al*, 2017) were described. The isolation of pure cyanidin-3-glycoside (C3G) as the major bioavailable form of anthocyanins, and the investigation of its anti-hypertensive potency has not been explored considerably. More so, animal studies and human trials have correlated a reduction in blood pressure parameters with the suppression of components of RAAS (Nwachukwu *et al*, 2015; Poulsen *et al*, 2019). Despite this, the mechanisms of action of RAAS that mediate the reduction in blood pressure are yet to be clearly proposed. Isolating anthocyanins and hypothesizing their mechanisms of action on RAAS may be judicious in the management of hypertension. Hence, the present study examined the anti-hypertensive potentials of anthocyanins from HS by evaluating their actions on the various components of RAAS.

## MATERIALS AND METHODS

**Drugs, Chemical and Reagents:** Captopril manufactured by Globela Pharma PVT. Ltd, India and Sodium pentobarbital from the marketers in Nigeria, Macjames Eco-

friendly Products, Nigeria, were acquired for the experiment. Sodium chloride, methanol and distilled water were gotten from the manufacturers – Sigma-Aldrich Chem. Company, St. Louis, USA. Anthocyanins were obtained locally from HS Calyx.

**Preparation of Drugs:** Captopril was reconstituted in distilled water to give the required dose of 30mg/kg/2ml body weight and was administered orally. Sodium pentobarbital (Nembutal) was used as anaesthesia at a dose of 100mg/kg b.wt given intraperitoneally. Sodium chloride was reconstituted as 8% NaCl and mixed with rat chow. Methanol was reconstituted as 80% methanol and used to extract the constituents of HS calyx. Anthocyanins extracted from HS calyx were used at graded doses of 50mg, 100mg and 200mg/kg body weight.

**Experimental Animals:** Thirty-five adult male Wistar rats, each weighing between 260g to 300g, were obtained from a local breeder in Akure, Nigeria. They were maintained under standard laboratory conditions at Biosolution Tech. Centre, Akure, fed with commercially formulated chow (Bendel Feeds and Flour Mills, Ewu, Edo state) and had tap water *ad libitum*. Excess feeds and water were removed and replaced daily. After two weeks of acclimatization, the animals were randomly divided into seven groups before the commencement of the experiment.

**Plant Collection and Authentication:** The fresh calyces of HS were collected from a vegetable garden in Akure, Nigeria. The plant specimen was identified and authenticated by Prof. Lawrence Okoror in the herbarium unit of Biosolution Centre, Akure. A specimen (AAh/207) was deposited in the herbarium.

**Preparation of Methanol Extract of HS Calyx:** One thousand grams (1000 g) of pulverized HS calyx was cold macerated in 2.5L of 80% methanol for 48hrs. The extract was filtered through filter paper (Whatman Int. Ltd, England). The filtrate was dried using a rotary evaporator (BUCHI Vacuum Controller, V-800) at 40°C under a reduced pressure for 3hrs. The resulting residue (13.5% w/w recovery) was kept under 4°C for the isolation of anthocyanins.

**Isolation of Anthocyanins:** Anthocyanins were isolated from the resulting residue of HS calyx using standard protocol. Extraction and purification of total anthocyanins were done according to the method described by Ologundudu *et al* (2010). The elution of the extract was done using sepabeads SP-207 resin column (Mitsubishi Chem. Japan). Then the eluate was collected with 60% ethanol and dried by a rotary evaporator at 40°C. Quantification of anthocyanins content in the purified extract was done by the pH differential method described by Lietal (2012).

$$\text{Anthocyanins content (mg/g)} = \frac{A \times mw \times df \times v \times 1000}{\epsilon \times L \times wt}$$

where:

A = absorbance value; mw = molecular weight of C-3-G, df = dilution factor; v = final volume;  $\epsilon$  = absorptive index of C-3-G; L = path length; wt = weight of extract.

The method described by Yang *et al* (2019) was used to determine the content of C-3-G form of anthocyanins using HPLC. The peak areas of anthocyanins from HS were compared with the chromatogram of standard anthocyanins.

#### Experimental Design and Induction of Hypertension:

The rats were divided into seven groups of five rats each according to the method described by Abubakar *et al* (2015) and Balogun *et al* (2016). Group 1 served as normotensive control. Groups 2 and 3 served as hypertensive control (negative untreated and positive treated with captopril 30mg/kg respectively). Groups 4, 5 and 6 served as treatment groups and were administered with graded doses of anthocyanins (50, 100 and 200mg/kg respectively). Group 7 received 100 mg/kg anthocyanins and 30mg/kg captopril. Administration of anthocyanins commenced at the end of six weeks of salt loading and lasted for 4 weeks between 8am and 9am. All drugs were administered daily by oral gavage.

Hypertension was induced by adding 8% sodium chloride to the feed of the rats daily for 6 weeks following the method described by Mojiminiyi *et al* (2012) and also Abubakar *et al* (2015). Weekly systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP) and heart rate (HR) were measured and recorded. Rats with SBP and DBP above 140 and 100mmHg respectively after three consistent readings were considered hypertensive and recruited for the study.

**Determination of blood pressure parameters:** An automated tail-cuff blood pressure meter, the CODA 11™ NIBP recording system (Kent Scientific Corporation, USA) was used to record blood pressure and heart rate in rats. Rats were placed in restraining holders with a nose cone to calm the animals. The restrainers were placed on a heating pad (32±2°C) to warm the rat's tail and maintain blood flow to the tail. The rats were placed in the restrainers for at least 5mins before determining their blood pressure. An average of three consistent readings was taken for each rat.

#### Protocol for serum collection and biochemical assays:

The rats were fasted for 12hrs and anaesthetized with sodium pentobarbital (100mg/kg b.wt, i.p) after the last measurement of blood pressure. Subsequently, 2.5ml of fasting blood samples were collected through the cardiac puncture into labeled sterile plain tubes. The samples were allowed to stand for 1hr at room temperature and then centrifuged at 3000rpm for 10mins. The serum was collected with a Pasteur pipette into sample bottles and stored under 4°C until required. All analyses were completed within 24hrs of sample collection.

**Evaluation of serum renin, ACE and aldosterone:** Renin concentration and ACE levels were estimated in the serum using assay kits according to the instructions of the manufacturer (Angiotenin IRIA kits, USA). Aldosterone was estimated in serum using Aldoct A k-2, diasorin, MN as described by the manufacturer.

**Ethical Clearance:** The procedures and techniques used in the study were in accordance with accepted principles for laboratory animal use and care by the United States National Institute of Health (US NIH Publication, 2011). Ethical

approval was obtained from Research Ethics Committee, College of Medicine, University of Nigeria, Enugu Campus. (Ref. No NHREC/05/01/2508B-FWA00002458-IRB00002323).

### Statistical analysis

The values obtained were presented as mean  $\pm$  SEM. The data were then analyzed using one way ANOVA followed by LSD posthoc test. Values of  $p < 0.05$  were considered to be statistically significant.

## RESULTS

**Total anthocyanins and cyaniding-3-glycoside (C-3-G) contents (in mg/g) and their percentage yield in H. sabdariffa calyx:** From each gram of H. Sabdariffa calyx, 9.71mg total anthocyanins were isolated – A yield of 0.97%; while 4.43mg C-3-G were isolated per gram of H. Sabdariffa calyx – a yield of 0.44% w/w.

**Changes in blood pressure and heart rate in all the groups in response to the administration of anthocyanins and captopril:** Treatment with anthocyanins for 4 weeks as presented in tables 1-3, significantly reduced blood pressure parameters in hypertensive rats when compared with their respective baseline values. The mean values of SBP, DBP,

MABP and HR in all the treatment groups were significantly reduced when compared with their corresponding baseline values. As seen in table 1, co-administration of anthocyanins and captopril caused maximum reduction in blood pressure and restored BP to normotensive values ( $p < 0.001$ ) especially when compared to the control group.

The mean values of SBP, DBP, MABP and HR of hypertensive rats after 4 weeks of treatment with anthocyanins were significantly reduced when compared with the hypertensive untreated group.

### Changes in the serum concentration of components of RAAS in all the groups in response to the administration of anthocyanins and captopril

As presented in tables 4-6, treatment with anthocyanins for four weeks significantly reduced serum level of some components of RAAS when compared with their respective baseline values. The mean values of serum ACE and aldosterone in all the treatment groups were significantly reduced (\* is  $p < 0.05$ , \*\* is  $p < 0.01$ , \*\*\* is  $p < 0.001$ ) when compared with their corresponding baseline values. The tables also show that the mean values of serum ACE and aldosterone after 4 weeks of anthocyanins treatment were significantly reduced ( $p < 0.05$ ) when compared with the hypertensive untreated group

**Table 1:**

Mean Systolic and Diastolic Blood Pressure (SBP and DBP) in mmHg before and after Administration of Anthocyanins

| GROUP           | SBP Before        | SBP After            | DBP Before        | DBP After           |
|-----------------|-------------------|----------------------|-------------------|---------------------|
| 1. Norm         | 122.42 $\pm$ 0.96 | 124.18 $\pm$ 1.62    | 78.30 $\pm$ 1.97  | 82.08 $\pm$ 1.26    |
| 2. Hyp-Untr.    | 195.28 $\pm$ 1.73 | 194.22 $\pm$ 1.90    | 139.02 $\pm$ 1.13 | 138.40 $\pm$ 0.01   |
| 3. Hyp-Cap.     | 192.50 $\pm$ 2.00 | 135.80 $\pm$ 1.64*** | 139.38 $\pm$ 1.38 | 87.58 $\pm$ 1.17*** |
| 4. Hyp-Ant. 50  | 194.54 $\pm$ 3.19 | 180.30 $\pm$ 1.59*   | 136.38 $\pm$ 2.11 | 144.56 $\pm$ 1.48*  |
| 5. Hyp-Ant. 100 | 195.60 $\pm$ 1.90 | 156.14 $\pm$ 2.26**  | 139.40 $\pm$ 0.53 | 100.80 $\pm$ 1.31** |
| 6. Hyp-Ant. 200 | 196.08 $\pm$ 1.58 | 141.04 $\pm$ 2.46*** | 139.48 $\pm$ 0.50 | 99.84 $\pm$ 0.28**  |
| 7. Hyp-Ant+Cap  | 192.64 $\pm$ 1.97 | 124.12 $\pm$ 1.51*** | 140.22 $\pm$ 0.34 | 79.46 $\pm$ 0.92*** |

All values are expressed as Mean  $\pm$  SEM (n=5 in each group); \* is  $p < 0.05$ , \*\* is  $p < 0.01$ , \*\*\* is  $p < 0.001$  vs corresponding baseline values

**Table 2:**

Mean arterial blood pressure (MABP) in mmHg before and after administration of Anthocyanins

| GROUP        | BASELINE (Before) | 4 <sup>TH</sup> WEEK (After) |
|--------------|-------------------|------------------------------|
| Norm         | 86.72 $\pm$ 0.84  | 84.64 $\pm$ 2.99             |
| Hyp-Untr.    | 153.30 $\pm$ 1.56 | 154.96 $\pm$ 2.15            |
| Hyp-Cap.     | 151.10 $\pm$ 0.97 | 97.46 $\pm$ 1.47**           |
| Hyp-Ant. 50  | 152.14 $\pm$ 1.20 | 130.44 $\pm$ 3.72*           |
| Hyp-Ant. 100 | 150.84 $\pm$ 0.91 | 126.22 $\pm$ 1.04**          |
| Hyp-Ant. 200 | 150.30 $\pm$ 1.41 | 111.22 $\pm$ 1.78**          |
| Hyp-Ant+Cap  | 150.48 $\pm$ 1.21 | 87.24 $\pm$ 3.05***          |

All values are expressed as Mean  $\pm$  SEM (n=5 in each group)

\* is  $p < 0.05$ , \*\* is  $p < 0.01$ , \*\*\* is  $p < 0.001$  vs corresponding baseline values

**Table 3:**

Mean Heart Rate (HR) in beats/minute before and after Administration of Anthocyanins

| GROUP        | BASELINE (Before) | 4 <sup>TH</sup> WEEK (After) |
|--------------|-------------------|------------------------------|
| Norm         | 364.60 $\pm$ 3.57 | 362.18 $\pm$ 3.76            |
| Hyp-Untr.    | 479.22 $\pm$ 2.48 | 476.52 $\pm$ 2.43            |
| Hyp-Cap.     | 475.10 $\pm$ 1.69 | 356.94 $\pm$ 1.82***         |
| Hyp-Ant. 50  | 472.80 $\pm$ 3.87 | 381.64 $\pm$ 1.08***         |
| Hyp-Ant. 100 | 476.50 $\pm$ 1.79 | 379.86 $\pm$ 0.29***         |
| Hyp-Ant. 200 | 477.80 $\pm$ 2.13 | 380.32 $\pm$ 0.20***         |
| Hyp-Ant+Cap  | 477.50 $\pm$ 2.48 | 372.94 $\pm$ 2.10***         |

All values are expressed as Mean  $\pm$  SEM (n=5 in each group)

\* is  $p < 0.05$ , \*\* is  $p < 0.01$ , \*\*\* is  $p < 0.001$  vs corresponding baseline values

**Table 4:**

Mean serum level of renin before and after administration of anthocyanins (ng/mL)

| GROUP        | BASELINE (Before) | 4 <sup>TH</sup> WEEK (After) |
|--------------|-------------------|------------------------------|
| Norm         | 2.06 $\pm$ 0.07   | 2.10 $\pm$ 0.05              |
| Hyp-Untr.    | 2.34 $\pm$ 0.07   | 2.34 $\pm$ 0.07              |
| Hyp-Cap.     | 2.12 $\pm$ 0.10   | 2.38 $\pm$ 0.10              |
| Hyp-Ant. 50  | 1.92 $\pm$ 0.04   | 2.32 $\pm$ 0.10              |
| Hyp-Ant. 100 | 1.96 $\pm$ 0.08   | 2.24 $\pm$ 0.09              |
| Hyp-Ant. 200 | 1.94 $\pm$ 0.07   | 2.20 $\pm$ 0.08              |
| Hyp-Ant+Cap  | 2.02 $\pm$ 0.04   | 2.38 $\pm$ 0.07              |

All values are expressed as Mean  $\pm$  SEM (n=5 in each group)

\* is  $p < 0.05$ , \*\* is  $p < 0.01$ , \*\*\* is  $p < 0.001$  vs corresponding baseline values

**Table 5:**

Mean Serum Concentration of ACE before and after Administration of Anthocyanins (pg/mol)

| GROUP        | BASELINE (Before) | 4 <sup>TH</sup> WEEK (After) |
|--------------|-------------------|------------------------------|
| Norm         | 1.24 $\pm$ 0.07   | 1.24 $\pm$ 0.08              |
| Hyp-Untr.    | 3.46 $\pm$ 0.02   | 3.48 $\pm$ 0.02              |
| Hyp-Cap.     | 3.20 $\pm$ 0.09   | 1.80 $\pm$ 0.06***           |
| Hyp-Ant. 50  | 3.10 $\pm$ 0.04   | 2.48 $\pm$ 0.05*             |
| Hyp-Ant. 100 | 3.02 $\pm$ 0.02   | 2.28 $\pm$ 0.02**            |
| Hyp-Ant. 200 | 3.14 $\pm$ 0.07   | 1.80 $\pm$ 0.06***           |
| Hyp-Ant+Cap  | 3.12 $\pm$ 0.04   | 1.70 $\pm$ 0.06***           |

All values are expressed as Mean  $\pm$  SEM (n=5 in each group)

\* is  $p < 0.05$ , \*\* is  $p < 0.01$ , \*\*\* is  $p < 0.001$  vs corresponding baseline values

**Table 6:**

Mean Serum Concentration of Aldosterone before and after Administration of Anthocyanins (mmol/l)

| GROUP        | BASELINE (Before) | 4 <sup>TH</sup> WEEK (After) |
|--------------|-------------------|------------------------------|
| Norm         | 1.80±0.06         | 1.86±0.04                    |
| Hyp-Untr.    | 4.60±0.03         | 4.58±0.04                    |
| Hyp-Cap.     | 4.64±0.02         | 4.42±0.13                    |
| Hyp-Ant. 50  | 4.60±0.03         | 2.82±0.02**                  |
| Hyp-Ant. 100 | 4.56±0.02         | 2.42±0.02***                 |
| Hyp-Ant. 200 | 4.66±0.02         | 2.44±0.07***                 |
| Hyp-Ant+Cap  | 4.52±0.02         | 2.38±0.02***                 |

All values are expressed as Mean±SEM(n=5 in each group)

\*is  $p < 0.05$ , \*\* is  $p < 0.01$ , \*\*\* is  $p < 0.001$  vs corresponding baseline values

## DISCUSSION

Evidence abounds that natural flavonoid from plants, including anthocyanins are associated with improved cardiovascular risk profile (Lelong *et al* 2015; Orororo *et al*, 2018). In our study, using HPLC, 4.43mg cyanidin-3-glycoside (C3G) of anthocyanins were isolated per gram of HS calyx, with a yield of 0.44% w/w. Li *et al* (2012) and Cortez *et al* (2015) have reported that C3G is the major bioavailable form of anthocyanins and that most of the biological effects of anthocyanins have been studied using C3G. In the present study, anti-hypertensive effects of anthocyanins from HS calyx on the RAAS were investigated using the salt-induced hypertensive model in rats. The result showed that the anthocyanins are effective as anti-hypertensive agents by significantly lowering blood pressure parameters and heart rate in hypertensive rats.

The anti-hypertensive potency of anthocyanins in the present study was comparable to captopril, a known ACE inhibitor. Previous studies have reported that HS possesses an ACE inhibitory effect (Ojeda *et al* 2010; Nwachukwu *et al*, 2015). Anthocyanins have been identified as one of the constituents responsible for the anti-hypertensive activities of HS Calyx (Herrera-Arellano *et al*, 2004). We found that anthocyanins at high doses and captopril, a standard anti-hypertensive drug, exert a similar anti-hypertensive action on blood pressure parameters of Wistar rats. Hence, the results in our present study suggest that anthocyanins from HS may be administered alone as an anti-hypertensive agent.

In the present study, co-administration of anthocyanins and captopril caused maximum reduction in blood pressure and restored BP to normotensive values. Hence, co-treatment of anthocyanins and captopril has a synergistic effect and may augment the anti-hypertensive potency of the primary drug. However, earlier workers proposed that co-administration of HS extract and captopril did not induce a further reduction in blood pressure compared to captopril alone (Shinta *et al*, 2019). The discrepancy in the action of anthocyanins and crude HS extract in combination with captopril may be due to other components in HS like quercetin which could lower the amount of captopril absorbed into the blood circulation (Barrenetrix *et al*, 2010). RAAS is a known physiologic regulator of blood pressure and it has been speculated that a reduction in blood pressure is associated with a down-regulation of components of the RAAS (Poulsen and Fenton, 2019). The attenuation of the three basic components of RAAS has been reported to be an important mechanism of action for blood pressure reduction (Munoz-Durango *et al*, 2016). In the

present study, anthocyanins at high doses, similar to captopril and a combination of anthocyanins with captopril, significantly reduced serum ACE in hypertensive rats. This finding is consistent with the known action of ACE inhibitors (Shinta *et al*, 2019) and HS extract (Ojeda *et al*, 2010). The observed reduced serum ACE by anthocyanins possibly reduced the conversion of Angiotensin I to Angiotensin II and consequently reduced the pressor response mediated by angiotensin II, leading to a reduction in BP (Nwachukwu *et al*, 2015). The ACE inhibitory effect of anthocyanins in this study was dose-dependent. The highest effect of anthocyanins on serum ACE was obtained in a combination of anthocyanins and captopril. The findings agree with Shinta *et al* 2019 who reported that HS extract used as a supplement with captopril may cause a synergistic anti-hypertensive action. In the current study, anthocyanins significantly reduced plasma aldosterone in hypertensive rats. Previous reports suggested that aldosterone levels decrease with ACE inhibitors (Nwachukwu *et al*, 2015). However, others have reported higher levels of aldosterone in hypertensive rats treated with ACE inhibitors (Sato *et al*, 2003) probably due to the escape of aldosterone from ACE inhibition (Sato *et al*, 2003). The observed reduced serum aldosterone by anthocyanins possibly reduced the reabsorption of fluid and electrolytes leading to a reduction in blood volume and blood pressure (Poulsen *et al*, 2019).

We found that anthocyanins, similar to captopril and a combination of anthocyanins with captopril did not significantly reduce plasma renin in hypertensive rats. However, studies have shown that HS caused a significant reduction in plasma renin levels of hypertensive rats (Joven *et al*, 2014). The lack of a significant reduction in plasma renin in the present study may be due to compensatory renin release resulting from reduced ACE and aldosterone (Ojeda *et al*, 2010). Hence, the present results suggest that renin levels in hypertensive rats treated with anthocyanins are not correlated with blood pressure reduction.

In conclusion, anthocyanins from *H. sabdariffa* calyx similar to captopril, exert a significant blood pressure-lowering effect in salt-induced hypertensive rats. The blood pressure reduction may be mediated by the reduction in serum ACE and plasma aldosterone. Thus, anthocyanins may be an effective anti-hypertensive agent either alone or in combination with a known anti-hypertensive drug.

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Full-Length Research Article

## Hepatoprotective and Renoprotective effect of *Moringa oleifera* Seed Oil on Dichlorvos-induced Toxicity in Male Wistar rats

Saka W. A., Adeshina O. A., Yusuf M. G., Omole A. I.

Department of Physiology, Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria

**Summary:** The liver and the kidney are vital organs of the body. Drug-induced toxicity is one of the most common problems encountered by these organs. The search for an effective medicine to treat this toxicity without any side effects has led to the use of traditional-based medicine. This study evaluated the effect of ethanolic extract of *Moringa oleifera* seed oil on hepatic and renal markers in dimethyl 2, 2-dichlorovinyl phosphate (DDVP, known as dichlorvos)-exposed Wistar rats. Twenty-one male Wistar rats were randomly divided into three groups of seven animals each. Group A served as the negative control and was not exposed to dichlorvos. Group B served as the positive control and was exposed to dichlorvos for 2 minutes but received no extract. Group C was exposed to the dichlorvos and received 300mg/kg of extract (*Moringa oleifera* seed oil) for 7 days before and 21 days after exposure. Exposure to DDVP led to a significant increase in hepatic & renal markers, inflammatory markers, decrease in plasma protein and alteration of plasma electrolyte. *Moringa oleifera* seed oil regulated and significantly enhanced plasma protein, reduced elevated level of hepatic & renal markers, inflammatory markers in the study sample. In addition, histopathology observation showed that *Moringa* seed oil was able to regenerate the hepatorenal damage on exposure to dichlorvos. *Moringa oleifera* seed oil exhibited hepatoprotective, nephro-protective properties and could be explored in nutrition and health.

**Keywords:** Hepatic, Renal, Histopathology, *Moringa oleifera*, Dichlorvos.

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\*Address for correspondence: E-mail: [mshehu84@gmail.com](mailto:mshehu84@gmail.com), [wsaka@lautech.edu.ng](mailto:wsaka@lautech.edu.ng); Tel: +2348063101718

Manuscript received- December- 2021; Accepted- April, 2022

DOI: <https://doi.org/10.54548/njps.v37i1.15>

### INTRODUCTION

The liver and kidney are crucial organs required by the body to maintain gastrointestinal homeostasis and bodily function. Other functions of these organs include regulation of internal milieu such as detoxification and excretion of toxic exogenous and endogenous metabolites and drugs (Ferguson *et al.*, 2008; Najafian *et al.*, 2014). Thus, its optimal function is essential for health and diseases (Saleem *et al.*, 2008). Various drugs and chemicals are used experimentally to induce liver and renal damage, which are assessed in the laboratory by physiological, biochemical, and pathological biomarkers. Hepatotoxin and Nephrotoxin, such as dichlorvos, malathion, gentamicin, acetaminophen, and Nickel, have been used for ages to cause hepatic and renal dysfunction in rats (Sohn *et al.*, 2009; Adeyemi and Elebiyo, 2014; Sief *et al.*, 2014; Dungca, 2016; Ajao *et al.*, 2017; Nwaichi *et al.*, 2019). Potential hepatoprotective and nephroprotective agents are screened for efficacy and safety by observing their effects on these preclinical models, and their utility in clinical conditions is predicted (Sharma and Paliwal, 2012).

