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Review Article

Essential Metals in the Brain and the Application of Laser Ablation-Inductively Coupled Plasma-Mass Spectrometry for their Detection

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Summary: Metals are natural component of the ecosystem present throughout the layers of atmosphere; their abundant expression in the brain indicates their importance in the central nervous system (CNS). Within the brain tissue, their distribution is highly compartmentalized, the pattern of which is determined by their primary roles. Bio-imaging of the brain to reveal spatial distribution of metals within specific regions has provided a unique understanding of brain biochemistry and architecture, linking both the structures and the functions through several metal-mediated activities. Bioavailability of essential trace metal is needed for normal brain function. However, disrupted metal homeostasis can influence several biochemical pathways in different fields of metabolism and cause characteristic neurological disorders with a typical disease process usually linked with aberrant metal accumulations. In this review we give a brief overview of roles of key essential metals (Iron, Copper and Zinc) including their molecular mechanisms and bio-distribution in the brain as well as their possible involvement in the pathogenesis of related neurodegenerative diseases. In addition, we also reviewed recent applications of Laser Ablation Inductively Couple Plasma Mass Spectrophotometry (LA-ICP-MS) in the detection of both toxic and essential metal dyshomeostasis in neuroscience research and other related brain diseases.

Keywords*: Metal dyshomeostasis, Bio-imaging, LA-ICP-MS, Neurodegenerative diseases, Essential metals, CNS*

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INTRODUCTION

Metals are found ubiquitously in the environment, they are present in air, land, water and various parts of the earth crust (Chen *et al.*, 2016). They form the major parts of the CNS and their critical role in several pathophysiological processes has been of keen interest to several researchers (Flora and Pachauri, 2010). Metals are categorized into essential and toxic metals based on their biological functions (Chen *et al*., 2016). Essential metals include copper (Cu), zinc (Zn), iron (Fe), manganese (Mn), lithium (Li), nickel (Ni), chromium (Cr), selenium (Se), and cobalt (Co). These trace metals are required in an adequate amount and are the key regulatory factors for many cellular activities and brain physiological processes (Lee *et al*., 2008; Becker *et al*., 2010; Chen *et al*., 2016; DeBenedictis *et al*., 2020). Although they are needed for normal brain activities, their deficit or excess through genetic, environmental or nutritional disposition may be linked with several neurological diseases. Their quantitative determination is of growing interest in brain research and biosciences and is relevant for studying many neurological diseases (Becker *et al*., 2007; Becker *et al*., 2010; Chen *et al*., 2016). Out of the aforementioned essential trace metals found in the brain, Zinc, Iron and Copper are the most significant players in both neurophysiology and neuropathology, particularly with regard to aging and neurodegenerative diseases, they constitute the major component of various proteins and enzymes essential for normal brain function and also connected to specialized brain activities (Que *et al*., 2008; Prashanth *et al*., 2015).On the other hand, toxic metals such as vanadium, arsenic, cadmium, lead, mercury, uranium and nickel are ubiquitous in nature, they are found freely in water, food and in green vegetation (Becker *et al*., 2007; Pohl *et al*., 2011; Tchounwou *et al*., 2012; Bhat, 2017; Bhat, 2019). Sources are also through some human activities such as heavy metal mining, crude oil processing, chemicals and toxic waste disposal as well as emission from industrial and electricity-generating (particularly coal-burning) activities (Arruti *et al*., 2010; Sträter *et al*., 2010; Pohl *et al*., 2011; Iqbal and Ahmed, 2019).

Toxic metals possess no functional role in human homeostasis and constitute a risk for most of the chronic neurodegenerative diseases (they elicit severe damages as they easily transverse the brain barrier, bind brain tissue to induce oxidative stress, block aquaporins, interfere with normal endocrine activities and displace essential cations such as zinc and magnesium (Chen *et al*., 2016; Becker *et al*., 2010). In addition, toxic metal exposure early in life pose the risk of lifelong behavioural, intellectual and physical impairment as well as accelerated brain ageing in young adults and children (Pohl *et al*., 2011; Calderón-Garcidueñas *et al*., 2012). Several age-related neurological disorders are strongly linked with disrupted metal homeostasis, thus, brain metal content and their spatial

distribution in the diseased brain is usually obtained and compared with that of controls. Therefore, besides the analysis of food, beverages and environmental samples, the study of elemental distribution in brain and biological tissues is of great importance (Becker *et al*., 2008; Becker *et al*., 2010). This will give a clue to the overall diagnosis of individuals with a metal poisoning symptomatology or dyshomeostasis. It will also inspire newer therapeutic strategies in the diagnosis and potential treatment of several metal induced neurological diseases.

Molecular Biology of Trace Metals in The Brain and Their Roles in Brain Function: Trace metals are micronutrients usually found in relatively small amount but highly needed for proper growth and function of a biological system (Zecca *et al*., 2004; Anderson *et al*., 2011). Being the major part of most vitamins and enzymes, they participate in key oxidative-reduction reactions that control several other cellular metabolic activities (Bartzokis *et al*., 2007; Que *et al*., 2008). In the CNS trace metals such as cobalt, copper, iodine, iron, manganese, molybdenum, selenium, and zinc combine with specific enzymes to catalyze several activities involved in various neurological processes e.g. Iodine is bound to thyrosine, colbat is a component of vitamin B12 and zinc has a special function in zinc metalloenzymes (Que *et al*., 2008). Although, trace metals are needed for proper brain development, their balances and transportation within the CNS is essential and strictly controlled by a complicated barrier system involving the blood-brain barrier (BBB), choroidal blood-cerebrospinal fluid barrier, blood-cerebrospinal fluid (CSF) barrier, and even CSF-brain barrier (Takeda *et al*., 2004; Strazielle and Ghersi-Egea, 2013; Hladky and Barrand, 2016). Since some essential trace metals need to be obtained from the environment in adequate amounts to optimize cellular metabolism, their homeostasis within the brain is dependent on the control of processes such as absorption, distribution, biotransformation, and excretion (Zheng and Monnot 2012; Fu *et al*., 2014). However, their excess or deficit as well as impaired homeostatic metabolic mechanism often generate oxidative stress with deleterious effects on the neurons and glia resulting in neurodegeneration and neurological dysfunction (Garza-Lombo *et al*., 2018). For example, low iron content has been related with brain disabilities such as pediatric stroke, pseudo-tumor cerebri, and cranial nerve palsy (Yager and Hartfield, 2002; Rangarajan and D'Souza, 2007), while aberrant iron, zinc, copper and calcium accumulation was associated with Alzheimer's disease brain (Leskovjan *et al*., 2009, 2011; Li *et al*., 2017; Grochowski *et al*., 2019), highly concentrated iron in neuro-melanin is implicated in dopaminergic mal-function in Parkinson's disease (Sian-Hülsmann *et al*., 2011; Depboylu *et al*., 2007), deficiencies in copper-binding proteins was also linked with neurological disorders such as Menkes and Wilson diseases (Squitti *et al*., 2012; Squitti *et al*., 2013; Strausak *et al*., 2001). The chemical reactivity, spatial distribution as well as biological functions of each essential trace element is quite variable within the CNS, iodine for example is low in content and less distributed, relative to elements such as iron and selenium which are enormous in volume and fairly evenly distributed in all regions of the brain. Others such as copper and zinc are also found to be highly enriched in some regions and nuclei (Bartzokis *et al*., 2007).

Molecular Biology, Bio-Distribution And Roles Of Copper: Copper is one of the essential transition metals needed by the brain. It is rated as the third most abundant trace metal in the CNS; it has an average neural concentration in order of 0.1 Mm (Linder and Hazegh-Azam, 1996; Stöckel *et al*., 1998; Gaggelli *et al*., 2006). It is unevenly distributed in different parts of the brain with higher accumulation in the grey matter when compared with the white matter (Dobrowolska *et al*., 2008). Additionally, high concentration of copper was specifically reported in some brain regions such as substantia nigra, locus coeruleus, dentate nucleus, basal ganglia, hippocampus, and cerebellum (Madsen and Gitlin, 2007; Becker *et al*., 2007b, Popescu *et al*., 2009a, Davies *et al*., 2013). The highest level of copper was found in the basal ganglia, while in glia cells, it was double fold higher in concentration when compared to that of the neuron, especially at the ventricular regions (Madsen and Gitlin, 2007; Becker and Salber, 2010).

Transportation of copper within the CNS is highly dependent on its oxidation state which enables it to be readily involved in several redox activities (Macreadie, 2008). The reduced form of copper is mostly transported and is found in higher concentration within the intracellular environment such as the neurons and glia cells, in contrast to the oxidized forms which are less in abundance and found mostly in extracellular spaces such as the blood serum, CSF and synaptic cleft (Macreadie 2008; Que *et al*., 2008). In the peripheral blood, copper ions are usually transported as free ions which transverses the BBB into the brain parenchyma from where it is utilized for several redox activities and subsequently released into the CSF. Choroid epithelial cells absorb copper from the CSF, and thus facilitate its clearance from the brain to maintain normal brain copper balance (Zheng and Monnot, 2012). Effective cellular copper transportation and homeostatic control is achieved by binding with specific protein transporters and a subset of intracellular proteins known as Cu chaperones which enhances its delivery for specific targets involved in biochemical activities. Upon entering the cell, the fate of copper ions includes; (1) entering into Cu-metallothionein storage pool, (2) incorporation into cytochrome c oxidase in mitochondria for energy generation (3) incorporation into cytoplasmic Cu/Zn SOD for antioxidation; and (4) conveyed to a P-type ATPase in the trans-Golgi network for secretion (Que *et al*., 2008; Zheng and Monnot, 2012; Grochowski *et al*., 2019). The membrane-associated Cu transporters include copper transporter-1 (CTR1), DMT1, and Cu exporter ATPases (ATP7A and ATP7B). The chaperones include antioxidant protein-1 (ATOX1), cytochrome oxidase enzyme complex (COX17), and Cu chaperone for SOD (CCS) (Harris, 2001). Current scientific research has demonstrated the existence of several of these protein transporters, in the Blood Brain Barrier (BBB) and Blood Cerebrospinal Fluid Barrier (BCB) where they facilitate the entering of copper ions into brain tissue (Choi and Zheng, 2009).

ATOX1 (formerly HAH1) is a copper chaperone belonging to a larger family of metallochaperone proteins that binds copper and convey it in a specific pathway manner within the CNS. In addition to their intracellular

copper ions delivery, they also play the additional role of preventing toxicity through removal of unused free copper ions in the brain. ATOX1 associates with the Cu-ATPases located in the trans-Golgi network to perform intracellular copper trafficking and also found widely distributed in the choroid plexus and brain capillary endothelial cells (Nishihara *et al*., 1998; Hamza *et al*., 2001; Hamza *et al*., 2003; Zheng and Monnot, 2012). COX17 is another copper chaperone widely distributed in the neuronal cells, but its existence is not yet confirmed in the BBB or BCB. COX17 mediates the delivery of cytosolic copper to cytochrome c oxidase of mitochondrion for energy metabolism (Kaler, 2011; Hamza and Gitlin, 2002). CCS is a chaperone required for the incorporation of Cu into Cu–Zn SOD in mammals for antioxidant defence (Culotta *et al*., 2006; Que *et al*., 2008; Grochowski *et al*., 2019). Metallothionein (MT) are cysteine-rich copper binding cytoplasmic proteins that chelate excess free copper ions due to much larger Cu-MT binding affinity relative to affinity for other metals such as zinc (Nishimura *et al*., 1992; Que *et al*., 2008; Ba *et al*., 2009). MT was reported to be widely expressed in BB and BCB and plays additional role of regulating intracellular Cu storage by binding Cu ions at the brain barriers (Que *et al*., 2008). ATP7A and ATP7B, variants of P-type ATPases are also copper chaperones that regulate cellular copper homeostasis, through the removal of excess copper ions within the brain cells via the trans-Golgi network secretory pathway (Zheng and Monnot, 2012). They also regulate the release of copper ions to cuproenzymes during neurotransmitter formation and metabolism and mediate uptake of copper ions into the brain from plasma through the BBB and CSF- brain barrier system (Grochowski *et al*., 2019; Zheng and Monnot, 2012). ATP7B specifically play essential role in excreting excess copper ions in the biliary system. While ATP7A is expressed ubiquitously in several brain regions such as the cerebellum and hippocampus, as well as the BBB endothelium, ATP7B are found mostly in the liver cells and in a specific few brain cells such as Purkinje neurons (Madsen and Gitlin, 2007; Zheng and Monno,t 2012), However, both ATP7A and ATP7B was reported to be well expressed in the apical membrane of the gut enterocytes, choroid plexus ependymocytes and capillary endothelium (Mercer et al., 2001; Choi and Zheng 2009; Merle *et al*., 2016). Copper transporter-1 (Ctr-1) is a representative member of copper transport protein family that is expressed widely in many tissues including the brain, specifically within BBB endothelium where they mediate copper uptake into the brain tissue from plasma (Que *et al*., 2008; Madsen and Gitlin, 2007; Zheng and Monnot, 2012). Their expression is usually upregulated in perinatal copper deficiency, however, in a situation of high cellular copper level, Ctr-1 becomes inactive and totally degraded (Madsen and Gitlin, 2007). Amyloid precursor protein (APP), DMT-1 and prion protein (PrP) are other abundant copper transporter proteins in the brain that are essential for the uptake and efflux of copper ions, thereby maintaining normal neural copper homeostasis (Madsen and Gitlin, 2007).

Copper as a redox active nutrient is required in optimal level to cope with high oxygen capacity and oxidative metabolism of brain tissue. As a main component or co-factor for various enzymes, it is essential for a series of protein/enzyme regulated biological functions including energy metabolism involving mitochondrial cytochrome c oxidase, protection against oxidative damage involving Cu/Zn superoxide dismutase (SOD1), modulation and biosynthesis of neuropeptide, regulator of iron metabolism as well as neurotransmitter and intracellular release of copper ion from mobile storage during neural activities (Scheiber and Dringen, 2013; Scheiber *et al*., 2014; Kozlowski *et al*., 2012; Sheykhansari *et al*., 2018).

Copper dyshomeostasis and neurodegenerative diseases: Copper is a redox metal that co-ordinates several biological activities, its accumulation in the brain may be toxic to the body cells if its homeostatic mechanism is not accurately regulated (Kozlowski *et al*., 2012; Emwas *et al*., 2013). Failure of well refined copper homeostatic control can lead to a number of neurodegenerative diseases such as Parkinson's disease (PD), Menkes disease, Alzheimer's disease (AD), familial amyotrophic lateral sclerosis (fALS), Prion diseases, and Wilson disease. In addition to this, higher ability of copper to bind ligands within the cells can also trigger unregulated cellular reactions leading to severed cell impairment and death (Kozlowski *et al*., 2012).

Involvement of copper ion in pathogenesis of Alzheimer's disease is attributed to abnormal binding of Cu ions with APP and the product of its cleavage (β amyloids) which lead to formation of intermediate metalloproteins (copper-amyloid complex) that triggers fenton-type reaction and rapid generation of highly reactive free radical such as hydroxyl radical (OH-) and hydrogen peroxide (H_2O_2) which mediate a number of repeated oxidative stress cycle that ultimately promote repeated plaque formation with subsequent accumulation in the brain including the extracellular fluids (Parthasarathy *et al*., 2014). In a number of studies, high level of copper and zinc were seen in the amyloid plaque and CSF from AD patients (Bolognin *et al*., 2011; Huzumi *et al*., 2011; Cardoso *et al*., 2013).

Prion disease is another neurodegenerative disorder that has been linked with copper ion dyshomeostasis, prion proteins are cell surface copper binding glycoprotein that has six attachment domains for copper ions (Nadal *et al*., 2009). Scientific evidence is available for the possible involvement of prion proteins in the regulation of brain copper metabolism including cellular signaling, anti-oxidation and buffering activities (Walter, 2009; Nadal *et al*., 2009). Studies have also revealed that, higher affinity of cellular form of prion proteins (PrPC) for free Mn^{2+} ions than Cu^{2+} ions may facilitate its modification into a typical toxic, pathological isoform (PrP- Sc), the resultant free unbound Cu^{2+} ions may further aggravate the disease pathogenesis through free radical generation and oxidative damage (Kozlowski *et al*., 2010). However, there are discrepancies in the physiological function of copper in the etiology of prion protein diseases, and thus makes the phenomenon not to be completely understood (Kozlowski et al., 2010; Thakur *et al*., 2011).

Parkinson's disease is a debilitating motor disorder characterized by progressive degeneration of dopaminergic neurons of the substantia nigra and intracellular deposition of lewy bodies, a misfolded form of α-synuclein protein (Paik *et al*., 1999; Uversky *et al*., 2001). Fibrillation of α-synuclein into a misfolded form is facilitated by its binding with several toxic and trace metals in the brain which promote free radical generation and oxidative stress. Self-

oligomerization of alpha synuclein was reported to be initiated by copper binding in the presence of toxic radicals such as H_2O_2 (Paik *et al*., 1999; Uversky *et al.*, 200). Paik *et al*. (1999) also reported copper II induced self-oligomerization of α-synuclein in the presence of coupling reagents such as dicyclohexylcarbodi-imide. Studies have reported that oligomer species of α-synuclein requires association with copper ions to induce neuronal death (Paik *et al*., 1999; Wright *et al*., 2009; Brown, 2009; Wang *et al*., 2010).

Familial amyotrophic lateral sclerosis (fALS) is genetically linked disorder which majorly affects motor neurons in the primary motor cortex, corticospinal tracts, brainstem and spinal cord (Rowland *et al*., 2001; Wijesekera and Leigh, 2009). fALS is believed to be either caused by gain of a novel toxic function of the protein or by mutation of essential gene encoding cytosolic Cu/Zn binding superoxide dismutase SOD, a metalloprotein that catalyzes the conversion of toxic superoxide anion radical O^- into hydrogen peroxide (Shibata *et al*., 2000; Shibata, 2001; Howland *et al*., 2002; Valentine *et al*., 2005), and is responsible for 20% of the inherited form of the disease (Deng *et al*., 1993; Goto *et al*., 2000).

Menkes and Wilson diseases are genetic disorders associated with the dysregulation of copper homeostasis (Llanos and Mercer, 2002; Polishchuk *et al*., 2019; Hartwig *et al*., 2019; Weiskirchen *et al*., 2019). It is caused by mutation in the Cu-transporter ATPase7B gene that encodes a protein responsible for the biliary efflux of copper ions, (Boaru *et al*., 2014). ATPase7B are involved in sequestration of excess Cu into bile, and CSF for excretion. They also incorporate excess copper into ceruplasmin apoproteins to avoid systemic toxicity (Boaru *et al*., 2014; Merle *et al*., 2016). Genetically defected ATPase7B copper (Cu) efflux pump resulted in impaired Cu excretion and gradual accumulation in the body organs (liver, brain and kidney) (Merle *et al*., 2016); excessive copper overload in the brain and liver ultimately resulted into clinical manifestation such as liver cancer and severe psychiatric and neurologic symptoms which are without specific therapy (Weiskirchen *et al*., 2019). Menkes disease is a storage copper disorder characterized by high accumulation of copper in non-hepatic tissue but deficit in the liver, the brain and the blood, due to mutation in the Menke- ATP7A gene encoding for Cu-transport protein, failure in ATP7A is responsible for the resulting systemic brain copper deficiency and reduced copper-containing enzymes (cuproenzyme) activity (Vulpe *et al*., 1993; Tumer 2013). In Menkes disease various ATP7A mutation induced deficiency in transportation of copper across the placenta, blood-brain barrier and gastrointestinal tract (Waggoner 1999; Strausak *et al*., 2001; Weiskirchen *et al*., 2019).

Molecular biology, bio-distribution and roles of Zinc in the brain: Zinc is a trace nutrient needed for normal brain function and maturation; it is rated as the 2nd most abundant trace element in the brain (Sensi *et al*., 2009; Kambe *et al*., 2015). It is a structural part of many proteins and co-factor of more than 300 enzymes involved in numerous cellular signaling pathways and functions, Zinc is found to be irregularly distributed in the brain but highly concentrated in some regions such as amygdala, neocortex, olfactory bulb, hippocampus, gray matter of the cortex and neurons (Takeda, 2000; Frederickson *et al*., 2001). In the brain, zinc exists in two forms; the most abundant static form (Zn^{2+}), constitutes up to 90% of the neuronal zinc and is usually tightly bound with numerous metalloproteins. The static or chelatable form plays structural roles in protein as well as structural and catalytic roles in enzymes; the labile or ionic form, constituting up to 10% are widely distributed within the presynaptic vesicles of zinc-dependent glutamatergic neurons (Que *et al*., 2008). The glutamate and zinc-releasing neuronal system forms the cortical–limbic associational network that unites limbic and cerebrocortical functions, and contains a vast number of glutamate- and zinc-releasing neurons with their cell bodies scattered within the cerebral cortex and limbic structures (Frederickson *et al*., 2001; Kozlowski *et al*., 2012). During neuronal activity the co-release of labile zinc at micromolar level with some classical neurotransmitter from the glutamatergic presynaptic vesicle resulted into modulation of their postsynaptic activity, for example Zn^{2+} has an inhibitory effect on N-methyl-D-aspartate (NMDA), $GABA_A$ and glycine inotropic receptors but highly activate α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) glutamate receptors and a specific metabotropic Zn^{2+}-sensing receptor GPR39 (Smart *et al*., 2004; Besser *et al*., 2009). The neuroprotective effect of zinc at physiological concentration have been reported, however deficit concentrations exceeding the physiological level was found to be highly neurotoxic, effective zinc transportation and homeostatic control is therefore required, to avoid brain cytotoxicity and damage (Fukada *et al*., 2011; Szewczyk, 2013; Maywald *et al*., 2017). Zinc transportation is usually mediated by a numerous number of zinc homeostatic regulatory proteins distributed widely in the brain tissue, they are classified into three major groups; ZnTs belonging to a larger family of SLC30 is a membrane bound protein that excretes cystosolic zinc from the cell or influx zinc ions from extracellular space into intracellular compartment or organelles (Huang *et al*., 2013; Kambe *et al*., 2015; Portbury and Adlard, 2017). The second group are the ZIP a member of zinc and iron-regulatory transporter proteins (SLC39 family) that are widely distributed in the brain and responsible for trafficking of zinc from the extracellular space or from intracellular vesicles to the cytoplasm (Cousins *et al*., 2006; Kambe *et al*., 2015). About 10 and 14 variants of ZnTs and ZIP that control zinc transportation in mammalian system have been identified (Cousins *et al*., 2006). The third group is the Metallothioneins (MT), low molecular weight proteins that have affinity for zinc metals.

Zinc dyshomeostasis and neurodegenerative diseases: The role of zinc as essential nutrients for normal brain function is being increasingly appreciated; however, studies have shown that zinc overdose resulting from defective homeostasis is linked with the pathophysiology of many neuropsychiatric diseases (Szewczyk *et al*., 2013; Portbury and Adlard, 2017). Clinical conditions such as epilepsy and stroke are associated with excessive influx of zinc into neurons that ultimately leads to excitotoxic neuronal death. On the other hand, zinc deficiency has been implicated to affect neurogenesis which triggers neuronal apoptosis and consequently leads to learning and memory impairment (Frederickson *et al*., 2005; Szewczyk *et al*., 2013). Effective transportation and homeostatic control to maintain

intracellular and extracellular zinc concentration at nontoxic level is achieved by a number of regulating proteins which includes membrane Zn^{2+} transporters proteins (ZnT and Zip), and metallothioneins (Sensi *et al*., 2009; Szewczyk *et al*., 2013; Portbury and Adlard, 2017). Alteration in any of these proteins have been implicated in the etiology of ageing and age related neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Alzheimer's disease (AD) (Frederickson *et al*., 2004; Szewczyk *et al*., 2013; Portbury and Adlard, 2017). Recently, alteration in ZnT and MT was implicated in ageing and progression of Alzheimer's disease (AD) (Yu *et al*., 2001; Wong *et al*., 2013).

Alzheimer's disease (AD) is one of the age related neurodegenerative diseases associated with Zinc dyshomeostasis. It is characterized by extracellular deposition of amyloid plaques and intracellular accumulation of Neurofibrillary tangles (NFT), the pathological hallmarks of the disease (Bolognin *et al*., 2011; Portbury and Adlard, 2017). Defective zinc homeostasis may contribute to pathogenesis of AD by promoting protein aggregation and deposition (Szewczyk *et al*., 2013), several lines of evidences have shown that zinc upregulation to toxic concentration (above 300 nM) in extracellular fluids can promote plaque formation in AD (Bush *et al*., 1994; Ha *et al*., 2007; Noy *et al*., 2008). Amyloid precursor proteins (APP) are abundantly distributed in the plasma membrane of neurons, with their functions virtually unknown, however their proteolytic cleavage yield Aβ peptides. Zinc availability is essential for APP function and metabolism especially in the regulation of its formation and processing (Grilli *et al*., 1996; Lee *et al*., 2009), located on the APP ectodomain is the cysteine-rich regions that constitute the binding site for Zn ions (Bush *et al*., 1994b; Que *et al*., 2008). The processing of APP also depends on activities of enzymes secretases (α, β and γ). The common route by which APP is processed in the brain is through the cleavage by α-secretase, within the Aβ region, producing sAPP (soluble amyloid precursor peptide) (Ling *et al*., 2003). Aberrant binding of zinc ions to APP may prevent the activities of α- secretases, to yield non-amyloidogenic peptide (soluble amyloid precursor peptide); further reduction in α-secretase activity facilitates the formation of defective Aβ peptides by its β and γ secretases counterparts (Wilquet and De, 2004). Several studies have highlighted the contribution of zinc dyshomeostasis in amyloid pathology of AD; a research study using ZnT3 knockout mice showed reduced vulnerability toward amyloid plaque deposition (Ritchie *et al*., 2003), while administration of copper-zinc chelator clioquinol was shown to prevent or ameliorate amyloid plaque aggregation in an additional study (Regland *et al*., 2001; Ritchie *et al*., 2003). Oxidative stress has also been suggested as another risk factor contributing to AD pathology (Butterfield *et al*., 2001; Jomova *et al*., 2010) ROS such as NO and peroxynitrate or exogenous oxidant could mediate mobilization of zinc ion from extracellular metallothioneins (MT) and zinc transporters (ZnT) which further upregulate intracellular zinc concentration to toxic level that triggers general tissue damage such as mitochondrion dysfunction and further promote ROS formation (Aizenman *et al*., 2000; Burdette and Lippard, 2003; Sensi *et al*., 2003; Bossy-Wetzel *et al*., 2004). Sensi *et al*. (2008) indicate ROS mediated Z^{2+} upregulation in AD neurons expressing mutant APP, presenilin-1 (PS-1) and tau. Another pathway through which zinc could be implicated in AD pathology is through hyperphosphorylation of tau proteins to generate neurofibrilary tangles another hallmark of AD. Studies have shown that zinc at micromolar concentration can promote NFT formation (Bjorkdahl *et al*., 2005; Pei *et al*., 2006; Mo *et al*., 2009) and the use of appropriate zinc chelator can effectively block hyperphosphorylation of tau (Sun *et al*., 2012).

Amyotrophic lateral sclerosis (ALS) is a chronic disorder characterized by the selective death of motor neurons (Rowland and Shneider, 2001). It is both familiar and sporadic in nature with the sporadic form constituting about 90% of the cases and 10%, the familiar form (Portbury and Adlard, 2017). Mutation in the gene encoding copper, zinc superoxide dismutase (SOD1) is responsible for about 20% of the inherited form of the disease. Mounting evidences are available, suggesting the involvement of zinc dyshomeostasis in the pathogenesis of ALS (Frederickson *et al*., 2005). Studies have shown that mutation of SOD genes result in loss of zinc from its active site and toxic gain of function in motor neurons (Frederickson *et al*., 2005; Roberts *et al*., 2007). Loss of zinc from SOD mutants has been reported to triggers peroxynitrate induced protein nitration, a toxic reaction presumes to contribute to selective death of motor neurons in ALS disease (Crow *et al*., 1997). In another study, deficiency of zinc in SOD mutant was observed to promote nitric oxide induced motor neuron degeneration in ALS disease (Estevez *et al*., 1999). In addition to SOD mutation, several studies have reported the involvement of metallothioneins (MTs) and zinc transporters (ZnTs) in the progression of ALS; a recent study has discovered downregulation of ZnT3 and ZnT6 in the spinal cord of ALS patient (Kaneko *et al*., 2015). Another study also recorded reduced expression of zinc metallothionein RNAs in the spinal cord of patient with sporadic form of ALS (Ishigaki *et al*., 2002; Hozumi *et al*., 2008b). In a study using a mutant SOD transgenic mouse, deficiency of MT1, MT2 or MT3 was shown to exacerbate ALS expression (Nagano *et al*., 2001; Puttaparthi *et al*., 2003). All these together have suggested the possible involvement of zinc dyshomeostasis in ALS disease pathogenesis.

Parkinson's disease is a chronic progressive neurological disease associated with defective motor system. Clinical symptoms develop gradually over time and include tremor, rigidity, postural instability, paucity of movement, behavioural and learning deficit and dementia which is associated with the late phase of the disease. Zinc deficiency has been detected in patients presenting with PD and the efficacy of appropriate zinc supplementation to reverse zinc shortage in animal model of PD has been demonstrated (Forsleff *et al*., 1999; Brewer *et al*., 2010). In addition, accumulation of zinc in specific brain regions associated with PD pathology such as substantia nigra, lateral putamen and caudate nucleus in patients expressing PD have been demonstrated (Dexter *et al*., 1991). Also Drosophila parkin mutants, a PD disease model expressing human PD phenotype with deficits such as, severely shorten life span and locomotor defect due to degenerated flight muscles were restored back to normal through zinc supplementation (Saini *et al*., 2010). Together all these

evidences have indicated the contributory role of zinc dyshomeostasis in the pathogenesis of PD.

Molecular biology, bio-distribution and roles of Iron in the brain: Iron is the most prevalent transition metal in the brain (Que *et al*., 2008; Beard *et al*., 2009). The brain being the organ with the highest rate of cellular metabolism requires iron as a major constituent of enzymes to carry out the process of oxygen transportation and metabolism (Cammack *et al*., 1990). Within the brain, iron is homogeneously distributed with the highest concentration found in the basal ganglia, thus suggesting basal ganglia as the major iron storage and distribution in the brain (Beard *et al*., 2009; Anderson and Erikson, 2011). In a cohort study conducted by Aoki et al. (1989), magnetic resonance imaging (MRI) of the brains of children and adolescents confirmed the substantia nigra, caudate nucleus, globus pallidus and putamen as the brain regions with highest iron concentration while the concentration remained relatively low in the cerebellum and cortex. Studies have also confirmed white matter as the major site of iron concentration within the brain with maximum influx occurring during rapid brain growth at the peak of myelinogenesis (Taylor and Morgan, 1990). Iron is also widely distributed in all cell types of the CNS including, microglia, oligodendrocytes, astrocytes and neurons, with oligodendrocytes having the highest concentration (Que *et al*., 2008). In the biological tissue, iron exists in two common oxidative states namely: +2 (ferrous) and +3 (ferric) oxidation states, other higher redox states are generated through several enzymatic catalytic cycles occurring in the cell (Que *et al*., 2008). With regard to the brain, iron participates in several neurological activities which includes involvement in the function and biosynthesis of neurotransmitters (Youdim, 1990; Loeffler *et al*., 1995), myelin formation (Beard *et al*., 1993; Que *et al*., 2008; Anderson and Erikson, 2011), cofactor for a variety of metalloenzymes and an essential role in neuronal function (Beard *et al*., 1993; Anderson and Erikson, 2011). Despite high need of iron in the brain, only a small quantity, about 5-10% is expected to be used for iron-dependent processes (Sigel *et al*., 2006), while the large portion of the unused (about 33-90%) is stored in ferritin. Due to abundance of iron in the brain and its high redox activities, tight homeostatic regulation is required to prevent oxidative damage to the cells by unlimited iron dependent Fenton reactions (Beard *et al*., 1993; Que *et al*., 2008). To avoid iron toxicity and deficiency, an elegant homeostatic system comprising transferrin, transferrin receptors, and ferritin are in place to ensure effective storage and well-timed release of iron to the cells. The mechanism of transportation of iron in the CNS is not fully comprehended, however both the transferrin-mediated and axoplasmic flow have been described as the most common pathway of iron into the neurons and grey matter (Dwork *et al*., 1990).

Iron initially absorbed from the gastrointestinal tract is integrated into ferritin and plasma transferrin for systemic storage and transportation. Influx of iron into the brain across the blood-brain barrier (BBB) is mediated through a general pathways involving transferrin (Tf), the transferrin receptor (TfR) localized on brain endothelial cells. Iron in oxidized state is incorporated into transferrin (Tf) and bound with transferrin receptor (TfR) to form TfFe2-TfR complex that is translocated across the BBB into the brain. Once in the brain, the influx of iron into the cell occurs through a few major pathways the choice of which is dependent on the cell type and brain region involved. Within the brain the resulting TfFe2-TfR complex is endocytosed into the cell through clathrin-coated endosomes, which undergoes acidification to liberate Fe3+ from transferrin, Fe3+ is further reduced to Fe2+ by an unknown mechanism and subsequently transported into mitochondria through mitoferrin by a mechanism mediated by divalent metal transporter-1 (DMT1), a mitochondrial iron transporter abundantly expressed in astrocytes. Within the mitochondrion, transported Fe^{2+} is utilized for the synthesis of heme and iron-sulfur clusters, while the remaining left over is incorporated into ferritin for storage. Other alternative pathway employed for iron uptake into brain cells include ferritin and ferritin receptors (FtR) (occurring in white matter in oligodendrocytes (Hulet *et al*., 1999; Hulet *et al*., 2000) the transferrin / transferrin receptor pathway (Hulet *et al*., 2000), lactoferrin mediated pathway which involves importing of iron into neuromelanin cells (Zecca *et al*., 2004) and divalent metal transporter-1 (DMT1) abundantly expressed in astrocytes.

Intracellularly iron homeostatic regulation is controlled through translational level with iron responsive elements (IREs) and iron regulatory proteins (IRPs) (Hentze and Kühn, 1996; Eisenstein, 2000). The nucleotide sequences of IREs are fully expressed on mRNA. The expression and the activities of TfR, Ft, and other iron metabolic regulatory proteins is controlled by IRP/IRE interactions. During intracellular iron depletion, the cell put up compensatory action and initiate: binding of IREs of TfR mRNA and Ft mRNA to IRPs to boost intracellular iron level by preventing iron degradation and reduce the population of iron stores in ferritin, while in the case of excess intracellular iron, conformational alteration in IRPs is initiated to prevent IRE binding and increase ferritin level required for excess iron sequestration or initiate TfR mRNA degradation to reduce subsequent iron influx into the cell. Other iron regulatory proteins whose expression is regulated by the IRP/IRE system include DMT1 and ferroportin-1 (FPN1) (Abbou and Haile, 2000; Dunn *et al*., 2007). FPN1 is an iron regulatory protein that controls efflux of iron from the cell. It is abundantly expressed in the brain (Abbou and Haile, 2000; Donovan *et al*., 2000; Burdo *et al*., 2001) and its over expression has been reported to result in intracellular iron deficiency (Abbou and Haile, 2000).

Iron dyshomeostasis and neurodegenerative diseases: Iron is an indispensable metal that is essential for several life processes and cellular functions, its level rises with age. Aberrant iron accumulation in the brain due to mis-regulated homeostasis is a characteristic of several neurological disorder such as Alzheimer's disease (AD) (Que *et al*., 2008; Li and Reichmann, 2016; Bjørklund *et al*., 2019). As a redox active element, iron is involved in several cellular activities, which if unregulated, may result in oxidative damage to macromolecule and cellular dysfunction (Belaidi *et al*., 2016; Eid *et al* 2017; Masaldan *et al*., 2018), evidences are available in the literature which show that abnormal iron accumulation in the brain promote protein aggregation through Fenton- type oxidation of

macromolecules (Zecca *et al*., 2004; Madsen and Gitlin, 2007; Que *et al*., 2008).

Parkinson's disease (PD) is a debilitating disease of the brain characterized by the accumulation of α-synuclein and degeneration of substantia nigra (SN) neurons (Que *et al*., 2008; Li and Reichmann, 2016; Costa-Mallen *et al*., 2017; Bjørklund et al., 2019). It affects about 2% of human population globally especially in ages well above 65 years (De Rijk *et al*., 1997). Clinical symptoms for PD include tremor, muscle rigidity, bradykinesia (slow movements), and deterioration of cognitive functions (Rinne *et al*., 2000). Both the genetic and the environmental factor have been strongly implicated in the etiology of PD, and several research studies have highlighted the role of environmental factors in the disease pathogenesis such as induction of parkinsonism in rats by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) exposure. Exposure to different environmental toxicant, exposure to toxic metals (Pb, V, Hg) as well as the essential trace element dyshomeostasis (iron, copper, manganese, zinc) have also contributed to the development of PD (Bjørklund *et al*., 2019). Evidences are available supporting impaired iron homeostasis as a function of elevated iron accumulation observed in PD, studies done on post mortem brain iron content using MRI and LA-ICP-MS bio-imaging revealed massive accumulation of iron in the SN of brain tissues obtained from various forms of PD (Li and Reichmann, 2016; Costa-Mallen *et al*., 2017; Bjørklund *et al*., 2019). *In-vivo* measurements of brain iron by magnetic resonance imaging (MRI) also confirmed the presence of increased iron deposition in the SN (Wallis *et al*., 2008; Rossi *et al*., 2013). Another study also detected reduction in the level of ferritin and neuromelanin (iron binding proteins) in the SN of PD individuals when compared with normal individuals (Connor *et al*., 1995; Zecca *et al*., 2002; Que *et al*., 2008). Further studies also found abnormally elevated iron accumulation in oligodendrocytes, astrocytes, microglia, and pigmented neurons and in the rim of Lewy bodies in PD patients. All these evidences confirm the association of disrupted iron homeostasis with the pathogenesis of PD. So far, the primary mechanism that is responsible for excessive iron accumulation in PD is insufficiently defined, however, disrupted BBB, α-synuclein aggregation, oxidative stress, mitochondrial dysfunction and iron dyshomeostasis have been suggested to be involved (Que *et al*., 2008; Li and Reichmann, 2016). Moreover, these factors together with iron accumulation constitute the process leading to neuro-inflammation and neuro-degeneration. In PD pathology the vicious cycle of mitochondrial injury, oxidative stress, iron dyshomeostasis and neuro-inflammation are closely interrelated with several other factors in PD (Li and Reichmann, 2016).

Interactions between excess Fe ions and various molecules in the brain are implicated in the pathology of PD. For example, the interaction of electrophilic ferric iron with dopamine in SN could be a major factor associated with neurotoxicity and neurodegeneration in PD (Que *et al*., 2008; Li and Reichmann, 2016). In the presence of elevated ferric iron, dopamine interacts with molecular oxygen to yield quinones and free oxygen radicals (ROS) which appeared to be toxic to SN cells (Zucca *et al*., 2014). Dopamine can be polymerized and oxidized directly to form a characteristic coloured neuromelanin or its other multiple toxic metabolites (Miyazaki *et al*., 2008; Zucca *et al*., 2014). The free reactive radical (ROS) generated promote protein carbonylation which subsequently triggers α-synuclein aggregation and Lewy body formation (Munch *et al*., 2000). Excess iron in SN may directly interact with α-synuclein and catalyse its aggregation into α-synuclein oligomer, while α-synuclein in excess may induce massive iron accumulation, excessive aggregated α-synuclein generated further exacerbate oxidative stress, mitochondrion impairment and iron dyshomeostasis (Devi *et al*., 2008; Que *et al*., 2008; Davies *et al*., 2011; Funke *et al*., 2013). Another example is the interaction of excess ferric ion with neuromelanin pigment, the end product of dopamine metabolism to form neuromelanin iron complex (NM-Fe^{3+}), which is seen in the degenerating neurons of the SN of PD patients (Jellinger *et al*., 1992; Zecca *et al*., 1996). Fe^{3+} stored in degenerating neurons of SN is released into extracellular environment where it interacts with microglia and trigger the release of neurotoxin that mediate neuro-inflammatory cascade. The release of NM-Fe^{3+} complex from degenerating neurons further triggers a cascade of events leading to neuronal death through microglial activation (Wilms *et al*., 2003; Zucca *et al*., 2014). Disrupted iron homeostasis seen in PD has also been attributed to mis-regulation of normal brain iron regulatory system, studies have shown changes in brain iron level in different forms of PD while the serum iron remain largely unaltered (Logroscino *et al*., 1997; Tórsdóttir *et al*., 1999; Costa-Mallen *et al*., 2015; Costa-Mallen *et al*., 2017). In addition, increase in the level of ferritin iron saturation as well as the level of lactoferrin and lactoferrin receptors, which are the potential source of iron storage in the brain, were detected in SN of PD patient in comparison with normal individual (Faucheux *et al*., 1995; Leveugle *et al*., 1996).

Alzheimer's disease (AD) is another degenerative disease of the brain strongly linked with dysfunctional iron homeostasis, and evidences are available showing association of aberrant iron accumulation with AD pathology (Connor *et al*., 1992; Smith *et al*., 2007; Bulk *et al*., 2018; Everett *et al*., 2018). Amyloid plaque and neurofibrillary tangles (NFT) which are pathology hallmarks of the disease were also found with high iron deposits (Connor *et al*., 1992). Studies on postmortem brain from AD patients revealed high iron accumulation especially in the hippocampus (Connor *et al*., 1992; Deibel *et al*., 1996). In addition to this, iron may also directly trigger β amyloid formation and aggregation through several pathway including oxidative stress which is built up in the cell by the activities of other redox metals (Zn, Cu) and thus promote oxidation and subsequent crosslinking of β amyloid species (Huang *et al*., 1999; Bush *et al*., 2003; Que *et al*., 2008; Jomova *et al*., 2010). Alteration of iron regulatory proteins involved in the removal of excess iron from the brain to prevent iron overload has also been implicated in promoting iron dyshomeostasis in AD (Connor *et al*., 1993; Guerreiro *et al*., 2015; Wan *et al*., 2011). This is further confirmed in an analysis on iron transportation and storage which revealed reduced iron mobilization in AD compared to normal individuals (Connor, 2018), the level of Divalent Metal Transporter 1 (DMT1) an iron importer is increased, while the level of ferroportin 1 (FPN1) and Ceruloplasmin (CP) cellular iron exporters were relatively low in AD brain (Connor *et al*., 1993; Wan *et al*., 2011; Guerreiro *et al*.,

2015). Intracellular iron distribution and accumulation may also affect the formation and processing of amyloid precursor protein (APP). The expression of iron responsive element (IRE) identified on the 5' end of APP mRNA, is suggestive of the role of iron in the regulation of APP formation and processing (Connor *et al*., 1993; Que *et al*., 2008; Ward *et al*., 2014), for example excess iron accumulation, has been reported to promote APP formation (Bodovitz *et al*., 1995). In addition, iron responsive element (IRE) on APP mRNA is involved in the translational processing of amyloid precursor protein (APP). Excess iron overload may trigger aberrant binding of iron responsive element and subsequently promote β amyloids formation and aggregation (Bodovitz *et al*., 1995; Rogers *et al*., 2002; Crichton *et al*., 2008; Caldwell *et al*., 2013). The common route of APP processing is non amyloidogenic pathway that involves proteolytic cleavage of APP by α and γ secretases to yield a neuroprotective extracellular soluble A β peptide, (Ling *et al*., 2003) and prevent the formation of β-amyloids however, in AD, APP cleavage by β and γ secreatases produced amyloidogenic fragments of β amyloid which subsequently aggregate to form plaque (Bodovitz et al., 1995; Silvestri. and Camaschella, 2008; Ward *et al*., 2014). Furthermore, the processing of APP is regulated by iron through furin (Hwang et al., 2006). Furin is a calcium-dependent proconvertase, produced in the endoplasmic reticulum (ER) and largely involves in promoting α-secretases cleavage of amyloid protein precursor (APP) to yield the sAPP neuroprotective form. However, excessive iron accumulation has been reported to decrease furin expression and enhanced β amyloids accumulation and aggregation in the brain (Bodovitz *et al*., 1995; Silvestri and Camaschella, 2008). Additionally, accumulated iron in neurofibrillary tangles (NFT) can mediate tau phosphorylation and aggregation (Yamamoto *et al*., 2002; Lovell *et al*., 2004; Chan and Shea, 2006; Castellani *et al*., 2012).

Amyotrophic lateral sclerosis (ALS) is another neurodegenerative disorder associated with aberrant iron trafficking and distribution, it is a debilitating progressive CNS disease characterized by gradual degeneration of motor neurons in the cerebral cortex, brain stem and the spinal cord. ALS affects mostly growing population with global incidence of up to 1/100,000 (Carrì *et al*., 2003; Goodall *et al*., 2008). ALS is categorized into familiar and the sporadic form, but both with similar clinical symptoms and pathological process (Portbury and Adlard, 2017; Sheykhansari *et al*., 2018). Iron as a cofactor is essential for various enzymatic catalyzed reactions in the brain (Hametner *et al*., 2013). A balanced brain iron homeostasis is essential to prevent deleterious effect on cell functions due to high accumulation, mis-regulation of iron may promote neuro-inflammation, mitochondrial impairment and oxidative stress (Carrì *et al*., 2003; Goodall *et al*., 2008; Hadzhieva *et al*., 2013; Tokuda *et al*., 2016), although the involvement of iron in the etiology of ALS is unclearly defined, however, redox capacity of iron to generate ROS has been proposed as one of the factors that initiate ALS pathology (Hametner *et al*., 2013). Moreover, mutation of the gene encoding copper-zinc dismutase (SOD), that constitutes about 20% of the familiar form of the disease has also been implicated in the pathogenesis of the disease (Yoshida *et al*., 2010). In normal conditions SOD is responsible for the catalytic conversion of toxic superoxide anion radical O^- into hydrogen peroxide through dismutation reactions (Shibata *et al*., 2000; Shibata, 2001; Howland *et al*., 2002;Valentine *et al*., 2005). SOD impairment result to reduction in dismutation activities and toxic accumulation of superoxide radicals that subsequently generates oxidative stress. Studies have demonstrated the ability of excess superoxide radicals O^- to remove iron from iron bearing proteins such as ferritin (Jeong *et al*., 2009; Jomova *et al*., 2010), the extracted iron is further incorporated into Fenton and Haber Weiss reactions to generate more free radicals such as OH^- and O_2^- which are toxic to brain cells (Wang *et al*., 2004; Jeong *et al*., 2009; Jomova *et al*., 2011). Furthermore, mutation of the genes controlling appropriate cellular iron homeostasis has been proposed as one of the predisposing factors to ALS (Zamboni *et al*., 2005). Mutation in Hfe with the associated hemochromatosis and decrease in Cu/Zn SOD1 activities have been implicated in ALS (Zamboni *et al*., 2005; Gemmati *et al*., 2006; Singh *et al*., 2010; Gemmati *et al*., 2012). There are several indications showing the involvement of aberrant iron homeostasis in the pathophysiology of ALS; assessment of iron state levels in ALS patient revealed high ferritin level associated with worsened muscle degeneration and shortened patients' survival (Goodall *et al*., 2008; Veyrat-Durebex *et al*., 2014; Nadjar, *et al*., 2012; Ikeda *et al*., 2012), abnormal iron accumulation has also been detected in the spinal cord of ALS patients (Yasui *et al*., 1993; Ince *et al*., 1994; Kasarskis *et al*., 1995; Markesbery *et al*., 1995), Furthermore, high iron concentration has been reported in the CSF of ALS patients (Hozumi et al., 2011). Using animal model of ALS, motor neuron degeneration due to aberrant iron deposition was reported in SOD transgenic mice (Winkler *et al*., 2014), The use of appriopriate iron chelator therapy to alleviate aberrant iron accumulation in a G93A-SOD1 murine model of ALS, resulted in neuroprotection and long life survival (Kupershmidt *et al*., 2009; Wang *et al*., 2011).

Multiple Sclerosis (MS) is a type of demyelinating CNS disorder associated with mis-regulated iron homeostasis. It is characterized by general disruption of iron regulatory mechanism controlled by oligodendrocytes. Oligodendrocytes are responsible for maintenance and myelin production, alteration in this regulatory process could lead to aberrant iron accumulation within the cell that triggers oxidative damage (Beard *et al*., 1993; Sheykhansari *et al*., 2018). Aberrant iron accumulation in the brain and associated oxidative stress is a component of MS pathology (Ferreira *et al*., 2017; Iranmanesh *et al*., 2013; Hametner *et al*., 2013). Studies have reported alteration in the normal cellular pattern of iron and transferrin due to cellular iron dyshomeostasis (Craelius *et al*., 1982; LeVine *et al*., 1997). Age related increase in iron accumulation was also seen in the white matter of MS subject (Hametner *et al*., 2013), Moreover, extensive glial degeneration including iron rich oligodendrocytes and myelin has been reported in MS lesion, the free iron liberated further exacerbates oxidative stress and leads to neurodegeneration (Uttara et al., 2009; Khare *et al*., 2014; Raymond *et al*., 2017), on the other hand, reduced iron accumulation with upregulated oxidative stress has been found in MS disease (Visconti *et al*., 2005; Crichton *et al*., 2008). Several studies have used experimental animal models to investigate the

pathophysiology of MS and their report have been documented, destructive blood brain barrier with excessive iron accumulation have been reported in animal model of experimental allergic encephalomyelitis (EAE) which is one of the clinical condition of MS (Forge *et al.*, 1998). In addition to this, endogenous administration of appropriate antioxidant has proven to reverse the clinical and pathological symptoms linked with experimental autoimmune neuritis in animal model of autoimmune demyelination (Hartung *et al*., 1988).

Methods of metal detection in the brain: Elemental or molecular mapping in biological tissue is of growing interest in different areas of biomedical research (Sussulini and Berker, 2015; Wu *et al*., 2011), in brain research it is mostly used for the detection of spatial element distribution and quantification in the brain. It is relevant in the study of neurodegenerative diseases (Hutchinson *et al*., 2005; Hare *et al*., 2009; Wang *et al*., 2010; Becker *et al*., 2010; Hare *et al*., 2010; Hare *et al*., 2014), proteomics (Wind *et al*., 2003; Becker *et al*., 2004; Feng *et al*., 2015) as well as aging and oncogenic research (Becker, 2005; Zoriy *et al*., 2006; Salber *et al*., 2007; Seuma *et al*., 2008; Fu *et al*., 2015). It also detects changes in metal distribution, homeostasis and contents within brain anatomical structures (Hare *et al*., 2017; Becker *et al*., 2012; Hare *et al*., 2012).

Presently, there are several analytical techniques available for detecting metals in biological system for the purpose of medical investigations, these include non-photometric techniques such as histochemical techniques (Wang *et al*., 2010; Hare *et al*., 2016), fluorescent method (Majumdar *et al*., 2012; Hare *et al*., 2015) and autoradiography (Wang *et al*., 2010; Becker and Salber, 2010); photometric methods such as flame photometry (Meloni *et al*., 2007; Elseweidy et al., 2008) and Atomic absorption spectroscopy (AAS) (Andrási *et al*., 1999; Grochowski *et al*., 2019) other surface analytical techniques include; X-ray spectroscopic techniques (e.g. X-ray photoelectron spectroscopy (XPS)(Briggs and Grant, 2012), scanning electron microscopy with energy dispersive X-ray analysis (SEM-EDX) (Lohrke *et al*., 2017;Pánik *et al*., 2018), proton-induced X-ray emission (PIXE) (Carmona *et al*., 2008; Nakazato *et al*., 2008), and imaging mass spectrometry such as secondary ion mass spectrometry (SIMS) (Chandra *et al*., 2016), and MALDI-MS (matrix assisted laser desorption/ionization mass spectrometry) (Seeley *et al*., 2011). However, these methods have a number of limitations such as non-multi-elemental capability, poor detection limits, poor lateral resolution, lower sensitivity for trace analysis and non-availability of quantification procedure when compared with spectrometry based method such as Laser ablation - inductively coupled plasma - mass spectrometry (LA-ICP-MS) a modern powerful micro-analytical technique with relatively lower matrix effect, high sensitivity, low detection limit and easy quantification and preparative procedure (Hattendorf *et al*., 2003; Hare *et al*., 2010). LA-ICP-MS imaging is a modern method applicable for measuring most of the biological relevant metals and their tissue concentration. The major goal of this review is to highlight the role of some essential metals in the brain and recent applications of LA-ICP-MS imaging in neuroscience, including brain diseases.

Mechanism of LA-ICP-MS: LA-ICP-MS is the most sensitive and widely used technique for *in situ* analysis of metals in cross sections of biological tissue (Becker and Jakubowski, 2009). It is of significant diagnostic importance in brain research, where it allows for the detection of absolute concentration and micro spatial and regional distribution of elements (metals, non-metals and metalloids) within the affected brain tissue. It is also essential for measuring the relative concentration of element within a large number of metals and metalloids (Mokgalaka and Gardea-Torresdey, 2006). A classical Laser ablation system is made up of three key components namely; (A) A high energy ultraviolet laser beam, (B) easily adjustable ablation stage, and (C) a detection system comprising of inductively coupled plasma mass spectrophotometer (ICP-MS). The detection system is of varied types depending on the type of mass analyzer used. However, the most widely used is the quadrupole (Q) based type consisting of a quadrupole mass filter (Potter 2008) with exceptional quality of high sensitivity and less design complexity when compared with the other types such as time of flight (TOF) and double – focusing sector-field (SF). A laser stage consists of a lens, an ablation chamber or cell, and adjustable platform to which is attached an optical microscope equipped with a charged coupled device (CCD) camera from where the cell can be effectively monitored and the material of interest concisely visualized. The mechanism of LA-ICP-MS involves the use of quadrupole (Q) or double-focusing sector field (SF) based mass spectrophotometer coupled with ultraviolet laser beam to vaporize materials from the surface of biological sample. A thin sliced section mounted on a glass slide is obtained and fixed into a sample holder located in a closed ablation chamber or cell. A high energy laser beam is focused unto the area of interest within the section, to generate ablated particulates which are transported in a continuous flow of inert carrier gas such as argon or helium into the inductively coupled plasma (ICP). With the extremely high thermal temperature and pressure of the ICP, the particles, through electromagnetic induction dissociated into ions, which was further extracted and directed into a high vacuum mass analyzer, from where ions are separated into different ones based on their mass - to - charge ratios (m/z). Finally, highly sensitive detection and quantification of the transmitted ions take place (Plates.1a and1b), (Durrant and Ward 2005; Mokgalaka and Gardea-Torresdey, 2006; Weiskirchen *et al*., 2019).

LA-ICP-MS Bio-imaging of normal brain : LA-ICP-MS metal bio-imaging is a unique technique that has provided a new insight in the study of several pathophysiological processes in brain research. Hare *et al*. (2016) used LA-ICP-MS bio-imaging to produce a three-dimensional atlas showing the distribution of Zinc (Zn), Copper (Cu) and Iron (Fe) by using aligned quantified images of these metals obtained from cerebrum and brainstem sections of a mouse brain. This atlas has thus contributed to the better understanding of these essential elements in the brain and further clarifies their function in neurobiology. Becker *et al*. (2005) also employed LA-ICP-MS imaging to produce spatial distribution of trace elements such as Zn and Cu in different layers of human hippocampus.

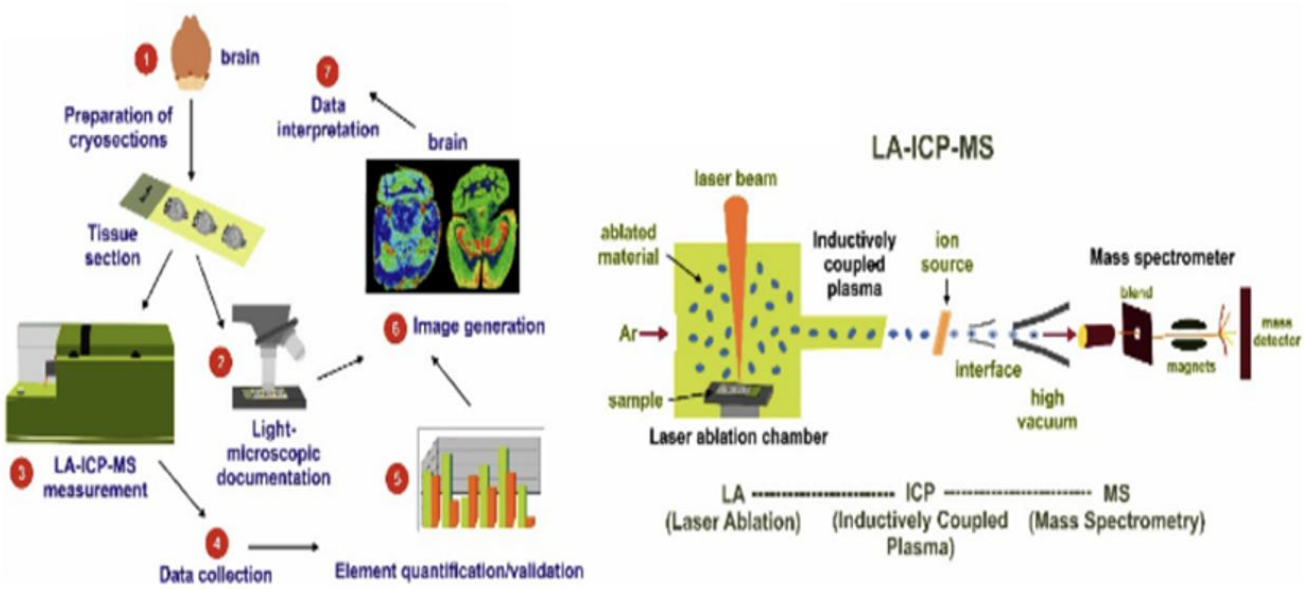

Plate 1 (a and b): Experimental workflow of bioimaging of elements in a brain section leading to a quantitative image: (Adapted from Weiskirchen *et al*., 2019).

In several research experiments, LA-ICP-MS techniques have been adopted to produce standard analytical methods required for data calibration, mostly used in two-dimensional mapping and quantitative assessment of essential trace elements and metals in sections of brain tissue (Becker *et al*., 2003; Becker *et al*., 2005; Zoriy *et al*., 2006; Pickhardt *et al*., 2006). In another follow up study, LA-ICP-MS was employed to reproduce series of quantitative data and images of Zn, Cu and Pb distribution in a numbers of measurements on adjacent sections and several other representative brain regions such as insula, central cortex and hippocampus from rat brain (Dobrowolska *et al*., 2008)

LA-ICP-MS Bio-imaging in Aging study: Metal dyshomeostasis or mis-regulation play essential role in brain aging and neurodegenerative disorders, in the context of ageing, complimentary potential of LA-ICP-MS with immunohistochemistry and autoradiography was used to study age related changes in copper distribution and the activities of cytoplasmic Cu-SOD in the brain of young (2-months), (7-9 months) and aged mouse (14-months). The analysis showed a progressive depletion of copper concentration, noticeable in the striatum and ventral cortex in the aged brain relative to the young brains, the regions with reduced Cu concentration also corresponded to the brain regions with reduced cytoplasmic Cu-SOD contents in the aged mouse. They concluded that decreased Cu content and SOD level may contribute to vulnerability of the aged brain to oxidative damage and neurodegeneration (Wang et al., 2010). In an additional study LA-ICP-MS bioimaging was employed to study the relative distribution of metals (Zn and Cu) in the brain of young (2-months) and old (14-months) mice. The analysis revealed massive accumulation of iron in the substantial nigra, the thalamus and the hippocampal CA 1 region of the older brain when compared to the young brain, while the zinc concentration largely appeared constant. This indicates that cerebral iron accumulation with age may contribute to age related neurodegeneration since iron catalyzes the formation of ROS; zinc enrichment observed in hippocampal CA3 of the young mice indicated the role of zinc in synaptic transmission (Becker *et al*., 2010).

Application of LA-ICP-MS in detecting brain metal dyshomeostasis

Bioavailability of essential trace metal is needed for normal brain function. However, abnormal distribution can influence several biochemical pathways in different fields of metabolism and cause characteristic neurological diseases (Hare *et al*., 2017). Involvement of metals in several neurophysiological and neuropathological events has prompted the study of their bio-distribution In most neurodegenerative disorders, the disease process is strongly linked with abnormal metal accumulations, with several evidences correlating aberrant metal deposition and neurodegeneration (Frederickson et al., 2004; Szewczyk *et al*., 2013; Portbury and Adlard, 2017). Metal overload or deficiency sometimes may result from usage of metal containing drugs such as lithium compounds or cisplatin as a cytostatic drug against some neurological conditions like depression and epilepsy or depletion of metal from therapy to reduce oxidative stress (e.g. in brain after stroke). Quantitative metal bio-imaging is therefore essential for the determination of proper brain function and prevention of certain neurological diseases. This field has therefore provided a unique understanding of brain biochemical architecture linking neuroanatomy, metal mediated processes, changes in metal homeostasis and disease formation (Hare *et al*., 2010; Grochowski *et al*., 2019).

In a study conducted by Boaru *et al*. (2014), (Plate .2) LA-ICP-MS was used to investigate cerebral metal accumulation in the brains of 10-24 months old ATP7B deficient mice, (animal model of experimental Wilson disease) and age matched wild types. Brain sections obtained from the respective animals were comparatively assessed for the multi-elemental distribution of Na, P, Mn, Fe, Cu and Zn. The analysis revealed insignificant difference in the level of Na and P, however, there was an

increased accumulation of Cu throughout the brain parenchyma but reduced deposition in the periventricular region, noticeable from 11 months of age. Also observed was upregulation of Zn concentration in brain regions with copper enrichment while Fe and Mn concentration remained relatively constant. Excessive Cu accumulation in specialized brain area of the ATP7B null mice is indicative of cognitive impairment and the deposition may be due to differential regional affinity to Cu within the brain. The reduced copper accumulation in the perivascular region is in line with the view that the perivascular region is an efflux compartment with low copper contents due to active transportation of Cu into CSF.

In another experiment, Matusch *et al*. (2010), studied multi-elemental distribution in the brain of mice sub-chronically intoxicated with MPTTP as a model for Parkinson's disease, 2 h, 7 d and 28 d post treatments, respective animals were sacrificed and subjected to investigation. The result showed massive depletion of Cu at the periventricular zone and fascia dentate at 2 h, 7 d. A recovery effect was observed at 28d post injury, indicated by increase in Cu concentration in affected brain regions. Also observed was an increase in Fe concentration in interpeduncular nucleus, but not in the substantia nigra, while the level of Zn and Mn were similar to that of the control. However, the level of C, P, and S. remained relatively unchanged at all the time points of treatment. This result confirmed the differential Cu and Fe regulation as well as their roles in Parkinson's disease.

Uerling *et al*. (2018) used LA-ICP-MS imaging to detect beneficial effect of adeno-associated virus (AAV) gene therapy to correct Cu dyshomeostasis using a mouse model of Wilson' disease (ATP7B transgenic mouse and untreated litter mates). After 14 weeks of treatment with AAV-AAT-co-miATP7B therapeutic agent, animals were sacrificed and together with the untreated litter mates were subjected to investigation. The result revealed marked reduction in the level of Cu in some brain regions including the cerebellar cortex, cerebellar white tract, corpus callosum, 3rd and 4th ventricles, and basal ganglia of the treated transgenic mice when compared with the untreated litter mates.

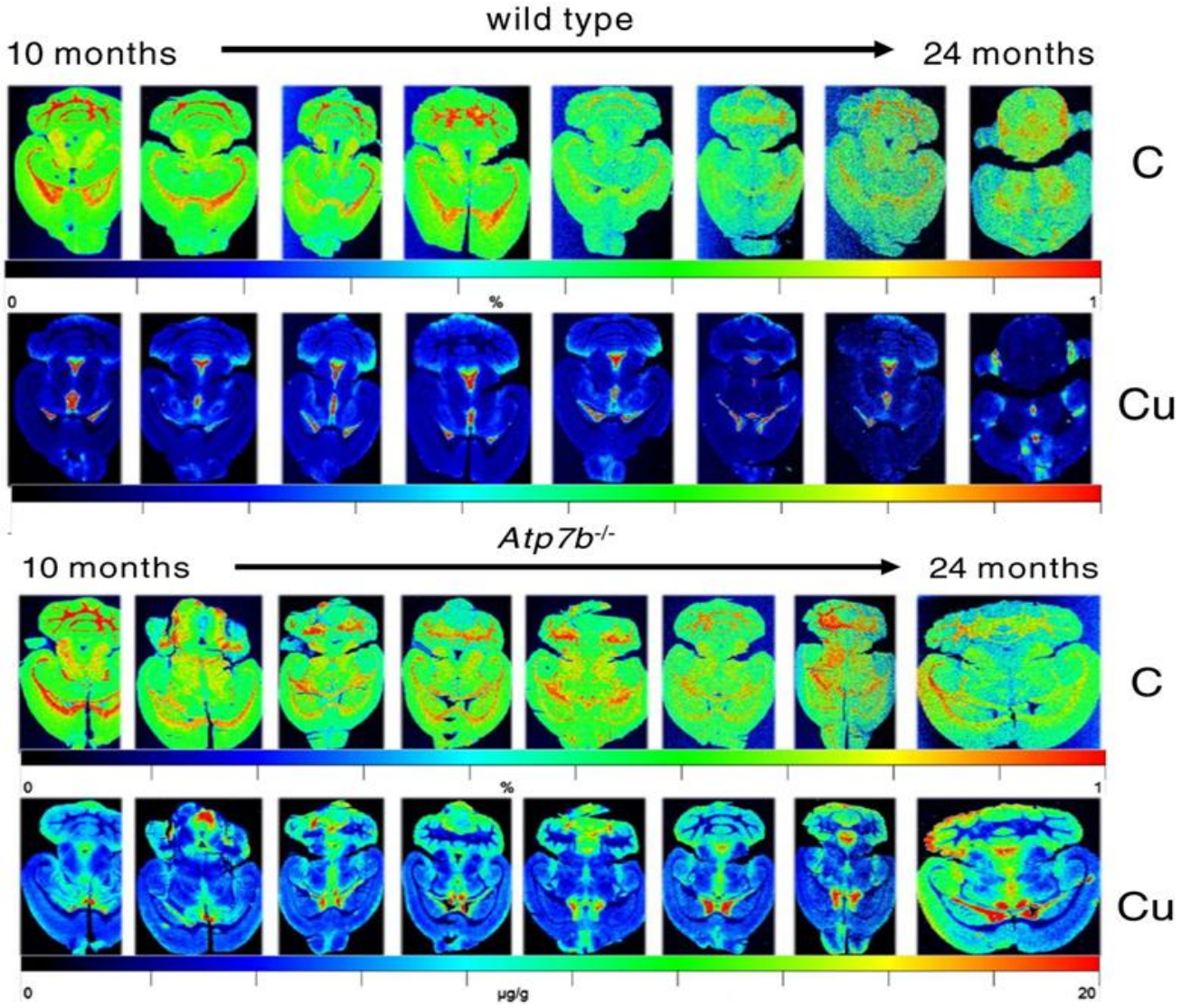

Plate 2:
Comparative assessment of Age-dependent cerebral copper accumulation (10-24 months) in Atp7b deficient mice and age-matched wild type as demonstrated by LA-ICP-MS. (Boaru *et al*., 2014).

Also observed was an unaltered content and distribution of other elements such as the Fe, Zn, Mn and Mg. The study suggested AAV gene therapy as an effective therapy for the treatment of cerebral copper overload in Wilson disease

A further advance of application of LA-ICP-MS in the study of neurodegenerative diseases was seen in a research conducted by Hutchinson *et al.* (2005) who employed the combination of LA-ICP-MS imaging and metal immunolabelling for the detection of β-amyloid distribution in the brain of aged TASTPM transgenic mice (model for Alzheimer) previously tagged with metal (europium) labeled secondary antibodies. The study detected a correlation between the β-amyloid deposits and the trace element content in the brain, this has provided a new insight into the study of metal tagged antibodies for imaging protein distribution one of such is seen in a study to detect neuronal susceptibility to pathological changes observed in Parkinsonism. In the study the effect of 6-hydroxydopamine (6-OHDA) neurotoxin on iron level and dopamine distribution is investigated in a wild-type C57BL/6 mouse by using antibodies previously tagged with gold particles (Hare *et al*., 2014; Ayton *et al.*, 2015).

