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Effects of Catechin, Quercetin and Taxifolin on Redox Parameters and Metabolites Linked with Renal Health in Rotenone-toxified Rats

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Summary: Nephrotoxicity, with the attendant risk of progression to kidney failure, is a growing problem in many parts of the world. Current orthodox treatment options for nephrotoxicity and kidney failure are limited and there is need for alternative or complementary approaches. This study aimed at evaluating the effect of three structurally related flavonoids, catechin, quercetin and taxifolin on renal redox and metabolite biochemical disturbances in rotenone intoxicated animals. Male Wistar rats were administered 1.5 mg/kg rotenone (s.c.) for ten days followed by post-treatment with catechin (5, 10 or 20 mg/kg), quercetin (5, 10, or 20 mg/kg) and taxifolin (0.25, 0.5 or 1.0 mg/kg) (s.c.), for 3 days. Renal redox indices and levels of renal-related metabolites (creatinine, urea and uric acid) were assessed after sacrifice of animals. Catechin, quercetin and taxifolin significantly attenuated rotenone-induced effects on oxidative stress markers and metabolites linked to renal health. Quercetin was clearly more effective than catechin. The activity demonstrated by taxifolin, despite being administered at the lowest doses, was compelling. The results highlight the potential of these phytochemicals in the management of renal dysfunction. The findings additionally suggest a correlation between the structure of the flavonoids and their activity but also indicate that additional structural considerations beyond conventionally acknowledged ones may be involved.

Keywords*:* Flavonoids, nephrotoxicity, oxidative stress, structure-activity relationship

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INTRODUCTION

The kidney performs several important functions including maintenance of homeostasis and regulation of the extracellular environment which involve detoxification and excretion of toxic metabolites and drugs (Kim and Moon, 2012). It is therefore a major target organ for exogenous toxicants. In nephrotoxicity, excretion is impaired owing to toxic effects of chemicals or drugs and this causes damage to the kidney (Kataria *et al*., 2015). The homeostatic function of the organ is impaired and serum levels of important metabolites and electrolytes are disturbed as the kidney becomes unable to rid the body of excess urine and wastes efficiently (Weber *et al*., 2017; Cao *et al*., 2018).

Rotenone is used as a broad-spectrum insecticide and pesticide. It occurs naturally in the seeds and stems of several plants (Gupta, 2012; Pamies *et al.*, 2018). Studies on the effect of rotenone toxicity in the pathogenesis of Parkinson's disease abound but information on its renotoxicity is scanty. Rotenone causes toxicity through inhibition of complex I of the respiratory chain and oxidative stress (Dorman, 2015; Neely *et al*., 2017)

The neurotoxic and nephrotoxic effects of exposure to chemicals in the environment, remains a topic of substantial current concern and interest. The National Institute for Occupational Safety and Health (NIOSH) reports that exposure to neurotoxic chemicals is one of the ten leading causes of work-related disease and injury and that over 25% of the chemicals for which the American Conference of Governmental Industrial Hygienists (ACGIH) has established threshold limit values (TLV) have demonstrated neurotoxicity and nephrotoxicity (Anetor *et al*., 2008; Arnold *et al*., 2016). To compound the problem, available therapies for the treatment and/or management of neurotoxicity and nephrotoxicity are merely symptomatic without addressing the root cause. In addition, they always cause further severe complications. There is therefore a need for viable alternatives. Medicinal plants and phytochemicals appear to be the most promising candidate over the years (Adil *et al*., 2016; Feriani *et al*., 2017).

Flavonoids are water-soluble, polyphenolic compounds found ubiquitously in plants, and are best

known for their multiple biological effects including antioxidant, anti-inflammatory, cardioprotective, anticancer, renoprotective, hepatoprotective as well as neuroprotective properties (Akinmoladun *et al.*, 2015; Kay *et al*., 2015). The biological efficacy of flavonoids has been linked to their structural properties and is related to the number of hydroxyl groups and additional groups on their flavane nucleus (Chen *et al*., 2018; Noshita *et al*., 2018). The structure-activity relationship analysis of phytochemicals can assist in optimizing their therapeutic potential and design of novel molecules with highly improved bioactivity. The aim of this study was therefore to evaluate the effect of the structurally-related flavonoids, catechin, quercetin and taxifolin (Figure 1) on renal redox and metabolite imbalances in rotenone-toxified rats with a view to delineating order of activity and any structure-activity relationships.

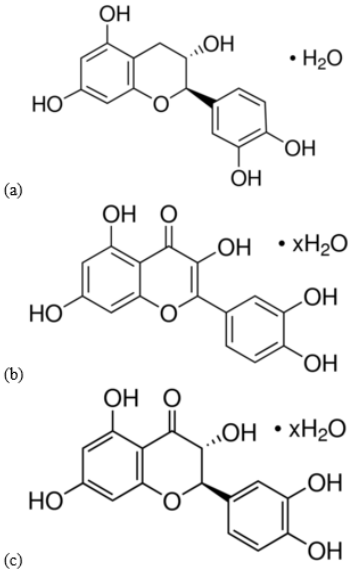

Figure 1: Chemical structures of (a) catechin, (b) quercetin and (c) taxifolin. (Akinmoladun et al., 2018).

MATERIALS AND METHODS

Chemicals and Reagents

Rotenone, (±)-catechin hydrate (trans-3,3′,4′,5,7-pentahydroxyflavane hydrate) ($C_{15}H_{14}O_6 \cdot xH_2O$), quercetin hydrate (3,3′,4′,5,6-pentahydroxyflavone hydrate) ($C_{15}H_{10}O_7 \cdot xH_2O$), (±)-taxifolin hydrate (3,3′,4′,5,7-pentahydroxyflavanone hydrate or dihydroquercetin hydrate) ($C_{15}H_{12}O_7 \cdot xH_2O$), 2,4-dinitrophenyl hydrazine (DNPH), xanthine, NAD^+, epinephrine, 2,4,5-tripyridyl-s- triazine (TPTZ), 2,4-dinitrophenyl hydrazine (DNPH), reduced nicotinamide-dinucleotide (NADH), 1-chloro-2, 4-dinitrobenzene (*CDNB*) and tetramethylbenzidine (TMB), were obtained from Sigma-Aldrich (St-Louis, MO, USA). Other chemicals and reagents used for this research were of analytical grade and obtained from standard sources.

Animal treatment and experimental groups

Male Wistar rats weighing 200±30 g housed at the Animal House of the Department of Biochemistry, The Federal University of Technology, Akure, Nigeria, were used for the study. They were fed standard rat chow and water *ad libitum*. The animals were divided into eleven groups with twelve animals per group. Animals were handled and used in accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

Rotenone, catechin, quercetin and taxifolin were dissolved in corn oil (vehicle) and administered subcutaneously to animals. Animals in group I (negative control) were administered vehicle (corn oil) only for 13 days. Group II (ROT) animals were administered 1.5 mg/kg rotenone (Thiffault *et al*., 2000) for 10 days followed by 3 days of administration of vehicle, and served as the positive control group. Animals in groups III ($ROT+CAT_5$), IV ($ROT+CAT_{10}$) and V ($ROT+CAT_{20}$) were administered 1.5 mg/kg rotenone for 10 days followed by 5, 10 and 20 mg/kg catechin (Vazquez Prieto *et al*., 2015; Tu *et al*., 2018), respectively, for 3 days. Animals in groups VI ($ROT+QUE_5$), VII ($ROT+QUE_{10}$) and VIII ($ROT+QUE_{20}$) were administered 1.5 mg/kg rotenone for 10 days followed by 5, 10 and 20 mg/kg quercetin (Nabavi *et al*., 2012; Vazquez Prieto *et al*., 2015), respectively, for 3 days while animals in groups IX ($ROT+TAX_{0.25}$), X ($ROT+TAX_{0.5}$) and XI ($ROT+TAX_{1.0}$) were administered 1.5 mg/kg rotenone for 10 days followed by 0.25, 0.5 and 1.0 mg/kg taxifolin (Arutyunyan *et al*., 2013; Wang *et al*., 2006), respectively, for 3 days. After the last treatment, animals were euthanized, the kidneys removed and processed for biochemical estimations. Smaller doses were used for taxifolin based on previous works and this appeared justifiable because of subsequent unpublished observations during investigations in our laboratory.