Dichlorvos, an organophosphate pesticide, is reported to cause toxicity to the reproductive system (Joshi *et al.*, 2003), pancreas (Hagar and Fahmy, 2002), kidney and spleen (Verma and Srivastva, 2003), liver and lung (Owoeye *et al.*, 2012), brain (Mogda *et al.*, 2009; Diwivedi *et al.*, 2010) and

immune system (Neishabouri *et al.*, 2004). The blood and other body fluids are the central transport system of the body, and any alteration caused by chemical intoxicant, pathogen, injury, and stress is reflected by changes in the blood picture and vital internal organs such as the liver and kidneys (Ihedioha *et al.*, 2004; Poiout-Belissent and McCartney, 2010). Like another organophosphate, the mechanism of toxicity of dichlorvos is the inhibition of acetylcholinesterase (AChE), which is attributed to the accumulation of acetylcholine, thus leading to excess stimulation of parasympathetic nerves (Wang *et al.*, 2004). This stimulation resulted in perspiration, nausea, lacrimation, vomiting, diarrhea, excessive bronchial secretion and death (ATSDR, 1997). Other effects on skeletal muscles and the nervous system include muscle cramps, muscle fasciculation, muscle weakness, flaccidity, drowsiness, fatigue, mental confusion, headache, convulsions, coma, and even death (ATSDR, 1997). However, new methods and drug investigations are needed for protective clinical treatment against hepatorenal damage caused by organophosphorus pesticide toxication (Ihedioha *et al.*, 2004). Several antidotes have been assessed for the regular treatment of organophosphorus pesticides poisoning (O.P.s), and the currently proposed drugs are atropine and pralidoxime chloride (Yadav *et al.*, 2012). Atropine has been used as an antidote against O.P.s for ages, as it effectively blocks the muscarinic receptors, but not nicotinic

receptors, against the toxic effects of AchE (Gunay *et al.*, 2010). Side effects of these drugs include headache, salivation, blurred vision, vomiting, etc. (CEPA, 1996). The need for effective medicine with minimal side effects has led to traditional-based treatment.

Medicinal plants have been essential in protecting the body system against harmful chemicals or drugs that may cause oxidative stress and alteration in antioxidant enzyme and non-enzymatic systems (Alabi *et al.*, 2005). Among many plants, *Moringa oleifera* is one of the best known and most widespread tree species crop of the Moringaceae family. *Moringa*, commonly known as 'drumstick tree' and horseradish tree, is a multipurpose tree used for human food, medicine, and oil production (Anwer *et al.*, 2007). It is cultivated in Africa, Central and South America, and South Asia (Ricardo, 2012). The leaves, flowers, roots, gums, fruits, and seeds are widely used in folk medicine for treating inflammation (Mahajan and Mehta, 2008), cardiovascular dysfunction, liver disease (Rao and Misra, 1998), hematological, hepatic, and renal dysfunction (Mazumder *et al.*, 1999). It is rich in phytochemicals such as kaempferol, rhamnetin, quercetin, chlorogenic acid, rutin, apigenin, ascorbic acid, and carotenoids (Anwar *et al.*, 2007; Karthivashan *et al.*, 2013). Leaves of this plant have been traditionally reported to possess a wide variety of biological activities, including antimicrobial, anti-inflammatory, anti-cancer, anti-diabetic, antispasmodic, stimulant, cough expectorant, and diuretic agent (Anwar *et al.*, 2007; Mbikay, 2012).

Several works of literature exist on the protective effects of the leaf, seed, and pod extract of *Moringa oleifera* against tramadol-induced nephrotoxicity, 7,12-dimethyl benzylanthracene (DMBA) induced renal cancer, and the GENT-induced nephrotoxicity, (Sharma and Paliwal, 2012; Ouédraogo *et al.*, 2013; Nafiu *et al.*, 2019). However, no scientific report is available on the protective effects of *Moringa oleifera* seed oil on liver and kidney toxicity. Hence, the present study was designed to investigate the protective effect of ethanolic extract of *Moringa oleifera* seed oil on dichlorvos-induced toxicity in the liver and kidney of male Wistar rats.

## MATERIALS AND METHODS

**Animals:** For this experiment, healthy male adult Wistar rats with weights ranging from 160-190g were bought from a private animal breeder around Taki in Ogbomoso North L.G.A, Oyo state. They were kept in well-ventilated plastic cages and housed in the animal house of the Department of Physiology, Faculty of Basic Medical Sciences, LAUTECH, Ogbomoso, Oyo State.

**Treatment:** The 21 rats were randomly divided into three groups as follows:

Group I: Negative control; consists of 7 rats that received only feed and clean water for 28 days.

Group II: Positive control (DDVP treated group); consists of 7 rats exposed to dichlorvos via inhalation at a dose of 1ml per rat daily and feed and water for 28 days.

Group III: DDVP and *Moringa oleifera* treated group; consists of 7 rats pretreated with *Moringa* seed oil at a dose

of 300mg/kg body weight for 7 days before and 21 days after exposure to the dichlorvos.

They were exposed to dichlorvos via inhalation at a dose of 1ml per rat daily and *Moringa* seed oil at a dose of 300mg/kg body weight for 21 days in addition to feed and water.

### Plant Materials and Preparation of Ethanolic Extract:

The oil of *M.oleifera* seed was extracted as Ogushina *et al.* (2014) previously reported with some modifications. Briefly, *Moringa oleifera* seeds used in the study were purchased at the New Waso market in Ogbomoso, identified and approved in the Department of Pharmacology and Therapeutics, Bowen University Teaching Hospital (BUTH), Ogbomoso, Oyo state. The husks of the seeds were crushed on a hard surface to obtain the sources. The seeds obtained were pounded into smaller particles using mortar and pestle. The particles obtained after hitting were poured inside a cone-shaped filter paper and then placed inside the extractor. 97% ethanol was run on the *Moringa oleifera* seed particles, and with the extractor being operational, at 78°C temperature, the oil in the seed was extracted and collected inside a beaker. The extract's cup is then placed inside an incubator at 37°C for preservation.

**Drugs and Chemicals:** The trade name of the local pesticide used for this study is **sniper™**. DDVP, containing 1000g/liter of 2,3-dichlorovinyl dimethyl phosphate (DDVP); manufactured by (Forward (Beinaj) Hepu Pesticide Co. Limited, China, for Saro Agrosiences Limited, Oyo State, Nigeria) was used. The pesticide, which contains dichlorvos as the active ingredient, was purchased from New Waso market, Ogbomoso, Oyo State.

**Exposure to DDVP:** Group B and C animals were exposed to 98.54g/m<sup>3</sup> of DDVP via inhalation, as Saka *et al.* (2020) reported. In addition, 1ml of the pesticide was soaked in cotton wool and placed in a desiccator. The rat was also placed inside the desiccator and allowed to inhale the dichlorvos for 2 minutes daily for 28 days to induce liver and kidney toxicity.

### Phytochemical analysis of *Moringa oleifera* seed oil:

Qualitative phytochemical analysis of *M.oleifera* seed oil was performed and reported as previously documented by Chitravadivu *et al.* (2009).

**Saponins:** 5ml of the seed oil was boiled in 20ml of distilled water in a water bath and filtered. Approximately 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously to obtain a stable, persistent foam. The resulting foam was then mixed with three drops of olive oil and shaken vigorously. The formation of emulsion indicated the presence of saponin

**Alkaloids:** 1ml of the seed oil was stirred with 5ml of 1% (v/v) aqueous HCl on a steam bath and filtered when hot. Distilled water was added to the residue, and then 1ml of the filtrate was treated with a few drops of Mayer's reagent, Wagner's reagent, and Dragendoff's reagent. Alkaloids were confirmed by forming a yellow color with Mayer's reagent, red precipitate with Dragendoff's reagent, and reddish-brown precipitate with Wagner's reagent

**Terpenoids:** 5ml of the seed oil was added to 2ml of chloroform. 3ml of concentrated H<sub>2</sub>SO<sub>4</sub> was then carefully added to form a layer. Reddish-brown discoloration of the interfaced was observed, indicating the presence of terpenoid.

**Steroids:** 2ml of acetic anhydride was mixed with 2ml of the seed oil, followed by the addition of 2ml of concentrated H<sub>2</sub>SO<sub>4</sub>. A color change confirmed the presence of steroids from violet to blue or green.

**Glycosides:** 5ml of the seed oil was mixed with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was then underplayed with 1ml of concentrated sulphuric acid. The presence of glycoside was confirmed by forming a violet-green ring below the brown circle.

**Flavonoids:** One milliliter (1ml) of 10% (w/v) NaOH was added to 3ml of the seed oil. The formation of yellow color confirmed the presence of flavonoids.

**Tannins:** One millimeter (1ml) of the seed oil was boiled with 20ml of distilled water in a test tube and filtered. Three drops of 0.1% ferric chloride were added to the filtrate. The formation of green color confirmed the presence of tannin.

**Anthraquinones:** Five millimeters (5ml) of the seed oil was mixed with 10ml of benzene and filtered. Five millimeters (5ml) of 10% NH<sub>3</sub> solution was added to the filtrate. The presence of anthraquinones was established by developing red color in the ammoniacal (lower) phase.

#### Sample collection and Preparation of Tissue

**Homogenate:** Animal sacrifice was performed using cervical dislocation, and the blood sample was obtained from the animal by cardiac puncture and collected into plain bottles (Saka *et al.*, 2011). Serum was obtained by centrifuging at 3000r.p.m for 15 minutes to obtain supernatant for biochemical assay.

#### Biochemical Assay

**Estimation of hepatic markers:** Alkaline phosphatase (A.L.P.), alanine aminotransferase (A.L.T.), aspartate aminotransferase (A.S.T.), albumin, globulin, and total protein were measured using standard laboratory kit according to manufacturer's guide.

**Estimation of renal markers:** Serum sodium, potassium, and calcium levels were determined using the Flame photometry method (410 flame photometer, Chiron Diagnostics) following the manufacturer's guidelines (Akhigbe *et al.*, 2008). Serum bicarbonate, chloride ion, urea, and creatinine were determined using the standard assay kit following back titration, electrometric method, diacetylmonoxime, and alkaline picrate methods.

**Estimation of Inflammatory markers:** Tumor necrosis factor-alpha (TNF- $\alpha$ ) and interleukin 6 (IL-6) markers of inflammation were assayed using ELISA kits (Elabscience Biotechnology Co, Ltd, U.S.A.) following the manufacturer protocols. At the same time, Myeloperoxidase (M.P.O.) was

determined using colorimetric methods (Akhigbe and Ajayi, 2000).

**Histopathological examination:** The organs (i.e., kidney and liver) were also excised, and the excised organs were washed using standard saline solution and later fixed in 10% formalin solution. A paraffin embedding technique was carried out, and sections were taken at 5mM thickness. Hematoxylin and eosin solutions were used to stain the cut-out tissue sections and then examined microscopically for histopathological changes (Preece, 1972).

**Ethical considerations:** Animals used for the research were treated humanely according to the institution's guidelines and criteria for humane care by the National Institute of Health Guidelines for the care and use of Laboratory Animals.

#### Statistical Analysis

Results obtained were expressed as mean  $\pm$  standard error of the mean (n=7). In addition, the data were subjected to a one-way analysis of variance (ANOVA), and differences between samples were determined by Tukey multiple comparison test using the Graph Pad Prism 5 (GraphPad Software Inc., San Diego, CA). While the level of significance used for the analysis was set at p<0.05nt.

## RESULTS

Table 1 shows the findings of the qualitative phytochemical studies. *Moringa oleifera* seed oil extract contained saponins, terpenoids, steroids, tannin, and anthraquinones in varying concentrations.

**Table 1:**  
Phytochemical analysis of Moringa seed oil

| Phytochemicals | Presence |
|----------------|----------|
| Saponins       | +        |
| Alkaloids      | -        |
| Terpenoid      | ++       |
| Steroids       | ++       |
| Glycoside      | -        |
| Flavonoids     | +++      |
| Tannins        | +        |
| Anthraquinones | ++       |

-not detected; + presence in low concentration; ++ present in moderate concentration; +++ present in high concentrations.

**Body weight changes:** The effects of dichlorvos and ethanolic extract of *Moringa oleifera* seed oil on body weight changes is depicted in Figure 1. Administration of dichlorvos decreased the weight of rats when compared to control. However, treatment with *Moringa oleifera* seed oil increased weight of the rats as compared to dichlorvos-induced rats.

**Estimation of Hepatic markers:** The effects of dichlorvos and ethanolic extract of *Moringa oleifera* seed oil on hepatic markers are summarized in Table 2. Administration of Dichlorvos induced extensive damage to the liver tissues, evident by the significant increase in alanine aminotransferase (A.L.T.), aspartate aminotransferase (A.S.T.), alkaline phosphatase (A.L.P.) and a corresponding decrease in albumin, globulin and total protein in the

pathogenic rats (Group B) when compared with control rats. On the other hand, treatment with ethanolic extract of *Moringa oleifera* seed oil (Group C) significantly decreases A.L.T., A.S.T.& A.L.P. In addition, it elevates the plasma protein level compared with the dichlorvos-induced rats.

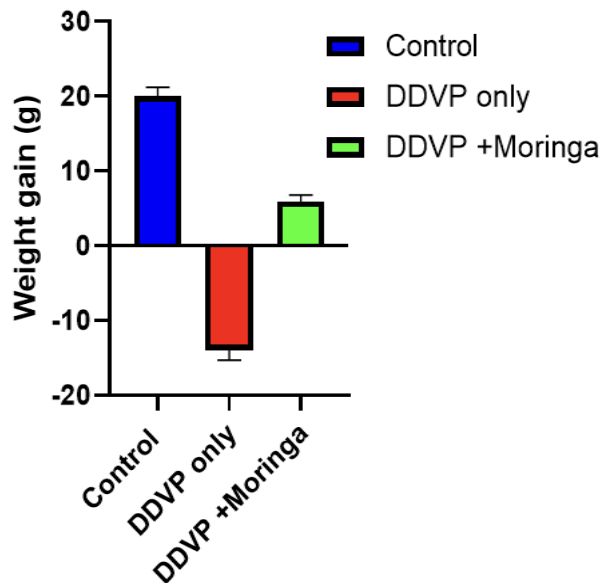


**Figure 1**

Effect of Ethanol Extract of *Moringa oleifera* on weight gain male Wistar rats.

**Table 2:**

Effects of ethanolic extract of *Moringa oleifera* seed oil on hepatic markers in experimental rats.

| GROUPS               | I               | II                           | III                          |
|----------------------|-----------------|------------------------------|------------------------------|
| AST (U/L)            | 82.03<br>± 7.91 | 97.29<br>± 2.64 <sup>a</sup> | 82.71<br>± 7.02 <sup>b</sup> |
| ALT (U/L)            | 27.33<br>± 1.59 | 54.44<br>± 3.50 <sup>a</sup> | 32.16<br>± 3.36 <sup>b</sup> |
| ALP (U/L)            | 31.31<br>± 1.68 | 55.53<br>± 0.49 <sup>a</sup> | 32.23<br>± 0.57 <sup>b</sup> |
| Albumin (g/dl)       | 1.74<br>± 0.09  | 1.08<br>± 0.04 <sup>a</sup>  | 1.68<br>± 0.05 <sup>b</sup>  |
| Globulin (g/dl)      | 1.32<br>± 0.16  | 0.57<br>± 0.07 <sup>a</sup>  | 1.14<br>± 0.14 <sup>b</sup>  |
| Total protein (g/dl) | 2.70<br>± 0.18  | 1.95<br>± 0.11 <sup>a</sup>  | 2.50<br>± 0.18 <sup>b</sup>  |

Values are expressed in Mean ± S.E.M. (n=7)

Groups with different superscript(s) are significantly different from other at  $p \leq 0.05$ . <sup>a</sup>Represent significant difference when compared with negative control. <sup>b</sup>Represent statistical significance when compared with positive control.

Group I = Negative control; Group II = Positive control; Group III = DDVP +300mg/kg of *Moringa oleifera* seed oil.

**Estimation of serum urea and creatinine:** The effects of dichlorvos and ethanolic extract of *Moringa oleifera* seed oil on serum urea and creatinine are summarized in Table 3. Dichlorvos administration induced extensive damage to kidney tissue. As a result, serum creatinine and urea significantly increased in the pathogenic rats (Group B) when compared with control rats ( $P < 0.05$ ). Similarly, there was a significant decrease in the elevated levels of serum creatinine after treatment with ethanolic extract of *Moringa*

seed oil (Group C) ( $P = 0.017$ ) and urea ( $P = 0.02$ ) when compared with dichlorvos-induced rats.

**Table 3:**

Effects of ethanolic extract of *Moringa oleifera* seed oil on renal markers in experimental rats.

| Group               | I                 | II                             | III                            |
|---------------------|-------------------|--------------------------------|--------------------------------|
| Creatinine (mg/dl)  | 0.44<br>± 0.07    | 0.96<br>± 0.06 <sup>a</sup>    | 0.62<br>± 0.10 <sup>b</sup>    |
| Urea (mmol/L)       | 9.08<br>± 1.08    | 16.65<br>± 1.27 <sup>a</sup>   | 11.02<br>± 1.63 <sup>b</sup>   |
| Sodium (ppm)        | 214.76<br>± 27.91 | 494.12<br>± 19.42 <sup>a</sup> | 340.98<br>± 57.50 <sup>b</sup> |
| Potassium (ppm)     | 2.62<br>± 0.10    | 0.65<br>± 0.06 <sup>a</sup>    | 2.10 ±<br>0.17 <sup>b</sup>    |
| Calcium (ppm)       | 14.63<br>± 0.46   | 10.84<br>± 0.75 <sup>a</sup>   | 14.16<br>± 0.26 <sup>b</sup>   |
| Bicarbonate (mEq/L) | 1.22<br>± 0.09    | 0.88<br>± 0.07 <sup>a</sup>    | 1.02<br>± 0.05                 |
| Chloride (mEq/L)    | 142.67<br>± 7.18  | 170.67<br>± 4.52 <sup>a</sup>  | 150.00<br>± 3.33 <sup>b</sup>  |

Values are expressed in Mean ± S.E.M. (n=7)

Groups with different superscript(s) are significantly different from other at  $p \leq 0.05$ ; <sup>a</sup>Represent significant difference when compared with negative control. <sup>b</sup>Represent statistical significance when compared with positive control.

Group I = Negative control; Group II = Positive control; Group III = DDVP +300mg/kg of *Moringa oleifera* seed oil.

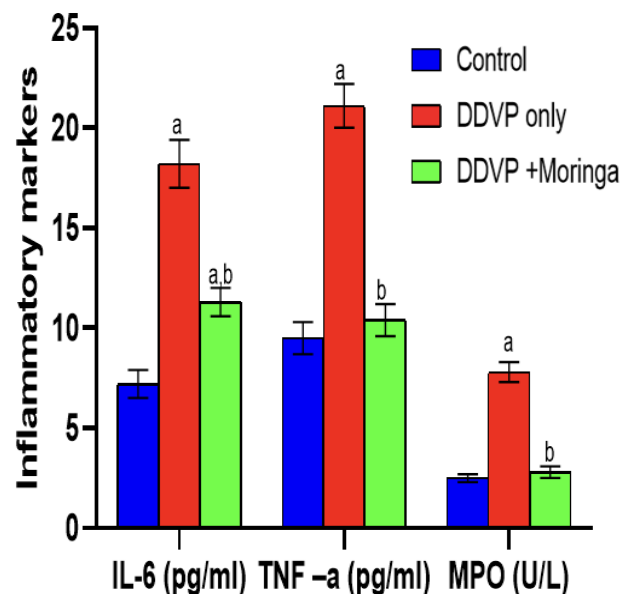


**Figure 2**

Effect of Ethanol Extract of *Moringa oleifera* on inflammatory markers in male Wistar rats.

**Estimation of inflammatory markers:** Exposure of male rats to DDVP led to a significant increase in M.P.O., TNF-α, and IL-6 compared to the control. However, treatment with *Moringa oleifera* seed oil significantly reduced elevated levels of these markers compared to DDVP-induced rats.

**Estimation of serum electrolytes:** The result (Table 3) showed a significant decrease in potassium, calcium, and bicarbonate ions ( $P < 0.05$ ) in the test group after

*Moringa oleifera* seed oil ameliorates dichlorvos-induced toxicity in rats

administration of dichlorvos as compared to the control rats. However, treatment with *Moringa* seed oil shows a significant increase in potassium ion, calcium ion ( $P < 0.05$ ), and a non-significant increase in bicarbonate ion ( $P > 0.05$ ) when compared with the dichlorvos-induced group. Also, there was a significant increase in sodium and chloride ions ( $P < 0.05$ ) in the test rats compared to control rats. On the other hand, treatment with *Moringa* seed oil significantly decreases sodium and chloride ions compared to dichlorvos-induced rats ( $P < 0.05$ ).

**Histology:** Photomicrograph of the liver section (Plate 1) of the standard control group (group A) showed normal central venules without congestion (white arrow), and the morphology of the hepatocytes appeared normal (blue arrow). In addition, the sinusoids seemed to be normal and not infiltrated (slender arrow); no pathological lesion was seen. Still, a photomicrograph of the liver section of group B induced with dichlorvos revealed a non-congested central venule with degenerated cells and macrophage within its lumen (white arrow), portal tracts with dilated portal venules seen (black arrow), the hepatocytes show hypochromic and severe necrosis (blue arrow), the focal area with degeneration and destroyed liver plates seen within the

parenchyma (green arrow). Finally, a photomicrograph of the liver section of group C pre-treated with 300mg/kg of *Moringa oleifera* seed oil before administration of dichlorvos showed mildly congested central venules (white arrow), the morphology of the hepatocytes appeared normal (blue arrow), the sinusoids appear mildly dilated but not infiltrated (slender arrow).

A photomicrograph of kidney sections of the standard control group (group A) showed typical kidney architecture, renal cortex with normal glomeruli, mesangial cells and capsular spaces (white arrows), normal renal tubules (blue arrow), and common interstitial spaces (blue arrow) but a photomicrograph of kidney section of group B induced with dichlorvos showed poor architecture, renal cortex with sclerotic glomeruli and widened capsular spaces (white arrow), collapsed renal tubules (blue arrow) and severe atrophic mesangial cells (black arrow).

A photomicrograph of the kidney section of group C pre-treated with 300mg/kg of *Moringa oleifera* seed oil before administration of dichlorvos shows the moderate architecture, renal cortex with normal glomeruli, mesangial cells and capsular spaces (white arrow), normal interstitial spaces (slender arrow) and attenuated renal tubules (blue arrow) when compared with DDVP-induced group

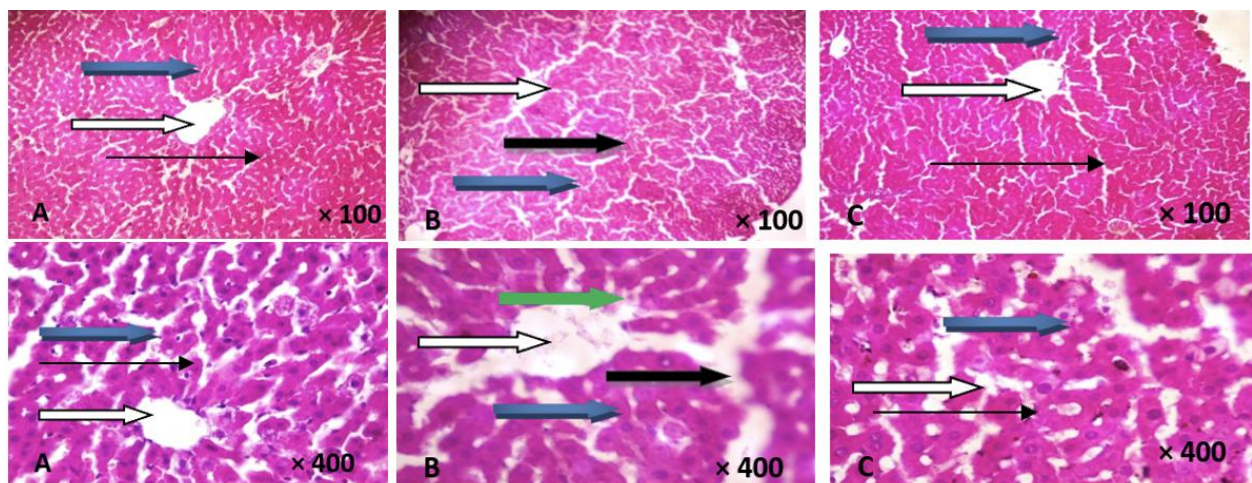


**Plate 1:**

Histopathological examination of the liver

A: Control, B: Dichlorvos-induced only, C: *Moringa oleifera* seed oil (300mg/kg)

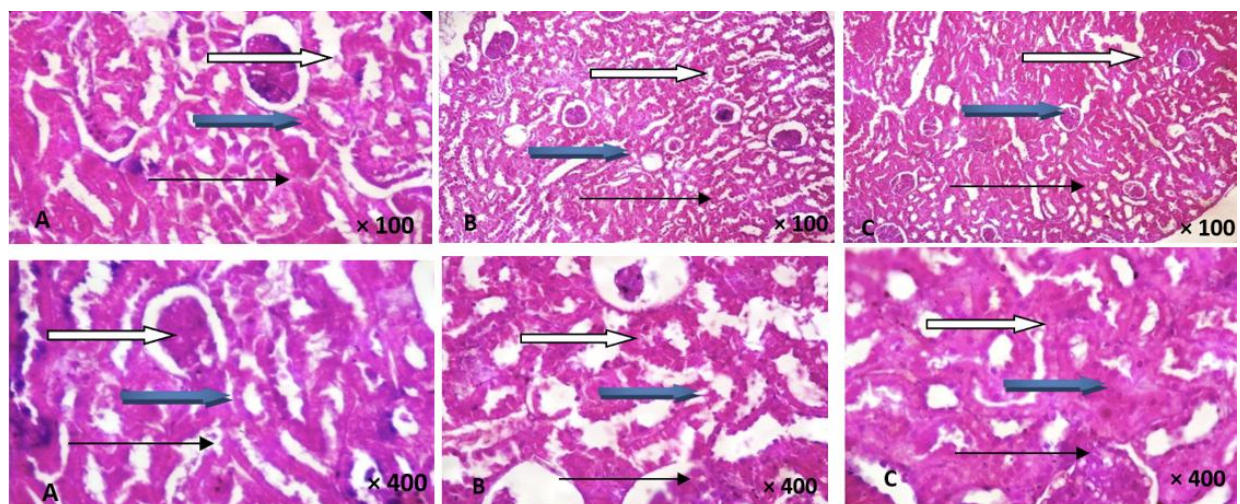


**Plate 2:**

Histopathological examination of the kidney

A: Control B: Dichlorvos-induced only C: *Moringa oleifera* seed oil (300mg/kg).