Another innovative application of LA-ICP-MS is employed in the identification of metals complexed with proteins (metalloproteins) such as phosphoproteins. The combination of LA-ICP-MS technique with proteome analysis through advanced biomolecular mass spectrometry techniques such as electrospray ionization mass spectrometry (ESI-MS) or Matrix-assisted laser desorption/ionization- mass spectrometry (MALDI) has provided a new opportunity for the identification of the detailed structure of metals bound to proteins (metal protein complexes) as well as detection of protein modification associated with several pathophysiological processes (Becker *et al*., 2010). An example of such is employed in the study of protein expression in animal model of Parkinson's disease unilaterally injected with 6-hydroxydopamine, the study revealed accelerated protein acetylation with changes in the striatum protein concentration in dopamine depleted animal when compared with the controls (Pierson *et al*., 2004). It is also useful in the identification of Zn-containing protein such as ATP synthase b-chain identification- in an Alzheimer's brain sample (Becker *et al*., 2006). In another interesting application by the Julich group, pulse from LA-ICP-MS was used for the identification of protein containing Cu, Zn and Fe in human brain tissue (Becker *et al*., 2005).

LA-ICP-MS Bio-imaging in toxic metal study; LA-ICP-MS imaging has also proven to be useful in toxicological study; toxic metals have no functional role in normal brain homeostasis but gradually accumulate in the brain tissue to elicit severe damage leading to chronic degenerative diseases. Lead (Pb) and other divalent cations have been shown to be involved in the damage of calcium- channel proteins which affect neuronal axons and synaptic release of neuro-transmitter (Marchetti, 2003), Pb and Mn have also been implicated in amyloid plaque aggregation (Yegambaram et al., 2015). The knowledge of toxic metal distribution in the brain is essential in both health and medical research where it provide relevant information needed for the study of pathophysiology and potential therapeutic treatment, In a recent study LA-ICP-MS imaging was used to show the distribution of Lead (Pb) and Uranium (U) in human glioblastoma multiform brain tumour (Zoriy *et al*., 2006), Berker *et al.* (2008) also used laser imaging to study the distribution of Uranium and Neodymium in post mortem rat brain tissue previously treated with these metals . The study showed high affinity of Uranium and neodymium for white matter fibres in contrast to its low binding with the grey matter as well as higher binding of these metals to the striatum than the cortex. This result is suggestive of myelinotoxic effect of these metals on the white tract and striatum neurons.

A time-course study (Plate 3) also used LA-ICP-MS imaging to study the distribution of vanadium metal a neuro-toxicant in the brain of mice following chronic exposure. The mass spectrometric analysis revealed gradual influx and accumulation of vanadium metal in several brain regions with an affinity for the olfactory bulb, brain stem and cerebellum and progressive clearance from the brain after withdrawal from the initial exposure. However, the molecular pathway involved in its clearance is unknown and needs to be further investigated. The author concluded that the brain regions with higher vanadium deposition correspond to the regions where distinct pathologies have been earlier reported in the literature (Folarin *et al*., 2017).

LA-ICP-MS bio-imaging in Neurodegenerative diseases and brain lesions: The use of LA-ICP-MS imaging techniques has been employed in the study of metal and elemental dyshomeostasis, diseases pathogenesis and the potential treatment of metal associated neurodegenerative diseases such as Alzheimer's, Parkinson's and Wilson's diseases (Berker *et al*., 2010). In an attempt to provide a novel approach for the assessment of white and grey matter iron accumulation in Alzheimer's diseases, a pilot study was conducted using LA-ICP-MS for the comparative analysis of white and grey matter iron level in an AD brain and control subject. The study detected intrusion of iron into grey matter of Alzheimer's brain when compared with the control. Upregulation of iron level observed in grey matter of the AD brain may be indicative of dysregulated iron homeostasis in vulnerable brain region or inflammatory response to chronic neurodegeneration (Hare *et al*., 2016).
LA-ICP-MS bio imaging techniques has a wide application in the study for detecting metal dyshomeostasis in brain lesions. In 2005, Becker and colleagues first conducted brain tumour study by using LA-ICP-MS for quantitative imaging and spatial distribution of copper, zinc, phosphorus and sulfur in the brain of rat (F344 Fisher rat) injected with F98 giloblastoma cells; the study demonstrated an association between the selected metals and the brain tumour growth. In another experiment by Becker and Salber, (2010), LA-ICP-MS bio-imaging was combined with immuno-histochemical and autoradiographic techniques to study elemental distribution and response of several brain cells to brain thrombosis induced by intense light, using a rat model for stroke. Result revealed massive accumulation of metals (iron, zinc and copper) at the thrombotic lesion as well as reactive gliosis and active neurogenesis at the region surrounding the lesion site.

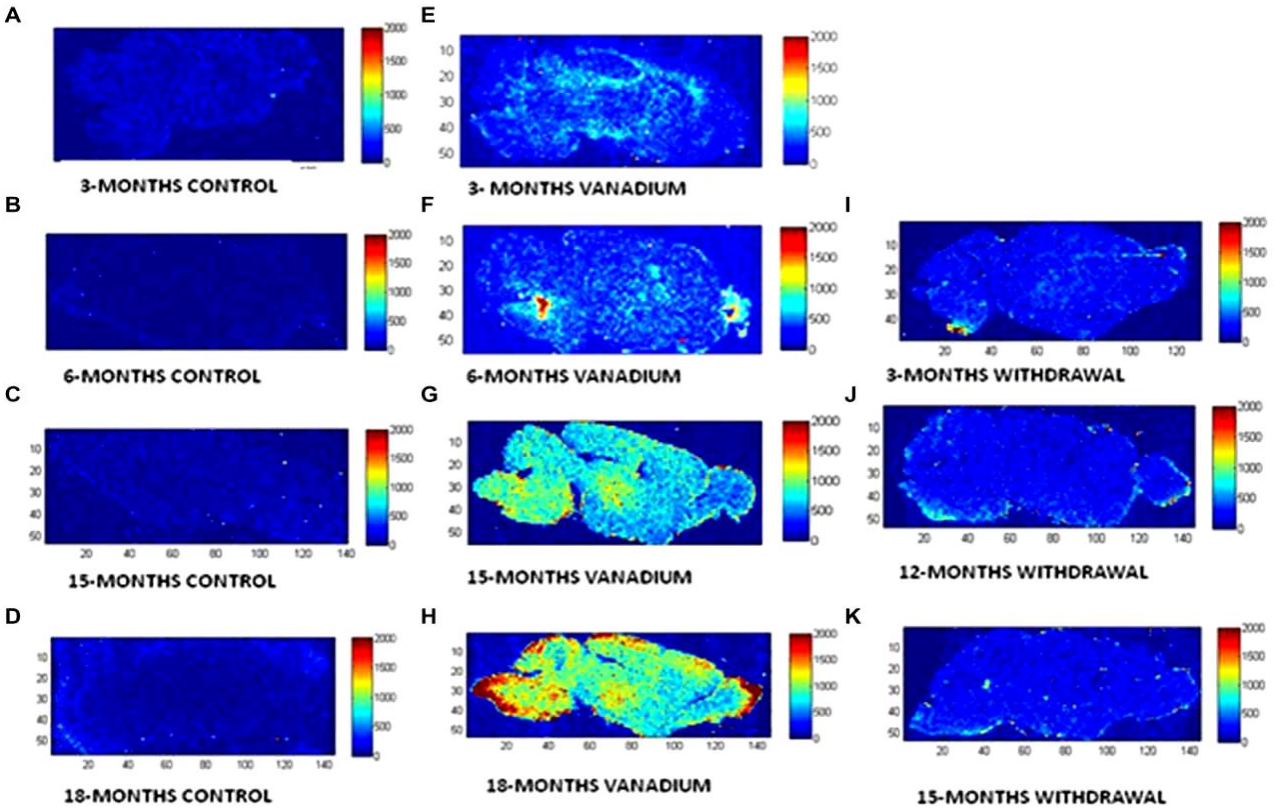

Plate 3:
Laser ablation-inductively coupled plasma-mass spectrometry (LA–ICP–MS) revealed the regional distribution and clearance of vanadium metal from mouse brain after chronic exposure and withdrawal from the initial exposure. (Folarin *et al.*, 2017).

A follow up study was also conducted by Zoriy *et al.* (2006) to examine the level of Zn, Pb and U in human glioblastoma Multiform brain sections. This study employed the complementary potential of LA-ICP-MS brain imaging and autoradiography imaging technique. Additional study by Becker *et al.* (2005) on small-sized brain tumors, also detected mark depletions of Cu and Zn around the tumor area indicating the pathophysiological role of these element in the tumour growth. Further study on comparative imaging of P, S, Fe, Cu, Zn and C was conducted by Zoriy *et al.* (2007) on thin sections of rat brain tumour, the analysis detected the relationship between the tumour boundaries and the regional elemental concentration and distribution.

LA-ICP-MS Bio-imaging in other neurological diseases: Application of LA-ICP-MS bio-imaging is also extended to the study of non neurodegenerative disorders. In an experiment using a mouse model of hypoxia, LA-ICP-MS imaging revealed massive accumulation of cobalt in the exposed brains when compared with the control, the elevated cobalt concentration strongly correlated with endoplasmic reticulum stress, myelin loss, axonal injury as well as vitamin B12 enrichment of the brain (Veasey *et al.*, 2013). Also using mouse model of traumatic brain injury LA-ICP-MS imaging of mouse brain subjected to a controlled cortical impact revealed immediate increase in the level of iron, copper and zinc which was extended till 28-days post injury (Portbury *et al.*, 2016). In another experiment, using animal model of post-traumatic stress disorder, changes in the zinc concentration and dyshomeostasis were detected using LA-ICP-MS bio-imaging. The result showed massive accumulation of Zn in the hippocampus and dentate gyrus of the stress exposed brains relative to the control, stress induced zinc accumulation in the hippocampus could be responsible for the physiological and behavioral deficit observed in this disorder (Sela *et al.*, 2017). LA-ICP-MS imaging of metals in the spinal cord has provided a new insight in the study of pathogenesis and development of target drugs for treating motor disorders such as amyotrophic lateral sclerosis (Robert *et al.*, 2014).

Conclusions and Perspectives: Over the years, LA-ICP-MS bio-imaging technique has gained global recognition and has been consistently employed in different areas of brain research due to its numerous outstanding features when compared with other metal bio-imaging methods. In addition, large list of recent references cited in this review has confirmed the wide application of LA-ICP-MS in several metal bio-imaging researches. Despite its wide use, LA-ICP-MS technique still has a major limitation with respect to calibration, which prevents it from being a front line analytical technique for achieving fast, precise and sensitive metal quantification. The following are the concluding remarks:

1. Sample preparation is a fundamental issue that must be highly considered when designing new experiments. Appropriate protocol must be put in place to prevent leaching of metal ions from brain sections that may likely occur during tissue storage and preparation

2. Formalin fixation a crucial process in any histochemical staining protocol usually results in chemical alteration as well as marked redistribution of trace element and metals in cut tissue section. This change may alter accurate interpretation of imaging data. Further study is needed to evaluate the likely effect of sample preparation on metal distribution; minimal sample handling is also recommended to avoid the chance of chemical alteration in the brain tissue.
3. Preparation of appropriate biological standard for matrix analysis and better understanding of fractionation and matrix effect is required for effective LA- ICP-MS bio-imaging analysis.
4. Comparative analysis of imaging data obtained from LA-ICP-MS technique and other metal bio- imaging method such as a synchrotron-based X-ray fluorescence microscopy (XFM) could improve data accuracy.
5. The study of cellular organelles and their biochemical processes could be enhanced by using higher spatial resolution instrument such as laser microdissection inductively coupled plasma mass spectrophotometry (LMD ICP-MS).
6. Complimentary potential of LA-ICP-MS bio-imaging techniques with other established biomedical imaging techniques such as magnetic resonance imaging (MRI) and metallomics has allowed for identification, quantification and better knowledge of the essential role of metalloproteins in health and in the pathophysiology of several neurological diseases.

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Full Length Research Article

Effect of Angiotensin Receptor Blockade on Plasma Osmolality and Neurohumoral Responses to High Environmental Temperature in Rats Fed a High Salt Diet

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Summary: Plasma osmolality (pOsmol) and neurohumoral signals play important roles in the pathophysiology of cardiovascular diseases. Our study investigated the effect of high environmental temperature (HET) on neurohumoral responses and pOsmol in rats fed a high salt diet (HSD), with and without angiotensin II receptor blockade (ARB), using telmisartan. Fifty-six male 8-week old Sprague-Dawley rats (95-110g) were randomly assigned into seven groups of 8 rats. These included control rats (I) fed with 0.3% NaCl diet (normal diet, ND); salt-loaded rats (II) fed with 8% NaCl (high salt) diet; ND rats (III) exposed to HET (38.5±0.5ºC) 4 hours daily per week; rats (IV) fed with 8% NaCl diet and exposed to HET daily. Others included rats (V) fed with 8% NaCl diet and treated with telmisartan (30mg/kg); ND rats (VI) exposed to HET and treated with telmisartan; rats (VI) fed with 8% NaCl diet, exposed to HET and treated with telmisartan. Plasma angiotensin II, aldosterone, vasopressin and norepinephrine (NE) concentrations were determined by ELISA technique; pOsmol from plasma K+, Na+ and Urea. HSD combined with HET in rats synergistically increased pOsmol ($P<0.001$) with an associated non-synergistic rise in fluid intake ($P<0.001$), fluid balance ($P<0.001$), plasma angiotensin II ($P<0.01$), aldosterone ($P<0.05$), NE ($P<0.001$) and vasopressin ($P<0.05$) concentrations compared to control. Telmisartan did not alter pOsmol in all the treated-rats, but normalized fluid intake levels and plasma vasopressin in the rats exposed to either HSD or HET *al*one. Prolonged exposure of rats to hot environment exacerbated the effect of excess dietary salt on pOsmol, with no effect on angiotensin II-mediated neurohumoral responses.

Keywords*: Plasma osmolality and neurohumoral responses to environmental heat and high dietary salt*

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INTRODUCTION

Cardiovascular diseases are reported to be on the rise worldwide, accounting for over 30% of all global deaths (Benjamin *et al*., 2017), with the majority of the reported deaths in 2015 occurring in developing countries (WHO, 2017). High environmental temperature (Moghadamnia *et al*., 2017) and high dietary salt (Mozaffarian *et al*., 2015) are independent risk factors in the pathophysiology of cardiovascular diseases. Sadly, global warming is raising environmental temperature worldwide, consequently increasing the population of workers exposed to hot environment (Nerbass *et al*., 2017). High dietary salt consumption is also ascending in many nations of the world, including African countries (Cappuccio *et al*., 2006; Mozaffarian *et al*., 2015). Our previous work demonstrated that high environmental temperature increased the severity of hypertension in animal models fed a high salt diet for 8 week by exacerbating sodium retention in the animals (Agbaraolorunpo *et al*., 2019). This is important as blood sodium level is a principal determinant of blood osmolality critical to body fluid homeostasis and cardiovascular adaptation. Earlier studies showed that both high environmental temperature and high salt diet independently increased plasma osmolality (Suckling *et al*., 2012).This evidently drives the development, progression and outcome of cardiovascular and chronic kidney diseases (Kaya *et al*., 2017; Ozsari, 2017).

High environmental temperature causes sweating and dehydration that contract plasma volume and raises blood osmolality to stimulate thirst and antidiuresis responses (de Wardener *et al*., 2004). This feedback mechanism is critical for the restoration of body osmotic balance and fluid volume (Thornton, 2018). Precisely, plasma osmolality is controlled by homeostatic mechanisms located outside the blood brain barrier (Baylis & Thompson, 1988). These mechanisms involve central osmoreceptors located in the brain to monitor blood osmolality; thirst centre that mediate thirst response; and paraventricular nuclei (PVN) and supraoptic nuclei (SON) of hypothalamus which produce vasopressin. In support of this, it has been demonstrated that lesion of these areas of the brain abolishes both vasopressin secretion and thirst responses to hyperosmolality in both humans and experimental animals (Baylis & Thompson, 1988; Johnson & Thunhorst, 1997). In an attempt to restore plasma osmolality, vasopressin limits fluid loss from the sweat gland (Nadel, 1985) and kidney (Cuzzo *et al*., 2020). Again, it has been shown that thirst sensations occurs in response

to exaggerated plasma osmolality and angiotensin II, with conversely attenuated plasma volume (Hughes *et al*., 2018). The link between alterations in plasma osmolality and sympatHETic outflow has also been demonstrated in a study examining centrally located sensing mechanisms (Kinsman *et al*., 2017).

Meanwhile, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor antagonists have both been shown to attenuate abnormal thirst drives (Sica, 2001). Likewise, blockade of angiotensin II type 1 receptors has been demonstrated to reduce water retention in dehydrated camels (Ali *et al*., 2012). But most worrisome, the efficacy of angiotensin II receptor blockade in the control of hypertension is marked with controversies with some study suggesting that administered alone, the drug is less effective among African population (Materson *et al*., 1993), ditto for ACE inhibitors (Brewster & Seedat, 2013).This dilemma may not be unconnected with the sensitivity of blood pressure to high salt diet among this race (Endo *et al*., 2009).This view is supported by the failure of angiotensin II blockers to control hypertension in salt-loaded animal models in our previous studies (Agbaraolorunpo *et al*., 2019) and other earlier studies (Susic *et al*., 2010). The physiological action of angiotensin II, such as sodium and water retention, aldosterone and vasopressin release is mediated by angiotensin type 1 receptor (AT1) receptor (Kaschina & Unger, 2003).Therefore, the inhibition of these receptor may modulate neurohumoral response to salt-induced and heat-induced alteration in plasma osmolality. This insight may help in further elucidating the plausible roles of plasma osmolality on blood pressure regulation by AT1 receptor blockade especially in individuals consuming high salt diets, with concomitant exposure to high environmental temperature.

MATERIALS AND METHODS

Animals: The study protocol was approved by the Ethics committee of College of Medicine of the University of Lagos (CMUL/HREC/11/18/471). Animal care and handling was done according to the National Research Council (US) Committee for the Care and Use of Laboratory (2011).

Fifty-six male Sprague-Dawley rats (8weeks, 95-110g) were supplied by Komad Farm, No 25 Old Abeokuta Lagos expressway. The rats were allowed to acclimatize for 2 weeks before the experimental procedures in standard cages at the animal house of Department of Physiology. The rats were maintained on a 12h dark/light cycle at ambient temperature of 25 ± 0.5 ^{o}C in the animal house. They were allowed access to standard rat chow and clean tap water ad libitum throughout the study.

Grouping of animals: The rats were randomly divided into 7 groups of 8 rats per group and subjected to different experimental conditions on the basis of dietary salt, environmental temperature and treatment with telmisartan (30mg/kg of animal's body weight).

Control (I): The rats were fed with normal salt diet (0.3% NaCl) and maintained at a room temperature of 25±0.5 ^{o}C for 8 weeks.

Salt (II): The rats were fed with high salt diet (8% NaCl) and maintained at a room temperature of 25 ± 0.5 ^{o}C for 8 weeks.

Heat (III): The rats were fed with normal diet (0.3%NaCl) and exposed to a high environmental temperature of 38.5 ± 0.5 ^{o}C 4 hours daily per week for 8 weeks.

Salt+Heat (IV): The rats were fed with high salt diet (8% NaCl) and exposed to a high environmental temperature of 38.5 ± 0.5 ^{o}C 4 hours daily per week for 8 weeks.

Salt+ARB (V): The rats were fed with high salt diet (8% NaCl), maintained at a room temperature of 25 ± 0.5 ^{o}C for 8 weeks and treated with telmisartan (30mg/kg per body weight) for 7 weeks.

Heat + ARB (VI): The rats were fed with normal diet (0.3%NaCl), exposed to a high environmental temperature of 38.5 ± 0.5 ^{o}C 4 hours for 8 weeks and treated with telmisartan (30mg/kg per body weight) for 7 weeks.

Salt+Heat+ARB (VII): The rats were fed with high salt diet (8% NaCl), exposed to a high environmental temperature of 38.5 ± 0.5 ^{o}C 4 hours daily per week for 8 weeks and treated with Telmisartan (30mg/kg per body weight) for 7 weeks.

Exposure of animals to high environmental temperature (HET): Heat-exposed rats were acclimatized to HET for one week starting from 30 ^{o}C to 35 ^{o}C with a daily temperature increase by 1^{o}C. Thereafter, the animals were exposed to HET at 38.5 ± 0.5^{o}C and relative humidity between 65 and 75 % using the method described by Barney & Kuhrt, (2016), with a slight modification of the temperature to a higher level of 38.5±0.5 ^{o}C. Exposure took place for 4 hours daily for 6 days/ week for 8 weeks in an environmental chamber from 9 a.m to 1p.m (Agbaraolorunpo *et al*., 2019). Environmental temperature was monitored with environmental thermometer. Rectal temperatures were determined, as index of core temperature, pre and post-exposure to HET with digital thermometer (Che Muhamed *et al*., 2016) .

Feeding of animals with high salt diet : The salt-loaded rats (Groups II, IV, V and VII) were fed with high salt diet as described by Sofola *et al*. (2002) for 8 weeks.

Treatment of animals with Angiotensin II receptor blocker: ARB-treated groups (V, VI and VII) were administered with 30 mg/kg/day telmisartan daily via oral gavage (Gohlke *et al*., 2001) for 7 weeks, starting from the 2nd week of the experiment. The volume dose was calculated as Volume = $\frac{dose(mg/kg) * weight\ of\ rat(kg)}{conc\ (mg/ml}$

The telmisartan (MSN Laboratory, India) was procured from Phillips Pharmaceuticals Ltd, Nigeria.

Blood collection: Retro-orbital puncture was performed on the experimental rats for the collection of blood sample. The samples were collected in heparin bottles. The blood samples were centrifuged at 3000 rpm for 15 minutes to separate plasma from whole blood. Plasma samples were kept in eppendorf tubes and stored at -25 ^{o}C in refrigerator until the assays of the respective peptides.

Urine collection: Twelve-hour urine output (V) in mls was determined at the end of 8th week of the experiment, from 7 p.m to 7 a.m. in a metabolic cage. Plasma Na^{+} in mmol/L was determined with ion selective electrode method (ISE 6000 analyzer, France), while plasma urea (mg/dl) was determined colorimetrically. Plasma osmolality was

determined by a method validated by (Martín-Calderón *et al.*, 2015). Net fluid gain was calculated by subtracting total urine loss from water intake, assuming that respiratory water loss and sweat loss at rest were negligible (Hiroshi *et al*., 1994).

Measurements of plasma Angiotensin II, Aldosterone, Norepinephrine, Arginine Vasopressin

Plasma Angiotensin II: Plasma angiotensin II concentrations were determined using Rat angiotensin II ELISA kit (MyBiosource.com, USA) according to the manufacturer's instruction. Briefly, 50µl standard was added to standard micro elisa plates, while 10 µl of plasma was added to testing well which was followed by the addition of 40 µl of sample diluent into plasma. 100µl of HRP-conjugate was added to each well (standard well and testing) and incubated at 37 ºC for 60 minutes. Thereafter, the plates were washed 5 times, followed by the addition of 50 µl of chromogen solution A and B respective at 37 ºC within 15 minutes in the dark for colour development (blue).50µL stop solution was then added into each of well to stop the reaction, resulting in colour change from blue to yellow. The absorbance was read at 450 nm in microplate within 15 minutes of adding the stop solution which was used to determine the concentration of angiotensin II.

Plasma Aldosterone: Plasma aldosterone concentrations were determined using Rat Aldosterone ELISA kit (MyBiosource.com, USA) according to the manufacturer's instruction. Briefly, 50 µl standard was added to standard microelisa plates, while 10µl of plasma was added to testing well which was followed by the addition of 40 µl of sample diluent into plasma. 100 µl of HRP-conjugate was added to each well (standard well and testing) and incubated at 37 ºC for 60 minutes. Thereafter, the plates were washed 5 times, followed by the addition of 50 µl of chromogen solution A and B respective at 37 ºC within 15 minutes in the dark for colour development (blue). A stop solution (50 µL) was then added into each of well to stop the reaction, which changed the colour from blue to yellow. The absorbance was read at 450nm in microplate within 15 minutes of adding the stop solution which was used to determine the concentration of aldosterone.

Plasma Norepinephrine: Plasma Norepinephrine concentrations were determined using Rat Norepinephrine ELISA kit (MyBiosource.com, USA) according to the manufacturer's instruction. Briefly, 50µl standard was added to standard micro elisa plates, while 10 µl of plasma was added to testing well which was followed by the addition of 40 µl of sample diluent into plasma. 100 µl of HRP-conjugate was added to each well (standard well and testing) and incubated at 37ºC for 60 minutes. Thereafter, the plates were washed 5 times, followed by the addition of 50 µl of chromogen solution A and B respective at 37 ºC within 15 minutes in the dark for colour development (blue). A stop solution (50 µL) was then added into each of well to stop the reaction, which changed the colour from blue to yellow. The absorbance was read at 450 nm in microplate within 15 minutes of adding the stop solution which was used to determine the plasma concentration of Norepinephrine.

Plasma Arginine vasopressin: Plasma vasopressin concentrations were determined using Rat Vasopressin ELISA kit (BioAim Scientific Inc, Canada) according to the manufacturer's instruction. 25 µl of ant-vasopressin antibody was added into all the wells.75 µl of assay diluent were pipetted into blank wells.50µl of assay diluent into 0 ng/ml standard.50µl and other graded preparation of standard solution.50 µl each of plasma samples were added to appropriate wells. 25 µl of biotinylated peptides was added into each well (except the blank well), sealed and incubated for 1 hour at room temperature The well was emptied and the content washed with buffer of 300µl four times.100µl of diluted streptavidin-HRP solution was added into each well, followed by the sealing of the well and incubation of the wells at room temperature for 45minutes, while gently shaking the wells. The washing of the wells with buffer solution was repeated as previously done.100 µl of TMB substrate solution was added into each well and incubated for 15 minutes at room temperature with gentle agitation in the dark to allow for colour development.50 µl of stop solution was added to the well to terminate the reaction. The absorbance in each well was read immediately within 30 minutes of stopping reaction and this was used to determine the concentration of the samples.

Statistical analysis: Data were presented as Mean ± SEM. Differences in experimental rat groups were compared with one-way ANOVA followed by Tukey post-hoc test. P<*0.05* was regarded as statistically significant. GraphPad 5 software package (GraphPad Software, California ,USA) was used for the analysis

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RESULTS

Influence of High Environmental Temperature (HET) on plasma osmolality (pOsmol) in salt-loaded rats (HSD) treated with ARB: In comparison with control rat group, pOsmol was significantly higher in the rat group fed a HSD (P<0.01), the group exposed to HET *al*one (P<0.05), as well as the group exposed to the combined factors (P< 0.001). Meanwhile, pOsmol was significantly higher in the rat group exposed to the combined environmental factors compared with the rats fed with HSD alone (P<0.05) and in the group exposed to HET *al*one (P<0.05). Meanwhile, ARB did not significantly moderate pOsmol in all the experimental rat groups (P>0.05) (Figure 1).

Influence of High Environmental Temperature on fluid intake, fluid balance and urine output in salt-loaded rats treated with ARB: In comparison with control rat group, fluid intake (Table 1) and fluid balance respectively (Figure 2) were significantly increased in rat group fed with high salt di*et al*one (P<0.01 and P<0.01) and in the group exclusively exposed to high environmental temperature (P< 0.001 and P<0.001) as well as in the group exposed to the two environmental factors (P< 0.001 and P< 0.001). Meanwhile, angiotensin II receptor blockade in the salt-loaded rat group, and in the group exposed to high environmental temperature, significantly reduced the salt-induced and the heat-induced rise in fluid intake (P<0.05 and P<0.001 respectively), ditto for the attenuation of the rise in fluid balance (P<0.05 and P<0.001 respectively).

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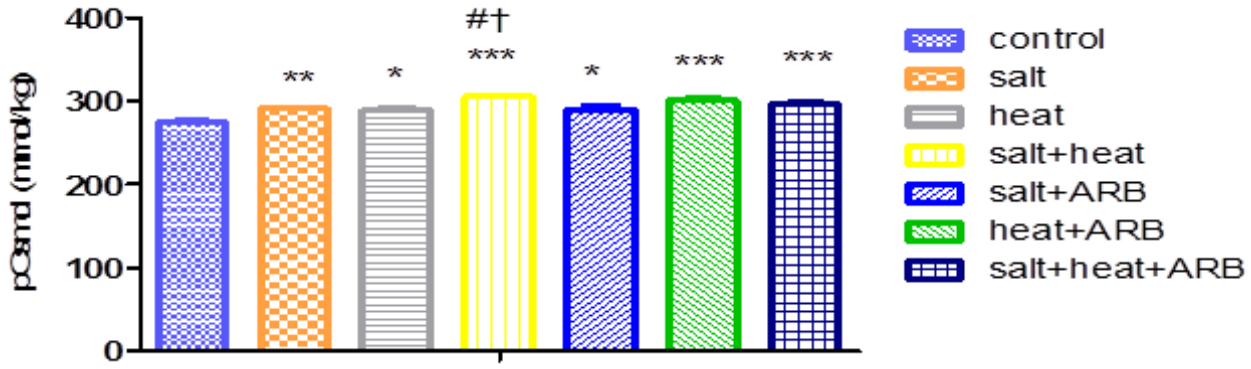

Figure1:
Plasma Osmolality in salt-loaded rats exposed to HET with and without ARB treatment.
*P<0.05, **P<0.01, ***P<0.001 vs control; #P<0.05 vs salt; †P<0.05 vs heat (n≥6).

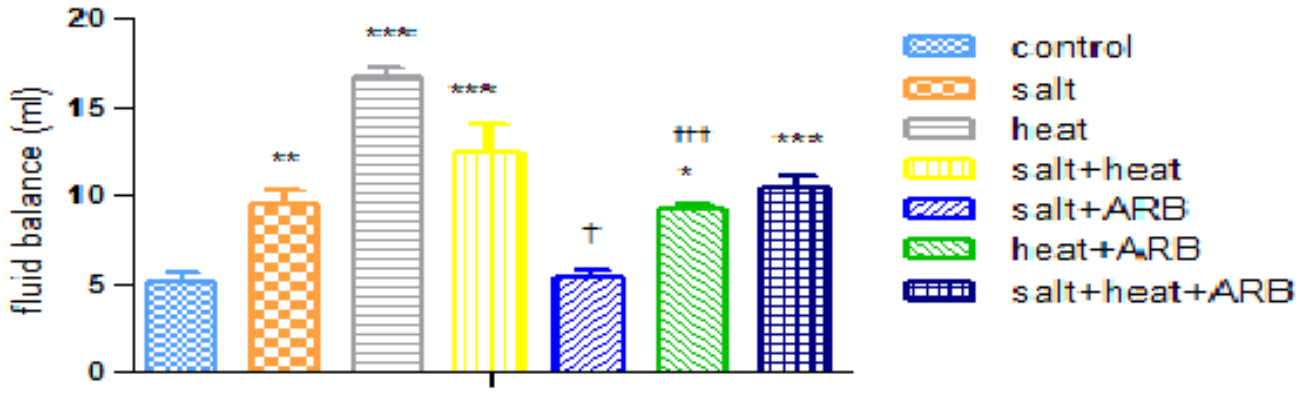

Figure 2:
Fluid balance in salt-loaded rats exposed to HET with and without ARB treatment:
**P<0.01, ***P<0.001 vs control; †††P<0.001 vs heat; † P<0.01 vs salt. Data presented as Mean ± SEM; One-way ANOVA followed by Tukey multiple comparison test (n≥7).

Table 1:
Effect of HET on Fluid intake and urine output in salt-loaded rats treated with and without ARB

Group	Fluid Intake (ml)	12-Urine output (ml)
control	9.6 ± 0.9	3.9 ± 0.7
Salt	18.04 ±1.1**	7.2± 0.8**
Heat	20.3 ±1.1***	2.2 ± 0.4##
Salt+Heat	20.0 ±1.3***	5.1 ± 0.8#
Salt+ARB	13.5± 0.3†	4.8 ± 0.3†
Heat+ARB	9.6 ±0.3†††	0.5 ± 0.2
Salt+Heat+ARB	15.5 ±1.3**†	3.4 ± 0.7

Fluid Intake: ***P<0.01,***P<0.001 vs control, † P<0.05 vs Salt , †† P<0.01 vs Heat;* ***Urine output:*****P<0.01 vs control, ##P<0.01 vs salt; Data presented as Mean ± SEM; One-way ANOVA followed by Tukey multiple comparison test (n≥7). Keys: HET, high environmental temperature; ARB, angiotensin receptor blocker.*

In contrast, ARB did not significantly attenuate fluid balance in the salt-loaded rats that were exposed to high environmental temperature, although it reduced their fluid intake significantly.
Furthermore, urine volume was significantly higher in rats fed a HSD compared to control rats (P<0.01) but this was attenuated in the ARB-treated rat fed a HSD (P<0.05). However, rats exposed to HET *al*one and combined with HSD had no change in their urine volume compared to control rats, ARB showed no modulatory effect under these conditions (Table 1).

Furthermore, urine volume was significantly higher in rats fed a HSD compared to control rats (P<0.01) but this was attenuated in the ARB-treated rat fed a HSD (P<0.05). However, rats exposed to HET *al*one and combined with HSD had no change in their urine volume compared to control rats, ARB showed no modulatory effect under these conditions (Table 1).

Influence of High Environmental Temperature on Neurohumoral responses in salt-loaded treated with rats with ARB: Rats exposed to HET *al*one had significant increase in plasma angiotensin II (P<0.05), aldosterone (P<0.001), arginine vasopressin (AVP) (P<0.001) and norepinephrine (P<0.001) compared with control rats. Similar increases were recorded in plasma angiotensin II (P<0.05), aldosterone (P<0.05), AVP (P<0.05) and norepinephrine (P<0.001) concentrations in the rats exposed to HET combined with high salt diet. However, only angiotensin II (p<0.05) and aldosterone (p<0.01) were

significantly elevated in the rats fed with high salt di*et al*one. Meanwhile, plasma angiotensin II, aldosterone, and AVP concentrations in the three groups of ARB-treated rats were comparable to the respective plasma levels in the control rats. Paradoxically, the increase in plasma norepinephrine was maintained in the ARB-treated rats fed with high salt diet ($P<0.05$) and in the rats exposed to HET ($P<0.05$) respectively compared with the control rat group. However, ARB normalised plasma aldosterone ($P<0.05$) and AVP ($P<0.01$) in the rats exposed to HET *al*one compared with the untreated rats in similar condition. AVP was similarly maintained at normal level ($P<0.01$) in the rats exposed to the combined factors compared with the untreated rats (Table 2).

Table 2:
Neurohumoral responses to dietary salt and environmental heat with and without ARB

Groups	Angiotensin II(pg/ml)	Aldosterone (pg/ml)	AVP(pg/ml)	NE(ng/ml)
control	619.3 ± 8.0	253.8 ± 15.9	1.80 ± 0.3	4.5 ± 0.4
salt	692.6 ± 12.7*	334.5 ± 13.3**	2.76 ± 0.6	5.6 ± 0.3
heat	675.6 ± 9.0*	353. ±18.5***#	5.4 ± 0.8***	9.2 ± 0.6***##
Salt+heat	692.9 ± 19.0**	320.1 ± 14.6*	4.16 ± 0.3*	9.9 ± 0.3***##
Salt+ARB	630.7±5.6	304.6 ± 7.9	2.33 ± 0.3	7.5 ± 1.2*
heat+ARB	625.0 ± 9.4	291.1 ± 13.7†	2.53 ± 0.3††	7.4 ± 0.6*
Salt+heat+ARB	673.6 ± 19.4	288.8 ± 4.8	2.06 ± 0.2†	6.2 ± 0.8††

Aldosterone**: *P<0.05,**P<0.01,P<0.001 vs control; #P<0.05 vs salt ;†P<0.05 vs heat ;*
AVP: ***P<0.05,P<0.001 vs control, †P<0.05 vs salt + heat, ††P<0.01 vs heat; **Norepinephrine**:*P<0.05,***P<0.001 vs control,##P<0.01 vs salt ,††P<0.001 vs salt + heat, ††. One-way ANOVA, followed by Tukey multiple comparison test (n ≥6)*

DISCUSSION

This present study investigated the effect of chronic exposure to high environmental temperature (HET) on plasma osmolality (pOsmol) and neurohumoral responses in rat models fed with high salt diet in the presence and absence of angiotensin receptor blocker (ARB), telmisartan. Our results revealed that chronic consumption of high salt diet (HSD) and prolonged exposure to high environmental temperature (HET) respectively and together increased pOsmol in our animal models. But most importantly, HET and HSD combined together in our experimental rats resulted in pOsmol level higher than that in either of the rats fed a HSD alone or exposed to HET *al*one. Surprisingly, angiotensin II receptor blockade, using telmisartan did not reverse the rise in pOsmol under the individual environmental condition as well as under the combined environmental conditions.

Our result is in line with previous study which showed that high salt diet increased pOsmol (Suckling *et al*., 2012), ditto for high environmental temperature (Gagnon *et al*., 2017). Most importantly, our study for the first time demonstrated the possible synergistic effect of these dual environmental factors on plasma osmolality when combined. This synergy possibly explains in part a probable mechanism that contributed to the exaggerated blood pressure and myocardial workload observed in our experimental animals fed a high salt diet in combination with exposure to high environmental temperature as reported in our previous work (Agbaraolorunpo *et al*., 2019).This view is supported by earlier studies which showed that increased blood osmolality resulted in elevated mean arterial pressure and heart rate (Gagnon *et al*., 2017; Kanbay *et al*., 2018). Furthermore, plasma sodium ion which has a direct relationship with high salt diet (Oloyo *et al*., 2016) and high environmental temperature (Allahverdi *et al*., 2013), possibly contributed to the increased plasma osmolality in this study. The increased plasma osmolality, otherwise referred to as hyperosmolality, when detected by SFO (subfornical organ) and OVLT (organum vasculosum) in the brain, could trigger series of homeostatic responses, including thirst (Hughes *et al*., 2018), sympatHETic nervous system activation and arginine vasopressin release (Leib *et al*., 2016), critical to body fluid volume expansion and blood pressure maintenance.

Therefore, the combined action of HSD and HET on pOsmol was expected to produce a corresponding synergistic effect on body fluid balance by increasing fluid consumption and reducing urine output. But our results revealed otherwise, showing that HSD and HET interactively potentiated fluid consumption and fluid balance, similar to the increases caused by either HSD (Bankir *et al*., 2017) or HET (Cuzzo *et al*., 2020), without any synergistic association. In agreement with earlier studies (Eriksson *et al*., 1984, Denton *et al*., 1985), urine output was also increased by HSD alone, but combined with HET, the increase was cancelled out in our animal models. These observations suggest that high environmental temperature possibly possess the potential to blunt salt-induced renal fluid loss, and this may play an important role in fluid retention. Interestingly, telmisartan normalized the increased fluid intakes in rat group exposed to the individual environmental factors and in the group exposed to the combined factors. Similarly, telmisartan significantly normalized the rise in fluid balance in the rats fed with excess salt diet and in the rats exposed to high environmental temperature respectively, with slight effect in the rats exposed to the combined environmental factors. This finding suggests a possible role for AT1receptor on thirst response and fluid balance adjustment (Sica, 2001) to the observed hyperosmolality caused by high salt diet and high environmental temperature respectively and together.

This plausible action of AT1 receptor blocker on body fluid homeostasis may in turn be modulated cardiovascular responses under these environmental conditions.

Evidently, the compensatory fluid volume adjustment to high salt diet and high environmental temperature was mediated by neurohumoral factors. These factors include angiotensin II, involves with thirst (Fitzsimons, 1998) and AVP release (Szczepanska-Sadowska *et al*., 2018); aldosterone, involves with renal sodium retention (Mulrow, 1999) and AVP, involves with fluid retention in the kidneys (Cuzzo *et al*., 2020) and sweat glands (Nadel, 1985). Hyperosmolality is also reported to increase sympatHETic nerves activity through osmosensitive neurons located in the forebrain circumventricular organs (Toney *et al*., 2003). Meanwhile, our current result showed that plasma angiotensin II and aldosterone levels were raised in the rats fed with high salt diet, similar to the inappropriate activation of these hormones reported by Gonsalez *et al*. (2018). But this was in disagreement with the suppressed levels of these hormones reported by Stocker *et al*., (2003) and Ramachandran *et al*. (2019).

Increases in circulatory angiotensin II and aldosterone were also noticed in the rat group exposed to HET in line with previous studies (Kosunen *et al*., 1976; Ma *et al*., 2001). Likewise, rats exposed to the combine environmental factors (HSD and HET) showed similar increases in angiotensin II and aldosterone, devoid of synergistic effect. These increases in circulatory angiotensin II and aldosterone were however less pronounced in the telmisartan-treated rats exposed to either of the individual factors or the combined factors. This finding indicates that ARB therapy moderately blunted angiotensin II and aldosterone responses in the rat groups fed with either HSD alone or combined with HET. This present finding contradicts previous results from earlier studies that reported increases in circulatory angiotensin II, following a prolonged therapy with angiotensin II receptor blockade (Nussberger *et al*., 1986; van den Meiracker *et al*., 1995). However, our present result aligned with findings from other works which demonstrated that long term AT1 receptor blocker therapy in hypertensive patients decreased plasma angiotensin II and aldosterone (Ichihara *et al*., 2001; Agata *et al*., 2006).

Furthermore, circulatory AVP and norepinephrine were significantly increased in the rats exposed to HET *al*one and combined with high salt diet, with no synergistic effect observed. Noticeably, high salt di*et al*one did not significantly increase AVP and norepinephrine in agreement with a study by Block *et al*., (1984), but contrary to studies by Kjeldsen *et al*., (1985) and Campese *et al*. (1982). Telmisartan did not attenuate the elevated norepinephrine level in the rats exposed to HET *al*one, but normalized AVP level. In contrast, telmisartan attenuated the increased plasma norepinephrine and AVP level in the rat group exposed to combined environmental factors, but paradoxically exaggerated the hitherto unchanged norepinephrine level in the group of rats fed a high salt di*et al*one. This finding suggests that interaction between high salt diet and high environmental temperature possibly improves AT1 receptor response to ARB therapy under the aforementioned condition. Given the roles play by norepinephrine (Kasparov & Teschemacher, 2008) and AVP (Matsuhisa *et al*.,2000) in the development of essential hypertension, it is not impossible that the effective blockade of the release of these two important peptides contributed to the effective blood pressure control in the rat group fed with high salt diet in combination with exposure to high environmental temperature as reported in our earlier work (Agbaraolorunpo *et al*., 2019).

Generally, blockers of AT1 receptor possess antiadrenergic potential to attenuate catecholamine level (Diz *et al*., 201; Balt *et al*., 2002). In support of this, earlier investigation revealed that AT1 receptor antagonist blunted Central Nervous SympatHETic activity in experimental animals with heart disease (Ramachandran *et al*., 2019). Averill *et al*. (1994) also demonstrated that losartan, an AT1 receptor antagonist, attenuated pressor and sympatHETic overactivity induced by angiontensin II in spontaneous hypertensive rats. Perhaps, the exaggerated plasma level of norepinephrine in the ARB-treated rats, fed a high salt di*et al*one, partly explains the unresponsiveness of salt-induced hypertension to ARB therapy in our previous study (Agbaraolorunpo *et al*., 2019) and in a study by Endo *et al*. (2009).

Furthermore, the increased plasma angiotensin II observed in this study likely promoted fluid intake and fluid gain in the hyperosmotic state of the rats fed a high salt diet and exposed to high environmental temperature respectively and together. This is supported by the attenuation of fluid intake by AT1 receptor blockade with telmisartan under this hyperosmotic condition. Similarly, Angiotensin-converting enzyme (ACE) inhibitors and Angiotensin II type 1 receptors antagonists have also been shown to attenuate abnormal thirst drives (Sica .,2001). Likewise, blockade of angiotensin II type 1 receptors was suggested to compromise water retention in a dehydrated camel (Ali *et al*., 2012). Therefore, it is not impossible that central inhibition of thirst and vasopressin by AT1 receptor inhibitor plays an active role in this process as suggested by our results. This is in conformity with earlier studies by Gohlke *et al*. (2002) and Nishimura *et al*., (2000) which demonstrated that peripherally administered ARB therapy sufficiently blocks centrally mediated action of angiotensin II.

Meanwhile, the rise in circulatory aldosterone associated with increased circulatory angiotensin II in this current study apparently promoted sodium ion retention. This ultimately contributed to the hyperosmolality caused by high salt diet and high environmental temperature. Although, the response of aldosterone was blunted by angiotensin receptor inhibition in line with a study by Nakamura *et al*.(2014), this action did not alter hyperosmolality in all the experimental rats treated with telmisarn as anticipated. This could be due to the attenuation of circulatory AVP level by ARB therapy, with the consequent suppression of AVP's antidiuretic action. This possibly compromised body fluid gain needed to normalize the observed hyperosmolality. The decline in circulatory AVP in this current study, was apparently counteracted by enhanced renal responsiveness to AVP as suggested by the decline in urine output in the telmisartan-treated rats. This could be a possible novel renal-defence mechanism geared towards ensuring fluid balance in the face of diminished thirst and plasma AVP possibly engendered by ARB therapy in a dehydrated state. This is supported by a study which proposed that renal action of circulatory AVP varies,

depending on the presence or absence of endogenous prostaglandin (Usberti *et al*., 1985).

Overall, chronic exposure to high environmental temperature synergized with high salt diet in our experimental rats to raise plasma osmolality, with the corresponding activation of fluid-regulating neurohumoral factors. Although, the combined effect of high salt diet and high environmental temperature on neurohumoral responses and body fluid balance was not additive, angiotensin II receptor blockade blunted these responses without correcting the exaggerated hyperosmolality. Therefore, patients on AT1 receptor blockers must be encouraged to take adequate water, given the tendency of this class of drug to blunt thirst and compromise body fluid balance with consequent hyperosmolality.

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Full-Length Research Article

Glycated Haemoglobin, Fasting Plasma Glucose, Plasminogen Activator Inhibitor Type-1, and Soluble Thrombomodulin Levels in Patients with Type 2 Diabetes Mellitus

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Summary: Diabetes mellitus has become increasingly prevalent over the years. The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunctions, and failure of different organs suggesting that the most effective tool to prevent complications is the effective control of hyperglycaemia itself. The study is set to determine the effect of glycemic control on plasminogen activator inhibitor type 1 (PAI-1), soluble thrombomodulin (STM) alongside fasting plasma glucose (FPG) and glycated hemoglobin (HbA1c) among type 2 diabetic subjects. One hundred diabetic subjects accessing care at the University of Calabar Teaching Hospital Calabar and 100 non –diabetics that served as controls were enrolled. Blood samples from participants were analyzed for FPG, HbA1c, PAI-1 and STM by standard methods. The result shows 74% of the diabetic to be females. Half of the diabetics were managed on only oral anti-diabetic drugs while the remaining half were either on insulin injection or a combination of oral and insulin injection. Poor glycemic control was observed in 56% of the studied subjects. The mean age of 54.69 ± 9.94 years for the diabetics was comparable to the age-matched controls (p=.097). Diabetics showed significantly higher FPG, HbA1c, PAI-1and STM (P=0.001) compared to control values. Correlations between STM, PAI 1 and glycated hemoglobin (figures 2 p=0.001, p =0.001) and STM, PAI-1 and FPG revealed significantly robust association (p=0.001, p=0.001). The study concludes that there is poor glycemic control among the treated diabetic subjects with PAI-1 and STM showing a very strong positive correlation with HbA1c than FPG.

Keywords*: Diabetes, Hyperglycemia, glycemic control, endothelial function*

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INTRODUCTION

Diabetes mellitus has been reported to be increasingly prevalent over the years (IDF, 2017). while vascular complications have been identified among the important morbidity and mortality factors (WHO, 2019). Persistently increased value of hyperglycaemia in diabetes is associated with long-term damage, dysfunction, and failure of different organs suggesting that the most effective tool to prevent complications from organ impairment in diabetes is the regulation of glycaemia itself. Owing in part to the inconvenience of measuring fasting plasma glucose levels or performing an oral glucose tolerance test, coupled with the day-to-day variability seen in glucose assessment, an alternative glucose measurement for the management of diabetes was introduced known as the glycated haemoglobin (HbA1c). Initially identified as "unusual" haemoglobin in patients with diabetes, glycated haemoglobin is now regarded as an objective measure of glycaemic control. Reports have shown that glycated haemoglobin correlates with coagulation derangements (WHO, 2011; ADA, 2018: Akwiwu *et al.,* 2020). Furthermore, recent endothelial studies done in diabetes suggest endothelial dysfunction, which is the hallmark of vascular diseases (Bretón-Romero *et al.,* 2018; Berra-Romani *et al.,* 2020).

The vascular endothelium, once thought to be simply a passive lining for blood vessels, is now recognized as a key determinant of vascular health. It has also become evident that endothelium is not an inert, single-cell lining covering the internal surface of blood vessels, but plays a crucial role in regulating vascular tone and structure (Iantorno *et al.,* 2014; Brakemier *et al.,* 2016). Other functions include the lining of the internal lumen of all the vasculature serving as an interface between circulating blood and vascular smooth muscle cells thus aiding as a physical barrier between the blood and tissues. Moreover, the endothelial cells facilitate a complex array of purposes in intimate interactions with the vascular smooth muscle cells, as well as cells within the blood compartment (Favero *et al.,*2014; Carrizzo *et al.,*2018). Therefore, injury or activation of the endothelium changes its regulatory functions and results in abnormal endothelial cell function. (Dogné *et al.,* 2018; Schiattarella *et al.,*2018).

Assessment of endothelial functions can be measured by the evaluation of endothelial cell markers. These Cell surface markers are proteins expressed on the surface of cells that often serve as indicators of specific cell types. Because endothelial activation often precedes overt endothelial dysfunction, biomarkers of the activated endothelium in serum or plasma may be detectable before

classically recognized markers of disease, and therefore, may be clinically useful as biomarkers of disease severity or prognosis in systemic infectious diseases (Page and Conrad, 2013) Predictors of endothelial dysfunction could improve the screening of individuals at increased risk, thus leading to the early diagnosis, appropriate treatment, as well as effective prevention of the complications of type 2 diabetes (Page and Conrad, 2013; Lau *et al.,* 2015). Plasminogen activating inhibitor-1 (PAI-1) is an endothelial damage marker and the primary enzyme inhibitor of plasminogen activation. Plasminogen is an acute-phase protein and precursor to plasmin, which digests fibrin thus playing a key role in the maintenance of the fibrinolytic system. Elevated levels of PAI-1 thus predispose to clot formation by inhibiting fibrinolytic activity and thus may be an early risk marker for disease progression (Pernow *et al.,* 2015; Lau *et al.,* 2015). Soluble thrombomodulin is another marker of endothelial damage. It represents the major substance of the protein C anticoagulant system (Chudy *et al.,* 2011). Thrombomodulin and activated protein C constitute a system that maintains vascular integrity as well as the thrombosis/ haemostasis balance. These roles are facilitated as the system provides anticoagulant, anti-inflammatory, and cytoprotective activities (Ikezoe, 2015). Although soluble thrombomodulin is yet to be extensively studied, its elevation is thought to be linked to widespread vascular damage and could be useful in the assessment of vascular complications in diabetes (Pernow *et al.,* 2015; Lau *et al.,* 2015). To understand more about the extent of activation of the endothelial cells concerning glycemic control, this study was done to determine FPG, HbA1c, PAI-1 and STM of diabetics and their possible relationships among diabetic subjects.

MATERIALS AND METHODS

Participants: A total of 200 participants were recruited for this study. They were made up of 100 persons with type 2 diabetes mellitus who were attending the clinic at the University of Calabar Teaching Hospital. Another 100 age and sex-matched healthy non-diabetic subjects drawn from the general population served as controls.

Ethical consideration: Ethical approval was obtained from the Health Research Ethics Committee (HREC) of the University of Calabar Teaching Hospital. Informed consent was obtained from each participant enrolled in the research and confidentiality was maintained. Bio-data and related information were obtained using a questionnaire.

Data collection: Pre-test counseling was administered to each respondent. Blood specimen was collected from each participant between 8am-9am in the mornings to minimize variability. Fasting plasma glucose was assayed by the glucose oxidase method (Randox, UK). In this method, the glucose oxidase enzyme catalyses the complete oxidation of glucose to produce hydrogen peroxide and gluconic acid. The hydrogen peroxide in the presence of the enzyme peroxidase is broken down and the oxygen released reacts with 4-amino-phenazone and phenol to give a pink coloured derivative whose absorbance is then measured in a colorimeter using a green filter.

Glycated haemoglobin was assayed by ion exchange resin method (Spectrum, Egypt). Glycosylated haemoglobin (GHb) has been defined operationally as the fast fraction haemoglobin HbA1 (HbA1a, A1b, A1c) which elutes first during column chromatography. The non-glycosylated haemoglobin, which consists of the bulk of haemoglobin has been designated HbAo. A haemolysed preparation of whole blood is mixed continuously for 5 minutes with a weakly binding cation-exchange resin. The labile fraction is eliminated during the haemolysate preparation and during the binding. During this mixing, HbAo binds to the ion-exchange resin leaving GHb free in the supernatant. After the mixing period, a filter separator is used to remove the resin from the supernatant. The percent glycosylated haemoglobin is determined by measuring absorbance of the glycosylated haemoglobin (GHb) and total haemoglobin fraction (THb). The ratio of the absorbance of GHb and THb of the control and test is used to calculate the percent GHb of the sample.

Both PAI-1 and soluble thrombomodulin were assayed using enzyme-linked immunosorbent assay kits from Bioassay Technology Laboratory, China. The PAI-1 present in the sample is added and binds to antibodies coated on the wells. Biotinylated Human PAI-1 antibody is added and binds to PAI-1 in the sample. Then streptavidin-HRP is added and binds to the biotinylated PAI-1 antibody. After incubation, unbound streptavidin-HRP is washed away during a washing step. The substrate solution is then added and colour develops in proportion to the amount of human PAI-1. The reaction is terminated by the addition of acidic stop solution and absorbance is measured at 450nm. The STM present in the sample is added and binds to antibodies coated on the wells. Biotinylated human STM antibody is added and binds to STM in the sample. Then streptavidin-HRP is added and binds to the biotinylated STM antibody. After incubation, unbound streptavidin-HRP is washed away during a washing step. Substrate solution is then added and colour develops in proportion to the amount of human STM. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450nm.

Data analysis: Data analysis was done using SPSS version 22.0. Student t-test was used for comparison of means while Pearson's correlation was used to establish a relationship between variables. Statistical significance was drawn at $p \leq 0.05$.

RESULTS

The assessed variables of both diabetic and non-diabetic subjects have been captured in Table 1. Approximately three-quarters (74%) of the diabetic subjects were females while the remaining one-third (26%) were males. Fifty percent (50%) of the diabetic subjects were on only oral anti-diabetic drugs, 31% on oral and insulin combined while 19% were on insulin alone. More than half (56%) of the studied subjects had poor glycemic control. Fifteen persons (15%) out of the 100 diabetic subjects had normal glyceamic control while 8% and 21% respectively represent good and fair glyceamic controls.

Table 1.
Measured Parameters of Diabetic and Control subjects

Parameters	Diabetic Subjects n = 100	Control n = 100	p-Value
Gender			
Males	26	26	
Females	74	74	
Treatment			
Oral drugs	50	-	
Insulin	19	-	
Oral & insulin	31	-	
Glycemic Control			
Normal	15	66	
Good	8	20	
Fair	21	14	
Poor	56	0	
Age (years)	54.69±9.94	52.40±9.50	0.097
FPG (mmol/l)	10.45±4.82	4.36±0.76	0.001
HbA1c (%)	7.97±1.80	5.56±0.86	0.001
PAI-1 (ng/ml)	13.26±4.13	4.86±1.54	0.001
STM (ng/ml)	8.77±0.65	3.18±0.28	0.001

The mean age of 54.69 ± 9.94 years for the diabetics was comparable to that of the age-matched controls (52.40±9.50 years). The FPG (10.45±4.82mmol/l), HbA1c (7.97±1.80%), PAI-1 (13.26±4.13ng/ml) and STM (8.77±0.65ng/ml) were significantly higher in diabetics compared to control values (4.36±0.76mmol/l, 5.56±0.86%,4.86±1.54ng/ml and 3.18±0.28ng/ml respectively). The chart in figure 1 represents the percentage impact of treatment types on glycemic control. Oral agents alone achieved more of good glycaemic control followed by oral/insulin combination and the least being insulin alone.
The correlation between STM and glycated hemoglobin in Fig. 2 (p=0.001, r=0.845) was stronger than that between STM and FPG (p=0.001, r=0.691). Same pattern was observed between PAI-1 and glycated hemoglobin (p =0.001, r=0.812) and PAI-1 and FPG (p=0.001, r=0.652) Fig 3.

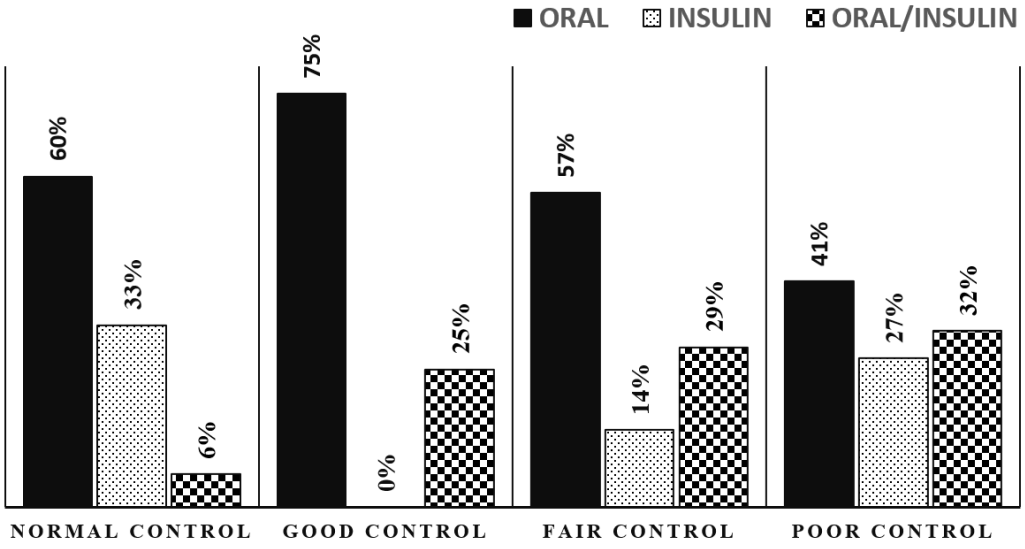

Figure 1
Categorisation of glycaemic control drug regimens

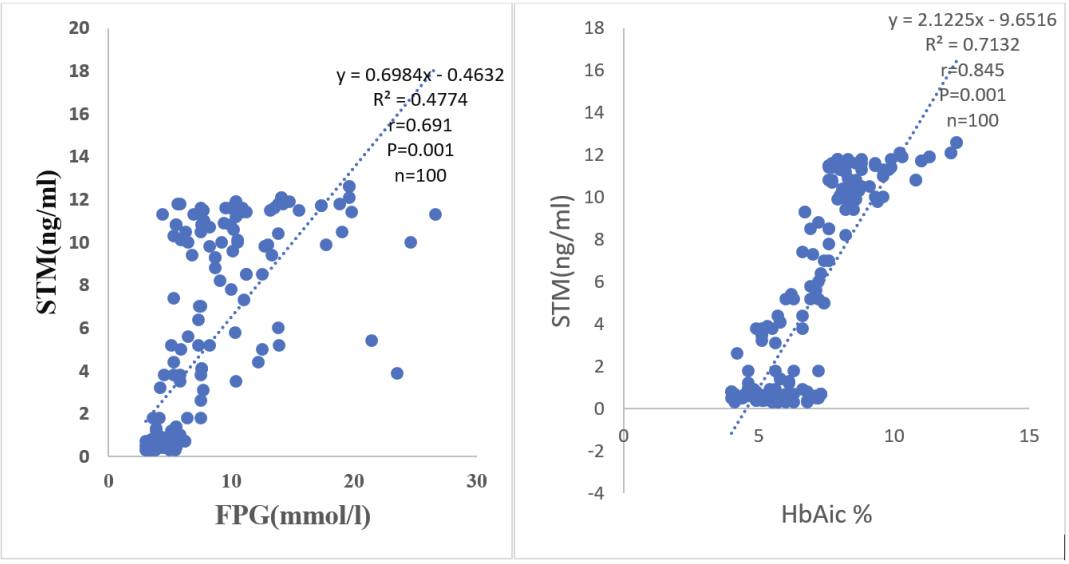

Figure 2:
Correlations of soluble thrombomodulin with fasting plasma glucose and glycated haemoglobin levels of diabetic subjects

DISCUSSION

Diabetes mellitus is a frequent and increasing public health problem mainly due to changes in dietary habits and general lifestyle during the last few years as well as rapid epidemiological transition (CDC, 2014; Sabir *et al.*, 2017; WHO, 2019). This study observed a mean age of 54.69±9.94 years and female preponderance of 74% within the studied population. Age and gender distribution differences in diabetes vary across the world. Depending on the region, distribution of these factors is influenced by prevailing risk factors as determined by socio-cultural and economic situations. These include poor dieting and physical inactivity particularly in low income countries with inadequate access to health care services (Kavanagh, *et al.*, 2010; Agardh *et al.*, 2011). Central obesity as well as high levels of estrogen and progesterone (both of which can reduce whole body insulin sensitivity) in women may also be implicated in the diabetic gender skew (Christensen *et al.*, 2008; Hilawe *et al*, 2013).

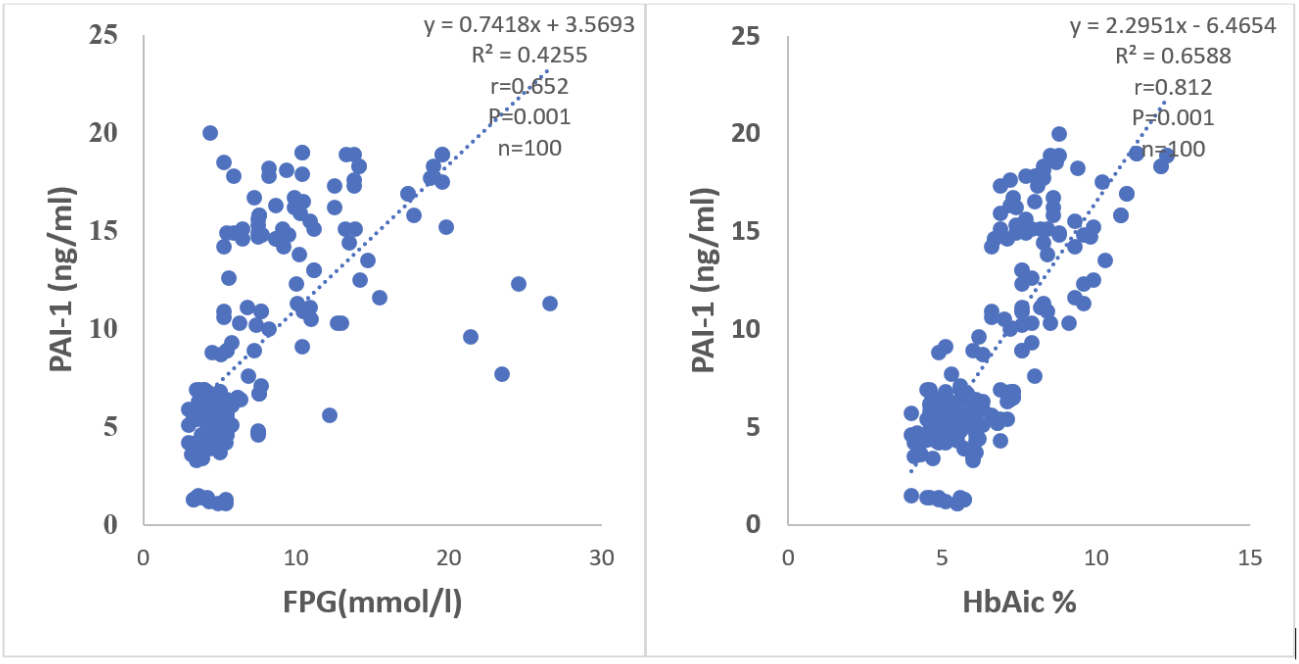

Figure 3:
Correlations of plasminogen activator inhibitor type-1 with fasting plasma glucose and glycated haemoglobin levels of diabetic subjects

This study measured fasting plasma glucose, glycated haemoglobin, plasminogen activator inhibitor type-1 and soluble thrombomodulin of subjects with type 2 diabetes on treatment. In proper management of diabetics, the primary goal is achieving glycaemic control. In this study, the glycated haemoglobin which is a measure of glycaemic control presents the results as, normal when patients value is <6.0%, good when the value is between 6.0-6.8%, fair when it is 6.8 -7.65% and poor when value is >7.65%). Findings of this study has shown that despite accessing diabetic care at the study center, attempts at maintaining near normal blood glucose levels among the diabetic subjects have not been very successful as only 15% of them had normal results while 56% had poor glycaemic control. Type 2 diabetes being a syndrome characterized by insulin resistance and increased hepatic glucose output (Kahn *et al.*, 2014; WHO, 2019), require medications that can correct one or more of the metabolic abnormalities. Prescriptions for the subjects are in the form of oral agents, insulin injection or combination of oral agents and insulin. The main goal is to achieve such therapeutic effects that will give good glycaemic control. It was observed as at the time of this study that half (50%) of the diabetics were on oral antidiabetic therapy and this achieved better glycaemic control than the combination therapy and insulin alone.

The current study also observed that glycaemic indices (FPG and HbA1c) of diabetics were significantly raised when compared with the control subjects. This format of result is expected as the test subjects were already known and managed diabetic subjects.

Furthermore, plasminogen activator inhibitor type-1 concentrations were observed to be raised significantly in diabetics. This may be due to its role as an acute phase reactant whose concentrations are raised in inflammatory conditions. Patients with diabetes also have insulin resistance, which contributes to inflammation that leads to the potential of causing endothelial dysfunction. Previous studies had observed correlation between plasma insulin and PAI-1 in different groups including obese subjects and non-insulin dependent diabetic patients (Rosenson *et al.*, 2011).

Soluble thrombomodulin is a marker of endothelial damage, representing major substance of the protein C anticoagulant system. Elevated levels of soluble thrombomodulin have been found in association with chronic diseases related to inflammation and endothelial dysfunction. More specifically, hyperglycaemia has been noted to induce increased expression of soluble thrombomodulin in the system (Kubisz, *et al*, 2010; Dietrich *et al.*, 2013; Elsalakawy *et al.*,2014; Hayden, 2019). Apparently, the elevated soluble thrombomodulin in diabetes as seen in this study is indicative of ongoing

endothelial injury induced by hyperglycaemia. In fact, elevated plasma concentrations of soluble thrombomodulin in patients with type 2 diabetes could be having deeper implications, such as widespread vascular damage (Chudy *et al.,* 2011; Brakemier *et al.,* 2016).

Apart from the observation that both fasting plasma glucose and glycated haemoglobin were significantly raised among the diabetic subjects when compared with the control subjects, there was also positive correlation between the endothelial cell markers with both fasting plasma glucose and glycated haemoglobin, (among the diabetics). However, glycated haemoglobin provided more sensitive relationships with the endothelial cell markers investigated than fasting plasma glucose. Thus, challenge in achieving glycaemic control is indicatively the more underlying mechanism for diabetic endothelial dysfunction rather than just the presence of hyperglycaemia. This obviously has implications in the areas of routine laboratory monitoring and effective management of type 2 diabetes. This study concludes that for better management of type 2 diabetes, at least one of either PAI-1 or STM alongside HbA1c should be used in the laboratory tests for the routine evaluation of affected persons.