Biochemical Estimations

The kidneys of the sacrificed rats were excised, washed in ice cold 1.15% (v/v) potassium chloride solution, blotted with filter paper and weighed. They were then homogenized in phosphate buffered saline PBS (pH 7.4) (1:10 w/v) using a Teflon homogenizer. The resulting homogenate was centrifuged at 10,000 x g at 4ºC for 30 min to obtain the supernatant which was used for biochemical analyses. The amount of protein in samples was estimated according to Lowry *et al*. (1951). Extent of lipid peroxidation was evaluated by measuring the formation of thiobarbituric acid reactive substances (TBARS) (Varshney and Kale, 1990). Protein carbonyl (PC) content in the kidney was determined according to the method of Levine *et al.* (1990). The method of Beutler *et al.* (1963) was followed in estimating the level of reduced glutathione

(GSH). Glutathione transferase (GST) activity was evaluated as previously described (Habig *et al.*, 1974). The ferric reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain (1996). The activity of superoxide dismutase (SOD) was determined as previously described (Kakkar *et al*., 1984). Xanthine oxidase activity was measured using a previously described spectrophotometric method (Prajda and Weber, 1975). Myeloperoxidase (MPO) activity was evaluated as previously reported (Eiserich *et al*., 1998). Lactate dehydrogenase (LDH) activity was assayed as previously described (McKee *et al*., 1972). Creatinine level was estimated using an assay kit obtained from Agappe Diagnostics (Switzerland) based on Bowers and Wong (1980). Urea and uric acid levels were estimated using assay kits obtained from Randox Diagnostics (Switzerland) based on Jung *et al*. (1975) and Krieg *et al*. (1986), respectively.

Statistical Analysis

Results were analyzed using appropriate analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. In all the tests, $p<0.05$ was taken as criterion for statistical significance. The statistical software used to analyze the data was GraphPad Prism 6.01 (GraphPad Software Inc., CA, USA).

RESULTS

While it appears difficult to directly compare the effects of all three flavonoids because of the different doses of taxifolin employed for the study, results obtained still present valuable insights into their relative efficacy. A direct comparison of catechin and quercetin is straightforward and should be seen as the main focus while taxifolin could be considered a reference compound.

Redox homeostasis was altered by rotenone intoxication as reflected in changes to enzymic and non-enzymic antioxidants and other oxidative stress indicators. There was significant increase in lipid peroxidation (Figure 2I) and protein carbonyl level (Figure 2II) coupled with reduction in GSH level (Figure 2III) and ferric reducing antioxidant power (Figure 2IV), in rotenone-administered, positive

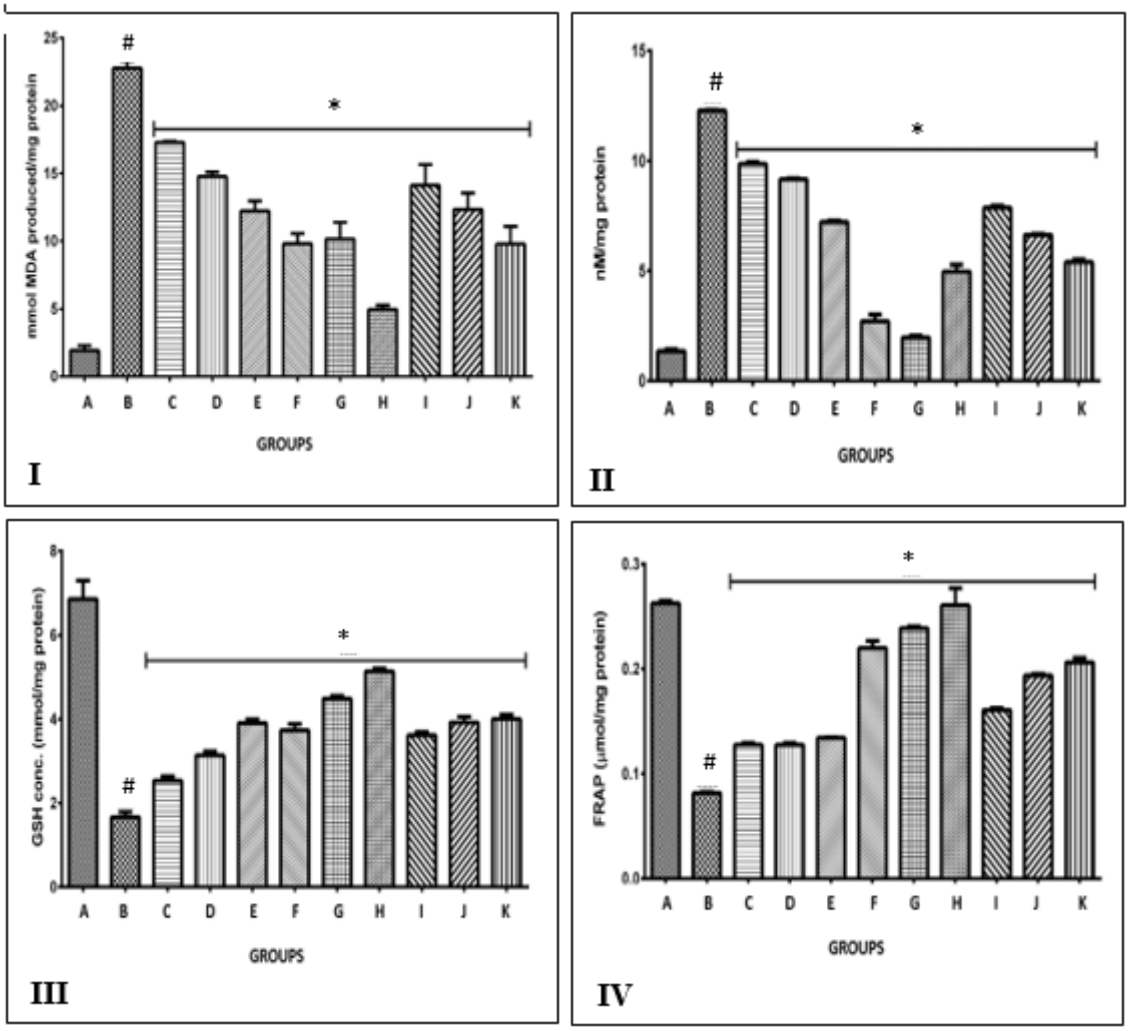

Figure 2: Effect of catechin, quercetin and taxifolin post treatment on (I) lipid peroxidation, (II) protein carbonyl content, (III) Reduced glutathione level and (IV) ferric reducing antioxidant power in kidneys of rotenone-intoxicated rats. Results are expressed as mean ± SD (n=12). $^{\#}p<0.0001$ vs control; $^{*}p<0.0001$ vs rotenone. . A= Control, B= ROT, C= ROT+CAT_5, D= ROT+CAT_{10} E= ROT+CAT_{20} F=ROT+QUE_5, G= ROT+QUE_{10}, H= ROT+$QUE_{20,}$ I= ROT+$TAX_{0.25}$, J= ROT+$TAX_{0.5}$, K= ROT+$TAX_{1.0}$. ROT: Rotenone; CAT: Catechin; QUE: Quercetin; TAX: Taxifolin