*Moringa oleifera* seed oil ameliorates dichlorvos-induced toxicity in rats

## DISCUSSION

The continuous use of pesticides has increased human exposure to environmental pollutants that may have acute and chronic adverse effects on health. In addition, synthetic chemicals in pesticides may contribute to long-term impacts on the environment. Humans are concomitantly exposed to insecticides whose toxicity profiles have not been delineated in homes. Dichlorvos, a highly hazardous chemical classified by the WHO as (Class I) (WHO, 1992), accumulate in humans and cause toxic effects in the various organ of the body (Tsitsimpikoua *et al.*, 2013). These organs are either affected by direct or indirect exposure to dichlorvos (Sheiner *et al.*, 2003). The effects of *Moringa oleifera* seed oil on dichlorvos-induced toxicity in liver and kidney organs of male Wistar rats were investigated in the study. This is done by assessing the liver enzyme activities, renal function tests, and inflammatory markers, which serve as the biomarkers for liver and kidney function, respectively.

The results of the acute exposure clearly show that dichlorvos has the potential to promote hepatic and renal damage. This was evident in the study by significant increases in hepatic and renal markers (Table 2;4). Hepatic impairment is characterized by an elevation in serum liver enzymes viz A.L.T., A.L.P., and A.S.T. A.S.T. and A.L.T. are the most common because they are readily released into the extracellular space by the hepatocytes (Ozer *et al.*, 2008). A.S.T. is a highly sensitive hepatocellular marker than A.L.T. (Al-Mammary *et al.*, 2002). However, the concentration of A.L.T. in the body is more than A.S.T. as the body generates more A.L.T. than A.S.T. (Mayne, 1996). Thus, the high levels of A.S.T., A.L.T., and A.L.P. observed in this study (Table 2) indicate the hepatotoxic effect of dichlorvos. This was in tandem with the previous report by Celik *et al.* (2009) and Garba *et al.* (2013), who suggested that dichlorvos causes liver damage in rats. Creatinine and urea are known biochemical markers of acute and chronic nephrotoxicity (Naggayi *et al.*, 2015). Thus, serum creatinine and urea levels are often considered reliable predictors of renal function (Yaman & Balikci, 2010; Naggayi *et al.*, 2015). The significant elevation is seen in the plasma level of urea, creatinine, and other renal markers in the untreated rats could be attributed to the nephrotoxic effect of dichlorvos, which further suggest impairment of the glomerular function and tubular damage of the kidneys (Gross *et al.*, 2005; Adeyemi and Akanji, 2012). This is consistent with the findings of Edelstein (2008), who posited elevated levels of the renal marker in dichlorvos exposed population. The degeneration and destruction observed in the hepatic and renal tissues can be linked to the generation of reactive oxygen species (R.O.S.) initiated by dichlorvos (Sharma and Singh, 2012).

It is a fact that excessive R.O.S. generation and oxidative stress may trigger an inflammatory response and vice versa. Oxidative stress usually enhances a variety of cell signaling pathways involved in inflammation (Khalil *et al.*, 2019). Exposure to DDVP led to marked increased inflammatory markers such as TNF- $\alpha$ , IL-6, and M.P.O., which play a central role in the development and progression of liver and kidney dysfunction. This aligns with previous reports, which confirm that although DDVP

acts via permanent binding and inhibition of acetylcholinesterase activity, most of its harmful biological effects are oxidative-stress mediated (Burke *et al.*, 2016; Pearson *et al.*, 2016; Wang *et al.*, 2016; Imam *et al.*, 2018).

The toxicity of dichlorvos did not spare the tissues (kidney and liver) under examination, as revealed by the histopathological results. Dichlorvos induced histological alterations in the liver of rats ranging from degenerated venules to necrotic hepatocytes. This might indicate hepatic damage, as Abdelhalim and Jarrar (2012) reported. Also, the histopathological examination of the renal tissue attacked by dichlorvos revealed many alterations ranging from tubular degeneration (distorted renal tubules) and atrophy of the glomeruli (glomeruli with obliterated capsular spaces) to congestion of renal blood vessels. This was in tandem with the report of Elhalwagy *et al.* (2008), Kalender *et al.* (2007), Sulak *et al.* (2005), and Mohnsen (2001) that pesticides cause various histopathological changes in the liver and kidney tissues of experimental animals.

From this study, *Moringa oleifera* seed oil has shown its potential in reducing and controlling stress response and played an enormous role in protecting the organs affected by dichlorvos exposure, as demonstrated by the photomicrograph histopathology results. However, it may require more time for complete recovery. This ability of *Moringa* seed oil to regulate the activities of these biomarkers can be attributed to the presence of bioactive compositions such as phenols, flavonoids, terpenoids, and saponins (Bharali *et al.*, 2003; Anwar *et al.*, 2007; Karthivashan *et al.*, 2013). These compounds scavenge R.O.S., chelate metal ions, and regenerate membrane-bound antioxidants. This finding agrees with Kumar and Pari, (2003) and Arabshahi *et al.* (2007). which demonstrated the antioxidant activity of *Moringa* extract. Furthermore, reports have shown that saponins possess tumor-inhibiting activity *in vivo* (Akindahunsi & Salawu, 2005). Thus, its presence can control human cardiovascular disease and reduce blood cholesterol. Other bioactive compounds found in *Moringa* seed oil are high pro-vitamin A, vitamin C, tocopherols, carotenoids, and anthocyanins, which explain their mode of action in the induction of antioxidant profiles in the present investigation. However, the underlying process is yet to be elucidated.

*Moringa* seed oil also contains the bioactive agent  $\beta$ -carotene, which supports healthy liver and kidney functioning, as well as flavonoids, shown to have anti-inflammatory, anti-cancer, anti-diabetic, antispasmodic, stimulant, cough expectorant, and diuretic agent (Bharali *et al.*, 2003; Anwar *et al.*, 2007; Kumar *et al.*, 2010; Mbikay, 2012; Minaiyan *et al.*, 2014). These features might have resulted in the observed hepato-protection against homeostatic imbalance imposed by dichlorvos exposure. Moreover, the maintenance of normal levels of serum electrolytes by *Moringa oleifera* seed oil is consistent with reported medicinal potential, including the improvement of the health of renal tissues and general well-being (Anwar *et al.*, 2007; Ndong *et al.*, 2007). The efficacy of *Moringa* seed oil in improving plasma protein, lowering elevated liver enzymes (A.S.T., A.L.T., and A.L.P.), and renal biomarkers (electrolyte, urea, and creatinine) were observed. All this may be attributed to phytochemicals present in the plants. The consumption of this plant oil may provide health benefits in terms of hepatoprotective, nephroprotective, and

anti-inflammatory effects (due to the presence of  $\beta$ -carotene, flavonoid, tannins, alkaloids, etc.) and scavenging of free radicals in the body (due to the presence of tocopherols, phenolics, and carotenoids) (Karthivashan *et al.*, 2013; Bhatnagar and Krisna, 2013)

In conclusion, this study revealed the harmful effect of dichlorvos exposure on liver and kidney organs. This was confirmed by the assay of liver and renal biomarkers, inflammatory markers, and histopathological examination of the organs. The Moringa seed oil also showed its regenerative potential in recuperating the damaged organs. Thus, Moringa seed oil possesses both hepatoprotective and nephroprotective potential, which depends on its bioactive compounds. This study has shown that Moringa seed oil can regulate and reduce impaired cellular integrity expressed in hepatic and renal markers in individuals that are constantly exposed to household pesticides (i.e., dichlorvos) and could be explored in the treatment of hepatic and renal conditions and also, in the protection of the hepatorenal integrity from some oxidative stress. Future studies are required to fully evaluate the contents of *Moringa oleifera* responsible for the protection against hepatotoxic and nephrotoxic agents.

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Full-Length Research Article

# Biochemical Studies on the Colorectal Tissues of Wistar Rats Treated with Methanol Leaf and Stem Bark Extracts of *Annona muricata* (Soursop)

Agu, K.C.<sup>1</sup> and \*Olubodun, S.O.<sup>2</sup>

<sup>1</sup>Department of Chemistry and Biochemistry, Central Michigan University, MI, United States

<sup>2\*</sup>Department of Medical Biochemistry, University of Benin, PMB 1154, Benin City, Nigeria.

**Summary:** *Annona muricata* (AMC), is a tropical plant species of the Annonaceae family known for its medicinal uses in ameliorating and/or protecting several organs and tissues in the body. In this study, we elucidated the influence of the methanol extracts of AMC leaf and stem bark on the biochemical and histological integrity of the colorectum. Different doses (100, 200, 400, 600, and 800mg of extract/kg body weight) of methanol extracts of the leaves and stem bark were orally administered to adult male Wistar rats of average weight (100 - 150 g) for 28 days. At the end of the experimental period, the rats were sacrificed and colorectal tissues harvested for analyses. Adenosine triphosphatase (ATPases), glucose-6-phosphate dehydrogenase (G6PD) lactate dehydrogenase (LDH) activities, and tissue protein (TP) concentration, were evaluated by colorimetric method using appropriate test kits. The results in the colorectal tissue analysed showed that total ATPase, LDH and G6PD (energy markers) activities increased significantly in the groups which received methanol leaf and stem bark extracts of AMC when compared with control. However, a general decrease was detected in TP and Na<sup>+</sup>/K<sup>+</sup>-ATPase activity but there was a twist in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in stem bark doses of 200mg/kg and 400mg/kg which showed significant increases in dose-dependent manner when compared with the control. Even though G6PD activity showed fluctuating patterns for both extracts, the highest dose (800mg/kg) reflected the most significant increase when compared with the control. The histology confirmed the toxicological results by the biochemical parameters. Under the condition of this study, we inferred that the methanol leaf and stem-bark extracts of AMC may significantly influence the enzymes by reducing and increasing total ATPase and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities depending on the doses and administration should therefore depend on the desired outcome.

**Keywords:** Adenosine triphosphatase, *Annona muricata*, Colorectum, Glucose-6-phosphate dehydrogenase, Lactate dehydrogenase

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\*Address for correspondence: [stella.olubodun@uniben.edu](mailto:stella.olubodun@uniben.edu); +2348023411948; +234 8051526364

Manuscript received- January 2022; Accepted- April 2022

DOI: <https://doi.org/10.54548/njps.v37i1.16>

## INTRODUCTION

Over the history of humans, natural products derived from plants and other by-products have been utilized in the treatment of different types of disease conditions. Numerous chemotherapeutic agents of foremost importance have derived either directly from plants natural products of plants or its derivatives. Outside these vital drugs, there are many other plant preparations including crude herbal concoctions which have been established to have promising results for the prevention, management, amelioration, and treatments of various disease conditions. One of such plant preparations is the one that was prepared from a plant widely seen and grown in subtropic and tropic regions of the world, and from the Annonaceae family (Qazi *et al.*, 2018; Handayani and Yuzammi, 2021; Hernández Fuentes *et al.*, 2022). One of the most popular species of this family is the *Annona muricata*, that is locally known and called Soursop. The plant's fruit-pulp, seeds, root-bark stem-bark and leaves extracts and many other species of Annonaceae (Handayani and Yuzammi, 2021; Hernández Fuentes *et al.*, 2022), have been studied broadly for its antioxidant properties as well as

the anticancer and anti-inflammatory effects (Agu *et al.*, 2017a; Agu and Okolie, 2017a; Agu and Okolie, 2017b). Phytochemical analyses have shown that a bioactive member of polyketide constituents known as acetogenins have been isolated from Annonaceous plant species, and lots of these compounds (including flavonones) are found in diverse parts of *Annona muricata* (Moghadamtousi *et al.*, 2013), and these compounds apart from reported medicinal properties against pathologic conditions, could also help to enhance (or even negatively affect) the integrity of cellular and tissue components, viz, membrane proteins, enzymes, as well as, intracellular biomolecules. Thus, this study was designed to examined the effects of the crude extracts of *Annona muricata* leaf and stem bark on colorectal tissues of Wistar rats, by investigating the colorectal tissue adenosine triphosphatase (ATPase; sodium-potassium, Na<sup>+</sup> K<sup>+</sup>-ATPase and calcium, Ca<sup>2+</sup>-ATPase) and energy markers, glucose 6 phosphate dehydrogenase (G6PD) as well as lactate dehydrogenase (LDH) activities as well as the Na<sup>+</sup>/K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-ATPase activities influenced by identified acetogenin and flavonone.

## MATERIALS AND METHODS

**Experimental rats:** One hundred and forty-four (144) adult male Wistar rats comprising between 100 g – 150 g body weight were utilized in this experimental study. These rats were purchased in the Department of Anatomy, University of Benin, Benin City and were acclimatized for two weeks at the animal house of the Department of Biochemistry, prior to the study. The animals were fed with water and standard rat chow ad libitum. The Research Ethics Committee Guideline Principles on Animal Handling of the College of Medicine, University of Benin (CMR/REC/2014/57) provided written approval for the study, which was strictly followed.

***Annona muricata* crude extract preparation:** A large quantity of the plant's fresh leaves and stem bark were collected from trees in house-hold gardens in Benin City and within the vicinity of the University of Benin in Edo State, Nigeria, and its identification was done at the Plant Biology and Biotechnology department of same university with the voucher authentication specimen number of UBHa 0205, deposited at the Department's Herbarium. The samples (leaves and stem bark) were exposed to room temperature (about 25°C) for 4 weeks, to dry and were separately pulverized. After macerating each pulverized plant sample in methanol for 48 hours, it was filtered through cheese cloth. The extracted materials were then concentrated in vacuo using a rotary evaporator to produce viscous gels that were air-dried to gel-like solids. Using distilled-deionized water as a solvent, the gel-like crude methanol extracts from both plant parts were reconstituted to produce a stock solution. Each reconstituted crude extract was kept in small-capped plastic containers in a refrigerator at -40°C prior to its used.

**Extract administration:** The extracts were administered via gavage, which served as an oro-gastric tube. The rats were given special care to avoid any oral or esophageal injuries.

**Sub-chronic toxicity assessment:** During period of usage, extracts were administered to the rats based on calculated doses per weight of rat (i.e. equivalent volume). This dose calculations were done weekly per weight of rats for the sub-chronic studies, as the weekly weights of the rats per group were recorded i.e. at day 0, day 7, day 14, day 21 and day 28. Untreated rats (group one) served as the control and was administered 2 ml of distilled water (Agu, 2016; Agu *et al*, 2017a; Pongri and Igbe, 2017).

**Observations in sub-chronic assessment (clinical signs and mortality):** The rats were observed for signs of weakness, increased or decreased appetite, weight loss and other physiological changes including mortality. Clinical signs to be assessed before dosing, immediately and 4hrs after dosing, include level of sedation, restlessness, change in nature of stool, urine and eye colour, excretion of worms, diarrhoea, haematuria, uncoordinated muscle movements etc. The animals were observed for toxic symptoms such as weakness or aggressiveness, anorexia, weight loss, diarrhoea, discharge from the eyes and ears, stridor and mortality (Fielding and Matheron, 1991; Vijayalakshmi *et al*, 2000; Suanarunsawat *et al* 2009).

### Experimental protocol for sub-chronic toxicity studies:

The methanol extracts of the leaves and stem-bark were administered at increasing doses from 100 mg/kg (group 2), 200 mg/kg (group 3), 400 mg/kg (group 4), 600 mg/kg (group 5) and 800 mg/kg (group 6). The group 1 rats which served as control, were given 2 ml of distilled water (0 mg/kg). Each group had six (6) rats each.

**Animal Sacrifice:** After the 28 days treatment prior to sacrifice, the rats were fasted overnight. Their weights were taken and then sacrificed after being anaesthetized with chloroform. Their abdomen was opened, and colorectal part of the intestine, i.e. about 1.5cm from the anus, was harvested. Part of the colorectal tissue was preserved in a plain sample container containing normal saline prepared with pH=7.0 phosphate buffer solution, while the other part of the colorectal tissue was preserved in formal-saline prior to tissue slide preparation for histological studies. The tissues stored in the normal saline-phosphate buffer solution were homogenized in phosphate buffer (pH=7.0) using mortar and pestle (0.5g of tissue per 5ml), centrifuged at 10,000g for five minutes and supernatant collected and preserved in a freezer for biochemical analyses.

**Biochemical analysis:** Colorectal ATPases assays (Total ATPase,  $Mg^{2+}$ ,  $Ca^{2+}$ , and  $Na^+/K^+$ -ATPases) were done using the procedure described by Adam-Vizi and Seregi (1982); colorectal tissue lactate dehydrogenase (LDH) activity, glucose-6-phosphate dehydrogenase (G6PD) activity, and tissue protein (TP) concentration, were investigated using ready-to-use Randox® kit method.

**Isolation and elucidation of compounds:** The compounds which served as the ligands that were docked against the ATPases had been isolated and elucidated, as well as, previously reported by Agu *et al*. (2017a).

### Molecular Docking Experiments

**Preparation of Protein:** The Protein data bank (PDB) ID of 4GL7, (the starting crystallography structure) for calcium ATPase and 1MO8 for rat alpha1 Na, K-ATPase nucleotide-binding domain) which was the requirement for docking was obtained from <http://www.rcsb.org> a protein data bank repository. Water and ligand coordinates were removed prior to docking, and the appropriate charges were added. The proteins were viewed in Discovery Studio to study the interactions of their 2-D amino acid residues.

**Preparation of the Ligand:** ChemDraw was used to create the 3D structures of the elucidated compounds, which were then converted to PDB format for docking studies using GaussView. The ligand molecules (compounds) were optimized appropriately.

**Molecular docking:** Molecular Docking calculations were performed using Autodock Vina. The modeled structures of calcium ATPase and nucleotide-binding domain of rat alpha1  $Na^+/K^+$ -ATPase, and elucidated compounds were loaded the Autodock/Vina and all the water molecules were removed prior to the upload. Protein snapshots were taken using PYMOL.

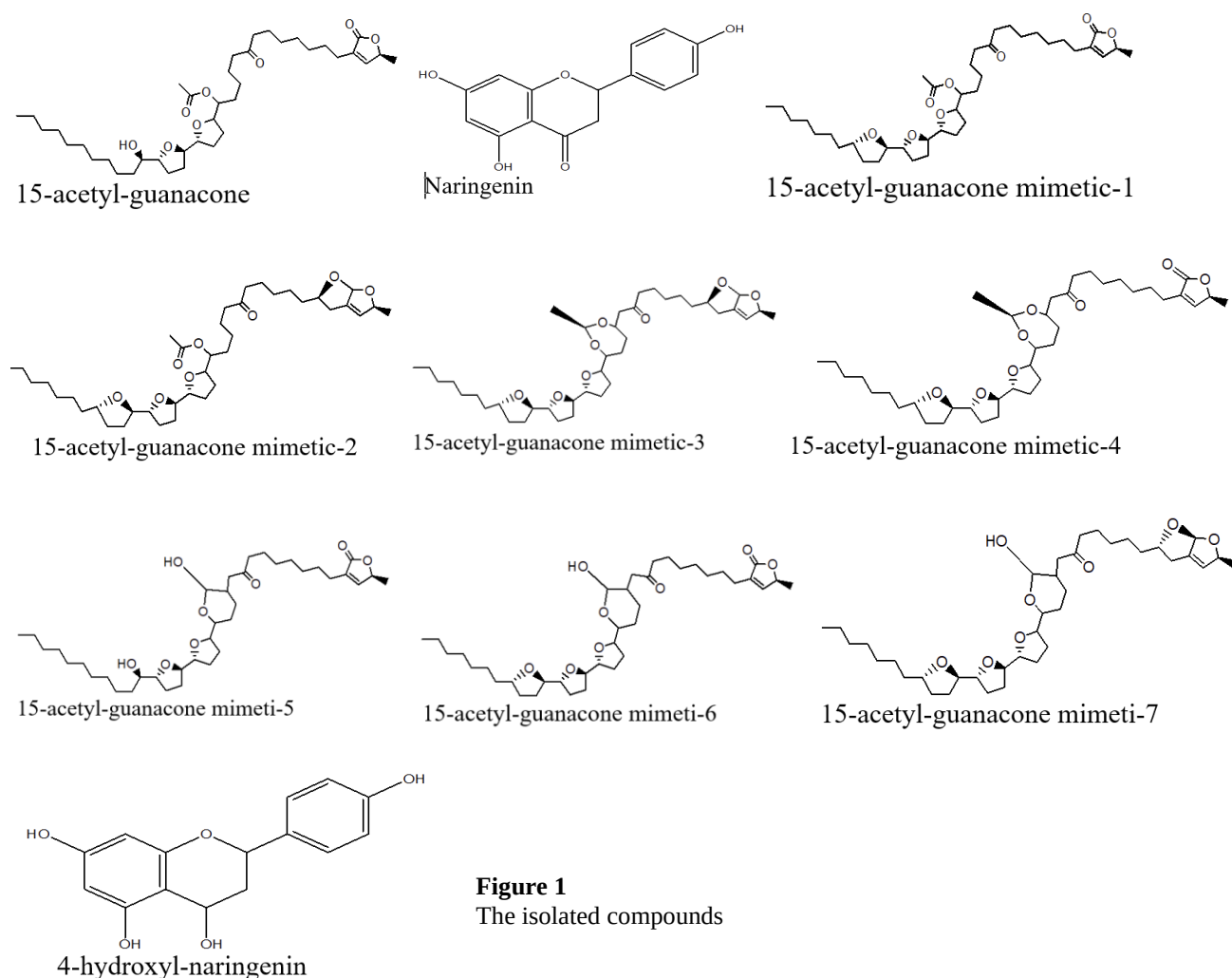


**Figure 1**  
The isolated compounds

**Statistical analysis:** Prior to descriptive analysis, data were entered into a Microsoft Excel spreadsheet (v.10). The data is presented as mean SEM and was analyzed with Duncan's multiple range analysis of variance, ANOVA. Pearson's correlation ( $p=0.05$ ) was used for correlation analyses in the Statistical Package for Social Sciences, SPSS®, Version 21.0, IBM Corp., Armonk, NY, USA. Graph Pad software®, Prism 5, Version 5.01, La Jolla, CA, USA, was used to create histograms and line plots. Significant values were defined as  $p < 0.05$

## RESULTS

Table 1 shows the results of the groups administered the leaf extracts of *A. muricata*. Total ATPase in group 3 and 6 increased significantly. While  $\text{Na}^+$ ,  $\text{K}^+$  ATPase of group 4 increased significantly, there was significant decrease in group 6 when compared with the control groups. Table 2 shows the results for the groups administered the stem-bark extracts of *A. muricata*. The total ATPase in groups 4 recorded a significant increase but a significant decrease was observed in group 6 ( $p < 0.05$ ). Also, while  $\text{Na}^+$ ,  $\text{K}^+$  ATPase showed an increase in group 3, group 6 recorded a decrease that was significant. The results recorded a generalized decrease in  $\text{Na}^+$ ,  $\text{K}^+$  ATPase activities at higher doses of the extracts administered. Thus, from the observation, it could be inferred that the groups

administered both extracts demonstrated a significant reduction in both total and  $\text{Na}^+$ ,  $\text{K}^+$  ATPase activities with subsequent significant increases close to the control (for stem-bark), at the highest dose of 800mg/kg ( $p < 0.05$ ); a response that may be termed bi-phasic to the extracts.

**Table 1:**  
Membrane-bound colorectal ATPase activities of rats administered methanol leaf extracts of *Annona muricata* ( $\times 10^{-1}$  U/mg wet weight of colorectal tissue)

| Assay/<br>Groups | Total<br>ATPase                 | $\text{Mg}^{2+}$ ATPase         | $\text{Ca}^{2+}$<br>ATPase     | $\text{Na}^+/\text{K}^+$ -<br>ATPase |
|------------------|---------------------------------|---------------------------------|--------------------------------|--------------------------------------|
| 1                | 10.66<br>$\pm 0.00^{\text{ab}}$ | 3.00<br>$\pm 0.00^{\text{abd}}$ | 1.23<br>$\pm 0.07^{\text{a}}$  | 4.91<br>$\pm 0.00^{\text{a}}$        |
| 2                | 8.10<br>$\pm 0.00^{\text{b}}$   | 3.91<br>$\pm 0.00^{\text{bd}}$  | 1.81<br>$\pm 0.00^{\text{b}}$  | 3.00<br>$\pm 1.00^{\text{bh}}$       |
| 3                | 14.67<br>$\pm 0.30^{\text{c}}$  | 6.92<br>$\pm 0.31^{\text{cf}}$  | 1.56<br>$\pm 0.00^{\text{c}}$  | 4.10<br>$\pm 2.60^{\text{cg}}$       |
| 4                | 11.98<br>$\pm 0.10^{\text{de}}$ | 3.27<br>$\pm 0.30^{\text{d}}$   | 1.78<br>$\pm 0.09^{\text{dg}}$ | 5.84<br>$\pm 0.00^{\text{dg}}$       |
| 5                | 10.32<br>$\pm 0.20^{\text{e}}$  | 1.10<br>$\pm 0.50^{\text{g}}$   | 1.95<br>$\pm 0.13^{\text{e}}$  | 2.98<br>$\pm 1.20^{\text{eh}}$       |
| 6                | 18.47<br>$\pm 3.10^{\text{f}}$  | 8.82<br>$\pm 0.60^{\text{f}}$   | 1.70<br>$\pm 0.10^{\text{fg}}$ | 1.00<br>$\pm 1.60^{\text{f}}$        |

The values are shown as mean  $\pm$  SEM ( $n = 6$ ). By one way Duncan's multiple range ANOVA, means with different superscripts are significantly different ( $p < 0.05$ ) down the column, with the control (group 1) taking the superscript 'a' alphabet.