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Research Article

Chronic Caffeine Ingestion Improves Memory and Learning and Increases Neuronal Population and Dendritic Length in the Hippocampus of Adult Mice

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Summary: Caffeine is the most widely consumed psychoactive drug in the world, ingested as a natural component of chocolate, coffee and tea, and as an added component to soda and energy drinks. Here we assessed behavioral changes caused by chronic caffeine administration as well as morphological changes within specific regions of the adult mice brain: the hippocampus and amygdala. Twenty-four adult male albino mice were randomly divided into three groups. Caffeine was administered daily by gavage for 8 weeks at a dosage of 20 mg/kg for the low dose (LD) group and 60 mg/kg for the high dose (HD) group while the third group served as control (CNT). After the period of administration, neurobehavioral tasks were carried out; Morris water maze for learning and memory open field test and elevated plus maze test for anxiety. The mice were sacrificed; their brain tissues were harvested and processed for H&E, Cresyl violet, and Golgi staining, and assessed qualitatively and quantitatively. Quantitative data from the neurobehavioural tests and neuronal cell counts were expressed as means ± standard errors of means and compared across the groups using analysis of variance (ANOVA). Significance was set at $p< 0.05$. Mice in the high dose group learned faster and had a significantly increased number of platform crossings in the Morris water maze test. There was, however, a slightly increased level of anxiety in the caffeine-treated mice, compared to controls. Histo-morphometric analysis revealed a significantly increased number of pyramidal neurons in the hippocampus in the low dose group, but a decreased neuronal count in the amygdala of the low dose and high dose groups compared to controls. The pyramidal neurons in the hippocampus of the caffeine-treated mice had increased apical dendritic length compared to the controls. Our findings strengthen the available data suggesting that prolonged caffeine intake improves cognition, and this process could be mediated by promoting the growth of dendrites and an increased number of neurons. However, this is coupled with an increased tendency to be anxiogenic.

Keywords*:* Anxiety, Amygdala, Caffeine, Cognition, Hippocampus

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INTRODUCTION

Caffeine, a non-selective adenosine receptor antagonist, is the most widely used psychoactive agent in the world. Adenosine is considered a 'fine-tune' neuromodulator that has a generally inhibitory effect on neuronal activity including synaptic transmission and plasticity (Ribeiro et al., 2003). As an adenosine receptor antagonist, caffeine tends to increase neuronal excitability which results in heightened arousal and attention (de Mendonca and Ribeiro, 2001). The A1 receptor subtype is the primary target of caffeine and is most prevalent in the hippocampus, a region of the limbic system that has been closely linked to learning, memory, and emotion (Fredholm et al. 1999). The hippocampus plays roles in memory formation and consolidation. Some studies have clearly shown that caffeine improves learning abilities, memory, and spatial orientation in various tests. The effect of caffeine on memory consolidation has recently been reported in human recognition memory whereby a single dosing of 200 mg caffeine administered to healthy adults after training was sufficient to improve the detection of subtle changes made to the training items 24h later (Borota et al. 2014). Watanabe and Ikegaya (2017) showed that caffeine reversibly increases Sharp Waves (SW; sharp waves being a biomarker for memory) in acute hippocampal slices in a dose-dependent manner. Caffeine induced about a 100% increase in the event frequency of SWs at concentrations of 60 and 200 μM. This effect was likely mediated via adenosine A1 receptors inhibition in either the Cornus Ammonis 3 (CA3) region or the dentate gyrus. The effects of caffeine on cognition are mediated primarily by blockade of the A_1 adenosine receptor (A_1R), as antagonists of A_1Rs enhance induction and stability of long-term potentiation (LTP) in hippocampal CA1 (Dunwiddie and Masino, 2001).

Studies have reported that higher levels of caffeine consumption in children (Ruxton, 2014) and young adult males (Trapp et al., 2014) correlates with increased anxiety. Rats administered acute caffeine (Ardais et al., 2014) also display increased anxiety while consuming caffeine during adolescence. Ample evidence suggest that caffeine consumption can exert anxiety in children, adolescent and adults, although sparse studies have examined the long-term

effects of chronic caffeine consumption on anxiety-related behaviors (Mahdi *et al.*, 2019).

With regards to memory, caffeine has been found to improve attention on personalized acute consumption in humans (Lanini *et al*., 2015), and moderate doses of caffeine (20 mg/kg) in rodents resulted in enhancement of memory (Almosawi *et al.*, 2018). Chronic consumption caffeine intake reportedly prevented the cognitive decline associated with aging by promoting the growth of dendrites and spines in the hippocampal neurons of the adult mice brain (Vila-luna *et al*., 2012). Chronic administration of caffeine is thought to be a better model for evaluating the consequences of regular coffee intake than acute administration of caffeine (Ribeiro et al, 2003).

The morphological changes occurring in the normal (non-diseased) brain following long term administration of caffeine has been so far, overlooked. This is important in order to determine if the caffeine effects will be long-lasting or easily reversible following termination of administration. In this study therefore, we examined the effect of chronic caffeine administration in normal adult mice, on learning, memory and emotionality, as well as the morphological changes that occur in regions of the brain that bring about these changes that is, the hippocampus for learning and memory, and the amygdala for emotions/anxiety.

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MATERIALS AND METHODS

Animals and groupings: Thirty young adult male albino mice, about 3 months of age and weighing between 22 – 27g, were obtained from the animal house of the University of Ibadan and left to acclimatize for a week. Ethical approval for the study was obtained from the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC/19/0006). The animals were then randomly assigned into three groups consisting of two experimental groups: low dose (n=10) and high dose (n=12) respectively and one control group (n=8). The mice in the low and high dose groups received 20 and 60 mg/ kg/ day of caffeine respectively (Olopade et al., 2021), obtained from Extra Pure Caffeine Anhydrous 98% powder (Laba Chemicals, USA) and dissolved in 0.3ml of tap water while the mice in the control group received the same volume of tap water only. The caffeine was administered daily by gavage, for eight weeks. Food and water were provided *ad libitum.*

Preliminary measurements: The body weights of the mice were measured weekly and they had a general physical examination, for general health, activity, and emotionality until the end of the study. Neurobehavioural assessments for cognitive functions - Morris water maze (MWM), and emotionality - Elevated plus maze (EPM) and Open-field tests, were carried out at the end of 8 weeks of caffeine administration. The tests were commenced 4 days before the termination of the caffeine administration because the Morris Water maze was carried out over 4 days. The sequence of neurobehavioural testing is given as follows: On the first day, the open field test was carried out, followed by the first day of the Morris water maze test. The next day, the Elevated Plus maze was carried out, followed by the Morris water maze test. On the third and fourth days, only the Morris water maze test was carried out.

Open Field Test: The open field is a white painted wooden box measuring 72cm by 72cm with black lines drawn on the floor to divide it into 18 cm by 18 cm squares. There is a centre square also measuring 18cm by 18cm. The mice were tested separately in the open field for a period of 10 minutes each to assess the following parameters of emotionality: grooming (that is, sets of heterogeneous constituents comprising face washing, body licking, paw licking, head and body shaking, scratching and genital licking), length of time spent in the centre square, length of time it spent freezing (i.e., staying in one position and not making any movements at all) and number of faecal boluses passed. These parameters were used to determine their anxiety level. On completion of the test for each mouse, the box was cleaned with 70% alcohol to prevent the subsequent mice from bias due to olfactory cues. (Olopade and Shokunbi, 2016).

Modified Morris Water Maze Test: The Morris water maze (MWM) tests hippocampal-dependent spatial learning and memory in rodents. The MWM consists of a circular pool of opaque water (120 cm in diameter, 30 cm in height) with a hidden circular escape platform (12 cm in diameter, 1 cm below the water level) which the mice must learn its location using contextual cues. The pool was marked North, South, East and West and the hidden platform was placed on the center of one of the four imaginary quadrants of the tank and maintained in the same position during all trials. Each mouse was dropped into the tank with its head facing the wall, allowed to swim freely and expected to search for the platform; the length of time it took to find the platform (in seconds) was recorded. If it did not find the platform after 120 seconds, the mouse was guided to the platform and allowed to stay there for 15 seconds. Each mouse went through four trials per day for three consecutive days. This test is a measure of the learning ability of the mouse. On the fourth day, a single probe trial was given to test the mouse's spatial memory retention in the water maze while the platform was removed. The mouse's memory of the initial location of the escape platform was measured by the time spent as well as its average speed in the target quadrant and the number of times it crossed the island zone where the platform was initially located. This was recorded as a test of memory retention/ability. (Angelucci *et al.*, 2002)

Elevated Plus Maze: The elevated plus maze tests anxiety-like behavior in rodents; it consists of two open arms (25 cm × 5 cm) and two enclosed arms of the same size at opposite sides of each other. The enclosed arms are surrounded by 15 cm high walls. The edges, 3 mm high, surround the open arms, minimizing the likelihood of animals falling from the apparatus. Both arms are 1m above the floor. Between the arms is a central square area (5 cm × 5 cm) where the mouse was placed. The entire apparatus was cleaned using 70% ethanol between subjects in order to eliminate olfactory clues. Each mouse was individually placed in the central square of the maze and allowed to freely explore the apparatus. The mouse behavior was recorded for the test period of 5 min and then analyzed. The number of entries per arm and the time spent in the open arms was recorded. An entry is recorded when all four paws enter the arm. The numbers of entries and time spent in the open arms reflect the general emotionality of the mice. A decreased number

of entrances into the open arms of the maze, as well as the decreased amount of time spent in them, are considered as a measure of anxiety-like behavior. (Almowasi *et al*., 2018).

Tissue processing and Stereology: The animals were euthanized at the end of eight weeks of caffeine administration after the neurobehavioural tests had been performed. Each mouse was anaesthetized with ketamine/xylazine (90/10 mg/kg) and transcardiac perfusion was done with 10% neural buffered formalin.

The mice brains were removed, post fixed in the same fixatives for 48 hours, processed, sectioned to reveal the *cornus ammonis* (CA) 1 region of hippocampus and amygdala, and stained using Haematoxylin and Eosin, and Cresyl stains. Random samples of framed regions of interest with an area of 417 µm by 459 µm in the hippocampus and amygdala were selected for neuronal counts, using two sections per animal and three animals per group.

A subset of three mice brains per group was selected for Modified Golgi stain according to the protocol previously described (Olopade et al., 2021), and used to study the dendritic arborization of the neurons in the *cornus ammonis* (CA) 1 region of hippocampus and amygdala. Silver impregnated neurons with well-defined cell body and processes were selected for qualitative morphological assessment. Their dendritic lengths were measured with the aid of computerized image analysis (ImageJ software version 1.46). This was done by placing a transparent grid with concentric rings of 10-mm spacing, over the picture of the neuron with its dendrites, the estimate of the total dendritic length was then taken by the software.

Statistical Analysis

Quantitative data from the scores in the behavioural test as well as data from the neuronal count and dendritic lengths from the histological slides are presented as mean ± SEM. D'Agostino-Pearson omnibus normality test was used to determine normality of the distribution of the means after which, the analysis of variance (ANOVA) test was used to compare the means. Turkey's multiple comparison post-hoc test was used to compare within the groups. The statistical significance was set at $p < 0.05$.

RESULTS

A total of 30 adult male mice were acquired for this experiment, 6 of which died before the end of the study - mainly from fight wounds. Two rats from the low dose group and four from the high dose group died before the end of the study, therefore 24 adult males were used in the analyses, eight in the low dose, and eight in the high dose group while eight served as controls. The mice in the control group were generally calmer than those receiving caffeine, which were hyperactive especially immediately after the caffeine administration.

Behavioural Assessment

Open field test: The parameters assessed in the open field task, related to emotionality were the time spent in center square and grooming.

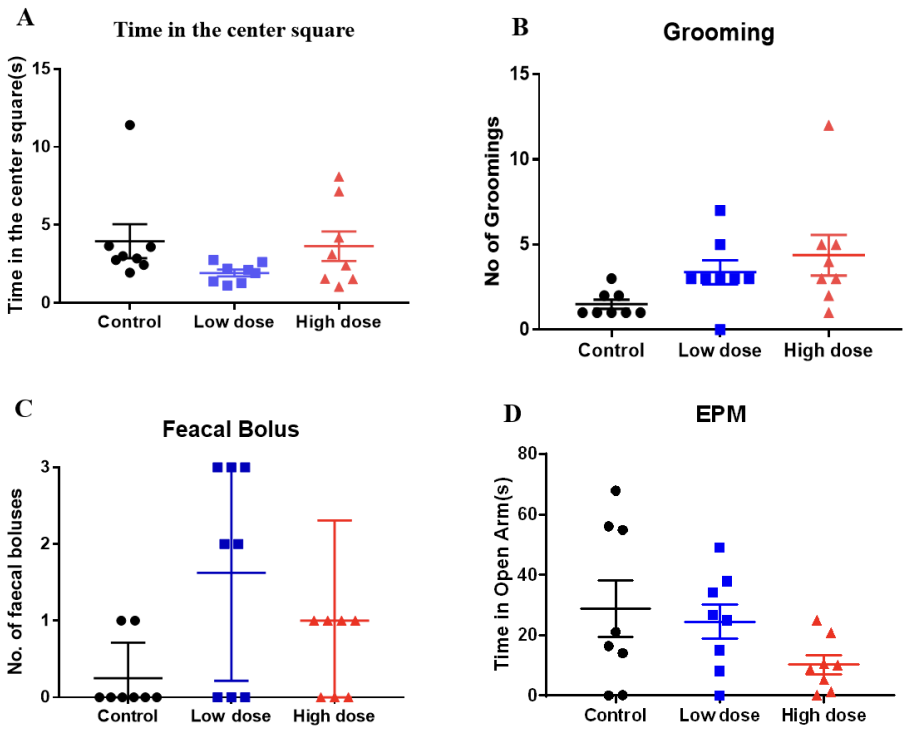

Figure 1:

Scatter plots showing the A) length of time the mice spent in the center square B) number of groomings and C) number of faecal boluses D) Time spent in the open arms of the elevated plus maze (EPM) across the groups.

There was no significant difference in the time spent by the mice in the centre square across the three groups ($F=1.699$; $p=0.20$ and $R^2=0.1393$). Similarly, no significant difference was observed in the number of faecal boluses passed by the mice across the groups. There was however a tendency towards more grooming in the experimental groups than in the controls, but it did not reach significant levels: control (1.5 ± 0.267); low dose (3.375 ± 0.706) and high dose (4.375 ± 1.194) ($F=3.20$; $p=0.06$ and $R^2=0.233$) (Fig. 1A-C).

There was no significant difference in time spent in the open arms of the elevated plus maze apparatus across the three groups (F=2.15, p=0.141 and R2 =0.169) (Fig. 1D).

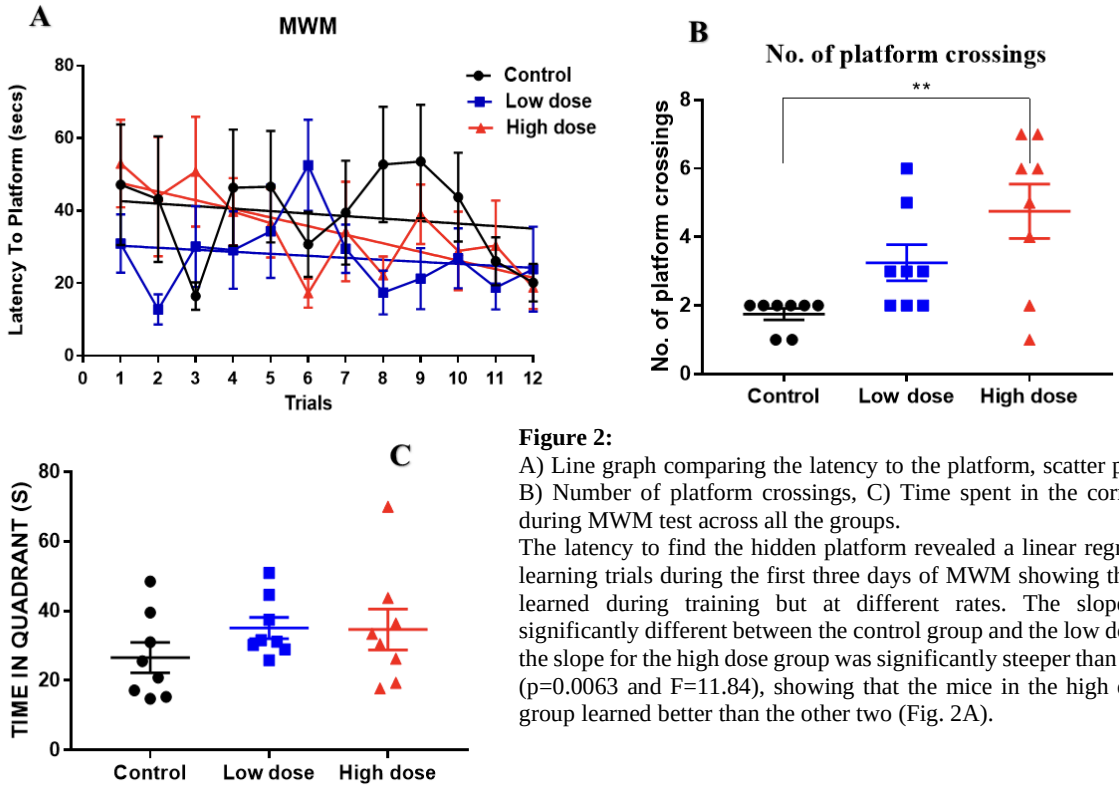

Figure 2:
A) Line graph comparing the latency to the platform, scatter plots showing B) Number of platform crossings, C) Time spent in the correct quadrant during MWM test across all the groups.
The latency to find the hidden platform revealed a linear regression in the learning trials during the first three days of MWM showing that all groups learned during training but at different rates. The slopes were not significantly different between the control group and the low dose group but the slope for the high dose group was significantly steeper than the other two (p=0.0063 and F=11.84), showing that the mice in the high dose caffeine group learned better than the other two (Fig. 2A).

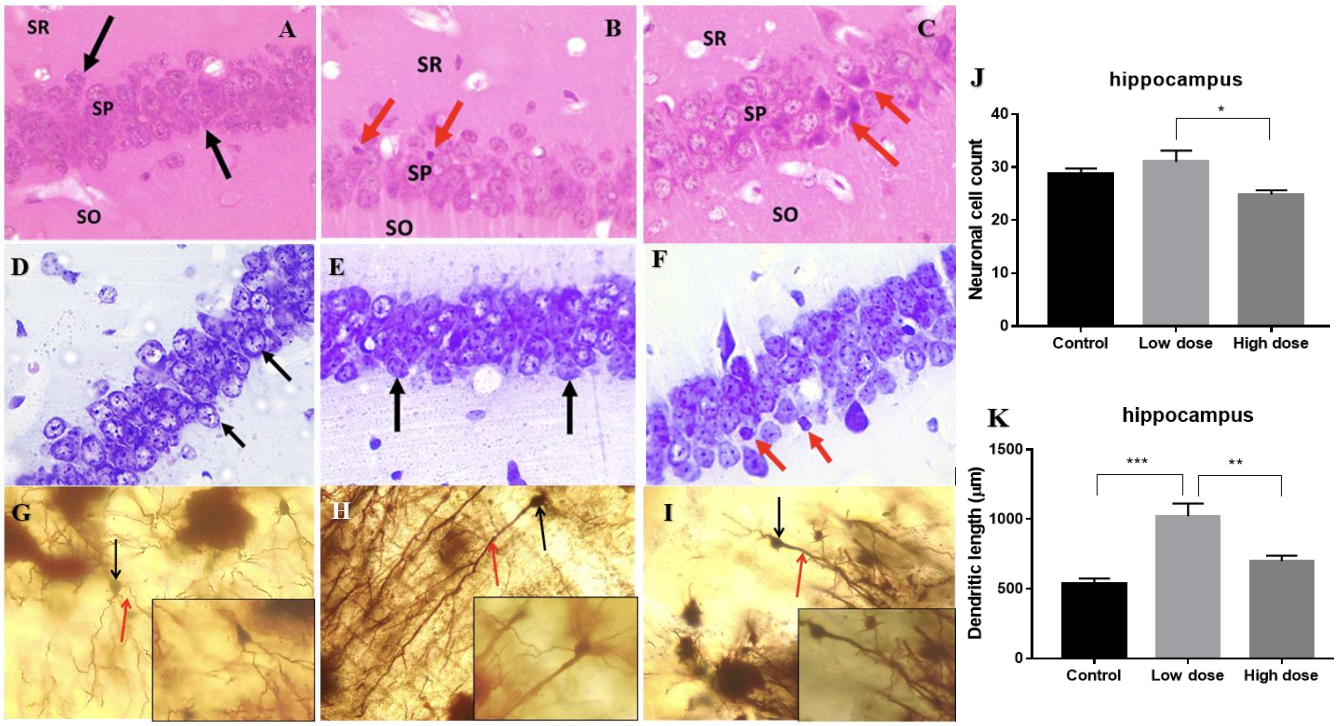

Figure 3:
Representative stained sections of CA1 region of the hippocampus of the mice brains. Top row: H&E stain (A) CNT; (B) LD; (C) HD. Middle row: Cresyl violet stain (D) CNT; (E) LD; (F) HD; Black arrow: normal pyramidal neurons; Red arrow: shrunken/darkly neurons undergoing pyknosis. Bottom row: Golgi stain (G) CNT (H) LD (I) HD. The black arrow shows the soma of the neuron while the red arrow shows the dendrites. (J) Bar chart representing the neuronal cell count across the three groups. (K) Bar chart representing the dendritic length across the three groups. Magnification: ×100(A-F); ×40 (G-I) SO: stratum oriens; SP: stratum pyramidialis; SR: stratum radialis.

The number of platform crossings is the measure of memory consolidation after the training trials in the Morris Water Maze test. The number of platform crossings was significantly higher in the high dose (4.75 ± 0.796) than the control (1.75 ± 0.164) and low dose (3.25 ± 0.523) groups. (F=7.2; p=0.004 and R^2 =0.407) (Fig 2 B). This showed that

memory retention of the mice in the high dose group was better than that of the mice in the low dose and control group. There was, however, no significant difference in time spent in the correct quadrant among animals in the three groups ($F=1.102$, $p=0.35$ and $R^2=0.09$) (Fig. 2C).

Histological Plates: The effect of caffeine on the cytoarchitecture of the hippocampus (CA 1) and amygdala were assessed. The brains of mice in the control and low dose groups had more compact, tightly packed neurons in the stratum pyramidialis of the CA1 region in the hippocampus than the ones in the high dose group which were sparse and appeared shrunken. Neuronal count was slightly increased in the low dose, but reduced in the high dose group, compared to the control. Thus, statistical analysis revealed a significantly decreased number of neurons in the high dose group compared to the low dose group ($p=0.03$) (Fig. 3).

The hippocampal pyramidal neurons in the control group had more arborization in the basal dendrites than the caffeine-treated groups, while the low dose caffeine group had longer apical dendrites (Fig. 3). The dendritic length of the hippocampal neurons of the mice in the low dose group was significantly greater than those in the control and high dose groups ($p<0.0003$).

Histological sections of the amygdala showed the intercalated cells especially in the control group with a lot of normal neurons. There was no visible difference in the structure of the cells across the groups. Cresyl violet photomicrograph shows black arrows that indicate normal neurons. The neuronal count in the amygdala of the mice in the low dose caffeine group was significantly less those in the control group ($p=0.014$) (Fig. 4).

DISCUSSION

In this study, we administered two different doses of caffeine to young adult male albino mice by oral gavage, as seen in Pechlivanova *et al,* (2012) and Olopade and Shokunbi (2018), for eight weeks. The high dose caffeine group exhibited significantly improved indices of learning and memory, and tended towards more anxious behaviour, compared to the control and low dose caffeine groups.

The high dose caffeine group learnt better with a steeper slope period than the other groups, and had better memory consolidation, shown by increased number of platform crossings. This contradicts previous studies like Angelucci *et al.,* (2002), who administered 10mg/kg of caffeine and Almosawi *et al.,* (2018), who gave 20mg/kg, however these two instances were in acute administration. Mahdi *et al.,* (2019) administered 20 mg/kg caffeine for one month and showed caffeine selectively increasing memory, but, in this study, only the high dose group showed significantly improved learning and memory. According to Dunwiddie and Masino (2001), it is very intriguing that unlike receptors of most of neurotransmitters, which once antagonised have pathological consequences, adenosine receptor antagonism apparently induces improvement in mental function and performance.

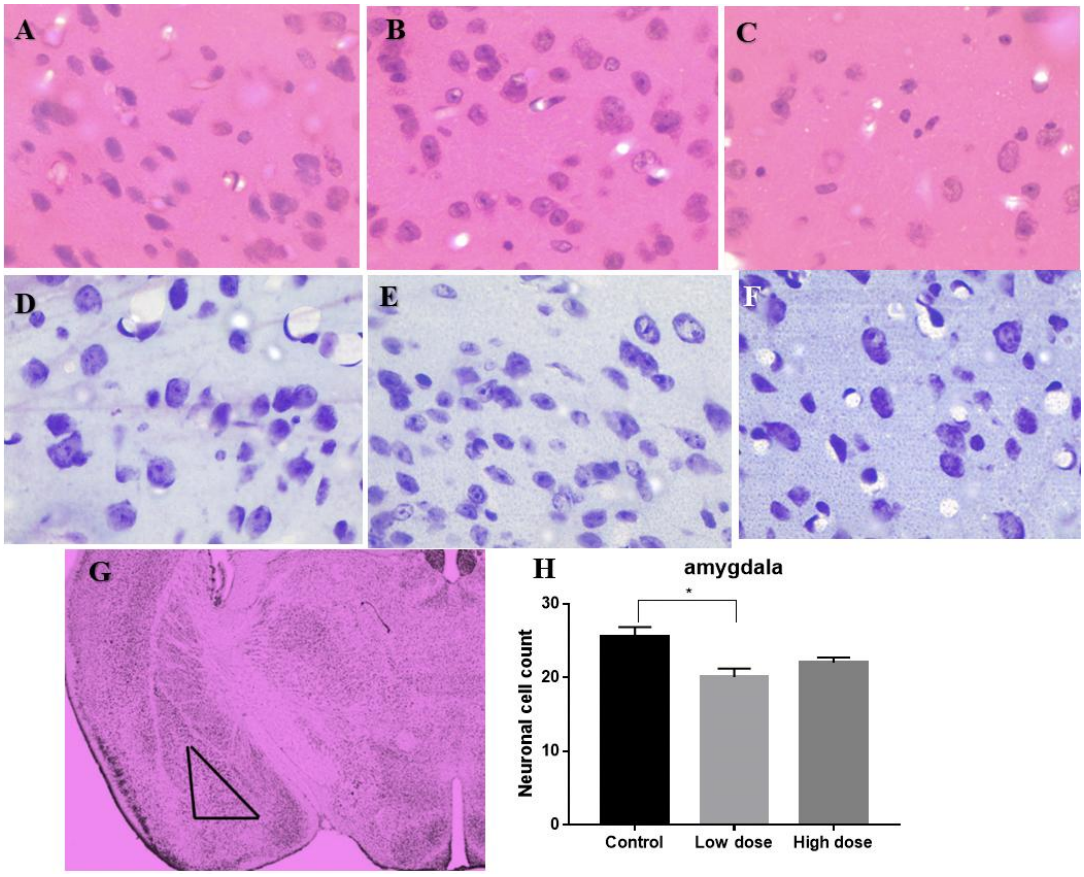

Figure 4:
Representative stained sections of the amygdala region of mice brain. Top row: H&E stain (A)CNT; (B) LD; (C) HD. Middle row: Cresyl violet stain (D) CNT; (E) LD; (F) HD; Black arrows indicate neuronal cells. Magnification ×100. Bottom row: H&E stain (G) Low magnification (x4) of the mouse brain, indicating the region of amygdala in a black triangle. (H)Bar chart representing the neuronal count across the groups.

The cognitive effects of caffeine are mostly due to antagonism of adenosine A1 receptors in the hippocampus and cortex, the brain areas mostly involved in cognition, however, caffeine also aids information processing and performance thus improving behavioural routines, arousal enhancement and sensorimotor gating (Fredholm et al., 1999).

The anxiety-like behaviours measured showed that experimental animals exhibited some degree of anxiety following caffeine administration, but its values did not reach significant levels. The mice in the low dose group spent the shortest time in the centre square of the open field, similar to the observations of Noschang *et al.,* 2009 and Vila-Luna *et al.,* 2012, supporting that subjects administered caffeine could develop anxiety in open spaces.

The Elevated Plus Maze (EPM) is considered sensitive to anxiety state of rodents, based on the principle that exposure to an elevated and open arm leads to an approach conflict that is stronger than that evoked by the closed arm (Pellow *et al.,* 1985). No significant differences were detected between control animals and those that had consumed caffeine in this study, but the high dose group spent the shortest time in the open arm of the EPM. This was similar to an earlier study which reported that chronic high dose caffeine treatment (50mg/kg) was associated with reduced time spent by mice in the open arms of an EPM in comparison with their controls (El Yacoubi *et al.,* 2000, Ardias *et al.,* 2014). It has been reported that long-term excessive caffeine intake can result in caffeine-induced anxiety disorders, which mimics organic mental disorders, such as panic disorder, generalized anxiety disorder, bipolar disorder, or even schizophrenia (Ribeiro et al., 2002).

The pyramidal cells in the CA1 region of the hippocampus were slightly increased in the low dose but reduced in the high dose group. The hippocampus is a region of the brain with high rate of expression of the adenosine receptors (Ribeiro *et al.,* 2002), which Ekong et al., 2017 reported had reduced hippocampal population cell count after chronic caffeine administration at 40mg/kg. The gradual loss of pyramidal neurons reported in the high dose group might be due inhibition of adult neurogenesis by caffeine as reported by Han et al., (2007).

In a human study carried out by Lanini *et al.,* 2016; acute caffeine dose of low to moderate consumption (25-300mg), was seen to sustain simple attention, improve subjective arousal and selectively enhance performance in executive updating (various cognitive abilities and behavior). Likewise, concerning chronic consumption, John-kozlow *et al.,* (2002) observed that elderly women (but not men) who consumed large amounts of caffeine throughout their lifetimes performed better on memory and other cognitive tasks than non-caffeine drinkers. These positive reports seen may be as a result of the increased cell numbers and increased length of the dendrites in the region of the brain concerned with memory such as we observed in the hippocampus in this study. Similar observations have been made in respect of the benefits of caffeine in prevention and alleviation of neurodegenerative disease such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Kolahdouzan and Hamadeh, 2017; Gökcen and Şanlier, 2019). Golgi-stained sections of the brains in this region revealed that hippocampal neurons of mice in the caffeine groups have significantly longer dendritic length compared to controls. This is similar to the report of Vila-Luna *et al.,* (2012).

The amygdala is a region of the brain concerned with emotions and anxiety symptoms (Vriend et *al*., 2016); recent discovery also links the amygdala to the sensorimotor cortex through functional circuits (Toschi *et al*., 2017). Examination of the amygdala in this study revealed normal neurons in both the control group and the experimental groups. However neuronal count revealed a reduction in the number of neurons in the low dose caffeine group. This reduction in neuronal count may be responsible for reduced oscillation stimulation pattern which may cause increased pathological functioning of the amygdala as seen in other antisocial personality disorder (Kolla *et al*., 2017; Henigsberg *et al*., 2019). In humans, high doses of caffeine have been shown to increase tension and symptoms of anxiety, nervousness and jitteriness (Stafford et al., 2007). Caffeine has also been reported to reliably decrease hand steadiness (Bovim *et al*., 1995) and hand tremors have been found to increase with caffeine users (Koller *et al.,* 1987). These behaviours may result from a reduction in the neuronal number as seen in this study, which thus alters structural and functional connectivity between the amygdala and other regions, like the medial prefrontal cortex (mPFC), as reported by Kim *et al*., 2011.

In this study, chronic caffeine consumption was shown to selectively bring about enhanced performance in learning and memory, but with a tendency towards anxiety-like parameters. When compared to acute administration of caffeine regardless of meal intake, caffeine of moderate doses (220-225 mg) decreased fatigue and improved simple and sustained attention and executive functions such as cognitive abilities responsible for the regulation of cognition and behavior (Lanini *et al*., 2016). Acute behavioural activities carried out by Almosawi *et al*., (2018) on mice with a similar concentration like this study but different administration route showed caffeine enhanced cognition and motor activities at low dose. Although the underlying mechanisms for the positive cognitive effect of chronic caffeine administration are not fully understood, Sallaberry *et al*., (2012) reported that modifications in brain-derived neurotrophic factor (BDNF) and related proteins in the hippocampus contribute to these effects on age-associated losses in memory encoding.

In conclusion, we have shown that the chronic administration of caffeine for eight (8) weeks promotes cognitive functions, learning and memory, but increased the level of anxiety in mice. These behavioural changes were associated with increased number and longer dendrites of hippocampal neurons but little structural change in the amygdala.

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Full-Length Research Article

Effects of Ruzu, a Polyherbal Mixture, on Neurobehaviour and Expression of Serotonin and Dopamine Transporters in Rats

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Summary: There is an increased possibility that combined herbal constituents may interact to increase toxicity and lower efficacy. Ruzu herbal bitters (RHB) is a blend of extracts from *Curculigo pilosa, Uvaria chamae*, and *Citrullus colocynthis*, each of which has been shown to possess important bio-effects. There is anecdotal evidence for efficacy of RHB in neurological disorders; however, there are no data on possible neurotoxic effects of RHB. Using behavioural, biochemical and molecular indices as surrogates of neurotoxicity, this study therefore evaluated the nervous system effects of RHB. Twenty male Wistar rats were grouped into two – a control group and RHB group (n=10). RHB (0.5ml/kg) was administered to the RHB group twice daily while control group took water (0.5ml/kg). Treatments lasted 6 weeks after which behavioural tests were carried out. Animals were subsequently sacrificed and the expression of serotonin transporter (SERT) and dopamine transporter (DAT) was determined in the striatum by immunofluorescence while specific activities of catalase, alkaline phosphatase and gamma glutamyltransferase were determined. In the elevated plus maze and light and dark box tests which are models of anxiety, animals treated with RHB showed significant anxiety compared to control. They also showed impaired locomotor activity in the open field and wire hang tests. The activity of catalase was significantly increased in the brain of the RHB treated rats while an increase in the expression of both DAT and SERT was observed in the striatum.

Keywords: *Ruzu, Polyherbalism, serotonin, dopamine, behaviour*

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INTRODUCTION

Polyherbalism started as a result of the inadequacy of the active phytochemical constituents of individual plants to provide attractive pharmacological action and attain the desirable therapeutic effects (Showande and Amokeodo, 2014). The concept of polyherbalism which involves the combination of several medicinal herbs in meticulous ratios has been proven to achieve greater therapeutic effectiveness (Karole *et al*. 2019). According to the World Health Organization, 80% of the world's population still rely majorly on the different species of plants for their health care (Parasuraman *et al*; 2014). Owning to cost effectiveness, perception of wonderful healing, curative, and preventiveness coupled with the notions for fewer side effects, the use of polyherbal formulations such as *Ruzu* herbal bitter is strongly encouraged in Nigeria (Oreagba *et al*. 2011).

Ruzu herbal bitters (RHB) is a poly-herbal mixture widely used as an anti-malaria, anti-typhoid, and anti-obesity concoction in Nigeria (Ogunlana *et al*. 2018). It has been claimed to be useful in the management of sexually transmitted diseases. It is also claimed to detoxify the kidneys, tone the liver, shrink fibroid tumor cells, lower high blood pressure and relieve waist, back and joint pains. As an aqueous preparation, RHB contains 40% *Curculigo pilosa* (Squirrel groundut) root, 20% *Uvaria chamae* (Bush Banana) stem, and 40% *Citrullus colocynthis* (Bitter Apple or Desert Gourd) bark.

The phytochemical investigation of *Curculigo pilosa*, which is used in the traditional treatment of arthritis, impotence, gastrointestinal and heart diseases, revealed that the plant has antioxidant, hepatoprotective, and neuroprotective activity (Nie *et al*. 2013). *Uvaria chamae*, has been reported to be effective in the treatment of sickle cell anemia, severe abdominal pains, cough, urinary tract and cerebral infections, and diarrhea (Oluremi *et al*. 2010). *Citrullus colocynthis* plant is widely known for its efficacy in the management of various diseases including diabetes, cough, asthma, jaundice, mastitis, cancer, leprosy, common cold, toothache, wound, joint pain, and gastrointestinal (Hussain *et al*. 2014).

The safety and efficacy of polyherbal mixtures has become a public health concern. The perception that "natural" equates to "safe" blinds users from the adverse effects of polyherbal mixture (Vidushi 2013). Although each of the RHB active constituents has been scientifically investigated and documented to be safe, the synergism in polyherbal mixtures such as RHB predisposes them to herb-herb interactions capable of affecting their pharmacological

or toxicological profile. Serious psychiatric and neurological effects have been associated with the use of herbal medicines (Ernst 2003). Furthermore, there are limited scientific data to support the claimed safety and therapeutic efficacy of polyherbal formulations like RHB. Hence, this study was designed to evaluate the neurobehavioural effects of *Ruzu* Herbal Bitters. This is important because the nervous system is a common site of natural products' toxicities (Harris & Blain 2004) and this toxicity is often manifested as both downstream (behaviour) and upstream (brain) disruptions. We evaluated the effects of RHB on anxiety, memory, and motor functions. It was also of interest to examine the expression of the serotonin and dopamine neurotransmitter transporters to have an insight into the molecular basis of any behavioural deviations caused by sub-chronic RHB.

MATERIALS AND METHODS

Animals: 20 Male Wistar rats (140g-160 g) were utilized for the study. The animals were acquired and housed at the animal house of the Department of Physiology, Ladoke Akintola University of Technology, Ogbomoso Oyo State, Nigeria. The animals were kept under standard laboratory conditions and housed in well ventilated plastic cages at room temperature and relative humidity with light and dark cycles (12hr/12hr). The animals were acclimatized for 2 weeks and were provided standard rat pellet and water *ad libitum* throughout the experiment. Animal management and experimental procedures were performed in accordance with the requirements of the university's guide for the use of laboratory Animals.

Materials: *Ruzu* herbal bitters (RHB) (NAFDAC Registration Number A7-1102L) was obtained from A2W Global Ltd., Lagos, Nigeria. Rat-reactive anti-SERT and anti-DAT rabbit polyclonal antibodies were purchased from Elabscience Biotechnology (USA). Anti-rabbit secondary antibody was purchased from R&D Systems (MN, USA). Every other reagents were of pure analytical grades from British Drug House (UK) and Sigma-Aldrich (USA).

Experimental design: After acclimatization, the animals were randomly grouped into two with each group made up of 10 animals. The first group (control) were given 0.5ml/kg of distilled water orally while the second group (RHB) received 0.5 ml/kg of RHB orally. This dose was extrapolated from the manufacturer-recommended adult daily dose. Treatment was twice daily (7am and 7pm) for six consecutive weeks. After the treatment period, animals were subjected to a battery of behavioural tests before being euthanized for whole brain harvesting.

Phytochemical screening: The qualitative phytochemical screening of *Ruzu* Herbal Bitters was conducted by adopting the methods as described by Odebiyi and Sofowora (1979) and Onwuka, (2005). The bioactive substances determined were Tannin, Saponin, Flavonoid, Cardiac Glycoside, Steroid, Terpenoids, Anthraquinones, Phenols, and Alkaloid.

Proximate Analysis: Proximate analysis was conducted on *Ruzu* Herbal Bitters to determine the nutritional value of the herbal bitter. Following the standard methods of Association of Official Analytical Chemists (AOAC, 2005), the proximate composition of the samples with respect to moisture content, Ash content, carbohydrate, crude protein, crude fiber, and crude fat were determined . Caloric value was calculated.

Behavioural Studies

Elevated plus maze: The elevated plus maze (EPM) has been documented to be very effective in assessing anxiety-like behavior (Komada *et al*., 2008). The EPM apparatus consists of two opposing open arms (50 × 10 cm) and two opposing closed arms (50 × 10 × 40 cm), elevated 50 cm above floor level. The arms are connected by a common central platform (10 × 10 cm) where the rat is placed. The edges, 6 mm high, surround the open arms, reducing the chances of rats falling from the apparatus.

Each animal was individually placed in the common central platform of the maze, with the animal facing one of the enclosed arms. Each animal was allowed to explore the maze freely for 5 mins. The rat's behavior was recorded by an overhead digital camera for the duration of the test and then video-analyzed by an assistant blinded to the study. The number of open and enclosed arm entries and time spent in each arm were scored. An entry is recorded when all four paws enter the arm. After each rat, the entire maze was cleaned using 70% ethanol before the next rat was put on the maze.

The numbers of entries into the open arms (OAE) and time spent in the open arms (OAT) reflect the general behavior of the rat. Anxiety-like behavior was deduced based on the less common entrance of the rat into the open arms of the maze and the decreased amount of time spent in the open arms of the maze.

Light and dark box test: The light/dark box test is based on the innate aversion of rodents to brightly illuminated areas and the spontaneous exploratory behavior of rodents in response to mild stressors like novel environment and light (Crawley and Goodwin 1980). The test is used to assess unconditioned anxiety and exploratory behavior. The light and dark box used was made of Plexiglass and consisted of two compartments which are equally divided. One compartment was covered outside with black cardboard making it the dark compartment while the other compartment was left uncovered making it the light compartment. The experiment was carried out in an isolated room away from noise, scents and movement.

Each animal was left to freely move from the first compartment to the second one through an open door between the compartments for 5 minutes. A rat was placed into the light box facing the open door, and the time the animal spent in each compartment was recorded by a video camera set up high above the plexiglass. The box was cleaned using 70% ethanol between rats to remove external scents, fecal deposits and urine.

Anxiolytic-like potential was deduced from increase in the time spent in the light chamber.

Open field test: In a bid to evaluated the general locomotor activity of the animals, they were subjected to an open field (OF) test. The OF apparatus is made of a wooden square box

(60 × 60 × 35 cm) and divided into nine equal squares (20 × 20 cm). Each animal was placed at the centre of the wooden box and allowed to explore the area freely for 5 minutes. Animals were filmed by an overhead digital camera. The distance walked in cm, analyzed for the whole arena (total distance) was quantified as the horizontal locomotor activity. The apparatus was cleaned with 70% alcohol after each trial.

Wire Hang Task: The wire hang task was used to assess forelimb strength as previously described (Jansone *et al*., 2016). Each rat was placed midway on a stainless-steel wire (90 cm length, 3 mm in diameter), mounted between two platforms at 60 cm above the ground. The performance was observed for a maximum of 2 minutes. Each rat was made to grasp the central position of the wire with its forepaws. The latency (s) to fall from the wire to the flat soft pad was measured. The trial was conducted three times for each rat with the longest duration used for the evaluation. Rats were allowed to rest for at least 3 minutes between consecutive attempts.

Oxidative Stress Status: Catalase Inhibition Assay was done to evaluate the oxidative stress status of the rats' whole brains. Catalase activity was determined using a colorimetric assay described in detail by Goth (Góth, 1991). The method is based on the formation of a yellow complex with molybdate and H_2O_2. Briefly, 50 µL of the supernatant of brain homogenate was added to 50 µL reaction mixture containing 50 mMH_2O_2 in sodium-potassium phosphate buffer (0.2 M, pH 7.4) in a 96-well microtiter plate. This was incubated for 3 minutes at 37°C. 100 µL ammonium molybdate (64.8 mM in H_2SO_4) was used to stop the enzymatic reaction, with the absorbance measured in a microplate reader at 405 nm (Micro READ 1000, Belgium).

Determination of Brain Markers: Activities of brain enzymes, such as gamma glutamyl transferase (GGT) as well as alkaline phosphatase (ALP) were assayed on a biochemical analyzer (Olympus AU-600, Tokyo, Japan) based on the protocols of commercial kits.

Immunofluorescence: Brain tissues from transcardially perfused (phosphate-buffered paraformaldehyde 4%) animals were removed and cryo-preserved in sucrose solution. Cryostat sections revealing the striatum were blocked with 1% BSA solution in PBS at room temperature for 60 minutes. Thereafter, the sections were incubated for 1 hour 30 minutes with rabbit anti-SERT/anti-DAT primary antibody which was diluted in blocking buffer (1:300). Subsequently, slides were incubated with anti-rabbit secondary antibody (1:200) for 60 minutes in a humidified dark chamber. Then slides were counterstained with Hoechst nuclear stain before being mounted with N-propyl gallate-supplemented glycerol/PBS solution. Mounted slides are stored at 4°C and away from light. Using SP-98-FL inverted fluorescent microscope (Brunel Microscope Limited), the slides were visualized. The images were analyzed, background noise was removed, and the mean optical density of the fluorescence signal were quantified with ImageJ software (NIH) to remove background noise and quantify.

Data Analysis: Video and data analyses were carried out by someone blinded to the experimental interventions. All values are expressed as mean ± SEM. Data were analyzed with GraphPad Prism, version 7.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Inter-group differences were analyzed with unpaired t-test while p values ≤ 0.05 were considered statistically significant.

RESULTS

Phytochemical screening: The result of the preliminary qualitative phytochemical screening of RHB showed that saponins, terpenoids, and alkaloids were strongly present in the polyherbal mixture. Flavonoids was found to be present in trace amount while tannins, cardiac glycosides, anthraquinones, steroids, and phenols were found to be absent.

Table 1
Phytochemical components of *Ruzu* herbal bitters

Component	Content
Saponins	++
Tannins	-
Flavonoids	+
Cardiac glycosides	-
Steroids	-
Anthraquinones	-
Terpenoids	++
Phenol	-
Alkaloids	++

Proximate analysis: Table 2 shows the proximate composition of RHB.

Table 2
Proximate composition of Ruzu herbal bitters

	Sample 1	Sample 2
Moisture (%)	74.44853	74.11395
CHO (%)	22.10301	22.45589
Calorific value (kj/100g)	476.7266	489.3274
Crude protein %	6.376324	6.778022
Crude fibre (%)	0.070667	0.071333
Ash (%)	0.525073	0.491159
Crude lipid (%)	0.029753	0.029735

Elevated plus-maze: As shown in Figure 1, RHB-treated animals had fewer open arm entries (RHB: 0.80±0.25 vs Control: 2.40±0.65; $p<0.05$), spent less time (seconds) in open arms (RHB: 6.90±1.48 vs Control: 24.60±2.70: $p<0.05$), and spent more time (seconds) in the closed arms (RHB: 295.90±1.28, $p<0.05$).

Light and dark box test: Figure 2 shows that the time (seconds) spent by the RHB-treated rats in the light compartment was less than that of the control (RHB: 130.40±11.33 vs Control: 151.40±10.23, $p>0.05$) although this was not statistically significant. Similarly, the RHB animals spent more time in the dark compartment than the control (227.20±7.52 vs Control: 215.50±9.22; $p>0.05$) although this also was not statistically significant.

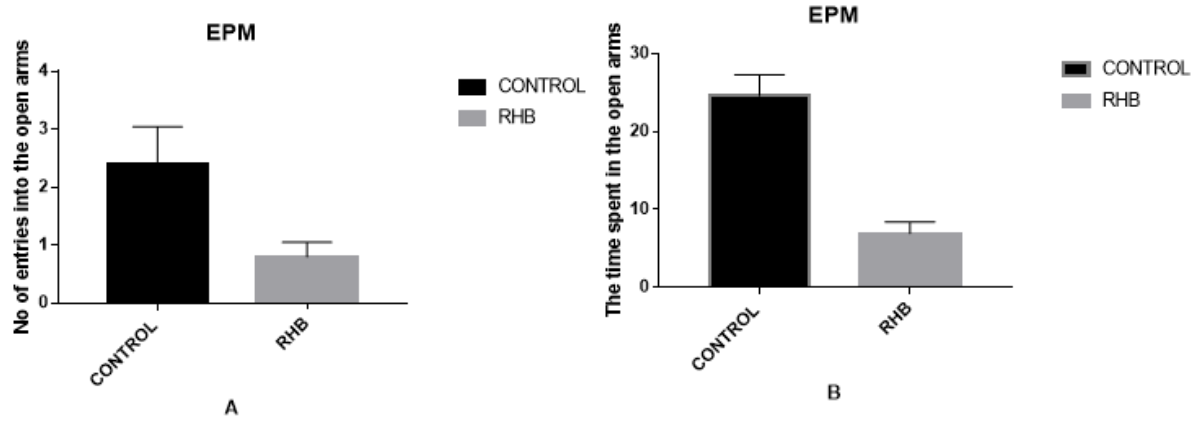

Figure 1
Elevated Plus Maze was used to asses anxiety. (A) The number of entries into the open arms during the test (B) The time spent in the open arms during the test. Histograms indicate the means±SD for the rats (n=10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters.

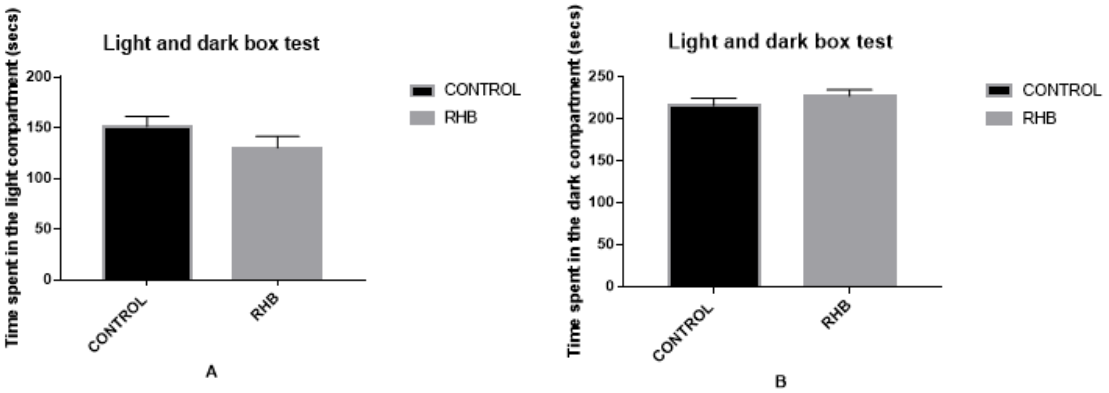

Figure 2
Light and Dark Box Test was used to assess anxiety. Histograms indicate the means±SD for the rats (n=10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters.

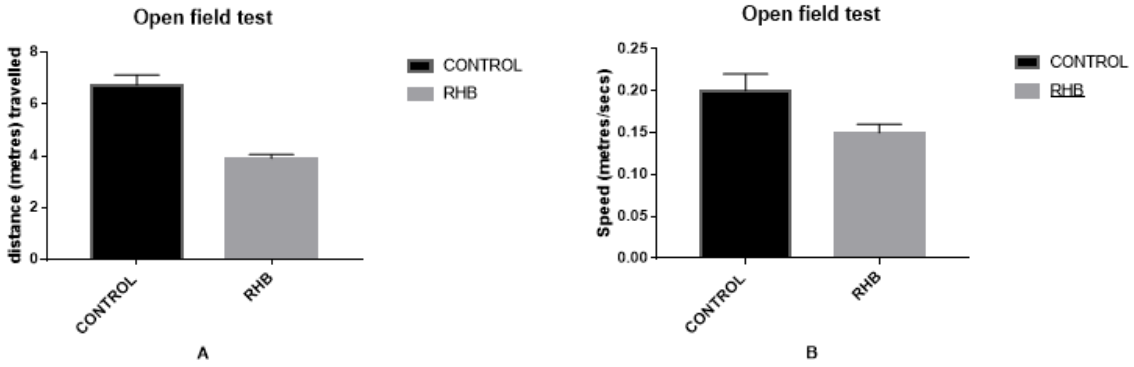

Figure 3
Open Field Test was used to assess general locomototry function. Histograms indicate the means ± SD for the rats. (n=10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters.

Open field test: The RHB animals displayed lower locomotive activity than the control as judged by the distance (metres) travelled (RHB: 3.90±0.15 vs Control: 6.73±0.40; p<0.05); they also moved at lower speed (RHB: 0.15±0.01 vs Control: 0.20±0.02, p<0.05) (Figure 3).

Wire hang test: The effect of Ruzu Herbal Bitters (RHB) on forelimb strength was assessed using the Wire hang task. As shown in figure 4, RHB animals displayed significantly lower wire-hang latency (seconds) than the control animals (RHB: 51.7±1.40 vs Control: 94.1 ±3.78, p<0.05).

Effects of RHB on oxidative stress markers in the brain: In the result of this study, the activity of Catalase in the brain of the RHB group (80.11±3.78 U/ mg protein) was significantly higher (p<0.05) compared to control group (41.2±5.172U/ mg protein) (figure 5). Alkaline phosphatase (ALP) was significantly lower RHB compared to control (RHB: 44.14±13.86 U/L vs Control: 63.34±8.654 U/L, p<0.05). The enzyme gamma glutamyltransferase (GGT) was also significantly lower in RHB than control group (RHB: 24.18±5.078 U/L vs Control: 29.3±5.86U/L, p<0.05) (Figure 6).

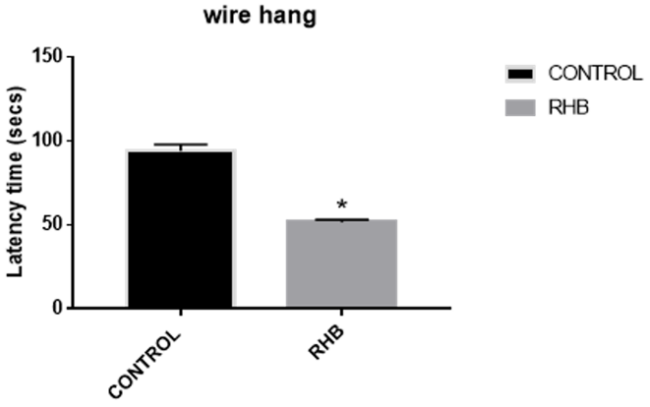

Figure 4
Wire Hang Task was used to assess forelimb strength. Histograms indicate the means ± SD for the rats (n = 10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters.

SERT and DAT expression: Immunofluorescence staining for DAT (Plate 1) and SERT (Plate 2) show that RHB animals had a statistically significant higher expression of DAT compared to control (RHB: 2.4±0.92 vs Control: 1.4±0.95, p<0.05) and a higher expression of SERT which was not statistically significant (RHB: 1.7±0.67 vs Control: 1.5±0.80, p>0.05) (Figure 7).

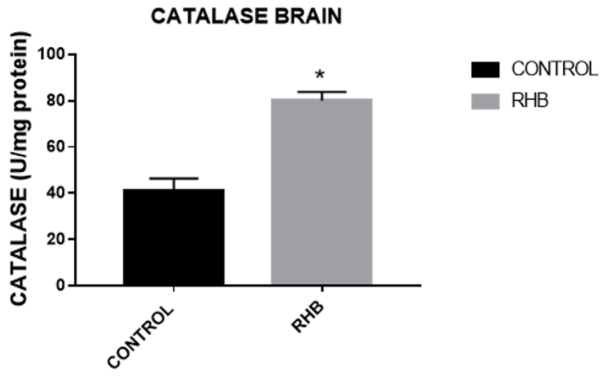

Figure 5
Effect of RHB on the activity of Catalase in the brain. Histograms indicate the means ± SD for the rats (n = 10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters

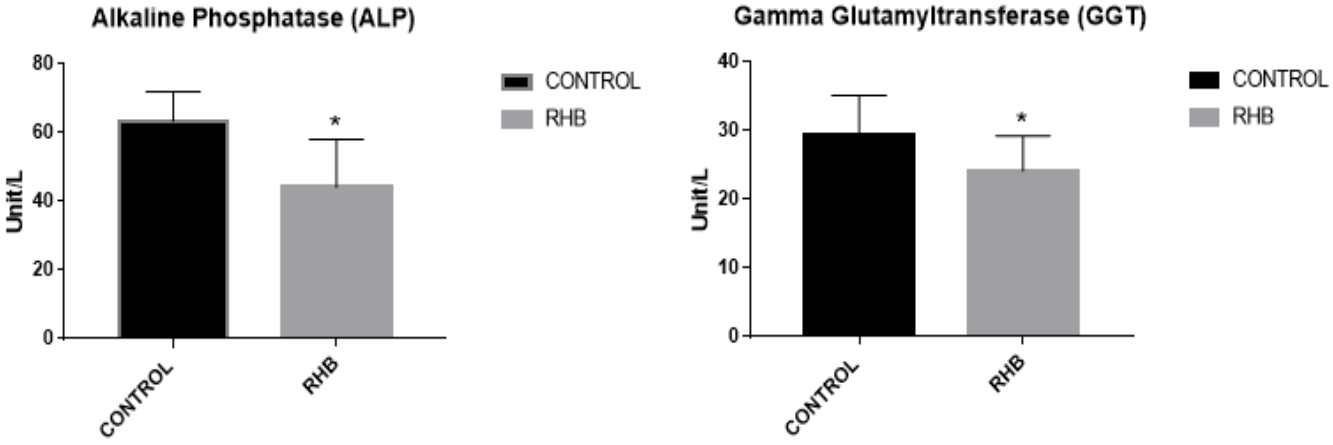

Figure 6
Effect of RHB on the activities of Alkaline Phosphatase and gamma Glutamyl transferase in the brain. Histograms indicate the means ± SEM for the rats (n = 10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters

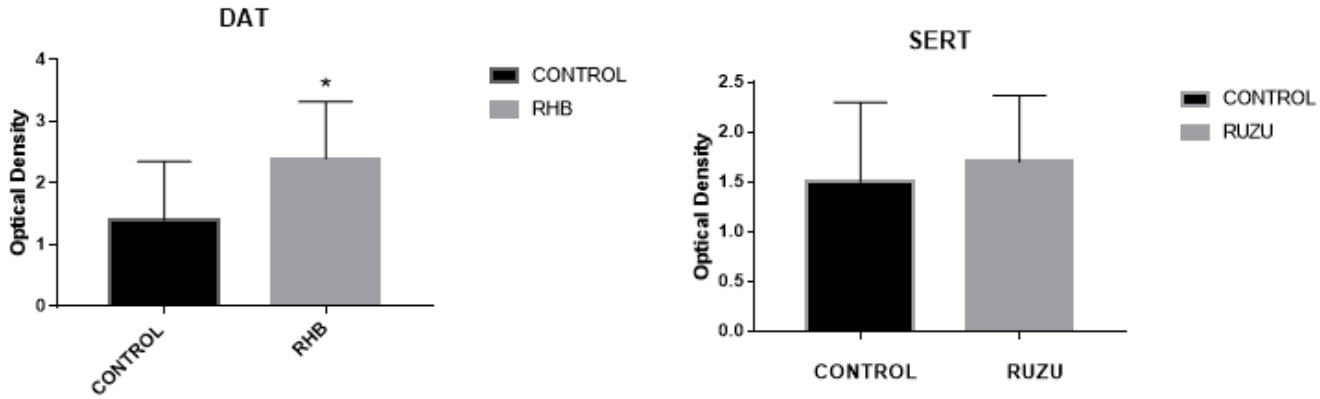

Figure 7
Effect of RHB on the expression of DAT and SERT in the brain. Histograms indicate the means ± SEM for the rats (n = 10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters

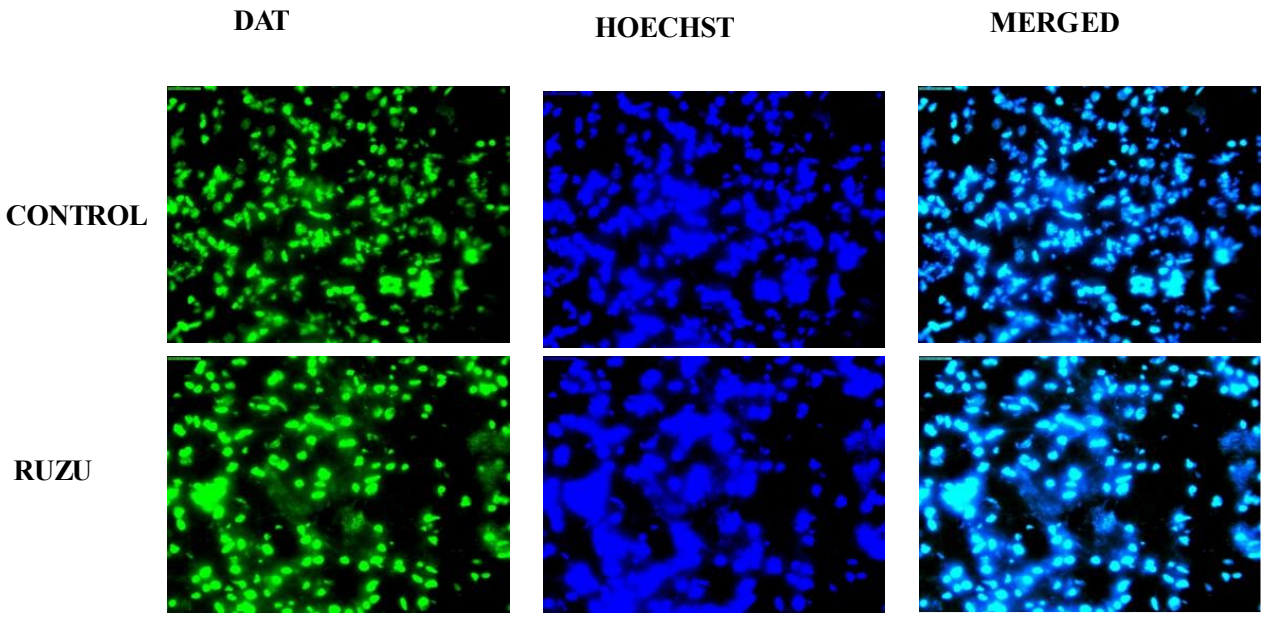

Plate 1
A photomicrograph of the brain of RHB treated animal compared with control animal showing DAT expression

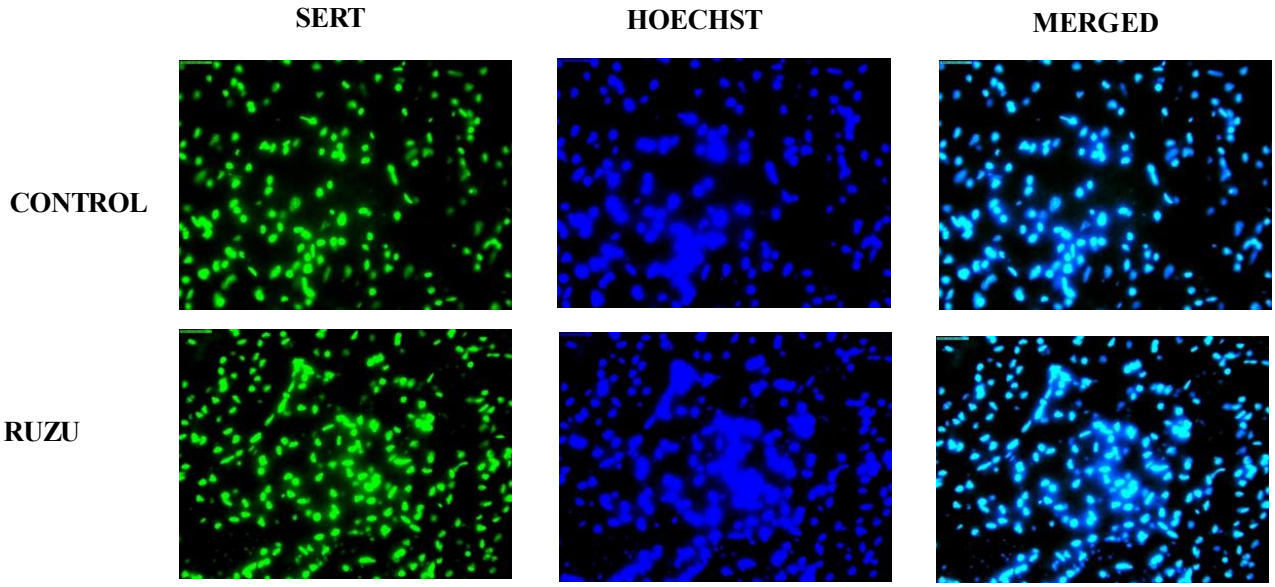

Plate 2
A photomicrograph of the brain of RHB treated animal compared with control animal showing SERT expression

DISCUSSION

The study evaluated possible neurotoxic effects of *Ruzu* Herbal Bitters (RHB) in rats by measuring animal behaviour, enzyme activities and neurotransporter expression as surrogates. Anxiogenic-like profile was observed in RHB treated rats; the observed anxiogenic-like profile is evidenced by the avoidance of the open arms by RHB-treated rats during the EPM. This behaviour is considered an anxiogenic behavior and a sign of anxiety (Belzung *et al*. 2001). In addition, animals treated with RHB spent lesser time and had fewer entries into the open arms, indicating an anxiogenic-like effect.

It is very likely that RHB contains extracts/active principles that could induce anxiety-like behaviour; some African herbal bitters have been similarly reported. For instance, the report of Woode *et al*., (2009), showed anxiogenic properties of a Ghanaian herbal extract with *Sphenocentrum jollyanum* as a major constituent. Aside compounded herbal bitters, individual herbal extracts such as *Agastache Mexicana* (Molina-Hernandez *et al*., 2000), *Visnea mocanera* (Hernandez-perez *et al*., 1995), *Myristica fragrans* (Sonavane *et al.,* 2002), and *Passiflora incarnata* (Elsas *et al.,* 2002) are known to elicit anxiogenic responses in animals. Interestingly, in some cases, these anxiogenic-like effects can occur side by side other positive effects such as anticonvulsant effects (Elsas *et al.,* 2002), and psychostimulant actions (Hernandez-perez *et al.,* 1995

The results of the Light and Dark Box Test (LDBT) showed that RHB-treated animals exhibited a preference for the dark compartment by spending less more time in the dark compartment and more time in the light compartment.

Although compared to the control animal the difference was not statistically significant, the data trend showed a clear leaning towards anxiety behaviour. Understandably, the LDBT suffers from high variability induced by age, weight, strain and genetics of animals (Bourin and Hascoet, 2003), but when combined with data from or physically with other tests such as EPM and open field, the data are a reliable predictor of anxiety-like states in rodents (Ramos *et al*., 2008). Therefore, put together with the EPM data, there is clear anxiogenic effects of RHB.

A decrease in locomotory and motor activity was observed in the rats treated with RHB; this was evidenced by the lower locomotive activity animals displayed during the open field test (OFT), an in vivo assay used in assessing the efficacy of therapeutic substances capable of improving locomotion and/or motor function (Malerba *et al*. 2011). The result of this study indicated that the rats treated with RHB covered shorter distances and also moved at lower speed. In addition, the rats displayed significantly lower wire-hang latency during the wire hang test (WHT), indicative of a decreased grip strength which can be attributed to a decrease in motor coordination and muscle tone (Carmela *et al*. 2012).

However, the data from OFT and WHT should be interpreted with a caveat: the possible sedative, depressant and muscle-relaxant activities of RHB were not independently studied. Depressant activity is physiologically linked to reduced motor activity (Bhosale *et al*., 2011) and this could account for reduced mobility and some muscular relaxation. This thinking is corroborated by the RHB phytochemical analysis data which show high presence of saponins, alkaloids, terpenoids and flavonoids. Several plants have been reported to have CNS depressant activity due to the presence of triterpenoids (Datta *et al*., 2004), saponins (Anandhan *et al*., 2010) and flavonoids (Datta *et al*., 2004; Anandhan *et al*., 2010). In addition, it has been hypothesized that triterpenoid saponins behave like benzodiazepines because of their agonistic/facilitatory activities at GABAA receptor complex (Chakraborty *et al*., 2010).

The activity of Catalase, a common enzyme which catalyzes the breakdown of hydrogen peroxide to oxygen and water, thus protecting the cell from oxidative damage caused by reactive oxygen species (ROS) (Chelikani *et al*. 2014) was significantly increased in the brain of the RHB treated rats, indicating that RHB has antioxidants capability. This correlates with the phytochemical investigation of one of the active components of RHB, *Curculigo Pilosa*, reported to have antioxidant activity (Nie *et al*. 2013).