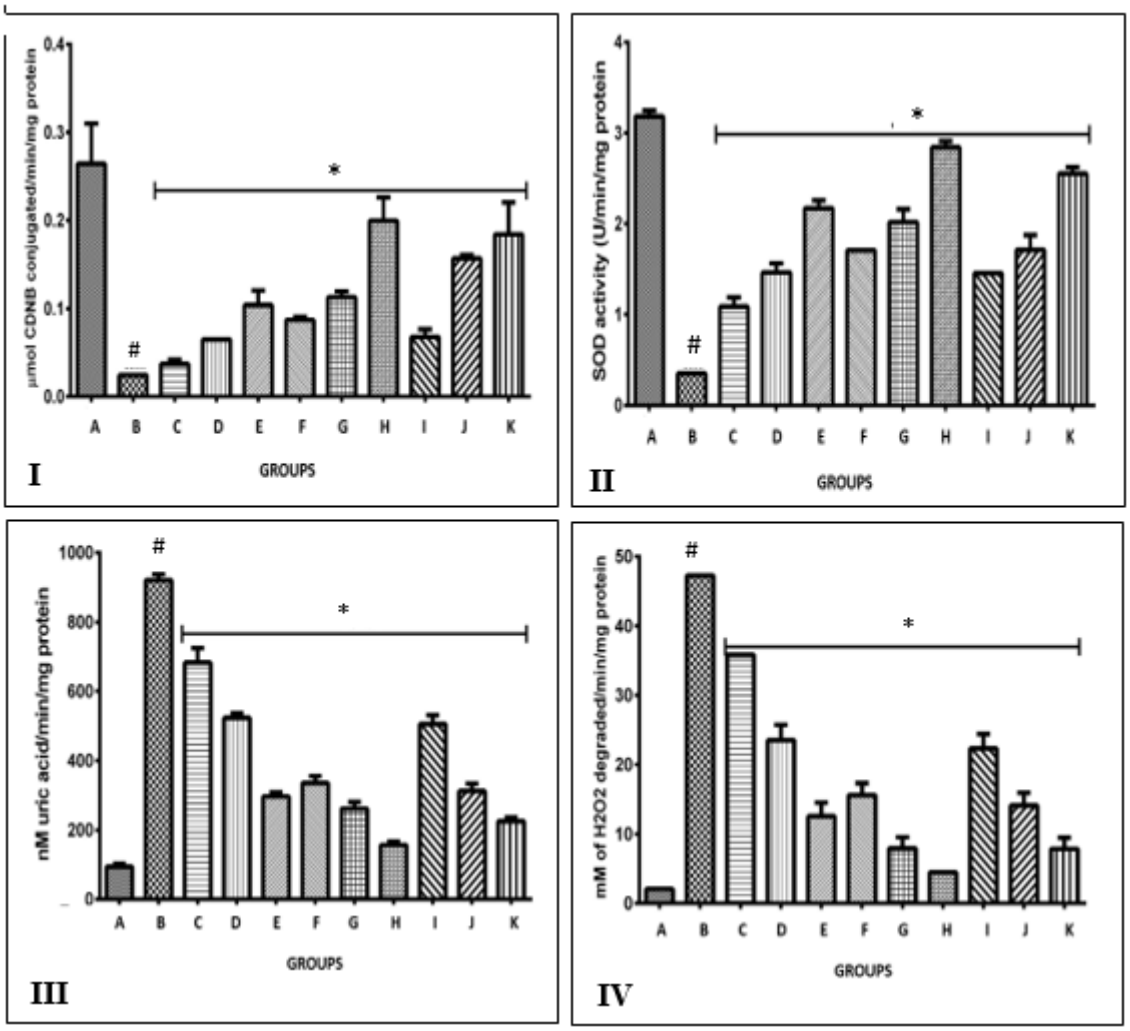

Figure 3: Effect of catechin, quercetin and taxifolin post treatment on renal glutathione transferase (I), superoxide dismutase (II), xanthine oxidase (III) and Myeloperoxidase (IV) activities in rotenone-intoxicated rats. Results are expressed as mean ± SD (n=12). #p<0.0001 vs control; *p<0.0001 vs rotenone. A= Control, B= ROT, C= ROT+CAT_5, D= ROT+CAT_{10} E= ROT+CAT_{20} F=ROT+QUE_5, G= ROT+QUE_{10}, H= ROT+QUE_{20}, I= ROT+$TAX_{0.25}$, J= ROT+$TAX_{0.5}$, K= ROT+$TAX_{1.0}$ ROT: Rotenone; CAT: Catechin; QUE: Quercetin; TAX: Taxifolin.

control group compared with the vehicle treated group (negative control). Rotenone-induced alterations to these parameters were significantly attenuated in the flavonoid treated groups in a dose dependent manner. Quercetin showed superior activity to catechin in tests evaluating extent of lipid peroxidation, protein carbonyl level, GSH level, and FRAP. At 20 mg/kg, quercetin showed the best protection in tests evaluating extent lipid peroxidation, GSH level, and FRAP. Quercetin at 10 mg/kg showed best activity in the test to determine protein carbonyl level with a tendency towards prooxidative effect at 20 mg/kg. Quercetin at 5 mg/kg showed better activity than 20 mg/kg catechin except in the test for GSH level where they appeared equipotent.Activities of the enzymic antioxidants, GST and SOD, were decreased by rotenone administration but restored by post-treatment with catechin, quercetin and taxifolin, dose-dependently (Figures 3I and 3II). In both assays, quercetin (20 mg/kg) displayed best activity and catechin (5 mg/kg), the least. The activity shown by quercetin (10 mg/kg) appear comparable with that shown by catechin (20 mg/kg). On the other hand, the activities of the prooxidant and pro-inflammatory enzymes, XO and MPO, were increased in rotenone-intoxicated control animals. This increase was corrected in animals post-treated with catechin, quercetin and taxifolin in a dose-dependent manner with quercetin demonstrating a clear superior activity to catechin (Figures 3III and 3IV). It could be observed that catechin, quercetin and taxifolin selectively regulated renal antioxidant and prooxidant factors to confer protection against rotenone induced redox imbalance. Antioxidant factors (GSH, GST and SOD) were augmented while prooxidant factors (lipid peroxides, XO and MPO) were suppressed by the flavonoids.

LDH activity (Figure 4I) was significantly increased due to rotenone administration but this was ameliorated by post-treatment with catechin, quercetin