**Table 2:**

Membrane-bound colorectal ATPase activities of rats administered methanol stem-bark extracts of *Annona muricata* ( $\times 10^{-1}$  U/mg wet weight of colorectal tissue).

| Assay/Groups | Total ATPase             | Mg <sup>2+</sup> ATPase  | Ca <sup>2+</sup> ATPase   | Na <sup>+</sup> /K <sup>+</sup> -ATPase |
|--------------|--------------------------|--------------------------|---------------------------|-----------------------------------------|
| Group 1      | 15.88<br>$\pm 0.02^a$    | 2.30<br>$\pm 0.00^{acd}$ | 10.44<br>$\pm 0.07^{acf}$ | 3.17<br>$\pm 0.02^{abef}$               |
| Group 2      | 7.85<br>$\pm 0.93^b$     | 4.94<br>$\pm 0.13^{be}$  | 4.03<br>$\pm 0.00^{be}$   | 2.17<br>$\pm 1.00^{bef}$                |
| Group 3      | 26.77<br>$\pm 5.10^c$    | 2.12<br>$\pm 0.30^c$     | 9.15<br>$\pm 1.00^{cf}$   | 15.51<br>$\pm 5.00^c$                   |
| Group 4      | 17.50<br>$\pm 0.40^{ad}$ | 2.68<br>$\pm 0.50^d$     | 6.30<br>$\pm 0.40^d$      | 8.60<br>$\pm 0.20^d$                    |
| Group 5      | 11.00<br>$\pm 1.61^e$    | 5.86<br>$\pm 0.00^e$     | 4.74<br>$\pm 1.00^e$      | 3.05<br>$\pm 1.00^{ef}$                 |
| Group 6      | 19.77<br>$\pm 1.60^f$    | 7.96 $\pm 1.20^f$        | 10.10<br>$\pm 1.77^f$     | 3.04<br>$\pm 1.10^f$                    |

The values are shown as mean  $\pm$  SEM ( $n = 6$ ). By one way Duncan's multiple range ANOVA, means with different superscripts are significantly different ( $p < 0.05$ ) down the column, with the control (group 1) taking the superscript 'a' alphabet

The colorectal tissue profile of some metabolic enzyme activities of groups treated with the leaf and stem-bark extracts are presented in Tables 3 and 4. There was a significant increase in LDH activity of the rats administered the leaf and stem-bark extracts as the concentrations increased through group 3 compared with the control that was not administered any extract(s). However, there were significant decreases in the observed LDH activity at the group 4 concentration for both the leaf and stem-bark, followed by a significant increase at the highest concentration (group 6).

**Table 3:**

Binding Affinities of the Ligands against Colorectal Proteins

| Receptor                              | Ligand                        | Affinity (Kcal/mole) | Dist. from RMSD l.b. | Best mode RMSD u.b. |
|---------------------------------------|-------------------------------|----------------------|----------------------|---------------------|
| Ca <sup>+</sup> -ATPase               | 15-acetyl-guanacone           | -6.60                | 5.931                | 11.633              |
|                                       | 15-acetyl-guanacone mimetic-1 | -5.90                | 4.337                | 6.877               |
|                                       | 15-acetyl-guanacone mimetic-2 | -7.10                | 4.802                | 6.593               |
|                                       | 15-acetyl-guanacone mimetic-3 | -6.80                | 2.296                | 5.379               |
|                                       | 15-acetyl-guanacone mimetic-4 | -5.80                | 1.834                | 2.548               |
|                                       | 15-acetyl-guanacone mimetic-5 | -6.50                | 3.168                | 5.146               |
|                                       | 15-acetyl-guanacone mimetic-6 | -7.70                | 2.049                | 3.050               |
|                                       | 15-acetyl-guanacone mimetic-7 | -7.40                | 23.821               | 27.089              |
|                                       | naringenin                    | -7.50                | 18.515               | 19.943              |
|                                       | 4-hydroxyl-naringenin         | -7.90                | 20.292               | 22.758              |
| Na <sup>+</sup> K <sup>+</sup> ATPase | Internal ligand               | -8.30                | 1.425                | 2.067               |
|                                       | 15-acetyl-guanacone           | -6.20                | 1.270                | 2.223               |
|                                       | 15-acetyl-guanacone mimetic-1 | -6.30                | 3.939                | 12.874              |
|                                       | 15-acetyl-guanacone mimetic-2 | -6.30                | 2.173                | 2.826               |
|                                       | 15-acetyl-guanacone mimetic-3 | -7.60                | 3.239                | 15.248              |
|                                       | 15-acetyl-guanacone mimetic-4 | -7.40                | 2.679                | 13.317              |
|                                       | 15-acetyl-guanacone mimetic-5 | -7.30                | 3.590                | 5.738               |
|                                       | 15-acetyl-guanacone mimetic-6 | -6.70                | 1.983                | 3.931               |
|                                       | 15-acetyl-guanacone mimetic-7 | -7.70                | 2.540                | 4.280               |
|                                       | Naringenin                    | -7.90                | 2.288                | 7.363               |
|                                       | 4-hydroxyl-naringenin         | -7.40                | 2.432                | 7.398               |
|                                       | ATP                           | -7.00                | 11.177               | 13.841              |

Binding affinities greater than 6.00 Kcal/mole is an indication of appropriate bonding of ligand on the protein surface (RMSD = root mean square distance). The results obtained for 15-acetyl-guanacone mimetic-7 and 4-hydroxyl-naringenin suggest that the number of rings and hydroxyl functional groups along the acetogenin and flavonone molecules increased the binding affinity with the colorectal tissue Ca<sup>+</sup> and Na<sup>+</sup> K<sup>+</sup>-ATPases when compared with 15-acetyl-guanacone (though, the binding affinity for the flavonone, naringenin, decreased when 4-1-, was converted to 4-hydroxyl functional group). The flavonone demonstrated to be a better binding compound to Na<sup>+</sup> K<sup>+</sup>-ATPase when compared with acetogenin

**Table 3:**

Colorectal Tissue Lactate Dehydrogenase (LDH), Glucose-6-Phosphate Dehydrogenase (G6PD) Activities and Tissue Protein (TP) Concentrations of AMC Leaf Extracts.

| Assay/Groups | LDH (U/mg of tissue)   | G6PD (U/mg of tissue)  | TP (g/mg tissue)       |
|--------------|------------------------|------------------------|------------------------|
| Group 1      | 1.03 $\pm 0.01^a$      | 8.55 $\pm 0.22^a$      | 1.23 $\pm 0.07^a$      |
| Group 2      | 1.40 $\pm 0.10^{b\ g}$ | 9.65 $\pm 0.20^{b\ g}$ | 1.81 $\pm 0.00^b$      |
| Group 3      | 1.50 $\pm 0.00^c$      | 5.58 $\pm 0.00^{c\ h}$ | 1.56 $\pm 0.00^c$      |
| Group 4      | 1.10 $\pm 0.20^{d\ h}$ | 7.03 $\pm 0.20^d$      | 1.78 $\pm 0.09^{d\ g}$ |
| Group 5      | 1.13 $\pm 0.20^{e\ h}$ | 5.86 $\pm 0.00^{e\ h}$ | 1.95 $\pm 0.13^e$      |
| Group 6      | 1.40 $\pm 0.19^{f\ g}$ | 9.65 $\pm 0.20^{f\ g}$ | 1.70 $\pm 0.10^{f\ g}$ |

The values are shown as mean  $\pm$  SEM ( $n = 6$ ). By one way Duncan's multiple range ANOVA, means with different superscripts are significantly different ( $p < 0.05$ ) down the column, with the control (group 1) taking the superscript 'a' alphabet

**Table 4:**

Colorectal Tissue Lactate Dehydrogenase (LDH), Glucose-6-Phosphate Dehydrogenase (G6PD) Activities and Tissue Protein (TP) Concentrations of AMC Stem-Bark Extracts.

| Assay/Groups | LDH (U/mg of tissue)   | G6PD (U/mg of tissue) | TP (g/mg tissue)     |
|--------------|------------------------|-----------------------|----------------------|
| Group 1      | 1.00 $\pm 0.00^{agh}$  | 4.10 $\pm 0.00^a$     | 3.40 $\pm 0.00^{ae}$ |
| Group 2      | 1.10 $\pm 0.01^{b\ i}$ | 2.20 $\pm 0.00^b$     | 2.90 $\pm 0.00^{bg}$ |
| Group 3      | 1.20 $\pm 0.00^{c\ g}$ | 8.21 $\pm 0.10^c$     | 2.48 $\pm 0.10^{ch}$ |
| Group 4      | 1.03 $\pm 0.10^{d\ h}$ | 5.00 $\pm 0.00^d$     | 2.08 $\pm 0.00^d$    |
| Group 5      | 1.49 $\pm 0.00^{e\ i}$ | 6.20 $\pm 0.10^e$     | 3.20 $\pm 0.20^{eg}$ |
| Group 6      | 1.50 $\pm 0.00^f$      | 7.64 $\pm 0.00^f$     | 3.40 $\pm 0.00^{ae}$ |

The values are shown as mean  $\pm$  SEM ( $n = 6$ ). By one way Duncan's multiple range ANOVA, means with different superscripts are significantly different ( $p < 0.05$ ) down the column, with the control (group 1) taking the superscript 'a' alphabet.

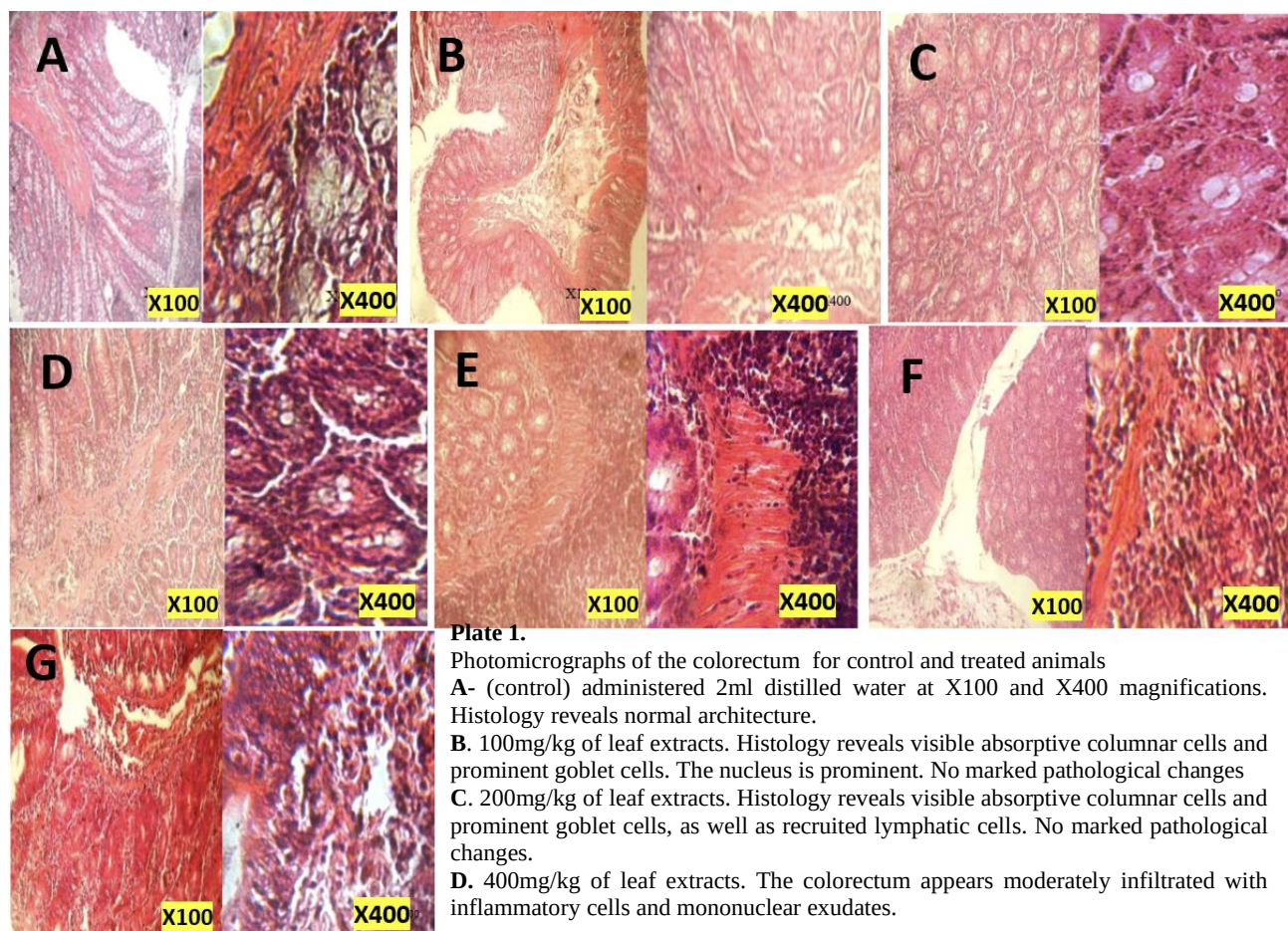


**Plate 1.**

Photomicrographs of the colorectum for control and treated animals  
**A-** (control) administered 2ml distilled water at X100 and X400 magnifications. Histology reveals normal architecture.

**B.** 100mg/kg of leaf extracts. Histology reveals visible absorptive columnar cells and prominent goblet cells. The nucleus is prominent. No marked pathological changes

**C.** 200mg/kg of leaf extracts. Histology reveals visible absorptive columnar cells and prominent goblet cells, as well as recruited lymphatic cells. No marked pathological changes.

**D.** 400mg/kg of leaf extracts. The colorectum appears moderately infiltrated with inflammatory cells and mononuclear exudates.

**E.** 600mg/kg of leaf extracts. The colorectum appears moderately infiltrated with inflammatory cells and mononuclear exudates

**F.** 800mg/kg of leaf extract. Greatly observed histological changes mostly associated with inflammatory exudates and mononuclear cell infiltration.

**G.** 800mg/kg of stem-bark extract. Varying degrees of histological changes mostly associated with inflammatory exudates and mononuclear cell infiltration

The G6PD activity for the rats administered the leaf extract demonstrated decreases through groups 3 and 5, with increased activity in group 6 rats, compared with the control; whereas the G6PD activity of the rats administered the stem-bark extract showed marked increased activity for the group 3 rats, when compared with the control. The LDH and G<sub>6</sub>PD activities for the leaf and stem-bark treated rats did not show any particular observable pattern of interest.

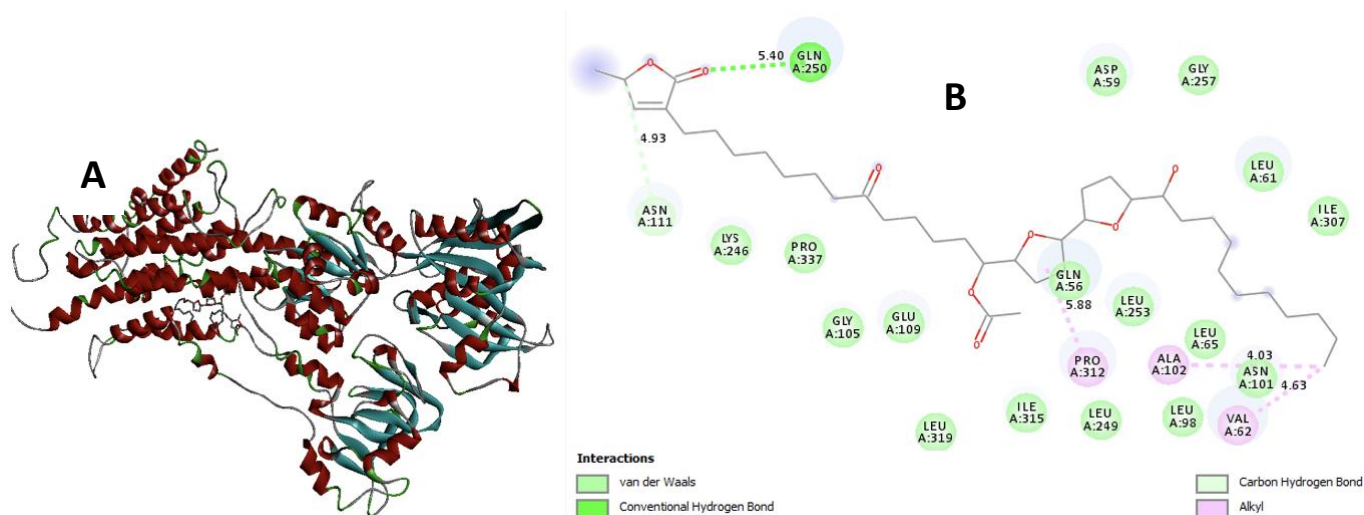
**Binding properties of the isolated compounds:** Binding properties of the isolated compounds: The binding properties between the isolated compounds of *Annona muricata* are presented in Table 5, Plate 1 and Figures 2 to 5.

Binding affinities greater than 6.00 Kcal/mole is an indication of appropriate bonding of ligand on the protein surface. The results obtained for 15-acetyl-guanacone mimetic-7 and 4-hydroxyl-naringenin suggest that the number of rings and hydroxyl functional groups along the acetogenin and flavonone molecules increased the binding affinity with the colorectal tissue Ca<sup>2+</sup> and Na<sup>+</sup> K<sup>+</sup>-ATPases when compared with 15-acetylguanacone (though, the binding affinity for the flavonone, naringenin, decreased when 4-1-, was converted to 4-hydroxyl functional group). The flavonone demonstrated to be a better binding compound to Na<sup>+</sup> K<sup>+</sup>-ATPase when compared with acetogenin (Table 5, Figures 2 - 5).

Photomicrographs of the colorectum for control and treated animals at X100 and X400 magnifications revealed normal architecture for control. In the 100mg/kg leaf extracts treated rats, histology revealed visible absorptive columnar cells and prominent goblet cells. The nucleus was prominent. However, there was no marked pathological changes. Rats treated with 200mg/kg leaf extracts, revealed visible, absorptive columnar cells and prominent goblet cells, as well as recruited lymphatic cells and no marked pathological changes. In the rats given 400mg/kg and 600mg/kg leaf extracts, the colorectum appeared moderately infiltrated with inflammatory cells and mononuclear exudates. For the rats treated with 800mg/kg leaf extracts and stem-bark extracts, there were varying degrees of histological changes mostly associated with inflammatory exudates and mononuclear cell infiltration (Plate 1, A - G)..

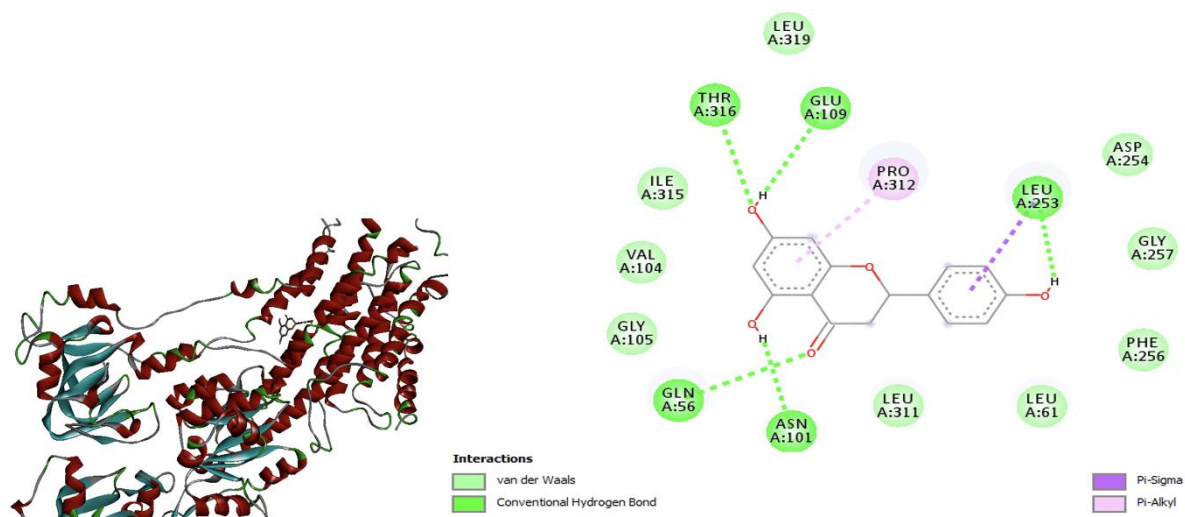
## DISCUSSION

The groups administered the AMC leaf extract showed a steady significant increase in the total ATPase activity and a dose-related significant decrease in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity at group 4, with subsequent sharp decrease at higher administered doses (groups 5 and 6). The stem-bark AMC extract group showed almost a similar trend.

**Figure 2**

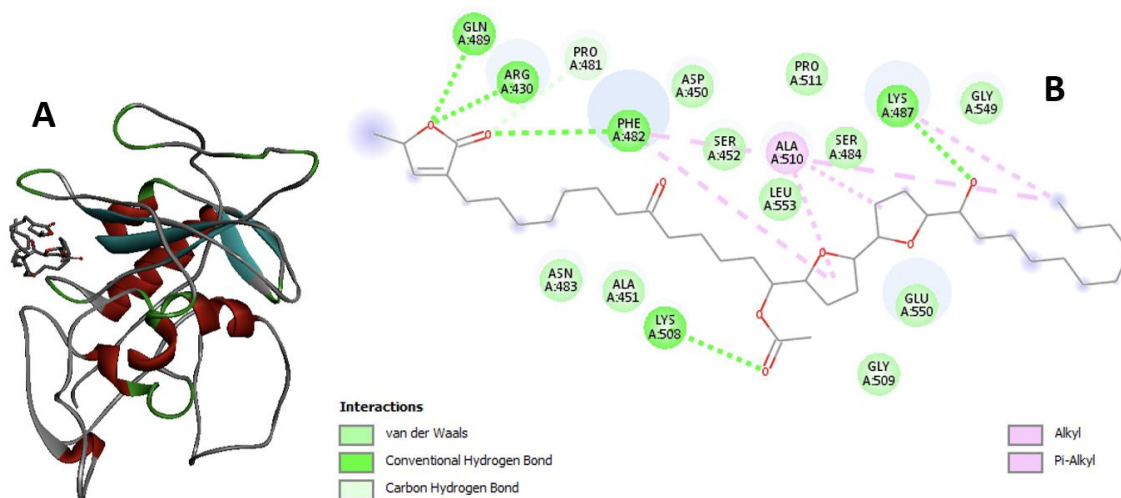
**A-** Three-D bonding between  $\text{Ca}^{2+}$ -ATPase and 15-acetyl-guanacine

**B-** Bonding association and active site amino acid residues between  $\text{Ca}^{2+}$ -ATPase and 15-acetyl-guanacine bonding

**Figure 3**

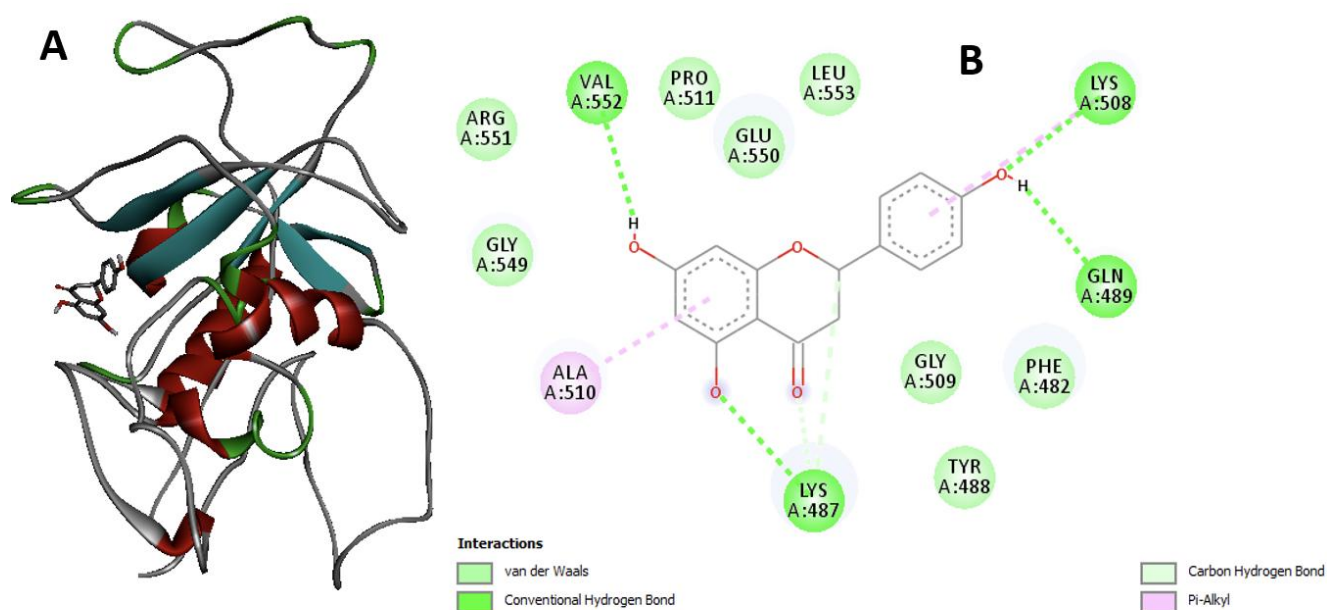
**A-** Three-D bonding between  $\text{Ca}^{2+}$ -ATPase and Naringenin

**B-** Bonding association and active site amino acid residues between  $\text{Ca}^{2+}$ -ATPase and Naringenin bonding

**Figure 4**

**A-** Three-D bonding between the nucleotide-binding domain of rat  $\alpha$ -1  $\text{Na}^{+}$   $\text{K}^{+}$ -ATPase and 15-acetyl-guanacine

**B.** Bonding association and active site amino acid residues between the nucleotide-binding domain of rat  $\alpha$ -1  $\text{Na}^{+}$   $\text{K}^{+}$ -ATPase and 15-acetyl-guanacine.