Dopamine transporter (DAT) which controls the dynamics of dopamine (DA) neurotransmission by directing its extracellular reuptake has been implicated in the etiology of many diseases including depression and attention deficit (Vaughan & Foster 2013). RHB treated animals showed an increased expression of the protein DAT. This might be responsible for the observed behavioral decrease in locomotory and motor activity. This is in accordance with the study conducted by Vaughan & Foster (2013) which stated that over-expression of DAT causes a moderate increase in DAT function, leading to fine motor impairment. The serotonin transporter (SERT) which plays a central role in serotonin neurotransmission has been implicated in depression and anxiety (Ni *et al*. 2003). RHB treated animals showed a non-significant increase in the expression of the protein SERT. An increased expression of the protein SERT is most consistently associated with reduced anxiety-like behavior (Holmes *et al*. 2003; Murphy and Lesch 2008; Olivier *et al*. 2008). This contradicts the observed anxiogenic behaviour evidenced by the results of the EPM from this study. However, the motor impairment observed in RHB treated rats might be responsible for the observed anxiogenic behavior as changes in baseline locomotor activity is capable of presenting a difficulty when interpreting the results of anxiety tasks (Line *et al*. 2011). For instance, the reduced locomotive activity in RHB treated rats might be responsible for the reduction in open arm entries during the EPM and thus cause them to appear anxious.

We conclude that RHB produces anxiogenic-like behaviour in rats, and this might result from phytochemicals with depressant-like activities. This is however without prejudice to the clear antioxidant effects of RHB.

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Research Article

Carpolobia lutea Root Extract Improved Steroidogenic Activity in Male Wistar Rats Exposed to Cadmium

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Summary: Cadmium (Cd) is known to affect reproductive functions adversely. *Carpolobia lutea* is a protective herbal derivative due to its antioxidant potential. This study investigates the steroidogenic activities of methanol extract of *Carpolobia lutea* root on cadmium-induced reproductive toxicity in male Wistar rats. *Carpolobia lutea* root was obtained in Ijare via Akure. The plant was authenticated at the herbarium of Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria, with FHI number 109784. The methanol extract *Carpolobia lutea* root (MCL) was obtained by Soxhlet extraction. Thirty male Wistar rats (150-170g) were used in this study (n=5) and treated as follows: Control, Cd (2 mg/kg), Cd+MCL (2 mg/kg+100 mg/kg), Cd+MCL (2 mg/kg+200 mg/kg), MCL (100 mg/kg), and MCL (200 mg/kg). The extract was administered orally for eight weeks, and a single dose of 2 mg/kg Cd was given intraperitoneally. Serum Follicle Stimulating Hormone (FSH), Luteinizing hormone (LH), testosterone levels, testicular hydroxysteroid dehydrogenases (HSDs) activities and Steroidogenic Acute Regulatory protein (StAR) expression were evaluated. Data were subjected to descriptive statistics and analysed using ANOVA at $p<0.05$. Serum FSH, LH, testosterone levels, 3β-HSD, 17β-HSD activities and StAR expression were significantly reduced ($p<0.05$) in Cd group. The co-administration of Cd with MCL (200mg/kg) significantly increased ($p<0.05$) serum FSH, LH, testosterone levels, 3β-HSD, 17β-HSD activities and StAR expression when compared with Cd group. *Carpolobia lutea* root extract improved steroidogenic activity in male Wistar rats exposed to cadmium.

Keywords: *Carpolobia lutea, hydroxysteroid dehydrogenases, Steroidogenic Acute Regulatory protein, Hormone, Wistar rat*

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INTRODUCTION

Steroidogenesis is the enzymatic reactions leading to the synthesis of male steroid hormones. One of the functions of the testes is male sexual hormones (androgens) synthesis. Androgens, especially testosterone is required for normal male fertility, frequency and presence of sexual phantasies, morning erections, the frequency of copulation and sexual activity (Chang *et al*., 2004; Zhang 2006; Xu *et al*., 2007). The testicular functions can be affected by some factors. Heavy metals exposure is one of the environmental factors that can affect testicular steroidogenesis.

Many recent studies have suggested that pathogenesis of male infertility is attributed to oxidative stress (Akinola *et al*., 2020). Spermatozoa produce small amounts of reactive oxygen species (ROS) in physiological conditions and various scavengers help reduce the concentration of these ROS in the seminal plasma. But, increased production and/or decreased clearance causes oxidative stress within the sperm, leading to reduced motility (Kao *et al*., 2008) and defective membrane integrity (Agarwal *et al*., 2003). One of the reactive oxygen species inducers is environmental exposure to toxicants.

The roles of heavy metals in the aetiology of reproductive dysfunctions have been studied for several years (Patrick, 2003). Heavy metals are among one of the most widespread potential chemical contaminants in the environment (Ragan and Mast, 1990). Some heavy metals like lead, cadmium (Cd), mercury and cobalt are toxic to reproductive functions (Anderson *et al*., 1992). Effect of these metals exposure on the testis is of enormous interest as occupational exposure to them impaired reproductive activities (Afonne *et al*., 2002). Cadmium is one of the major heavy metals that increase in ecological systems through mining, smelting and industrial activities. The major sources of human exposure to this metal include food, cigarette smoke, alcoholic beverages and underground water (Jarup *et al.,* 1998). Testes are exquisitely sensitive to cadmium toxicity (Anders, 1990). Stoh *et al.* (2001), El-Demerdash *et al.* (2004) and Massanyi *et al.* (2007) reported that cadmium caused testicular damage, reduction in serum testosterone level, and reproductive dysfunction in Wistar rats. Acute cadmium chloride exposure causes significant reproductive dysfunction via generation of free radical and increased oxidative stress leading to histological alteration, (necrosis, edema etc.) and spermatological damage (decreased sperm motility and sperm concentration, and increased abnormal sperm cells) (Akinola *et al*., 2020). Cadmium toxicity is associated with severe damage to various organs,

particularly the testes, in both humans and animals (Fouad *et al*., 2009). Cadmium disrupt the reproductive capacity by causing serious testicular degeneration, seminiferous tubule damage and necrosis in rats (Burukoğlu and Bayçu, 2008).

Carpolobia lutea G. Don (family: Polygalaceae) is also known as cattle stick (English), Ikpafum (Ibibio, Southern Nigeria), Agba or Angalagala (Igbo, Eastern Nigeria) and Oshunshun (Yoruba, Western Nigeria) (Etekudo, 2003). It is a shrub of about 5cm tall and widely found in tropical Africa (Akpan *et al*., 2012). Ethno-botanically, various parts of the plant have been affirmed to be used in curing several diseases. The leaves have also been used to promote childbirth (Ajibesin *et al*., 2008; Muanya and Odukoya, 2008), while the root bark has been implicated to be used for treating rheumatism, fever, general pain and insanity (Ajibesin *et al*., 2008). The dried stem bark is usually taken as snuff to cure a migraine (Nwidu *et al*., 2011). Furthermore, the root decoction is reputed in Western and Southern Nigeria as ‘‘Ogun aleko’’ meaning sex stimulating tonic or aphrodisiac (Nwafor and Bassey, 2007; Ajibesin *et al*., 2008).

Aphrodisiacs modes of action are majorly classified into three types, which increase libido; potency; or sexual pleasure. *Carpolobia lutea* root extract has been used in folk medicines of different Nigerian cultures to energise, vitalise and improve sexual function, and physical performance in male (Yakubu and Jimoh, 2014; Dare *et al.*, 2015).

Therefore, the present study was designed to examine the possible ameliorative and protective effects of methanol extract *of Carpolobia lutea* (MCL) root against Cd-induced testicular dysfunction in male Wistar rats.

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MATERIALS AND METHODS

Plant Collection and Extraction: *Carpolobia lutea* root was obtained from Ijare a village *via* Akure, Ondo state. The plant was authenticated at the herbarium of Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria, with FHI number 109784. The root was air dried and pulverised. The pulverised root (5.20 kg) was subjected to Soxhlet extraction using pure methanol. The methanol root extract of *C. lutea* was concentrated at 4^0C in a rotary evaporator. The remaining extract was finally dried in a vacuum oven at 30^0C for 2 hours to ensure the removal of any residual solvent. The yield of the powdery mass was 87.88g (1.69% yields).

Animal and Experimental Design: Adult male rats of Wistar strain (150 to 170 gram) housed in well-ventilated cages in the Central Animal House, College of Medicine, University of Ibadan were used for this study. They were maintained under standard laboratory conditions of 12-hour light and 12-hour dark cycle and were fed with standard commercial rat pellets (Ladokun feeds Limited, Ibadan, Nigeria) and allowed access to water *ad libitum*. They were acclimatized for two weeks. The procedures in this study conformed to the guiding principles for research involving experimental animals as recommended by the Declaration of Helsinki as well as the Guiding principles in the Use and Care of Animals (American Physiological Society, 2002).

Thirty male Wistar rats were randomly divided into six groups with five animals per group and treated as follows: Group 1 were given distilled water (Control), group 2 were given Cd (2 mg/kg *b.w.*) single dose intraperitoneally, groups 3 and 4 were pre-treated with Cd (2 mg/kg *b.w.*) single dose intraperitoneally before treating with MCL 100 mg/kg *b.w.* and 200 mg/kg *b.w.*) orally for 8 weeks respectively, groups 5 and 6 were treated with MCL 100 mg/kg *b.w.* and 200 mg/kg *b.w.*) orally for 8 weeks respectively..

Twenty-four hours after the last administration, the animals were anaesthetised with 50 mg/kg *b.w.* of sodium thiopentone. Blood was collected from all the animals via cardiac puncture for serum levels of gonadotropin and testosterone. The testes were harvested and used for determination of androgenic enzymes activities and steroidogenic acute regulatory protein (StAR) expression.

Serum Hormone Analysis: Serum was obtained from a blood sample collected and used for testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) analysis. The analysis was carried out using an Enzyme-Linked Immunosorbent Assay (ELISA) based on the manufacturer’s manual. (Ulloa-Aguirre and Timossi, 1998; Oyeyemi *et al*., 2019).

Assay of Testicular Androgenic Enzyme Activities: The testicular 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was measured according to the method of Talalay (1962). The testicular tissue of each animal was homogenized in 15% spectroscopic grade glycerol (BDH, Mumbai, India) containing 5 mmol potassium phosphate (Loba, Mumbai, India) and 1 mmol EDTA (Organon, Calcutta, India.) at a tissue concentration of 100 mg/ml. The homogenized mixture was centrifuged at 10,000 × g for 30 min at 4° C. The supernatant (1 ml) was mixed with 1 ml of 100 µmol sodium pyrophosphate buffer (pH 8.9) and 30 µg of dehydroepiandrosterone (Sigma) in 40 µl of ethanol and 960 µl of 25% BSA (Sigma), making the incubation mixture a total of 3 ml. The enzyme activity was measured after addition of 0.5 µmol of NAD (Sigma) to the tissue supernatant mixture in a U 2000 spectrophotometer (Hitachi, Japan) cuvette at 340 nm against a blank (without NAD). One unit of enzyme activity was the amount causing a change in absorbance of 0.001/min at 340 nm.

The activity of testicular 17β-hydroxysteroid dehydrogenase (17β-HSD) was done according to Jarabak *et al*. (1962). The same supernatant prepared for the assay of 3β-HSD (above) was used. The supernatant (1 ml) was mixed with 1 ml of 440 µmol sodium pyrophosphate buffer (pH 10.2), 40 µl of ethanol containing 0.3 µmol of testosterone (Sigma) and 960 µl of 25% BSA (Sigma), making the incubation mixture a total of 3 ml. The enzyme activity was measured after addition of 1.1 µmol NAD (Sigma) to the tissue supernatant mixture in a U 2000 spectrophotometer cuvette at 340 nm against a blank (without NAD). One unit of enzyme activity was equivalent to a change in absorbance of 0.001/min at 340 nm.

Extraction and Amplification of Steroidogenic Acute Regulatory Protein Gene Expression by Polymerase Chain Reaction: Testicular DNA was purified using Zymo research DNA extraction kit. *Taq* Polymerase chain reaction (PCR) was carried out on purified testicular DNA samples. The DNA samples were amplified using

Steroidogenic Acute Regulatory protein (StAR) primer (Forward -CGT GGC TGC TCA GTA TTG AC and backward- AGT CCT TAA CAC TGG GCC TC) which was designed with Primer 3 software, and its specificity was ascertained with the help of National Centre of Biotechnology Information (NCBI)-Blast.

Digital Image Analysis: The PCR plate was analysed and quantified using ImageJ software (Version 1.49, National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis: Results are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to assess the statistical significance of the data, and Fisher´s Least Significant Difference (LSD) test was used for post hoc analysis (Multiple comparisons). P<0.05 was considered significant.

RESULTS

Effect of methanol extract of *Carpolobia lutea* root on serum testosterone level in male Wistar rats exposed to cadmium: Table 1 shows that serum testosterone level was significantly decreased (p<0.05) in Cd (2 mg/kg) and Cd+MCL (100 mg/kg) treated groups when compared with control. There was a significant increase (p<0.05) in serum testosterone level of MCL (200 mg/kg) group when compared with the control group. Serum testosterone level was significantly increased (p<0.05) in Cd+MCL (200 mg/kg) group when compared with Cd (2 mg/kg) treated group.

Effect of methanol extract of *Carpolobia lutea* root on serum follicle stimulating hormone level in male Wistar rats exposed to cadmium

Table 2 shows that serum follicle stimulating hormone was significantly decreased (p<0.05) in Cd (2 mg/kg) treated group, while there was a significant increase (p<0.05) in MCL (200 mg/kg) group when compared with control group. Alternatively, there was a significant increase (p<0.05) in Cd+MCL (200 mg/kg) group when compared with Cd (2 mg/kg) treated group.

Effect of methanol extract of *Carpolobia lutea* root on serum luteinizing hormone level in male Wistar rats exposed to cadmium: Serum luteinizing hormone was significantly decreased (p<0.05) in Cd (2 mg/kg), Cd+MCL (100 mg/kg) and Cd+MCL (200 mg/kg) treated groups when compared with control. Alternatively, there was significant increase (p<0.05) in Cd+MCL (200 mg/kg) group when compared with Cd (2 mg/kg) treated group (Table 1). .

Table 1:
Effects of Methanol Extract of *Carpolobia lutea* root on Serum Testosterone, Follicle Stimulating Hormone and Luteinizing Hormone in Male Wistar Rats Exposed to Cadmium

S/N	Groups	Control	Cd(2 mg/kg)	Cd+MCL (100 mg/Kg)	Cd+MCL (200 mg/Kg)	MCL (100 mg/Kg)	MCL (200 mg/Kg)
1	Testosterone	8.6±0.58	3.8 ± 0.52*	5.5±0.58*	8.5±0.86^{+}	8.3±0.46	11.8±1.01*
2	FSH	6.4±0.42	3.1 ± 0.47*	4.0±0.24*	7.4 ± 0.48^{+}	7.8± 0.76	12.5±1.26*
3	LH	12.1±0.44	7.9 ± 0.58*	9.3±1.11*	10.3±0.20*$^{+}$	11.8±0.37	12.3± 0.20

*Values expressed in mean ± SEM, *,+ p<0.05 show a significant difference when compared with control and Cd respectively.*

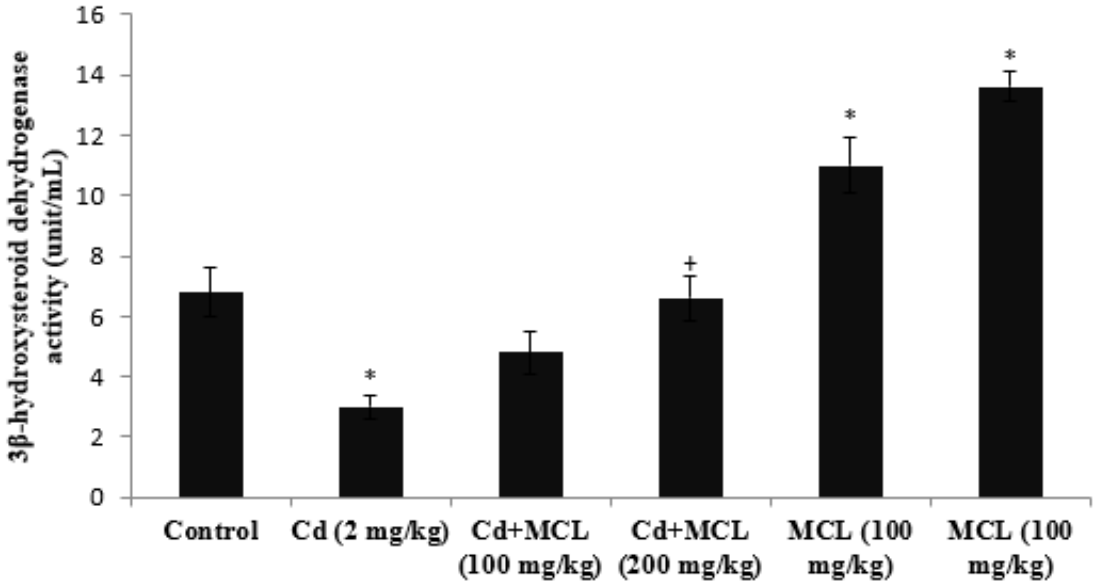

Figure 1:
Effect of Methanol extract of Carpolobia lutea root on 3β-hydroxysteroid dehydrogenase activity in male Wistar rats exposed to cadmium
*Data expressed in mean ± SEM, *,+ p<0.05 show a significant difference when compared with control and Cd respectively.*

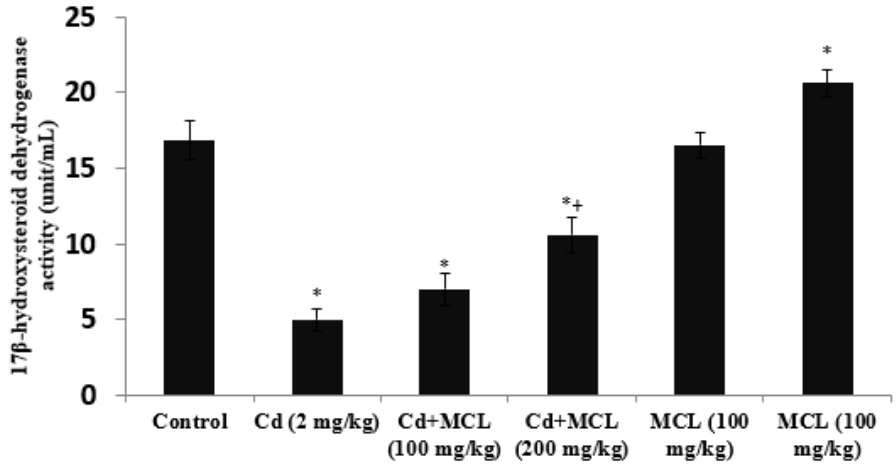

Figure 2:
Effect of methanol extract of *Carpolobia lutea* root on 17β-hydroxysteroid dehydrogenase in male Wistar rats exposed to cadmium
*Data expressed in mean ± SEM, *,+ p<0.05 show a significant difference when compared with control and Cd respectively.*

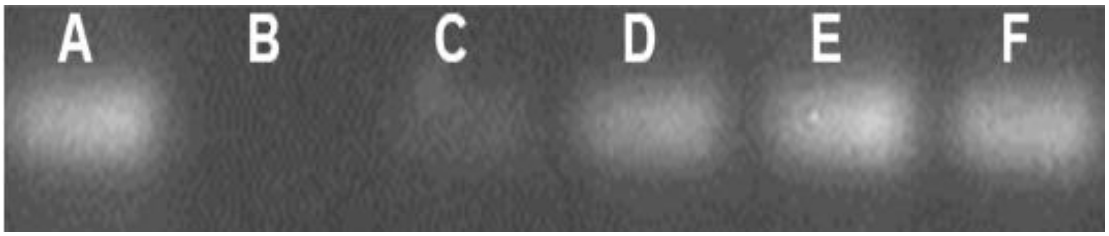

Plate 1:
Effect of methanol extract of *Carpolobia lutea* root on testicular steroidogenic acute regulatory protein expression in male Wistar rats exposed to cadmium
Lanes: A: Control; B: Cd (2 mg/kg); C: Cd+MCL (100 mg/kg); D: Cd+MCL (200 mg/kg); E: MCL (100 mg/kg) F: MCL (200 mg/kg)

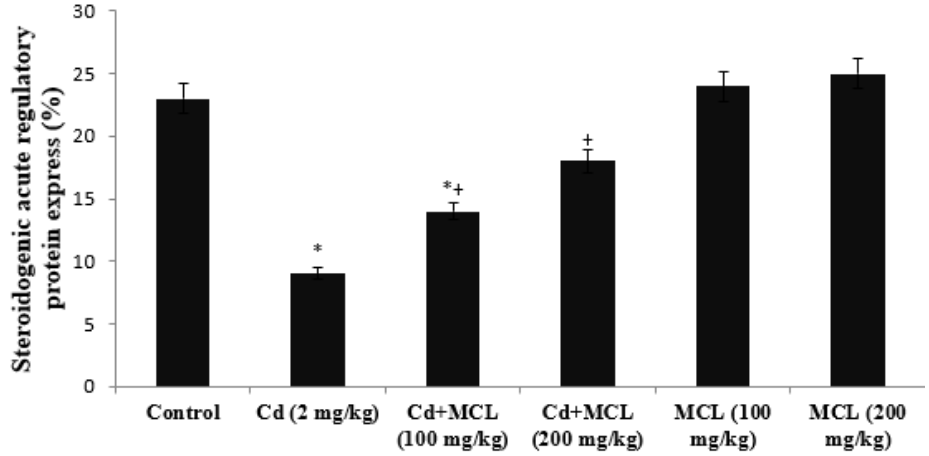

Figure 3:
Effect of methanol extract of *Carpolobia lutea* root on testicular steroidogenic acute regulatory protein expression in male Wistar rats exposed to cadmium
*Data expressed in mean ± SEM, *,+ p<0.05 show a significant difference when compared with control and Cd respectively.*

Effect of methanol extract of *Carpolobia lutea* root on 17β-hydroxysteroid dehydrogenase in male Wistar rats exposed to cadmium: Figure 2 shows that 17β-hydroxysteroid dehydrogenase activity was significantly decreased ($p<0.05$) in Cd (2 mg/kg), Cd+MCL (100 mg/kg) and Cd+MCL (200 mg/kg) treated groups when compared with control. Also, there was a significant increase ($p<0.05$) in MCL (200 mg/kg) when compared with control. Alternatively, there was a significant increase ($p<0.05$) in Cd+MCL (200 mg/kg) group when compared with Cd (2 mg/kg) treated group

Effect of methanol extract of *Carpolobia lutea* root on testicular steroidogenic acute regulatory protein expression in male Wistar rats exposed to cadmium: Plate 1 and Figure 3 show the qualitative RT-PCR products and relative expression of testicular steroidogenic acute regulatory protein (StAR) expression. Steroidogenic acute regulatory protein expression was moderately expressed in the control, Cd+MCL (200 mg/kg), MCL (100 mg/kg) and MCL (200 mg/kg) groups while it was mildly expressed in Cd (2 mg/kg) and Cd+MCL (100 mg/kg) groups respectively (Plate 1). The figure 3 shows that the StAR expression was significantly reduced ($p<0.05$) in Cd (2 mg/kg) and Cd+MCL (100 mg/kg) groups when compared with control group, while significant increase ($p<0.05$) was observed in Cd+MCL (200 mg/kg) group when compared with control.

DISCUSSION

The present study demonstrated that Cd toxicity induced serious alterations in the testes, which were protected against by co-administration of methanol extract of *Carpolobia lutea* root. Cd administration induced significant decreases in the serum levels of testosterone, FSH, LH, androgenic enzymes activities and expression of steroidogenic acute regulatory protein (StAR) in the rat. Furthermore, methanol extract of *Carpolobia lutea* root ameliorated all alterations induced by Cd toxicity. Cadmium is a toxic metal that is widely distributed in the environment (Singh *et al*., 2012). Humans are exposed to Cd toxicity by either inhalation or ingestion; skin absorption of Cd is relatively insignificant (Zalups and Ahmad, 2003; Mead, 2010). Cd is reported to be contained in industrial emissions, cigarette smoke and agricultural fertilizers (Zalups and Ahmad, 2003). It facilitates early oxidative stress, disrupts hypothalamic pituitary testicular axis and testicular steroidogenesis (Massanyi *et al*., 2007). Cd accumulation may also occur in the testes, where it causes testicular oxidative stress by two mechanisms i.e. by reacting with the sulfhydryl groups of various proteins or by glutathione depletion (Valko *et al*., 2005). It has also been previously reported that Cd acts through overproduction of ROS and enhanced lipid peroxidation (Sen Gupta *et al*., 2004). In addition, chronic Cd toxicity leads to impairment of the H_2O_2 removal system, which leads to inhibition of steroidogenesis in the Leydig cells due to an accumulation of H_2O_2 (Diemer *et al*., 2003). The result of this study showed a significant reduction in serum testosterone level in cadmium-treated rats. This result is consistent with the previous reports of Habeebu *et al*. (1998), Stoh *et al*. (2001), Massanyi *et al*. (2007) who reported a reduction in testosterone levels and reproductive dysfunction in Wistar rats exposed to cadmium. Also, Yang *et al*. (2003) and El-Demerdash *et al*. (2004) also reported that in the male gonad, cadmium could cause testosterone suppression, failure of spermiation, reduced sperm motility, increased incidence of Leydig cell tumours, and at high doses testicular damage. The marked decrease in the serum levels of testosterone detected in the present study may also possibly be due to Cd-induced decreased synthesis and availability of cholesterol for steroidogenesis. Consequently, decreases in cholesterol biosynthesis result in downregulation of steroid biosynthesis (Barlow *et al*., 2003). Testosterone level was significantly increased in cadmium group treated with high dose of C. lutea extract. Cadmium has been proposed to reduce testosterone production by testes through feedback inhibition of hypothalamic-pituitary-testicular axis (Waalkes *et al*., 1997). Therefore, treatment of C. lutea root extract with cadmium may prevent the feedback inhibition of the hypothalamic-pituitary-testicular axis, and hence facilitate testosterone synthesis in the testes. C. lutea root extract may avert the inhibitory effect of cadmium in testosterone biosynthesis in Leydig cells. It has been reported that cadmium decreases serum testosterone level in male Wistar rats and this may be a reflection of the accumulation and direct toxic effect of the cadmium in the testes (Salama and El-Bahr, 2007).

Cadmium is known to directly destroy the hypothalamus-pituitary-gonadal axis, thus destroying the secretory organs of hormones (Massanyi *et al*., 2007) and compromising hormonal release. As regards the serum levels of the gonadotrophins, both follicle stimulating hormone (FSH) and luteinizing hormone (LH) were significantly reduced in cadmium-exposed rats. The reduction in serum FSH and LH in cadmium-exposed rats may indicate destruction in the pituitary testicular axis (Sadik, 2008). The observed reduction in serum FSH and LH hormonal level of cadmium-exposed rats may be responsible for observed decreased in serum testosterone since gonadotrophins induce the signals for testosterone synthesis (Habeebu *et al*., 1998; Massanyi *et al*., 2007). The administration of *Carpolobia lutea* root extract reversed observed reduction in serum FSH and LH in cadmium-exposed rats. The antioxidant activity of *Carpolobia lutea* leaf extract (Nwidu *et al*., 2012) may be responsible for protecting pituitary gland from cadmium toxic and enhance gonadotrophins secretion.

In testicular steroidogenesis, 3β - and 17β - hydroxysteroid dehydrogenase are the prime enzymes that play a critical regulatory function in testicular androgenesis (Ghosh *et al*., 1990; Jana and Samanta, 2006). The diminution in these enzymes by cadmium treatment in our study is in agreement with the findings of others (Sen-Gupta *et al*., 2004; Sadik, 2008). A decrease in serum concentration of testosterone in cadmium-treated rats may occur due to the inhibition of these testicular androgenic enzymes activities because these enzymes are involved in the regulation of testosterone biosynthesis (Ghosh *et al*., 1990; Jana *et al*., 2005). The activity of *C. lutea* root extract in improving these steroidogenic enzymes activities may be associated with cytochrome P450 side-chain cleavage complex (P450scc) because cadmium has been reported to decrease (P450scc) (Sen-Gupta *et al*., 2004). Also, C. lutea root extract may prevent cadmium to bind with the thiol group of proteins and enzymes (Hassoum and Stohs, 1996) hence increase the activity of 3β - and 17β - hydroxysteroid dehydrogenase. The observed increase in serum testosterone level in *C. lutea* root extract co-administered with cadmium treated rats may be due to the stimulatory action of these testicular androgenic enzyme activities since these enzymes are responsible for the regulation of testosterone biosynthesis (Sen-Gupta *et al*., 2004, Sadik, 2008). Moreover, the increase of testicular androgenic enzymes in animals co-treated with C. lutea root extract and cadmium may be as a result of raised in serum level of LH, the prime regulator of testicular androgenic enzymes activities (Kerr and Sharpe, 1986).

The steroidogenic acute regulatory (StAR) protein has been shown to perform a critical function at this step via the transfer of cholesterol (Lin *et al*., 1995). It has been reported that a decrease in StAR activity is associated with a decrease in steroidogenesis (Adel *et al*., 2016). In this study, the steroidogenic acute regulatory protein (StAR) was mildly expressed in the cadmium treated rats. Zhang and Jia, (2007) opined that mechanistic studies have revealed that cadmium inhibits progesterone synthesis in granulosa cells by down-regulation of StAR and p450scc. The same article reported that co-treatment with 8-bromo-cAMP blocked the decline in progesterone secretion, indicating that cadmium exerts its action by interfering with cAMP synthesis and signalling, which in turn leads to a reduced expression of StAR and p450scc. The delivery of substrate cholesterol to the inner

mitochondria membrane in which the P450SCC enzyme is located is considered to be the rate-limiting step in steroidogenic cascade (Stocco and Clarke, 1996). The upregulation of StAR expression in the C. lutea root extract plus cadmium may be responsible for the observed increase in the serum testosterone level. Steroidogenic acute regulatory protein (StAR) up-regulation by C. lutea extract might be attributed to its ability to prevent interference of cadmium with testicular luteinizing hormone receptor, messenger ribonucleic acid, and cyclic adenosine monophosphate synthesis and signalling (Lin *et al*., 1995; Gunnarsson *et al*., 2003; 2007).

From the present results, it can be concluded that administration of methanol extract of C. lutea root can improve testicular steroidogenesis in cadmium-exposed male Wistar rats via steroidogenic activity and gonadotrophin.

In conclusion, methanol extract of C. lutea root ameliorated Cd-induced alterations in reproductive hormones, androgenic enzymes activity and StAR protein expression. Future studies are required to outline the direct beneficial effect of methanol extract of C. lutea root and its availability for treatment of testicular dysfunction during Cd toxicity.

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Full Length Research Article

Ethanol Extract of *Camellia sinensis* Elicited Hypoglycemic but Lacked Antimalarial Properties in *Plasmodium berghei*-Infected Diabetic Mice

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Summary: The *in vivo* antimalarial and antidiabetic potential of *Camellia sinensis* (ECS) extract in alloxan-induced diabetic and *Plasmodium berghei*-infected mice were investigated. Eighty-four BALB/c mice divided into sets 1 & 2 infected with *P. berghei* and 2 & 3 injected with alloxan received either distilled water, ECS (300mg/kg), Chloroquine (CQ-10mg/kg) or Metformin (250mg/kg). Results showed significant increases ($p<0.05$) in percentage parasitaemia of *P. berghei*-infected mice treated with ECS and *P. berghei*-diabetic mice. Furthermore, ECS significantly decreased ($p<0.05$) blood glucose and PCV in diabetic and *P. berghei*-diabetic mice. *ECS* regenerated pancreatic islet cells in *P. berghei*-infected-diabetes but lacked appreciable antimalarial activity.

Keywords: *antimalarial activity, hypoglycemic activity, ethanolic extract of Camellia sinensis, Plasmodium berghei, mice*

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INTRODUCTION

The high incidence and associated detrimental effects of diabetes mellitus has been a major medical challenge; it results to the poor physical and psychological state as well as the attendant morbid condition in diabetic patients (Moodley *et al.*, 2015). WHO reported the increased number of diabetics in double-fold in the past few years with 9% of total population above 18 years suffering from this disease (Alwan, 2010). If there is no improvement, it is projected that by year 2030, the number of diabetic patients would be 552 million (Whiting *et al.*, 2011). In Nigeria, diabetes mellitus is the commonest endocrine disorder in medical practice and is the major cause of morbidity and mortality with grave personal, social and economic consequences (Kim *et al.*, 2009). Diabetes is a heterogenous disorder characterized primarily by impaired hormone secretion, fat, protein and carbohydrate metabolism due to insufficient amount of insulin production or reduced sensitivity of tissue to insulin (Pistrosch *et al.*, 2015). It is associated with secondary complications including hypertension, neuropathy, retinopathy, cardiomyopathy, atherosclerosis, stroke, coronary ischemia etc (Rutter and Nesto, 2011). The generation of reactive oxygen species through the pro-inflammatory cytokine pathways has been implicated in the exacerbation of the secondary complications of diabetes (Chow *et al.*, 2004). Several reports have further demonstrated reduction in antioxidant activities in diabetes condition with consequent progression into oxidative stress leading to diabetic complications (Lopes de Faria *et al.*, 2011).

Camellia sinensis has received much attention because of its beneficial health effects such as anti-oxidant, anti-carcinogenic (Rashidi *et al.*, 2017; Jayashree *et al.*, 2017), and antimicrobial activities (Nibir *et al.*, 2017). It also contains phytochemicals with neuro-protective activity (Bai *et al.*, 2017; Ben *et al.*, 2016). The anti-infection (Nibir *et al.*, 2017) and antiviral (Burkard *et al.*, 2017) potential of *Camellia sinensis* catechins have been documented (Burkard *et al.*, 2017). As a reno-protective agent, green tea ameliorates diabetes and the related-complications whereas its flavonoids inhibit angiogenesis in several diseased settings including diabetic retinopathy (Peixoto *et al.*, 2015). Also, hyperglycemia and diabetes complications are controlled by EGCG which lowers insulin level, and plasma glucose as well as liver and kidney weights (Sampath *et al.*, 2017). Re-sensitization of insulin-resistant muscle to insulin by EGCG was recently studied (Pournourmohammadi *et al.*, 2017). *Camellia sinensis* was also reported to have a protective effect on experimental pancreatitis and promotes insulin sensitivity (Nomura *et al.*, 2015).

Recently, *Camellia sinensis* extract and its individual catechins have shown antimalarial activity against asexual blood-stage parasites (Sannella *et al.*, 2007). In malaria endemic region like Nigeria, there are incidence of diabetic patients that were presented with malarial infection. The availability of glucose for cellular functions becomes a problem during malaria infection. However, there is dearth of information on the effects of *C. sinensis* in *Plasmodium berghei* infected diabetic mice especially this particular specie that was grown in Nigeria expected to have certain characteristics different from the imported ones resultant from factors including geographical location and period of harvest etc which may influence pharmacological properties. Therefore, the purpose of this study was to evaluate the effect of ethanol extract of *Camellia sinensis* in *Plasmodium berghei* infected diabetic mice.

MATERIALS AND METHODS

Animal maintenance: Male albino mice weighing 23-28g were obtained from Central Animal House of the University

of Ibadan. The animals were acclimatized for two weeks before commencing the experiment. The animals were divided into nine groups of 8-10 per cage and fed with clean water *ad libitum* under a 12-hour light/dark cycle.

Preparation of ethanol extract *of Camellia sinensis* (ECS): *Camellia sinensis* extract was prepared as described by (Babu *et al.,* 2002). Briefly, the leaves were obtained from Cocoa Research Institute of Nigeria, Ibadan, Oyo State, Nigeria. The leaves were air dried and powdered in a miller. The powdered tea was extracted with 95% ethanol (for a high recovery of catechin) for 2 days with constant marceration and filtered on a Whatman No 1 filter paper. The pooled tea solution was evaporated in vacuo below 45^0C to obtain a residue. The dried extracts were stored at 4^0C.

Antidiabetic activity: Diabetes was induced in male mice fasted for 24 hours by single intraperitonial injection of 200 mg/kg body weight of alloxan monohydrate dissolved in 0.01M sodium citrate buffer (pH 3.0) (Xi-Qun *et al.,* 2005). Blood glucose levels of mice were determined after 72h with AccuCheck glucometer and animals with blood glucose levels $\geq$200mg/dl were considered diabetic and used for the study (Elased *et al.,* 1996). The diabetic mice were divided into six groups (IV – IX) of ten mice each as follows; Group IV diabetic mice (untreated) were given distilled water, Group V and VI were given 300mg/kg body weight ECS and 250 mg/kg body weight metformin, respectively. Non fasting blood was collected daily from these mice throughout the treatment and a week post-treatment. All treatments were administered orally to the mice. The remaining 3 groups (VII–IX) of diabetic animals were inoculated with malarial parasites and received treatments as stated below under antimalarial activity.

Induction of parasites and antimalarial activity: Mice in groups I-III (Healthy, n=10) and VII-IX (diabetic, n=10) were innoculated with a chloroquine sensitive *Plasmodium berghei* (ANKA strain). After 72hrs post-infection, thin films were made, fixed with methanol and stained with 10% Giemsa stain and used to monitor the parasitemia daily using X100 magnification of a light microscope. Treatments commenced when about 10% parasitemia were established in the mice which took about 72 hours. Animals in groups I and VII were administered distilled water, groups II and VIII received 300mg/kg ECS while groups III and IX received chloroquine.

Blood collection: Blood samples were collected from the tail of the mice in order to determine percentage parasitemia, packed cell volume (PCV) and blood glucose level. Two mice were sacrificed before treatment, 24 hours after treatment and a week post-treatment in all the groups. The liver and pancreas of these animals were collected and stored in 10% formalin and later used for histopathological analyses.

Histopathological evaluation: Tissue samples from the liver and pancreas were trimmed, processed by paraffin wax sections of 4µm thickness cut, stained with haematoxylin and eosin for light microscopic examination. Photomicrographs of relevant stained sections were taken and documented.

Statistical Analysis: Data were analyzed using the GraphPad prism v6.0 (Graphpad software, San Diego, CA, U.S.A). P-values less than 0.05 were considered significantly different. All the values were expressed as mean ± standard deviation.

RESULTS

Figure 1A shows the effect of treatment with ECS and chloroquine in malaria infected mice. The parasite infected untreated group had their parasitaemia increased with days of infection until death while the parasite infected mice treated with the standard drug, chloroquine had a rapid decrease in parasitaemia in the course of treatment compared with ECS treatment that had no effect on parasitaemia. Percentage parasitaemia in infected untreated group was observed to increase significantly ($p< 0.05$) on days 4 to day 7 when compared with day 0. Conversely, in infected chloroquine treated group, it decreased significantly ($p<0.05$) on day 4 and had completely cleared by day 14. A significant increase ($p<0.05$) in percentage parasitemia was also observed in malaria – ECS (300mg/kg) treated mice on days 4,5,6,7 and 14 when compared with day 0. The same pattern was observed (Figure1B) in parasitised diabetic mice given the same treatment.

Table 1 shows that malaria infected diabetic animals untreated group had the least mean survival time while the malaria-infected chloroquine treated animals had the highest mean survival time. The mean survival time for malaria-infected -diabetic animals treated with *Camellia sinensis* extract was low and similar to that of malaria untreated group. Similar pattern was observed in untreated parasitized diabetic mice.

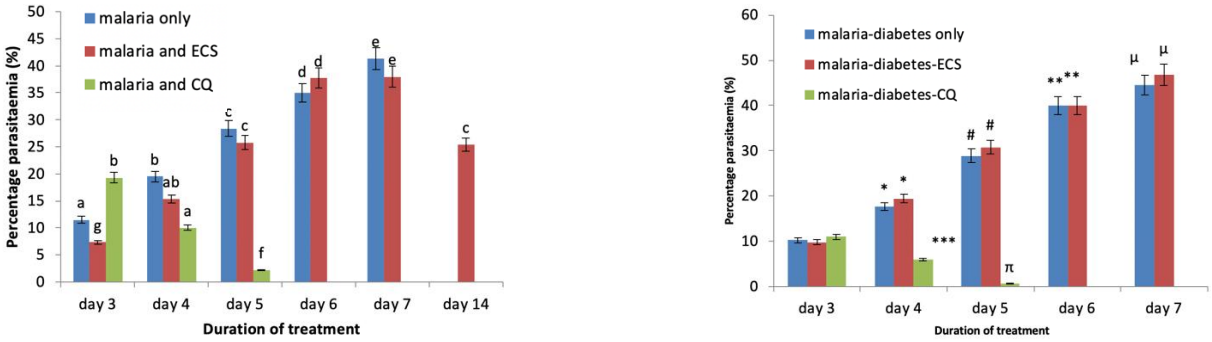

Figure 1
The effect of treatment with ECS on percentage parasitaemia in (A) mice infected with *P. berghei (B) P. berghei* infected diabetic mice. Values are expressed as Mean ± SD. Values with different letters, (*), (µ), (π), (#) and (**) are significantly different. ECS- Ethanol extract of *Camellia sinensis*, CQ- chloroquine

Table 1
The mean survival time of various treated groups

Group	Days (Mean±S.D)
Malaria untreated	9.9 ± 4.8
Malaria and *Camellia sinensis*	11.7 ± 2.0
Malaria and Chloroquine	31.4 ± 2.8*
Malaria-Diabetes untreated	7.8 ± 3.0
Malaria-Diabetes *Camellia sinensis*	9.1 ± 2.3
Malaria-Diabetes Chloroquine	30.5 ± 3.2*

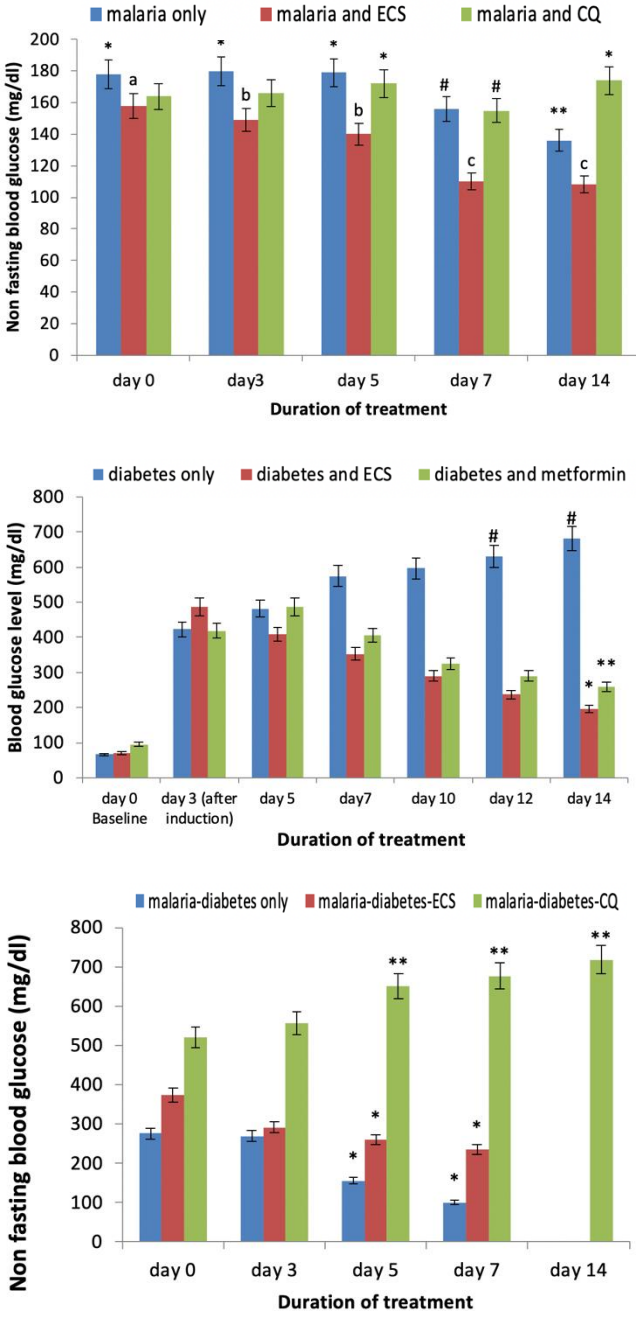

Figure 2
The effects of ECS on blood glucose level in (A) mice infected with *P. berghei*(B) diabetic mice (C) *P. berghei* infected diabetic mice
Values are expressed as Mean ± SD. Values with different letters, (*), (#) and (**) are significantly different. ECS- Ethanol extract of Camelliasinensis, CQ- chloroquine

Figures 2A and B show the effect of administration of ECS on blood glucose concentration in mice infected with malaria and diabetes. A significant decrease ($p<0.05$) in blood glucose level was observed in mice treated with ECS compared with infected untreated and chloroquine-treated groups. No changes were observed in the blood glucose level of mice treated with chloroquine and the untreated group at days 3, 4 and 5. However, the blood glucose level of the untreated group started to decrease steadily as the percentage parasitaemia increased. The blood glucose of mice treated with chloroquine increased progressively till day 14. Interestingly, blood glucose level in mice treated with the extract decreased relatively faster than that of the untreated group.

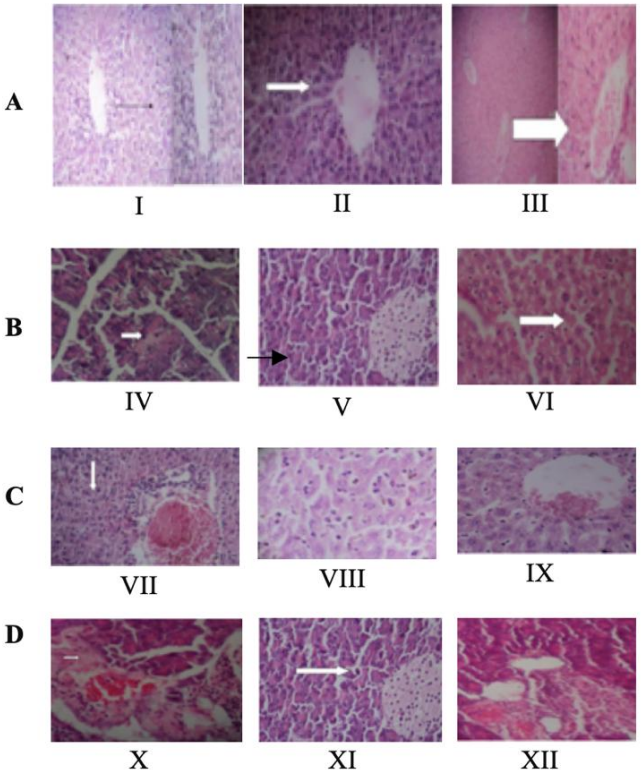

Plate 1
(A) Photomicrograph of the liver of P. bergheimice (I) without treatment (II) treated with 300mg/kg Camellia sinensis (III) treated with 10mg /kg chloroquine. I - shows sinusoidal dilation with perivascular mononuclear infiltration, centrilobular necrosis and kupfer cell hyperplasia with hemosiderosis.II-shows sinusoidal dilation with mononuclear cell infiltration.Perivascular, foci, kupfer cell hyperplasia with hemosiderosis.III-massive hepatic degeneration, mild perivascular mononuclear infiltration with Kupffer cell hyperplasia and mild hemosiderosis. X 100 Hematoxylin and eosin stain, (H&E).
(B) Photomicrograph of the pancreas of diabetic mice (IV) without treatment (V) treated with 300mg /kg Camellia sinensis, and (VI) treated with 250mg/kg metformin. IV- shows massive islet cell necrosis and degeneration. V-shows Mild islet cell necrosis and degeneration and VI-.Shows apparently normal islet cells.X 100 Hematoxylin and Eosin stain, (H&E).
(C) Photomicrograph of the liver ofP. berghei diabetic mice (VII) without treatment (VIII) treated with 300mg/kg Camellia sinensisand (IX) treated with 10mg/kg CQ. VII - shows kupffer cell hyperplasia with marked focal perivascular lymphocytic infiltration. VIII-shows sinusoidal dilation with perivascular mononuclear cell infiltration, centrilobular necrosis and kupffer cell hyperplasia with hemosiderosis.IX-shows sinusoidal dilation and kupffer cell hyperplasia with mild hemosiderosis. X 100 Hematoxylin and eosin stain, (H&E).
(D) Photomicrograph of the pancreas of P. berghei diabetic mice. VII without treatment, VIII treated with 300mg/kg Camellia sinensis and IX treated with 10mg /kg chloroquine. VII - shows massive islet cell necrosis and degeneration. VIII - shows mild islet cell necrosis and degeneration. IX - shows severe pancreatic islet necrosis. X 100 Hematoxylin and eosin stain, (H&E)

Figure 2C shows a significant decrease ($p<0.05$) in blood glucose in diabetic mice treated with ECS or metformin when compared to the untreated group. There was a significant decrease ($p<0.05$) in blood glucose level in untreated and extract treated groups when compared with chloroquine group. The blood glucose level in untreated mice decreased as the percentage parasitaemia increases. Chloroquine did not have effect on control of blood glucose level as there was a significant increase in blood glucose level in the chloroquine treatment group compared to that of the extract or metformin. Similarly, the blood glucose concentration of mice in the malaria infected diabetic ECS treated group decreased.

Table 2 shows the pattern of PCV changes in the *P. berghei* infected mice. The PCV decreased steadily in both untreated and ECS treated *P.berghei* infected mice. There was no significant difference ($p>0.05$) in PCV when these two groups were compared. PCV values were similar among all groups up till day 3. However, by days 7 and 14, PCV was significantly lower in ECS treated and untreated groups when compared with the chloroquine group. The pattern of PCV changes was similar in *P. berghei* infected diabetic mice (Table 3).

Table 2A: The packed cell volume (%) in *P. berghei* infected mice treated with ECS and chloroquine (CQ)

Days	Malaria untreated	Malaria and ECS	Malaria and CQ
Day 0	45.6 ± 2.7	44.3 ± 1.1	43.0 ± 4.6
Day 3	44.4 ± 3.0	46.3 ± 5.2	42.7 ± 3.7
Day 7	32.5 ± 8.0*	22.3 ± 1.1*	39.0 ± 5.7
Day 14	22.7 ± 2.3*	22.0 ± 1.0*	41.0 ± 7.8

Values are Mean ± SD. *significantly different from control. ECS – Extracts of *Camellia sinensis*

Table 2B: The packed cell volume (%) in diabetic mice treated with ECS and metformin

Days	Diabetes untreated	Diabetes and ECS	Diabetes and metformin
Day 0	45.4 ± 3.4	48.6 ± 2.9	45.3 ± 4.6
Day 3	45.6 ± 3.6	46.1 ± 3.0	46.6 ± 2.5
Day 7	44.1 ± 2.6	46.6 ± 2.4	44.9 ± 4.0
Day 10	43.9 ± 2.9	44.4 ± 5.5	48.0 ± 2.0
Day 14	42.1 ± 2.0	46.1 ± 3.3	41.4 ± 6.8

The PCV was not significantly affected in the diabetic mice by non-treatment or treatment with ECS or metformin from day 0 to 14

Table 2C : The packed cell volume of malaria infected diabetic mice treated with ECS or chloroquine

Day s	Malaria – Diabetes untreated	Malaria – Diabetes and ECS	Malaria – Diabetes and CQ
Day 0	45.4 ± 5.1	47.7 ± 4.4	44.6 ± 3.5
Day 3	39.6 ± 1.1	45.4 ± 3.5	40.0 ± 3.3
Day 7	23.4 ± 9.3	35.1 ± 4.7	40.0 ± 3.5
Day 14*	-	-	44.2 ± 2.4

The PCV of malaria – diabetic untreated mice decreased steadily up to 7 days. The PCV of the ECS treated malaria and diabetic mice were significantly reduced when compared to the untreated mice while the PCV of the CQ-treated malaria-diabetic mice was not significantly affected up to day 14.

*All the mice in the malaria-diabetic untreated group and malaria-diabetes extract group had died by day 14

Table 3

The percentage chemo-suppression (%) by ECS in malarial-infected diabetic and malarial -infected only male mice.

Days	Mal + ECS (%)	Mal + CQ (%)	Mal + Diab + ECS (%)	Mal + Diab + CQ (%)
3	36.5	68	4	-8
4	22	49	-10	7
5	9	92	-7	98
6	-8	100	0.3	98
7	8	100	-5	100
14	33	100	-	100

DISCUSSION

Hypoglycemia is implicated in malarial infection; for instance, infection with the lethal murine parasite *P yoelii YM* or the nonlethal parasite *P chabaudi* induces severe hypoglycemia as a result of hyperparasitemia and hyperinsulinemia (Elased *et al.,* 1994). Moreover, injection of parasitized blood cells to mice induces hypoglycemia (Taylor *et al.,* 1992). Similarly, extracts are capable of synergizing with insulin in enhancing glucose uptake in adipocytes *in vitro* (Taylor *et al.,* 1992) and of inducing insulin secretion from isolated pancreatic islets (Elased *et al.,* 1996). The present study was used as a model to investigate the effects of ethanolic extract of *Camellia sinensis* (ECS) on parasitized diabetic mice by evaluating the blood glucose levels.

The present study reports the anti-diabetic effects of ECS in alloxan-induced diabetic mice. This study showed that 300mg/kg of the ECS was effective for the treatment of diabetes. It was observed that the blood glucose level of diabetic mice was steadily reduced when compared to that of untreated diabetic mice such that the treated mice became hypoglycemic (reference point $\geq$ 200mg/kg) by day 14. The reduction of blood glucose level by the standard drug (metformin) and ECS followed the same pattern. This finding corroborates the report of Sabu *et al.,* (2002) that oral administration of green tea polyphenols reduced the serum glucose tolerance in alloxan– induced diabetic rats and also increased the antioxidant potential in the rats. Wu *et al.* (2004) also reported rapid normalization of blood glucose level in diabetic rats by green tea extract, and suggested that green tea extract protected the β-cells against the streptozotocin toxicity by regenerating the damaged cells. The anti-diabetic effect of the water-soluble polysaccharide fraction has also been documented (Han *et al.,* 2011). Furthermore, Sun *et al.,* (2016) reported that green tea polyphenols inhibited salivary amylase, intestinal sucrase and α-glucosidase, suggesting that the inhibitory activity of catechins against carbohydrates digestibility may be responsible for its ability to reduce blood glucose levels in diabetic rats, and these mechanisms may be responsible for the anti-hyperglycemic effect of green tea

Furthermore, the significant decrease ($p<0.05$) in blood glucose level in *P.berghei* infected diabetic mice treated with ECS and untreated *P.berghei*infected diabetic group when compared with chloroquine treated group might be due to the regeneration of the islet cells seen after 24 hours post-treatment. As the blood glucose of the untreated *P.berghei* diabetic group reduced, the percentage

parasitemia increased implying that the parasites might be utilizing the glucose for its metabolism.

Malaria parasites do not store glycogen or other reserve polysaccharides at the intra-erythrocytic stage (Elased *et al.,* 1995). As a result, the growth and reproduction of *Plasmodia* depend on simple sugars (notably glucose). Olszewski and Llinas (2011) observed that *P. falciparum* and *P. vivax*-infected erythrocytes required addition of glucose for their maintenance *in vitro* and they detected only maltose as an adequate substitute for glucose. Similarly, Kirk *et al.,* (1996) noticed the severity of infection of *P. falciparum* was intensified by glucose uptake. In contrast, many reports have shown that normal erythrocytes used very little of the added metabolite, oxygen, glucose and several other sugars as well as glycerol *in vitro* (Olszewski and Llinas, 2011). Thus, the sudden increase in glucose consumption by erythrocytes infected with a malarial parasite is resulted from an alteration in the permeability barrier of the erythrocyte membranes since chicken, duckling and mouse erythrocytes demonstrated a low sugar uptake. Thus, the presence of the malaria parasite promotes sugar uptake in malaria-infected erythrocytes by changing the permeability characteristics of the host cell.

The reduction of the elevated blood glucose by green tea and its catechins was reported in both type 1 and type 2 diabetic animals (Sabu *et al.,* 2002; Thipubon *et al.,* 2015). Green tea was shown to enhance the basal and insulin-stimulated glucose uptake of rat adipocytes (Wu *et al.,* 2004). Epigallocatechin gallate (EGCG) inhibit intestinal glucose uptake by sodium-dependent glucose transporter SGLT1 (Waltner-Law *et al.,* 2002) and mimic insulin by decreasing the expression of genes that control gluconeogenesis (Thipubon *et al.,* 2015). The antihyperglycemic effect of green tea extract may be linked to the increased glucose uptake, inhibition of intestinal glucose transporter and repression of genes that control gluconeogenesis (Kobayashi *et al.,* 2000; Musial *et al.,*2020).

Observation from the present study indicated that graded doses of the ECS administered to parasitized mice in a pilot study carried out in our laboratory revealed that the extract does not have any effect on parasite clearance in mice. The data in Figure 1 showed that the parasite infected untreated group had their parasitaemia increased steadily with days of infection until death which was similar to the findings in the ECS treated mice group. The pattern of result was similar in alloxan diabetic mice infected with *P. berghei*. This result correlates with the report of (Thipubon *et al.,* 2015) that epicatechin did not inhibit the growth of *P. berghei* in mice when administered for 4 consecutive days but it is at variance with the findings of Sannella *et al.,* (2007) that gallated catechins, epicatechin gallate and epigallocatechin gallate inhibited *Plasmodium falciparum* (strains NF54, K1 and 3D7) growth, with IC_{50} values between 10 and 40µM *in vitro*. The less IC_{50} values in excess of 100–300µM of ungallatedcatechins revealed its less potency. Sannella *et al.,* (2007) could not determine a precise mechanism of antimalarial action for catechins, but established that anti-folate mechanism of action was unlikely.

The PCV of *P.berghei*infected mice treated with ECS like that of untreated *P.berghei* infected mice were significantly decreased ($p<0.05$) when compared with the chloroquine treated group (Table 2). Since the ECS does not have antimalarial property. It is not surprising that there was a similarity in the PCV values of malaria - untreated and ECS-treated mice as Plasmodium infection is known to induce anaemia. In the study with *P.berghei* diabetic mice, the PCV of the untreated and that of ECS-treated animals decreased significantly ($p<0.05$) when compared with chloroquine-treated mice. The rate of PCV reduction was significantly different for untreated *P.berghei* diabetic mice and that of ECS treated *P.berghei* diabetic mice (52% and 23%), respectively. Elased *et al.,* (1994) reported that malaria lowers the blood glucose in diabetic animals and diabetes exerts a partial control over parasitaemia, and consequently anaemia, probably via an activating effect on macrophages. The PCV of untreated diabetic mice and that of the ECS treated diabetic group was similar to that of the metformin treated group. This is probably because diabetes does not have direct effect on red cell lysis.

In conclusion, our findings indicated that ethanol extract of *Camellia sinensis* possesses hypoglycemic activity as documented by blood glucose lowering effects in alloxan-induced diabetic mice and *P.berghei* infected diabetic mice. It however does not seem to have anti-malaria activity as portrayed by its inability to attenuate the parasitaemia in *P.berghei* infected ECS treated and *P. berghei* infected, diabetic ECS treated.

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Full-Length Research Article

Ethanol Extract of *Salacia nitida* Root Bark Ameliorates Lipid Peroxidation and Hepatosplenomegaly in *Plasmodium berghei* Malaria-Infected Mice

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Summary: The root bark of *Salacia nitida* L. benth (celastraceae) is used as a remedy for malaria and typhoid fever in the Southern part of Nigeria. This study is designed to evaluate the effect of treatment with ethanol extract from the root bark of *S. nitida* on lipid peroxidation, hepatomegaly, and splenomegaly in *Plasmodium berghei* malaria-infected mice. Thirty *P. berghei*-infected and six uninfected mice were used for the study. 280, 430, and 580 mg/kg b. w/day of ethanolic extract and 4 mg/kg b. w/day of artesunate were administered orally to infected mice in groups B, C, D, and E, while 4 ml/kg b. w/day of physiological saline was given to infected untreated mice in group A and the uninfected untreated mice in group F. Treatments were administered for five days. Levels of malondialdehyde were measured as means of assessing lipid peroxidation. Weights of experimental animals, liver, spleen were recorded, and the length of spleen was taken by planimetry. Animal's liver and spleen-body weight ratios were determined. Results from the study showed a significant decrease in levels of malondialdehyde and a significant increase in body weights. Significant decreases were observed in liver and spleen weights, lengths of the spleen, and organ-body weight ratios of malaria-infected treated mice. This study confirmed anti-plasmodic activity of ethanol extract of *S. nitida* root bark probably via reduced lipid peroxidation and hepatosplenomegaly in *P. berghei*-infected mice, as it is seen in its ability to attenuate lipid peroxidation and hepatosplenomegaly in mice.

Keywords*:* Hepatomegaly, Splenomegaly, Malaria, Lipid peroxidation, Organ-body weight ratio, *Salacia nitida*.

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INTRODUCTION

Malaria is one of the tropical diseases mostly reported worldwide. In 2008, malaria was reported to be responsible for 20% of childhood deaths in Africa (WHO, 2008). Malaria is caused by *Plasmodium* species that are introduced into the host's body by infected female *Anopheles* mosquitos. Malarial schizogony takes place in the liver at the pre-erythrocytic stage with the primary schizonts developing into the merozoites. In the erythrocytic phase, merozoites invade the erythrocytes, which manifest into malaria. Features of malaria infection that appear with haemolysis of erythrocytes include hepatomegaly, fever, malaise, splenomegaly, nausea, weight loss, etc. (Cheesbrough, 2004; Perlmann and Troye-Blomberg, 2007).

Malaria has been associated with hepatosplenomegaly (Cook, 1994). Hepatomegaly results from the congestion of the sinusoids (Bruce-Chwatt, 1978), haemozoin pigments, hepatic sinusoidal infiltrations and dilatation by lymphocytes, and abnormally increases in the number of kuffer cells (Lichtenberg, 1989; Kamal and Rodney, 1996). On the other hand, splenomegaly is due to the presence of cytoplasmic particles, parasites, toxins, and nuclear remnants that are left behind in the spleen as a result of erythrophagocytosis of old and deformed erythrocytes, vascular swellings, depositions of iron, and edema (Buffet *et al*. 2011; Dennis *et al*., 2012; Krucik, 2016), and hyperplasia of the lymphoid tissues (Marsden and Hamilton, 1969).

The production of oxygen free radicals by malaria parasites in the body cause lipid peroxidation, which is the oxidative deteriorations of polyunsaturated fatty acids (PUFA) of cell membrane phospholipids, causes nucleic acid injury and damages to protein structure (Prabha *et al*., 1990; Omodeo-Sale *et al*., 2003). Lipid peroxidation led to the formation of a mixture of lipid hydroperoxide and aldehyde end-products such as malondialdehyde (MDA) (Poli and Parola, 1997; Girotti, 1998), and causes some structural and functional alterations in red cell membranes such as changes in permeability and fluidity, leading to haemolysis (Chen *et al*., 1995), thus making hemoglobin available to the parasites for degradation. Proteolysis of haemoglobin by malaria parasites provide free amino acids for the parasite protein synthesis and haem in which Fe^{2+} is oxidized to Fe^{3+}, and electrons produced reacting with molecular oxygen to form oxygen radicals (Gamboa and Rosenthal, 1996; Francis *et al*., 1997) that lead to oxidative stress and inflammation. As a result of oxidative stress, lipid peroxidation and accelerated aging of erythrocytes occur, thus contributing to anaemia.

The dependence on medicinal plants for the treatment of malaria is gaining more recognition because of the lack of health facilities in the rural areas and the high cost of drugs. *Salacia nitida*, a member of the celastraceae family is one

of the medicines used traditionally to treat malaria in Nigeria. Decoctions of root bark of *S. nitida* are orally taken for treatments of malaria. The root bark of *S. nitida* has been reported to contained alkaloids such as spartein, lunamarine and ribalinidine, tannins, sapogenin, flavonoids such as epicatechin, catechin, rutin, and kaempferol, phenol, phytate, and anthocyanin which are bioactive phytochemicals (Nwiloh *et al*., 2016). The antimalarial activity of the root bark of *S. nitida* has been reported (Nwiloh *et al*., 2017). Therefore, this research is designed to study the amelioration of lipid peroxidation and hepatosplenomegaly in *P. berghei* malaria-infected mice treated with ethanol extract from the root bark of *S. nitida*.

MATERIALS AND METHODS

Chemicals and Drug: Chemical reagents, assay kits, and drug used for the study are of analytical grade. MDA assay kit was provided by Randox Laboratories Ltd (USA) and the antimalarial drug used was artesunate tablet, 50 mg (Artesunat ®) (Mekophar chemical pharmaceutical Joint-Stock Company, Vietnam).

Collection of plant material and preparation of extract: *Salacia nitida* was collected in February 2016, from Diidi farm in Nyogor-Beeri, Khana local government area in Rivers State, Nigeria, and the plant was identified by Dr. N. L. Edwin-Wosu of the Department of Plant Science and Biotechnology, Faculty of Science, University of Port Harcourt, Rivers State, Nigeria (voucher number UPHV-1033). The plants were uprooted and the roots were washed in clean water and air-dried under shade. The barks were removed from the root and reduced to smaller bits with machete onto a clean leather material and air-dried again under shade for one week and pulverized with a grinding machine (corona-16D).

Extraction was done according to the method reported by Nwiloh *et al* (2017), using Soxhlet extractor with 300 g of powder root bark material and 250 ml of ethanol at a temperature of 80^{o}C with a water bath for about 18 hours. The extract was then concentrated with a water bath regulated at 60^{o}C and dry extract stored in a refrigerator regulated at 4^{o}C until required for use. The dosages of ethanol extract from the root bark of *S. nitida* and artesunate used were calculated according to OECD (2000) and WHO (2015).

Experimental animals and Ethics: The study was conducted according to the United States National Institute of Health (NIH) "Guides on Care and Use of Laboratory Animals" (NIH, 1986) and the guidelines on the use of laboratory animals of the University of Port Harcourt (UPH/BCH/AEC/2016/015). Thirty-six (36) healthy Swiss mice of mixed-sex, weighing between 28g - 37g, and randomly selected out of eighty (80) healthy Swiss mice procured from the Department of Pharmacology, College of Medicine, University of Port Harcourt, were used for the study. Physical appearances and feeding behavior of the mice were used to ascertain their health status and were also confirmed malaria-free with rapid diagnostic test strips (Access Bio Inc, NJ, USA). They were housed in plastic cages and maintained under standard environmental conditions of humidity, room temperature of about 26.5 ^{o}C and, 12 hours' light/12 hours' darkness cycle, with free access to animal feed and clean water *ad libitum* for two weeks.

Acute toxicity test: An acute toxicity test was carried out according to Lorke (1983). Initially, twelve mice divided into three groups of four mice each were used. Doses of 100, 200, and 300 mg of the ethanol extract were orally administered to healthy mice in groups 1, 2, and 3 respectively. The mice were monitored for 24 hours for signs of toxicity. No toxicity was observed, so, a second phase was designed with another 12 mice, further divided into another three groups each containing four mice. The mice were orally given 400, 700, and 1000 mg of the ethanol extract and were monitored for another 24 hours for signs of toxicity. The geometric mean of the least dose that killed at least one mice and the highest dose that did not kill any mice was used to calculate the LD_{50} (Akhila et al., 2007).

Inoculation of mice: *Plasmodium berghei* (NK-65) obtained from the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos State, Nigeria, was used for the study. They were contained in five (5) donor mice and maintained at the World Bank-Assisted Malaria and Phytomedicines Research Laboratory, University of Port Harcourt. Before inoculation, levels of parasitaemia in donor mice were determined by cutting the tip of the tail of donor mice with sterile pairs of scissors and blood extruded into a small beaker containing 0.5 ml normal saline. A drop of diluted infected blood from the donor mice was placed on the rapid diagnostic malaria test strips (Access Bio Inc, NJ, USA) and the intensity of colour produced was used to ascertain the level of parasitaemia (Moody 2002; Cheesbrough, 2004; Tarazona *et al*., 2004). During inoculation, all donor mice with higher levels of parasitaemia (Moody 2002; Cheesbrough, 2004; Tarazona *et al*., 2004) were sacrificed by cervical dislocations, and blood was collected by cardiac puncture into a 50 ml beaker, using sterile disposable syringe and needle to avoid any variability in parasitaemia. 0.2 ml of parasitized blood from donor mice were added to 9.8 ml of normal saline. 0.2 ml of the infected diluted blood containing $1x10^{7}$ *P. berghei* infected red blood cells (RBC) obtained from donor mice were injected intraperitoneally into 30 experimental mice on day one (Akuodor *et al*, 2010), and randomly divided into five (5) groups labelled A, B, C, D, and E containing six (6) infected mice each, while another six (6) healthy mice were placed in group F to serve as a reference control.