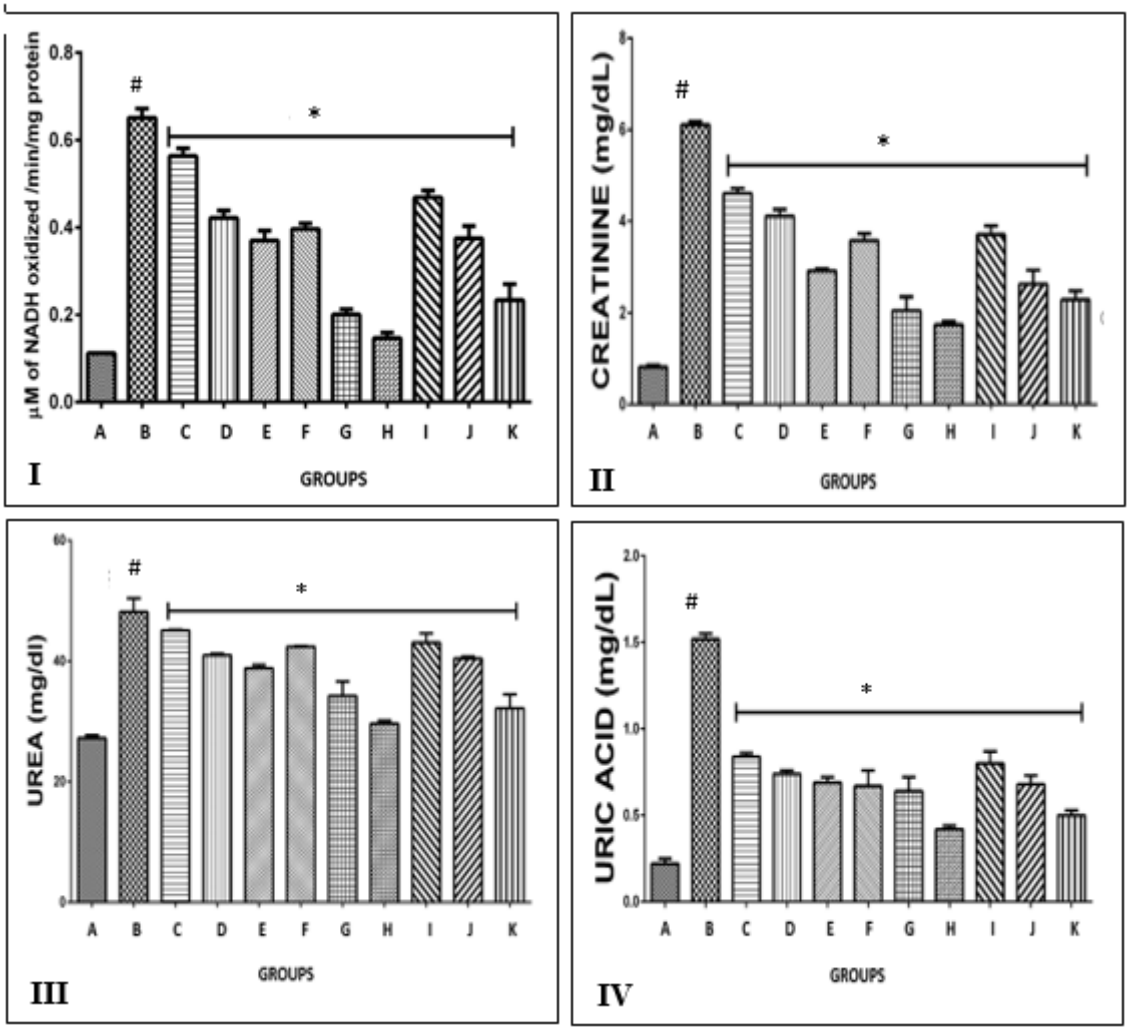

Figure 4: Effect of catechin, quercetin and taxifolin post treatment on lactate dehydrogenase activity in kidney (I), serum level of creatinine (II), serum Urea level (III) and Serum level of uric acid (IV) of rats subjected to rotenone intoxication. Results are expressed as mean ± SD (n=12). #p<0.0001 vs control; *p<0.0001 vs rotenone. A= Control, B= ROT, C= ROT+CAT_5, D= ROT+CAT_{10} E= ROT+CAT_{20} F=ROT+QUE_5, G= ROT+QUE_{10}, H= ROT+QUE_{20}, I= ROT+$TAX_{0.25}$, J= ROT+$TAX_{0.5}$, K= ROT+$TAX_{1.0}$ ROT: Rotenone; CAT: Catechin; QUE: Quercetin; TAX: Taxifolin.

and taxifolin. The performance of catechin and quercetin revealed the same trend of superior activity of quercetin with 5 mg/kg quercetin showing comparable activity to 20 mg/kg catechin. Renal functional imbalance was obvious in the positive control group administered rotenone without flavonoid post-treatment. Serum concentrations of creatinine, urea and uric acid which are principal metabolites that give insight on renal health, were significantly increased in rotenone-administered positive control group when compared with that of the negative control group. However, the rotenone-induced increase in the level of these metabolites was significantly corrected by post-treatment with catechin, quercetin and taxifolin in a dose-dependent fashion (Figures 4II, 4III and 4IV). Again, in all three tests, quercetin, especially at 20 mg/kg demonstrated the best activity compared to catechin.

Taxifolin, at the doses employed showed remarkable activity in this investigation. For example, in the lipid peroxidation test, the activity of 1 mg/kg taxifolin surpassed that of catechin even at the highest dose and was comparable to that of 5 and 10 mg/kg quercetin. In the test for protein carbonyl level, 1 mg/kg taxifolin showed superior activity to catechin at 20 mg/kg and in the FRAP test, 0.25 mg/kg taxifolin showed better activity than 20 mg/kg catechin. Also, taxifolin at 0.5 mg/kg proved superior to 10 mg/kg quercetin in the glutathione transferase assay while in the assay for myeloperoxidase activity, and determination of creatinine and urea levels, 1 mg/kg taxifolin appear equipotent with 10 mg/kg quercetin and superior in the uric acid test.

DISCUSSION

Rotenone, a potent mitochondrial toxin blocks mitochondrial complex I (NADH: ubiquinone oxidoreductase) activity causing accumulation of a large number of free radicals and ROS which leads to oxidative damage (Bonet-Ponce *et al*., 2016). Renal

tissues are vulnerable to injury by rotenone, probably because of their high metabolic state, active enzymes, and massive oxygen demand (Meng *et al*., 2016; Jiang *et al*., 2017). Flavonoids are best known for their antioxidant property and can prevent oxidant-induced injury in various ways (Taşlı *et al*., 2018).

The effects of rotenone on MDA level, protein carbonyl content, GSH level, FRAP score, and activities of GST, SOD, XO, MPO and LDH observed in this study, are consistent with the pathological features of nephrotoxicity (Feriani *et al*., 2017; Jiang *et al*., 2017; Taşlı *et al*., 2018). In rotenone renotoxicity, free radical overload resulting from mitochondrial dysfunction causes oxidative stress seen in a drastic reduction in the level of the non-enzymic antioxidant, GSH as well as decreased activities of the enzymic antioxidants, SOD and GST (Magaji *et al*., 2012). This is also accompanied by an increase in protein carbonyl content, a consequence of protein denaturation by the free radicals. Attenuating effect of catechin, quercetin and taxifolin on rotenone-induced redox disturbances indicates their potential to mitigate mitochondrial dysfunction and ensuing redox disturbances.

FRAP is an index of the antioxidant capacity of various biological samples (Wootton-Beard *et al*., 2011) while XO and MPO are prooxidant enzymes. Xanthine oxidase generates reactive oxygen species such as superoxide radical and hydrogen peroxide when it catalyzes the oxidation of hypoxanthine to xanthine. Therefore, increase in the activity of xanthine oxidase indicates further accumulation of free radicals (Romagnoli *et al*., 2010). MPO generates reactive oxygen species and produces hypochlorous acid from hydrogen peroxide and chloride anion during neutrophil respiratory burst (Degrossoli *et al*., 2018). MPO also oxidizes tyrosine to tyrosyl radical using hydrogen peroxide as an oxidizing agent (Dai *et al*., 2018; Degrossoli *et al*., 2018). Both hypochlorous acid and tyrosyl radical are cytotoxic and pathogenicidal but hypochlorous acid may also cause oxidative damage in host tissue if over-produced (Tian *et al*., 2017; Dai *et al*., 2018). The modulation of FRAP and the activities of XO and MPO by the flavonoids under consideration, further confirmed their redox-stabilizing property. In addition to being pro-oxidant enzymes, XO and MPO have been implicated in inflammatory processes (Zhao *et al*., 2017; Aldemir *et al*., 2018). Therefore, the effect of catechin, quercetin and taxifolin on the activities of XO and MPO may also reflect their anti-inflammatory potential (Magaji *et al*., 2012; Topal *et al*., 2016; Kalai-Selvi and Nagarajan, 2018).