**Figure 5**

A - Three-D bonding between the nucleotide-binding domain of rat alpha-1 Na<sup>+</sup> K<sup>+</sup>-ATPase and Naringenin

B- Bonding association and active site amino acid residues between the nucleotide-binding domain of rat alpha-1 Na<sup>+</sup> K<sup>+</sup>-ATPase and Naringenin

The result of this study is in agreement with the findings of Senthil-Nathan *et al.* (2005) who reported significant inhibition of ATPase activity in different animal species exposed to different contaminants. Muzzanti *et al.* in Olubodun and Eriyamremu (2014) stated that a decrease in Na<sup>+</sup>/K<sup>+</sup>-ATPase is a reflection of qualitative changes in the phospholipid elements of cells. Rauchova *et al.* (1995) demonstrated that membrane microviscosity changes can affect the kinetic properties of membrane-bound enzymes. There is the possibility that the inhibited ATPase activities may be responsible for the relative inflammatory exudates and mononuclear cell infiltration observed in the histology of this study (Olubodun and Eriyamremu, 2014; Clausen *et al.*, 2017).

The observed inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase activities for the leaf (and also, stem-bark) especially above 400mg/kg of the administered extracts could be linked to the diarrhea observed during the acute and sub-chronic toxicity studies, and thus suggests an inhibition of the membrane-bound Na<sup>+</sup>/K<sup>+</sup>-ATPase –Osmo-regulatory coupled mechanism of the colorectal cells (colonocytes), where Na<sup>+</sup>, Cl<sup>-</sup>, and water are lost into luminal content (chyme) of the colon for excretion (Clausen *et al.*, 2017; Müller *et al.*, 2018).

The Na<sup>+</sup>/K<sup>+</sup>-ATPase pump is located on the basolateral side of the colonocytes which creates a means of Na<sup>+</sup> as well as, Cl<sup>-</sup> ions transport (electrochemical gradient and secondary active as well as, down-hill drag, respectively), and also passive water transport through the brush border side of the colonocytes by an osmotic diffusion. This Na<sup>+</sup>/K<sup>+</sup>-ATPase pump is relevant in the whole of this process as the [Na<sup>+</sup>] and [K<sup>+</sup>] are about 142mEq/L and 4mEq/L in the extracellular fluid, respectively, and 10mEq/L and 140mEq/L in the intracellular fluid, respectively (Guyton and Hall, 2016). The primary active exchange of three Na<sup>+</sup> ions out of the colonocytes for two

K<sup>+</sup> ions into the colonocytes creates a net negative charge inside the colonocytes, which facilitates a subsequent secondary transport of Na<sup>+</sup>, and co-transport of Cl<sup>-</sup> ions into the colonocytes from the luminal chyme through the brush border into the colonocytes. It is also possible that at a very high dose, the leaf AMC extract may be acting as an irritant to the cells lining the colorectal lumen, or contains osmotically active phyto-molecules, thus initiating a “diluting-effect”, where Na<sup>+</sup>, Cl<sup>-</sup>, and water are passed from the plasma (blood vessels) via the colorectal cells into the colorectal lumen so as to “dilute” the irritating metabolite present in the leaf AMC and facilitate its excretion in a very watery form, resulting in diarrhea (Clausen *et al.*, 2017). It is also possible that the leaf (and stem-bark) methanol extracts contain diuretic compound which resulted in the observed increase in urine excreted as well as diarrhea. Along with the excreted watery feces was observed some worms, especially tape worm, suggesting that *Annona muricata* methanol extracts (leaf and stem-bark) possess anti-helminthic properties.

Studies of the influence of both extracts of AMC on colorectal tissue protein and some energy markers (LDH and G6PD) showed that, there were dose-dependent increases and decreases in colorectal tissue protein for the groups administered the leaf and stem-bark extracts respectively. Colorectal tissue LDH activity increased in a dose-dependent pattern, for the groups administered the leaf and stem-bark extracts of AMC. The colorectal tissue G6PD activity demonstrated fluctuating patterns for all the AMC extracts but showed a significant increase with the highest dose (800mg/kg) when compared with the control groups for both extracts. An increase in LDH activity was reported for plant seedlings exposed to 7 days of anoxia (Chen and Quallis, 2003). Kulkarni and Chavan (2013) however, reported a decrease in LDH activity also in two plant species

subjected to waterlogging stress. The LDH results of the study is consistent with previous reports (Xu *et al.*, 2012; Olubodun and Eriyamremu, 2020). However, it is at variance with the report of Kulkarni and Chavan (2013) and the Davis-Roberts pH-stat hypothesis. The high LDH activity of the colorectal tissues of the groups administered the leaf and stem bark extracts of AMC when compared with the control group may indicate elevated energy demand to overcome the stress induced in the rats by the crude extracts of AMC. The limitation or absence of oxygen as a result of the crude extracts may have induced conversion of aerobic respiration to anaerobic respiration (fermentation) hence increase in the lactate dehydrogenase activity (Xu *et al.*, 2012; Chikezie *et al.*, 2018; Olubodun and Eriyamremu, 2020; Farhana and Lappin, 2022). Conversely, the increase in LDH activity at the highest administered dose (800mg/kg) suggests an increase in the cellular glycolytic-lactic acid pathway, which may have ultimately led to an increase in the colorectal cellular pH level due to lactic acid accumulation. This observation was corroborated by the observation of the histology (plate 1). This may have also had a far-reaching biochemical effect on the colorectal ATPase status. Though, no observable effect could be deduced from the colorectal tissue protein (TP).

The fluctuating pattern observed for G6PD in this study is consistent with Liu *et al.* (2007) also reported fluctuating (increase and decrease) movement after certain amounts of contamination in plants. The increment in G6PD interaction may have increased NADPH production in the colorectal tissues, resulting in tissue tolerance to the administered extracts. The increase in NADPH levels may have stimulated the production of nitrate reductase-dependent Nitric Oxide (NO), which has been shown to regulate tissue growth and development and enhance or boost iron homeostasis (Liu *et al.* 2007). The upsurge in G6PD activity may have exacerbated the increase in antioxidant enzyme activity observed in an earlier report (Agu and Okolie, 2017b, Agu *et al.*, 2017b). Increased antioxidant enzyme activities may have aided in the scavenging of reactive oxygen species (ROS) produced by the extracts (Agbafor *et al.*, 2017). Glucose-6-phosphate dehydrogenase activity may have played an important role in protecting rat colorectal tissues from oxidative stress caused by both crude extracts and establishing transient colorectal tolerance to the crude extracts (Liu *et al.*, 2007; Olubodun and Eriyamremu, 2015; Agbafor *et al.*, 2017).

Additionally, the methanol extracts of both plant parts showed histological changes in the colorectal tissues in all animals with marked changes in the highest dose. The control group had a properly aligned cellular morphology whereas, the other groups exhibited presentations of distorted cellular morphologies marked by increased inflammatory bodies. The dysmorphic presentations were significant for the group 8 rats. Several studies have reported histopathological modulations in different organs exposed to various plant species with more effects on the concentrated doses (Ngueguim *et al.*, 2016; Samir *et al.*, 2017).

In conclusion, the influence of both extracts of AMC on colorectal tissue protein and some energy markers (ATPase, LDH and G6PD) showed that, there were dose-dependent increase in colorectal tissue protein for the groups administered the leaf and stem-bark extracts respectively.

However, after a certain dose, the effects were reversed. Colorectal tissue LDH activities increased in a dose-dependent pattern, for the groups administered both extracts. The colorectal tissue G6PD activity demonstrated fluctuating patterns for the extracts but showed a significant increase with the highest dose (800mg/kg) when compared with the control. We inferred that the methanol leaf and stem-bark extracts of AMC may significantly influence the enzymes by reducing and increasing total ATPase and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities depending on the doses and administration should therefore depend on the desired outcome.

#### Acknowledgments

“This Research was funded by TETFund Research Fund Grant (2018/2019)”. The Tertiary Education Trust Fund (TETFund) Nigeria (2018/2019 Research Projects Batch 13; Ref: TETFUND/DESS/UNI/BENIN/2018/RP/VOL. 1), is acknowledged for the financial support. The authors are grateful to University of Benin for releasing the funds and the Department of Medical Biochemistry and Biochemistry, University of Benin, for technical support.

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Full-Length Research Article

## Hypoglycemic, Hypolipidemic and Antioxidant Potentials of Ethanolic Stem Bark Extract of *Anacardium occidentale* in Streptozotocin-Induced Diabetic Rats

Bamisaye, F.A.<sup>1</sup>, Ajuwon, O.R.<sup>1</sup>, Ibrahim, R.B.<sup>2</sup>, Sulyman, A.O.<sup>2</sup>, Jubril, A.O.<sup>2</sup>

<sup>1</sup>Department of Biochemistry, Faculty of Science, Federal University Oye Ekiti, P.M.B. 373, Oye-Are Road, Oye Ekiti, Ekiti State, Nigeria.

<sup>2</sup>Department of Biochemistry, School of Basic Medical Sciences, College of Pure and Applied Sciences, Kwara State University, Malete, Kwara State, Nigeria.

**Summary:** Diabetes mellitus is one of the most widespread diseases affecting the world's population causing substantial morbidity, mortality and long-term complications. This study was designed to investigate possible hypoglycemic, hypolipidemic and antioxidant effect of ethanolic stem bark extract of *Anacardium occidentale* in streptozotocin (STZ)-induced diabetic rats. Twenty-eight STZ (60 mg/kg body weight)-induced diabetic, male albino rats were randomly distributed into Groups II-V (7 rats each) and orally administered with water, metformin (14.2 mg/kg), 200 mg/kg *Anacardium occidentale* extract and 400 mg/kg *Anacardium occidentale* extract respectively daily for 15 days. Group I rats were untreated with STZ and serves as control all under the same sham handling. Blood samples were taken for measurement of fasting blood glucose (FBG) and lipid profile. Liver and kidney tissue samples were taken for determination of glycemic indices (glucose and glycogen), as well as redox status markers such as malondialdehyde (MDA), total glutathione (GSH), activities of superoxide dismutase (SOD) and glutathione-s-transferase (GST). Results showed that treatment with 200 and 400 mg/kg *Anacardium occidentale* stem bark extract reversed hyperglycemia and hyperlipidemia induced by STZ similar to what was observed with the standard drug, metformin. Similarly, both extract concentration produced a significant reduction in MDA while the activity of SOD and GST, as well as concentration of GSH were elevated. This study suggested that ethanolic stem bark extract of *Anacardium occidentale* at 200 and 400 mg/kg can ameliorate diabetes and its associated complications via its hypoglycemic, hypolipidemic, antioxidant and free radical scavenging properties.

**Keywords:** *Anacardium occidentale*, Antioxidant, Diabetes, Hypoglycemic, Hypolipidemic

©Physiological Society of Nigeria

\*Address for correspondence: olawale.ajuwon@fuoye.edu.ng; +234-8054127724

Manuscript received- October 2021; Accepted- March 2022

DOI: <https://doi.org/10.54548/njps.v37i1.17>

### INTRODUCTION

Diabetes mellitus is one of the most widespread diseases affecting the world's population causing substantial morbidity, mortality and long-term complications. Available evidence indicated that diabetes currently affects an estimated 429 million people worldwide, and with the prevalence increasing alarmingly, the figure is projected to increase to around 625 million people by 2045 (IDF, 2017). In Nigeria, diabetes is becoming very common and currently rising to epidemic proportions. With an estimated 3.9 million people living with diabetes and a prevalence rate of 4.3% among adult populations, Nigeria has the highest incidence of diabetes in sub-Saharan Africa (Dahiru *et al.*, 2016). This upsurge in rate may be attributed to changing behavioral and lifestyle risk factors including bad eating habits, alcohol consumption, cigarette smoking and nonchalant attitude towards comprehensive and routine medical check-up.

Classified as either insulin-dependent diabetes mellitus (IDDM), also known as type 1 diabetes mellitus (T1DM) or non-insulin-dependent diabetes mellitus (NIDDM) or type 2

diabetes mellitus (T2DM). T1DM, characterized by inability to produce insulin because of autoimmune destruction of pancreatic  $\beta$  cells, accounts for about 5 to 10% of diabetes (Niedowicz and Daleke, 2005) while T2DM is characterized by a dysfunctional carbohydrate, lipid and protein metabolism resulting from a progressively impaired insulin secretion and/or insulin resistance (Ajuwon *et al.*, 2018). Whether resulting from defect in insulin production, insulin utilization or both, diabetes leads to decreased uptake and utilization of glucose in the skeletal muscle, liver and adipose tissues resulting in uncontrollable hyperglycemia.

Accumulating evidence has shown that reactive oxygen species (ROS) production and generation of oxidative stress are considered as key factors in the pathophysiology of diabetes and diabetic complications (Ajuwon *et al.*, 2018). Reports have shown that overproduction of reactive oxygen species (ROS) may result in  $\beta$ -cells dysfunction as a result of decrease in  $\beta$ -cells mass attributable to oxidative stress-induced apoptosis (Wali *et al.*, 2014). Furthermore, there are studies linking overproduction of ROS to insulin resistance,

impairment in insulin synthesis and  $\beta$ -cell insulin secretion (Robertson *et al.*, 1992; Kim *et al.*, 2008; Karunakaran *et al.*, 2013; Yang *et al.*, 2014).

Treatment for diabetes relied on pharmacological agents such as insulin, sulfonylureas, metformin and others to inhibit the hyperglycemic condition. However, the efficiency of these conventional antidiabetic agents is not completely guaranteed as indicated by increase in morbidity and mortality rate in diabetic patients (Asrafuzzaman *et al.*, 2017). Furthermore, the commonly used antidiabetic agents cause various side effects, such as weight gain, hypoglycemia, fluid retention, skin rash or itching, tiredness or dizziness, diarrhea, liver disease and heart failure (Akindele *et al.*, 2015; Xu *et al.*, 2018), thus limiting their use. These drawbacks, and the fact that about 80% of people affected by diabetes lives in low- and middle-income countries, where accessibility to adequate medical care may not be available, have made the development of new antidiabetic agents which can control hyperglycemia with fewer side effects very essential. Since hyperglycemia-induced oxidative stress has been fingered in the pathogenesis of diabetes and diabetic complications, it is therefore suggested at least in theory, that medicinal plants, which are known sources of natural antioxidants could be beneficial in the treatment and management of diabetes.

*Anacardium occidentale*, commonly known as Cashew, is a member of the Anacardiaceae family, and it is found all over Nigeria. The tree is rich in anthocyanins, carotenoids, ascorbic acid (vitamin C), flavonoids and other polyphenols, as well as mineral components. Reports from Nigerian folk medicine indicated that the stem bark extract of *A. occidentale* is used in the treatment of malaria fever, asthma, dysentery, toothache, sore gum, dyspepsia, impotence, urinary disorders and syphilis (Saidu *et al.*, 2012; Agedah *et al.*, 2010). Similarly, fruit, juice and the nut oil derived from *A. occidentale* are used in folk remedies for cancerous ulcers and elephantiasis (Saidu *et al.*, 2012). Evidence has shown that *A. occidentale* displayed a broad spectrum of biological effects, with reports indicating antioxidants, anti-inflammatory (Olajide *et al.*, 2004), antimutagenic (Melo-Cavalcante *et al.*, 2011), antiulcerogenic, antibacterial, antifungal and larvicidal (Behravan *et al.*, 2012) effects among others. Though, the various parts of *A. occidentale* are widely used in folk medicine in the treatment of various ailments including diabetes, however, scientific reports of the use of its stem bark extract for diabetes treatment is still limited. Therefore, in this study, we report on the antioxidant, hypoglycemic and hypolipidemic activities/potentials of the ethanolic stem bark extract of *A. occidentale*.

## MATERIALS AND METHODS

### Plant materials and authentication

The stem bark of *A. occidentale* was obtained in the vicinity of Kwara State University, Malete. The plant was identified and authenticated at the Department of Plant and Environmental Biology, College of Pure and Applied Sciences, Kwara State University, Malete, Nigeria.

**Chemicals and Reagents:** Kits for lipid profile assays were obtained from Randox Limited, London. All other chemicals and reagents used are of analytical grades and obtained from Sigma Aldrich, Johannesburg, South Africa.

**Preparation of extract:** The stem bark of *A. occidentale* was harvested, cut into small pieces and oven-dried at 40°C until constant weight was obtained. The dried stem bark (200 g) was powdered, soaked with 2000 ml of absolute ethanol for 72 hours with intermittent shaking. It was filtered with micelles cloth and then concentrated to dryness with water bath at 45°C. A yield of 46.5 g (23.25%) was obtained. The dried powdered filtrate extract was stored in an air tight container at 4°C in the refrigerator. A calculated amount of residue was weighed and re-constituted in distilled water to give the required doses of 200 and 400 mg/kg body weight of the extract used to treat the animals in this experiment.

**Animals:** Male Wistar rats (6 weeks old) weighing 135 - 150 g were obtained and housed at the Animal Unit of the Department of Biochemistry, Kwara State University, Malete, Kwara State. The animals were housed under controlled environment, maintained at a temperature of between 22-24°C, with a 12 h light-dark cycle and 50-54% humidity. The rats were fed standard rat pellet *ad libitum* and had free access to tap water. Animals were acclimatized under this condition for 10 days before the commencement of the experiment. All rats used received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

**Induction of diabetes mellitus:** Male Wistar rats (135 - 150 g) were injected intramuscularly with freshly prepared STZ (60 mg/kg body weight (Sigma Aldrich, South Africa) in 0.1 M citrate buffer (pH 4.5). Blood samples were taken from the tail tip after 72 h of STZ injection and glucose levels determined using an AccuChek glucometer (Roche, Cape Town, South Africa). Rats with blood glucose levels above 180 mg/dL were selected as diabetic and used in this study.

**Study design:** The rats were randomized into 5 experimental groups of 7 rats each and treated accordingly as shown below:

- i. **Control group:** Received a single intramuscular injection of citrate buffer and have access to food and drinking water for 15 days.
- ii. **Diabetes Mellitus (DM) group:** Received a single intramuscular injection of STZ (60 mg/kg body weight) and have access to food and drinking water for 15 days.
- iii. **Diabetes + Metformin (DM + MTF) group:** Received a single intramuscular injection of STZ (60 mg/kg body weight) and treated with 14.2 mg/kg body weight of a standard drug, metformin orally for 15 days. Rats also have free access to food and drinking water.
- iv. **Diabetes + 200 mg/kg extract (DM +200 mg.kg) group:** Received a single intramuscular injection of STZ (60 mg/kg body weight) and treated with 200 mg/kg body weight of *A. occidentale* stem bark ethanolic extract orally for 15 days. Rats also have access to food and drinking water.

- v. **Diabetes + 400 mg/kg extract (DM +400 mg.kg) group:** Received a single intramuscular injection of STZ (60 mg/kg body weight) and treated with 400 mg/kg body weight of *A. occidentale* stem bark ethanolic extract orally for 15 days. Rats also have access to food and drinking water.

The general conditions of the rats were monitored daily throughout the study. Fasting blood glucose (Accu-Chek, Roche, South Africa) and body weight of the rats were recorded on day 1, 3, 5, 10 and 15. At the end of the experimental period, fasted (16 h) animals in all the groups were sacrificed under mild ether anesthesia. About 5 ml of blood was collected via the abdominal aorta into collection tubes with EDTA (1.5 mg/dL of blood) to obtain plasma. Plasma was separated immediately by centrifugation at 3,000 g for 10 min, transferred into properly labelled vials and stored frozen (-20° C) until used for analysis. The liver and kidney were removed, washed twice with ice-cold PBS (10 mM phosphate buffered saline pH 7.2) to remove residual blood, blotted to dry, weighed and stored frozen (-20° C).

**Preparation of liver and kidney homogenates:** Liver and kidney tissues were separately homogenized on ice in 5 volumes of cold 0.25 M sucrose solution. The homogenate of each was transferred into tubes and centrifuged at 10,000 g for 10 min. The supernatant of each was collected, divided into aliquots, and stored at -20° C until used for analyses of lipid peroxidation, antioxidant enzymes activity and glutathione status.

### Biochemical assays

**Plasma lipid Profiles:** Plasma samples were used for the determination of triglyceride (TG), total cholesterol (TC) and high density lipoprotein (HDL)-cholesterol (HDL-C). TG, TC and HDL-C were measured using commercially available kits (Randox Laboratories, UK) according to manufacturer's instructions.

**Hepatic glucose and glycogen:** Hepatic glucose was estimated using the glucose oxidase-peroxidase method (Trinder, 1969). Hepatic glycogen was determined by a method previously described by Musabayane *et al.* (2005). Briefly, about 1 g of liver tissue was homogenized in 2 ml of 30% potassium hydroxide (300 g/L) and boiled at 100 °C for 30 min, and then cooled in ice saturated sodium sulphate. Glycogen was precipitated with ethanol, pelleted, and resolubilized in distilled water. Glycogen content was determined by treatment with anthrone reagent and the absorbance measured at 620 nm.

**Lipid Peroxidation:** The extent of lipid peroxidation was assessed in terms of thiobarbituric acid reactive substances (TBARS), using malondialdehyde (MDA) as standard by method of Beuge and Aust (1978). Briefly, 1 ml of liver or kidney homogenate was mixed with 2 ml of the TCA-TBA-HCl reagent [15% (w/v) TCA, 0.375% (w/v) TBA and 0.25 N HCl]. The mixture was heated in a boiling water bath for 15 minutes, cooled on ice and centrifuged at 3,500 x g for 3 min. at 4° C. The absorbance of the supernatant was read at 535 nm and the MDA concentration of the sample calculated using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$  and expressed as  $\mu\text{mol MDA/g tissue}$ .

**Superoxide dismutase activity:** The activity of superoxide dismutase (SOD) was determined according to method described by Das *et al.* (2000). Briefly, 1.4 ml aliquot of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer, pH 7.4, 0.075 ml of 20 mM L-Methionine, 0.04 ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM hydroxylamine hydrochloride and 0.1 ml of 50 mM EDTA) was added to 100  $\mu\text{l}$  of liver or kidney homogenate and incubated at 30° C for 5 min. Then 80  $\mu\text{l}$  of riboflavin was added and the tubes were exposed to two 20W-Philips fluorescent lamps arranged parallel to each other for 10 min. After the exposure time, 1 ml of Greiss reagent (mixture of equal volume of 1% sulphanilamide in 5% phosphoric acid) was added and absorbance of the colour formed measured at 543 nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

**Glutathione-S-Transferase Activity:** Glutathione-S-Transferase (GST) activity was determined according to a modified method of Habig *et al.* (1974). This method is based on the reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH), catalysed by GST. To 900  $\mu\text{l}$  of reaction mixture (comprising of 700  $\mu\text{l}$  of 0.1 mol/L potassium phosphate buffer (pH 5.5), 100  $\mu\text{l}$  of 25 mmol/L CDNB and 100  $\mu\text{l}$  of 50 mmol/L GSH) was added 100  $\mu\text{l}$  of liver or kidney homogenate. After adding the sample, the formation of the product was monitored for 5 min. at 340 nm. The results were reported as micromole per milligram protein ( $\mu\text{mol/mg protein}$ ).