Treatment of malaria-infected mice: Seventy-two (72) hours after infections, all the *P. berghei* malaria-infected mice were treated by oral administration with 0.2 ml of ethanol extract from the root bark of *S nitida* and artesunate according to the protocol stated below.

Group A - contained six (6) *P. berghei* malaria-infected mice were given 4 ml/kg b. w/day of physiological saline (negative control group, NC),

Group B - contained six (6) *P. berghei* malaria-infected mice were given 280 mg/kg b. w/day of ethanol extract from the root bark of *S. nitida*,

Group C - contained six (6) *P. berghei* malaria-infected mice were given 430 mg/kg b. w/day of ethanol extract from the root bark of *S. nitida*,

Group D - contained six (6) *P. berghei* malaria-infected mice were given 580 mg/kg b. w/day of ethanol extract from the root bark of *S. nitida*,
Group E- contained six (6) *P. berghei* malaria-infected mice were given 4 mg/kg b. w/day of artesunate (positive control group, PC), and
Group F - contained six (6) healthy mice were given 4 ml/kg b. w/day of physiological saline (reference control group, RC).

Treatments were done by 8.00 am once daily for five consecutive days, using oral metal gavage. The mice were also allowed free access to food (grower's marsh) and clean water *ad libitum*. At the end of the treatment period, all the mice were sacrificed by cervical dislocations, and blood was collected by cutting the jugular veins with sterilized steel disposable scalpel (Nwiloh *et al*., 2009; George *et al*., 2012).

Determination of weights of experimental animals: The experimental mice were weighed before and after treatments with a digital analytical weighing balance (Mettler Toledo Type BD6000, Mettler-Toledo GmbH, Greifensee, Switzerland), and the weights obtained were recorded in grams.

Determination of malondialdehyde (MDA): The serum from the blood that were pooled into labeled dry free sample bottles was used for evaluation of MDA. Malondialdehyde was determined according to the method in the manual that accompanies the assay kit by dispensing 0.4 ml of serum into each of the six labeled centrifuge tubes, and the same amount of distilled and deionized water was dispensed into a centrifuge tube labeled as blank. 1.6 ml of Tris-KCl buffer was pipetted into all the tubes, to which was added 0.5 ml of 30% trichloroacetic acid (TCA), and 0.5 ml of 0.75% thiobarbituric acid (TBA). Tubes were placed in a beaker containing ice cubes for about 5 minutes and then centrifuged at 3000 g for 15 minutes. Supernatants were decanted into cuvettes accordingly, and the absorbance read spectrophotometrically at 532 nm against the blank. The MDA was estimated as absorbance of test sample – absorbance of blank/1.56 x 10^5 M^{-1} cm^{-1}, and result obtained expressed as nmol MDA ml^{-1}.

Determinations of hepatomegaly and splenomegaly experimental mice: The sacrificed mice were dissected with t aid of a steel disposable scalpel, and the liver and spleen remov for assessment of levels of hepatomegaly and splenomegaly, usi a pair of scissors and placed on tissue paper. The liver and sple were then weighed with a digital analytical weighing balance, a their weights were recorded in grams. The length of each splee was planimetrically done and recorded in mm. Liver-body weigh (LBWR) and spleen-body weight ratios (SBWR) of experimental animals were calculated according to Lazic *et al*. (2020), using the formula;

Organ to body weight ratio =
Weight of organ (g)/body weight of the animal (g).

Data analysis: Results were expressed as mean values ± standard error of means (SEM). The data obtained were statistical analyzed using one-way analysis of variance (ANOVA) with the SPSS version 22 statistical package. Multiple comparisons were done using Scheffe's post hoc test to compare differences between results. Results were considered significant at 95% confidence level ($p < 0.05$).

RESULTS

Ethanol extract from the root bark of *S. nitida* was safe at the doses administered to experimental mice. The extract was not lethal to mice and no gross behavior or physical changes were observed in the experimental animals.

From Fig.1 below the level of MDA slightly increased in the malaria-infected mice in group A (NC) when compared to healthy mice in group F (RC). There was a decrease in MDA levels in the *P. berghei* malaria-infected mice in groups B treated with 280 mg/kg b. w/day of extract, C treated with 430 mg/kg b. w/day of extract, D treated with 580 mg/kg b. w/day of extract, and those in group E (PC) treated with 4 mg/kg b. w/day of artesunate.

From figure 2 below, it was observed that the body weights of *P. berghei* malaria-infected untreated mice in group A decrease significantly ($p < 0.05$) from day 1 through day 8 when compared to the healthy mice in the reference control group (group F). Weights of infected treated mice in groups B through E decrease significantly ($p < 0.05$) on day 3, and which significantly increased ($p < 0.05$) on day 8 after treatments with 280, 430, 580, and 4 mg/kg b. w/day of extract and artesunate compared to the *P. berghei* malaria-infected untreated mice in group A, and non-significantly ($p > 0.05$) compared with *P. berghei* malaria-infected treated mice in groups B through E on the 3rd day.

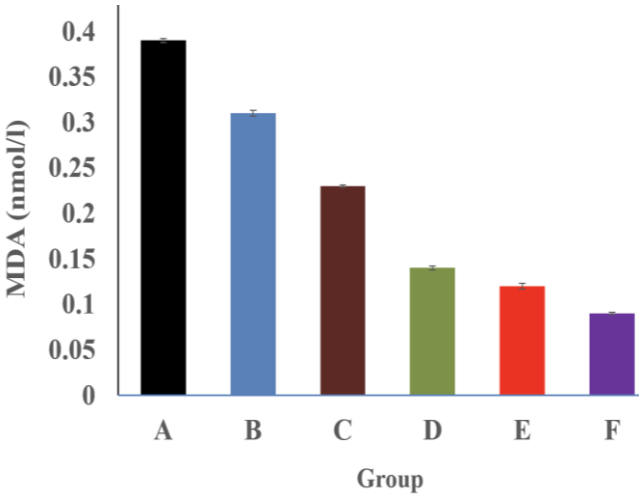

Figure 1:
Effect of ethanol extract of the root bark of *S. nitida* on MDA (nmol/l) in *P. berghei*-infected mice. n = 6. A = 4 ml/kg b. w/day of saline, B = 280, C = 430, and D = 580 mg/kg b. w/day of extract, E = 4 mg/kg b. w/day of ART, and F = 4 ml/kg b. w/day of saline.

The results for weights of liver and spleen in the experimental mice are displayed in figure 3. The figure showed a significant increase ($p < 0.05$) in the weights of liver and spleen in the group A (NC) mice when compared to healthy mice in group F (RC). There was a significant decrease ($p < 0.05$) in mean weights of liver and spleen in the *P. berghei* malaria-infected mice in groups B through E that were treated with different graded doses of extract from *S. nitida* root bark and artesunate.

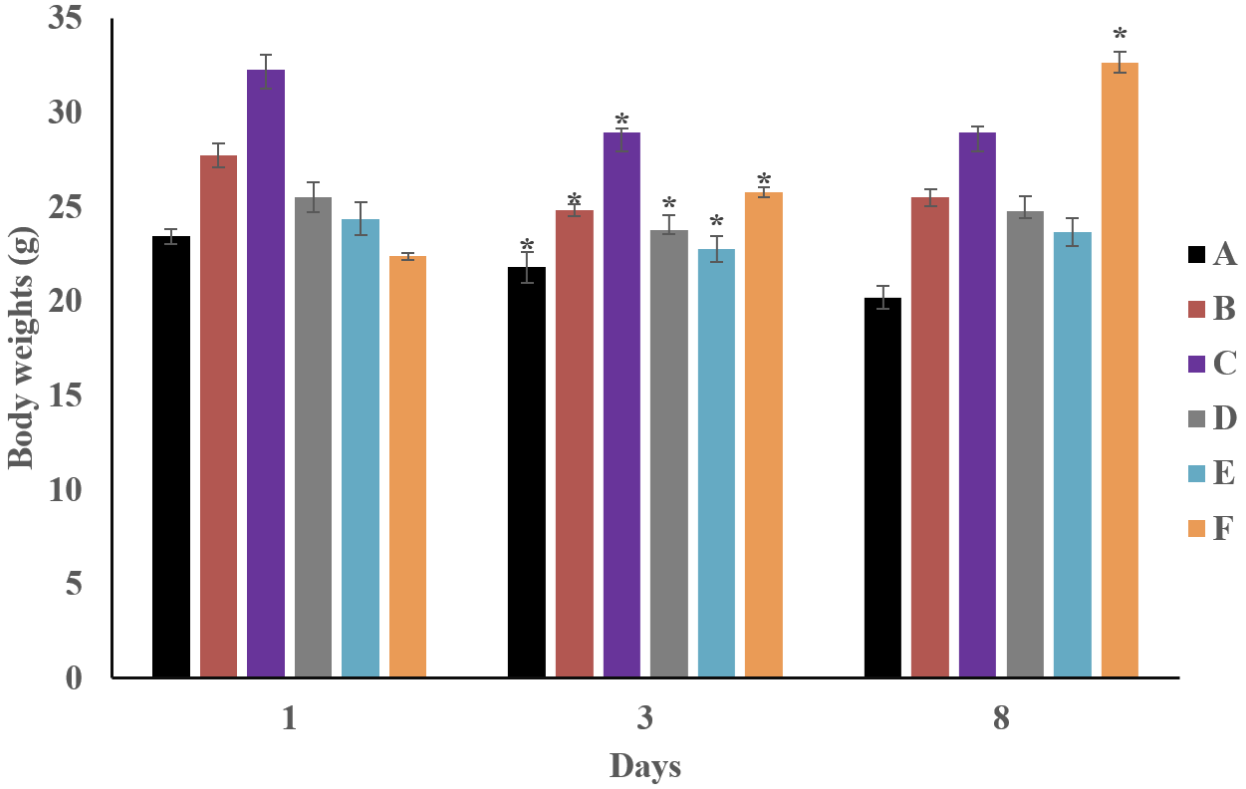

Figure 2:
Effect of ethanol extract from the root bark of *S. nitida* on body weights of *P. berghei* malaria-infected mice; n= 6. A = 4 ml/kg b. w/day of saline, B = 280, C = 430, and D = 580 mg/kg b. w/day of extract, E = 4 mg/kg b. w/day of ART, and F = 4 ml/kg b. w/day of saline; * = values are significant ($p < 0.05$).

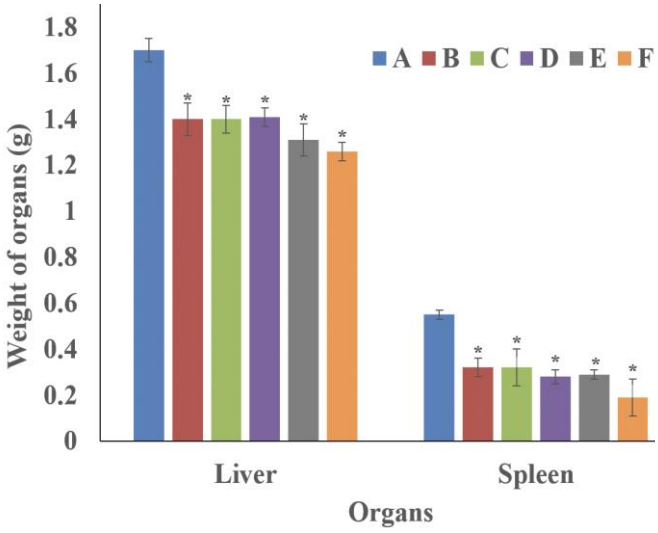

Figure 3:
Effect of ethanol extract from the root bark of *S. nitida* on weights of liver & spleen (g) in *P. berghei* malaria-infected mice; n= 6. A = 4 ml/kg b. w/day of saline, B = 280, C = 430, and D = 580 mg/kg b. w/day of extract, E = 4 mg/kg b. w/day of ART, and F = 4 ml/kg b. w/day of saline; * = values are significant ($p < 0.05$).

Results showing various length of spleens in the experimental mice investigated are shown in figure 4. The mean length of the spleen in malaria-infected mice in the negative control (group A) showed a significant ($p < 0.05$) increase when compared to those of healthy mice in reference control (group F). It was seen also from the figure that there is significant ($p < 0.05$) decrease in the length of spleens in the *P. berghei* malaria-infected mice treated with 280, 430, 580 mg/kg b. w/day of extract in groups B, C, D, and E that were given 4 mg/kg b. w/day of artesunate compared to those of the *P. berghei* malaria-infected untreated mice in group A and the healthy mice in group F.

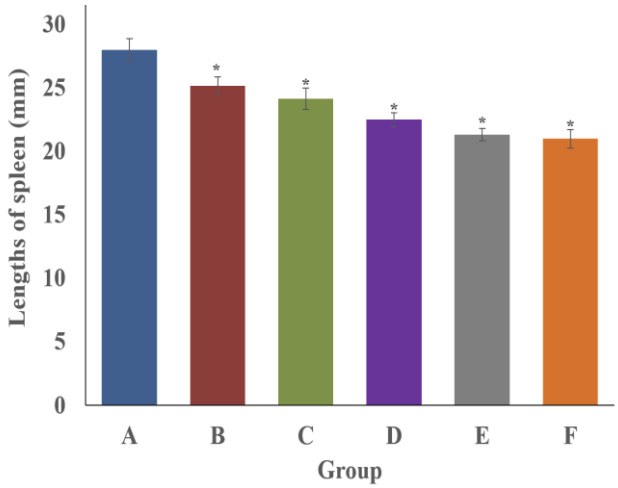

Figure 4:
Effect of ethanol extract from the root bark of *S. nitida* on length of spleen (mm) in *P. berghei* malaria-infected mice; n= 6. A = 4 ml/kg b. w/day of saline, B = 280, C = 430, and D = 580 mg/kg b. w/day of extract, E = 4 mg/kg b. w/day of ART, and F = 4 ml/kg b. w/day of saline; * = values are significant ($p < 0.05$).

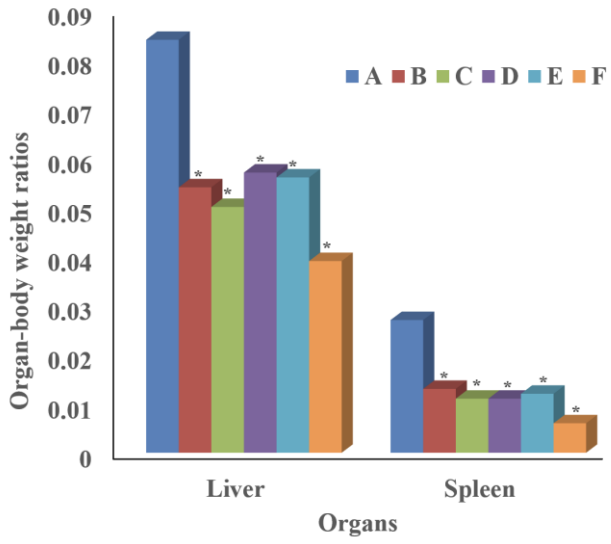

Figure 5:
Effect of ethanol extract of the root bark from *S. nitida* on organ-body weight ratios in *P. berghei* malaria-infected mice; n= 6. A = 4 ml/kg b. w/day of saline, B = 280, C = 430, and D = 580 mg/kg b. w/day of extract, E = 4 mg/kg b. w/day of ART, and F = 4 ml/kg b. w/day of saline; * = values are significant ($p < 0.05$).

It was observed from figure 5, that administration of extract and artesunate to the *P. berghei* malaria-infected mice in groups B through E brought about a significant ($p < 0.05$) decrease in both liver-to-body weight and spleen-to-body weight ratios (LBWR and SBWR) when compared to experimental mice in the negative control (NC) and reference control (RC) groups respectively.

DISCUSSION

The observed non-toxic effect of the extract is an indication that the root bark extract is very safe. Lipid peroxidation is one of the disorders that take place when malaria parasites infect erythrocytes (Omodeo-Sale *et al*., 2003), and malondialdehyde (MDA) is the biomarker of lipid peroxidation. The observed increase in MDAs in the malaria-infected untreated mice in group A might be due to the activities of malaria parasites in the RBCs, initiated by free radicals, and decreased antioxidant activity that resulted in inflammations of cell membranes (Kiklugawa *et al*., 1984). This increase could also be used as a biomarker in severe malaria diagnosis. It is also noticed that the increase in MDAs was ameliorated by the administration of ethanol extract from the root bark of *S. nitida*. The presence of phytochemical compounds like flavonoids, tannins, phenols, phytates, and anthocyanins with antioxidant and anti-inflammation activities, in the *S. nitida* root bark extract might be implicated in this study (Santos *et al*., 1998; Seeram and Nair, 2002; Okwu, 2004; Han *et al*., 2007; Buzzini *et al*., 2008; Oomah *et al*., 2008). Rutin and kaempferol, which are present in the extract (Nwiloh *et al*., 2016), are inhibitors of lipid peroxidations (Husain *et al*., 1987; Robak and Glyglewski, 1988). Also, to be implicated are the quinoline alkaloids present in the root bark extract, which are known metal chelators and antioxidants (Fernandez-Bachiller *et al*., 2010a).

The results obtained for the effect of ethanol extract from *S. nitida* root bark on body weights of experimental mice are in agreement with the report of Dikasso and colleagues (2006a), working with *Asparagus africanus*. Weight loss is one of the features of malaria and may be due to a reduced appetite for food (Perlmann and Troye-Blomberg, 2007).

Hepatomegaly and splenomegaly are also features of malaria (Cook, 1994). The observed increase in mean weights of liver and spleen, and length of the spleen in group A mice are shreds of evidence of liver and spleen enlargement (hepatomegaly and splenomegaly). Hepatomegaly observed in the group A mice might be due to sinusoidal infiltration with lymphocytes and congestion, and abnormally increase in the number of kuffer cells due to malaria parasites (Bruce-Chwatt, 1978), while the splenomegaly might also be due to the presence of cytoplasmic particles, malaria parasites, oxidized haemozoin and nuclear remnants left behind in the spleen as a result of erythrophagocytosis of old and deformed parasitized erythrocytes (Buffet *et al*. 2011; Dennis *et al*., 2012; Krucik, 2016). This finding is in tandem with the reports of Lawson and colleagues (1969), and that of Lacelle (1970), that abnormal erythrocytes encounter difficulty in traversing the cords and sinuses of the spleen, which result in congestion in the spleen. Splenomegaly also accelerates the clearance of old erythrocytes (Wyler *et al*., 1981; Looareesuwan *et al*., 1987). Hepatosplenomegaly in this study might also be caused by inflammation resulting from malaria. The dark-red colour and hardness of the liver and spleen might be due to inflammations and accumulation of malaria pigments in capillaries and sinusoids (Bruce-Chwatt, 1978). Enlargements of liver and spleen observed in this work are indications of hepatomegaly and splenomegaly as a result of *P. berghei* parasitized and damaged erythrocytes, anaemia, hepatic sinusoidal congestions, and splenic vein thrombosis (Buffet *et al*. 2011). The results obtained also showed that treatments of malaria in the infected mice in groups B through E, with ethanol extract from the root bark of *S. nitida* and artesunate, ameliorated the hepatosplenomegaly.

The organ-to-body weight ratio is another useful index for assessing the toxic effect of substances on the body (Nirogi et al., 2014; Lazic *et al*., 2020), that may cause diseases. The increase in liver-to-body weight and spleen-to-body weight ratios (LBWR and SBWR) recorded in this work for the *P. berghei* malaria-infected untreated mice in group A (NC) are indications of malaria infections in these mice. The decreased in LBWR and SBWR that was seen in the *P. berghei* malaria-infected treated mice in groups B through E, as a result of treatments with ethanol extract from the root bark of *S. nitida* and artesunate also supported the fact that the extract is antimalarial and anti-inflammatory in actions. These actions might be due to the presence of some pharmacologically bioactive compounds with antimalarial, antioxidant, and anti-inflammatory activities in the root bark of *S. nitida* extract. Hepatomegaly and splenomegaly were resolved once the infection was cleared, an observation that is in tandem with the reports of Greenwood (1987), Sowunmi *et al*. (2001), and Vennervald *et al*. (2005). Therefore, ethanol extract from the root bark of *S. nitida* ameliorates lipid peroxidation and hepatosplenomegaly in *P. berghei*-induced malaria-infected mice, which explains the reason for its common use traditionally in Southern Nigeria, for treatment of malaria.

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Full-Length Research Article

The Hypotensive Effect of the Aqueous Calyx Extract of *Hibiscus Sabdariffa* may occur through the Attenuation of Autonomic Nervous System Activity

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Summary: This study tested the hypothesis that the hypotensive effect of the aqueous calyx extract of *Hibiscus sabdariffa* (HS) occurs through autonomic mechanisms that may be associated with a reduction in the double product (DP) of the heart. Following ethical approval and informed consent, the Harvard step test (HST) was performed in healthy subjects (n=14) to activate the autonomic nervous system before and after the oral administration of 15mg/kg HS. The blood pressure (BP) and heart rate (HR) responses were measured and DPs and the mean arterial pressure (MAP) were calculated. Results were expressed as mean ±SEM. Paired t-test and one-way ANOVA with a posthoc Bonferroni test were used for statistical analyses. $P<0.05$ was considered significant. HST without HS resulted in a significant rise in MAP, HR and DP (112.6±2.7mmHg, 97.7±2.5/min and 12630.0±642 mmHg.bpm) from the basal values (98.5±2.3mmHg, 76.5±2.0/min and 8730.7±354.9 mmHg.bpm, $P<0.001$, $P<0.01$ and $P<0.001$ respectively). In the presence of HS, HST-induced changes (ΔMAP=7.8±1.6mmHg; ΔHR=8.1±1.6/min; ΔDP= 1113.6±103.4 mmHg.bpm) were significantly dampened compared to its absence (ΔMAP= 13.3±2.6mmHg; ΔHR=17.0±3.7/min; ΔDP= 3899.3±287.2 mmHg.bpm; $P<0.001$, $P<0.01$ and $P<0.0001$ respectively). The HST-induced increase in BP, HR and DP suggest sympathetic nervous system (SNS) activation and parasympathetic nervous system (PNS) withdrawal associated with an increased cardiac O_2 consumption and workload. These were dampened by HS suggesting that its hypotensive effect occurs through the inhibition of SNS activation, PNS withdrawal and an associated reduction in cardiac O_2 demand and workload.

Keywords: *Autonomic nervous system, blood pressure, double product, Harvard step test, heart rate, Hibiscus sabdariffa calyces*

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INTRODUCTION

Hibiscus Sabdariffa linn (Malvaceae) is planted in most parts of the world and cultivated for its leaf, fleshy calyx, seed or fibre (Dalziel, 1937). *Hibiscus sabdariffa* calyces extract (HS) is used in many parts of the world to make a red-coloured beverage which is called *sobo, soborodo or zobo* in Northern Nigeria. Its red hue and that of French wine appear indistinguishable. Indeed huge bales of HS calyces are exported from Senegal to France to service the wine industry. Hence, HS may help explain, at least in part, the so called "French paradox". It also serves as an antihypertensive agent in Nigerian folk medicine. This has been validated in animal (Onyenekwe *et al.*, 1999; Odigie, Ettarh and Adigun, 2003; Mojiminiyi *et al.*, 2007; Mojiminiyi *et al.*, 2012) and human studies (Haji-Faraji and Haji-Tarkani, 1999; Herrera-Arellano et al., 2004; Herrera-Arellano et al., 2007; Mckay *et al.*, 2010).

HS contains organic acids such as citric and malic acids (Ali *et al.*, 2005). In addition, it is rich in anthocyanins (Ali *et al.*, 2005). Anthocyanins are thought to be the compounds responsible for producing its anti-hypertensive effect (Herrera-Arellano *et al.*, 2007; Mckay *et al.*, 2010). There is also evidence that its polyphenol and hibiscus acid contents are important in carrying out its antihypertensive action (Carvajal-Zarrabal *et al.*, 2005; Hopkins *et al.*, 2013). The mode of action of HS is gradually being delineated. Some of its known actions include antioxidant (Wang, Cao and Prior, 1997; Hopkins *et al.*, 2013), diuretic (Mojiminiyi *et al.*, 2000), cholesterol-lowering (Ochani and D'Mello, 2009), acetylcholine-like, and histamine-like relaxant effects, and direct vasorelaxant action (Adegunloye *et al.*, 1996), inhibition of calcium influx (Ali *et al.*, 2005; Ajay *et al.*, 2007) and angiotensin-converting enzyme inhibitory actions (Ojeda *et al.*, 2010). In addition, HS has antimicrobial (Portillo-Torres *et al.*, 2019), hepatoprotective, immunomodulatory, antiparasitic and anti-cancer effects (Izquierdo-Vega *et al.*, 2020).

Furthermore, HS has been reported to lower both systolic blood pressure (BP) and heart rate (HR) in normotensive and hypertensive rats (Mojiminiyi *et al.*, 2007) and normotensive humans (Aliyu *et al.*, 2014). This suggests that its hypotensive effect may be associated with a reduction in the cardiac rate-pressure product or double product (DP) which is a product of systolic BP (SBP) and HR (i.e. SBP x HR). However, this is yet to be investigated. The cardiac DP is a surrogate measure of cardiac O_2 demand and workload (Katz and Feinberg, 1958; Kitamura *et al.*,

1972). It also correlates strongly with left ventricular mass (Hermida, Fernández and Ayala, 2001).

The autonomic nervous system is very important in the control of BP in health and disease (Guyenet, 2006). In spite of this, its role in the BP lowering effect of HS is yet to be well studied. In an earlier study, we reported that the hypotensive effect of HS was brought about through the attenuation of the discharge of the sympathetic nervous system (SNS) (Aliyu *et al.,* 2014). In that study, two models were used to activate the autonomic nervous system with and without HS. The methods used were the cold pressor test and hand grip or isometric exercise (Aliyu *et al.,* 2014). In the present study we have used a form of dynamic exercise, the Harvard step test, to activate the autonomic nervous system in the presence and absence of HS in order to further investigate the role of autonomic mechanisms in the hypotensive effect of HS. In addition, we have calculated the cardiac DP in order to delineate its role in the hypotensive effect of HS.

MATERIALS AND METHODS

Plant materials: The dried red calyces of HS were purchased from the Talata Mafara Central Market, Zamfara State, Nigeria. These have been 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: The dried calyces of HS were pounded into powdery form and 500g of it was mixed with 2.7 Litres of hot water (50 ^{o}C) in a conical flask. It was then mixed thoroughly using a magnetic stirrer overnight and filtered. The filtrate was decanted and evaporated to dryness in a water bath at 60 ^{o}C leaving a powdery extract**.**

Tableting of HS powdery extract: The extract was prepared into tablets containing 500mg of extract per tablet using the wet granulation method as described earlier (Aliyu *et al.,* 2014). The granules were tableted using a single pouch machine type (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 all experiments were performed in accordance with the Principles of the Declaration of Helsinki.

Subjects: 14 apparently healthy male subjects aged 26.6±1.0 years weighing 62.3±2.0 kg volunteered for the study. Their apparently healthy status was deduced from a questionnaire administered to the subjects.

Inclusion criteria: Subjects who were healthy and indicated no history of cardiovascular, renal, endocrine and other diseases were included.

Exclusion criteria: Subjects who were not on any medication that affects BP and HR and were neither consuming alcohol, tobacco, nor caffeine-containing beverages, nor involved in strenuous exercise 24hrs before the test were recruited for the study.

Harvard step test (HST): Brouha's protocol for Harvard step test (Brouha, Graybiel and Heath, 1943) was used in this study with slight modifications. The nature of the HST was first explained to the subjects. They were then made to rest for at least 30 minutes after which their basal blood pressure (BP) and Heart rate (HR) were measured as follows. The BP and HR were measured using the HuBDIC EchoMax plus BP-400 digital sphygmomanometer (HuBDIC Co. Ltd., Gyeonggi-do, Korea) with the cuff at the same level with the heart. These were regarded as casual BP and HR. Serial BP and HR recordings were then measured at 10- minute intervals until three almost identical readings were obtained. The last of these measurements were taken as the basal BP and HR (Wood *et al.,* 1984). The basal DP was calculated from the systolic BP and HR by multiplying these parameters together (Katz and Feinberg, 1958; Kitamura *et al.,* 1972). The HST was then performed on the subjects. The subjects stepped up onto and back down from a platform at a rate of thirty completed steps per minute (1s up, 1s down) for 5 minutes or until exhaustion and the BP and HR were measured immediately after the exercise. The highest of these were regarded as the peak BP and HR. The peak DP was also calculated. The change (Δ) in BP, HR and DP was calculated for each subject by subtracting the basal value of each parameter from the peak value as follows:

Change (Δ) in BP=peak BP-basal BP,
Change (Δ) in HR=peak HR-basal HR and
Change (Δ) in DP=peak DP-basal DP.

The subjects then rested for 1hour by which time their BP and HR had returned to basal levels. They were then given HS tablets at a dose of 15mg/kg orally and the procedure repeated 1hour post HS consumption. The mean arterial pressure (MAP) was calculated using the formular (Jaja *et al.,* 2000):

Mean arterial pressure=diastolic BP+1/3 pulse pressure
Where pulse pressure=systolic-diastolic BP (i.e. systolic minus diastolic BP)

Data Analyses: The data were presented as Mean±SEM. Paired student t-test was used to analyse the data except when three groups were compared. In the latter case one way ANOVA and a posthoc Bonferoni test was used. $P< 0.05$ was considered statistically significant.

RESULTS

Table 1 shows the demographic and physiological characteristics of the subjects who volunteered for the study. The basal BP parameters and HR compared to the peak parameters during the Harvard step test in the subjects are presented in table 2. There was a significant rise in the BP ($P<0.001$) parameters and HR ($P<0.01$) during the HST compared to the corresponding basal values.

Table 3 shows the peak BP parameters and HR of the volunteers during the Harvard step test with and without HS. The BP parameters and HR fell significantly ($P<0.001$ and $P<0.01$ respectively) in the presence of HS compared to its absence.

Table 1:
The demographic and physiological characteristics of apparently healthy human subjects who volunteered for the study. Values are presented as Mean ± SEM. n=14.

Parameter	Values
Age (years)	26.6±1.0
Sex	Male
Height (meters)	1.71±1.0
Weight (kg)	62.3±2.0
BMI (kg/m^2)	21.1±2.1

BMI = Body mass index

Table 2:
The basal systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP) and heart rate (HR) in apparently healthy human subjects compared to the peak parameters during the Harvard step test (HST). Values are presented as Mean ± SEM. n=14.

Parameter	Basal	Peak (HST)
SBP (mmHg)	127.8±2.6	147.8±3.0[a]
DBP (mmHg)	83.9±2.2	95.8±3.0[a]
MAP (mmHg)	98.5±2.3	112.6±2.7[a]
PP (mmHg)	38.9±2.0	52.0±3.2[a]
HR (beats/min)	76.6±2.0	97.7±3.9[b]

[a]*= P<0.001 peak blood pressure values during HST vs. basal blood pressure values.*
[b]*= P<0.01 peak heart rate values during HST vs. basal heart rate values.*

Table 3:
The peak SBP, DBP, MAP, PP and HR in apparently healthy human subjects during the HST with and without administration of HS extract. Values are mean ± SEM. n=14.

Parameter	HST	HST+HS
SBP (mmHg)	147.8±3.0	128.6±2.7[a]
DBP (mmHg)	95.8±3.0	84.0±3.0[a]
MAP (mmHg)	112.6±2.7	98.4±2.5[a]
PP (mmHg)	52.0±2.8	39.6±2.6[a]
HR (beats/min)	97.7±3.9	86.3±3.0[b]

[a] *= P<0.001 peak blood pressure values during HST vs. corresponding HST + HS values.*
[b]*= P<0.01 peak heart rate values during HST vs. corresponding HST + HS values.*
SBP= Systolic Blood Pressure; DBP= Diastolic blood pressure; MAP= Mean arterial pressure; PP = Pulse pressure; HR= Heart rate.

Figures 1A-E show the changes (Δ) between the peak and basal values of each parameter during the Harvard step test with and without HS. Taken together, the changes in the blood pressure parameters and HR were significantly (P<0.001 and P<0.01 respectively) lower in the presence of HS compared to its absence.

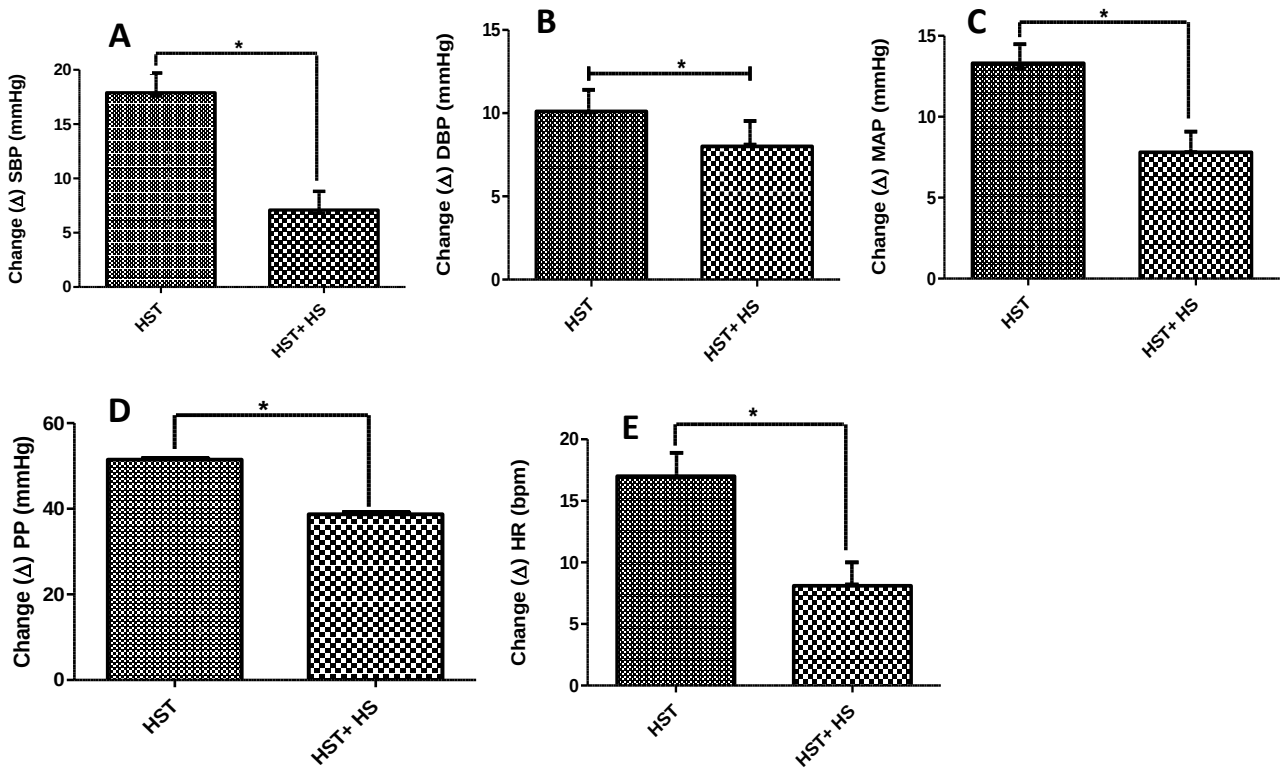

Figure 1
Change (Δ) between peak SBP and basal SBP during Harvard step test with and without HS (A). *=P<0.001 HST+HS vs. HST. SBP= systolic blood pressure; HS = *Hibiscus sabdariffa* calyx extract; HST = Harvard step test without HS and HST+HS = Harvard step test with HS. Change (Δ) between peak DBP and basal DBP during Harvard step test with and without HS (B). *=P<0.001 HST+HS vs. HST; DBP=diastolic blood pressure. Change (Δ) between peak MAP and basal MAP during Harvard step test with and without HS (C). *=P<0.001 HST+HS vs. HST; MAP=mean arterial pressure. Change (Δ) between peak PP and basal PP during Harvard step test with and without HS (D). *=P<0.001 HST+HS vs. HST. PP = pulse pressure. Change (Δ) between peak HR and basal HR during Harvard step test with and without HS (E). *=P<0.01 HST+HS vs. HST; HR = Heart rate.

Figure 2 shows the effect of HS on the DP of the volunteers. The DP rose significantly ($P<0.001$) during the Harvard step test compared to the basal value. However in the presence of HS (i.e. HST+HS), it fell significantly ($P<0.001$) compared to the Harvard step test alone. Also, the DP during the Harvard step test in the presence of HS (HST+HS) did not differ significantly from the basal value (Figure 2).

Figure 3 shows the change (Δ) in DP between the peak DP and basal DP during the Harvard step test with and without HS. The change fell significantly ($P<0.0001$) in the presence of HS compared to its absence.

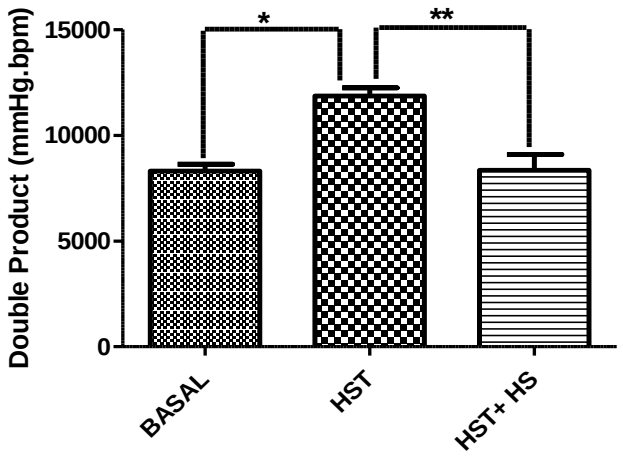

Figure 2:
Basal DP and peak DP during Harvard step test with and without HS. *=P<0.001 HST vs. BASAL; **=P<0.001 HST+HS vs. HST. DP = double product, HS = *Hibiscus sabdariffa* calyx extract; HST = Harvard step test without HS; HST+HS = Harvard step test with HS.

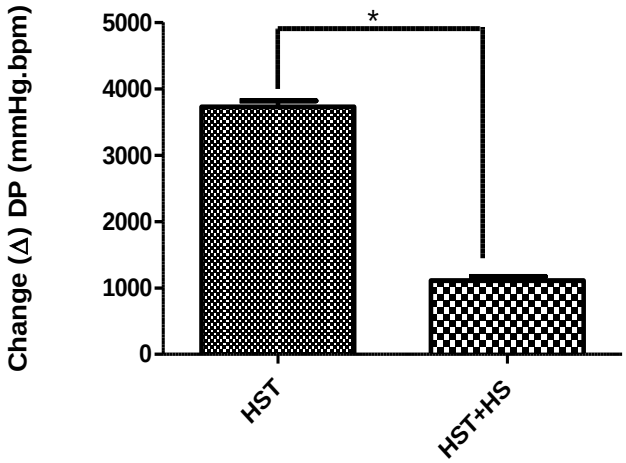

Figure 3:
Change (Δ) between peak DP and basal DP during Harvard step test with and without HS. *=P<0.0001 HST+HS vs. HST. HS = *Hibiscus sabdariffa* calyx extract; HST = Harvard step test without HS; HST+HS = Harvard step test with HS; DP = Double product.

DISCUSSION

The main finding of the present study is that HS reduces BP and HR via autonomic mechanisms associated with the sympathetic and parasympathetic nervous systems. In addition HS significantly lowered the cardiac rate pressure product or double product. These findings suggest that the hypotensive effect of HS may occur through the reduction of the discharge of the sympathetic nervous system (SNS) and inhibition of parasympathetic withdrawal and these are associated with a reduction in cardiac O_2 demand and workload.

During dynamic exercise, such as HST, the stroke volume, HR, and consequently cardiac output increase, whilst the peripheral resistance decreases. These haemodynamic changes result in a moderate increase in BP which produces a rise in blood flow to the skeletal muscle. This results in increased O_2 and nutrient supply to the contracting muscle (Pardeshi and Kirtikar, 2016). The peak BP, HR and DP obtained during the HST were significantly higher than the basal values of these parameters recorded before the start of the exercise. This is consistent with the rise in BP, HR and DP associated with dynamic exercise. Furthermore, the peak BP, HR and DP during the HST fell significantly in the presence of HS compared to its absence again suggesting that the rise in these parameters were attenuated in the presence of HS. In addition, the changes (Δ) in BP, HR and DP obtained during the administration of HS were significantly smaller compared to its absence. The change (Δ) was obtained by subtracting the basal values of these parameters from their peak values. The change (Δ) is a measure of vascular reactivity (Wood *et al.,* 1984) and is directly proportional to muscle sympathetic nerve activity (Cui *et al.,* 2011). Taken together, these findings suggest that HS may reduce vascular reactivity, attenuate the discharge of the SNS and inhibit the parasympathetic withdrawal associated with dynamic exercise. In addition, the decrease in the change in DP in the presence of HS suggests that it reduces myocardial O_2 demand and workload. Hence these may be some of the mechanisms by which HS acts. This is consistent with the earlier finding from our laboratory (Aliyu *et al.,* 2014) suggesting that HS may act through the attenuation of the discharge of the SNS.

The cardiovascular responses accompanying dynamic exercise (HST) described above are mediated by the autonomic nervous system through a rise in the activity of the SNS and inhibition of the activity of parasympathetic nervous system (PNS) (Murphy *et al.,* 2011). This occurs through the following mechanisms namely: the arterial baroreflex, the exercise pressor reflex and the central command (Smith, Mitchell and Garry, 2006; Murphy *et al.,* 2011). While evidence from the current study suggests that HS may act by inhibiting the activity of the SNS and by inhibiting PNS withdrawal, the precise mechanisms by which it does so remain to be seen. In other words, does it work by acting on the arterial baroreflex, or the exercise pressor reflex or the central command or through a combination of these? This suggests that more work is required to answer these questions. However, the extract contains anthocyanins (ACNs) which increase the elaboration of nitric oxide (NO) from the vascular endothelium (Bell and Gochenaur, 2006), as well as regulate the expression and function of endothelial NO synthase

(eNOS) (Vendrame and Klimis-Zacas, 2019). NO is largely responsible for endothelium-dependent relaxation. ACNs also stimulate soluble guanylate cyclase resulting in the relaxation of the smooth muscles in the vasculature. This also results in a rise in cyclic guanosine monophosphate (cGMP) which inhibits the contraction of the vascular smooth muscle by preventing intracellular Ca^{2+} release (Vendrame and Klimis-Zacas, 2019). In addition, ACNs increase the bioavailability of NO by preventing the destruction of NO by reactive oxygen species due to its antioxidant effect (Vendrame and Klimis-Zacas, 2019). This ensures vasodilation and fall in blood pressure. Furthermore ACNs reduce the synthesis of vasoconstrictor molecules such as angiotensin II, endothelin-I and thromboxanes thereby lowering the BP (Parichatikanond, Pinthong and Mangmool 2012; Vendrame and Klimis-Zacas, 2019).

Another interesting finding in this study is that HS acts acutely, within 1 hour of its administration, to carry out its effect. A similar finding was observed in the earlier work from our laboratory (Aliyu *et al.,* 2014). However, this finding needs to be confirmed by additional studies. To the best of our knowledge, the only drug known to lower BP acutely is sublingual nifedipine (Furberg, Psaty and Meyer, 1995).

However, the results of the present study are not in agreement with those of Adegunloye *et al*. which indicated that the hypotensive effect of HS may not be mediated by the inhibition of the sympathetic nervous system (Adegunloye *et al.,* 1996). In that study, the bilateral carotid occlusion (BCO) test was used to activate the sympathetic nervous system in rats. The subsequent rise in BP and HR in response to BCO with and without HS was similar making them to conclude that the sympathetic nervous system may not be involved in the action of HS (Adegunloye *et al.,* 1996). The discord between their findings and those of the present study may be due to differences in the methods used to stimulate the sympathetic nervous system. Although BCO may be used in animals as a method for stimulating the sympathetic nervous system, it cannot be used in man for ethical reasons. The present study used the Harvard step test which meets the ethical requirements for human studies and has therefore been used consistently by several authors in man (Leung *et al.,* 2013; Pardeshi and Kirtikar, 2016). The discord between the findings of Adegunloye *et al*. (1996) and the present study may also be attributable to differences in species used. Unpublished work from our laboratory indicates that the rise in BP and HR following the performance of BCO in the presence of HS compared to its absence are not different in rats thereby affirming the latter notion. It may also suggest that, perhaps, HS may not be acting through the baroreceptor mechanism since BCO tests mechanisms that are associated with the arterial baroreflex by occluding the common carotid arteries proximal to the carotid sinus baroreceptors.

The reduction in DP by HS seen in this study is another interesting observation. As far as we know, our study is the first to report this observation. DP is an index of cardiac O_2 demand as well as cardiac workload (Katz and Feinberg, 1958; Kitamura *et al.,* 1972). It also correlates highly with the left ventricular weight (Hermida, Fernández and Ayala, 2001). The DP rose significantly from the basal value during the HST. However, HS significantly lowered it back to the basal level. Furthermore, HS significantly reduced the change in DP that occurred during HST. These findings suggest that HS lowers myocardial O_2 demand and myocardial workload during dynamic exercise to levels similar to basal conditions. This implies that HS enables the heart to utilize O_2 more efficiently as well as reduce the cardiac workload during dynamic exercise. It is not clear why this is so but a plausible reason may be that HS may dilate the coronary arteries, as it dilates arteries elsewhere (Adegunloye *et al.,* 1996; Abubakar *et al.,* 2019), thereby increasing blood flow to the heart. This finding also suggests that HS may be useful in treating cardiac diseases such as angina pectoris as drugs used in the treatment of such diseases act by reducing DP (Jackson *et al.,* 1980; Kambara *et al.,* 1984). However actual experiments need to be done to confirm these speculations.

The present study has some flaws. Firstly, the BP and HR measurements were not blinded. Ideally, the person measuring the BP and HR should not have known what was administered to the subjects in order to make the study more objective. The subjects should also not have known what was administered to them. In other words the study should have been double blinded. Secondly, a placebo should have been administered to the subjects before the administration of HS. Alternatively, it could have been administered to a separate control group. The present design made each subject his own control without the administration of a placebo. It is a truism that a double blind placebo controlled design would have been more appropriate for this study. These flaws will be addressed in future.

Another limitation of the study is the small number of subjects used. In addition, heart rate variability (HRV) would have proved useful in determining the autonomic mechanisms underlying the effects of HS. However, the equipment required to perform HRV was not available to us.

In summary, the blood pressure, heart rate and double product rose during the HST, a form of dynamic exercise. The rise in these parameters were significantly inhibited in the presence of HS compared to its absence. Since the rise in BP, heart rate and double product are mediated by autonomic mechanisms associated with sympathetic activation and parasympathetic withdrawal, it is concluded that HS may be acting by inhibiting sympathetic activation and parasympathetic withdrawal and this action is associated with a reduction in the cardiac demand for oxygen and cardiac workload.

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Full-Length Research Article

Aqueous Extracts of *Daucus carota* (Linn) Protected the Developing Cerebellum of Wistar Rats Against Perinatal Arsenic-Induced Oxidative Stress

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Summary: The neuroprotective effects of the aqueous extract of *Daucus carota* (Dc) tuber against arsenic-induced oxidative damage on the developing cerebellum of Wistar rats were studied. Twenty-five pregnant rats (110-200g) were divided into five groups (n=5) – control received distilled water; Arsenic (As); Dc (200mg/kg); Dc (200mg/kg) +As; Vitamin C (Vc) (100mg/kg) +As. The pregnant rats in all the groups were treated orally from the first day of pregnancy to postnatal day 21. The Dc extract and Vc were administered one hour before the administration of As. Body weight of the pups on days 1, 7, 14, 21 and 28 were recorded, while neurobehavioural (forelimb grip strength and negative geotaxis) tests were done on day 21 pups. The rats were sacrificed and cerebellar tissues were collected for oxidative stress, histological (H and E), and immunohistochemical studies. Decreased forelimb grip strength, increased lipid peroxidation and decreased glutathione, glutathione peroxidase, catalase and superoxide dismutase was observed in the As group compared with the control and other treated groups. Histologically, the cerebellar cortex of the As pups showed persistent external granular layer (EGL) on postnatal day 21, reduced thickness of the molecular layer (ML) on postnatal day 28, pyknotic and depleted Purkinje cells compared with the control and other treated rats. Immunohistochemical evaluations of the cerebellar cortex showed astroliosis in the As-treated group on day 21 pups compared with the control and other treated groups. Aqueous extracts of *Daucus carota* and Vitamin C reversed the toxicity caused by arsenic. From the results of the study, arsenic-induced oxidative stress with morphological alterations in the perinatal developing rat cerebellum. Extracts of *Daucus carota* exhibited antioxidant activity as such may be a potential neuroprotective agent.

Keywords: Arsenic, *Daucus carota*, oxidative stress, developing cerebellum, Purkinje cells

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INTRODUCTION

The nervous system, in which the cerebellum is an important part of, is one of the earliest systems to develop embryologically but completes its developmental process postnatally (Hill *et al*., 2018), and as a result, it is highly prone to any form of injury in prenatal and postnatal life. The cerebellum lies in the posterior cranial fossa of the cranial cavity and functions in the control of smooth and skilful movements, balance, and higher cognitive and emotional functions (Hall *et al*., 1995; Imosemi and Osinubi, 2011; Hashimoto and Hibi, 2012; Buckner, 2013; Balaei *et al.,* 2017). The cerebellum is highly susceptible to oxidative stress during prenatal and perinatal periods (Lopez, *et al.,* 2009). Neurotoxins disrupt the function of the nervous system by destroying brain cells or nerves which carry signals around the body. Although metals are required for normal homeostasis, when taken in excess amounts, they accumulate in the brain, inducing neurotoxicity which eventually results in neurodegeneration (Chen *et al*., 2016). Arsenic is a naturally occurring metalloid found in groundwater (Izah and Srivastav, 2015; WHO, 2017) and from anthropomorphic sources (Ettinger *et al.,* 2017). Arsenic, a neurotoxin (Sodini, 2015), poses a major public health issue which has been linked to impairments in cognitive development (WHO, 2017). Arsenic toxicity continues to be a problem because it is difficult to have a global assessment of the burden of arsenic toxicity due to its long latency period and in most cases where water is polluted, there are usually more than one pollutants (Tyler and Allan, 2014; Chitsulo, 2018; UNICEF-WHO, 2018). In Nigeria, studies have shown that the level of arsenic in drinking water (Egbinola and Amanambu, 2014; Odebunmi, *et al.,* 2014; Izah and Srivastav, 2015; Etim, 2017) is higher than the WHO recommended level (10µg/L) (WHO, 2017) with the acceptable limit of 0.01 mg/L recommended by Standard Organization of Nigeria (SON) (Izah and Srivastav, 2015). Arsenic can induce oxidative stress by alternating between oxidation states of metals or by interaction with antioxidants, thereby increasing inflammation, accumulation of free radicals in cells and causing cellular dysfunction (Halliwell and Whiteman, 2004). Arsenic is capable of crossing the blood-placenta and blood-brain barriers, adversely effecting pregnancies leading to low-birth-weight, spontaneous abortions, infant death, foetal loss (Milton *et al.,* 2005; Rahman *et al.,* 2007; Rahman *et al.,* 2008; Kile *et al.,* 2016) and causing a disruption in neurological development (Benedetti, 1996; Ding, *et al.,* 2013; Tyler and Allan, 2014; Punshon, *et al.,* 2015; Sodini, 2015; WHO, 2017; UNICEF-WHO, 2018; Htway, *et al.,* 2019). The cerebellum has been shown to be highly susceptible to environmental insults and

oxidative damage (Kern and Jones, 2006). In a developmental study, maternal exposure to arsenic was reported to have altered the developmental process of the cerebellum by slowing down the proliferation of cells especially in the granular layer (Ding, *et al.,* 2013).

Neurological disorders have significantly been on the rise in developing and developed countries (Malomo, *et al.,* 2004; Ajibade, *et al.,* 2015) and in a bid to reduce this rising burden and as an alternative to synthetic drugs, plants are being employed as they are rich sources of phytochemicals (Prajna and Hedge, 2018). A regular intake of vegetables and fruits is believed to be effective in improving antioxidant activities in the brain (Potter, *et al.,* 2011; Yuan, *et al.,* 2017). *Daucus carota* Linn (Carrot) is a root vegetable from *Apiaceae* family (Mani, *et al.,* 2010; Silva Dias, 2014; El-masry and El-rhman, 2017; Praja and Hedge, 2018) that is grown globally and is available all year round. It is rich in carotenoids, flavonoids, polyacetylenes, vitamins and minerals (Silva Dias, 2014) such as ascorbic acid, and tocopherol (Prajna and Hedge, 2018). Consumption of *Daucus carota*, either as food or as juice, has been reported to prevent cancerous growths, impaired vision, control diabetes, blood pressure and boosting the immune system (Silva Dias, 2014; Praja and Hedge, 2018).

The characteristic orange colour of *Daucus carota*s is as a result of their rich content of beta-carotene (an antioxidant), and *Daucus carota*s are also rich sources of vitamins A, B, C and K as well as other minerals (Ca, F, Se, and Mg) (Bjarnadotti, 2015; Bystricka, *et al.,* 2015; Praja and Hedge, 2018). *Daucus carota* contains phytochemicals such as phenolic acid, flavone and flavonols (Alarcón-Flores, *et al.,* 2015). Fruit vegetable juice from *Daucus carota* has been shown to up-regulate mRNA levels in the brain (Yuan *et al.,* 2017) and protect tissues against toxins (Nayeem, *et al.,* 2010; Lee, *et al.,* 2011; El-masry and El-rhman, 2017). Praja and Hedge (2018) reported the cytotoxic, antioxidant, anti-diabetic, antimicrobial, gastro-protective, nephron-protective, cardio-protective, and anti-inflammatory effects of *Daucus carota*.

The primary targets of arsenic toxicity are the gastrointestinal tract, the heart, the brain and kidneys (Benedetti, 1996), Chronic exposure to arsenic may be deleterious to the developing brain (Tyler and Allan, 2014; Sodini, 2015) with the cerebellum highly vulnerable to such intoxication and poisoning (Manto, *et al.,* 2012). Humans and animals cannot survive without water, however, consumption of high amounts of arsenic or small amounts in water over a long period of time exposes man to a wide range of diseases and affects the developing cerebellum (Ding, *et al.,* 2013). This study therefore, evaluated the protective effects of aqueous extracts of *Darcus carota* on arsenic-induced oxidative stress in the postnatal developing cerebellum of rats.

MATERIALS AND METHODS

Drug/Plant materials: Fresh *Daucus carota* (carrots) were bought from Bodija market, Bodija, Ibadan and identified at the Forestry Research Institute of Nigeria (FRIN), Forestry Hill, Jericho, Ibadan with FHI: 112306. Standard vitamin C supplement, batch number 20VCII, manufactured by Nuel Pharm Ltd, Owode-Egba, Ogun State, Nigeria, was purchased from a reputable Pharmarcy in Ibadan. Sodium Arsenite, 10.1g of 98% produced by Qualigens (Shanon Co. Clare, Ireland) was bought from Ad-Folak scientific limited, Ibadan, Nigeria.

Procedure for aqueous extraction of *Daucus Carota*: The samples of *Daucus carota* (carrot) was washed with clean tap water and chopped into pieces. The chopped pieces (2.170kg) was blended using a laboratory electric blender Warring commercial blender, USA, sieved using muslin clothe and filtered. The filtrate was concentrated using a rotary evaporator at 40°C. The concentrate was air-dried in a glass desiccator in order to have the extract in a paste-like form. The final weight was determined as 80.50g.

Animals: Twenty-five (25) female albino Wistar rats, weighing 110-200g, were purchased from the Central Animal House of the College of Medicine, University of Ibadan. The animals were housed in the animal house of the Department of Veterinary Physiology and Biochemistry, University of Ibadan and acclimatised (12 hours light/dark cycle) in well-ventilated cages for two weeks with access to clean tap water and rat chow *ad libitum*. The rats were mated and pregnancy confirmed by the presence of vaginal plug and smear, and this was then regarded as the first day of gestation. All animals received humane care according to criteria outlined in the Guide for the Care and Use of Laboratory Animals published by the US Department of Health and Human Services, Washington (PHS, 1996).

Experimental design: The pregnant rats were divided into five groups (n=5) rats as follows; I- Rats received distilled water and served as the control group; II- Rats received 10% of LD50 (41mg/kg) of arsenic as sodium arsenite daily (Bashir *et al.,* 2006); III: Rats received 200mg/kg/body weight of aqueous extract of *Daucus carota* daily (Sodimbaku *et al.,* 2016); IV- Rats received aqueous extract of *Daucus carota* 200mg/kg per body weight and 10% of LD_{50} (41mg/kg) of arsenic daily; V-Rats received 100 mg/kg of Vitamin C (Okey and Ayo, 2015) and 10% of LD_{50} (41mg/kg) of arsenic daily.

Extracts of vitamin C was administered one hour before the administration of arsenic. All treatments were orally done using oral cannula from the first day of pregnancy to postnatal day 21.

Gross morphological studies: After birth, the body weights of days 1, 7, 14, 21 and 28 pups was recorded and the pups, sacrificed.

Neurobehavioural Tests

On day 21 for each group, forelimb grip strength and negative geotaxis tests were done.

Forelimb grip strength test: This test involves the forepaws of the rats being placed on a horizontally suspended metal wire (measuring 2 mm in diameter and 1m in length), placed one meter above a landing area filled with soft bedding. The length of time each rat was able to stay suspended before falling off the wire was recorded with a stopwatch. A maximum time of 2 minutes was given to each rat after which it was removed. This test reflects muscular strength in the animals (Tamashiro *et al*., 2000).

Negative geotaxis: The unlearned response to gravitational cues is referred to as negative geotaxis (Mortz and Alberts, 2005). This test was done to measure balance/equilibrium. Pups of day 21 were placed head down on a 45° (forty-five degrees) inclined plane and then observed for the time taken to orient in a head-up direction. Time taken was recorded using a stopwatch.

Sacrifice of animals and collection of samples: Pups of days 1, 7, 14, 21 and 28 from the various groups were sacrificed by quick cervical dislocation, and the brain dissected out and weighed. Some of the cerebella of day 21 pups were preserved in phosphate-buffered saline (PBS) at 4^{O}C and at pH 7.4 for oxidative stress and antioxidant assay, while others of days 1, 7, 14, 21 and 28 pups were fixed in 10% formol-saline for histomorphological and immunohistochemical (day 21pups) evaluations.

Oxidative stress and Antioxidant assay: The cerebella of the control and treated day 21 pups were homogenized in eight volumes of 50 mM of Tris-HCl buffer (pH 7.4) containing 1.15% potassium chloride. The homogenate was, centrifuged at 10,000 g for 15 minutes at 4 ºC. The supernatant collected from the centrifugation was used for the estimation of Malondialdehyde (MDA), Reduced Glutathione (GSH), Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx). The level of MDA determined lipid peroxidation according to the method described by Varshney and Kale (1990), Reduced glutathione was determined at 412 nm in a colorimeter using the method described by Beutler et al., (1963). Catalase activity (CAT) was measured spectrophotometrically by the method of Claiborne (1985). Superoxide dismutase (SOD) activity was determined by the method of Misra and Fridovich (1972) and Glutathione peroxidase (GPx) activity was measured according to the procedure of Rotruck et al., (1973) with some modifications.

Histological preparations: Cerebellar tissues from the pups of all groups were fixed in 10% formo-saline, processed employing routine paraffin embedding and stained with Haematoxylin and Eosin for histological evaluation. The slides were examined and evaluated under a 500-pixel Leica digital binocular microscope.

The following parameters were evaluated in the cerebellar cortex; thickness of the external granular layer (EGL) and molecular layer (ML), and Purkinje cell density and astrocyte population using the computer software, image-j.

Immunohistochemistry: Cerebellar tissues were immunostained with Glial fibrillary acidic protein (GFAP) for astrocyte population (neuroglia) using the Avidin biotin immunoperoxidase method. Briefly, cut formalin-fixed paraffin sections were treated with 3% hydrogen peroxide (H_2O_2) for 15min, to block endogenous peroxidase. Then, washed in phosphate-buffered saline (PBS) and treated with GFAP primary antibody (GFAP, mouse monoclonal antibody 1:100 dilution, Leica Biosystems Inc. Ilinois, USA) at room temperature for 60min. The sections were washed in 3 changes of PBS for 5min each, incubated with horseradish peroxidase (HRP) secondary biotinylated anti-mouse antibodies and washed in 3 changes of PBS for 5mins. The sections were then incubated with diaminobenzidine (DAB) for 3 to 5min and counterstained with Haematoxylin solution for 2 mins and blued briefly. Sections were dehydrated in alcohol, cleared in xylene and mounted in DPX. Images were captured from the cerebellar cortex with a 500-pixel Leica binocular microscope. Astrocyte population was counted using the software, image-j.

Statistical analysis: Data collected was further analysed as mean±SEM employing the one-way analysis of variance (ANOVA) followed by Tukey Posthoc for multiple comparisons using the GraphPad prism 6.0 at $p<0.05$.

RESULTS

Effect of treatment on Body weight: There was no significant difference in the mean body weight of all treated groups compared with the control group at $p>0.05$ (Table 1).

Effect of treatment on Brain Weight: There was no significant difference in the mean brain weight of all treated groups compared with the control group at $p>0.05$ (Table 2).

Table 1: Effect of treatment on body weight (g) of pups of the control and treated groups

Group	Day 1	Day 7	Day 14	Day 21	Day 28
Control	7.50±0.32	8.78±0.27	14.54±0.31	25.44±0.69	30.08±0.42
As	6.78±0.29	8.26±0.16	14.66±0.60	25.26±1.44	28.36±0.74
Dc	6.88±0.35	8.52±0.29	14.98±0.57	23.04±0.52	28.64±0.97
DcAs	7.00±0.27	9.02±0.31	14.52±0.80	24.38±0.70	28.90±0.67
VcAs	6.76±0.24	8.18±0.19	14.58±0.64	23.30±0.51	30.30±0.36

Values (n=5) are expressed as Mean±SEM As- Arsenic, Dc- Daucus carota, DcAs- Daucus carota+Arsenic, VcAs- vitamin C+Arsenic. The result showed that arsenic administered at 41mg/kg did not significantly affect the body weight of the treated pups studied, p>0.05.

Table 2: Effect of treatment on body weight (g) of pups of the control and treated groups

Group	Day 1	Day 7	Day 14	Day 21	Day 28
Control	0.25±0.02	0.68±0.01	1.13±0.05	1.26±0.04	1.46±0.03
As	0.23±0.01	0.65±0.03	1.08±0.06	1.24±0.04	1.44±0.03
Dc	0.23±0.01	0.65±0.02	1.11±0.05	1.23±0.05	1.36±0.02
DcAs	0.25±0.02	0.66±0.01	1.08±0.07	1.24±0.04	1.39±0.02
VcAs	0.25±0.02	0.63±0.01	1.13±0.05	1.21±0.04	1.43±0.02

Values (n=5) are expressed as Mean±SEM As- Arsenic, Dc- Daucus carota, DcAs- Daucus carota+Arsenic, VcAs- vitamin C+Arsenic. There was progressive increase in the brain weight of the pups, however, exposure to 41mg/kg arsenic did not significantly affect the brain weight of control and treated groups, p>0.05.

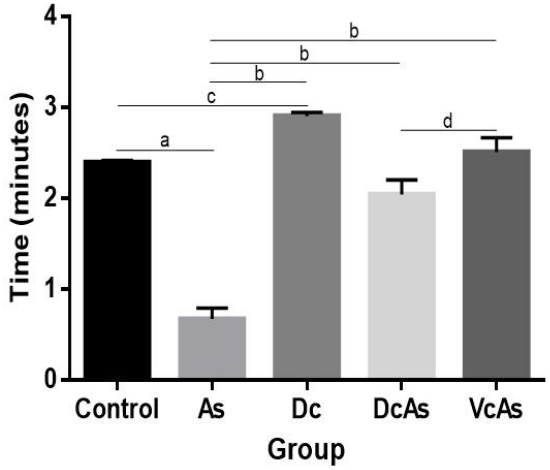


Figure 1:
Forelimb grip strength test. Values (n=8) are expressed as mean±SEM, As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota* + arsenic, VcAs- vitamin C + arsenic. Arsenic, administered at 41mg/kg decreased the forelimb grip strength, while Dc extract and Vc increased the forelimb grip strength.[a]p<0.05 vs control; [b]p<0.05 vs As; [c]p<0.05 vs Dc; [d]p<0.05 vs VcAs.

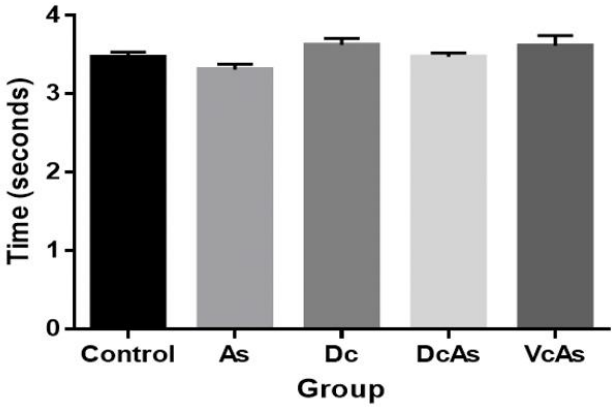


Figure 2: Negative geotaxis in seconds of pups of day 21. Values (n=8) are expressed as mean±SEM, As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota*+arsenic, VcAs- vitamin C+arsenic. Arsenic, administered at 41mg/kg arsenic did not significantly affect negative geotaxis in control and treated groups at the level of this study, p>0.05.

Table 3:
Oxidative stress markers and antioxidants in the cerebellum of the control and treated pups on day 21

Group	LPO µmol/mg tissue	GSH (µg/mg tissue)	CAT (Unit/mg tissue)	SOD Unit/mg tissue	GPx µg/mg tissue
Control	0.18±0.01	0.81±0.03	0.38±0.01	0.70±0.05	44.72±0.60
As	0.29±0.01[a]	0.41±0.04[a]	0.21±0.02[a]	0.46±0.05[a]	31.54±3.37[a]
Dc	0.15±0.02[b]	0.74±0.04[b]	0.32±0.02[b]	0.68±0.01[b]	43.04±2.50[b]
DcAs	0.16±0.01[b]	0.66±0.02[b]	0.31±0.03[b]	0.61±0.01[b]	38.96±2.74
VcAs	0.18±0.01[b]	0.66±0.06[b]	0.26±0.01[a]	0.58±0.01	40.40±1.02

Values (n=5) are expressed as Mean±SEM As- Arsenic, Dc- Daucus carota, DcAs- Daucus carota+Arsenic, VcAs- vitamin C+Arsenic. Arsenic exposure at 41mg/kg significantly caused increased lipid peroxidation, decreased GSH concentration as well as SOD, CAT and GPx activities. Daucus carota and Vc reversed the oxidative marker and antioxidants evaluated, [a]p<0.05 vs control; [b]p<0.05 vs As.

Effect of treatment on forelimb grip strength test: A shorter time was spent in the forelimb grip strength by the arsenic-treated pups on day 21 compared with the control and other treated rats at p<0.05 (Figure 1).

Effect of treatment on Negative geotaxis: The time (in seconds) taken for the pups of day 21to turn against gravity on an inclined (45°) flat surface was not significantly different at p>0.05 in both the control and treated groups (Figure 2).

Effect of treatment on Oxidative stress markers and antioxidant assays: A significant increase in lipid peroxidation and decrease in glutathione, catalase, superoxide dismutase and glutathione peroxidase was seen in the developing cerebellum of arsenic-treated pups on day 21 compared with the control and other treated groups at p<0.05. Catalase activity was significantly decreased in Vc+As-treated group compared with the control pups on day 21 at p<0.05 (Table 3).