Rotenone-induced increase in LDH activity was an indication of nephrotoxicity and renal injury (Hsiao *et al*., 2009; Piel *et al*., 2015). Impairment of the electron transport chain as a result of complex I inhibition (Birsoy et al., 2015) drastically reduces ATP synthesis. This leads to increased anaerobic respiration and accumulation of lactic acid because the physiological system switches to energy production through conversion of pyruvate to lactic acid, a reaction catalyzed by LDH (Piel *et al*., 2015). The studied flavonoids remarkably attenuated rotenone-induced alteration to LDH activity suggesting the protection of renal tissue from rotenone toxicity.

Rotenone toxicity elevated serum levels of creatinine, urea, and uric acid which are important metabolites associated with renal health (Sindhu *et al*., 2015; Amin *et al*., 2017). Creatinine, an anhydride of creatine, is formed by spontaneous and irreversible reaction during skeletal muscle metabolism. Serum creatinine is a kidney related variable that indicate renal toxicity or damage (Sindhu *et al*., 2015; Amin *et al*., 2017). Urea is formed by the liver and considered the main end product of protein catabolism in carnivorous and omnivorous species. Serum urea levels can be a reliable indication of renal function as a decrease in the rate of excretion of urea produces an increase in the concentration of serum urea, a key event in nephrotoxicity (Hassan *et al*., 2017). Uric acid is produced by the breakdown of purines and by synthesis from 5-phosphoribosyl pyrophosphate (5-PRPP) and glutamine. Uric acid is passed in the urine in humans but in other mammals, it is further oxidized to allantoin before excretion. Accumulation of uric acid as a result of poor excretion and elimination is an indication of nephrotoxicity (Sindhu *et al*., 2015; Amin *et al*., 2017). The increased serum levels of these kidney function markers may be related to oxidative stress and inflammation from rotenone toxicity (Pedraza-Chaverrí *et al*., 2003; Silan *et al*., 2007; Soliman *et al*., 2007; Sindhu *et al*., 2015) and the ameliorative action of the flavonoids indicates their free-radical scavenging, antioxidant and anti-inflammatory properties (Abdel-Raheem *et al*., 2009).

Many studies have examined rotenone toxicity on the central nervous system, especially in the pathogenesis of Parkinson's disease, but few have investigated the effects of rotenone on the kidney. The ability of rotenone to cause kidney injury was reported by Jiang *et al*. (2017) who carried out histopathological assessment along with other evaluations. Results from their study showed that rotenone caused dilation of the renal corpuscles and tubules, denuded epithelial lining and cytoplasmic blebs as well as congestion in the epithelial cells. The impairment in the renal cells could account for the significant increase in the serum concentrations of creatinine, urea and uric acid observed in our study following rotenone induction.

Quercetin apparently showed the best ameliorative activity in all tests when compared to catechin. Quercetin possesses the classical advantageous structural features adduced for strong bioactivity of flavonoids. These include C2-C3 double bond, 3-OH

group and 4-keto group on the C-ring, all of which are absent in catechin (Rice-Evans *et al*., 1996; Csepregi *et al*., 2016). Results of the present study agree with previous reports on the superior bioactivities of quercetin compared to catechin (Hayek *et al*., 1997; Jaiswal and Rizvi, 2014; Murakami *et al*., 2015). Quercetin has also been rated to be a more bioactive flavonoid than taxifolin although the main structural difference between the two is the absence of the C2-C3 double bond in the latter. Purported reasons for this have been advanced in previous reports (Makena *et al*., 2009; Weidmann, 2012; Osorio *et al*., 2013).

As earlier stated, although a direct comparison of the activity of quercetin and catechin seem implausible in the context of the present study, the overall activity demonstrated by taxifolin is compelling. This appear a bit intriguing since the C2-C3 double bond which is present in quercetin but lacking in taxifolin is a key advantageous structural feature for strong bioactivity in flavonoids (Trouillas *et al*., 2006). The remarkable activity shown by taxifolin at the low doses employed could point to the involvement of yet unclarified factors in the structure-activity relationships of flavonoids and warrants further investigation.

In conclusion, the results of this study demonstrated the positive modulation of redox environment and metabolites associated with renal health by catechin, quercetin and taxifolin in rotenone-toxified rats and pointed to the renoprotective effects of post-treatment with the flavonoids.

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Coconut Water Prevents Renal and Hepatic Changes in Offspring of Monosodium Glutamate-Treated Wistar Rat Dams

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Summary: Monosodium glutamate (MSG) is a widely-consumed taste enhancer which has been implicated in the aetiology of renal and hepatic dysfunction in adults and their offspring. There is increasing evidence on the therapeutic properties of Coconut Water (CW) in kidney and liver disorders. This study investigated the effects of CW on renal and hepatic functions in offspring of MSG-fed dams. Twelve female Wistar rats (120 – 140 g) were grouped into four as follows; Control (10 ml/Kg distilled water), MSG (0.08 mg/Kg), CW (10 ml/Kg) and MSG+CW. Treatments were given orally daily commencing two weeks prior to mating, throughout mating and gestation until parturition. All dams received standard rodent diet and drinking water *ad libitum* throughout the study. After weaning on Post-Natal Day (PND) 28, serum was obtained from offspring for assay of liver and renal function. Histological analysis of the livers and kidneys were performed on both dams and offspring. There was no significant difference in liver enzymes, urea, creatinine and albumin levels amongst the offspring on PND 28. However, liver and kidney sections from MSG dams and their offspring showed early degenerative changes which were not evident in renal and hepatic tissues from CW and MSG+CW dams and offspring. These observations suggest that coconut water protects against monosodium glutamate-induced renal and hepatic dysfunction in dams and offspring.

Keywords*:* Monosodium glutamate, *Cocos nucifera* water, Foetal programming, Kidney, Liver.

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INTRODUCTION

Monosodium glutamate (MSG), the sodium salt of glutamic acid, is a commonly used food additive. Though the arguments for and against the safety of MSG have remained inconclusive, but still ongoing (Walker and Lupien, 2000; Ataseven *et al*., 2016), MSG is generally considered safe for human consumption even during pregnancy and lactation (Walker and Lupien, 2000; Freeman, 2006; Beyreuther *et al*., 2007). This is quite confusing as there have been reports of sensitivity reactions to MSG (ie. the MSG symptom complex) and its consumption has also been associated with growth retardation, neuronal degeneration and endocrine dysfunctions in neonates (Olney, 1969; Bakke *et al*., 1978; Yang *et al*., 1997; Walker and Lupien, 2000).

Changing cultural and societal habits is always a tall order, hence discouraging the consumption of MSG during pregnancy, especially in the absence of conclusive facts, may not be achievable. To forestall any possible side effects to offspring, a more practical solution is to recommend an acceptable antidote to mothers. Coconut water was explored in this study. Coconut water is the liquid endosperm of the *Cocos nucifera* fruit. *Cocos nucifera* is a monospecie palm, commonly found in the tropics and its fruit's endosperm and its fruit's endosperm is a highly nutritious, non-toxic drink (Agyemang-Yeboah, 2011; Prades *et al.*, 2012a). The protective and therapeutic effects of coconut water have been reported in virtually every body system and in several disease states; including those affecting the renal and hepatic systems (DebMandal and Mandal, 2011; Yong *et al.*, 2009). In addition, Coconut water has been reported to prevent the foetal programming effects of harmful maternal diets on renal and hepatic functions of offspring (Lans, 2007; Kunle-Alabi *et al.*, 2015; 2016). Hence, the aim of this study was to investigate the effects of coconut water on the livers and kidneys of offspring from dams fed a nutritional daily dose of monosodium glutamate and their offspring.