**Total glutathione:** Total glutathione (GSH) concentration was determined in samples according to the method of Boyne and Ellman (1972). Briefly, 1 ml of homogenate was treated with 4.0 ml of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl dissolved in 100 ml of distilled water). After centrifugation, 2 ml of the supernatant was mixed with 0.2 ml of 0.4 M  $\text{Na}_2\text{HPO}_4$  and 1 ml of DTNB (5, 5' -Dithio-bis-(2-nitrobenzoic acid) reagent (40 mg DTNB in 100 ml of aqueous 1% trisodium citrate). Absorbance was read at 412 nm within 2 min. GSH concentration was expressed as  $\mu\text{mol/g tissue}$ .

### Statistical analysis

Values were expressed as mean  $\pm$  standard deviation (SD). Differences between group mean were estimated using one-way analysis of variance (ANOVA) followed by Tukey's test for all pairwise comparison. Results were considered statistically significant at  $p < 0.05$ . All the statistics were carried out using MedCalc v 12.2.1 software (MedCalc software bvba, Mariakerke, Belgium).

## RESULTS

Figure 1 shows the effect of stem bark extract of *A. occidentale* on body weight in control and STZ-induced diabetic rats. The average body weight of all the animals in the five experimental groups did not differ significantly ( $p < 0.05$ ) before the administration of STZ. Rats in the control group showed a consistent gain in body weight over the period of the experiment.

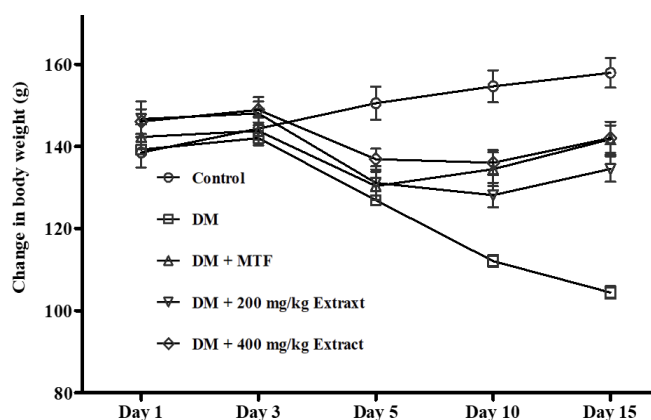


**Figure 1**

Effect of ethanolic stem bark extract of *Anacardium occidentale* on body weight of STZ-induced diabetic rats. Data points represent mean  $\pm$  standard deviation. **Note**, DM = diabetic rats; MTF = metformin.

By the 5th day of the experiment, body weight of animals in the diabetes untreated (DM), diabetes + metformin (DM + MTF) and diabetes + extract (DM + 200 mg/kg and 400 mg/kg) groups was significantly ( $p < 0.05$ ) reduced compared to control animals. This weight loss continued until the end of the experiment. At the end of the experiment, DM + 200 mg/kg and DM + 400 mg/kg extract treated rats showed sign of recovery from body weight loss ( $134.49 \pm 3.11$  vs  $104.40 \pm 3.99$  g and  $141.99 \pm 4.02$  vs

$104.40 \pm 3.99$  g respectively) when compared with DM group rats. The improvement in body weight induced in the DM + 400 mg/kg extract group is similar to what was observed in the DM + MTF group ( $141.99 \pm 4.02$  vs  $141.75 \pm 3.33$  g).

The effect of the *A. occidentale* stem bark extract on markers of glycemic control in control and STZ-induced diabetic rats is shown in Figure 2. The fasting blood glucose of the control rats were within the range of 90-100mg/dL throughout the study period. Intraperitoneal injection of STZ resulted in a 3-fold (3.06 – 3.20) increase in fasting blood glucose (FBG) level across the treatment groups by day 3 of the study (Figure 2a), and reaches its peak by day 5 across all the groups. Starting from the 10<sup>th</sup> day of the study, administration of both 200 mg/kg and 400 mg/kg body weight of *A. occidentale* induced a significant ( $p < 0.05$ ) decrease in the FBG level of the diabetic rats to various degrees. The effect of the ethanolic stem bark extract of *A. occidentale* on hepatic glucose and glycogen concentration of STZ-induced diabetic rats was also assessed. STZ injection resulted in a significant ( $p < 0.05$ ) increase and decrease in hepatic glucose and glycogen concentration, respectively (Figure 2b and 2c). Both the 200 mg/kg and 400 mg/kg body weight extract reversed the increase in hepatic glucose concentration similar to what was observed with the standard drug, metformin. STZ-induced reduction in hepatic glycogen was also reversed in both extract treatment groups similar to the level observed in the control group.

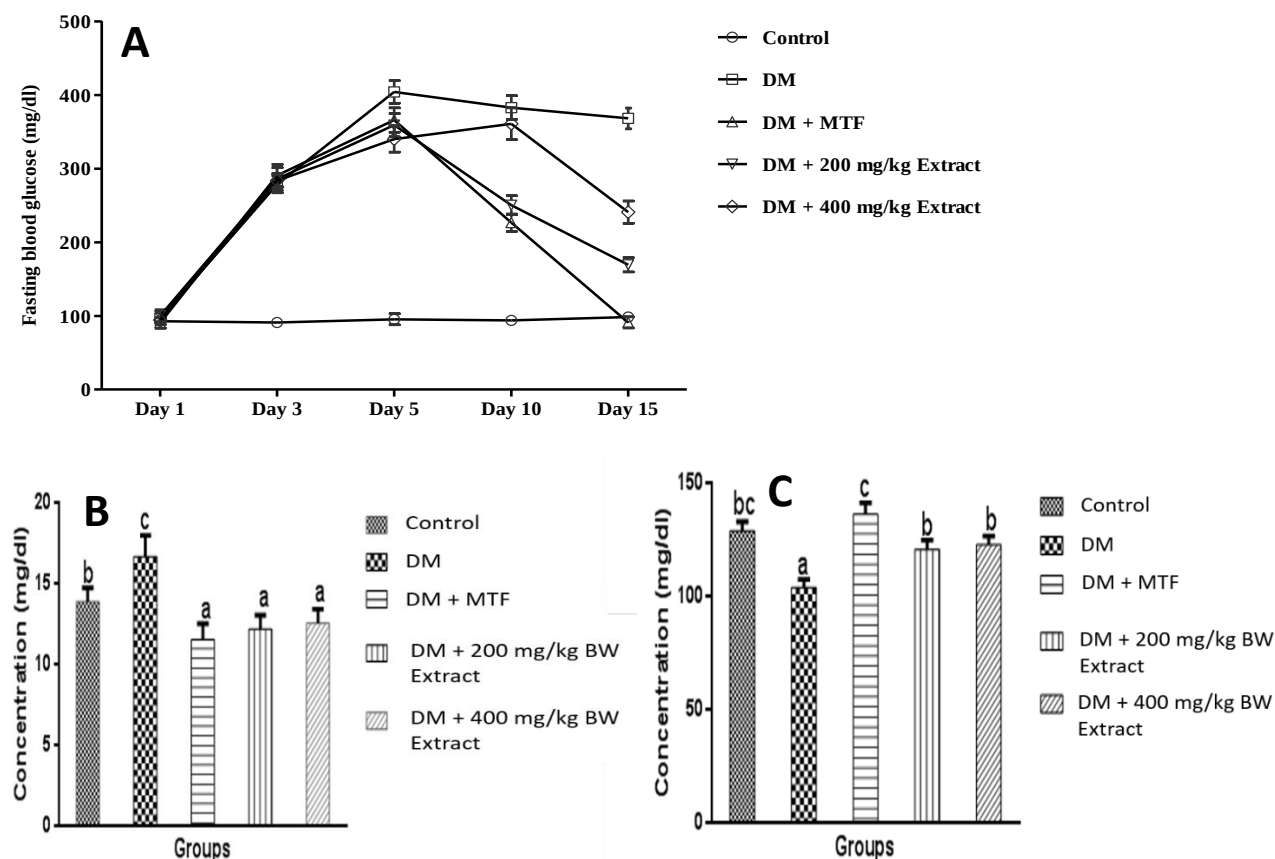
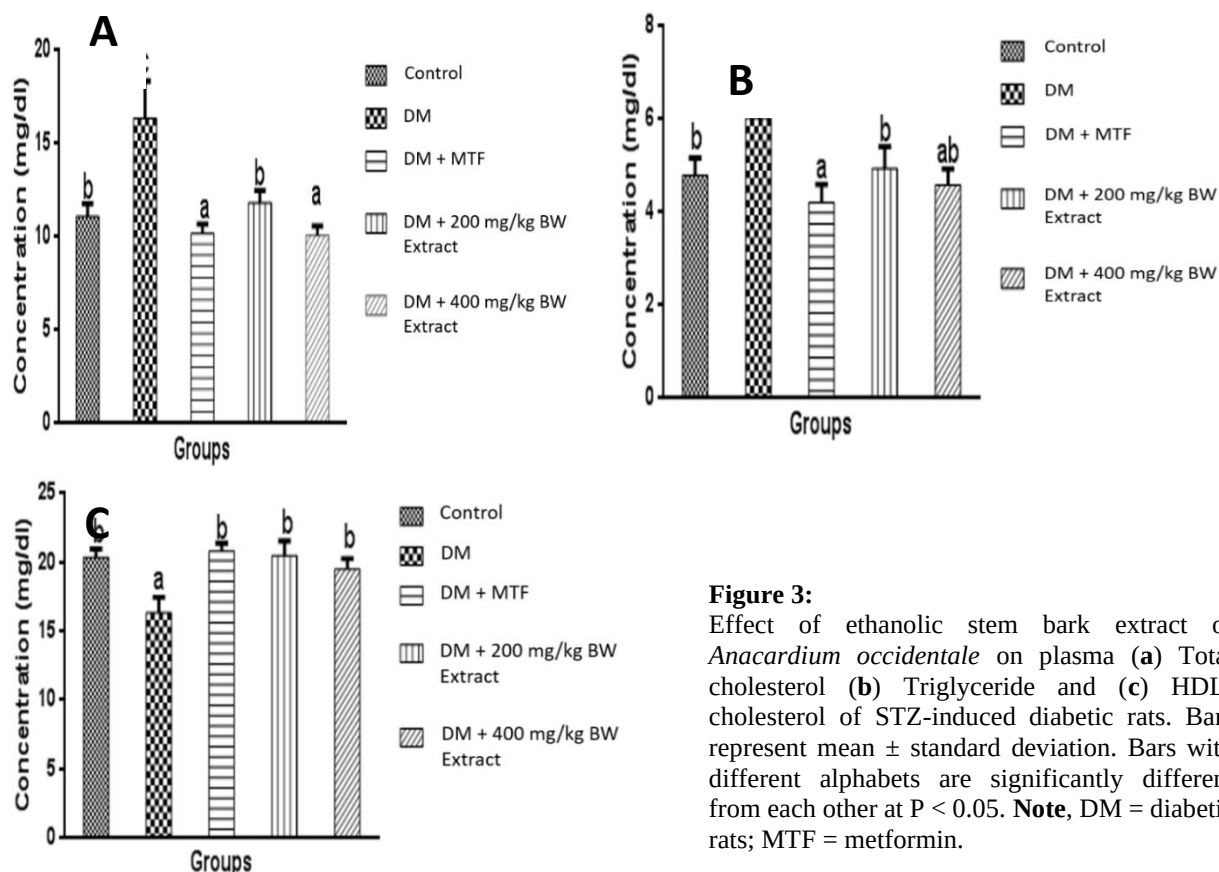


**Figure 2:**

Effect of ethanolic stem bark extract of *Anacardium occidentale* on change in (a) Fasting blood glucose (b) Hepatic glucose and (c) Hepatic glycogen concentration of STZ-induced diabetic rats. Data points and bars represent mean  $\pm$  standard deviation. Bars with different alphabets are significantly different from each other at  $P < 0.05$ . **Note**, DM = diabetic rats; MTF = metformin.

**Figure 3:**

Effect of ethanolic stem bark extract of *Anacardium occidentale* on plasma (a) Total cholesterol (b) Triglyceride and (c) HDL-cholesterol of STZ-induced diabetic rats. Bars represent mean  $\pm$  standard deviation. Bars with different alphabets are significantly different from each other at  $P < 0.05$ . **Note**, DM = diabetic rats; MTF = metformin.

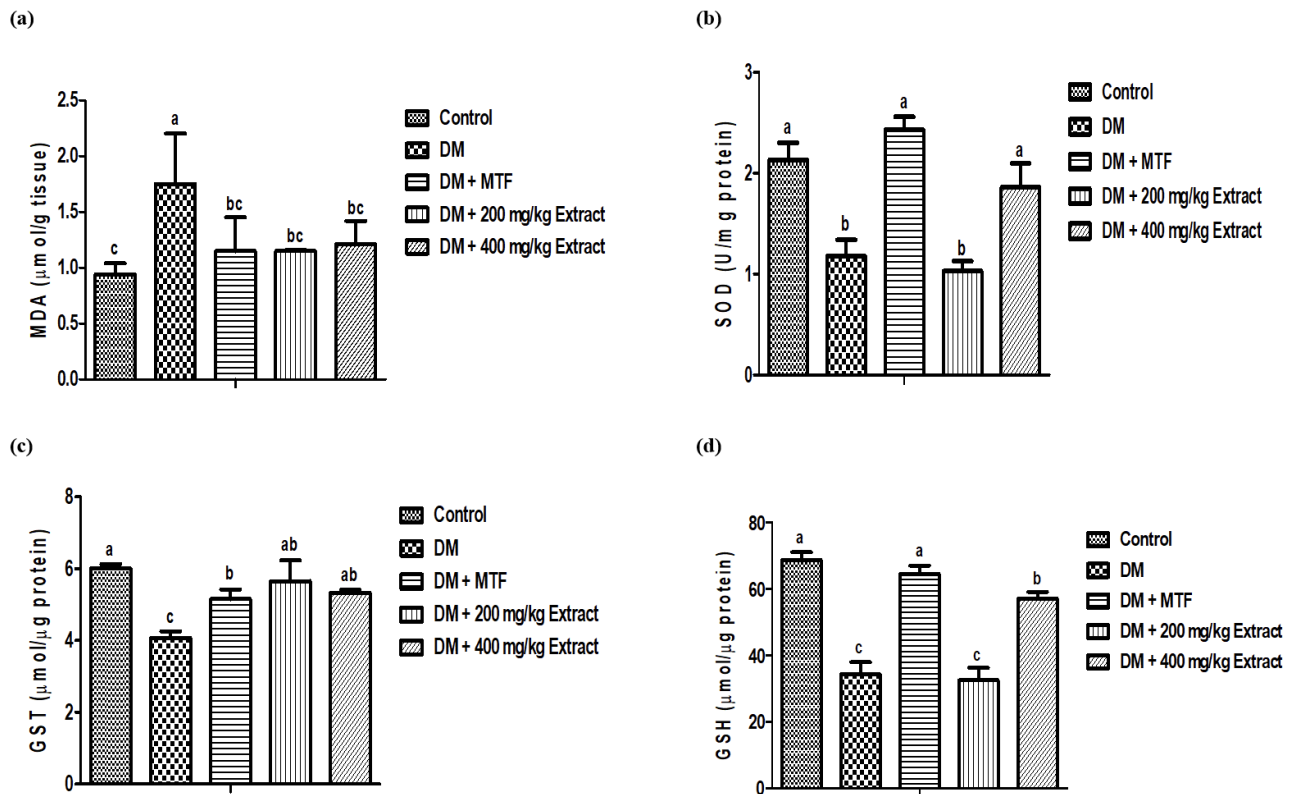
Next, we assessed the effect of the ethanolic stem bark extract of *A. occidentale* on plasma lipid profile in STZ-induced diabetic rats. The level of plasma total cholesterol (Figure 3a) and triglyceride (Figure 3b) was significantly ( $p < 0.05$ ) increased while HDL-cholesterol (Figure 3c) was reduced in diabetic untreated (DM) group when compared with control animals. Treatment with both 200 mg/kg and 400 mg/kg body weight extracts reversed the observed changes by significantly ( $p < 0.05$ ) reducing the increase in plasma total cholesterol and triglycerides, as well as increasing ( $p < 0.05$ ) plasma HDL-cholesterol to a level comparable with what was observed in either the standard drug (MTF) group or the control group.

Figures 4 and 5 showed the effect of ethanolic stem bark extract of *A. occidentale* on antioxidant indices and lipid peroxidation level in the liver and kidney of STZ-induced diabetic rats. Malondialdehyde, the marker of lipid peroxidation was significantly ( $p < 0.05$ ) increased in the liver (1.86-fold) and kidney (1.63-fold) of STZ-induced diabetic rats, as shown in figure 4a and figure 5a respectively. Administration of 200 mg/kg and 400 mg/kg body weight of *A. occidentale* extract was able to reverse the elevation in MDA level induced by STZ in both liver and kidney similar to what was obtained with the standard drug, metformin. Other indices of antioxidant status assessed indicated that in the liver, diabetes induction with STZ significantly ( $p < 0.05$ ) decreased the activity of SOD (1.80-fold) and GST (1.47-fold), as well as total glutathione level (2.00-fold). GST activity was restored to the level obtained in the control and metformin treated rats as a result of the administration of 200 mg/kg and 400 mg/kg body weight of *A. occidentale* extract (Figure 4c). The 400 mg/kg body weight extract, when administered to diabetic rats

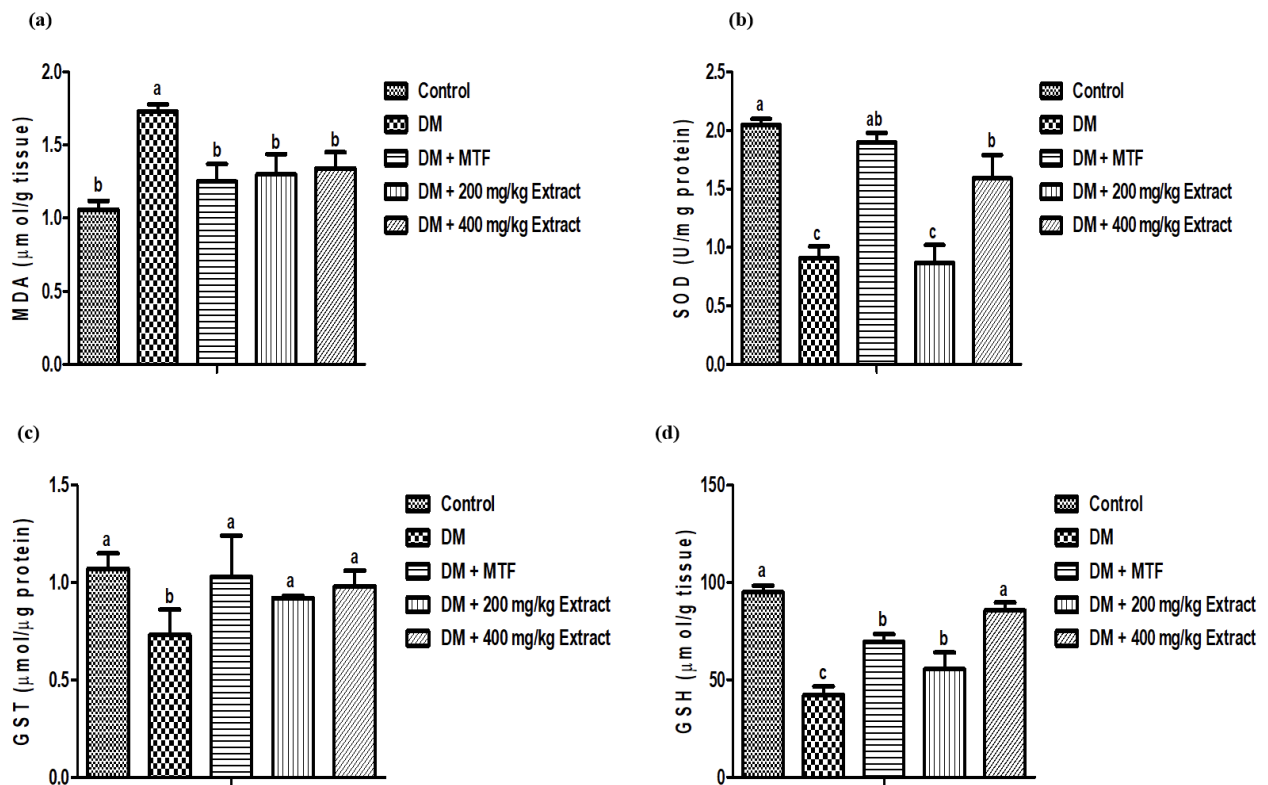
reversed the decrease in activity of SOD and glutathione concentration to a level comparable to what was obtained in either the control or metformin group (Figures 4b and 4d). Similarly, in the kidney, STZ injection resulted in a significant ( $p < 0.05$ ) reduction in the activity of SOD (2.25-fold), GST (1.47-fold), as well as in the concentration of total glutathione (2.26-fold) as shown in Figures 5b, 5c and 5d respectively. Only the 400 mg/kg body weight extract was able to reverse the reduction in SOD activity to the level obtained in the control and metformin group when administered to diabetic rats (Figure 5b). Both 200 mg/kg and 400 mg/kg body weight stem bark extract of *A. occidentale* ameliorated the pro-oxidant effect of STZ by significantly ( $p < 0.05$ ) improving the GST activity and total glutathione concentration when administered to diabetic rats (Figures 5c and 5d). Both extract concentrations restored the GST activity and glutathione concentration to the level observed in the control rats.

## DISCUSSION

Diabetes is a metabolic disease resulting from defective insulin secretion and/or resistance to the action of insulin in the peripheral tissues. Characterized by sustained hyperglycemia as a result of decrease in uptake and utilization of glucose in the skeletal muscle, liver and adipose tissues, diabetes is rising to epidemic proportions globally. Unfortunately, the efficacy of orthodox drugs for the treatment of diabetes cannot be completely guaranteed and majority of them are known to have serious side effects, thereby limiting their use.

**Figure 4:**

Effect of ethanolic stem bark extract of *Anacardium occidentale* on liver (a) Lipid peroxidation (b) SOD activity (c) GST activity and (d) Total GSH concentration of STZ-induced diabetic rats. Bars represent mean  $\pm$  standard deviation. Bars with different alphabets are significantly different from each other at  $P < 0.05$ . **Note:** GSH = total glutathione; GST = glutathione-s-transferase; SOD = superoxide dismutase; DM = diabetic rats; MTF = metformin.

**Figure 5:**

Effect of ethanolic stem bark extract of *Anacardium occidentale* on kidney (a) Lipid peroxidation (b) SOD activity (c) GST activity and (d) Total GSH concentration of STZ-induced diabetic rats. Bars represent mean  $\pm$  standard deviation. Bars with different alphabets are significantly different from each other at  $P < 0.05$ . **Note:** GSH = total glutathione; GST = glutathione-s-transferase; SOD = superoxide dismutase; DM = diabetic rats; MTF = metformin.

Therefore, the development of alternative antidiabetic agents is of outmost importance. Recently, the pharmacological benefits of natural products from plants has been gaining a lot of attention, as these plants are believed to be important sources of new chemical substances with therapeutic potentials. In Nigeria, several hundreds of plants are used traditionally in the management of diabetes, however, there are very limited scientific data to back up this therapeutic claims. Therefore, this study was designed to investigate the potential hypoglycemic, hypolipidemic and antioxidant effects of ethanolic stem bark extract of *Anacardium occidentale*.

The diabetic state has been associated with severe body weight loss, and we observed a significant reduction in the body weight of STZ-induced diabetic animals compared with control animals in this study. Evidence has shown this to be due to degradation of fats and structural proteins, as well as to decrease in protein content in muscular tissue by proteolysis induced by insulin deficiency (Subash Babu et al., 2007; Ahmad et al., 2014). When diabetic animals were treated with both 200 mg/kg and 400 mg/kg body weight of ethanolic stem bark extract of *A. occidentale*, they showed an improvement in body weight loss. The reversal of body weight loss in the extract-treated diabetic animals may be due to the ability of phytochemicals in the extract to reverse proteolysis, gluconeogenesis and glycogenolysis (Ahmed et al., 2014).

The streptozotocin diabetic model is a widely used and accepted model of diabetes that is comparable to the human diabetic state. STZ is a pancreatic  $\beta$ -cells toxic agents that enters the  $\beta$ -cells via the glucose transporter 2 (GLUT 2) and can induce sustained hyperglycemia within few days of administration (King and Bowe, 2016). STZ causes alkylation of DNA and subsequently, PARP activation, NAD<sup>+</sup> depletion, cellular ATP content reduction and inhibition of insulin production follows (Asrafuzzaman et al., 2017). We observed sustained elevated levels of fasting blood glucose in STZ-induced diabetic animals when compared with control animals throughout the experimental period. Similarly, STZ administration resulted in elevated level of glucose and lowered level of glycogen in the hepatic tissue of diabetic animals. Results from our study also showed that the stem bark extract of *A. occidentale* showed hypoglycemic effect by reversing the alteration in carbohydrate metabolism associated with the diabetic state as reported earlier (Saidu et al., 2012). The hypoglycemic effect shown by *A. occidentale* stem bark extract may be due to its ability to stimulate key enzymes involved in carbohydrate metabolism, thus increasing glycogen storage in the liver, glucose uptake by the muscle and/or inhibition of gluconeogenesis (Miranda-Osorio et al., 2016). Lower dose of STZ (60 mg/kg body weight) as used in our study has been reported to produce an incomplete destruction of the  $\beta$ -cells despite the animals having stable, long-lasting hyperglycemia (Asrafuzzaman et al., 2017), thus there could be many surviving  $\beta$ -cells, making regeneration possible. Although we did not measure plasma insulin level in our study, however we are tempted to speculate that the hypoglycemic activity of *A. occidentale* stem bark extract observed in this study may be due to its ability to enhance the regeneration of pancreatic  $\beta$ -cells as previously reported (Bassey et al., 2012), and hence potentiate insulin release from the regenerated  $\beta$ -cells. Furthermore, the

hypoglycemic action of the ethanolic stem bark extract of *A. occidentale* observed in our study is comparable to that of the conventional drug, metformin, which mode of action involved reduction of the hepatic glucose production as well as potentiation of insulin sensitivity (Rena et al., 2017).