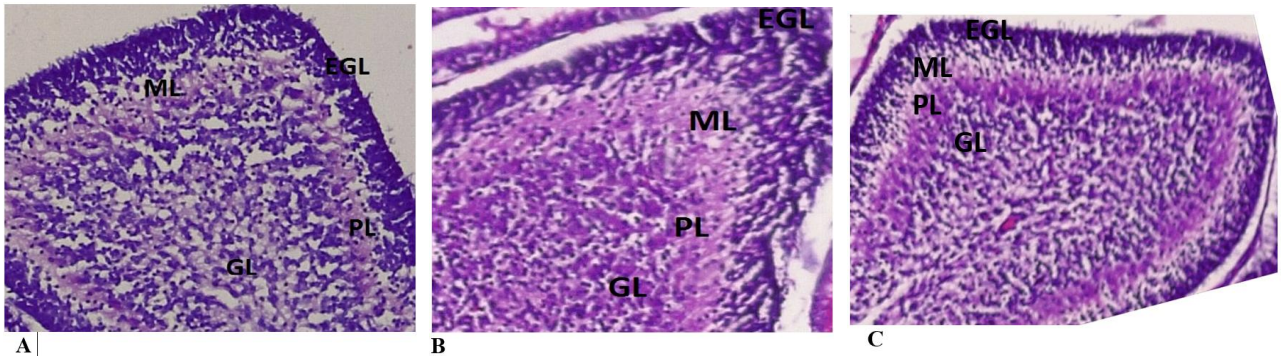
Microscopic observations of the cerebellar cortex Histological analysis: There was no significant difference in the thickness of the external granular layer (EGL) of the cerebellar cortex in the control and treated rat pups on postnatal day 7 at p>0.05. However, a significantly increased EGL thickness was seen in arsenic-treated pups on day 14 compared with the control and *Daucus carota* groups at p<0.05 (Table 4, Plates 1 and 2).

Table 4:
Effect of treatment on the thickness of the external granular layer of the cerebellar cortex on postnatal day 7 and 14.

Group	Day 7 EGL (µm)	Day 14 EGL (µm)
Control	86.00 ±16.31	24.00±2.45
As	80.00 ±4.47	42.00±7.35[a]
Dc	80.00 ±15.17	24.00±2.43[b]
DcAs	69.00 ±5.83	34.00±2.49
VcAs	68.00 ±18.44	30.00±2.41

Values (n=5) are expressed as mean ± SEM. EGL- External Granular Layer, As- arsenic, Dc- Daucus carota, DcAs- D. carota + arsenic, VcAs- vitamin C + arsenic. [a]p<0.05 vs control; [b]p<0.05 vs As.

Effect of treatment on cerebellar cortex of day 21 pups: Employing routine paraffin-embedding, H and E staining technique, the cerebellar cortex of day 21 pups of the control and treated rat pups showed the normal cytoarchitecture of Molecular layer (ML), Purkinje layer (PL) and Granule layer (GL). However, there was persistent 2-3 cell layer thick external granular layer (EGL) in the cerebellar cortex of the As-treated, and one cell layer thick in the DcAs and VcAs pups compared with the control on day 21 (Plate 3).



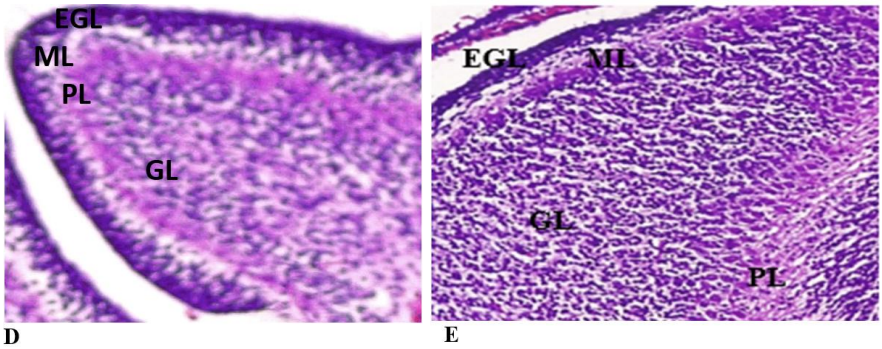


Plate 1:
Photomicrographs of the cerebellar cortex of day 7 pups H&E ×100. (A): Control, (B): As, (C): Dc group, (D): DcAs group, (E): VcAs group. EGL- external granular layer; ML- molecular layer; PL- Purkinje cell layer; GL- granular layer. As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota* + arsenic, VcAs- vitamin C + arsenic.

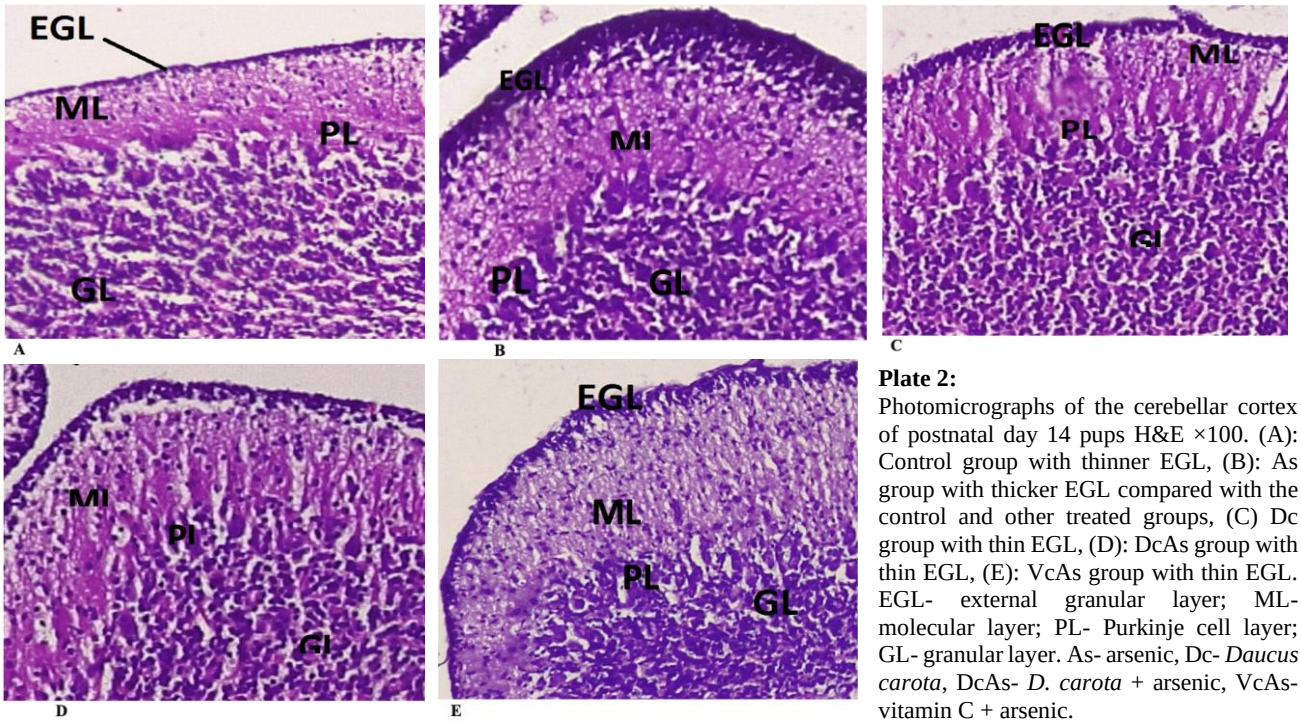


Plate 2:
Photomicrographs of the cerebellar cortex of postnatal day 14 pups H&E ×100. (A): Control group with thinner EGL, (B): As group with thicker EGL compared with the control and other treated groups, (C) Dc group with thin EGL, (D): DcAs group with thin EGL, (E): VcAs group with thin EGL. EGL- external granular layer; ML- molecular layer; PL- Purkinje cell layer; GL- granular layer. As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota* + arsenic, VcAs- vitamin C + arsenic.

Effect of treatment on the thickness of the molecular layer and Purkinje cell density of the cerebellar cortex on postnatal day 28: A significant decrease in the thickness of the molecular layer (ML) and density of the Purkinje cells of the cerebellar cortex was observed in the As, DcAs and VcAs groups on postnatal day 28 compared with the control- and Dc-treated rat pups at $p<0.05$. However, a significant increase in the thickness of the molecular layer (ML) and density of the Purkinje cells of the cerebellar cortex was seen in the Dc and DcAs groups compared with the As-treated pups on day 28 at $p<0.05$ (Table 5, Plate 4).

Table 5:
Effect of treatment on the thickness of the Molecular layer and Purkinje cell density of the cerebellar cortex in the control and treated rats on postnatal day 28.

Group	ML thickness (μm)	Pc density/1.3mm
Control	280.80±4.24	41.00±1.00
As	215.20±3.18[a]	18.80±1.53[a]
Dc	256.20±5.37[b]	32.80±3.71[b]
DcAs	246.30±8.43[ab]	29.20±2.71[ab]
VcAs	229.80±7.83[a]	27.00±2.35[a]

Values (n=5) are expressed as mean±SEM, ML- Molecular Layer, Pc- Purkinje cell, As- arsenic, Dc- Daucus carota, DcAs- D. carota + arsenic, VcAs- vitamin C + arsenic. [a]p<0.05 vs control; [b]p<0.05 vs As.

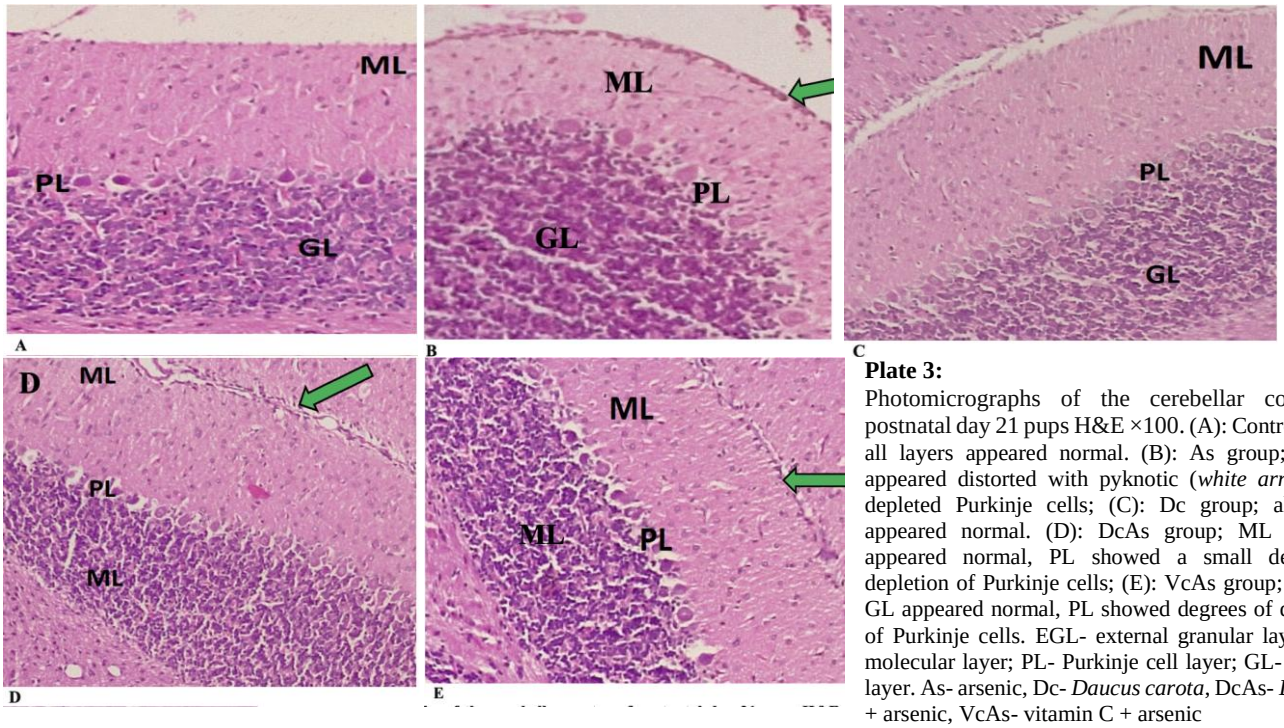


Plate 3:
Photomicrographs of the cerebellar cortex of postnatal day 21 pups H&E ×100. (A): Control group; all layers appeared normal. (B): As group; the PL appeared distorted with pyknotic (*white arrow)* and depleted Purkinje cells; (C): Dc group; all layers appeared normal. (D): DcAs group; ML and GL appeared normal, PL showed a small degree of depletion of Purkinje cells; (E): VcAs group; ML and GL appeared normal, PL showed degrees of depletion of Purkinje cells. EGL- external granular layer; ML- molecular layer; PL- Purkinje cell layer; GL- granular layer. As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota* + arsenic, VcAs- vitamin C + arsenic

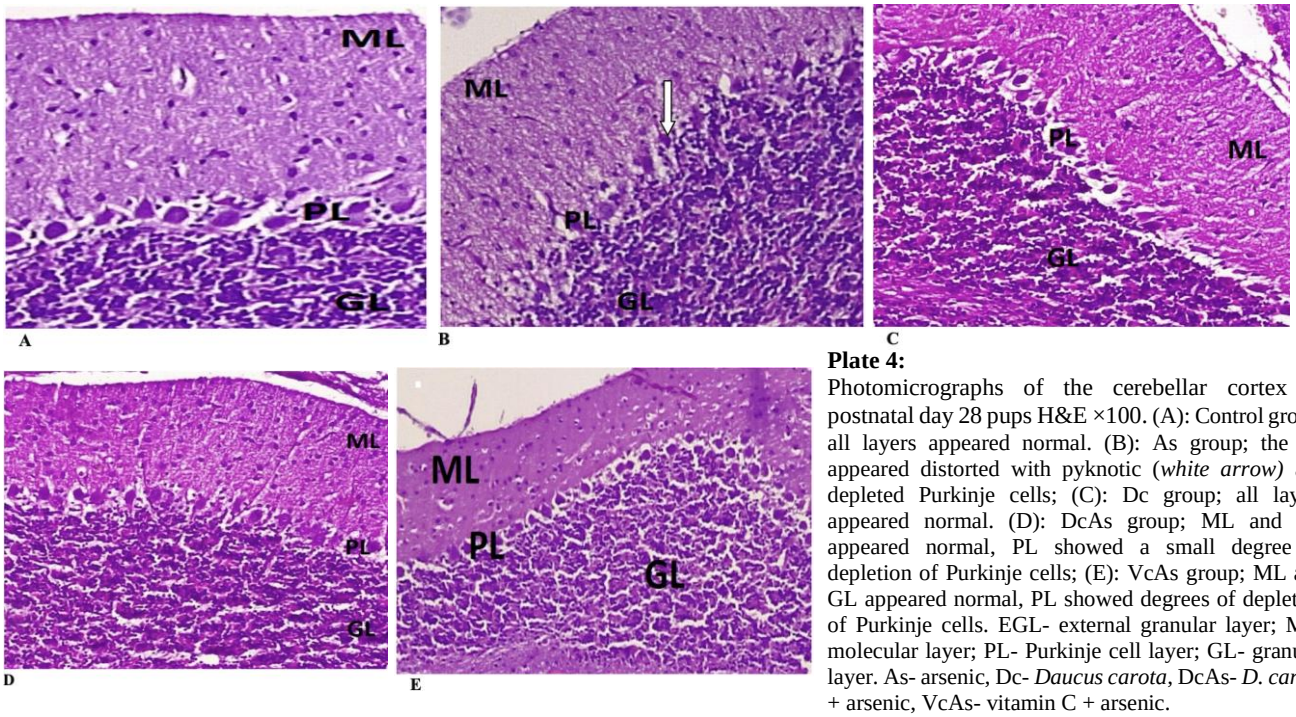


Plate 4:
Photomicrographs of the cerebellar cortex of postnatal day 28 pups H&E ×100. (A): Control group; all layers appeared normal. (B): As group; the PL appeared distorted with pyknotic (*white arrow)* and depleted Purkinje cells; (C): Dc group; all layers appeared normal. (D): DcAs group; ML and GL appeared normal, PL showed a small degree of depletion of Purkinje cells; (E): VcAs group; ML and GL appeared normal, PL showed degrees of depletion of Purkinje cells. EGL- external granular layer; ML- molecular layer; PL- Purkinje cell layer; GL- granular layer. As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota* + arsenic, VcAs- vitamin C + arsenic.

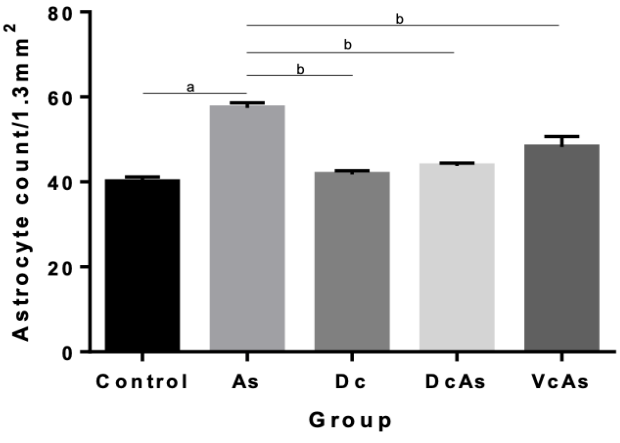


Figure 3:
Astrocyte population in the cerebellar cortex of day 21 pups. Values (n=5) are expressed as mean±SEM, As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota*+arsenic, VcAs- vitamin C+arsenic. [a]p<0.05 vs control; bp<0.05 vs As

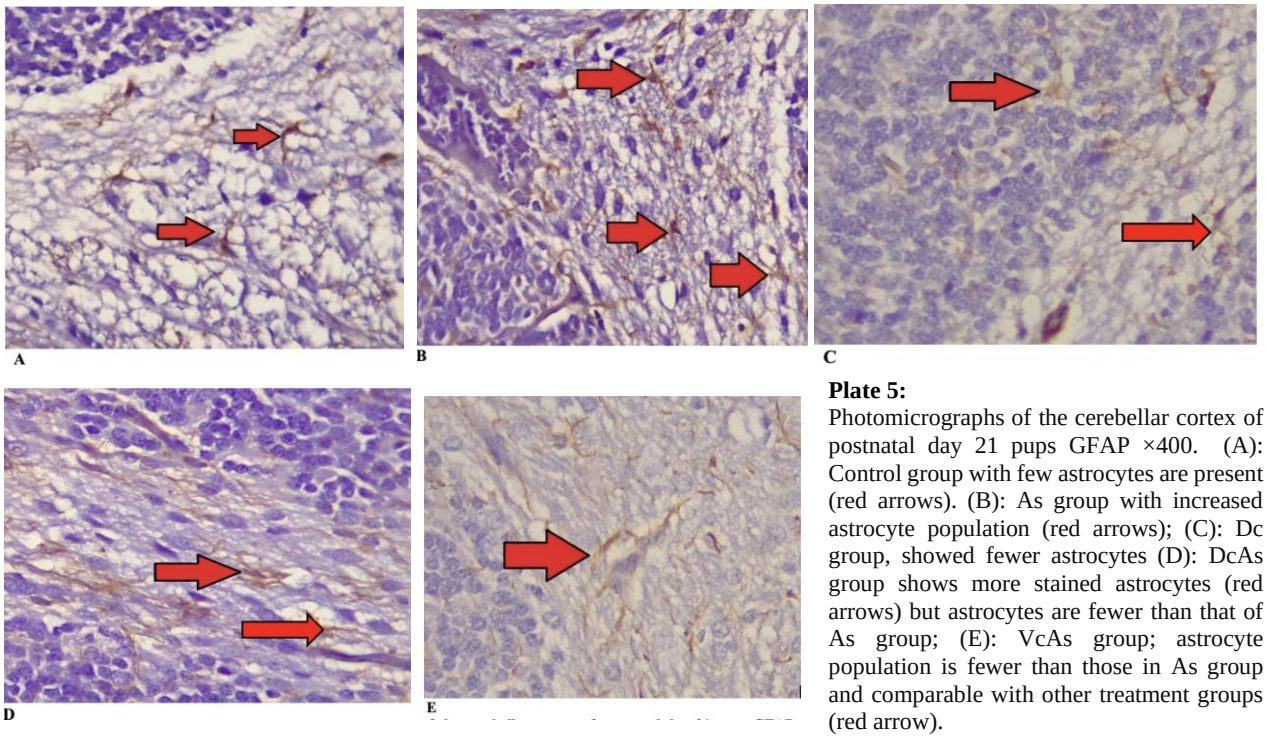


Plate 5:
Photomicrographs of the cerebellar cortex of postnatal day 21 pups GFAP ×400. (A): Control group with few astrocytes are present (red arrows). (B): As group with increased astrocyte population (red arrows); (C): Dc group, showed fewer astrocytes (D): DcAs group shows more stained astrocytes (red arrows) but astrocytes are fewer than that of As group; (E): VcAs group; astrocyte population is fewer than those in As group and comparable with other treatment groups (red arrow).

Effect of treatment on Astrocyte Count on Postnatal Day 21: There was significant increase in astrocyte population in the cerebellar cortex of the As-treated pups compared with the control and other treated pups on day 28 at $p<0.05$ (Figure 3 and Plate 5).

DISCUSSION

In this study, the protective effects of *Darcus carota* (Dc) on arsenic-induced neurotoxicity on the postnatal developing cerebellum was observed. The results from our study showed that the body and brain weight of the control and treated rat pups was not significantly affected. These findings are in agreement with the reports of Grosicki and Kowalski (2006); Potter *et al*. (2011). Experimental studies have linked the toxic effect of arsenic to a decrease in body and organ weight (Avani and Rao, 2009; Kozul-Horvath *et al.*, 2012; Negishi *et al.*, 2013; Tolins *et al.*, 2014; Barai *et al.*, 2017). Herrera *et al.* (2013) observed that there was no decrease in body or organ weight in perinatal arsenic-treated pups. Other researchers have also supported that body and organ weight are unaffected in pups whose dams were prenatally and or perinatally exposed to arsenic (Gandhi *et al.*, 2011; Ince *et al.*, 2012; Aung *et al.*, 2016). Reduction in body weight has been reported to be more in adult rats than in pups (Bikashvili *et al.*, 2017) and differences observed may be as a result of a diet which can reduce the effect of arsenic toxicity (UNICEF-WHO, 2018).

Neurobehavioural evaluations showed decreased forelimb grip strength, an indication of decreased muscular strength and tone in the arsenic-treated group resulting in muscular weakness. Muscle coordination and orientation, a function of negative geotaxis involving the re-orientation against gravity was not significantly affected. Arsenic toxicity has been shown to affect the development of the cerebellum (Ding *et al.*, 2013), affecting smooth and skilful movements, balance, emotion and cognition (Buckner 2013; Balaei *et al.*, 2017). Research has shown that oxidative stress induces generation of ROS which causes increase in protein loss, reduction of force generation and increased muscle atrophy (Gumucio and Mendias, 2013). Increased muscular strength and tone observed in the group administered Dc and vitamin C may be attributed to their antioxidant and protective properties.

Oxidative stress occurs as a result of a distressing imbalance in the production of reactive oxygen species and antioxidant activity (Betteridge 2000; Halliwell and Whiteman, 2004). It has been proposed that oxidative stress may be the mechanism by which arsenic toxicity exerts its effects (Tolins *et al.*, 2014) and the cerebellum is susceptible to oxidative damage especially during prenatal and perinatal periods (Lopez *et al.*, 2009). The elevated level of MDA and reduction of GSH concentration as well as SOD, CAT and GPx activities in the developing cerebellum of arsenic-treated day 21 pups indicated oxidative stress. Cerebellar tissue is known to be highly sensitive to damage by free radicals because of its high concentration of polyunsaturated fatty acids, low concentration of cytosolic antioxidants and high use of oxygen (Ebokaiwe et al., 2013). The results indicated that arsenic induced oxidative stress in the brain of the arsenic exposed pups. These findings are similar to those of other researchers (Rao and Avani, 2004; Bashir *et al.*, 2006; Ince *et al.*, 2012; Herrera *et al.*, 2013; Mundey *et al.*, 2013; Negishi *et al.*, 2013; Bonetto *et al.*, 2017; Khodayar *et al.*, 2019). Antioxidants have the ability of reducing free radicals and preventing oxidation. Day 21 pups administered extracts of Dc and vitamin C had reduced MDA levels and elevated GSH concentration as well as SOD, CAT and GPx activities suggesting antioxidant activity of Dc, which helped in mitigating the oxidative damage induced by arsenic toxicity in the developing rat cerebellum. The results suggested that Dc demonstrated ameliorative effect against lipid peroxidation probably due

to the high antioxidant activity associated with its high phenolic content (Dada et al., 2017).

Neurons and astrocytes expend a high amount of oxygen in carrying out their function (Gandhi and Abramov, 2012) and may be the major target of arsenic neurotoxicity (Piao *et al.,* 2005; Ma *et al.,* 2010). These may be the reasons behind the histomorphometric observations found in this study. The developing cerebellar external granular layer (EGL) on postnatal day 14 arsenic-treated pups was found to be significantly thicker than that of the control group, although the difference was not significant on postnatal day 7. Ding *et al.* (2013) reported a decreased thickness of the cerebellar EGL on postnatal days 10 and 13 rats treated with arsenic. The thicker EGL observed in the arsenic-treated pups on day 14 may be due to delayed differentiation of the EGL because the EGL is a highly metabolic layer which disappears in humans at approximately two years of age and in mouse, at about postnatal days 20 (Hatten and Heintz, 1995; Altman and Bayer, 1985; Imosemi and Osinubi, 2011; Butts *et al.,* 2014). The external granular layer (EGL) consists of highly metabolic cells whose differentiations result in the synthesis of the outer stellate, basket, Golgi type II and granule cells of the cerebellar cortex. In this study, there was complete disappearance of EGL in the control and other treated groups on day 21 pups but 2-3 layers thick of EGL persisted in arsenic-treated group. The mechanism for the persistent EGL in the arsenic-treated group is not clearly understood but delayed maturation of cells of the EGL have been reported by some workers (Malomo et al., 2004; Imosemi and Osinubi, 2011) which may be due to the oxidative stress induced by arsenic. The molecular layer (ML) of the cerebellar cortex is usually smaller than the other cortical areas in early development but shows a rapid increase between postnatal days 9-25. It becomes the most superficial layer when the external granular layer disappears (Heinsen 1977). There are numerous synapses of the Purkinje cells (Pc) with basket and stellate cells in the ML layer (Balaei, *et al.,* 2017). In the present study, it was observed that arsenic significantly decreased the thickness of the ML in pups of day 28. Ding *et al.,* (2013) reported that arsenic cause condensation, vacuolation and nuclear fragmentation in the ML, causing reduction in the thickness of the ML. The Purkinje cells of the cerebellum serve as the sole output neuron of the cerebellar cortex, therefore they are highly important (McKay and Turner, 2005; Balaei, *et al.,* 2017) and are easily affected by genetic and environmental insults (Minai, 2014). It was observed in this study, that the number of Purkinje cells was significantly reduced in the arsenic-treated pups on day 28. The mechanism involved in the loss of Purkinje cells is not completely clear but arsenic has been reported to generate ROS which inhibits the activity of antioxidant enzymes in some tissues, inducing oxidative stress and causing cell deaths. Co-administration of extracts of Dc and vitamin C with arsenic increased the ML thickness and density of the Pc count, probably by its free radical scavenging activity and preventing further oxidative damage of the ML and Pc of the developing cerebellum.

Astrocytes play an important role in protecting the blood brain barrier and they also provide biochemical support to other neurons (Tolins, *et al.,* 2014). In this study, arsenic toxicity caused an increase in astrocyte population probably by the generation of free radicals in the developing cerebellum on postnatal day 28 resulting in astrogliosis. Cantanzaro *et al.* (2010) and Tolins *et al.* (2014) reported that arsenic exposure to rats increased apoptosis in primary culture of astrocytes. Research has shown that oxidative stress can up-regulate the expression of glial fibrillary acidic protein (GFAP) and ROS in astrocytes thereby promoting excessive reactive astrogliosis and glial scar formation (Sofroniew, 2009). *Daucus carota* containing phenolic compounds and flavonoids, and vitamin C reduced astrogliosis probably by scavenging the free radicals generated by arsenic toxicity.

From our results, perinatal arsenic exposure induced oxidative stress, affected forelimb grip and caused morphological changes (delayed maturation and differentiation of the EGL, reduced thickness of the ML, loss of Pc and astolgliosis) in the developing cerebellum of rats, and that extracts of *Daucus carota* and Vitamin C reduced the rate at which arsenic induced oxidative stress as such may be a potential neuroprotective agent.

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Full-Length Research Article

Ficus vogelii Methanol Leaf Extract Possess Anti-Arthritis, Anti-Inflammatory and Membrane Stability Potentials in C57BL/6J Mice

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Summary: The study evaluated the membrane-stabilizing potentials in red blood cells and anti-inflammatory properties in C57BL/6J mice of the methanol leaf extract of *Ficus vogelii*. Animals were treated orally with different doses of the extract (50, 100, 200 mg/kg) for 30 days and their blood was measured for membrane stability at different saline concentrations. Diclofenac (12.5 mg/kg) or Indomethacin (10 mg/kg) was used as standard in the anti-inflammatory studies. The mean corpuscular fragility (MCF) values and their corresponding percentage stabilization increased significantly ($p \leq 0.05$) in the treatment groups compared to the negative control. Treatment of mice with 50, 100 and 200 mg/kg of the extract significantly ($p \leq 0.05$) inhibited carrageenan-induced paw oedema in mice. The highest dose (200 mg/kg) showed lower anti-inflammatory activity compared to Diclofenac (12.5 mg/kg). Daily administration of the extract significantly ($p \leq 0.05$) suppressed adjuvant-induced paw arthritis by day 15 and 30 post arthritis induction. *Ficus vogelii* extract inhibited granuloma formation significantly. The anti-inflammatory effects of methanol leaf extract of *Ficus vogelii* on granuloma formation were comparable to that of Indomethacin (10 mg/kg). In summary, this study showed that the methanol leaf extract of *Ficus vogelii* possessed membrane-stabilizing potentials and anti-inflammatory properties, therefore, providing further proof that the leaves contain an active compound with potent anti-inflammatory activity.

Keywords*: Ficus vogelii*, membrane stability, arthritis, anti-inflammatory, oedema, C57BL/6J mice

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INTRODUCTION

The cell membrane that surrounds a cell is selectively permeable, allowing only certain molecules to enter and exit the cell. It is well known that mammalian cells are equipped with extensive repair mechanisms that help to constantly maintain the integrity of the cell membrane, thus highlighting the importance of the cell membrane to the survival of cells (Dias and Nylandsted, 2021). In events that lead to the compromise of the health of the cell membrane, the cell becomes susceptible to acute or chronic injuries or completes disruption that may lead to cell death (Cooper and McNeil, 2015; Boye and Nylandsted, 2016). Known cell membrane repair mechanisms includes; membrane fusion and replacement strategies (via exocytosis-mediated repair), removal of damaged membranes (by endocytosis-mediated repair or shedding), and protein-driven membrane remodelling and wound closure (Dias and Nylandsted, 2021). However, these repair mechanisms do not occur individually but co-operate in unison for maximum repair to be effective (Moe *et al*., 2015; Jimenez and Perez, 2017; Dias and Nylandsted, 2021). In all, a rise in the intracellular calcium level, which the cell interprets as danger, is the key stimulus that initiates the cell membrane repair mechanism, intended to rapidly reseal the wound (Draeger *et al*., 2011; Dias and Nylandsted, 2021).

Understanding the process of membrane repair had enabled the identification of therapeutic strategies and boosting of membrane repairs in diseases related to poor membrane integrity (Boye and Nylandsted, 2016). Different therapeutic strategies which are mainly the use of membrane stabilising agents and exogenous expression of several recombinant proteins are currently in use. Membrane stabilization strengthens or reinforces the cell membrane to become stronger and able to withstand influences from the outside environment (Anosike *et al.*, 2012). The use of membrane stabilizers in the management of inflammation and consequently pain, both acute and chronic is gaining wide acceptance (Boniface *et al.*, 2014). They have been used for many years to treat painful conditions such as trigeminal neuralgia, complex regional pain syndrome, and headaches (Honoro *et al.*, 2018). Poloxamer 188, a membrane-stabilising agent is a multi-block copolymer surfactant which seals cell membrane defects in various cell types caused by different types of injuries (Murphy *et al*., 2010; Gu *et al*., 2013; Inyang, *et al*., 2020). Most anti-inflammatory drugs have been reported to have membrane stabilising effects on cell membranes (Ogbomade *et al.*, 2019). The anti-inflammatory actions of steroids such as Vamorolone have been used as a membrane stabilising agent (Sreetama *et al*., 2018; Dias and Nylandsted, 2021). Furthermore, proteins such as the MG53, dysferlin, GRAFI and the annexins have shown enhancement of cell

membrane repairs (Benevolensky *et al*., 2000; Matteo and Moravec, 2000; Cai *et al*., 2009; Lenhart *et al*., 2015).

Inflammatory response of tissues is a defensive and protective mechanism aimed at removing the harmful stimuli causing injuries and subsequently initiates healing processes to the injured tissue (Maldini *et al*., 2009). In the treatment of the consequences of inflammatory conditions, steroids and non-steroidal anti-inflammatory drugs are commonly used. An effective anti-inflammatory drug should potentially inhibit the development of inflammation with fewer side effects on the integrity of the tissues or system (*Fakhrudin et al., 2014*). However, the unpleasant side effects of these agents such as gastrointestinal ulcers (Goldsby *et al*., 2003) have necessitated the search for newer, safer and more effective conventional and herbal-derived anti-inflammatory agents.

Studies have shown that several herbal derived drugs have the properties to be classified as anti-inflammatory agents (Chi *et al*., 2001; Mohini *et al*., 2012; Lui *et al*., 2014; Sarkar *et al*., 2016; Ren *et al*., 2017). Furthermore, some herbal extracts have both anti-inflamatory and membrane stabilising activities (Oyedapo *et al*., 2004, 2020; Arawwawala *et al*., 2010; Debnath *et al*., 2013). The aim of this research work was to evaluate the membrane stabilising effects and anti-inflammatory activities of the extract of the leaf of *F. vogelii* in order to relate its medicinal activities to natural and bioactive constituents. The leaf of *Ficus vogelii*, a commonly used green-leafy vegetable in various dishes in Northern Cross River State of Nigeria is traditionally believed to guarantee good health and well-being. Its ethanol extract is used by adults for well-being, while its aqueous extract is used for weaning children and for treatment of pediatric anemia (Igile *et al.*, 2018). Relevant literature discovered that traditionally, there are claims that the bark and root are used in treating urinary tract infection, asthma, diabetes, malaria, cardiovascular diseases, kidney diseases and cough (Igile *et al.*, 2018).

MATERIALS AND METHODS

Extraction of Plant materials: *F. vogelii* leaves were obtained from Akamkpa Local Government Area in Cross River state, Nigeria were identified at the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Enugu State, Nigeria. The leaves were dried at room temperature (25 °C) without direct sunlight and grinded. The ground leaves (1.5 kg) were extracted by cold maceration with 80 % methanol (Sigma Aldrich, Germany) at room temperature for 48 hours then filtered with a Whatman No. 1 filter paper. The filtrate was concentrated with a rotary evaporator (Büchi, Switzerland) at 40 °C and stored in a refrigerator at 4 °C.

Animal and experimental design: All experiments with animals were approved by the Ethical Committee of the University of Nigeria, Nsukka (Approval Number: UNNRA019/V0411). C57BL/6J mice of both sexes, aged 6 weeks, purchased from the Department of Veterinary Animal Laboratory, University of Nigeria, Nsukka. The mice were acclimatized for 10 days in polyethylene cages with free access to water and a 15 % fat diet in a controlled environment and relative humidity, with a 12 h light-dark cycle. All animals were humanely handled and their welfare respected throughout this study as stipulated in the 1964 Helsinki Declaration, as amended (World Medical Association, 2013)

Acute toxicity test: Thirty (30) mature albino mice of both sexes (27.15±2.31 g) were weighed and randomly separated into 5 groups (A–E) of 6 rats per group. Groups A–D were dosed orally with varying doses (100, 500, 1000, and 2000 mg/kg) of the methanol leaf extract *of F. vogelii* respectively while group E (5th group) was given an equivalent volume of distilled water (10 ml/kg). The mice were allowed access to feed and water *ad libitum* for 72 h and observed for signs of toxicity and death**.**

Osmotic fragility of Red Blood cells in C57BL/6J mice: Twelve (12) mature mice of both sexes (27.15±2.31 g) were weighed and randomly separated into 4 groups (A–D) of 3 rats per group. Group A-C were treated orally with 50, 100 and 200 mg/kg of methanol leaf extract of *Ficus vogelii* respectively. Group D was given distilled water and served as a negative control. Blood was collected from mice in each group into heparinized tubes after a 30-days treatment. For each blood sample, test tubes were numbered from 1 to 17. Five ml of buffered NaCl ranging from 0.1 to 0.85% was placed in tubes 1 through 16. Five ml of distilled water was placed in tube 17. 0.1 ml of blood was added to each tube and mixed gently and then incubated at 37 °C for 1 h. the tubes were centrifuged at 2000 rpm for 10 min to sediment any intact red cells. The haemoglobin content of the supernatant was measured at 540 nm with a spectrophotometer using the 0.85%-saline tube as a blank and the distilled water tube as the 100%-haemolysis as standard. The highest value of optical density which corresponded to an incubation concentration of 0.1% NaCl was taken as 100% haemolysis. Percentage lysis was calculated using the formula:

$$\frac{\text{Abs of test}}{\text{Abs of standard}} \times 100$$

% haemolysis was plotted against % NaCl concentrations. The mean corpuscular fragility (MCF), which is the concentration of NaCl producing 50 % lysis was extrapolated from the graphs and recorded. Also, % stabilization was calculated.

Anti-Inflammatory Studies

Carrageenan-induced paw edema: Thirty (30) mice of both sexes were placed in groups of 6 mice per group and treated orally with either methanol leaf extract of *F. vogelii* at 50, 100, 200 mg/kg, Diclofenac (12.5 mg/kg) or distilled water, 60 min prior to an injection of 1 % carrageenan (Ramchandran *et al*., 2002) into the plantar tissue of the right hind paw. The contra-lateral hind paws were injected with 0.1 ml of normal saline as control. Paw edema volume was measured using an Orchid digital plethysmometer at 0, 1, 2 and 3 h after carrageenan injection.

Adjuvant-induced polyarthritis: The arthritic syndrome was induced in mice by an injection of 0.1 ml of Freund's complete adjuvant into the sub-plantar region of the right hind paw according to methods of Ramchandran *et al.* (2002) and Kasture *et al*. (2001). Twenty-five mice of both sexes were grouped into 5 groups of 5 animals each. The

groups were orally treated with either methanol leaf extract of *F. vogelii* at 50, 100, 200 mg/kg, Diclofenac (12.5 mg/kg) or distilled water (0.03 ml/10 g) for 30 days. Plethysmographic determination of paw volume was performed on both injected and contra-lateral foot. Paw volume after 18 h of adjuvant injection was taken as sub-acute phase of inflammation and that of the 15th and 30th days were observed as an index of chronic inflammation.

Cotton pellet-induced granuloma test: Cotton pellet induced granuloma test was performed as described by Niemegeers *et al.* (1975). Formation of granuloma was induced by subcutaneous implantation of 50 mg of sterile cotton pellets into the left and right axillae of each mouse under pentobarbitone (35 mg/kg) anaesthesia. Twenty-five mice of both sexes were grouped into 5 groups of 5 animals each. Post cotton pellet implantation, each group was treated once daily with either the extract (50, 100 and 200 mg/kg) respectively, or indomethacin (10 mg/kg) or distilled water intraperitoneally for a period of 7 days. On day 8, the animals were euthanized using chloroform and the pellets surrounded by granuloma tissues were carefully dissected out. The moist pellets were weighed and dried at 60 °C for 24 h. The dry pellets were then weighed to obtain their dry weights. The weights of the granuloma formed were calculated as the difference between the wet and dry weights (Udegbunam *et al.*, 2011).

Data Analysis

Median Corpuscular Fragility (MCF) values and % stabilization was calculated by the one-way analysis of variance (ANOVA) using unpaired student's t test. The student t-test was applied at 5 % confidence level. The mean oedema size and granuloma weights of the treated groups and those of the control were compared using one-way analysis of variance. All analyses were done in the SPSS version 16.0. Duncan multiple range test was used to separate variant mean at $P < 0.01$ or 0.05.

RESULTS

Yield and Acute toxicity test: The total yield of the extract was 72 g. Acute toxicity studies revealed no extract-induced mortality or overt serious clinical manifestation even at the highest test dose of 2000 mg/kg.

Median Corpuscular Fragility (MCF) values and % stabilization in C57BL/6J mice treated with methanol leaf extract of *F. vogelii*: The methanol leaf extracts of *F. vogelii* significantly and dose dependently reduced the corpuscular fragility and increased cell membrane stabilization in the test animals. The percentage reductions in corpuscular fragility were 0.277±0.023, 0.317±0.028, 0.387±0.042 for the 50, 100 and 200mg/kg of the extract while the percentage stabilization were 25.34, 43.44 and 75.11 respectively (Table 1).

Effect of methanol leaf extract of *F. vogelii* on carrageenan-induced paw edema in C57BL/6J mice: The effect of the doses (50,100,200mg/kg) of the methanol leaf extracts of *F. vogelii* were comparable to those of the reference drug (diclofenac) and was more pronounced at 3h after the injection of the carrageenan (Table 2).

Table 1:
Median Corpuscular Fragility (MCF) values and % stabilization in C57BL/6J mice treated with methanol leaf extract of *F. vogelii*

	Dose (mg/kg)	MCF (% [NaCl])	stabilization
Ficus vogelii	50	0.277±0.023	25.34[a]
	100	0.317±0.028	43.44[a]
	200	0.387±0.042	75.11[b]
Control	-	0.221±0.015	

The MCF values and their corresponding % stabilization were increased in the treatment groups as compared to the negative control. The highest dose (200 mg/kg) gave the highest % stabilization.

Table 2:
Effect of methanol leaf extract of *F. vogelii* on carrageenan-induced paw oedema in C57BL/6J mice

Treatment	Dose (mg/kg)	Paw volume (ml)			
		0 h	1 h	2 h	3 h
Ficus vogelii	50	0.35 ±0.01	0.35 ±0.02	0.44 ±0.01*	0.48 ±0.03*
	100	0.34 ±0.03	0.33 ±0.01*	0.50 ±0.02	0.51 ±0.03
	200	0.35 ±0.02	0.34 ±0.01*	0.45 ±0.02*	0.44 ±0.02*
Diclofenac	12.5	0.36 ±0.03	0.44 ±0.02	0.31 ±0.02*	0.31 ±0.02*
Distilled water	0.03ml/10g	0.33 ±0.01	0.53 ±0.03	0.59 ±0.03	0.63 ±0.04

*Values are expressed as mean±S.E.M.; n = 6; *P <0.01 compared with negative control*

Effects of methanol leaf extract of *F. vogelii* on Adjuvant-induced paw arthritis in C57BL/6J mice: The result shows a dose dependent effect of leaf extracts of *F. vogelii* on paw inflammation when compared against the positive control. The volume of the paw oedema decreased as the phases progressed from the 0h to Day 30. The doses significantly decreased the paw size at day 30 when compared against the previous phases (Table 3).

Table 3:
Effects of methanol leaf extract of *F. vogelii* on Adjuvant-induced paw arthritis in C57BL/6J mice

Treatment	Dose (mg/kg)	Paw volume (ml)			
		0 h	18 h	Day 15	Day 30
Ficus vogelii	50	0.34 ±0.02	0.58 ±0.03*	0.51 ±0.32	0.45 ±0.02*
	100	0.33 ±0.02	0.56 ±0.02*	0.51 ±0.03	0.41 ±0.03**
	200	0.33 ±0.03	0.59 ±0.03	0.47 ±0.02*	0.42 ±0.02**
Diclofenac	12.5	0.34 ±0.01	0.48 ±0.02**	0.44 ±0.02**	0.35 ±0.01**
Distilled water	0.03ml/10g	0.34 ±0.02	0.77 ±0.04	0.80 ±0.04	0.77 ±0.02

*Values are expressed as mean±S.E.M.; n = 5. *P<0.05; **P<0.01 compared with negative control*

Effect of methanol leaf extract of *F. vogelii* on Granuloma weight in C57BL/6J mice: The methanol leaf extracts of *F. vogelii* significantly and also dose-

dependently reduced the granuloma weight and increased percentage inhibition in mice. The granuloma weights were 0.24±0.03, 0.16±0.02 and 0.11±0.01 for the 50, 100 and 200mg/kg of the extract while the percentage inhibition were 11.11, 40.74 and 59.26 respectively (Table 4).

Table 4:
Effect of methanol leaf extract of *F. vogelii* on Granuloma weight in in C57BL/6J mice

Treatment	Dose	Granuloma wt (g).	% inhibition
Distilled water	1 ml/kg	0.27 ± 0.03 _	
Indomethacin	10 mg/kg	0.09 ± 0.02*	66.67*
Extract	50 mg/kg	0.24 ± 0.03	11.11
Extract	100 mg/kg	0.16 ± 0.02*	40.74*
Extract	200 mg/kg	0.11 ± 0.01*	59.26*

Values are expressed as mean±S.E.M. or %;
*n = 5. *P<0.05; **P<0.01 compared with negative control.*

DISCUSSION

The present study showed that oral administration of 50 mg/kg, 100 mg/kg and 200 mg/kg of the methanol leaf extract of *F. vogelii* significantly reduced the percentage hemolysis of the erythrocytes in comparison to the control. The inhibition of cell membrane lysis in this study could be attributed to the anti-inflammatory activities of the leaf extract as reported in some other studies with extracts (Mounnissamy *et al*., 2008; Debnath *et al*., 2013; Oyedapo *et al*., 2020) Furthermore, the highest dose of 200 mg/kg showed most significant percentage of stabilization and membrane protective capacity with a relatively low flagiligram. This suggests that the methanol leaf extract of *F. vogelii* confers significant level of cell membrane protection to the cells when compared to the control. Similar result was obtained from works on *Pilostigma thonningii*, a plant used in the treatment of various diseases characterized with anaemia, jaundice and fatigue (Fongang *et al*., 2014). Furthermore, osmotic fragility tests are used clinically as a diagnostic and management tool in hereditary spherocytosis, elliptocytosis and other red cell membrane disorder as a measure of cell viability (Brown *et al*., 1983; Da Costa *et al*., 2013). However, they may also be useful in the interpretation of inflammatory and prolonged malfunctions due to high blood pressure and other oedema-associated diseases in poor countries where automated blood-cell counters are readily available. Thus the efficacy of any membrane stabilizing agent is dependent on its ability to either reduce the harmful effects or restore the physiology of the cell membrane that has been disrupted by the toxicant. This is so since the free radicals (superoxide, hydroxyl radicals, hydrogen peroxide, nitric oxide radical) produced as a result of oedema during inflammation sometimes all act to disintegrate cell membranes.

Upon inducement of paw oedema by carrageenan test, the results showed marked reduction with the extract of *F. vogelii* when compared with group treated with the diclofenac and distilled water which served as positive and negative controls respectively. This suggests that the extract may have reduced the oedema, pain and inflammation that is normally associated with tissue injury. Pain and inflammation accompanies tissue damage or injury. Most analgesics act on the peripheral or central nervous system where they either block the generation of impulses at the chemoreceptor sites of pain or raise the threshold of pain. Analgesics also alter the physiological responses to pain, suppress anxiety and apprehension which are essential to the repair process (Chandana *et al.,* 2011). The pathway of the analgesic abilities of the *F. vogelii* which were in phases in this work may not be known presently, however, the analgesic properties have been highlighted in this study. Similar result was reported on a related plant, *Ficus glomerata*, where the early phase of analgesia was attributed to the release of histamine and serotonin and the later phases sustained by the leukotriene and prostaglandins (Menezes *et al.,* 2011). Flavonoids and tannins have been reported to inhibit PG synthesis as identified in work done on *Ficus religiosa* (Yaso *et al.,* 2018) as most NSAIDs have well balance anti-inflammatory and ulcerogenic activities which can be attributed to its PG synthase inhibitor activities.

The extract of *F. vogelii* when used in the treatment of the inflammatory conditions of synovitis showed promising results with pronounced effects. These effects (decrease in paw volume) increased in subsequent days when compared to the group administered diclofenac and the distilled water serving as controls. Even though the group administered diclofenac showed more effects in the Adjuvant-induced paw arthritis test, the *F. Vogelii l*eaf extract showed significant reduction in the induced inflammation and synovitis, thus implying that the extract was able to suppress inflammation and synovitis. Rat adjuvant induced arthritis is a commonly used animal model for the studies of NSAIDs and disease modifying anti-rheumatic drugs (Srinivasa *et al.,* 2019). Development of arthritis is divided into phases. It starts with the induction phase without any evidence of synovitis, followed by early synovitis and finally late synovitis with progressive joint destruction. An effective anti-rheumatic drug should be able to block one or more of these phases. *F. vogelli* at the dose of 200 mg/kg showed very effective prevention of systemic inflammation as seen in the reduced destruction of joints. Similar findings were seen in works where extracts of *Ficus benghalensis* roots where used to alleviate arthritis in rats (Bhardwaj *et al*., 2016). This activity can be attributed to its tannins and flavonoid contents.

The extracts of the *F. vogelii* showed marked decrease in granuloma weight with increased administered dose when compared to the group administered indomethacin which served as the positive control group. Inflammation is known to be an integral part of the body's defence mechanism. Furthermore, inflammation and granuloma normally develop within several days with the proliferative phase of inflammation involved with the production of macrophages, fibroblasts and neutrophils which subsequently leads to formation of granuloma. The marked level of inhibition with increased doses and subsequent decrease in weight of the granuloma indicates that the proliferative phase was effectively suppressed by the extract. This is in tandem with work by Arul *et al* (2005) on *Morus indica.* This ability of the methanol extract of *F. vogelii* to suppress the proliferative phase of inflammation may be because of inhibition of the mediators of inflammation such as histamine, serotonin as well as prostaglandin.

In conclusion, the efficacy of *F. vogelii* as an effective therapeutic agent for treatment of cases of inflammation and

stabilization of anaemic patient have been shown in this work and hence, authenticates its folkloric use in treatment of pediatric anaemia. It also shows that methanol leaf extract of *F vogelii* possess a modulatory potentials on changes that occur within the erythrocyte and vascular system in cases of acute inflammation by creating changes to the mediators of inflammatory reaction. Further studies are required to elucidate the mechanism of its anti-inflammatory properties and also possible isolation of active constituent responsible for the stated properties.

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Full-Length Research Article

Comparative Effects of Methanol and Aqueous Extracts of *Corchorus olitorius* Plant on Haematology and Some Reproductive Indices of Male Wistar Rats

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Summary: *Corchorus olitorius* is a vegetable plant/shrub and the leaves are very nutritious and rich in vitamins, minerals and dietary fibers. The study was carried out to evaluate the effect of the aqueous and methanol leaf extracts of this plant on some male reproductive indices in male Wistar rats Forty-five mature male rats of about 12 weeks old, each weighing about 120g were used for this study, kept in a cage and fed with commercial rat pellets and water was given at ad-libitum. They were randomly divided into three groups A, B and C of 15 rats per group. The leaf extract (250mg/kg) was administered orogastrically once daily for 21 days. Group A and B were treated with methanol and aqueous leaves of Corchorus extract respectively and Group C was given distilled water and served as the control. Five rats per group were sacrificed and the following analysis was carried out namely, haematology, testosterone assay and semen. In this study, it was observed at the first-week post-treatment that there was a significant ($p \leq 0.05$) decrease in the PCV and haemoglobin values of group A (Methanol treated) rats compared to the control group but the values later increased at the second week and third-week post-treatments. There was also a significant decrease in spermatozoa motility in the methanol treated groups A and aqueous treated B compared to the control group C (distilled water treated) at the first, second and third-week post-treatments. Also, with the results of the serum testosterone level of group A and B compared to group C. The value was higher in group A followed by group B whereas, at the second week and third week, there was no significant difference in the values of the serum testosterone levels compared to the control groups. It is therefore concluded that the methanol and aqueous extract of *Corchorus olitorius* leaves significantly decreased sperm motility in male albino rats hence could decrease male fertility.

Keywords: *Corchorus olitorius, Wistar rats, fertility, spermatozoa, testes. Reproductive indices.*

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INTRODUCTION

Corchorus olitorius is commonly known as Nalta jute, Tossa jute (Nyadamu *et al*., 2017), Jew's mallow (Duke and James, 1979) West African sorrel and bush okra. It is a species of shrub in the family Malvaceae. It is the primary source of jute fibres (Holm *et al.*, 1997). Some Nigerians names for the crop include Ewedu in (Yoruba), Achingbara in (Igbo) and Rama in the Hausa Language. The plant grows well in the lowland tropics, up to an elevation of around 700 meters (Tindall, 1983). They are reported to tolerate annual precipitation between 400 and 4290mm, and an annual average temperature range of 16.8 to 27.5^{o}C (Ghorai, 2008). Phytochemical screening on both methanol and aqueous extracts revealed some constituents common to both solvent extracts except alkaloids which were absent in methanol extracts (Barku *et al.*, 2013).

Several studies were conducted on the chemical constituents of *Corchorus olitorius*. These chemicals include cardiac glycosides (Rao *et al*., 1972, Sanilova and Lagodich, 1998) Triterpene chemical constituents by Manzoor *et al.* (1971 and 1979) and Mukherjee *et al.* (1998), Ionones chemical constituent by Yoshikawa *et al.* (1997), Phenolics chemical constituents by Yoshikawa *et al*. (1997), Azuma *et al*. (1999) and Mukherjee *et al*. (1998) and the lignin content of a mature *Corchorus olitorius* stem by Tanmoy *et al*. (2014). This plant is important as a nutritive vegetable rich in K, Ca, P, Fe ascorbic and carotene as reported by Azuma *et al.* (1999).

The leaves and young fruits are used as a vegetable, the dried leaves are used for tea and as a soup thickener and the seeds are edible. Young leaves are added to salads whilst older leaves are cooked as a pot-herb. The leaves quickly become mucilaginous when cooked. The dried leaves can be used as a thickener in soups (Facciola, 1990). It is an important leaf vegetable in Cote d'Ivoire, Benin, Nigeria, Cameroon, Sudan, Uganda, Kenya and Zimbabwe. It is also cultivated and eaten in the Caribbean and Brazil, in the Middle East and India, Bangladesh, Japan and China. In Nigeria, the leaves are boiled to make a sticky, mucilaginous sauce which is served with balls of cassava that are otherwise rather dry (Grubben, 2004).

The leaves are demulcent diuretic febrifuge and tonic (Chopra and Nayar, 1986; Nishiumi *et al.*, 2005). They are used in the treatment of chronic cystitis, gonorrhoea and dysuria (Chopra and Nayar, 1986). A cold infusion is said to restore the appetite. Injection of Olitoriside an extract from the plant, markedly improve cardiac insufficiencies and have no cumulative attributes; hence, it can serve as a substitute for strophanthin (Ghorai, 2008). The methanol

extract of its leaves has been reported to show a broad-spectrum antibacterial and it has wound healing properties (Barku *et al.,* 2013). It is speculated by Oyedeji *et al.* (2013) that the plant has a deleterious effect on some reproductive functions in male albino rats also that *Corchorus olitorious* is known to contain a high level of iron and folate which are useful for the prevention of anaemia (Oyedele *et al.,* 2006). Furthermore, the different parts of *Corchorus olitorious* were found to exhibit diverse biological activities. The leaves of *Corchorus olitorious* were reported to exhibit antioxidant (Oboh *et al.,* 2009) antitumor (Furumot *et al.,* 2002), gastroprotective (Al-Batran *et al.,*2013) antibacterial and antifungal (Iihan, *et al.,* 2007) anti-inflammatory and analgesic activities (Das *et al.,* 2010).

Wistar rats are an outbred strain of albino rats belonging to the species Rattus norvegicus. The strain was developed at the Wistar Institute for use in biological and medical research and is notably the first rat strain developed to serve as a model organism. (Clause 1988). The rats because of their marked anatomical homology to the human cardiovascular system (Casteleyn *et al.* 2017), proved valuable in psychological studies of learning and other mental processes (Vandernbergh, 2000), a study also found rats to possess metacognition, a mental ability previously documented in humans as reported by Foote *et al*. (2007) and Wallinford *et al. (*2010).

Pal *et al.* (2009) reported some antifertility effects of methanol extract of *Corchorus olitorious* while Oyedeji *et al*. (2013) reported the antifertility effect of the aqueous extract of the plant. Thus, the study aimed to compare the effects of methanol and aqueous extracts of *Corchorus olitorious* leaves on some reproductive indices in male Wistar rats.

MATERIALS AND METHODS

Animals: Forty-five adult male rats of about 12 weeks old, each weighing 120g were used for this study. They were kept in cages at the experimental Animal Unit at the Faculty of Veterinary Medicine, University of Ibadan. They were fed with commercial rat pellets and water was given *ad libitum.*

The rats were grouped into 3 (A, B and C) of 15 rats each.
Group A- was treated with methanol extract
Group B- was treated with aqueous
Group C- was the control which was given distilled water.
250mg/kg of the plant extracts were used for these experiments and were administered orogastrically (Oyedeji, *et al*., 2013). The experimental rats were sacrificed at weeks 1, 2 and 3 post-treatment.

Ethical consideration: The research proposal was approved by the Animal Care and Use Research Ethics Committee (ACUREC) of the University of Ibadan. The andrological examination was carried out on the rats and they were acclimatized for 2 weeks with other veterinary attention.

Plant Collection & Identification: The fresh leaves of *Corchorus olitorious* plant were collected from the University farm and the botanical identification was done at the Department of Botany Herbarium, the University of Ibadan with Botanical ID no UIH-22616.

Plant Extraction: The leaves of the plant were plucked and cleaned. The leaves were then air-dried for two weeks after which 200g of the dried leaves were pulverized.

For Aqueous extraction, 1000g of the grounded dried sample was weighed, transferred into a glass container and 10 litres of distilled water was added, stirred every two hours with a glass rod and allowed to stay for 24 hours. The solvent (now containing the extract) was collected using a muslin bag and the filtrate was further filtered using 1mm Whatman filter paper. the filtrate was then concentrated with the aid of a rotatory evaporator set at 50^{o}c, after which the extract was further concentrated using a vacuum oven set at 50^{o}C with a pressure of 700mmHg. The total yield from the aqueous extraction was 84.931 with a percentage yield of 8.5%.

For Methanol extraction, 1000g of the dried sample was transferred into a glass container and 5 litres of pure methanol was added, stirred every two hours with a glass rod and allowed to stay for 72 hours. The resulting solution was collected using a muslin bag and the filtrate was further filtered using 1mm Whatman filter paper. This process was repeated twice with another 5 litres of pure methanol added each time to the shaft. The combined filtrate was then concentrated with the aid of a rotatory evaporator set at 40^{o}C, after which the concentrate was further concentrated using a vacuum oven set at 40^{o}C with a pressure of 700mmHg.

Administration of the extracts: 0.2 ml of 250mg/kg of the leaf extracts was given to the rats. The rats were picked up properly and a syringe and cannula were used to administer the plant extracts orogastrically

The infraorbital lateral cantus method was used which entailed blood being collected from the orbital sinus as described by Jain, (1986). The rats were anaesthetized in a glass chamber containing ether soaked in cotton wool. Anaesthesia was achieved in about 2 minutes. Capillary tubes used were filled with the blood samples up to about plain 2/3 and were analyzed using the method of Jain, (1986) and Coles (1989).

Blood samples from the rats were collected, 3ml in a sample bottle with ethylenediaminetetraacetic acid (EDTA) and 2ml in a plain bottle slanted to facilitate separation of serum for biochemical analysis.

Semen Collection: The male rats were put into a glass chamber containing cotton wool soaked in ether and allowed to lose consciousness. The testicles were exteriorized through a mid- caudoventral abdominal incision with a sterile scalpel blade. Sperm cells were then collected from cauda epididymis and analyzed as described by Oyeyemi and Fayomi (2011) and Zemjanis (1970).

Bodyweight, testicular & Epididymal Biometry: Testicular parameters like the width of the testis, length of individual right and left testis were measured using digital vernier callipers. The bodyweight of the rats and the weight of the epididymis, individual right and left testes were taken using a digital scale.

Statistical Analysis: - All data obtained were expressed as means with the standard errors and one-way analysis of variance was used to compare means and the significance was reported as ($P \leq 0.05$).

RESULTS

Semen characteristics: Comparison of results of groups within weeks revealed that the first-week post-treatment for semen characteristics value of spermatozoa motility is significantly higher in group C which is the control group than group A which was treated with methanol extract and that of group B which was treated with aqueous extract but the value of spermatozoa motility of group A is significantly higher than the value of group B.

The results for the second week (Figure 2) revealed that sperm motility decreased significantly in groups A and B compared to group C whereas there is a non-significant difference in sperm liveability in all the groups.

The results for third-week semen characteristics (Figure 3) revealed that there was a significant decrease in sperm motility of groups A and B compared to group C. The value is higher in group C followed by group B which is followed by group A. There is no significant difference in sperm liveability in all the groups.

Group A showed a progressive decrease over the three weeks (88.75, 75.00 and 68.00%) while there was a non-significant decrease in the percentage of sperm liveability. While group B revealed a progressive decrease of sperm motility and liveability at the first and second week after treatment but there was a non-significant increase at the third week in both parameters. There was a non-significant change in the values in group C for both sperm motility and liveability.

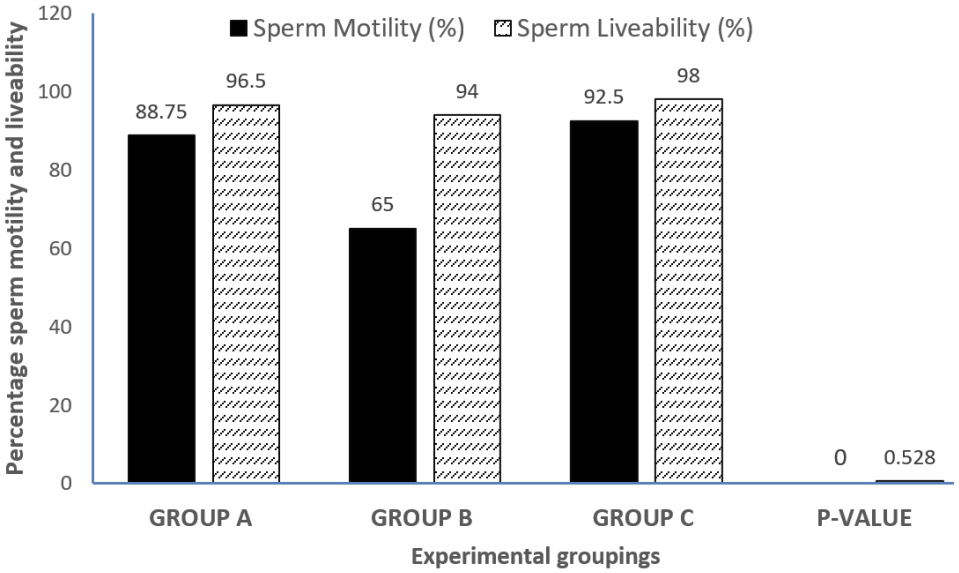

Figure 1
Semen characteristics after one week of oral administration of aqueous extract of *Corchorus olitoriorus*

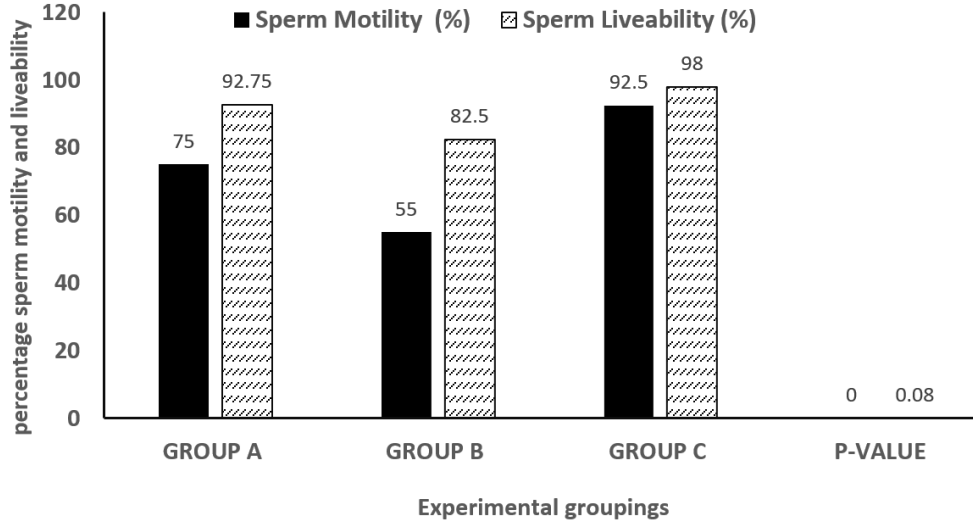

Figure 2
Semen characteristics after two week of oral administration of *Corchorus olitorius.*

Spermatozoa morphological abnormalities: The results for first-week morphological abnormalities (Table 2) that there is no significant difference in spermatozoa morphological abnormalities in the treated groups compared to the control group. The conspicuous spermatozoa morphological abnormalities observed were tailless head, headless tail, rudimentary tail, bent tail, curved tail, curved midpiece, bent midpiece and looped tail.

Haematology: The haematology values for the first-week post-treatment (Table 5) revealed that packed cell volume and haemoglobin concentration of group A were significantly lower than that of groups B and C. The Red blood cells of group A was significantly higher than that of group B which is also significantly higher than that of group C.

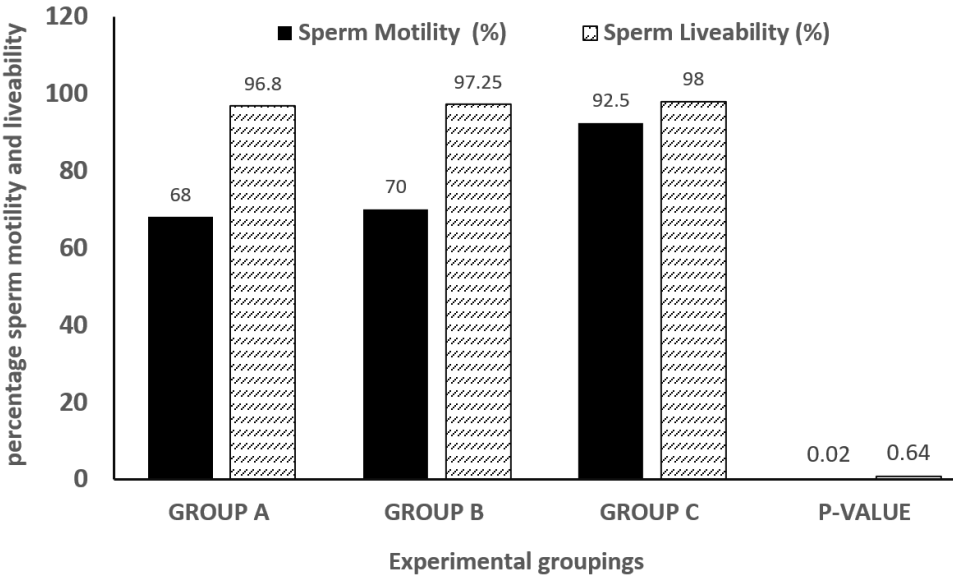

Figure 3
Semen characteristics after the third week of oral administration of *Corchorus olitorius.*

Table 1.
Comparison of values of motility and livability across the three weeks

	First Week			Second Week			Third Week		
Parameter	A	B	C	A	B	C	A	B	C
Sperm Motility (%)	88.75± 3.15	65.00± 2.89	92.50± 2.50	75.00± 2.89	55.00± 2.89	92.50± 2.50	68.00± 3.74	70.00± 4.08	92.50± 2.50
Sperm Livability (%)	96.50± 0.87	94.00± 3.08	98.00± 0.00	92.75± 3.20	82.50± 4.33	98.00± 0.00	96.80± 0.73	97.25± 0.75	98.00± 0.00

Table 2
Results For Sperm Cell Morphological Abnormalities after one Week Post Treatment

GROUP	GROUP A	GROUP B	GROUP C	P-VALUE	REMARKS
Tailless head (%)	4.00±5.78 [a]	4.50±0.65 [a]	4.25±0.75 [a]	0.87	NS
Headless tail (%)	4.00±0.41 [a]	4.75±0.63 [a]	4.75±0.25 [a]	0.44	NS
Rudimentary tail (%)	1.50±0.29 [a]	2.00±0.41 [a]	2.25±0.48 [a]	0.44	NS
Bent tail (%)	8.00±0.58 [a]	8.00±0.41 [a]	8.50±0.65 [a]	0.77	NS
Curved tail (%)	9.25±0.48 [a]	8.25±0.63 [a]	8.75±0.75 [a]	0.55	NS
Curved midpiece (%)	9.25±0.48 [a]	7.50±0.50 [a]	8.25±0.25 [a]	0.05	NS
Bent midpiece (%)	9.50±0.29 [a]	8.75±0.48 [a]	8.75±0.63 [a]	0.48	NS
Looped tail (%)	2.50±0.29 [a]	2.50±0.29 [a]	1.75±0.48 [a]	0.29	NS
Total abnormal cells	48.00±1.22 [a]	46.25±0.48 [a]	47.25±2.66 [a]	0.77	NS
Total cells	403.75±2.40 [a]	407.50±3.23 [a]	403.75±2.39 [a]	0.55	NS

NS=Not significant, S=Significant, [abc]=Means in the same row with different superscript differ significantly($p<0.05$).