MATERIALS AND METHODS

Experimental Animals

All procedures involving animals in this study conformed to the guiding principles for research involving animals as recommended by the guidelines for laboratory animal care of the National Institute of Health (NIH publication no. 85-23, revised 1996). Twelve female Wistar rats weighing 120 - 140 g obtained from the Central Animal House, College of Medicine, University of Ibadan and four proven-

breeder male Wistar rats weighing 140 - 150 g obtained from the Laboratory for Reproductive Physiology and Developmental Programming, Department of Physiology, College of Medicine, University of Ibadan were used for the study.

Monosodium Glutamate

Monosodium glutamate (MSG) of approximately 99% purity (Caraway Foods International Nigeria Limited) was used for this study. The MSG was dissolved in distilled water and administered at a dose of 0.08 mg/Kg body weight daily (Zia *et al.*, 2014).

Coconut Water

Coconut fruits were obtained from a local farm in Ibadan, Oyo state, Nigeria and authenticated at the Department of Botany, University of Ibadan, Ibadan, Nigeria. Fresh coconut water was obtained daily by drilling a hole through the germinal pore of each fruit. Coconut water was administered orally at a dose of 10 ml/Kg body weight daily which has previously been reported to protect offspring from the effects of adverse maternal diet (Kunle-Alabi *et al*., 2015).

Experimental protocol

The female rats were divided into four groups of three rats each and their respective treatments are listed as follows;

1. **Control** (10 mL/Kg distilled water)
2. **MSG** (0.08 mg/Kg monosodium glutamate)
3. **CW (**10 mL/Kg coconut water)
4. **MSG+CW** (0.08 mg/Kg monosodium glutamate and 10 mL/Kg coconut water).

Treatments were given daily via oral gavage starting two weeks before mating commenced, during mating and throughout the gestation period. All treatments were stopped at parturition.

Mating

The female animals were paired with the proven breeder males at the ratio 1:3 (male to female). Mating was confirmed by the observation of spermatozoa in vaginal smears after which the dams were separated into individual breeding cages.

Sample collection

Dams were allowed to litter naturally at which time (i.e. parturition) all treatments ceased. They were allowed to nurse their own respective offspring until Post-Natal Day (PND) 28 (Oštádalová and Babický, 2012; Lutsyk *et al.*, 2013). On PND 28, one male and one female offspring were randomly selected from each dam and were anesthetised along with the dam using sodium thiopentone (Tanimura *et al.*, 1967; Nasrolahi *et al.*, 2013). Blood was then collected from the offspring through cardiac puncture into plain bottles and centrifuged to obtain serum for analysis. The kidneys and livers of the dams and offspring were and fixed post-mortem and fixed in 10% formalin for histological analysis.

Kidney and Liver Function Tests

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, creatinine and albumin were determined using spectrophotometry kits according to the manufacturer's instructions (Randox Labs., UK).

Histological analysis

Tissues collected from the dams and offspring were dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax. Serial sections of five microns thickness were obtained using a rotatory microtome. The deparaffinised sections were stained routinely with Haematoxylin and Eosin (H&E) and viewed under a light microscope.

Statistical analysis

Data were presented as mean ± standard error of mean (mean ± SEM). They were analysed using ANOVA and Fisher's post-hoc test as appropriate on SPSS (version 20) software. Only p values less than 0.05 ($p<0.05$) were considered statistically significant.

RESULTS

Liver and kidney assays

Maternal intake of monosodium glutamate and/or coconut water before and during pregnancy did not cause any significant alteration in serum alkaline phosphatase, alanine aminotransferase, urea, albumin and creatinine levels (Table 1).

Liver histology of dams and offspring

Histological sections from the livers of dams in Control and CW groups showed normal architecture of the hepatocytes, sinusoids and interstitial tissue (Plate 1). The hepatocytes from liver sections of MSG dams showed signs of degeneration (Plate 1). Liver sections from the MSG offspring showed vacuolation within

Table 1: Effects of maternal monosodium glutamate and coconut water consumption on liver and kidney function assessments of offspring.

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Urea (mg/dL)	Creatinine (mg/dL)	Albumin (g/dL)
Control	44.2±1.5	31.5±1.3	110.8±3.5	14.7±0.4	0.7±0.1	3.1±0.3
MSG	45.2±1.9	32.5±0.4	116.7±3.6	15.5±0.4	0.7±0.1	3.2±0.1
CW	43.5±2.2	30.2±1.2	106.0±1.9	14.5±0.5	0.7±0.1	3.4±0.2
MSG+CW	46.7±1.4	33.2±0.6	112.3±4.1	15.8±0.5	0.7±0.1	3.4±0.3

MSG – monosodium glutamate, CW = Coconut water, AST – aspartate aminotransferase, ALT – alanine aminotransferase, ALP – alkaline phosphatase. Data represent mean ± SEM. n=6.

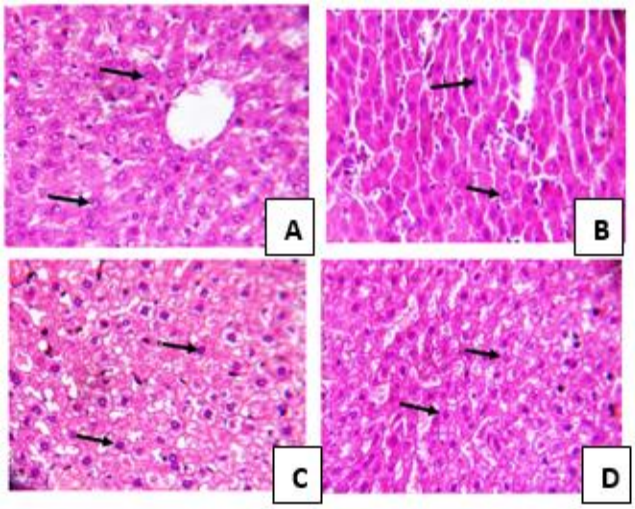

Plate 1: Photomicrographs of longitudinal section of livers from (A) control, (B) Monosodium, (C) Coconut Water and (D) Monosodium+ Coconut Water treated mothers. H&E. Magnification x 400. Hepatocytes (black arrows) appear normal.

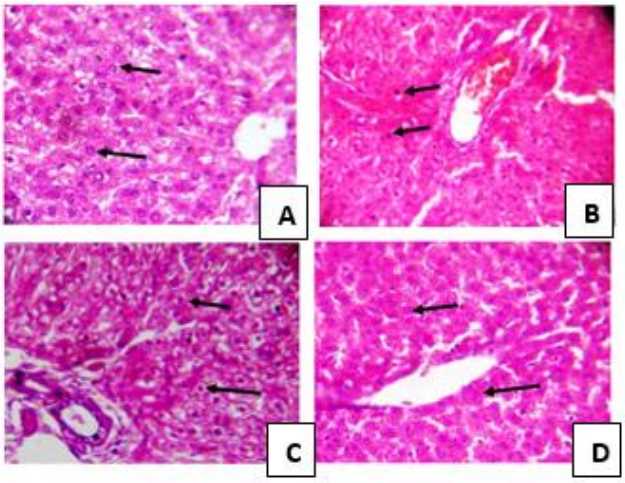

Plate 2: Photomicrographs of longitudinal section of livers from offspring of (A) control, (B) Monosodium, (C) Coconut Water and (D) Monosodium+ Coconut Water treated mothers. H&E. Magnification x 400. Hepatocytes (black arrows) show vacuolation in MSG group and appear normal in other groups.