Dyslipidemia is one of the main risk factors for coronary artery disease and cerebrovascular disease (Baena-Diez et al., 2011). Hypercholesterolemia and hypertriglyceridemia are the two most common lipid abnormalities in the pathogenesis of diabetes. In this study, we observed an increased level of total cholesterol and triglyceride, with a concomitant reduction in HDL-cholesterol level in STZ-induced diabetic animals. This observed STZ-induced dyslipidemia may be due to increased intestinal absorption and increased cholesterol biosynthesis, as well as increased fatty acid mobilization from adipose tissue in the diabetic animals (Subash Babu et al., 2007; Watcho et al., 2012; Ahmed et al., 2014). Administration of ethanolic stem bark extract of *A. occidentale* lowers total cholesterol and triglyceride while HDL-cholesterol level was increased in diabetic rats. This result is consistent with previous studies that have demonstrated hypolipidemic effect for *A. occidentale* (Olatunji et al., 2005; Anyaegbu et al., 2017). Hyperglycemia-induced oxidative stress and its resultant cellular effects are well established in several studies (Fiorentino et al., 2013). In the diabetic state, ROS production overwhelms the cell antioxidant defense resulting in damage to macromolecules. Peroxidation to membrane lipids is one of the manifestation of oxidative stress in STZ-induced diabetic animals. In the current study, diabetes-induced lipid peroxidation was assessed by determining the hepatic and kidney malondialdehyde (MDA) levels, and these were found to be significantly elevated in the diabetic rats. Our result is similar to several studies that have previously shown that biomarkers of oxidative damage including MDA were elevated in the liver and kidney of chemical-induced diabetic rats (Palmer et al., 1998; Van Dam et al., 2001; Nain et al., 2012; Akindele et al., 2015). We also observed that administration of *A. occidentale* stem bark extract at 200 mg/kg and 400 mg/kg of body weight, decreases MDA level in the liver and kidney of the diabetic rats in a manner comparable to that of the antidiabetic drug, metformin. This finding suggests that administration of ethanol stem bark extract of *A. occidentale* prevents lipid peroxidation in STZ-induced diabetic rats. This is similar to earlier reports where methanol stem bark extract of *A. occidentale* reduced MDA level in triton X-100 induced hyperlipidemic and fructose-induced diabetic rats (Olatunji et al., 2005; Anyaegbu et al., 2017).

Antioxidant enzymes such as SOD, CAT and GST produced by mammalian cells as defense against ROS generation are closely related to lipid peroxidation. Evidence abound of association between change in antioxidant enzymes activity and oxidative stress-mediated injury in STZ-induced diabetic rats (Palsamy et al., 2010; Alkan and Celik, 2018; Uddand Rao et al., 2019). Similar to those reports, we observed a reduction in the activities of SOD and GST in the liver and kidney of diabetic rats in our study. The superoxide anion ( $O_2^{\cdot-}$ ) is the chief free radical that has been implicated as one of the toxic mediators responsible for STZ-induced oxidative damage to macromolecules. SOD, a metalloprotein, is the key enzyme involved in protection of cells which spontaneously dismutate  $O_2^{\cdot-}$  to hydrogen

peroxide ( $\text{H}_2\text{O}_2$ ). The  $\text{H}_2\text{O}_2$  produced by SOD is then decomposed to water and oxygen by CAT, a hemoprotein localized in the peroxisomes. GST is a family of cytosolic isoenzymes that catalyzes the conjugation of glutathione with a variety of reactive electrophilic compounds, thereby neutralizing their active electrophilic sites. Therefore, the observed reduction in the activities of SOD and GST may be as a result of glycation (Yan and Harding, 1997) and ROS-induced inactivation of the enzymes (Kono and Fridovich, 1982). GSH is the predominant, non-enzymatic intracellular redox buffer which functions as a direct free radical scavenger, as cofactor for many enzymes, including GST and in maintenance of membrane protein thiol. In agreement with previous studies (Palsamy *et al.*, 2010; Alkan and Celik, 2018; Uddand Rao *et al.*, 2019), we also observed a decline in the concentration of total glutathione (GSH) in the liver and kidney of the STZ-induced diabetic rats compared to control. The reduction in tissue GSH concentrations might be alluded to the ability of GSH to directly scavenge free radicals and other peroxidation products generated in the diabetic rats. Furthermore, the lower levels of GSH could be as a result of decreased synthesis and/or increased degradation of GSH (Loven *et al.*, 1986; Uddand Rao *et al.*, 2019). Results from our study further showed that administration of *A. occidentale* ethanolic stem bark extract at 200 mg/kg and 400 mg/kg body weight was able to reverse the decrease in the activities of SOD and GST, as well as the reduction in GSH concentration in both the liver and kidney. This ameliorative effect, especially with the 400 mg/kg body weight extract is also observed to be similar to what was obtained with the antidiabetic drug, metformin. The increase in GSH and improvement in antioxidant enzymes activities under *A. occidentale* extract supplementation had been reported previously in the liver and kidney of Triton X-100 induced hyperlipidemic rats (Anyagbu *et al.*, 2017). These results clearly showed that ethanolic extract of *A. occidentale* possess potent antioxidant activity. Evidence has shown that *A. occidentale* stem bark extract is rich in total phenolic and total flavonoid contents (Anyagbu *et al.*, 2017), and there are reports of presence of several phenolic compounds in the stem bark and leaves extract of *A. occidentale* (Encarnacao *et al.*, 2014; 2016). Therefore, the antioxidant effects shown by *A. occidentale* stem bark extract could be ascribed to the ability of its individual bioactive components to scavenge and/or quench free radicals generated due to diabetic induction, and boost the synthesis of GSH and other key enzymes.

In conclusion, results of the present investigation indicate that ethanolic stem bark extract of *A. occidentale* showed ameliorative effect in STZ-induced diabetic rats. This is demonstrated by its ability to (1) reverse hyperglycemia and dyslipidemia induced by diabetes and (2) prevent lipid peroxidation and maintaining redox status in the liver and kidney of the diabetic rats. This suggests that ethanol extract of *A. occidentale* may be of benefit as an adjuvant therapy in the management of diabetes. However, future studies are necessary to fully understand the specific mechanisms of the ameliorative effect shown by this extract.

#### Acknowledgement

The authors are grateful to Kwara State University of Science and Technology and Federal University, Oye-Ekiti, Nigeria for support and use of facilities.

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Full length Research Article

# The Effect of Acute Administration of the Aqueous Calyx Extract of *Hibiscus sabdariffa* on Blood pressure, Heart rate and Double Product of Apparently Healthy Human Subjects during Different Postures

Aliyu, B. <sup>1</sup>, \*Mojiminiyi, F.B.O. <sup>1</sup>, Oyeniyi, Y.J. <sup>2</sup>, Isezuo, S.A. <sup>3</sup>, Alada, A.R.A <sup>4</sup>

Departments of <sup>1</sup>Physiology, <sup>2</sup>Pharmaceutics and <sup>3</sup>Medicine Usmanu Danfodiyo University, Sokoto, Nigeria

<sup>4</sup>Department of Physiology, University of Ibadan, Ibadan, Nigeria

**Summary:** The aqueous calyx extract of *Hibiscus sabdariffa* (HS) is widely consumed as a beverage in Northern Nigeria and other parts of the world. HS has been reported to lower blood pressure (BP) in animals and man. However, not much is known about the effect of HS on BP in different postures. We tested the hypothesis that HS may lower BP, heart rate (HR) and heart rate-pressure product or double product (DP) by attenuating the discharge of the autonomic nervous system in different postures. Experiments were performed in accordance with the Principles of the Declaration of Helsinki. Following ethical approval and informed consent, BP and HR were measured in different postures (supine, sitting and standing) in apparently healthy human subjects (n=20) before and after (+HS) the oral administration of 15mg/Kg HS. Mean arterial pressure (MAP; taken as representative BP) and DP were calculated. Results are expressed as mean  $\pm$  SEM. Paired t test and ANOVA with a post hoc Bonferroni test were used for statistical analyses.  $P < 0.05$  was considered significant. In the supine position MAP, HR and DP were significantly ( $P < 0.0001$  each) reduced in the presence of HS ( $85.6 \pm 1.7$  mmHg,  $72.1 \pm 1.1$  /min and  $8716 \pm 320$  mmHg.bpm) compared to its absence ( $89.6 \pm 2.0$  mmHg,  $73.7 \pm 1.6$  /min and  $8921 \pm 444$  mmHg.bpm). Similar trends were observed in the sitting position in the presence of HS ( $85.4 \pm 2.7$  mmHg,  $73.7 \pm 1.8$  /min and  $9098 \pm 345$  mmHg.bpm vs its absence:  $91.4 \pm 2.3$  mmHg,  $77.1 \pm 1.9$  /min and  $9388 \pm 478$  mmHg.bpm;  $P < 0.0001$ ,  $P < 0.0001$  and  $P = 0.007$  respectively) and in the standing position (+HS:  $89.3 \pm 2.0$  mmHg,  $78.1 \pm 1.8$  /min and  $10164 \pm 230$  mmHg.bpm vs its absence:  $94.3 \pm 2.1$  mmHg,  $81.8 \pm 2.3$  /min and  $10742 \pm 268$  mmHg.bpm;  $P < 0.0001$ ,  $P < 0.0001$  and  $P = 0.007$  respectively). In the absence of HS, HR and DP were significantly higher in the standing posture ( $81.8 \pm 2.3$  /min,  $10742 \pm 268$  mmHg.bpm) compared to the sitting ( $77.1 \pm 1.9$  /min,  $9388 \pm 478$  mmHg.bpm;  $P < 0.05$  and  $P < 0.0001$  respectively) and the supine ( $73.7 \pm 1.6$  /min,  $8921 \pm 444$  mmHg.bpm;  $P < 0.001$  each) postures while the BP remained similar. A similar trend was observed across the three postures in the presence of HS although the parameters were significantly lower. It is concluded that HS lowered BP, HR and DP by modulating autonomic mechanisms through the inhibition of both parasympathetic withdrawal and sympathetic nervous system discharge across the postures. Also the standing posture is associated more with a higher sympathetic nervous system discharge and a higher cardiac oxygen demand and workload than the sitting and supine postures in the absence or presence of HS.

**Keywords:** *Hibiscus sabdariffa* calyces, Posture, Blood pressure, Heart rate, Double product, Autonomic nervous system

©Physiological Society of Nigeria

\*Address for correspondence: [frank.mojiminiyi@udusok.edu.ng](mailto:frank.mojiminiyi@udusok.edu.ng); +2347065748556

Manuscript received- December 2021; Accepted- April 2022

DOI: <https://doi.org/10.54548/njps.v37i1.18>

## INTRODUCTION

The calyces of *Hibiscus sabdariffa* linn (family: Malvaceae) are used in many parts of the world, including Sokoto and much of Northern Nigeria, to make a delectable beverage locally called soborodo. The calyx extract of *Hibiscus sabdariffa* (HS) is also used in folk medicine in the treatment of hypertension. This has been scientifically validated by some studies (Mojiminiyi *et al.*, 2007; Mojiminiyi *et al.*, 2012). Changes in position of the human body are known to affect cardiovascular, endocrine, and renal functions (Norsk, 1992). While assuming the standing posture from sitting, for example, blood is pooled to the

lower extremities due to gravitational forces. This leads to decreased venous return, stroke volume, arterial blood pressure and subsequent decrease in blood flow to the brain (Olufsen *et al.*, 2005). This reduction in arterial blood pressure stimulates the baroreceptors located in the carotid sinus and aortic arch to a lesser extent, leading to withdrawal of parasympathetic discharge and activation of sympathetic discharge through the baroreflex-mediated autonomic control (Olufsen *et al.*, 2005). The parasympathetic withdrawal induces fast increase in heart rate, while the sympathetic activation causes a slower increase in vascular resistance, vascular tone, cardiac contractility and a further increase in heart rate (Smith and Kampine, 1990; Bullock *et*

al., 2001; Guyton and Hall, 2011). These responses combine to increase the blood pressure back to normal and ensure that blood flow to the brain is preserved. So the standing or erect posture may be associated with a state of parasympathetic withdrawal and activation of the sympathetic nervous system.

Other postural changes appear to affect blood pressure and heart rate as well. In the recumbent or horizontal posture, changing from the prone to supine and vice versa are associated with changes in blood pressure and heart rate (Pump *et al.*, 2002; Tabara *et al.*, 2005; Watanabe *et al.*, 2007) with little or no change in the balance of the autonomic nervous system (Watanabe *et al.*, 2007). However changes from the recumbent or horizontal posture (prone or supine) to the vertical (sitting or standing) are associated with significant differences in the balance of autonomic discharge to the heart, such that there is a shift towards the dominance of the sympathetic nervous system in the sitting position (Watanabe *et al.*, 2007). In general, the parasympathetic nervous system (PNS) seems to be predominant in the recumbent postures while the sympathetic nervous system (SNS) appears to be predominant in the vertical postures (Watanabe *et al.*, 2007). From the above, it is apparent that maintenance of blood pressure and heart rate in the different postures are dependent on the activity of the autonomic nervous system. An earlier work from our laboratory suggests that one mechanism by which HS lowers blood pressure is by inhibiting the discharge of the SNS (Aliyu *et al.*, 2014). We therefore hypothesized that HS, being an inhibitor of the SNS, may lower the blood pressure and heart rate in the vertical postures of standing and sitting and perhaps in the recumbent posture as well.

The cardiac heart rate-pressure product or double product (DP) is the product of heart rate and systolic blood pressure. It is an index of cardiac oxygen consumption and workload (Katz and Feinberg, 1958; Kitamura *et al.*, 1972; Hermida *et al.*, 2001). Since HS lowers both heart rate and systolic blood pressure (Mojiminiyi *et al.*, 2007; Aliyu *et al.*, 2014), it is conceivable that it may lower DP too. However, this is yet to be examined.

Consequently, this study tested the hypotheses that HS may lower BP, heart rate (HR) and double product (DP) by attenuating the discharge of the autonomic nervous system across the postures and that this effect may be associated with a reduction in myocardial oxygen consumption and workload.

## MATERIALS AND METHODS

**Plant materials:** The dried red calyces of HS were obtained from the Talata Mafara Central Market, Zamfara State, Nigeria. The dried red calyces of HS were previously identified and a voucher specimen (voucher number PCG/UDUS/MLV 001) deposited in the herbarium of the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria (Aliyu *et al.*, 2014).

**Extraction procedure:** This was done as described earlier (Aliyu *et al.*, 2014). Briefly, the dried calyces of HS were pounded into coarse powder. 500g of the powder was mixed with 2.7 litres of hot water (50°C) in a conical flask and

stirred continuously with a magnetic stirrer. It was left overnight and later filtered using a filter paper (0.5µ). It was then decanted and evaporated to dryness in a water bath at 60°C leaving a powdery extract.

**Tableting of powdery extract of HS:** The powdery extract was then prepared into tablets containing 500mg of extract per tablet using the wet granulation method as earlier described (Aliyu *et al.*, 2014). The granules were tableted using the single pouch machine (ART 400 Eureka GmbH, Germany).

**Ethical Clearance:** Before the commencement of the study, ethical approval was obtained from the ethical committee of Specialist Hospital Sokoto, Nigeria, and informed consent was obtained from the subjects. The protocol number of the ethical committee approval was: SHS/SUB/133/1. Indeed, the experiments were performed in accordance with the Principles of the Declaration of Helsinki.

**Participants:** 20 apparently healthy male human volunteers aged 29.9±1.6 years and weighing 67.3±2.7 kg were randomly selected for the study. They were not on any medication that affects blood pressure and heart rate and were not consuming alcohol, tobacco or caffeine-containing beverages. They were also not involved in strenuous exercise twenty four hours before the test. Participants were excluded if they had cardiovascular, renal or endocrine diseases.

**Data acquisition:** The blood pressure and heart rate were measured using the EchoMax plus BP-400 digital sphygmomanometer (HuBDIC Co. Ltd., Gyeonggi-do, Korea) with the cuff at the level of the heart. In order to minimize the impact of cardiovascular adjustments during postural changes on our observations, the subjects were made to maintain each posture for at least 5 minutes before the blood pressure and heart rate were measured (Watanabe *et al.*, 2007). Since we started with the supine position the subjects were allowed to rest for a minimum of twenty-five minutes in the supine position (Nardo *et al.*, 1999) in order to allow sufficient time for the blood pressure and heart rate to reach the resting level after which these parameters were recorded in this position. They were then asked to sit up and the parameters measured after more than a five-minute rest (Tabara *et al.*, 2005). They were then asked to stand up, and the blood pressure and heart rate recorded after more than five minutes. Thereafter, the HS tablets were given orally at a dose of 15mg/kg and the subjects were allowed to rest. One hour after HS administration, the above procedures were repeated.

The mean arterial pressure (MAP) was calculated using the formula:

Mean arterial pressure =  
diastolic blood pressure + 1/3 pulse pressure  
(Ganong, 1991; Jaja *et al.*, 2000)

Where pulse pressure = systolic - diastolic blood pressure

In addition, the heart rate-pressure product or double product (DP) was calculated by multiplying the heart rate and the systolic blood pressure (Katz and Feinberg, 1958; Kitamura *et al.*, 1972; Hermida *et al.*, 2001).

**Statistical analysis:** Data were presented as Mean  $\pm$  SEM. Paired student t-test was used to analyse the data that fell in pairs i.e. before the administration of HS compared to after its administration. However, when data from the three different postures were compared, ANOVA with a Bonferroni post hoc test was used for analysis.  $P < 0.05$  was considered statistically significant.

## RESULTS

The systolic (SBP), diastolic (DBP), mean arterial pressure (MAP), heart rate (HR) and double product (DP) obtained in the supine position in the absence and presence of HS are shown in table 1. The SBP, DBP, MAP, HR and DP were significantly ( $P < 0.0001$  each) lower in the presence of HS (Supine +HS) compared to its absence (Supine).

Table 2 shows the blood pressure parameters, heart rate and DP obtained in the sitting position in the absence and presence of HS. The blood pressure parameters and heart rate were significantly ( $P < 0.0001$  each) lower in the presence of HS compared to its absence. In addition, the double product was significantly ( $P = 0.007$ ) lower in the presence of HS compared to its absence.

**Table 1:**

The Blood pressure, heart rate and double product of apparently healthy subjects ( $n=20$ ) in the supine position in the absence and presence of 15mg/kg HS

| Parameter      | Supine          | Supine+HS        |
|----------------|-----------------|------------------|
| SBP (mmHg)     | 123.0 $\pm$ 2.0 | 120.2 $\pm$ 1.9* |
| DBP (mmHg)     | 72.9 $\pm$ 2.0  | 68.4 $\pm$ 1.8*  |
| MAP (mmHg)     | 89.6 $\pm$ 2.0  | 85.6 $\pm$ 1.7*  |
| HP (beats/min) | 73.7 $\pm$ 1.6  | 72.1 $\pm$ 1.1*  |
| DP (mmHg/min)  | 8921 $\pm$ 444  | 8716 $\pm$ 320*  |

\*\*= $P < 0.0001$  standing + HS vs standing

\*= $P = 0.007$  standing + HS vs standing

SBP= Systolic Blood Pressure; DBP= Diastolic Blood Pressure

MAP= Mean Arterial Blood Pressure; HR= Heart Rate

DP= Heart rate-pressure product or double product

The blood pressure parameters, heart rate and DP obtained in the standing position in the absence and presence of HS are shown in Table 3. The blood pressure parameters and heart rate were significantly ( $P < 0.0001$  each) lower in the presence of HS compared to its absence. Furthermore, DP was also significantly ( $P = 0.007$ ) lower in the presence of HS compared to its absence.

The SBP, DBP, MAP, HR and DP obtained in the supine, sitting and standing positions in the absence of HS are shown in figures 1 to 5. The blood pressure parameters (SBP, DBP and MAP) followed the same trend. They were highest in the standing position, followed by sitting and were least in the supine posture. However these parameters did not differ significantly across the three postures (Figs. 1-3). The HR was significantly ( $P < 0.05$ ) higher in the standing position compared to the sitting (Fig. 4). It was also significantly ( $P < 0.001$ ) higher in the standing posture compared to the supine (Fig. 4). The HR, however, showed no significant difference between the sitting and supine positions. The DP or heart rate-pressure product (Fig. 5) was also significantly higher in the standing posture than in the supine ( $P < 0.001$ ) and sitting ( $P < 0.05$ ) postures.

**TABLE 2:**

The Blood pressure, heart rate and double product of apparently healthy subjects ( $n=20$ ) in the sitting position in the absence and presence of 15mg/kg HS

| Parameter      | Supine          | Supine+HS        |
|----------------|-----------------|------------------|
| SBP (mmHg)     | 124.9 $\pm$ 2.2 | 122.3 $\pm$ 2.3* |
| DBP (mmHg)     | 75.1 $\pm$ 2.1  | 70.0 $\pm$ 1.8** |
| MAP (mmHg)     | 91.4 $\pm$ 2.3  | 85.4 $\pm$ 2.7** |
| HP (beats/min) | 77.1 $\pm$ 1.9  | 73.7 $\pm$ 1.0** |
| DP (mmHg/min)  | 9388 $\pm$ 478  | 9098 $\pm$ 345*  |

\*\*= $P < 0.0001$  sitting + HS vs sitting

\*= $P = 0.007$  sitting + HS vs sitting

SBP= Systolic Blood Pressure; DBP= Diastolic Blood Pressure

MAP= Mean Arterial Blood Pressure; HR= Heart Rate

DP= Heart rate-pressure product or double product

**TABLE 3:**

The Blood pressure, heart rate and double product of apparently healthy subjects ( $n=20$ ) in the standing position in the absence and presence of 15mg/kg HS

| Parameter      | Supine          | Supine+HS         |
|----------------|-----------------|-------------------|
| SBP (mmHg)     | 126.7 $\pm$ 2.3 | 123.3 $\pm$ 2.5** |
| DBP (mmHg)     | 78.1 $\pm$ 2.3  | 72.4 $\pm$ 2.1**  |
| MAP (mmHg)     | 94.3 $\pm$ 2.1  | 89.3 $\pm$ 2.0**  |
| HP (beats/min) | 81.8 $\pm$ 2.3  | 79.3 $\pm$ 2.0**  |
| DP (mmHg/min)  | 10742 $\pm$ 268 | 10164 $\pm$ 230*  |

\*\*= $P < 0.0001$  standing + HS vs standing

\*= $P = 0.007$  standing + HS vs standing

SBP= Systolic Blood Pressure; DBP= Diastolic Blood Pressure

MAP= Mean Arterial Blood Pressure; HR= Heart Rate

DP= Heart rate-pressure product or double product

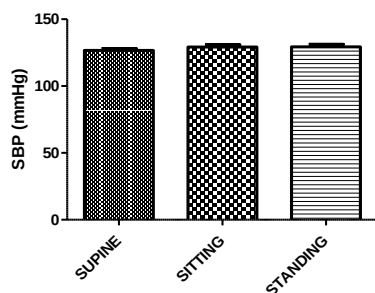


**Figure 1:**

The SBP (systolic blood pressure) of apparently healthy subjects ( $n=20$ ) in the absence of HS in the supine, sitting, and standing positions. The SBP showed no significant difference across the postures.

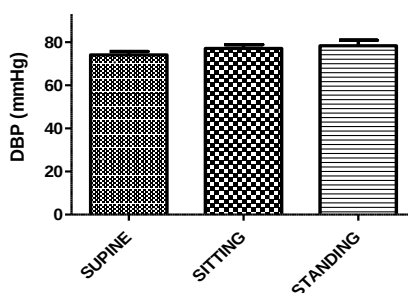


**Figure 2:**

The DBP (diastolic blood pressure) of apparently healthy subjects ( $n=20$ ) in the supine, sitting, and standing positions in the absence of HS. The DBP did not show a significant difference across the postures.

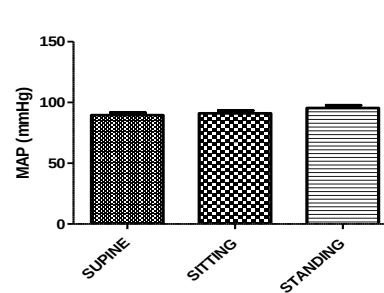


**Figure 3:**

The MAP (mean arterial pressure) of apparently healthy subjects ( $n=20$ ) in the supine, sitting, and standing positions in the absence of HS. The MAP showed no significant difference across the postures.