Table 3
Results for Sperm Cell Morphological Abnormalities (%) After The Second Week of Treatment.

GROUP	GROUP A	GROUP B	GROUP C	P-VALUE	REMARKS
Tailless head	4.50±0.65 [a]	3.75±0.48 [a]	4.25±0.75 [a]	0.36	NS
Headless Tail	4.25±0.48 [a]	4.00±0.41 [a]	4.75±0.25 [a]	0.96	NS
Rudimentary Tail	2.00±0.41 [a]	2.25±0.48 [a]	2.25±0.48 [a]	0.10	NS
Bent Tail	8.50±0.65 [a]	8.75±0.75 [a]	8.50±0.65 [a]	0.05	NS
Curved tail	8.25±0.48 [a]	8.50±0.29 [a]	8.75±0.75 [a]	0.21	NS
Curved midpiece	9.00±0.41 [a]	8.00±0.41 [a]	8.25±0.25 [a]	2.05	NS
Bent midpiece	8.50±0.29 [a]	7.75±0.48 [a]	8.75±0.63 [a]	1.15	NS
Looped tail	1.75±0.48 [a]	2.25±0.48 [a]	1.75±0.48 [a]	0.36	NS
Total abnormal cells	46.75±0.63 [a]	45.25±1.03 [a]	47.25±2.66 [a]	0.38	NS
Total cells	403.75±2.39 [a]	406.25±2.39 [a]	403.75±2.39 [a]	0.36	NS

N=Not significant, S=Significant, [abc]=Means in the same row with different superscript differ significantly($p<0.05$).

TABLE 4
Results for Sperm Cell Morphological Abnormalities after Third Week of Treatment

GROUP	GROUP A	GROUP B	GROUP C	P-VALUE	REMARKS
Tailless head	5.00±0.41 [a]	5.00±0.41 [a]	4.25±0.75 [a]	0.63	NS
Headless tail	4.50±0.65 [a]	4.25±0.48 [a]	4.75±0.25 [a]	0.27	NS
Rudimentary tail	2.25±0.48 [a]	2.25±0.48 [a]	2.25±0.48 [a]	0.00	NS
Bent tail	8.75±0.48 [a]	9.50±0.29 [a]	8.50±0.65 [a]	1.11	NS
Curved tail	9.00±0.82 [a]	9.00±0.41 [a]	8.75±0.75 [a]	0.05	NS
Curved midpiece	9.50±0.50 [a]	8.25±0.63 [a]	8.25±0.25 [a]	2.21	NS
Bent midpiece	9.25±0.85 [a]	9.00±0.41 [a]	8.75±0.63 [a]	0.15	NS
Looped tail	2.25±0.48 [a]	2.00±0.41 [a]	1.75±0.48 [a]	0.30	NS
Total abnormal cells	50.50±2.10 [a]	49.25±1.38 [a]	47.25±2.66 [a]	0.60	NS
Total cells	402.50±2.50 [a]	403.75±3.75 [a]	403.75±2.39 [a]	0.06	NS

NS=Not significant, [abc]=Means in the same row with different superscript differ significantly (p<0.05).

Table 5.
Haematology Result after one Week of Treatment

GROUP	GROUP A	GROUP B	GROUP C	P-VALUE	REMARKS
PCV (%)	31.60±2.86[a]	38.40±1.60[b]	40.40±1.81[b]	0.03	S
HB (%)	10.47±0.90 [a]	12.80±0.53 [b]	12.72±0.46 [b]	0.04	S
RBC (x106uL)	8.83±0.37[a]	8.97±0.17[b]	6.72±0.27 [c]	0.00	S
WBC (x106uL)	8790.00±648.34 [a]	10240.00±342.56 [b]	5490.00±573.67 [c]	0.00	S
PLATELET	3.15 x 10^5±178239.61 [a]	1.45 x 10^5±5991.66 [a]	1.116 x10^5±25486.47 [a]	0.36	NS
LTM (%)	63.40±2.46 [a]	65.40±1.86 [a]	67.20±1.39 [a]	0.42	NS
NEUT	28.80±2.24 [a]	28.00±2.07 [a]	28.40±1.50 [a]	0.960	NS
MON (%)	3.40±0.51 [a]	3.40±0.51 [b]	1.80±0.20 [a]	0.03	S
EOS (%)	4.40±0.24 [a]	3.20±0.73 [b]	2.60±0.24 [a]	0.05	NS

NS=Not significant, S=Significant, [abc]=Means in the same row with different superscript differ significantly(p<0.05).
PCV=Packed cell volume, HB=Haemoglobin concentration, RBC=Red blood cell, WBC=White blood cell, LYM=Lymphocyte, NEUT=Neutrophils, MON=Monocytes, EOS=Eosinophils

Table 6.
Haematology Result after Second Week of Treatment

GROUP	GROUP A	GROUP B	GROUP C	P-VALUE	REMARKS
PCV (%)	43.40±1.57 [a]	40.00±1.64 [a]	40.40±1.81 [a]	0.33	NS
HB (%)	14.06±0.52 [a]	13.26±0.53 [a]	12.72±0.46 [a]	0.21	NS
RBC (x106uL)	7.12±0.17 [a]	6.54±0.21 [a]	6.72±0.27 [a]	0.22	NS
WBC (x106uL)	4350.00±359.17 [a]	4620.00±285.31 [a]	5490.00±573.67 [a]	0.21	NS
PLATELET	1.044 x10^5±6423.39 [a]	6.78x10^5±16144.35 [a]	1.116x10^5±25486.47 [a]	0.18	NS
LTM (%)	68.80±1.07 [a]	66.20±1.71 [a]	67.20±1.39 [a]	0.22	NS
NEUT	26.80±1.24 [a]	30.20±1.88 [a]	28.40±1.50 [a]	0.45	NS
MON(%)	1.60±0.40 [a]	2.20±0.37 [a]	1.80±0.20 [a]	0.34	NS
EOS (%)	2.80±0.20 [a]	1.40±0.51 [a]	2.60±0.24 [a]	0.46	NS

NS=Not significant, [abc]=Means in the same row with different superscript differ significantly(p<0.05).
PCV=Packed cell volume, HB=Haemoglobin concentration, RBC=Red blood cell, WBC=White blood cell, LYM=Lymphocyte, NEUT=Neutrophils, MON=Monocytes, EOS=Eosinophils
The Haematology results for week 3 (table 7) revealed that there is a non-significant difference in the parameters of groups A and B compared to the control group except for lymphocytes and neutrophils in which there is a significant decrease in the values of groups A and B compared to the control group C.

The white blood cell value was significantly lower in group A than that of group B which is also significantly lower than that of group C. The platelets value for group A was higher than that of group B which was also higher than that of group C but the values were not significantly different. The lymphocytes value for group A is non-significantly lower than that of group B which is also non-significantly lower than that of group C. The values of Neutrophils, Lymphocytes and Eosinophils were not significantly different.

Haematology values for the second-week post-treatment (Table 6) revealed that the Packed cell volume for group A is non-significantly higher than group B and group C whereas the value for group B is lower than that of group C. The Haemoglobin concentration in group A is non-significantly higher than that of group B which is also non-significantly higher than that of group C. The Red blood cell value for group A is non-significantly higher than that of groups B and C whereas the value for group C which is the control group is non-significantly higher than that of group B. The White blood cell value for group A is non-significantly lower than that of group B which is also non-significantly lower than that of group C. The Platelets value for group A is non-significantly higher than that of group B whereas the value for group C which is also the control group is non-significantly higher than that of groups A and B. The lymphocytes value for group A is non-significantly higher than that of groups B and C whereas that of group C which is the control group is non-significantly higher than that of group B.

Table 7
Haematology results after third week of treatment

GROUP	GROUP A	GROUP B	GROUP C	P-VALUE	REMARKS
PCV (%)	38.00±1.55 [a]	41.75±2.25 [a]	40.40±1.81 [a]	0.39	NS
HB (%)	12.80±0.44 [a]	14.15±0.79 [a]	12.72±0.46	0.19	NS
RBC (x106uL)	6.29±0.24 [a]	7.08±0.39 [a]	6.72±0.27 [a]	0.22	NS
WBC (x106uL)	4310.00±532.07 [a]	5450.00±250.00 [a]	5490.00±573.67 [a]	0.21	NS
PLATELET	$1.2378x10^5$±12018.32 [a]	$1.235x10^5$±14239.03 [a]	$1.116x10^5$±25486.47 [a]	0.87	NS
LTM (%)	60.40±1.72 [a]	60.50±2.50[a]	67.20±1.39[b]	0.03	S
NEUT	67.20±1.39[a]	60.50±2.50[b]	28.40±1.50 [c]	0.03	S
MON (%)	1.80±0.37 [a]	1.25±0.25 [a]	1.80±0.20 [a]	0.37	NS
EOS (%)	1.80±0.37 [a]	2.25±0.48 [a]	2.60±0.24 [a]	0.31	NS

N=Not significant, S=Significant, [abc]=Means in the same row with different superscript differ significantly(p<0.05).
PCV=Packed cell volume, HB=Haemoglobin concentration, RBC=Red blood cell, WBC=White blood cell, LYM=Lymphocyte, NEUT=Neutrophils, MON=Monocytes, EOS=Eosinophils

The Neutrophils value for group A is lower than those of groups B and C but the value of group B is higher than that of group A and the control group C. The monocytes value for group A is lower than that groups B and C but group B value is higher than that of the control group C. There is non-significant difference between the values of the monocytes and Eosinophils in all the groups.

Haematology results from animals treated orally with aqueous and methanol extracts of *Corchorus olitorius:*
The result of group A (Table 8) revealed an increase in the Packed cell volume and Haemoglobin concentration at both the second and third weeks compared to the first week while there is a progressive decrease in the Red blood cells, White blood cells and the Eosinophils from the first week to the third week after treatment. There was a decrease in platelets at the second and third weeks compared to the first week. There was an increase in neutrophils in the third week compared to the first and second week.

Table 8
Haematology Result of Group A after First Week, Second Week and Third Week Post Treatment.

GROUP	1ST WEEK	2nd WEEK	3rd WEEK
PCV (%)	31.60 ±2.86[a]	43.40 ±1.57 [a]	38.00 ±1.55 [a]
HB (%)	10.47 ±0.90 [a]	14.06 ±0.52 [a]	12.80 ±0.44 [a]
RBC (x106uL)	8.83 ±0.37[a]	7.12 ±0.17 [a]	6.29 ±0.24 [a]
WBC (x106uL)	8790.00 ±648.34 [a]	4350.00 ±359.17 [a]	4310.00 ±532.07 [a]
Platelet	$3.156x10^5$ ±178239.61 [a]	$1.044x10^5$ ±6423.39 [a]	$1.2378x10^5$ ±12018.32 [a]
LYM (%)	63.40±2.46 [a]	68.80±1.07 [a]	60.40±1.72 [a]
NEUT	28.80±2.24 [a]	26.80±1.24 [a]	67.20±1.39[a]
MON (%)	3.40±0.51 [a]	1.60±0.40 [a]	1.80±0.37 [a]
EOS (%)	4.40±0.24 [a]	2.80±0.20 [a]	1.80±0.37 [a]

[abc]=Means in the same row with different superscripts differ significantly(p<0.05).
PCV=Packed cell volume, HB=Hemoglobin concentration, RBC=Red blood cell, WBC=White blood cell, LYM=Lymphocyte, NEUT=Neutrophils, MON=Monocytes, EOS=Eosinophils

Haematology result of group B (Table 9) revealed a progressive increase in the Packed cell volume, Hemoglobin concentration, and Neutrophils from week one to week three post-treatment whereas there is a decrease in the Red blood cells, White blood cells, Platelets, Monocytes and Eosinophils at the second-and third-week post-treatment compared to the first week.

The haematology result of group C revealed that there is no difference in the haematology parameters from the first to the third-week post-treatment.

Table 9
Haematology Result of Group B after First Week, Second Week and Third Week post-treatment.

GROUP	1st WEEK	2ND WEEK	3RD WEEK
PCV (%)	38.40 ±1.60[b]	40.00 ±1.64 [a]	41.75 ±2.25 [a]
HB (%)	12.80 ±0.53 [b]	13.26 ±0.53 [a]	14.15 ±0.79 [a]
RBC (x106uL)	8.97 ±0.17[b]	6.54 ±0.21 [a]	7.08 ±0.39 [a]
WBC (x106uL)	10240.00 ±342.56 [b]	4620.00 ±285.31 [a]	5450.00 ±250.00 [a]
Platelet	$1.45x10^5$ ±5991.66 [a]	$6.78x10^5$ ±16144.35 [a]	$1.235x10^5$ ±14239.03 [a]
LTM (%)	65.40 ±1.86 [a]	66.20 ±1.71 [a]	60.50 ±2.50[a]
NEUT	28.00 ±2.07 [a]	30.20 ±1.88 [a]	60.50 ±2.50[b]
MON (%)	3.40 ±0.51 [b]	2.20 ±0.37 [a]	1.25 ±0.25 [a]
EOS (%)	3.20 ±0.73 [b]	1.40 ±0.51 [a]	2.25 ±0.48 [a]

[abc]=Means in the same row with different superscripts differ significantly(p<0.05).
PCV=Packed cell volume, HB=Haemoglobin concentration, RBC=Red blood cell, WBC=White blood cell, LYM=Lymphocyte, NEUT=Neutrophils, MON=Monocytes, EOS=Eosinophils

Table 10
Haematology Result Of Group C After First Week, Second Week And Third Week Post Treatment

Group	1st Week	2nd Week	3rd Week
PCV (%)	40.40±1.81 [b]	40.40±1.81 [a]	40.40±1.81 [a]
HB (%)	12.72±0.46 [b]	12.72±0.46 [a]	12.72±0.46 [a]
RBC (x106uL)	6.72±0.27 [c]	6.72±0.27 [a]	6.72±0.27 [a]
WBC (x106uL)	5490.00 ±573.67 [c]	5490.00 ±573.67 [a]	5490.00 ±573.67 [a]
PLATELET	1.116 x10 [5] ±25486.47 [a]	1.116 x10[5] ±25486.47 [a]	1.116 x10[5] ±25486.47 [a]
LYM (%)	67.20 ±1.39 [a]	67.20 ±1.39 [a]	67.20 ±1.39 [b]
NEUT	28.40 ±1.50 [a]	28.40 ±1.50 [a]	28.40 ± 1.50 [c]
MON (%)	1.80±0.20 [a]	1.80±0.20 [a]	1.80±0.20 [a]
EOS (%)	2.60±0.24 [a]	2.60±0.24 [a]	2.60±0.24 [a]

N=Not significant, S=Significant, [abc]=Means in the same row with different superscript differ significantly ($p<0.05$).
PCV=Packed cell volume, HB=Haemoglobin concentration, RBC=Red blood cell, WBC=White blood cell, LYM=Lymphocyte, NEUT=Neutrophils, MON=Monocytes, EOS=Eosinophils

Serum testosterone level: Results for serum testosterone level for 1st week, 2nd week and 3rd -week post-treatment (figure 4) revealed at the first week that there is a significant difference in the serum testosterone level of groups A and B compared to group C. The value is higher in group A followed by group B. It was observed that at the second week, there was no significant difference in the values of the serum testosterone levels compared to the control group. At the 3rd week, there was no significant difference in the serum testosterone levels in treatment groups A and B compared to the control group C.

Testicular biometry: The testicular biometry result for the first week (Table 11) revealed that the bodyweight value of group C which is the control group is non-significantly higher than those of groups A and B. There is also no significant difference statistically between the weight of the left testis, and right testis compared to that of the control group. There is also no significant difference between the lengths of the left testis and right testis and also the diameter of the left testis and right testis.

The result for the second week of testicular biometry (Table 12) revealed that there is no significant difference in all the parameters of the testicular biometry in the treated groups' A and C compared to the control group C.

The result for the third-week post-treatment (Table 13.) revealed that there was no significant difference in all the parameters of the testicular biometry in the treated groups' A and B compared to the control group C.

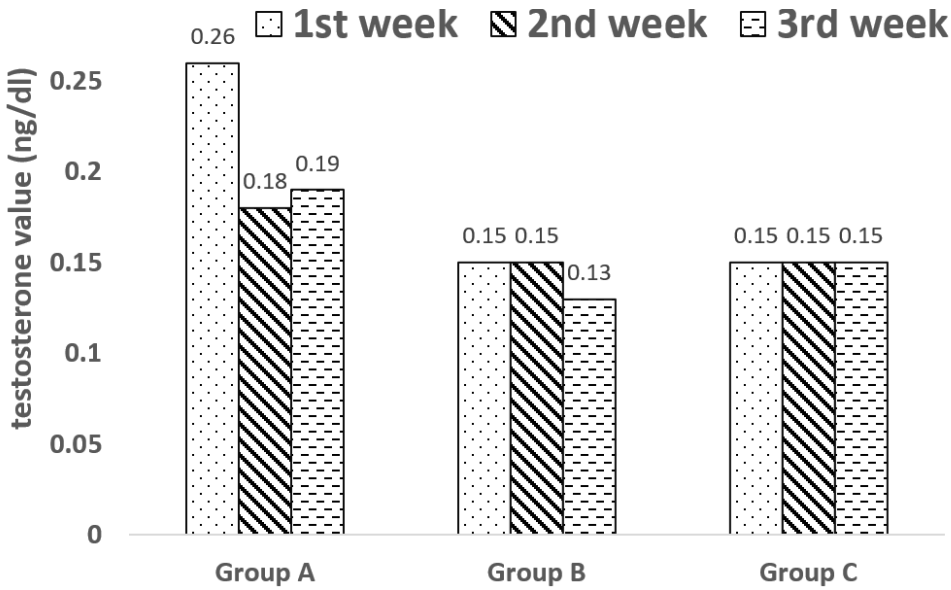

Figure 4
Serum Testosterone Level Results After the First, Second and Third Week of Treatment.

Table 11.
Bodyweight, Left Epididymal Weight And Testicular Biometry Results After First Week Of Treatment

GROUP	GROUP A	GROUP B	GROUP C	P-VALUE	REMARKS
Body Weight (g)	100.40±8.72 [a]	102.80±0.67 [a]	118.80±12.80 [a]	0.32	NS
Left Epididymis	0.12±0.02 [a]	0.16±0.02 [a]	0.16±0.05 [a]	0.57	NS
Left Testis Weight (g)	0.54±0.13 [a]	0.76±0.05 [a]	0.61±0.15 [a]	0.43	NS
Right Testis Weight(g)	0.67±0.09 [a]	0.77±0.05 [a]	0.62±0.16 [a]	0.62	NS
Left Testis Length (cm)	14.67±0.69 [a]	15.93±0.58 [a]	13.26±1.97 [a]	0.35	NS
Right Testis Length(cm)	14.97±0.74 [a]	15.82±0.37 [a]	13.64±1.54 [a]	0.34	NS
Left Testis Diameter	7.81±0.29 [a]	8.85±0.33 [a]	8.00±0.92 [a]	0.43	NS
Right Testis Diameter	8.26±0.56 [a]	8.99±0.22 [a]	8.05±0.89 [a]	0.55	NS

NS=Not significant, [abc]=Means in the same row with different superscript differ significantly($p<0.05$).

Table 12.
Body Weight, Left Epididymal Weight and Testicular Biometry Results After Second Week Of Treatment

Group	Group A	Group B	Group C	P-Value	Remarks
Body Weight (g)	131.80±10.29 [a]	106.00±3.46 [a]	118.80±12.80 [a]	0.21	NS
Left epididymis	0.21±0.03 [a]	0.13±0.02 [a]	0.16±0.05 [a]	0.25	NS
Left Testis Weight (g)	0.95±0.04 [a]	0.72±0.12 [a]	0.61±0.15 [a]	0.14	NS
Right Testis Weight(g)	0.94±0.04 [a]	11.59±10.85 [a]	0.62±0.16 [a]	0.39	NS
Left Testis Length (cm)	14.88±0.98 [a]	14.75±1.56 [a]	13.26±1.97 [a]	0.72	NS
Right Testis Length (cm)	14.88±0.98 [a]	14.75±1.56 [a]	13.64±1.54 [a]	0.63	NS
Left Testis Diameter	8.43±0.74 [a]	8.34±0.92 [a]	8.00±0.92 [a]	0.93	NS
Right Testis Diameter	8.59±0.85 [a]	8.36±0.92 [a]	8.05±0.89 [a]	0.91	NS

NS=Not significant, [abc]=Means in the same row with different superscript differ significantly(p<0.05).
The result for the third-week post-treatment (Table 13.) revealed that there was no significant difference in all the parameters of the testicular biometry in the treated groups' A and B compared to the control group C.

Table 13.
Body Weight, Left Epididymal Weight And Testicular Biometry Result After Third Week Of Treatment.

GROUP	GROUP A	GROUP B	GROUP C	P-VALUE	REMARKS
Body Weight (g)	114.89±3.75 [a]	108.87±7.24 [a]	118.80±12.80 [a]	0.73	NS
Left epididymis weight (g)	0.20±0.03 [a]	0.23±0.02 [a]	0.16±0.05 [a]	0.54	NS
Left Testis Weight (g)	0.85±0.08 [a]	0.89±0.07 [a]	0.61±0.15 [a]	0.21	NS
Right Testis Weight (g)	0.82±0.07 [a]	0.87±0.06 [a]	0.62±0.16 [a]	0.27	NS
Left Testis Length (cm)	16.43±0.57 [a]	17.47±0.59 [a]	13.26±1.97 [a]	0.11	NS
Right Testis Length (cm)	15.97±0.72 [a]	16.90±0.63 [a]	13.64±1.54 [a]	0.15	NS
Left Testis Diameter	9.33±0.33 [a]	9.64±0.31 [a]	8.00±0.92 [a]	0.19	NS
Right Testis Diameter	8.98±0.24 [a]	9.51±0.34 [a]	8.05±0.89 [a]	0.27	NS

NS=Not significant, [abc]=Means in the same row with different superscript differ significantly(p<0.05).

DISCUSSION

In haematology**,** it was observed that at the first-week post-treatment that there was a significant (p<0.05) decrease in the PCV and haemoglobin values of group A compared to group C which is the control group but the value later increased at the second week and third-week post-treatment. This may be due to the newness of the methanol extract in their system. Also, there was a significant decrease in the values of lymphocytes at the third week of both groups A and B compared to the control group C. The values obtained for RBC and showed non-significant effects in the treatment of rats with Methanol and Aqueous extracts of *Corchorus olitorius* on red blood cells (RBC) counts when compared with the control. This is an indication that there was no destruction of red blood cells and no change in the rate of production of RBC (erythropoiesis). This also shows that both the methanol and aqueous extracts of the plant do not have the potential to stimulate erythropoietin release from the kidneys, which is the humoral regulator of RBC production (Polenakovic and Sikole, 1996). The non-significant effects of the treatment of rats with 250mg/kg of both extracts also indicate that there was no change in the oxygen-carrying capacity of the blood and the amount of oxygen delivered to the tissues since RBC is very important in transferring respiratory gases (De Gruchy, 1976). It has been reported that values of RBC and associated parameters lower than normal ranges are indicative of anaemic conditions while higher values are suggestive of polycythemia (American Diabetes Association, 2000), thus, the treatment of rats with methanol and aqueous extracts of the plant may not have the potential to induce anaemia or polycythemia.

It was observed in the values for body weight, testicular and epididymal biometry that there was no significant (p≥0.05) difference in the body weight of the experimental animals during the period of the experiments. It was also observed that there is a non-significant difference in the weights of the left testes, right testes, the length of both the left and right testes and also the diameter of the testes in all the groups for the period of the experiments. There is also a non-significant difference in the weight of the left epididymis in the experimental groups compared to the control group.

In semen characteristics, there was a significant decrease in spermatozoa motility in the treated groups' A and B compared to the control group C at the first, second-and third-week post-treatments. This is in agreement with the treatment of male albino rats with ethanol extract of *Aegle marmelos* leaf (Chauhan and Agarwal, 2008).

It was observed that there was no significant difference in the spermatozoa morphology abnormalities in the experimental groups compared to the control group for the duration of the experiments

There is a significantly high value in the testosterone level in group A which was treated with methanol extract when compared to the control group at first-week post-treatment but there was no significant difference in the values at the second and third weeks. This is contrary to the reduction in the levels of serum testosterone level, reported when the crude methanol extract of *Quassia amara* was administered to male albino rats (Raji and Bolarinwa, 1997) and also when methanol extract of *Abelmoschus esculentus* fruit was administered to male albino rats (Olatunji-Bello *et al*., 2009).

In conclusion, the methanol and aqueous extracts of *Corchorus olitorius* leaves significantly decreased sperm motility in male albino rats. Therefore, prolonged use or administration of *Corchorus olitorius* could decrease male fertility. Meanwhile, its effect on human reproduction is not known but considering the findings in this study, infertile males undergoing fertility treatments are advised to take *Corchorus olitorius* plant with caution. Also, in this study, Methanol and Aqueous extracts of *Corchorus olitorius* leaves increased the Packed cell volume and Haemoglobin concentration and therefore can be used to treat anaemia in both animals and humans but with caution.

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Short Communication

Teratogenic Effect of Aqueous Leaf Extract of *Aspilia africana* on The Dentate Gyrus of Wistar Rat Fetuses

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Summary: *Aspilia africana* is an herbal plant widespread in Africa used for medicinal purposes and also used by pregnant women for health-related issues. This study was aimed at investigating the teratogenic effect of aqueous leaf extract of *Aspilia africana* on the dentate gyrus of Wistar rat fetuses. Twenty (20) female adult rats weighing between 190-205g were used for this study. The rats were divided into four groups; control, low dose, medium dose and high dose with each group containing five rats. Pregnancy was induced by caging the female rats with sexually matured males. The presence of vaginal plug and tail structure in the vaginal smear the following morning confirmed coition, and it was regarded as day 0 of pregnancy. The control group was given distilled water. The low dose, medium dose, and the high dose groups received 750mg/kg, 1000mg/kg, and 1250mg/kg body weight of aqueous leaf extract of *Aspilia africana* through an orogastric tube from day 7-11 of gestation. On the 20th day of gestation, the animals were sacrificed using chloroform-inhalation method. Their fetuses were harvested via uterectomy, the brain was excised and fixed in 10% buffered formalin, and then routine histological processes were carried out. Staining was done using Haematoxylin and Eosin method, histometric measurements were measured**.** Histological observation of the dentate gyri of experimental groups revealed marked distortion, reduction of the polymorphic layer, hyperplasia and hypertrophy of cells in the molecular and granular layer especially in the high dose group whose mothers received 1250mg/kg of the extracts. Histometric analysis revealed reduction of cell diameter, total number of cells in the square of pyramidal cells and packed density of cells especially in the group whose mothers were treated with high dose of plant extract. The result suggests high doses of aqueous leaf extract of *Aspilia africana* may be teratogenic to the dentate gyrus of Wistar rat fetuses.

Keywords: *Dentate gyrus, Hyperplasia, Hypertrophy, Neurogenesis, Memory loss, Teratogenic*

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INTRODUCTION

The dentate gyrus is an organ found in the brain which is responsible for learning and formation of new memories. It takes part in the relay of synaptic transmission in the hippocampus and is known to take part in the formation of new episodic memories (Amaral *et al*, 2007; Saab *et al*, 2009), spontaneous exploration of novel environments (Saab *et al*, 2009) and other several functions (Scharfman, 2007). It is one of the few regions of the adult brain where adult neurogenesis takes place in many species of mammals (Cameron and McKay, 2001; Piatti *et al*, 2013; Benarroch, 2013; Brickman *et al*, 2014; Zhang and Zhang, 2018). Neurogenesis is thought to play a role in the formation of new memories (Kitabatake *et al*, 2007; Deng *et al*, 2010). According to Nakashiba *et al* (2012), new memories could preferentially use newly formed dentate gyrus cells, providing a potential mechanism for distinguishing multiple instances of similar events or multiple visits to the same location. Several researches have been carried out on effect of some plants on the dentate gyrus (Jahanshashi *et al*, 2007; Marzban *et al*, 2011). 70-80% of Africa's population relying on traditional medicines, the importance of the role of medicinal plants in the healthcare system cannot be ruled out (Mander and Breton, 2006). Nigeria has a great variety of natural vegetation which is used in trado-medicine to cure various ailments. In Nigeria, *Aspilia africana* is known by different names like "edemedong" in Efik and Ibibio, "orangila" in Igbo, "yunyun" in Yoruba, and "toozalin- yan-maata in Hausa (Single, 1965). *Aspilia Africana* has been shown to exhibit a wide range of biological activities including antiviral, fungicide and antibacterial activities and for the treatment of several ailments such as gonorrhea, tuberculosis, cough, rheumatic pains, stomach trouble, corneal opacity, wounds healing (Iwu, 1993; Okoli *et al*, 2007; Okwuonu *et al*, 2008; Christian *et al*, 2012; Komakech *et al*, 2019)

The dentate gyrus is of particular interest as new dentate granule cells (GCs) are generated continuously in the adult mammalian brain (Cameron and McKay, 2001; Ming and Song, 2011). Impairment of the dentate gyrus leads to memory loss as information cannot be consolidated into the working memory. It also leads to stress and depression due to increase in neurogenesis in the dentate gyrus which leads to physiological effects of stress and depression. There is paucity of literature on the teratogenic effect of aqueous extract of *Asipilia africana* on the dentate gyrus of albino rat fetuses, hence the study.

MATERIALS AND METHODS

Breeding of Animals: Twenty (20) adult Wistar rats of weighing between 180-200g were used for the research work. The rats were obtained from the Faculty of Basic Medical Sciences animal house, University of Calabar, Calabar. They were kept in standard wooden cages with iron nettings and kept in the animal house of the Department of Anatomical Sciences, Faculty of Basic Medical science, University of Calabar, Calabar, Nigeria. The animals were randomly divided into four groups and were kept in a stable and standard environmental condition facilitated by proper ventilation and approximate room temperature (of about 25-27ºc photo period 12 hours natural light. 12 hours dark and humidity 45-50%) throughout the duration of the experiment. The animals were fed daily and regularly with vital feed (grower mesh) obtained from Vital Food Company No: 44 Nelson Mandela Street Calabar and water were given *ad libitum*. Cleaning and replacement of beddings were done regularly to maintain a good hygienic status for the animals.

Ethical Consideration: Approval was given by the Faculty of Basic Medical Sciences Committee on animal use and care, University of Calabar to carry out this research work following laid down rules and guidelines of the institution in the use of medicinal plants and animal models.

Preparation of *Aspilia africana* Leaf Extract: Fresh leaves of *Aspilia africana* were picked at the University of Calabar farm, Calabar, Cross River State of Nigeria. The plant was identified and authenticated by a botanist at the Botany Department, University of Calabar, Calabar. The harvested fresh leaves were washed with clean water to remove dirt and air dried for two weeks. The dried leaves were homogenized with the aid of electric blender into fine powder in New Chemistry Laboratory, Department of Chemistry University of Calabar. One hundred and seventy-six grams (176g) of powdered leaves was soaked in one thousand two hundred millilitres (1200mls) of distilled water for 48hours in the research laboratory of the Biochemistry Department of the University of Calabar, Calabar. It was filtered first with sieve basket and the chess cloth and later Whatman number one filter paper. The filtrate was concentrated in a water bath which was regulated to 50ºc and yielded a thick-semi solid paste of brown colour which is the crude extract. The extract was stored in a refrigerator from where it was taken from for oral administration.

Experimental Protocol: The animals were randomly selected and divided into four groups labelled control, low dose, medium dose and high dose, with each group consisting of five (5) rats. The oestrous cycle of the animals was checked with normal saline to be sure that they were due for mating. Each female rat's oestrous cycle was determined by daily vaginal lavages and at oestrous, each rat was caged overnight with a sexually active male rat of the same strain. The morning after a vaginal smear showed the presence of sperm in the female tract, the process of spermatozoa signified day zero of pregnancy.

Extract Administration

Control: The control rats were given grower mesh and distilled water only.

Low Dose: The rats were administered with 750mg/kg of the aqueous extract of *Aspilia africana* per body weight.

Medium Dose: The rats were administered with 1000mg/kg of the aqueous extract of *Aspilia africana* per body weight.

High Dose: The rats were administered with 1250mg/kg of the aqueous extract of *Aspilia africana* per body weight.

The extract was administered to each animal in the experimental group based on their body weight and administration was done using the oral route through orogastric intubation from days 7-11 of gestation respectively. The rats were sacrificed on the 20th day of pregnancy using chloroform inhalation method. The fetuses of each group were collected after opening the anterior abdominal wall, fixed and then processed for histological studies. Histometric measurements were measured such as diameter of cells, total number of cells in the square and packed density of cells.

Diameter of Cells: The diameter of the pyramidal cells of the dentate gyrus were calculated using the formula;

Diameter of a cell= Axial ratio X calibration constant

Total number of cells in the square: Stained sections were focused under 40X magnification objective lens of the microscope. Counting was done under 400X magnification. The pyramidal cells of the dentate gyrus were counted. The cell region for calculation was selected using random selection technique from the serial section from each group.

Packed density of cells: The packed cell density was calculated using the method of Ramar and Sarawathi (2013).

RESULTS

Table 1 shows the histometric results of the diameter of cells, total number of cells in a square and packed density of cells in the control and experimental dentate gyrus of fetuses. There was a significant reduction in the parameters measured in the experimental groups when compared to control especially in high dose group fetuses whose mothers were treated with 1250mg/kg body weight of extract.

Table 1:
Histometric characteristics of the fetal dentate gyrus in the various groups

Groups	Diameter of cells (µ)	Total number of cells in the square	Packed density of cells per square (x10cubic/mm
Control	3.98±0.16	982.40±24.50	128.52±5.25
Low dose	3.20±0.18	760.50±12.40	104.83±6.27
Medium dose	2.94±0.29[a]	698.50±17.5[a]	96.35±8.25[a]
High dose	2.48±0.27[b]	486.28±10.6[b]	82.64±8.06[b]

Values are mean ± SEM (n=5)

[a] Significantly different from control at p≤0.05 b- significantly different from control at p≤0.01

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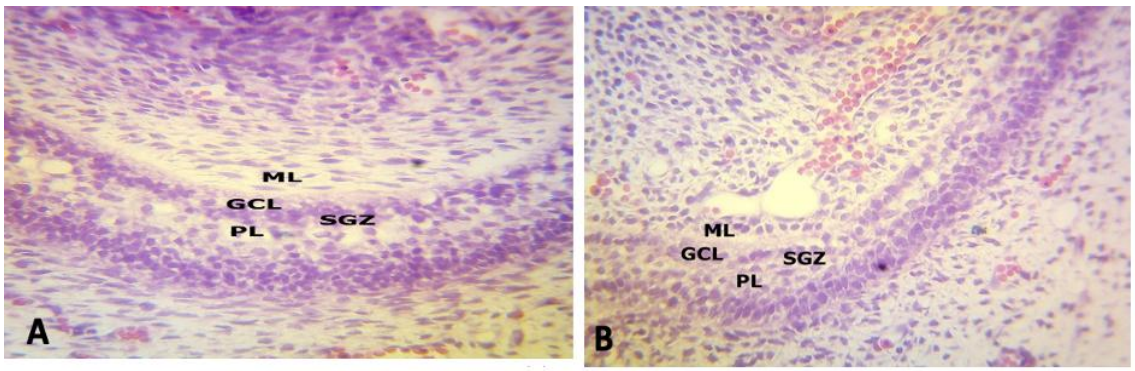

Plate 1:
Photomicrograph of the dentate gyrus of control and low dose group which received 750mg/kg 0f aqueous leaf extract of *Aspilia africana* (H & E × 400 for all plates).
A. Control dentate gyrus showing its three distinct layers; molecular layer (ML), polymorphic layer (PL), and granular layer (GL) with the subgranular zone (SGZ).
A. Dentate gyrus of low dose group showing the molecular layer (ML), polymorphic layer (PL), and granular layer (GL) and the subgranular zone (SGZ) with marked distortion and reduction of the polymorphic layer (PL), slight hypertrophy of cells in the molecular layer (ML) and distortion of cells in the granular layer (GL).

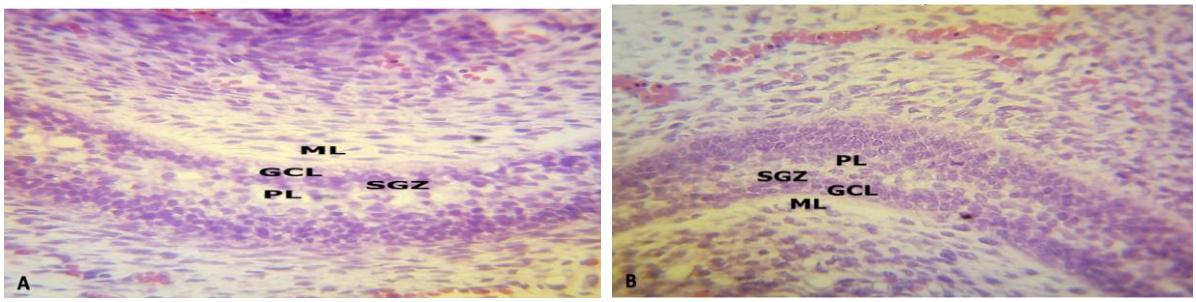

Plate 2:
Photomicrograph of the dentate gyrus of control and medium dose group which received 1000mg/kg 0f aqueous leaf extract of *Aspilia africana* (H & E × 400 for all plates).
A. Control dentate gyrus showing its three distinct layers; molecular layer (ML), polymorphic layer (PL), and granular layer (GL) with the subgranular zone (SGZ).
A. Dentate gyrus of low dose group showing the molecular layer (ML), polymorphic layer (PL), and granular layer (GL) and the subgranular zone (SGZ) with marked distortion and reduction of the polymorphic layer (PL), slight hypertrophy of cells in the molecular layer (ML) and distortion of cells in the granular layer (GL).

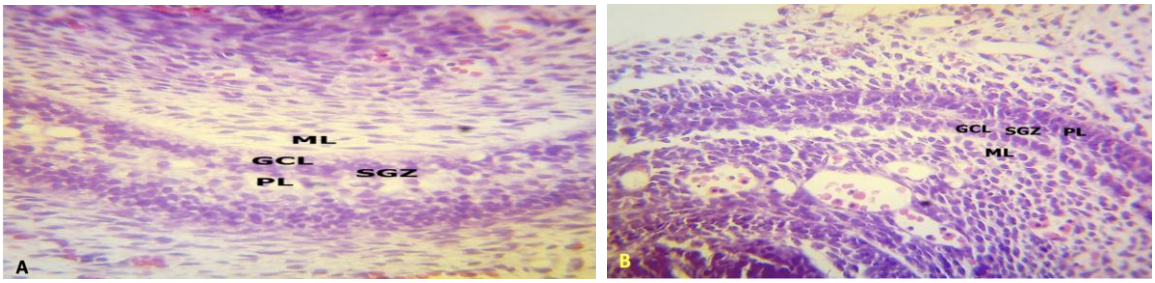

Plate 3: Photomicrograph of the dentate gyrus of control and high dose group which received 1250mg/kg 0f aqueous leaf extract of *Aspilia africana* (H & E × 400 for all plates).
A. Control dentate gyrus showing its three distinct layers; molecular layer (ML), polymorphic layer (PL), and granular layer (GL) with the subgranular zone (SGZ).
A. Dentate gyrus of low dose group showing the molecular layer (ML), polymorphic layer (PL), and granular layer (GL) and the subgranular zone (SGZ) with marked distortion and reduction of the polymorphic layer (PL), slight hypertrophy of cells in the molecular layer (ML) and distortion of cells in the granular layer (GL).

DISCUSSION

Medicinal plants are the bedrock of primary health care for majority of world's population since the beginning of civilization (Pan *et al*, 2014). The dentate gyrus is an organ found in the brain responsible for learning and formation of new memories and composed of unidirectional projections dispersed towards CA3 pyramidal cells of the hippocampus (Treves *et al*, 2008). In rats, approximately 85% of the granule cells are generated after birth (Bayer, 1974); while, in humans, it is estimated that granule cells begin to be generated during gestation weeks 10.5 to 11, and continue being generated during the second and third trimesters after birth and all the way into adulthood (Bayer *et al*, 1982; Eriksson *et al*, 1998; Bayer *et al*, 2008). Studies have shown that after destroying about 90% of their dentate gyrus cells, rats had extreme difficulty in maneuvering through a maze they had been through, prior to the lesion being made. When

being tested a number of times to see whether they could learn a maze, the results showed that the rats did not improve at all, indicating that their working memories were severely impaired. Rats had trouble with place strategies because they could not consolidate learned information about a maze into their working memory, and, thus, could not remember it when maneuvering through the same maze in a later trial.

Every time a rat entered the maze, the rat behaved as if it was seeing the maze for the first time (Xavier 2009). From this study, it has been shown that aqueous leaf extract of *Aspilia africana* affects the dentate gyrus by causing hyperplasia, hypertrophy and distortion of the molecular, polymorphic and granular layers which received 750mg/kg, 1000mg/kg and 1250mg/kg body weight of the extract. The effect was dose dependent and may have been due to the effect of the chemical constituent of the extract. The teratogenicity may affect learning and formation of new memories.

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Short communication

Hippocampal Astrogliotic Reduction in Scopolamine Hydrobromide-Induced Alzheimer's Cognitive Dysfunction Wistar Rats Following Administration of Aqueous Extract of *Telfairia occidentalis* (Hook F.) Seeds

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Summary: Astrocytes are small star-shaped glial cells that maintain normal human brain physiology including secretion of several active compounds and the formation of blood brain barrier. Reactive astrocytes support regenerating axons and also actuate some genes responsible for the induction of synapse formation. In this study, the effect of aqueous extract of *Telfairia occidentalis* seeds on hippocampal astrogliosis was done using scopolamine-induced Alzheimer's type cognitive dysfunction Wistar rats. Thirty Wistar rats of 6 weeks of age (180-200g) were randomly grouped into five designated A, B, C, D and E. Each group contained six rats. Alzheimer's type cognitive dysfunction was induced in groups B to E by administering intraperitoneally, 1 mg/kg body weight of scopolamine for seven days before Donepezil and the aqueous extract of *Telfairia occidentalis* seeds for fourteen days. Twenty-four hours after the last administration, the animals were sacrificed; their brain tissues perfused and stained with glial fibrillary acidic protein (GFAP) dye. Results revealed prominently stained astrocytes with their processes intact (group A). Some densely stained numerous *astrogliosis* with hypertrophied fibres were noticed in group B. Group C demonstrated prominent astrocytes with hypertrophied fibres; group D, moderately stained *astrogliosis* with hypertrophied fibres while group E showed numerous astrocytes with prominent nuclei and hypertrophied fibres. In conclusion, there was a reduced hippocampal *astrogliosis* mostly in group D treated with *Telfairia occidentalis* which may have neutralized oxidative stress and enhanced learning and memory in the Wistar rats of the present study.

Keywords: *Alzheimer, Astrogliosis, Hippocampus, Scopolamine hydrobromide, T. Occidentalis seeds, Wistar rats*

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INTRODUCTION

Alzheimer's disease (AD) is a chronic neuro-degrading disorder with cognitive and recall impairment, chaperoned by intracellular neurofibriliary entangles and extracellular amyloid plaques (Zheng *et al*., 2007). The global occurrence of nervous disease among individuals > 60 years is between 5-7% (Prince *et al.*, 2013). A study reported that AD is the most frequent type of dementia before blood vessel dementia (Akter *et al.*, 2012). More so, there are light and discord information on the prevalence of dementia and its subtypes in the sub-Saharan Africa (Prince *et al.*, 2013) with far-reaching implications on public health policies. While some studies revealed a reduced occurrence of dementia in the sub-Saharan Africa (Yusuf *et al.*, 2011; Prince *et al.*, 2013), others reported similar occurrence compared to those in the western world (Paddick *et al*., 2013). Research also revealed obvious difference in the occurrence of AD in men and women with two third of Alzheimer's disease patients being women (Alzheimer's Association, 2017). In 2010, the global economic implication of dementia was estimated to be eight hundred and eighteen billion dollars which may reach one trillion dollars in 2018 (Prince *et al.*, 2016).

Due to high cost of management and treatment of AD, the use of plants such as pumpkin seeds (*Telfairia Occidentalis*) as an alternative became paramount to scientists. Pumpkin seeds are very common in most parts of Nigeria. The plant is characterised with pharmacological activities such as antioxidants, antidiabetic, antibacterial, anti-inflammatory and antifungal effects (Nkosi *et al*., 2006). Pumpkin seeds have been documented to possess neuroprotective effects and enhanced learning and memory (Eru *et al*., 2020; 2021).

The endogenous (glutathione secreted by the neuronal cells) and exogenous antioxidants from plants such as *T. occidentalis* seed help to neutralize the redundant free radicals, defend the cell from poisonous substances and also, chip in to avert disease (Pharm-Huy *et al.*, 2008). The free antioxidant property of *T. Occidentalis* seed may provide safer option, hence, the necessity to probe the outcome of aqueous extract of *T. occidentalis* seeds on hippocampal astrogliosis using scopolamine hydrobromide-induced Alzheimer's type cognitive dysfunction Wistar rats.

MATERIALS AND METHODS

Ethical consideration: Ethical approval was obtained from the Faculty of Basic Medical Sciences, College of Medical Sciences, University of Calabar, Calabar, Nigeria (Approval number: FAREC-FBMS 042ANA3719) in line with the principles of laboratory animal care (NIH publication NO. 85-23, revised 1985) as well as specific National laws applied.

Animals: A total of thirty adult female and male Wistar rats of 6 weeks of age (180-200g) were bought from the University of Calabar animal farm, kept in animal room in the Department of Anatomical Sciences, for two weeks under standard conditions of temperature (27ºC – 30ºC) for acclimatization. The animals were fed with raw chow manufactured by the Agro Feed Mill Nigeria Ltd, Calabar and allowed access to drinking water. After acclimatization, the experimental rats were randomly grouped into five, each containing six rats designated A, B, C, D and E.

Plant extract preparation: Fresh *Telfairia occidentalis* seeds were obtained from the Watt market, Calabar, Cross River State, Nigeria. The fresh *Telfairia occidentalis* seeds were identified, authenticated and registered with voucher number: HERB/BOT/UCC/322 in the Department of Botany, University of Calabar, Calabar. The seeds were removed from shell, washed to free debris, chopped into smaller pieces and air-dried in the laboratory. The dried samples were blended into powder using blender (with model number Bravo3JARS Mixer grinder) with 1600 g of the powder seed soaked in 1000 mls distilled water for twenty-four hours. The mixture was then filtered using chess cloth and the Whatman No.1 filter paper. The solution was obtained and concentrated to a syrupy residue at 40ºC-50ºC using man-made thermostatic water bath (with model number F.NR: 1508.0271) and kept in a cool dry place for use during administration.

Induction of Alzheimer's type cognitive dysfunction: Alzheimer's type cognitive dysfunction was induced to the rats in groups B, C, D and E through intraperitoneal injection of 1.0 mg/kg body weight of scopolamine hydrobromide (SHB) for seven days.

Determination of LD_{50}: The LD_{50} of aqueous extract of *Telfairia occidentalis* seeds was established to be > 7000 mg/kg according to Lorke's method. The dose of aqueous extract administration was determined using 12.5% (875 mg/kg) and 25% (1750 mg/kg) of the established LD_{50} (7000 mg/kg body weight of aqueous extract of *Telfairia occidentalis* seeds).

Plant extract and Donepezil administration: Group A served as the negative control and received animal feed with water *ad libitum*; group B served as the positive control and received 1.0 mg/kg body weight of SHB only; group C received 1.0 mg/kg body weight of SHB and 1.0 mg/kg body weight of Donepezil, group D received 1.0 mg/kg body weight of SHB and 875 mg/kg body weight of aqueous *Telfairia occidentalis* seeds while group E received 1.0 mg/kg body weight of SHB and 1750 mg/kg body weight of aqueous *Telfairia occidentalis* seeds for fourteen days.

Tissue processing and staining procedure: Twenty-four hours after the last administration, the animals were sacrificed with their brain tissues perfused and processed immunohistochemically. Serial paraffin sections of 5 µm thick were deparaffinised and dehydrated. The endogenous peroxidise activity was blocked with 0.05% hydrogen peroxide for 30 minutes. The slides were washed for 5 minutes in phosphate-buffered saline at a pH of 7.4. Sections were later placed in a 0.01M citrate buffer (pH 6) in a microwave for 5 minutes. The slides were incubated in 1% Bovine serum albumin for 30 minutes at 37ºC. Two drops of antibodies were applied to the sections and then incubated for 90 minutes at room temperature. Glial fibrillary acidic protein (GFAP) was then applied to the sections. The anti-mouse immunoglobulins conjugated to a peroxidise-labelled dextran polymer. The slides were incubated in 3,3'-diaminobenzidine for 15 minutes. The slides were counterstained with Mayer's haematoxylin and then dehydrated, cleared, mounted with dibutylphthalate polystyrene xylene (DPX) and observed under light microscope.

RESULTS

Sections of the hippocampus of adult Wistar rats in the negative control (group A) revealed prominently stained astrocytes and their processes (Plate 1A) while group B treated with 1mg/kg body weight of SHB showed densely stained numerous astrogliosis with hypertrophied fibres (Plate 1B). Group C treated with 1mg/kg body weight of SHB and Donepezil showed prominent astrocyte with hypertrophied fibres (Plate 1C), group D treated with 1mg/kg body weight of SHB and 875 mg/kg of *Telfairia occidentalis* revealed moderately stained astrogliosis with hypertrophied fibres (Plate 1D) while group E treated with 1mg/kg body weight of SBB and 1750 mg/kg of *Telfairia occidentalis* revealed numerous astrocytes with prominent nuclei and hypertrophied fibres (Plate 1E).

Immunohistochemical staining showed few of the GFAP positive astrocytes in the hippocampus of the negative control rats (Plate 1). In contrast, reactive astrocytes (astrogliosis) and hypertrophied fibres were markedly increased in the hippocampus of the SHB treated group (Plate 2).

DISCUSSION

The result of this study is in line with Anderson *et al.*, (2016) who reported considerable proliferation of astrocytes seen sequel to trauma when the reactive response produced a protective scar around the injury. Reactive astrocytes from other groups treated with Donepezil and *T. occidentalis* seeds were markedly increased in the hippocampus (plates 3-5) when compared with the negative control (plate 1) but less reactive when compared with the positive control treated with SHB (plate 2).

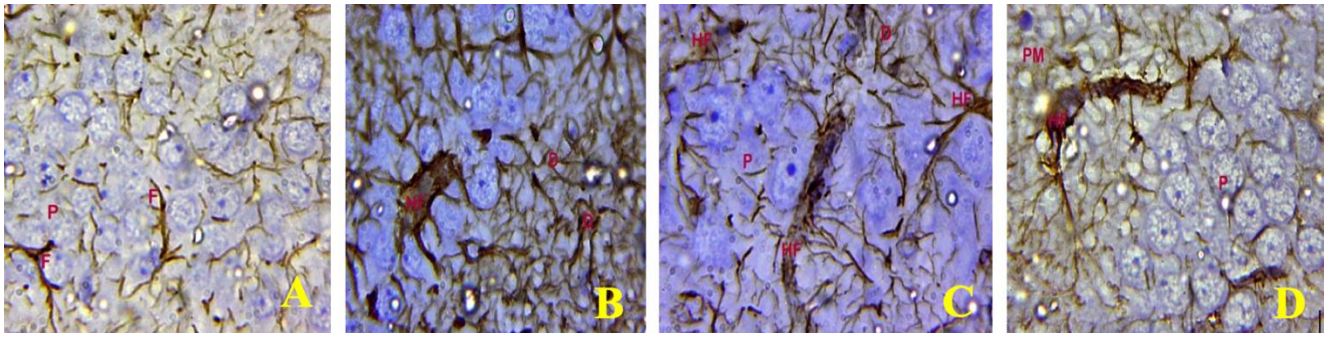

Plate 1:

A. Photomicrograph of a section of the hippocampus negative control (group A) showing prominently stained fine astrocyte processes and fibres.

B. Photomicrograph of a section of the hippocampus positive control (group B) treated with 1mg/kg body weight of SHB showing numerous astrocytes with prominently stained extensive processes and interdigitations in addition to astrocyte proliferation (astrogliosis).

C. Photomicrograph of a section of hippocampus (group C) treated with 1mg/kg body weight of SHB and Donepezil showing astrocytes with prominently stained interdigitating and hypertrophic fibre.

D. Photomicrograph of a section of hippocampus (group D) treated with 1mg/kg body weight of SHB and 875 mg/kg body weight of *Telfairia occidentalis* showing astrocytes with moderately stained and hypertrophic fibres coupled with astrocytes proliferation (astrogliosis).

E. Photomicrograph of a section of hippocampus (group E) treated with 1mg/kg body weight of SHB and 1750 mg/kg body weight of *Telfairia occidentalis* showing numerous astrocytes with prominent nuclei and extensive processes with hypertrophic fibres.

Astrocytes are characteristic star-shaped cells in the brain and spinal cord (Verkhratsky and Butt, 2013). In reaction to destruction inflicted on the central nervous system, astrocytes alter from their normal quiescence state to a so-called reactive state. This process of reactive gliosis is noted by morphological variations (hypertrophy), functional changes and profound increase in the expression of astrocytes specific intermediate filament and the GFAP (Middeldop and Hol, 2011). In this research, a tremendous rise in the GFAP-positive astrocytes was observed in SHB treated group (plate 2) compared to the control animals (plate 1). Immunohistochemical results from this study showed reduced expression of the GFAP positive reactive astrocytes in groups C, D and E (plates 3-5). Reactive astrocytes accompany every acute injury and chronic neurological disease that exists in two different states of activation; A_1 and A_2. Reactive astrocytes while providing trophic support to the generating axons (Anderson *et al.*, 2016), can also inhibit axon regeneration (Silver and Miller, 2004). However, the consumption of antioxidants through diets and supplements is expected to remove reactive oxygen species from the living system and provide health benefits (Zhang *et al.*, 2011) as *T. occidentalis* has been documented to have antioxidants and minerals that aid normal brain function. The reduction in GFAP expression cells in these treated groups may be attributed to the antioxidant potentials of the *T. occidentalis* seeds hence, resulting to cellular regenerations. In conclusion, hippocampal astrogliosis induced by SHB was observed to be reduced mostly in group D following the administration of aqueous extract of *Telfairia occidentalis* seeds. The extract may neutralize oxidative stress, and increase cellular regeneration with enhanced learning and memory in Wistar rats.

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ABSTRACTS OF THE PROCEEDINGS OF THE XXXIXth ANNUAL SCIENTIFIC CONFERENCE OF THE PHYSIOLOGICAL SOCIETY OF NIGERIA

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Effects of Low-Volume, High-Intensity Interval Exercise on BDNF isoforms levels in Sedentary Healthy Men

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Exercise and dietary modifications help improve energy balance and glucose control. High-intensity interval exercise (HIIE) induces an increase in serum brain-derived neurotrophic factor (BDNF) with a corresponding decrease in its precursor (proBDNF). ProBDNF possesses biological activities opposite those of BDNF. However, only a few reports on the effect of HIIE on proBDNF compared to extensive studies on the BDNF. Normal, healthy, male adults performed six sessions of HIIE in 2 weeks. Venous blood was collected, from which fasting blood sugar, glycated haemoglobin, serum insulin, BDNF and proBDNF were measured using ELISA. Marked decrease in markers of glycemic control was recorded following the intervention. A reduction in proBDNF and an increase in BDNF were also observed post-exercise. We found a strong negative relationship between BDNF and FBS post-exercise. HIIE causes an increase in serum BDNF and a reduction in serum proBDNF among sedentary male subjects.

Gastric Acid Secretion and Pump Activities in Healthy and Streptozotocin-induced Diabetic Rats Treated with Sodium Metavanadate

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Gastrointestinal tract (GIT) functions have been described as one of the mechanisms the body receives beneficiary or toxic agents. Exposure to vanadium, a heavy metal has been linked to modulate GIT functions in healthy and diabetic state. However, mechanisms underlying this modulatory role of vanadium, remain vague. This study investigates the effect of vanadium treatment on gastric acid secretion in healthy and diabetic conditions. Fifty male Wistar rats (100-130g) were randomized into 2 experimental conditions (healthy and diseased) with 5 major groups (n=5) each. Healthy condition composed of control and varying-doses of sodium-metavanadate (20mg/kg/p.o, 40mg/kg/p.o, 60mg/kg/p.o and 80mg/kg/p.o) and disease condition include diabetes(DM)-induced (65mg/kg/i.p Streptozotocin-STZ) and groups concomitantly treated with doses of sodium-metavanadate listed above. After 8weeks, Gastric acid secretion-GAS (continuous perfusion method), Gastric tissue H^+K^+ATPase and Na^+K^+ATPase pump activities were assessed spectrophotometrically. Body weight and blood glucose level were measured daily. Data were analyzed using descriptive statistics and ANOVA α 0.05. Body weight significantly increased in vanadium-treated diabetic groups. Blood glucose level was significantly reduced in 20 and 40mg/kg/p.o as well as in 20, 40, 60 and 80mg/kg/p.o vanadium-treated groups in healthy and DM-induced conditions respectively. Basal and stimulated GAS was significantly reduced in DM-induced condition but only in stimulated GAS in healthy condition vanadium-treated groups. Interestingly, Gastric H^+K^+ATPase and Na^+K^+ATPase activities were mitigated in 20, 40 and 60mg/kg/p.o vanadium-treated groups in DM-induced conditions. Vanadium treatment at 20, 40 and 60 mg/kg/p.o in healthy and Streptozotocin-induced diabetes caused a decreased gastric acid secretion due to inhibition of the H^+/K^+ATPase activities.

HbA1c Associated *G6PD* G202A Polymorphism in Type II Diabetes Mellitus Patients Attending Murtala Muhammad Specialist Hospital, Kano.

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Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia. In hyperglycemic cases, haemoglobin binds irreversibly with glucose to form glycated haemoglobin (HbA1c), an accepted diagnostic test for type 2 diabetes mellitus (T2DM) used for monitoring glycaemic control in patients with diabetes. Some genetic variants influence HbA1c levels, by acting via the erythrocytic pathway (erythrocytic variants) or through the glycemic pathway (glycemic variants). Glycemic variants

increase the risk of developing diabetes, while erythrocytic variants have no association with the risk of diabetes. *G6PD* G202A is a single nucleotide polymorphism (SNP) associated with HbA1c, which lowers HbA1c levels irrespective of blood glucose levels. Alterations in HbA1c caused by this variant lead to massive under-diagnosis of T2DM. There is no study on the association between this SNP and HbA1c in diabetic Nigerians, hence the need for this study. Thirty-two non-diabetic and 54 diabetic male adult subjects were recruited after signing informed consent. Venous blood was collected from which fasting blood glucose and glycated haemoglobin were measured. DNA was extracted from whole blood, amplified and amplicons digested using the PCR-RFLP. These were visualised using a UV transilluminator. Data were analyzed using SPSS version 20 and SHEsis online software. The genotypes of *G6PD* G202A in diabetics were statistically significant (HWE $p<0.05$), with a minor allele frequency of 20%. *G6PD* G202A polymorphism showed association with HbA1c in diabetic subjects. *G6PD* G202A SNP is associated with HbA1c diabetic Nigerians.

Effect of Vanadium Treatment on Some Hematological and Serum Biochemical Variables in Normal and Diabetic Rats

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Vanadium, a trace element, has been known as a building material for bones and teeth. Its promising usefulness in the management of diabetes has also been reported. However, there are conflicting reports on the toxicity of Vanadium in its various forms. In this study, the effect of vanadium treatment on hematological parameters and serum biochemical variables were studied in normal and Streptozotocin-induced diabetic (STZ-DM) rats. FiftyMale Wistar rats (100-130g) were divided into 5 groups, (n=5): Control, 20mg/kg/p.o, 40mg/kg/p.o, 60mg/kg/p.o and 80mg/kg/p.o sodium metavanadate exposed for 2weeks. In another experiment, diabetes was induced with 65mg/kg Streptozotocini.p. and exposed to sodium metavanadate for 2week with groups as in the first experiment. Body weight gain and blood glucose levels were determined daily. Blood was obtained through retro-orbital puncture for hematological and serum biochemical assay and were assessed spectrophotometrically. Data were analyzed using descriptive statistics and ANOVA at α 0.005. Significant decrease in Body weight and Blood glucose level was observed in vanadium treated groups in both experiments. Packed Cell Volume, Hemoglobin and Red Blood Cell levels decreased significantly in the normal+vanadium 40-mg/kg/p.ogroupand diabetic+vanadium 80mg/kg/p.o treated groupscompared with control. Blood total protein values were significantly increased in the diabetic control and diabetic+vanadium treated groups compared with control. There was decrease in serum Alanine transferase, Alanine phosphatase and Aspartate aminotransferase of diabetic+vanadium treated groups compared with diabetic control. Orally administered Vanadium (at lower doses) proved useful in reduction of Blood glucose level in health/disease and improved serum biochemical variables of diabetic rats.

EFFECT OF *CURCUMA LONGA* SUPPLEMENTS ON COGNITIVE FUNCTION IN SWISS ALBINO MICE

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Dementia is an age-related mental disorder and a characteristic symptom of various neurodegenerative disorders. *Curcuma longa (C. longa)* contains many pharmacological and chemical important compounds which have shown many beneficial effects. This study aimed at evaluating the effect of *Curcuma longa* on cognitive function of Swiss albino mice. A total of sixteen (16) mice of both sexes weighing between 24 – 30 grams were used for the study. The mice were divided into four groups of four mice each (n=4). Groups I served as control and received 10 ml/kg distilled water; groups II, III and IV were given 5%, 10% and 20 % of *Curcuma longa* for 14 days respectively. Y maze and novel object recognition task were used to assess spatial working memory and social memory respectively. We observed that the 5% *C. longa* (77.60 ± 10.15) group showed significant ($p < 0.05$) improvement in percentage alternation compared to control group (64.40 ± 5.99). We also found out that the 5% *C. longa* supplemented group showed significant ($p < 0.05$) increase in both short term memory (-14.08 ± 3.26) and discriminative index (-0.26 ± 0.07) when compared to control group (-31.55 ± 2.65) and (-0.33 ± 0.07) respectively. Thus, *C. longa* supplement at 5% improves spatial working memory, short term memory and discriminative index of Swiss albino mice.

Showcasing Diversification of Physiology arouse Students Interest in the Field of Science

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“Scientific Literacy” defined as the ability of the society to understand science and its applications to their daily activities, can only be achieved through effective science communication. The present study is a science outreach focused on school children and first-year undergraduate physiology students, to imbibe in them the fundamentals of physiology as a course of study and clarifying its diversifications. Professors of different specializations in physiology were invited to share their experiences in the

field and expatiate on the various opportunities available for trained physiologists. Data were collected with the aid of questionnaires, which were designed so that we could evaluate the physiological knowledge of the participants before and after the orientation exercise by the professors. The results obtained from the analyzed data supported the aim of the research being fulfilled. Specifically, the interest of the school children was observed to be escalated after they had learned about the diversifications of physiology as a field of study. Moreover, the undergraduate students demonstrated an enhanced understanding of the basics of physiology. Conclusively, effective communication of science should be encouraged among prospective young scientists, to promote the awareness of scientific research in our society.

Antidepressant Activity of Methanol Leaf Extract of *Ziziphus Mauritiana* Lam: Involvement of Inflammatory Mechanism

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The leaves of *Ziziphus mauritiana* have been reported to be used in the management of depressive illnesses in traditional medicine in Northern Nigeria. However, due to little scientific proof the present study therefore attempts to establish the involvement of inflammatory system in the antidepressant activity of the extract using *Bacillus Calmette Guérin* (BCG) induced depressive like behaviour model. Mice were administered with BCG (0.2 mg/kg, i.p) followed by administration of the graded doses of the extract (25, 50, 100 and 200 mg/kg) for 21 days. Body weight and temperature were measured on daily basis. Exploration behaviour at 4th, 8th 24th and 48th hours, immobility time at 7th, 14th and 21st day, were all assessed using open field test, and tail suspension test, decrease in immobility time ($p<0.001$), increase in body weight ($p < 0.004$), decrease in body temperature ($p<0.001$), increase in number of line crossing ($p<0.001$) in the extract treated groups were observed in a dose non-dependent manner. It may be concluded therefore that the anti-depressant activity of methanol leaf extract of *Ziziphus mauritiana* may possibly involve an anti-inflammatory mechanism.

Effect of Acute Cocoa (*Theobroma Cacao*) Consumption on Blood Pressure and Pulse Rate During Cold Pressor Test

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Studies have shown that cocoa (*Theobroma cacao*) possesses the potential to lower blood pressure (BP) in man through a number of mechanisms. However, the role of autonomic mechanisms in the BP-lowering effect of cocoa is unknown. This study was designed to investigate the BP lowering effect of acute administration of cocoa through the modulation of the Autonomic Nervous System (ANS). Following ethical approval and informed consent, a randomized double-blind placebo-controlled study was conducted using 45 apparently healthy subjects, aged 20-28years. The overall effect of cocoa was assessed using the Cold Pressor Test (CPT), a standard test used to stimulate the ANS. The test was performed before and after oral administration of either 28g of cocoa powder dissolved in boiled water (198ml) or the placebos. Digital sphygmomanometer was used in obtaining values. One way ANOVA with a post-hoc Tukey test was used to analyze differences between the groups while Paired t-test was used for analysis within the groups. Results were expressed as Mean±SEM and $P<0.05$ was considered significant. It was observed that the CPT activated ANS. CPT caused significant rise in SBP, DBP, MAP from the basal values to the peak values ($P<0.05$, $P<0.01$, $P<0.01$ respectively) but not in pulse pressure and PR. However, cocoa beverage was not able to dampen the change (Δ) that occurred within the respective test-groups ($P>0.05$). The result therefore suggests that acute cocoa administration does not lower BP and pulse rate through modulation of the ANS.

Cardiovascular Response to Acute Exercise in Young Adults under Varying Environmental Conditions

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Prolong moderate intensity exercise in hot environment has been reported as an efficient way for athletes to acclimatize to heat, however, little effort is paid on how these environmental phenomena could impact on cardiovascular responses to exercise. The present study therefore, aimed to assess the difference in cardiovascular parameters at various environmental temperature conditions at rest and after exercise. Seventy three Male and female students from the Department of Human Physiology, Bayero University Kano were recruited for the study. Subjects' body temperature and cardiovascular parameters were examined, using appropriate instruments, in warm room (33ºC±1ºC) and then in cold room (22ºC±1ºC), before and after a modified Harvard step test. Data were analyzed using SPSS V20.0 with paired samples t-test as comparative statistic, and summarized using mean±SEM. From the findings, pre-exercise body temperature, systolic blood pressure, pulse pressure, mean arterial pressure and heart rate were observed to be higher in hot environment while diastolic blood pressure appeared higher in the cold environment. Following exercise, cardiovascular parameters significantly increased in cold environment, but not in hot environment, also, we found no significant effect of gender on cardiovascular response to acute exercise in the various environmental conditions. In conclusion, the present study has demonstrated that cold environment provides better cardiovascular responses to acute exercise.