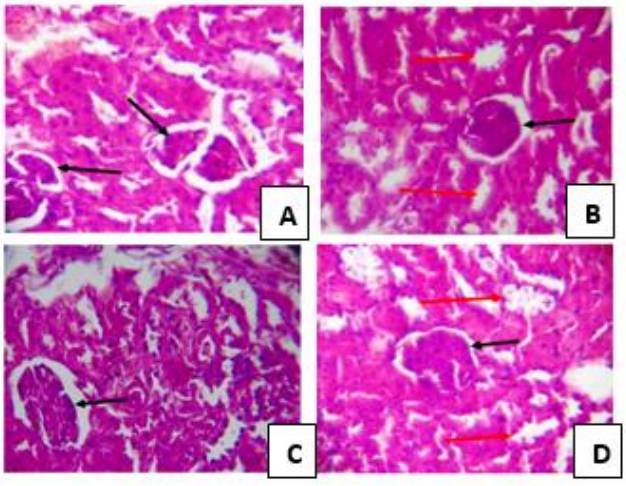

Plate 3: Photomicrographs of transverse section of Kidney from (A) control, (B) Monosodium, (C) Coconut Water and (D) Monosodium+ Coconut Water treated mothers. H&E. Magnification x 400. Glomeruli (black arrows) appear normal. Medullary interstitium shows vascular congestion (red arrows) in MSG and MSG+CW groups.

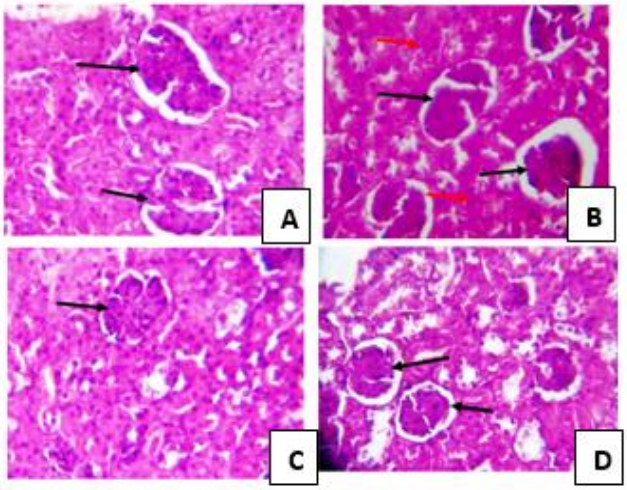

Plate 4: Photomicrographs of transverse section of Kidney from offspring of (A) control, (B) Monosodium, (C) Coconut Water and (D) Monosodium+ Coconut Water treated mothers. Glomeruli (black arrows) appear normal. Medullary interstitium shows disruption in parenchymal architecture (red arrows) in MSG group.

the cytoplasm of the hepatocytes (Plate 2). Kidneys from dams in all groups appeared normal (Plate 3). However, renal tissues from offspring of MSG dams (Plate 4) showed vascular congestion and parenchymal degeneration within the medulla. While the kidneys from offspring of dams in other groups appeared normal (Plate 4).

DISCUSSION

This study investigated the effects of maternal consumption of the popular food additive, monosodium glutamate (MSG), on renal and hepatic functions of dams and offspring, and the actions of coconut water on these effects. Monosodium glutamate is generally believed to be safe for consumption even during the perinatal period (Rezaei *et al*., 2013; Rogers, 2016). However, other reports have demonstrated the disruption of renal and hepatic functions by monosodium glutamate (Ortiz *et al*., 2006; Tawfik and Al-Badr, 2012), which suggest a need for plausible protective interventions. The concept of foetal programming of disease in postnatal life postulates that maternal dietary exposures permanently alter the manner in which various phenotypes are expressed (Langley-Evans and McMullen, 2010; Kamimae-Lanning *et al*., 2015). Hence, the need to explore the effects of maternal monosodium glutamate on renal and hepatic functions of offspring and dosed dam.

The results from the serum assays of markers of renal and hepatic functions [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, creatinine and albumin], suggest that maternal monosodium glutamate consumption does not cause any adverse effects on renal and hepatic functions of offspring. This corroborates reports that monosodium glutamate

is non-toxic and safe for consumption during pregnancy as it is not alleged to cross the placental barrier (Walker and Lupien, 2000; Beyreuther *et al*., 2007; Rezaei *et al*., 2013; Rogers, 2016). However, the histological presentations of the kidneys and livers in both the dams treated with monosodium glutamate and their offspring suggest otherwise.

The degenerative changes observed in hepatic and renal tissues of dams and offspring, in the absence of concomitant biochemical derangements, serve as early pointers of degenerative changes. Early detection of systemic disorders, especially those involving the liver and kidneys, is essential to improving prognosis and health outcomes. As an early response, hepatocytes may swell due to fluid influx secondary to insults which could lead to membrane disruption and subsequent release of liver enzymes into the circulation (Hayashi and Fontana, 2014). This is described as hydropic degeneration and is usually characterised by destruction of the cytoplasm due to leakage of hydrolytic enzymes from the lysosomes (Gerlyng *et al*., 1993). Vacuolation, which is swelling of the cytoplasm of the hepatocytes, might indicate acute and subacute liver injury (Gerlyng *et al*., 1993; Vásquez *et al.*, 2014). The kidneys play an indispensable role in homeostasis and their function begins almost as soon as they develop (Rosenblum, 2008; Sirin and Susztak, 2012). Thus, the earliest form of detection of systemic disorders is best observed at the cellular level (Korman *et al.*, 1974; Chen *et al*., 2011; Angulo *et al*., 2015). However, the impracticability of this may be one of the reasons for the high occurrence of renal and hepatic disorders worldwide. Global statistics show that the prevalence of renal diseases is 8-16% (excluding high-risk populations who have a prevalence >50%) (Jha *et al*., 2013), while that of hepatic diseases is 25% (Younossi *et al*., 2016). It is also important to note that the clinical reference ranges for serum levels of liver enzymes are relatively wide and dependent on the level of damage of hepatocytes. Thus, an appreciable degree of liver damage must have occurred for a significant change in the enzyme level can be detected. This suggests that apparently normal biochemical indices, as were observed in this study, are not a reliable depiction of correspondingly normal renal and hepatic functions. Consequently, the kidneys and livers of monosodium glutamate-fed dams and their offspring showed visible lesions which may not be detected using routine clinical tests until later in life, at which time irreversible damage may have occurred.

These results also underscore the fact that monosodium glutamate consumption is detrimental to renal and hepatic functions not only in adults, as has previously been reported (El-Meghawry El-Kenawy *et al.*, 2013; Sharma, 2015), but despite conflicting reports, also in their offspring. This consequently suggests that monosodium glutamate may traverse the placental barrier and subsequently alter renal and hepatic functions in offspring. Interestingly, Pitkin *et al.* (1979) had earlier suggested that monosodium glutamate crosses the placental barrier, and more recently, Park and Choi (2016) corroborated this finding. It has been suggested that monosodium glutamate is "metabolically compartmentalised" in the body (Brosnan *et al*., 2014). This explains, to some extent, its limited impact within the tissues without significant effects on circulating biochemical markers. The mechanisms whereby monosodium glutamate causes damage within kidney and liver tissues is still unclear, however, the induction of oxidative stress has been proposed (Tawfik and Al-Badr, 2012). Sharma (2015) reviewed this proposal and concluded that monosodium glutamate indeed up-regulates oxidative stress possibly via enzymatic activation of cellular processes (specifically; α-ketoglutarate dehydrogenase, glutamate receptors and cystine-glutamate antiporter).