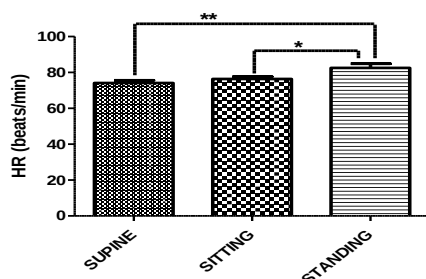


**Figure 4:** The HR (heart rate) of apparently healthy subjects (n=20) in the supine, sitting, and standing positions in the absence of HS. The HR was significantly higher in the standing position than in the supine and sitting positions. \*= $P<0.05$  standing vs sitting; \*\*= $P<0.001$  standing vs supine

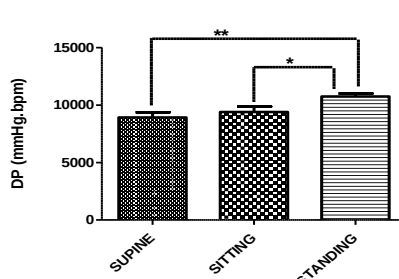


**Figure 5:** The DP (Double Product or heart rate-pressure product) of apparently healthy subjects (n=20) in the supine, sitting, and standing positions in the absence of HS. The DP was significantly higher in the standing position than in the supine and sitting positions.

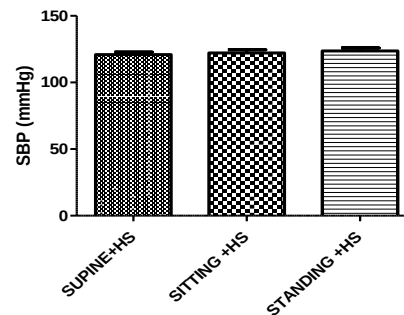


**Figure 6:** The SBP (systolic blood pressure) of apparently healthy subjects (n=20) in the supine, sitting, and standing positions in the presence of HS. The SBP showed no significant difference across the postures.

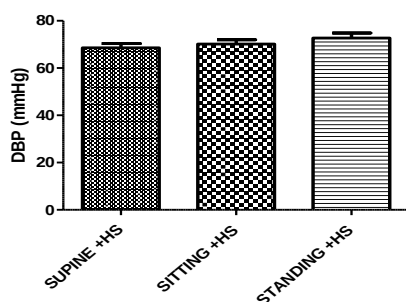


**Figure 7:** The DBP (diastolic blood pressure) of apparently healthy subjects (n=20) in the supine, sitting, and standing positions in the presence of HS. The DBP showed no significant difference across the postures.

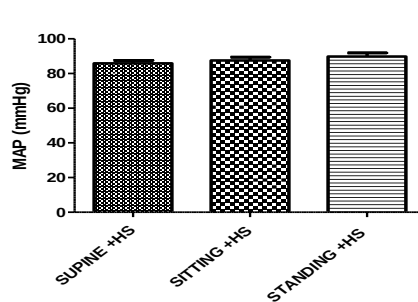


**Figure 8:** The MAP (mean arterial blood pressure) of apparently healthy subjects (n=20) in the supine, sitting, and standing positions in the presence of HS. The MAP showed no significant difference across the postures

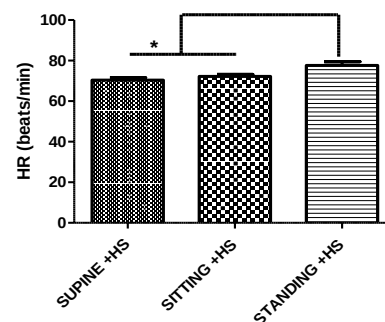


**Figure 9:** The HR (heart rate) of apparently healthy subjects (n=20) in the supine, sitting, and standing positions in the presence of HS. The HR was significantly higher in the standing position than in the supine and sitting positions. \*= $P<0.001$  standing +HS vs supine + HS and standing +HS vs sitting +HS

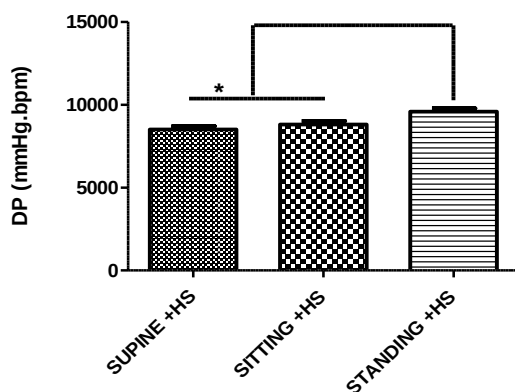


**Figure 10:** The DP (Double Product) of apparently healthy subjects (n=20) in the supine, sitting, and standing positions in the presence of HS. The DP was significantly higher in the standing position than in the supine and sitting positions. \*= $P<0.001$  standing + HS vs supine +HS and standing + HS vs sitting +HS

The SBP, DBP, MAP, HR and DP obtained in the supine, sitting and standing positions in the presence of HS are shown in figures 6 to 10. The blood pressure parameters (SBP, DBP and MAP) also followed a similar trend as in the absence of HS. They were highest in the standing posture, followed by sitting and were least in the supine position. However, these parameters showed no significant difference across the three postures (Figs. 6-8). The HR was

significantly ( $P<0.001$  each) higher in the standing position compared to the sitting and supine postures although it did not differ between the sitting and supine postures (Fig. 9). The DP was significantly ( $P<0.001$  each) higher in the standing position compared to the supine and sitting postures (Fig. 10). However, the DP did not differ significantly between the supine and sitting postures.

## DISCUSSION

The major finding of this study is that HS consistently lowered blood pressure, heart rate and double product across the postures studied. An additional finding is that in the absence of HS, HR and DP were significantly higher in the standing posture compared to the sitting and the supine postures while the BP remained similar across these postures. Furthermore, in the presence of HS a similar trend was observed for these parameters although the values were significantly lower. To the best of our knowledge, this study is the first to report that HS lowers the BP, HR and DP across the different postures.

This study found that HS lowered BP, HR and DP in the supine position compared to its absence. Since the supine posture is associated with a predominance of the parasympathetic nervous system discharge to the heart (Watanabe *et al.*, 2007), this finding suggests that HS may act by stimulating the parasympathetic nervous system, or

inhibiting parasympathetic withdrawal, resulting in the lowering of BP, HR and DP.

The vertical postures of sitting and standing are associated with parasympathetic withdrawal and sympathetic activation of the heart (Watanabe *et al.*, 2007). HS reduced the BP, HR and DP in these postures suggesting that it might be acting by inhibiting parasympathetic withdrawal and inhibiting the discharge of the SNS. So the notion that HS might act by inhibiting the SNS activation as hypothesized in this study appears to be correct.

The decrease in DP caused by HS across the postures as seen in this study is an interesting observation that is being reported for the first time to the best of our knowledge. DP is a measure of cardiac work load as well as myocardial O<sub>2</sub> consumption (Katz and Feinberg, 1958; Kitamura *et al.*, 1972) and it closely relates more with the left ventricular mass than the daily blood pressure mean (Hermida *et al.*, 2001). In this study HS significantly lowered the DP across the supine, sitting and standing postures compared to its absence. This suggests that HS reduced the cardiac workload and myocardial O<sub>2</sub> consumption across the postures. This finding implies that HS enables the heart to utilize O<sub>2</sub> more efficiently as well as reduce the cardiac workload in the different postures. It is not clear how HS does this. A likely explanation may be that since HS is a known vasodilating agent (Adegunloye *et al.*, 1996; Abubakar *et al.*, 2019), it may be dilating the coronary arteries thereby leading to an increase in blood flow to the heart.

A comparison of the BP parameters across the postures in the absence and presence of HS showed no significant difference. It is not clear why this is so but it may suggest that the autonomic control of BP may be similar across these postures. However, the HR and DP were significantly higher in the standing posture compared to the sitting and supine postures in the absence and presence of HS. While changing posture from the supine to sitting and from sitting to standing, the immediate rise in HR is due to parasympathetic withdrawal followed later by sympathetic activation (Smith and Kampine, 1990; Bullock *et al.*, 2001; Guyton and Hall, 2011). Since the HR was measured at least five minutes after the assumption of the posture by which time the autonomic adjustments are over, the increased HR may be due to the predominant autonomic system associated with that posture. Since the predominant autonomic system associated with standing is the sympathetic nervous system (Watanabe *et al.*, 2007), the increased HR seen in the standing posture compared to the sitting and supine may be due to the increased discharge of the SNS. DP tended to follow a similar trend with HR since it is the product of HR and systolic pressure implying that the increased DP seen in the standing posture compared to the sitting and supine may also be due to the increased discharge of the SNS associated with standing. The increased SNS discharge associated with the standing position may be an effort to increase the cardiac output and ensure blood supply to the brain thereby overcoming the gravitational forces predominant in this position compared to the sitting and supine postures. Although the HR and DP were reduced in the presence of HS the significant difference in these parameters remained in the standing position compared to the sitting and supine postures.

The findings of this study showing that the BP parameters did not differ significantly across the postures in the absence of HS is in agreement with the findings of Silvani *et al.*, (2017) with respect to systolic pressure. These workers reported no difference in systolic pressure across the supine, sitting and standing postures. However our findings are not in agreement with the findings of Silvani *et al.*, (2017) with regards to the diastolic pressure. They found that the diastolic pressure was significantly higher in the vertical postures of standing and sitting compared to supine. They also reported that the BP was significantly higher in the standing compared to the sitting posture (Silvani *et al.*, 2017). Tabara *et al.*, (2005), also found that the blood pressure increased significantly in the sitting position compared to supine which is also not in accord with our findings. The disparity between our findings and those of these earlier workers may be due to differences in experimental protocol. It may also be due to the fact that they used older subjects than us. Silvani *et al.*, (2017) used subjects with a mean age of 39±5 years while Tabara *et al.*, (2005) used subjects with a mean age 50±11 years some (33.9%) of whom were hypertensive. We used younger subjects with a mean age of 29.9±1.6 years all of whom were normotensive. In addition, our findings concerning HR in the absence of HS are dissimilar to those of Tabara *et al.*, (2005) who reported that the heart rate increased in the sitting posture compared to supine. Tabara *et al.*, (2005) however, did not study subjects in the standing posture.

HS tablets were used in this study in order to ensure that the exact dose required was administered to the subjects. This was also done in our earlier study (Aliyu *et al.*, 2014). One of the limitations of this study was that direct evidence of the involvement of the PNS and SNS through microneurography and/or heart rate variability techniques was not provided. This is because we do not have the equipment to perform these techniques due to poor funding. But it will be interesting to confirm the findings of this study using microneurography and/or heart rate variability techniques.

This study provides further evidence that the blood pressure lowering effect of HS may occur through the modulation of the autonomic nervous system across the postures of supine, sitting and standing. This supports the earlier finding from our laboratory suggesting that the hypotensive effect of HS was due to the inhibition of the discharge of the SNS (Aliyu *et al.*, 2014). HS may also lower BP and HR through other mechanisms including: angiotensin-converting enzyme inhibitory action (Ojeda *et al.*, 2010), diuretic (Mojiminiyi *et al.*, 2000; Alarcon-Alonso *et al.*, 2012), cholesterol-lowering (Ochani and D'Mello, 2009), direct vasorelaxant effect through acetylcholine-like and histamine-like actions (Adegunloye *et al.*, 1996), through Ca<sup>2+</sup> influx inhibition (Ajay *et al.*, 2007; Alsayed *et al.*, 2020), through antioxidant effects (Wang *et al.*, 1997; Hopkins *et al.*, 2013) and through the nitric oxide-cyclic Guanosine Monophosphate (NO-cGMP) relaxant pathway (Ajay *et al.*, 2007; Sarr *et al.*, 2009).

In summary, the BP, HR and DP of apparently healthy normotensive subjects were significantly lowered by HS across the supine, sitting and standing postures. In addition, in the absence and presence of HS, the HR and DP were significantly higher in the standing posture compared to the sitting and supine postures while the BP remained

unchanged. It is concluded that HS may lower BP, HR and DP through the modulation of autonomic nervous system activity predominant in each posture. Such autonomic modulation may involve stimulation of the parasympathetic nervous system or inhibition of parasympathetic withdrawal and inhibition of the discharge of the sympathetic nervous system. These autonomic effects of HS are associated with a reduction in cardiac oxygen demand and reduction in cardiac workload as evidenced by its lowering of the DP. In addition, the standing posture, regardless of the presence or absence of HS, is associated predominantly with increased sympathetic nervous system discharge to the heart which is accompanied by an increase in cardiac oxygen demand and cardiac workload compared to the sitting and supine postures.

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Short communication

## Changes in Serum Electrolytes, Urea and Creatinine in *Nicotiana tabacum*-treated Rats

\* Nwaji A.R.<sup>1,2</sup>, Ante I.A.<sup>2</sup>, Nwoke F.K.<sup>1</sup>, Inwang U.<sup>1</sup>

<sup>1</sup>Department of Physiology, Faculty of Basic Medical Sciences, Alex Ekwueme Federal University, Ndufu-Alike, Ebonyi State-Nigeria

<sup>2</sup>Department of Physiology, Faculty of Basic Medical Sciences, University of Calabar, Calabar-Nigeria.

**Summary:** Tobacco, a product of *Nicotiana tabacum* (*N. tabacum*) is composed of nicotine as its primary active ingredient. Nicotine has been reported to be the major cause of tobacco addiction with consequent renal implications. Therefore, this study was conducted to investigate the effect of the aqueous extract of *N. tabacum* on serum electrolytes, urea and creatinine levels as indices of renal function in male Wistar rats. Eighteen male Wistar rats weighing between 140 to 230g were used for this study. The animals were divided randomly into three groups: A, B and C; with each group containing 6 rats. Group A served as control while Group B and C were orally administered sublethal doses of 20 and 30 mg/kg body weight of the *N. tabacum* extract respectively once per day for 21 days. At the end of the experimental observations, the animals were sacrificed and blood samples were collected for biochemical assay. The results showed a significant increase ( $p < 0.05$ ) in the serum concentration of sodium, potassium and urea levels of rats treated with the extract when compared with the control. However, serum concentrations of chloride, bicarbonate and creatinine showed no significant appreciable differences between the treated groups and the control group ( $p < 0.05$ ). In conclusion, the study showed that aqueous extract of *Nicotiana tabacum* is associated with renal dysfunction with consequent hypernatremia and hyperkalemia, and may also suggest impaired urea clearance by the kidneys in male Wistar rats.

**Keywords:** Creatinine, electrolytes, kidney, *Nicotiana tabacum*, urea, Wistar rats

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\*Address for correspondence: E-mail: [zubbynwaji@yahoo.com](mailto:zubbynwaji@yahoo.com); Tel: +2348114105954

Manuscript received- February 2022; Accepted- May 2022

DOI: <https://doi.org/10.54548/njps.v37i1.19>

### INTRODUCTION

The kidney plays a primary role in homeostasis by excreting urea and creatinine (Walter, 2004 and Stuart, 2011). This it achieves through filtration of waste products from the blood stream and converting the ultra-filtrate to urine. Also, by regulating blood volume, and maintaining the acid-base balance (pH) and levels of electrolytes, especially sodium and potassium. Thus, according to Valerie and Scanlon, (2007), compromising kidney function can cause pernicious health effects due to build-up of unwanted substances in the body system.

The most commonly requested biochemical tests are urea and electrolytes because they provide crucial information on renal function, chiefly in homeostasis and excretion. The major factor in determining the estimated glomerular filtration rate, which is the gold standard marker of kidney health are the levels of creatinine (Blann, 2014). Also, glomerular filtration rate and levels of electrolytes, urea and creatinine give a strong indication of kidney function and can guide treatment (Blann, 2014).

Tobacco is a product of *Nicotiana tabacum* and has nicotine as its main phytochemical (Di-Chiara, 2000 and Harvey et al., 2004). Nicotine has been reported to be an addictive drug and the leading cause of tobacco addiction. Tobacco is derived from any plant of the genus *Nicotiana* of the Solanaceae family and has been reported to affect several

vital organs of the body. For instance, Nwaji et al., (2021) reported that *Nicotiana tabacum* impaired serum testosterone and testicular weight in rats. The smokeless tobacco has different names in Nigeria. It is called Anwuru in Igbo, Taba in Yoruba and Hausa languages (Thomson, 2008).

Smokeless tobacco is in two forms according to Aduema et al., (2012), viz: 'Tobacco snuff' and 'Chewing tobacco'. Also, according to Ureme et al., (2007), tobacco snuff is the powdered form blended with potash as the main additive in Nigeria and is being recommended as a substitute for cigarette since it is free of hazardous elements such as tar and carbon monoxide (Russel et al., 1980). For this sole reason, people now believe that using smokeless tobacco is safer than smoking it. However, this assumption is false because smokeless tobacco can as well induce nicotine addiction. (Dempsey, 2001).

An understanding of tobacco's effects on renal function can clearly point towards novel approach to its use or disuse at both social and clinical settings with references to renal implications. However, there is dearth of literature on the sub-lethal effects of its graded doses on the renal system. This study thus sought to investigate the effect of *Nicotiana tabacum* on serum electrolytes, urea, and creatinine as indicators of renal function.

## MATERIALS AND METHODS

### Collection and Identification of plant

Leaves of *Nicotiana tabacum* were collected, identified and authenticated by experts at the Alex Ekwueme Federal University, Ndufu-Alike, Ebonyi State.

**Extraction of Sample:** 100grams of the plant material was extracted by maceration in 1000ml of distilled water with intermittent agitation (8hrs/day) for 3days using mechanical shake. The mixture was filtered afterwards using Whatman no. 1 filter paper. The filtrate concentrate by rotary evaporation to dryness and solid residue was obtained. The dark brown paste residue was transferred to an airtight bottle and refrigerated until use. The concentration of the aqueous extract was determined using this formula:

$$\text{Concentration} = \text{Weight of leaves (g)} / \text{Quantity of water (ml)}$$

And the actual dose administered was obtained using this formula:

$$\text{Dose (ml)} = \text{Dosage (mg)} / \text{Body weight (kg)} / \text{Concentration (mg/ml)}$$

**Experimental Design:** 18 male rats of Wistar strain weighing between 140 to 230g were used for this experiment. The animals were acclimatized for two weeks at the Animal House of the Department of Physiology, Alex-Ekwueme Federal University, Ndufu-Alike. The animals were divided randomly into three groups, containing 6 rats each. The experimental design consisted of three groups denoted Group A, B and C. Group A served as the control while groups B and C were orally treated with 20 and 30 mg/kg body weight doses of the *N. tabacum* extract respectively.

The experiment was conducted following the humane treatment for the care and use of laboratory animals.

The animals were kept under optimal laboratory conditions with 12 hour light/dark cycle. The animals were fed with normal rat chow and allowed access to water *ad libitum* for three weeks.

**Blood sample collection:** The extracts were administered orally for three weeks using oropharyngeal cannula. On the last day of the experimental protocol, the animals were sacrificed and blood samples were collected via cardiac puncture into sample bottles. Serum was obtained by centrifuging at 3000 rpm for 15 minutes.

**Determination of serum electrolytes, urea, and Creatinine:** Serum levels of sodium and potassium were

determined using the Flame photometry method (Chiron Diagnostics) by strictly following the manufacturer's guidelines. Serum bicarbonate, urea, and creatinine levels were determined by titrimetry, diacetyl monoxime, and alkaline picrate methods respectively using the standard assay kit. These assays were carried out at the Chemical Pathology unit of the Alex-Ekwueme Federal University Teaching Hospital, Abakaliki (AE-FUTHA), Ebonyi State.

### Statistical Analysis

Data were expressed as mean  $\pm$  SEM. Data were analyzed using Graph pad prism 7.0 software. Differences in means were compared using ANOVA and Students T-test. P-values  $< 0.05$  were taken as statistically significant.

## RESULTS

### Changes in Serum Electrolyte, Urea and Creatinine in

***Nicotiana tabacum*-treated Rats:** The effect of *Nicotiana tabacum* on serum electrolytes ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$  and  $\text{HCO}_3^-$ ), urea and creatinine concentration are shown in Table 1 below. Administration of *Nicotiana tabacum* extract has no significant effect ( $p < 0.05$ ) on the serum chloride ion, bicarbonate and creatinine concentrations. There was a significant increase in the serum sodium ion concentration of rats fed with 30mg/kg body weight of the extract when compared with the control group. Also, there was a significant increase ( $P < 0.05$ ) in the potassium ion concentration of all the treated groups when compared with the control. There was also a significant increase in the serum urea concentration of the treated group which received higher dose (30mg/kg body weight) of the extract when compared with the control group.

## DISCUSSION

Nicotine is the primary and most important bioactive constituent of Tobacco (*Nicotiana tabacum*) (Harvey *et al.*, 2004). Electrolytes at the right levels are required for the proper functioning of the central nervous system, endocrine and musculoskeletal and digestive systems. The kidney is the principal organ that regulates electrolytes, urea and creatinine levels thereby maintaining homeostasis. Thus, making urea and electrolytes the most commonly requested biochemical tests to assess renal function, principally in excretion and homeostasis. According to Blann, (2014), the serum levels of electrolytes, urea and creatinine give a strong indication of kidney function and can guide diagnostics and medical treatment.

**Table 1:**

Changes in serum electrolyte, urea and creatinine in *Nicotiana tabacum*-treated rats. Values are expressed as mean  $\pm$  SEM (n=6)

| Group | Parameters               |                         |                          |                                        | Urea (mmol/l) | Creatinine (mmol/l) |
|-------|--------------------------|-------------------------|--------------------------|----------------------------------------|---------------|---------------------|
|       | Electrolytes             |                         |                          |                                        |               |                     |
|       | Na <sup>+</sup> (mmol/l) | K <sup>+</sup> (mmol/l) | Cl <sup>-</sup> (mmol/l) | HCO <sub>3</sub> <sup>-</sup> (mmol/l) |               |                     |
| A     | 143.7±0.3                | 4.6±0.1                 | 108.7±0.8                | 24.0±0.5                               | 4.4±0.1       | 76.0±1.1            |
| B     | 142.5±1.1                | 5.1±0.1**               | 109.8±0.5                | 21.2±1.2                               | 4.7±0.4       | 77.0±2.4            |
| C     | 145.2±0.4*               | 5.2±0.2*                | 108.5±0.6                | 24.2±1.4                               | 5.1±0.2**     | 79.3±1.1            |

Key: n=6, \*\*  $P < 0.01$ , \*  $P < 0.05$  vs. ctrl group,  $\text{Na}^+$  (sodium ion),  $\text{K}^+$  (potassium ion),  $\text{Cl}^-$  (chloride ion),  $\text{HCO}_3^-$  (bicarbonate)

The result of this study shows that aqueous leaf extract of *Nicotiana tabacum* caused elevated serum sodium, potassium as well as increased serum urea concentrations. However, serum levels of chloride, bicarbonate and creatinine concentrations showed no appreciable differences between the treated groups and the control group.

The result of this study is in pact with previous findings (Eliasson *et al.*, 1993; Yuka *et al.*, 2012; El-Sayed *et al.*, 2013 and Munzir *et al.*, 2015) who reported an increase in serum levels of sodium, magnesium, potassium and urea levels amongst smokers.

Some researchers also reported decreased serum and urinary creatinine values amongst smokers (Eliasson *et al.*, 1993). However, Munzir *et al.*, (2015) reported that there was an elevated value of serum creatinine amongst smokers. This is not consistent with our findings. Eliasson *et al.*, (1993) also reported decreased urinary creatinine, increased serum phosphate and increased urinary potassium amongst snuff users.

Hypernatraemia (Raised sodium) can be caused by a salt-rich diet or dehydration. It can also be caused by low blood volume, resulting from insufficient drinking or excessive loss of water in urine, sweat or diarrhoea (Blann, 2014). Also, raised potassium (hyperkalaemia) may be due to renal problems such as failure to excrete, acidosis (high pH) or potassium being released from damaged cells, such as red bloods cells.  $\text{HCO}_3^-$  (bicarbonate) is also important in determining the pH of the blood, thus, indicating acidosis or alkalosis.

The main excretory product of body's biochemical metabolism is urea, while creatinine is a more specialized product of the breakdown of protein. Thus, urea and creatinine molecules help with the excretion of excess nitrogen in the body. Urea is a good marker of acute renal disease while creatinine is useful as a longer-term marker of renal function (Blann, 2014). Moreso, the elevated serum urine concentration according to Ritz *et al.*, (1998) might be due to the fact that nicotine (an active ingredient in *Nicotiana tabacum*) increases renovascular resistance which will lead to the fall in renal plasma blood, filtration fraction and glomerular filtration rate (GFR)

The decrease in GFR will, therefore, lead to a decrease in distal tubular flow rate and thus, it is not surprising that it may bring about increased urea reabsorption in the distal convoluted tubule of the kidney (JoAnn and Robert, 2011). It has been suggested that several mechanisms may be involved in inducing renal vasoconstriction and vascular damage. For instance, nicotine has been reported by Ritz *et al.*, (1998) and Gambaro *et al.*, (1998) to increase plasma levels of vasoconstrictors such as catecholamines, vasopressin, endothelin-1 and arginine.

In conclusion, the results of the study suggest that aqueous extract of *Nicotiana tabacum* impaired renal handling of electrolytes with consequent hypernatremia and hyperkalemia and may also suggest impaired urea clearance by the kidneys in male Wistar rats.

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