Dietary zinc deficiency induces oxidative stress and alters development in fruit flies: a preliminary study

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Zinc is an important micronutrient whose dietary deficiency has been associated with later life diseases. Due to the probable epigenetic influence, this preliminary study was carried out to assess the developmental changes in wild type (w^{1118}) *Drosophila melanogaster* (fruit flies) nurtured on a zinc-chelated diet. Graded doses (50,100,150, and 200µmol/L) of N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylenedidiamine (TPEN), were added to the diets of the fruit flies to induce zinc deficiency. Gravid female flies were transferred onto the graded TPEN supplemented diets for 24hrs and laid eggs were allowed to develop on the diet until adulthood. Adult flies were transferred and cultured on a standard cornmeal diet for seven days. After three days on a standard diet, three female flies were randomly selected per group and transferred to the egg chamber for fecundity assay. Flies were removed after 4hrs and eggs were counted under a stereomicroscope. This was repeated for three consecutive days. No eclosion was observed in vials of 200µmol/L TPEN. Metal analyses showed reduced zinc levels in flies fed 50, 100, and 150µmol/L TPEN compared to control in both males and females. The Fecundity rate was significantly reduced in flies fed on 100 and 150µmol/L TPEN compared to control. There was a significant reduction in the levels of catalase and total antioxidant capacity at 50, 100, and 150µmol/L compared to control. Moreover, TPEN fed flies showed elevated malondialdehyde levels compared to control counterparts. Collectively, findings from this study buttress the importance of zinc in development, the implication of dietary zinc deficiency on oxidative stress, and the suitability of the Drosophila model to study the developmental and metabolic role of zinc.

Exposure to Light-at-Night is Associated with Metabolic and Haematological Impairments in Sleep-Restricted Male Wistar Rats

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While shift work model researches had shed light regarding the health implications of light-at-night (LAN) exposure, extrapolation onto general population will be unreliable. Our study therefore aimed at evaluating the independent effect of LAN exposure on metabolic and haematological parameters in male Wistar rats. Sixteen male Wistar rats (aged 8-10 weeks), weighing 100g±12g were randomly, but equally, divided into control and LAN exposure groups. Gentle handling was used to induce SR while LAN was instituted using a customized light rack system for six weeks. Fasting weight and blood sugar were obtained using a digital weighing scale and Glucometer respectively. Lipids were analyzed using their respective Randox kits and chemistry Autoanalyser. Malondialdehyde, catalase and superoxide dismutase activities were assayed while Full blood counts and CD 4^+ T-cells were determined using automated analyzers. Data were analyzed using SPSS $V_{20.0}$ and summarized using Mean±SEM. Student's t-test was used to investigate differences between the groups and $p \leq 0.05$ was considered as statistically significant. Our findings have demonstrated that although LAN exposed rats eat less feeds (1296g vs. 1347g), they are observed to have gained body weight significantly (p=.010) higher than controls (65g vs. 37g). LAN exposed rats also have statistically higher (p=.019) fasting blood sugar levels than controls (122 mg/dl vs. 111 mg/dl). In contrast, TriG index, HDL, AIP and markers of oxidative stress are found to be statistically similar between the groups. In conclusion, LAN exposure have demonstrated tendencies to cause obesity, diabetes and increased risk of adverse cardiovascular events.

Effects of Fruit Pulp Methanolic Extract of *Azanza Garckeana* (Goron Tula) on Isolated Rabbit Ileum and Normal Defaecation of Adult Male Wistar Rats

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This study investigated the effect of fruit pulp methanolic extract of *Azanza garckeana* on normal defaecation of adult male Wistar rats and isolated rabbit ileum. Twenty rats were grouped into four: Group I (control) received the vehicle (10% Tweene 80), while Groups II-IV received 300, 600 and 1,200 mg/kg of the extract orally, respectively. The rats were then placed in individual cages lined with clean white papers and observed for four hours. Also, with the aid of an electronic transducer connected to a microdynamometer, the effect of the extract was tested on spontaneous contractions of the isolated rabbit ileum. The results show that the extract caused no significant changes in total stool weight, frequency and diarrhoeal scores of the rats at the end of the four hours period. However, there was a significant increase in the stool weight and frequency among the 300 mg/kg extract-treated rats at the end of the first hour, which significantly phased out therafter. No significant changes were observed in all the parameters among the other groups. However, the extract (especially at 3.4 mg/mL) caused a marked reduction in strength of contractions of the isolated ileum, which was not blocked by atropine or propranolol. These results indicate that the extract may not have any significant effect in normal defecation or diarrhoea, rather, it could have a potential benefit of decreasing it in diarrhoeic states.

Urinalysis as an Adjunct in Assessing Malaria Severity

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Malaria diagnoses is based on microscopy and RDT, with RDT being the only option in some settings. Healthcare providers rely on their clinical acumen in determining severity of the infection in the absence of microscopy. This study correlated urinary abnormalities to parasite density in malaria using Dipstick. Pregnant women (n = 150) attending Murtala Muhammed Specialist Hospital Kano, comprising 100 women with positive Giemsa-stained blood films for malaria and 50 healthy controls were recruited. Malaria was classified into mild, moderate and severe, based on parasite density. Biochemical components of their urine were assessed using Medi-Test Combi 10 dipstick. The results showed significantly higher urine parameters in the case than in control groups ($P < 0.05$). However, Bilirubin, Urobilinogen, Blood, Ketones and Protein were positively correlated with malaria parasite density. Proteinuria was highest in severe malaria, then moderate, then mild (310.16 ± 10.03mg/dl), (190.96 ± 8.0mg/dl) (170.19 ± 7.8 mg/dl). Bilirubinuria was also highest, in severe malaria, (3.96 ± 0.31) moderate (1.95 ± 1.2) then mild (1.07 ± 0.63). Similarly, highest Urobilinogenuria was found in severe (9.10 ± 3.03mg/dl), then moderate (6.60 ± 1.0mg/dl) then mild malaria parasitaemia (3.19 ± 1.8 mg/dl). Ketones also followed similar pattern (3.16 ± 0.03), (1.90 ± 2.0) and (1.0 ± 0.8) for severe, moderate and mild parasitaemia respectively. Haematuria in severe malaria parasitaemia was also highest in (170.16 ± 5.03 Ery/µl), then moderate (30.96 ± 4.0 Ery/µl) and lowest in mild (9.02 ± 0.8 Ery/µl). Alongside RDT, urinalysis may serve as adjunct in identifying severe malaria parasitaemia, especially in resource constrained settings

The Effects of L-Citrulline on the Relaxation of Acetylcholine Induced Contraction on Intestinal Smooth Muscle

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When bowel motility is affected, digestion and absorption of nutrients is compromised and movements of the intestinal contents are impeded. These results in a host of signs and symptoms such as nausea, reflux, regurgitating, bloating and diarrhea. L-Citrulline is a non-protein amino acid compound that is important in the urea cycle in the liver and kidneys. It is mainly metabolized in the kidney and converted into arginine. This study examined interventions that can be used to displace the uncoordinated contractions of the intestinal smooth muscle by bringing about relaxation using L-citrulline. Adult wistar rats (n= 10) weighing between 200-250 g were euthanized and the abdomen was opened, a segment of the small intestine (ileum) were excised and placed in Tyrode solution. The tissues were cut into segments of 2 cm each and then suspended in organ baths. The tissue segments were suspended from a stationary hook within the organ bath and connected to a force transducer that measured the tension generated by the tissues. The contractions and relaxations were recorded with the use of the data acquisition software power lab. L-citrulline caused a relaxation in Ach induced vessels. There was no significant difference in the rate of relaxation of atropine and L-citrulline in both administration of 1.0 ml ($p= 0.237$) and 2.0 ml ($p= 0.136$). These results therefore suggest that L-citrulline when supplemented will bring about a relaxation in the same way as atropine.

Modulatory Role of Antioxidant Vitamins (A and E) in Cyanide Induced Liver and Brain Damage in Adult Male Albino Mice

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Cyanide is a potent neurotoxic substance that can initiate series of intracellular reactions leading to oxidative stress. To evaluate the effect of sub lethal administration of potassium cyanide (KCN)on lipid peroxidation and some antioxidant enzymes in adult male albino mice and possible ameliorative role of vitamins A and E. Thirty five adult male mice weighing between 18-22 g were used. An acute toxicity study was carried out to determine LD_{50} using Locke method. The animals were randomly divided into five groups (n = 7) as follows; group I (control, received deionized water), group II (1.5 mg/kg KCN), group III (1.5mg/kg KCN + 25 mg/kg vitamin A), group IV (1.5 mg/kg KCN + 50 mg/kg vitamin E) and group V (1.5 mg/kg KCN + 25 mg/kg vitamin A + 50 mg/kg vitamin E). Treatment groups was carried out daily through oral gavage for administration of potassium cyanide while vitamins A and E were administered intraperitoneal (IP) for 28 days and on the last day, the animals were sacrificed and isolation of tissues for biochemical assays of malondialdehyde, superoxide dismutase, catalase, acetyl cholinesterase and serum liver enzymes. From acute toxicity studies, LD_{50} was calculated to be 15 mg/kg. The results obtained indicated significant ($p < 0.05$) increase in MDA levels, indicating lipid peroxidation in the cyanide group (3.30±0.19 nMol/mg) compared to the vitamin treated group V (1.82±0.21 nMol/mg). There was a significant ($p < 0.05$) decrease in inhibition of superoxide dismutase in group II (1.30±0.07 µ/mg). Furthermore, a decrease in catalase inhibition was recorded in group II (30.81±1.43 mmol/min/ µ/mg) in comparison to group III (41.60±1.96 mmol/min/ µ/mg). Acetylcholinesterase enzyme activity was significantly increased in group II (32.10±0.90 nmol/min/mg) as compared to the vitamin treated group V (16.20±0.90 nmol/min/mg). Liver enzymes AST recorded high levels in the potassium cyanide treated group

(155.20±6.44 U/L) when compared to control (45.80±1.77U/L). Antioxidant vitamins (A and E) played an important role in ameliorating the oxidative stress poised by cyanide through stimulating the antioxidant defence system.

Assessment of Respiratory Function Parameters Among Suya Sellers In Gwale Local Government, Kano, Nigeria

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Wood smoke has been associated with a wide range of health effects including lung dysfunction leading to respiratory symptoms and diseases. Suya sellers are often exposed to high levels of wood smoke and oil fumes in the course of their job and hence are at risk of lung dysfunction. The aim of this study was to assess lung volumes and capacities of Suya sellers in Gwale LGA Kano using Wet spirometry. Twenty participants consisting of 10 Suya sellers and 10 healthy controls were recruited for the study. Wet spirometry was performed following standard protocol. Data were analyzed on SPSS version 23.0 and results presented as mean±SD. P value ≤ 0.05 was considered statistically significant. The mean age of the Suya sellers and that of controls were 30.0±6.38 and 29.20±2.09 (p = 0.725) years respectively. The Suya sellers had significantly lower respiratory rate compared to the controls (13.20 ± 1.14 vs 13.20 ± 1.14, p = 0.025), though both were within normal limits. However, there was no statistically significant difference in lung volumes and capacities between the Suya sellers and controls. In conclusion, the Suya sellers had significantly lower respiratory rate but there was no difference in lung volumes and capacities between the two groups.

Evaluation of Renal and Liver Functions in Pregnant Women with Malaria Parasitemia Attending Gombe Specialist Hospital, Gombe State, Nigeria

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Malaria, a vector-borne disease is an ancient disease and is now regarded as a life threatening disease. A study to evaluate the renal and liver function parameters in pregnant women with malaria parasitemia attending Gombe specialist hospital, Gombe State, Nigeria May-July, 2019 was conducted to determine the prevalence malaria parasite in pregnant women, assess the interaction between malaria parasitaemia and kidney function in pregnant women, and assess the interaction between malaria parasitaemia and liver function in pregnant women. A cross-sectional study involved a sample size of 384 (284 with gestational malaria as cases and 100 healthy pregnant women as controls) was carried out among volunteering consented pregnant women. Data analysis was carried out using Statistical Package for Social Sciences version 20.0. Bivariate and multivariate analyses were performed and P-value of < 0.05 was considered significant. Biochemical parameters revealed significant decrease in the levels of serum urea, sodium and bicarbonate. Furthermore, the study found significant increase (P < 0.05) in the activities of the enzymes Alanine transaminase (152.62± 12.5) and alkaline phosphatase (66.88+ 6.29) for the pregnant women with malaria parasitaemia. Similarly, urea (20.66+6.12) and creatinine, decrease significantly (p < 0.05) among the pregnant women with malaria parasitaemia. In conclusion, the prevalent species of the parasite is plasmodium faciparium result in the renal and liver functions parameters changes.

Effects of the interaction between Age, Trimester and Malaria Parasitemia on Haematological Parameters of Pregnant Women Attending Gombe Specialist Hospital

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Malaria is major public health problem in Sub-Saharan Africa. Most of the infections are affect mainly pregnant women and children. Blood sample was collected from 384 pregnant women. 284 were tested positive and 100 as controls, effects of malaria on the following haematological parameters were determined which includes WBC, RBC and PLTC, PCV, Haematocrit, Differential Leucocyte Count, MCV, MCH and MCHC. A period of 3 months was used for blood sample collection. Data analysis was done using statistical package for social science version 20.0. Bivariate and Multivariate analysis were performed P-value < 0.05 was considered significant. 284 (74%) pregnant women infected with malaria, age 21-25 (40.8%) had the highest rate of infection, the lowest was ≥ 31 years (9.9%). First trimester had the highest rate (41.9%) and third trimester had the lowest rate of infection (20.1%). In some of the Haematological parameter, the total WBC, absolute Lynphocytes and monocytes were significant higher in the malaria infected patients than in non-infected pregnant women (P < 0.05). There was no significant difference in the basophil count between the malaria positive and the controls (P > 0.05). There was a significant difference in the levels of WBC, PLTC and MCV, MCH and MCHC; insignificant difference between the first, second and third trimester of the positive cases and negative. In conclusion, malaria infection during pregnancy has adverse effects on maternal haematological parameters, and anaemia is the most common consequence of *P. falciparum* malaria infection in pregnant women

Some Haematological Parameters of Aqueous Stem Extract of *Anisopus Mannii* in Alloxan-induced Diabetic Wistar Rats

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The financial burden of management of diabetes has become globalized and this has become paramount for developing countries to search for diabetic drugs which are cost effective with minimal side effects. The aim of this study is to determine the effects of aqueous stem extract of anisopus

mannii on some haematological indices on alloxan induced diabetic wistar rats. This study was carried out on twenty five wistar rats of both sexes weighing 150 to 300g (8-10 weeks old). They were randomly divided into five groups of five rats each. Diabetes mellitus was induced by a single intraperitoneal injection of Alloxan monohydrate at 150mg/kg. The LD50 for the aqueous stem extract was > 5000 mg/ dL. The results showed no statistical significance ($p < 0.05$) in RBC; Hb and PCV at the various doses of aqueous stem extract of Anisopus *mannii* compared to the diabetic control respectively. However, the results showed a significant ($p < 0.05$) increase of total white blood cell count at the dosage of 100 mg/ Kg and 400 mg/ Kg of Anisopus *mannii* stem extract respectively. In addition, there was a significant ($p < 0.05$) increase in the level of monocytes at 200 mg/ kg and 400 mg/ Kg compared to the diabetic control; with associated significant ($p < 0.05$) increase in the level of lymphocytes at all doses of stem extract compared to the diabetic control respectively. In conclusion, Anisopus *mannii*, has no effect on haematological indices in alloxan induced diabetic wistar rats.

Anti-Ulcerogenic Activities of *Zea Mays* (Corn) Diet in Male Wistar Rats

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Gastric ulcer caused by imbalance between protective and aggressive factors is one of the most common disorders of the gastrointestinal tract. *Zea mays* L. (Corn) is a source of nutrition with natural phytochemical compounds. Previous study reported that the methanolic extract of *Zea mays* (Zm) decreases gastric acidity and inhibited percentage gastric ulceration. This study therefore investigated the underlying gastroprotective mechanisms of the Zm diet. Sixty male wistar rats were used, randomly grouped into 6; 1(Control), 2(Ulcerated), 3, 4, 5 (prefed with Zm diet -55%, 65%, 75%) and 6 (cimetidine- 40mg/kg) for 28 days. Gastric ulcer was induced by oral administration of indomethacin (40mg/kg) and animals were sacrificed after 4 hours. Hematological variables and gastric blood flow was determined prior induction of ulcer. The stomach was excised and cleaned, it was then homogenized and centrifuged for biochemical assays. Increased PCV, HBconc, RBC and gastric blood flow was observed in Zm diet group. There is a significant reduction in gastric ulcer area in all treated groups. Significant increase in gastric tissue mucin content and gastric mucosal SOD, NO and PGE2 levels and significant reduction in the MDA was observed in the Zm diet and cimetidine group. Results were buttressed with histological analysis. Results obtained from this study suggests that Zm diet may have anti-ulcerogenic properties by enhancing antioxidant enzymes as well as mucosal blood flow via increased hematological variables, nitric oxide and prostaglandin E2 mechanisms.

Effect of Bevi-mix (Artificial Sweetener) and its Major Constituent Aspartame on the Integrity of Gastric Mucosa in Male Wistar Rats.

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Bevi mix (BM) is a powdered drink consisting of artificial sweetener of which aspartame (ASP) its major constituent. Aspartame consumption has been associated with a risk factor for diseases due to the formation of its metabolites. The effect of BM and ASP on the integrity of gastric mucosa in male Wistar rat were investigated. Forty-two male Wistar rats were divided into 6 groups (n = 7). Group 1 (1 ml/kg distilled water), Group 2 and 3 (BM at 330 and 660 mg/kg bw), Groups 4-6 (ASP at 20, 40 and 80mg/kg bw). Gastric juice was collected via pyloric ligation, gastric acid, pepsin, mucus component (total sialic acid content) and mucus gel were evaluated. Administration of BM at 660 mg/kg and ASP at 80 mg/kg caused a significant ($P < 0.05$) increase in volume of gastric juice but the titratable acidity and total acid output were significantly ($P < 0.05$) increased. The groups given aspartame showed significant ($P < 0.05$) increases in titratable acidity and total acid output. There was significant ($p <0.05$) increase in pepsin concentration in all groups given BM and ASP. Mucus secretion was significantly ($p<0.05$) decrease with BM at 660mg/kg, aspartame 40 mg/kg or 80 mg/kg. Bevi mix at 660 mg/kg and ASP at 80 mg/kg caused a significant ($P < 0.05$) increase in free sialic acid and bound sialic acid. In conclusion, BM at higher doses and all doses of ASP increases gastric acid secretion, pepsin concentration, free sialic acid and decreased mucus secretion which may decrease the integrity of the gastric mucosa.

Effect of Bevi-mix (Artificial Sweetener) and its Major Constituent Aspartame on Oxidative stress parameters, Kidney electrolyte and Liver enzymes in Male Wistar Rats.

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Bevi mix (BM) is a powdered drink consisting of artificial sweetener of which aspartame (ASP) its major constituent. The effect of BM and ASP on oxidative stress biomarkers, liver enzymes and kidney electrolytes in male Wistar rat were investigated. Forty-two Wistar were divided into 6 groups (n = 7). Group 1 (1 ml/kg distilled water), Group 2 and 3 (BM at 330 and 660 mg/kg bw), Groups 4-6 (ASP at 20, 40 and 80mg/kg bw). Blood sample, kidney and the liver homogenate were analyzed. Serum SOD and GSH were decreased by BM (660mg/kg) and ASP significantly ($P \leq 0.05$), BM 330 mg/kg caused a significant ($P \leq 0.05$) increase in GSH. The activity of ALT was increased by BM at 330 mg/kg but all the doses of aspartame significantly ($P < 0.05$) decreased it; while the activity of AST was not affected by BM groups however both doses of BM increase the activity of ALP level. The ASP groups showed a significant ($p< 0.05$) increase in AST and ALT. BM 330 or 660 mg/kg caused a significant ($p < 0.05$) increase in the kidney Na^+ but no significant ($p < 0.05$) change in kidney K^+ level. ASP at 20, 40 or 80 mg/kg caused a significant ($p < 0.05$) increase in kidney Na^+ and K^+ levels. The BM 1and

ASP groups showed a significant ($p < 0.05$) increase in kidney bicarbonate ion level. In conclusion, higher doses of BM and ASP increases increases Na^+ and H_2CO^- ion levels and causes oxidative stress and decreasing serum antioxidant enzymes in the rats.

Evaluation of Effects of Hexane and Methanol Extracts Of *Trigonella Foenum-gaecum* L. in Isolated Rabbits Heart

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Trigonella Foenum-gaecum L. contains a broad spectrum of therapeutic properties and it's use as galactagogue and antidiabetic among others is recoded in traditional medicine practice. Experiment shows that Methanol and Hexane extracts' affect blood pressure and myocardial relaxation which may be either by the combined effects of myocardial depression, restorative function of the plant high vitamin C content or/ and muscarinic receptor mediated vascular smooth muscle relaxation. Atropine (25 mg/ml) at all tolerable doses did not block the hypotensive effect of both extracts but rather potentiated the effect of the extracts. The methanol and hexane extracts of the seeds of Trigonella Foenum-gaecum L. decreases both the force and rate of myocardial contraction in a concentration dependent manner. The result of this experiment on Isolated Rabbit Prefused Heart indicated that the plant Trigonella Foenum-gaecum L. has vascular smooth muscle relaxation with potent blood pressure lowering properties, and therefore, could lead the pharmaco-physiological credence to folkloric, as well as ethnomedical use of the plant in management and/or control of high blood pressure.

Pathophysiology and Histological Effect Of Spitting Cobra's Venom *(Naja Naja)* In The Eyes

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Snakebite is associated with diverse Pathophysiology due to the magnitude of variation in venom compositions that is observed Worldwide. Spitting cobra can spit venom towards the eyes of the predator as a defense mechanism, causing painful and potentially blinding ocular envenoming. Six albino mice were used for the study which were divided in to two per group, venom of *naja naja* were exposed at different range of 2-5cm length same concentration. The eyes were rinsed with normal saline and analgesic administered to reduce the pain, and corneal thickness was assessed. The effect of the venom on selected enzymes activity in the serum/liver of albino rats induced with the snake venom was studied and the histopathology. The result shows elevated AST (75%) and ALT(60%) level on experimental animals significant change was observed as compared with the control group ($P>0.05$). Naja naja venom induced significant corneal edema and epithelial necrosis. In this experiment Naja naja venom was capable of inducing a high rate of vascular permeability or pathophysiological effect in the eyes, hepatic and cardiac tissues alterations.

Cinnamon Reverses Depressive-Like Symptoms of Mice In Open-Space Forced Swim Test Model

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Depression is a mental disorder characterized by depressive episodes, such as low mood, low self-esteem, feeling of guilt, and poor concentration. Depression has a high comorbidity with cognitive impairments. Studies have shown that cinnamon has anti-inflammatory and anti-diabetic potentials. Therefore, the aim of the research was to assess the antidepressant effect of cinnamon on open-space forced swim-induced depression in mice. Twenty-five (25) Swiss albino mice were grouped into five groups (n=5). Group 1: control (negative control), Groups 2, 3 and 4 received graded doses of Cinnamon 12.5, 25, and 50 mg/kg, group 5 received fluoxetine 20 mg/kg orally. The animals were subjected to Open Space Forced Swim Test (OSFST), Open Field Test (OFT), Y-maze test, and Novel Object Recognition Test (NORT). Administration of cinnamon showed decrease immobility time (behavioural despair) in OSFST compared to control and fluoxetine groups ($p < 0.05$). Similarly, Cinnamon 12.5, 25 and 50 mg/kg showed statistically significant decrease in the number of entries into the Y-maze arms compared to control and fluoxetine groups ($p < 0.05$). Furthermore, cinnamon improved spatial short-term memory as observed by an increase in the spontaneous alternation and percent alternation in Y-maze test compared to the control and fluoxetine groups ($p < 0.05$). Also, cinnamon 25 mg/kg showed a significant increase in rearing in OFT compared to control and fluoxetine groups ($p < 0.05$). However, no statistically significant effect was observed in the discrimination ratio of NORT between cinnamon administered groups and the control group. In conclusion, cinnamon has shown antidepressant-like effect in open-space forced swim-induced depression in mice.

Cinnamon Improves Spatial Short-Term Memory and Attenuates Anxiety-Like Symptoms of Mice Exposed To Chronic Unpredictable Mild Stress

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Depression is a state of low mood and aversion to activity. Studies have shown that cinnamon has antioxidant, anti-diabetic and anti-inflammatory potentials showing its possible anti-depressant effects. This study is aimed at evaluating the antidepressant like effect of cinnamon in

mice exposed to chronic unpredictable mild stress (CUMS) model of depression. Thirty (30) Swiss albino mice were grouped into six groups (n=5). Group 1: received distilled water (DW) 10 ml/kg, Group 2: exposed to chronic unpredictable mild stress (CUMS). Group 3, 4, and 5 received graded doses of cinnamon extract 12.5, 25, and 50 mg/kg, respectively. Group 6 received fluoxetine 20 mg/kg orally. Groups 2,3,4,5 and 6 were subjected to tail suspension test (TST) 24 hours before, after 2-weeks and at the end of CUMS, respectively. Thereafter the mice were subjected to open field test (OFT), Y-maze, and novel object recognition test (NORT). This study revealed no statistically significant difference in the TST. However, a statistically significant increase was observed in line crossing of OFT between fluoxetine and DW group (p<0.05). Similarly, comparison between fluoxetine and cinnamon treated groups showed significant difference in the line crossing (p<0.05). Cinnamon 12.5 mg/kg showed significant increase in rearing of OFT when compared with CUMS and fluoxetine groups at p<0.05. In number of entries in to the arms in Y-maze, a significant effect was observed in cinnamon 12.5 and 50 mg/kg compared to DW group (p<0.05). More so, significant effect was also seen in spontaneous alternation ratio in Y-maze in cinnamon 50 mg/kg as compared to CUMS group (p<0.05). In NORT, no significant effect was observed. In conclusion, cinnamon showed antidepressant-like effect in mice exposed to CUMS.

Correlation between Body Mass Index (BMI) and Cognitive Function among 400 level Students of

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Obesity affects our body in different manner and considered as an important public health problem. As documented by some previous studies that Various cognitive functions of brain might get affected by high body mass index (BMI > 30 Kg/m2), previous researches has found that height is correlated with cognitive function at older ages, and also that there is correlation between weight and cognitive function. This study aims to study the correlation of BMI, height and weight with cognitive performance of 400 level Students, Department of Human Physiology, Ahmadu Bello University Zaria. A total of 69 400 level Physiology Students were selected in random manner in the medical college. All study subjects were informed about tests to be performed. The subjects height and weight were taken and the BMI was calculated. Digit Symbol Substitution Test (DSST) and Montreal Cognition Assessment Test (MoCA) were used to check cognitive functions in the subjects. There was no statistically significant relation between BMI and cognitive function score of the subjects (p > 0.05). DSST showed a slight but insignificant negative correlation with height (p = 0.12), while MoCA showed positive and statistically significant correlation with height (p = 0.03). **:** There was no statistically significant relation between weight and cognitive function score of the subjects (p > 0.05). This study showed that height is associated with cognitive function, that taller people have higher cognitive function than the shorter one, whereas BMI and weight have no relation with cognitive function.

Effects of Melatonin on Gastric Ulcer Parameters, Gastric Mucus Secretion and Oxidative Stress Induced by Water Immersion Restraint Stress in Male Wistar Rats.

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The effects of melatonin on gastric ulcer parameters, gastric mucus secretion and stomach oxidative stress markers induced by water immersion restraint stress in Wistar rats. Male Wistar rats, restrain cages, thermostatically controlled water bath, Ketamine, Alcian Blue, Sucrose Solution, Sodium Acetate, Magnesium Chloride, Diethylether, 10% Formalin, Eosin, 2%Phenolphthalein, 0.01 NaoH Solution. The rats experimented on were kept fasting for 48hrs starting from the 3rd day of administration in separate restrain cages to ensure complete emptying of the stomach. During sacrifice the rats were first made unconscious through the intra-peritoneal administration of ketamine hydrochloride to immobilize them on the restraint board. The water immersion restraint-stress was then achieved by restraining the rats on a board and lowering the restraint board into a thermostatically-controlled water bath at a temperature of 23 ± 0.5ºC, to the levels of xiphoid process with the head vertically up which lasted for 3.5 hours. Data were processed using IBM SPSS statistics version 20.0. All results obtained are expressed as the mean ±SEM. The significance level was set at P < 0.05. Melatonin produced a significant (p<0.05) decrease in gastric ulcer score with the active group 64.26±78.54 and pretreated group 32.75±27.54 with preventive indices of 100% respectively. Gastric mucus secretion on the active group with 0.1±0.02 and pretreated group with 0.1±0.23. Histologically the gastric mucosa of animals in the study showed a severe necrosis of the epithelial cells of the active group than observed in the pretreated study. The administration of melatonin did increase the stomach tissue MDA and SOD while it decreased Catalase activity in the pretreated group more than the active group. This study concluded that melatonin showed protective effects on ulcer parameters, mucus secretion.

Age-Related Peculiarities of the Effect of Oxidative Stress on Expression of Fibroblast Growth Factor-2(FGF-B) in Indomethacin-Induced Peptic Ulcer in Wistar Rats

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Peptic ulcer is an acid-induced lesion of the digestive tract, which occurs due to imbalance between protective and aggressive factors. Fibroblast growth factor-β(FGF-β), being a member of Fibroblast Growth Factors (FGFs), plays a pivotal role in repair processes of many organ systems, including the gastrointestinal tract (GIT), which has highcell turnover; along the length of the GIT, FGFsmediate cell proliferation, differentiation, epithelial cell restitution, and stem cell maintenance. The aim of this study was to determine the effect of age and oxidativestress on fibroblast growth factor in Indomethacin-induced gastric ulcer in Wistar rats. Three (3) groups of Wistar *Rats* (n=10) of aged 5, 8 and 12 months were used; peptic ulcer was induced after a 24-hours fasting, prior to administration of Indomethacin. After animals were sacrificed using ketamine as anesthesia, stomach was removed, stored in phosphate buffer for histology studies. The FGF-β was assessed using enzyme-linked immunoassay, while SOD was assessed using spectrophotometry. There was no significant statistical difference in Mean FGF-β concentration in groups 1, 2 and 3. It was concluded that, age and oxidative stress had no effect in the expression of FGF-β in Indomethacin-induced peptic ulcer in Wistar rats.

Alpha-Lipoic Acid Attenuates Depressive Symptoms in Mice Exposed to Chronic Unpredictable Mild Stress

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Depression is the most common psychiatric illness that involves mood disturbances affecting many brain regions. Alpha-lipoic acid (ALA) is an antioxidant that plays an essential role in mitochondrial energy metabolism and neurotransmitter modulation. Hence, this research was aimed at assessing a possible antidepressant effect of ALA in mice exposed to chronic unpredictable mild stress (CUMS). Twenty-five **(**25) Swiss albino mice weighing between 20-26 g were grouped into five groups (n=5). Group 1: which served as control received normal saline (NS) and was exposed to CUMS, Groups 2, 3, and 4 received graded doses of ALA (100, 200, and 400 mg/kg respectively), Group 5 received fluoxetine (20 mg/kg). Daily administration was done through oral gavage. The animals were subjected to open field (OF) and staircase (SC) tests after induction of depression using CUMS. Thereafter, brain and blood samples of the mice were collected for serotonin, brain-derived neurotrophic factor (BDNF), catalase, superoxide dismutase (SOD), and malondialdehyde (MDA) analysis. Treatment with ALA 200 mg/kg significantly decreased immobility time compared to CUMS + NS group ($p \leq 0.05$) in the tail suspension test. Similarly, fluoxetine 20 mg/kg significantly increased brain serotonin level and decreased BDNF level compared to CUMS + NS group ($p \leq 0.05$). However, ALA did not significantly affect brain serotonin and BDNF levels ($p > 0.05$). In the OF test, a significant decrease was observed in the number of line crossings in ALA 100, 200, and 400 mg/kg and fluoxetine 20 mg/kg administered groups when compared with CUMS + NS group ($p \leq 0.05$). However, in SC test and oxidative stress biomarkers, no significant effect was observed ($p > 0.05$). In conclusion, ALA showed a promising antidepressant-like effect in mice subjected to CUMS murine model of depression by decreasing immobility time.

Effects of Hydro-Ethanol Leaf Extract of *Cissus Aralioides* on Prolactin and Thyroid Stimulating Hormone in Female Wistar Rats.

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One option that have been employed to tackle the situation is the use of herbs. *Cissus aralioides* leaves are well known and mostly used by women who are expecting fruitfulness. The aim of this study is to ascertain the effects of hydro-ethanol leaf extract of *cissus aralioides* on prolactin and thyroid stimulating hormone in female wistar rats. A total of 20 rats weighing between 150mg to 190mg were used for the study. The animals were divided into four groups with five animals per group. Group 1 (control) were administered 5mls of distil water for 30days, group to received 150mg/kg of *C. arlioides,* group 2 received 300mg/kg of extract and 3 received 600mg/kg of extract. Statistical analysis was done using SPSS version 24 with ANOVA. $P < 0.05$ was said to be significant. The study revealed that there is significant increase in prolactin when 150mg/kg of extract was given and significant decreased when extract of 600mg/kg was administered when compared with control. Also, for TSH, there is significant decreased in TSH when 150mg/kg of extract was administered and significant increase in TSH when extract of 300mg/kg and 600mg/kg when administered compare to the control. *Cissus aralioides* leaves are well known and mostly used by women who are expecting fruitfulness. The study shows significant increase in prolactin at low dose and significant decreased at high

dose. Again, there is significant decreased in TSH at low dose and significant increase at high dose.

Effect of Aqueous Extract of *Allium Sativum* on Some Red-Blood Cell Indices in Phenyhydrazine-Induced Anemic Wistar Rats

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Phenylhydrazine is a drug known to cause hemolysis in rats. *Allium sativum*a widely consumed plant has been used in traditional medicine to cure several illnesses. The aim of the study was to evaluate the effect of aqueous extract of *Allium sativum* on packed cell volume (PCV), hemoglobin (Hb), red blood cell (RBC) count, mean corpuscular volume (MCV), mean corpuscular hemoglobin(MCH) andmean corpuscular hemoglobin concentration (MCHC) in phenylhydrazine-induced anemic rats. A total of 20 Wistar rats weighing 120±10grams were used for the study. 15 out of the animals were administered with phenylhydrazine to induce anemia which was confirmed on the third day.All animals were then divided into four groups (n=5)and received treatments orally and daily for 14days. Group 1 (control group): Normal rats treated with distilled water, group 2: Anemic rats treated with distilled water, group 3: Anemic rats treated with 200mg/kg of extract, group 4: Anemic rats treated with 400mg/kg of extract.At the end of the study, blood sample was collected and placed in EDTA bottles. Level of PCV and RBCcountof group 2 animals were significantly lower than the control group while the level PCV and RBC count of group 3 and group 4 were significantly higher compared to group 2. Other parameters were not significantly different in all groups. It was concluded that administration of *Allium sativum*ameliorated the negative effect of phenylhydrazine on PCV and RBC count.

Effect of Acute Cocoa Consumption on the Sympathetic Nervous System during Hand Grip Exercise

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It has been shown that cocoa (*Theobroma cacao*) lowers blood pressure (BP) in man through a number of mechanisms (Corti et al., 2009). However, the role of the autonomic nervous system (ANS) in the BP-lowering effect of cocoa is yet to be investigated. This study was aimed to address this gap in knowledge. A randomized double-blind placebo-controlled study was conducted using 45 apparently healthy subjects aged 20-28years after obtaining informed consent and ethical clearance. The effect of cocoa was investigated using the hand grip exercise (HGE) which is a standard test used in activating the ANS. The HGE was done before and after the oral consumption of either 28g of cocoa powder as beverage (198ml) or the placebos. A digital sphygmomanometer was used to measure the BP. Paired t-test was used to analyze the effect within groups (before and after administrations) while One-way ANOVA with a post-hoc Tukey test was used to analyze differences between the groups. Results were expressed as Mean±SEM. $P<0.05$ was considered significant. The HGE induced ANS activation. HGE caused significant increases in systolic BP (SBP), diastolic BP (DBP), mean arterial pressure (MAP) and pulse rate (PR) from basal to peak levels ($P<0.001$ respectively) but not in pulse pressure. Cocoa beverage could only dampen the change (Δ) in the SBP ($P<0.05$). The result therefore suggests that cocoa beverage administered acutely could hold some potential in the management of SBP through the modulation of the ANS.

Effects of Different Doses of Lipopolysaccharide on Tail Suspension, Forced Swimming and Open Field Tests in Mice

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Lipopolysaccharides (LPS) is the major outer membrane constituent of Gram-negative bacteria Lipid A part is known to be responsible for the toxic effects of infections with Gram-negative bacteria (Wang and Quinn, 2010). Several evidence point to the involvement of Gram-negative bacteria in the pathophysiology of depression. It stimulates production of pro-inflammatory cytokines, immune mediators and oxidative stress in various cell types (Park *et al.*, 2017; Gomes *et al.*, 2017). Mice were randomly divided into four groups (n = 5). Group I received normal saline, group II received LPS (0.5 mg/kg), group III received LPS (1 mg/kg), group IV received LPS (2 mg/kg), all intraperitoneally once, 24 h later the depressant-like effects of LPS was evaluated using tail suspension test (TST), forced swimming test (FST) and open field test (OFT) at 24 h interval. A single administration of LPS (0.5 mg/kg, i.p.) significantly increased the immobility time in TST and FST (*$P<0.05$).* It also decreased frequency of rearing, line crossing and centre crossing in OFT. Single administration of LPS (1 mg/kg and 2 mg/kg i.p.) significantly increased immobility time in TST. LPS (1 mg/kg and 2 mg/kg i.p.) significantly decreased frequency of Rearing, line crossing and centre crossing in OFT (*$P<0.05$).* These results demonstrated that 0.5 mg/kg, i.p. LPS is the most suitable dose for acute induction of depression in LPS model.

Antitrypanosomal Activities and Haematological Profile Of Crude Extract of the Stem Bark of *Acacia Nilotica* Plants Against *Trypanosoma Brucei* Infected Wistar Rats.

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This study was aimed at investigating antitrypanosomal activities and haematological profile of crude extract of the stem bark of *Acacia nilotica* plants against *Trypanosoma brucei brucei* infected Wistar rats with a view to determining the; antitrypanosomal activities of crude extract of *Acacia nilotica* against *Trypanosoma brucei brucei* infected Wistar rats and haematological profile of *Trypanosoma brucei brucei* infected Wistar rats, before and after administration of crude extract. The methanolic extracts of the plants was administered to the Wistar rats intraperitoneally daily and the parasitaemia count was determined using the rapid matching method. PCV, WBC and differential counts were determined before and after the administration to ascertain any significant differences. The findings show that the stem barks of *Acacia nilotica* crude extracts (100, 200, 300 and 400mg/kg body weight) had antitrypanosomal activity. Parasites were cleared from circulation within 12 days of treatment. Haematological indices of *Acacia nilotica* in *Trypanosoma brucei brucei* infected Wistar rats showed that there was no statistical significant change in the packed cell volume, white blood cells and differential counts before and after treatment with all doses of the crude extracts. The crude extracts of the stem bark of *Acacia nilotica* exhibits trypanocidal effect at various doses which is often associated with reduction in experimental African trypanosomiasis, and the lack of statistical significance in the Haematological indices may be due to some degree of maintenance of blood value parameters by the extracts since trypanosomiasis is associated with a rapid decline in PCV, haemoglobin concentration.

Protective Effects of Hydro-Ethanolic Extract of *Nigella Sativa* (Black Seed) on Some Haematological Parameters of Lead-Induced Poisoning in Albino Rats.

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Heavy metal toxicity such as Lead(Pb) has been proved to be a major threat due to several health risk associated with it, including RBC destruction. This present study investigated the protective effects of *Nigella sativa* on some haematological parameters of Lead induced poisoning in Albino rats. Twenty five adult Albino rats were divided and treated as follows; Group I (Control): 2ml/kg of distilled water, Group II (Negative control): 60mg/kg of Lead acetate only, Group III, IV and V: 200 mg/kg, 400 mg/kg and 800 mg/kg of *Nigella sativa* seed extract together with 60 mg/kg of Lead acetate solution respectively for 36days, Blood samples were collected and analysed for hematological parameters which included Red blood cell count(RBC), packed cell volume(PCV), Haemoglobin(Hb) concentration, platelet count, white blood cell(WBC) profile and haematological indices using an automated digital blood analyser. The study revealed that *Nigella sativa* prevented a significant ($P<0.05$) decrease in RBC count, Hb concentration, PCV while WBC, Lymphocyte and platelet count were significantly ($P<0.05$) increased in the treated group when compared with the negative control. In conclusion, *Nigella sativa* offers protective effects on hematological parameters against Lead poisoning in albino rats.

Evaluation of Oxidative Stress Biomarkers in Male Wistar Rats Treated With Indomie Instant Noodle Seasoning

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Small amounts of reactive oxygen species are necessary for spermatozoa to acquire fertilizing capabilities; however, oxidative stress has a negative effect on male reproductive capacity. Therefore this study was designed to evaluate the effects of indomie instant noodle seasoning (INS) on oxidative stress biomarkers in Wistar rats. Fifteen (15) male Wistar rats were used for this study. The animals were randomly divided into three groups of five animals each (n=5) and treated as follows via oral gavage for 8 weeks. Group 1 (control) was given distilled water (1ml/kg), group 2 was given INS (3083mg/kg) while group 3nreceived INS (6170mg/kg). At the end of the experiment, animals were euthanized and sera from blood sample obtained and used for biochemical analysis using ELISA kits for the antioxidant enzymes and spectrophotometric method for MDA. Serum malondialdehyde (MDA) concentration was significantly higher ($P<0.0.5$) in group 3 compared to control; 37.00±1.71 vs 32.92±1.86. There was also a significant decrease ($P<0.05$) in level of superoxide dismutase (SOD) in group 3 compared to control; 13.02±0.73 vs 17.95±0.58. Serum glutathione peroxidase (GPx) was significantly higher ($P<0.05$) in group 3 compare to control; 31.35±0.71 vs 21.90±0.39. However, there was a significant decrease ($P<0.05$) in the level of serum catalase (CAT) in group 2 and 3 compared to the control; 15.95±1.01 and 12.97±0.60 vs 19.75±0.66 respectively. In conclusion, treatment with INS significantly increased serum MDA concentration and GPx level with consequent decrease in endogenous SOD and CAT level in male Wistar rats.

Serum Testosterone Level, Sperm Analysis and Testicular Histomorphological Evaluation in Male Wistar Rats Treated With Indomie Instant Noodle Seasoning

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Fear has increased in recent years over the possible deleterious effects of fast food condiments on fertility. Therefore, this study was designed to evaluate the effects of Indomie Instant Noodle Seasoning (INS) on male reproductive functions in Wistar rats. Fifteen (15) male Wistar rats were used for this study. The animals were randomly divided into three groups of five animals each (n=5) and treated as follows via oral gavage for 4 weeks: Group 1 served as the control and was given distilled water (1 ml/kg), Group 2 was given INS (3085mg/kg) while Group 3 received INS (6170 mg/kg). At the end of the experiment, animals were euthanized and sera from blood sample obtained and used for biochemical analysis while testicular tissues were excised and used for histological studies. Although serum testosterone levels were lower in INS treated groups compared to the control, it was however not significant. Percentage of active cells was significantly lower (P) in group 2 and 3 compared to control. Percentage of viable cells was significantly lower (P) in group 3 compared to control; 32.00±2.35 vs 60.75±3.93. There was a significant decrease (P) in total sperm count in the treated groups compared to control; 36.50±2.63 and 11.50±0.87 vs 46.05±3.16.Testicular histology showed that the treated groups had irregular seminiferous tubules with epithelial sloughing, cellular degeneration and fibrosis relative to the control. In conclusion, INS reduced serum testosterone with declining sperm characteristics in male Wistar rats.

Effect of Sleep Restriction on Cardiometabolic and Haemoinflammatory parameters in Adult Male Wistar Rats

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While insufficient sleep remains an under-recognized public health issue across the globe, there have been paucity and heterogeneity of data regarding its cardiometabolic and haemoinflammatory implications. We therefore aimed at evaluating the impact of chronic sleep restriction on cardiometabolic and haemoinflammatory parameters in male Wistar rats. Sixteen (16) male Wistar rats (aged 8-10 weeks) were randomly assigned into control or sleep restriction (SR) groups (n=8). Gentle handling was used to induce SR for six weeks. Fasting weight and blood sugar were obtained and lipids were analyzed using their respective Randox kits. Malondialdehyde levels, catalase and superoxide dismutase activities were assayed. Full blood counts and CD 4^+ T-cells counts were determined using automated analyzer. Data were analyzed using SPSS $V_{20.0}$ and compared using Student's t-test, with level of significance set at $p \leq 0.05$. Our findings have demonstrated that chronic sleep restriction cause significant initial weight loss, increase in feeds consumption and percentage increase in FBS (32% vs 15%). We also noted the TriG index of SR rats to be significantly higher (6.22) than that of controls (5.62). In addition, a significant reduction in monocytic counts, MLR and absolute CD4+ cell counts among the sleep restricted rats was observed. Our findings have provided objective evidence that, over the course of 6 weeks, 5 hours of sleep restriction had caused body weight gain, hyperglycaemia, insulin resistance, and impairment in immunoinflammatory status, hence, could be a risk factor for developing cardiometabolic syndrome and immune related disorders.

Hepatotoxicity and Hematological Assessment in Adult Male Wistar Rats Following the Administration of Indomie Instant Noodle Seasoning

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The liver is a site where waste products of metabolism are detoxified through processes such as amino acid deamination. Measurement of liver enzymes remains the most practical tool to diagnose liver damage. Therefore, this study was designed to evaluate the effects of indomie instant noodle seasoning (INS) on serum levels of liver enzymes and some haematological parameters in wistar rats. Fifteen (15) male Wistar rats were used for the study. The animals were ramdomly divided into three groups of five animals each (n=5) and treated orally for eight weeks: Group 1 (control) was given distilled water (1 ml/kg). Group 2 was given INS (3083 mg/kg) while group 3 received INS (6170 mg/kg). At the end of the experiment, animals were euthanized and sera from blood samples were obtained and used for biochemical analysis. Serum analine aminotransferase (ALT) was significantly higher ($p<0.05$) in group 3 compared to control. There was significant increase ($p<0.05$) in serum alkaline phosphatase level in group 2 and 3 compared to control. Serum aspartate aminotransferase was also significantly higher ($p<0.05$) in group 2 and 3 compared to control. Red blood cells (RBC) and platelets counts were significantly higher ($P<0.05$) in group 3 compared to control. In conclusion, INS increased serum liver enzymes alongside RBC, WBC and platelets counts in adult male wistar rats.

Assesment of Effect of Repeated Cold Exposure on Some Cardio-Respiratory Parameters and Two Points Threshold among Frozen Fish Workers at Kofar Nasarawa Kano Municipal.

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People who live and work in cold areas have a higher incidence of respiratory disease, hypertension and related cardiovascular diseases. People working in frozen fish processing and storage centers as in Kofar Nassarawa Kano, have repeated and prolong exposure to cold. However, there is paucity of researches that assess physiological parameters among these group of workers especially in Kano. This is a cross sectional that assessed some cardiorespiratory parameters and two point threshold among frozen fish

workers at Kofar Nassarawa, Kano State compared with controls. Systolic and diastolic blood pressure were assessed using sphygmomanometer and stethoscope by auscultatory method, Peak expiratory flow Rate (PEFR) using Peak flow meter, Oxygen Saturation and Pulse rate using fingertip Oximeter, and two points threshold using weber compass and meter rule. The result indicated that the frozen fish workers have significantly lower (P = 0.01) PEFR (359.04 ± 55.14) and SPO_2 (88.00 ± 18) compared to Controls with PEFR and SPO2 values of (409.63 ± 53.27) and (97.00 ± 9.00) respectively. They were also found to have significantly higher (P = 0.01) diastolic blood pressure (75.00 ± 27) and two point threshold (20.00 ± 25.00) compared to control group with DBP of (70.01 ± 30) and two point threshold of (10.00 ± 9.01). A significant (P = 0.03) negative correlation (r = -0.31) between hours of exposure and PEFR was also established. In conclusion, Exposure to cold among frozen fish workers may cause a decrease in PEFR, SPO2, increase in DBP and altered tactile discrimination.

Two Weeks Fasting Improved Anthropometric and Serum Lipid Parameters in Obese Women in Kano, Nigeria

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Obesity is a leading risk factor for many cardiovascular diseases. Fasting is believed to improve indices of obesity; however, reports on effects of fasting on indices of obesity and serum lipid have been inconsistent. The aim of this study was to determine the effects of two weeks fasting on anthropometric and lipid parameters in obese women in Kano, Nigeria. Twenty participants consisting of ten each of obese and normal weight women were recruited for this cross sectional analytical study. All participants were assessed for anthropometric and lipid parameters at the end of the first two weeks of 2021 Ramadan fasting and same were repeated four weeks thereafter. Participants abstained from all forms of food and water from 5:15am to 6:15pm daily during the fasting period. All measurements were done using standard protocols. Data were analyzed on SPSS version 23.0, student independent t test was used to determine mean values of quantitative variables between obese and non-obese participants while paired t test was used for before and after fasting comparison, and p value ≤ 0.05 was considered significant. Obese participants were significantly older than the normal weights (p = 0.048). Obese participants had significant improvement in weight (p = 0.001), body mass index (p = 0.001), waist circumference (p = 0.005), hip circumference (p = 0.001), systolic blood pressure (p = 0.001), diastolic blood pressure (p = 0.001), mean arterial pressure (p = 0.001), and triglycerides (p = 0.02) after two weeks fasting period. Similar changes were observed among normal weight participants with regards to weight (p = 0.001), body mass index (p = 0.001), waist circumference (p = 0.004), triglycerides (p = 0.030), and total cholesterol (p = 0.001). In conclusion, two weeks fasting improved anthropometric and some lipid parameters in obese and normal weight women. Modified fasting regimen can therefore be used as a non-pharmacological treatment of obesity.

Hematological Profile of Obese Male Wistar Rats Fed With Medium Chain Triglyceride- Ketogenic Diet

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Obesity which is characterized by elevated body mass index has been positively correlated with haematological indices. Medium chain triglyceride-ketogenic diet (MCT-KD) is a ketogenic diet containing medium chain fatty acid with numerous potentials. The aim of the study was to evaluate the level of haematological indices in obese Wistar rats fed with MCT-KD. Twenty male Wistar rats were divided into four groups of five animals each. Group I animals were fed with normal diet feed (NDF; fat 17%, protein 26%, Carbs 57%), Group II were fed with MCT-KD only (fats 71%, protein 18%, Carbs 11%) Group III were fed with high fat diet only (HFD; fat 54%, protein 19% Carbs 27%) while Group IV were fed with both HFD and MCT-KD on alternate days for 3 weeks. The results showed a significant increase ($p \leq 0.05$) in total white blood cell (WBC) count in MCT-KD and HFD+MCT-KD groups when compared with NDF group. The total WBC count in HFD only group was reduced significantly ($p \leq 0.05$) when compared with MCT-KD+HFD group. However, differential WBC count did not show significant difference ($p > 0.05$) in all the groups. There was also no significant difference ($p > 0.05$) in RBC count, platelet count, PCV and haemoglobin concentration in all the groups. It can be concluded from this study that MCT-KD causes total leucocytosis in obese male Wistar rats.

Body Weight and Blood Glucose Dysregulation in Wistar Rats Fed With High Fat Diet And Medium Chain Triglyceride- Ketogenic Diet

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Medium chain triglyceride-ketogenic diet (MCT-KD) is a diet of high fat and low-carbohydrate, formulated with medium chain triglyceride as the major fat component, which posses' plethora of therapeutic effects. This study was design to evaluate the protective effect of MCT-KD against obesity-induced body weight and blood glucose dysregulation in Wistar rats. Twenty male Wistar rats were divided into four groups of five animals each. Group I animals were fed with normal diet feed (NDF; fat 17%, protein 26%, Carbs 57%), Group II were fed with MCT-KD only (fats 71%, protein 18%, Carbs 11%) Group III were fed with high fat diet only (HFD; fat 54%, protein 19% Carbs 27%) while Group IV were fed with both HFD and MCT-KD on alternate days for 3 weeks. The results showed a significant increase ($p \leq 0.05$) in body weight in the groups fed with MCT-KD only and HFD only when compared to the animals fed with NDF. However, the animals fed with

both MCT-KD and HFD on alternate days did not show any change in body weight ($p > 0.05$). There was a significant increase ($p \leq 0.05$) in blood glucose level in MCT-KD only group compared to other groups. MCT-KD and HFD fed animals however did not show any change ($p > 0.05$ in blood glucose level when compared to NDF and HFD group. It can be concluded from this study that MCT-KD prevents HFD-induced increase in body weight and blood glucose, but it increases them by itself when given alone.

Association of Micro-albuminuria with Retinopathy and Glycaeted Haemoglobin among Type 2 Diabetes Patients attending a Hospital in Bauchi, Nigeria

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Type 2 diabetes is associated with microvascular complications, which can be detected early using specific biomarkers such as micro-albuminuria to detect early stages of renal impairment. Little research was done in this environment to evaluate micro-albuminuria among type 2 diabetes patients. This study aimed to determine the prevalence of micro-albuminuria among patients with type 2 diabetes and evaluate its relationship with some selected diabetic complications. A total of 643 male and female subjects were recruited into this cross-sectional study, comprising of 314 diabetics and 329 non-diabetics matched for age and sex as controls. Socio-demographic and clinical information was obtained during an interview. Microalbuminuria and glycaeted haemoglobin were determined using Micro-albustistics and Quo lab HbA1c analyser, respectively. Data were expressed as mean ± S.E.M. or proportion, compared using t- test or Mann-Whitney u test, using IBM SPSS Statistics version 20.0. Association was determined using Chi-square; *P* values < 0.05 were considered significant. Micro-albuminuria was detected among 30.9% and 17.6% of diabetics and controls, while elevated glycaeted haemoglobin (> 6.5%) was seen in 68.5%, and 4.9% of the diabetics and controls, respectively. Prevalence of retinopathy, visual disturbance and decreased urine volume was significantly higher among the diabetics compared to the controls. Among the diabetics, micro-albuminuria was associated with abnormal glycaeted haemoglobin, retinopathy and decreased urine volume. Prevalence of microalbuminuria is high (30.9 %) among subjects with type 2 diabetes. Microalbuminuria, a consequence of renal microvasculopathy, occurs concurrently with other microvascular complications elsewhere in the body.

Dromedary Camel DNA and its Downstream Applications as Affected by Anticoagulants and Short-term Storage

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Precision in molecular research and diagnosis relies heavily on the quality, quantity, purity, reproducibility, and scalability of DNA extraction across the world. In the current study, blood samples were collected from 10 male dromedary camels brought to the Kano Main Abattoir during the early hours of the sampling day. They were collected in containers with EDTA, heparin, fluoride, citrate or no anticoagulant. Genomic DNA was extracted from the samples and assessed for quality and quantity using UV-V is spectrophotometer and agarose gel electrophoresis. PCR and RFLP were conducted. Trials were conducted on sampling day (fresh samples) and three days after storage at ambient, refrigerated and frozen conditions. The highest DNA yield was observed in the frozen samples across all the anticoagulants and lowest in those under ambient condition except for citrate which had the lowest yield in the refrigerated samples. In terms of purity, citrate-treated samples had the highest DNA purity under refrigeration (A_{260}/A_{280} =1.99) followed by heparin in fresh samples (A_{260}/A_{280} =2.0), and frozen fluoride- and refrigerated EDTA-treated samples (A_{260}/A_{280} =2.16 each). DNA and amplicons were visible on agarose gel electrophoresis image across anticoagulant type on Day 1 and only visible for EDTA- and citrate-treated samples after 3 days. Restriction digestion was successful only for EDTA and citrate treatments. Citrate was considered the best anticoagulant for PCR and RFLP in frozen samples. Therefore, the present study revealed, for the first time in dromedary camel in Nigeria, the effects of anticoagulants and storage on downstream applications.

Exogenous Nicotinamide Administration Modulates some Neurobehavioural Outcomes in Animal Model of Diabetic Neuropathy.

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Nicotinamide, the water-soluble amide form of vitamin B3, is a key component of the metabolic pathway involved in the production of Nicotinamide adenine dinucleotide (NAD+). Interest of the scientific community on nicotinamide has grown over time due to its suspected effect in preventing the progress of diabetic neuropathy. Based on the presented antidiabetic effect of nicotinamide, this study was designed to test the hypothesis that exogenous nicotinamide administration does not modulate neurobehavioural outcomes in animal model of neuropathic

pain. Thirty six (36) apparently healthy male Wistar rats weighing 100-150g were divided into six experimental groups (n = 6). Groups IV, V and VI were administered oral nicotinamide (250mg/Kg, 500mg/Kg and 1000mg/Kg respectively), while the other groups served as controls. Neuropathy was induced by single IP injection of 150mg/Kg of Alloxan and maintained for six weeks. Result of the study showed that there was no statistically significant effect on hot plate pain and mechanical pain, formalin induced inflammation and motor coordination, but the effects on blood glucose level, tail flick pain, depression, oxidative stress, lipid profile and serum electrolytes were statistically significant. In conclusion, the present study showed that nicotinamide may be useful in improving some of the neurobehavioural biochemical deficits induced by diabetic neuropathy.

Assessment of stress perception among students of Faculty of Basic Medical Sciences, Bayero University, Kano, Nigeria

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In pursuit of education, students are vulnerable to problems associated with personal, social and academic spheres, and these problems predispose them to academic stress. Self-reported academic stress has been reported to be influenced by sex of the students among other characteristics. However, studies of this kind are scarce in this environment. This study, therefore, investigated stress perception among students of Basic Medical Sciences, Bayero University, Kano, and determined its gender difference. Perceived stress was determined using the perceived stress scale (PSS) questionnaire; socio-demographic and anthropometric data was also collected. Using IBM, SPSS, statistics version 20.0, categorical variables were summarized using frequencies and percentages while numerical data was expressed as mean standard deviation. Gender difference in stress perception was compared using students t-test, were $P < 0.05$ was considered significant. A total of 383 students participated in the study, among which 41.0% were males and 59.0% were females; with 72.3% of them aged between 20 – 25 years. Majority of the students (89.8%) were single, and lived in urban areas (72.1%). About 49.9%, 23.0% and 37.2% were in their second, third and fourth years of study; and 67.9% had normal BMI. Most of the students (71.8%) had moderate perceived stress, while the others had low (25.1%) or high (3.1%). There was no difference ($P = 0.37$) in PSS scores of males (16.70 ± 5.36) and females (17.21 ± 5.70) students. It was concluded that majority of the students experience moderate stress in similar measure among males and females.

Modulatory Role of N-Acetyl-Cysteine on Gastric Mucosal Lesion and Haemato-Biochemical Changes in Albino Wistar Rats Subjected to Indomethacin Treatment

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Gastric ulcer is caused by multifaceted etiological factors such as environmental and indiscriminate use of non-steroidal anti-inflammatory drugs (NSAIDs) such as Indomethacin. N-acetyl-cysteine (NAC) is an antioxidant that protects the lipid bio-membrane against oxidative stress. This study investigated the Effect of NAC on Gastric Mucosal Lesion and Haemato-Biochemical Changes in Albino Wistar Rats Subjected to indomethacin treatment. Twenty (20) adult male rats, were divided into five (5) groups; Group I (Control): Received distilled water/kg/Bdw, Group II: Indomethacin 40 mg/kg in 0.5 % carboxymethylcellulose (Ulcer group), Group III: Received 2.5 ml/kg of 0.5% CMC, Group IV: Received NAC 500 mg/kg/Bdw orally + Indomethacin (500 mg/kg), Group V: Received Ranitidine 50 mg/kg/Bdw + Indomethacin (40 mg/kg). All treatment lasted for 7 days. Three hours after last treatment, rats were humanely sacrificed. The stomach and blood samples were collected for physical and biochemical analysis. Data was analysed using ANOVA and $p < 0.05$ was considered significant. The P index of NAC in indomethacin induced ulcer is found to be 75 %. A significant increase ($p < 0.05$) in final body weight was observed in Indomethacin group, when compared to the control, CMC and Ranitidine + Indomethacin groups. A significant ($p < 0.05$) increase in INOS concentration was observed in all treatment group, when compared to the control. In conclusion, we surmise that acute administration of Indomethacin increased body weight of rats, which was decreased by CMC and Ranitidine treatments, while NAC treatment failed to improve haemato-biochemical changes in adult Wister rats.

Evaluation of Chronic Shisha Smoke Inhalation on Some Long-Term Memory Assessment in Adult Male Mice

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Shisha is a flavoured tobacco designed to be smoked in a water-pipe. There is paucity of information on the effects of *Shisha* smoke on the different forms of long-term memory. The aim of the study was to evaluate the effect of *Shisha* smoke inhalation on some long-term memory models in adult male mice. Twenty male mice were divided into 4 groups of five mice each. Group I (control): fresh air; group II: exposed to bonged *Shisha*; group III: exposed to unbonged *Shisha*; group IV: exposed to activated charcoal smoke only. Each group was exposed for thirty minutes daily for seven weeks. Long-term memory was assessed using elevated plus maze (EPM), novel object recognition test (NORT) and Barnes maze (BM). Bonged Shisha significant decrease (0.03) percentage preference when

compared to control in NORT. There was a significant (0.02) increase in number of entries to incorrect holes in the bonged *Shisha* group (7.80 ± 0.86) when compared with control (5.20 ± 0.86) in BM, and there was statistically significant (0.01) decrease in acetycholinesterase level activity in the bonged *Shisha* group (62.21 ± 1.22) when compared with control (79.37 ± 2.39). There was no statistically significant difference in anxiety related spatial memory in EPM when compared with the control. The outcomes of this study suggest that bonged *Shisha* smoke may be neurotoxic to the brain as a result of combined effect of various toxicants emanating from different *Shisha* smoke constituents used in the set-up that may lead to potential memory impairment.

Effects of acute and chronic citric acid ingestion on serum malodialdehyde (MDA), lipid profile and haematological parameters of adult male mice

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Citric acid (DanTsami) is a flavoring agent and preservative which is widely used to give sour taste to food and soft drinks. Over the years, numerous studies have been conducted into possible links between soft drink intake and medical problems, the results of which however remain highly contested. Eighteen (18) adult male Wister rats were randomly divided into three groups of six (6) animals each; Control group, acute treatment group (ATG) and chronic treatment group (CTG). ATG and CTG were administered 10mg/kg body weight of citric acid for one week and four weeks respectively. The data collected was analysed using SPSS Version 23. Our study revealed significant decrease in MDA, Total cholesterol, HDL and LDL in both ATG and CTG treatment groups compared with the control group. The result shows statistical significant decrease in MCH, and MCHC and significant increase in platelet count. Citric acid has both hypolipidemic and anti-oxidant effects on the body.

Oral Acute Toxicity Studies on Stem Bark Extract of *Bombax Costatum* on Wistar Albino Rats

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Bombax costatum is mostly found in grassland areas of West Africa. In Nigeria and other countries, it is usually consumed as food or for the treatment of different ailments such as epileptic seizures (Oyen, 2011) and wound healing (Oyen, 2011). Although *Bombax costatum* is frequently consumed as food or as traditional medicine, there is dearth of data on the toxicological effect of the plant and thus the need for this studies. The stem bark of *Bombax costatum* was collected from Jere LGA, Borno State and extracted using three different solvents; chloroform, ethanol and n-Hexane. The up-and-down procedure as explained by the Organization for Economic Co-operation and Development (OECD, 2008) guideline 425 was employed to determine the acute oral toxicity profile of *Bombax costatum* stem bark extracts. Twelve rats were divided into 4 groups (n=3). Group 1 was administered with distilled water, Group 2 5000mg/kg of chloroform extract, Group 3 5000mg/kg of ethanolic extract and Group 4 5000mg/kg of n-Hexane extract of *Bombax costatum* respectively. All administrations were done orally once and the rats were monitored for 14 days for any sign of toxicity and euthanized on day 15. Blood samples was collected and evaluated for biochemical and hematological parameters. No mortality was recorded, though mild weakness and lack of appetite was observed in the first hour of administration. No statistically significant differences was observed in the serum concentration of sodium, potassium, chloride, creatinine and hematological parameters among all the groups of rats when compared to the control. There is also no statistically significant difference in concentration of liver enzymes. However, a statistically significant decrease in the levels of bicarbonate and increases in the levels of urea was observed in chloroform extract treated group when compared to the control group of rats. There is also increase in urea concentration in n-Hexane extract treated rats compared with the control rats while the concentration of total bilirubin shows a statistically significant decrease in the chloroform and n-Hexane extract treated rats compared with the control. This study shows that chloroform, ethanol and n-Hexane extracts of *Bombax costatum* have high degree of safety and the LD_{50} is greater than 5000mg/kg body weight.

Effect of Methanol Extract of Goron Tula (*Azanza Garckeana*) Fruit Pulp on Some Neurobehavioural Assessment in Male Wistar Rats

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GORON TULA (*Azanza garckeana*) is an edible fruit used as a fertility enhancer contains phenol, alkaloid, saponin, ethylacetate, flavonoid, tannin. *A. garckeana* possesses high mineral content. The study evaluated the effect of methanol extract of *A. garckeana* fruit pulp on some neurobehavioural assessment in male Wistar rats. A total of twenty adult Wistar rats divided into four groups (n=5): Group I (Control); 10% tween 80 only; group II: *A. garckeana* 300 mg only; group III: *A. garckeana* 600 mg only; group IV: *A. garckeana* 1200 mg only. At the end 21th day (3 weeks), neurobehavioural assessment were performed. Data obtained were analyzed using Graphpad prism 5.03 for windows using ANOVA followed by tukey's *post-hoc* test. Values of $p < 0.05$ were considered statistically significant. *A. garckeana* (300 mg/kg) significantly ($p < 0.05$) improved motor endurance and significantly ($p < 0.05$) alleviated

anxiety-like behaviour in male Wistar rats when compared to control group. A significant ($p < 0.05$) improvement in learning acquisition was observed in *A. garckeana* (1200 mg/kg) when compared to control group. The finding in this present study concludes that *A. garckeana* improves motor endurance, learning acquisition and alleviates anxiety-like behaviour in adult male Wistar rats.

Antioxidant Effects of Fermented Ginger Rhizome in High Fat Diet Induced Type 2 Diabetes in Rabbits

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Type 2 diabetes is one of the most distressful metabolic disease in the world, its onset and advancement is strongly associated with oxidative stress among other clinical indicators. The aim of this work is to evaluate the antioxidant effects of fermented ginger supplementation in high fat diet induced type 2 diabetes in rabbits. Twenty (15) male rabbits (5 weeks of age) divided into four groups (n=5) were used; Group I (Normal control) was treated with standard animal feed (SAF). Group II and III comprises of diabetic animal model (DAM) groups treated as follows: Group II; treated with SAF only, Group III; treated with SAF + fermented ginger (12.5%) supplements. High fat diet (SAF = 69% + Cholesterol = 1% + Ground nut meal = 20% + ground nut oil = 10%) was fed to rabbits for eleven weeks to ascertain diabetic animal model (DAM), Thereafter experimental treatment protocol last for six weeks. At completion of the treatments, animals were sacrifice and serum was used for laboratory assessments of superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) concentrations, data obtained were statistically analyzed. The results revealed a significant increase in catalase activity and a decreased MDA concentration in the supplement treated group when compared to that of group II. This results indicate a suppressed lipidperoxidation activity and a triggered antioxidant status. This experiment strongly revealed antioxidant-like activity of the supplement, this could be attributed to secondary metabolites of certain phytochemicals such as phenolic, saponins, trypsin and flavonoids among other bioactive compounds found in the supplement as revealed in the results of its preliminary phytochemical screening. Further work to validate these effects could facilitate the use of the supplement as a composite in formulating diet for type 2 diabetic patients.