The histological analysis also exhibited the reno-protective and hepato-protective effects of coconut water in monosodium glutamate-treated dams and their offspring. The protective properties of coconut water against disorders of the renal and hepatic system have been widely reported (Loki and Rajamohan, 2003; Bhagya *et al.*, 2010; Nwangwa *et al.*, 2012; Pinto *et al*., 2015). The ability of coconut water to prevent against maternal diet-induced foetal programming of renal function has also been reported (Kunle-Alabi *et al*., 2016). Coconut water has numerous properties which may enable it perform this function. These include; antioxidant, anti-inflammatory and growth modulatory actions (Ajeigbe *et al*., 2011; Prades, 2012b; Pinto *et al*., 2015). The mechanisms of foetal programming are highly dynamic and have therefore not been fully elucidated (Chmurzynska, 2010). However, studies have shown that dietary interventions can play a significant role in their prevention (Junien, 2006; Gueant *et al.*, 2013; Kunle-Alabi *et al.*, 2016; 2017).

In conclusion, maternal monosodium glutamate consumption adversely alters renal and hepatic architecture in mothers and offspring. This disruption of the kidney and liver begins early in the life of offspring, and may not be detected until irreversible functional damage has occurred to these organs. Coconut water prevents the kidney and liver damage induced by maternal monosodium glutamate consumption in both mothers and offspring.

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Adiposity Measures in Metabolic Syndrome among Hausas in Kano, Northern Nigeria

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Summary: Ethnic variations exist in the relationship of adiposity indices with metabolic syndrome (MetS). There are however, limited studies on the usefulness of body adiposity index (BAI) and visceral adiposity index (VAI) among Hausas of Kano, Northern Nigeria. The aim of the study was to determine the relationship of measures of adiposity to the components of MetS in Hausas of Kano. The study included 465 (266 males and 199 females) subjects, with mean age of 34.4 years and 32.0 years for males and females respectively. Anthropometric measures were obtained using standard protocols. Visceral adiposity was estimated using sex specific VAI. Fasting blood sample was obtained for serum analyses of lipid profiles, glucose, protein and uric acid. Pearson's correlation was used to test the association between adiposity measures with MetS indices while Student's t test was used for group comparison. The results of the study showed that the adiposity indices significantly correlate with metabolic syndrome indices. Visceral adiposity index was superior to other adiposity measures and Waist to hip ratio was the strongest anthropometric correlate of MetS components. In conclusion, WHR is the strongest anthropometric correlate of MetS components. Body adiposity index, NC and HC are weaker adiposity tools. Visceral adiposity index is superior to all other adiposity tools.

Keywords*:* Adiposity measures, metabolic syndrome, metabolic syndrome biomarkers, Hausas, Northern Nigeria.

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INTRODUCTION

Body adiposity is documented to be tightly linked with cardio-metabolic risk factors and metabolic syndrome (Akuyam et al., 2009) which are leading causes of death in both developed and developing countries (Mahmoud et al., 2010). The metabolic syndrome is a cluster of interrelated common clinical disorders, including hypertension, hyperglycemia, glucose intolerance and dyslipidaemia in addition to obesity (Moller and Kaufman, 2005). It is defined based on the presence of three or more of the following criteria: abdominal obesity with waist circumference >94cm for men or > 80cm for women (Grundy et al., 2005), triglycerides >150 mg/dl, high density lipoprotein cholesterol (HDL-cholesterol) < 40 mg/dl for men or <50 mg/dl for women (Bergman et al., 2006), blood pressure >130/85 mmHg (Tremblay et al., 2004) and fasting glucose >100 mg/dl (Grundy et al., 2005).

Rapidly rising prevalence levels of the MetS in developed and developing countries and the associated high mortality and morbidity are forcing scientists to review promising therapeutic agents and population specific anthropometric criteria for defining its phenotype (Matsuzawa, 2005). Robust evidences in the literature indicate that the various anatomic reserve of adipose tissues donot carry the same burden of metabolic risk (Bergman et al., 2011; Amato et al., 2014). It is also documented that that race and ethnicity affects both adiposity measures (Tulloch-Reid et al., 2003) and pattern of relationship with metabolic parameters (Duerenberg et al., 1998). There is currently an ongoing controversy on the particular adiposity measure which has the best discriminatory strength and confers the highest metabolic risk (Bergman et al., 2011; Mbanya et al., 2015). Conflicting results have been reported from different races and ethnic groups showing variation in the strength of correlation of the various adiposity measures with the different components of the MetS (Bergman et al., 2011; Mbanya et al., 2015). It is becoming a popular notion in recent time that adiposity and metabolic risk do not follow a universal trend and therefore MetS risk prediction must take into account ethnicity and population peculiarities. On this

note, many ethnic groups or populations such as South Asians, Chinese, and Aboriginals among others have identified adiposity measures that are germane to their population in terms of correlation and prediction of metabolic syndrome indices (Razak et al., 2007). Similarly, the validity of adiposity measures in various disease conditions and their population and ethnic variability have been reported among Nigerians (Charles-Davies et al., 2012)

Visceral adiposity index (VAI) is a recently derived index to measure visceral fat based on the knowledge of waist circumference (WC), plasma HDL, triglycerides and BMI (Amato and Giordano 2014). VAI has been adjusted for gender and is based on the formula proposed by Amato and Giordano (2014). The body adiposity index (BAI) is also a relatively new adiposity measure which was described and subsequently validated (Bergman et al., 2011). It estimates percentage of body adipose tissue in both sexes without numerical correction and has the advantage of not requiring a gender-specific calculation making this surrogate index very convenient for practical use. The usefulness of this adiposity measure has been tested in other populations and revealed varying degree of correlation with MetS components (Amato et al., 2010; Bergman et al., 2011; Amato and Giordano, 2014). Such studies are scarce in the Nigeria literature, we therefore seek to investigate the usefulness of BAI and VAI and compare with other measures of body adiposity in terms of their relationship with the different components of MetS, uric acid and adiponectin. The aim of this study is to identify the adiposity markers that are most germane to the Hausas of Kano in MetS.

MATERIALS AND METHODS

Study Location and participants

Systematic random sampling technique was employed in selecting 465 original Hausas of Kano based on a history of at least two parental generation being Hausas from Kano. Participants were recruited from outpatient units of Murtala Muhammad Specialist Hospital, Khadija Memorial Hospital, Shehu-Uran clinic, General Hospital Dawakin-Tofa and the old campus of Bayero University, Kano. The study included only subjects in the age range of 18 years to 68 years. Subjects with pregnancy, abdominal or pelvic space occupying lesions, congenital and / or acquired spinal deformity were however excluded. Subjects that were on medications that could interfere with any component of metabolic syndrome were also excluded. Ethical approval was obtained from Kano State Hospitals Management Board and written informed consent obtained from the subjects. The study was conducted commenced October, 2016 and ended September, 2017.

Anthropometry

Height was measured to the nearest 0.1cm as the vertical distance between the standing surface and the vertex of the head while the subject was standing erect in the frank forth plane and without shoes using a stadiometer. The weight was measured in kilograms using a digital weighing scale while the subject is in light clothes. The body mass index was be calculated by dividing the weight in kilograms by the square of the height in meters and the result expressed in kg/m^2. Waist circumference was measured in centimeter with a non- stretchable plastic tape horizontally placed over the unclothed abdomen at the narrowest point between the lowest rib and the iliac crest. Hip circumference: was measured while the subject is standing erect with the feet fairly close together; pockets emptied and the tape passed around the point with the maximum circumference over the bottom (Lean et al., 1995). WHR and WHtR were obtained by dividing waist circumference by hip circumference and height respectively. Neck circumference: was measured in centimeter with a non- stretchable plastic tape horizontally placed over the unclothed neck at the level of the thyroid cartilage (Lean et al., 1995).

Estimation of Visceral and Body Adiposity Index

Visceral adiposity was estimated using sex specific visceral adiposity index (Amato and Giordano, 2014):

$$VAI(Male) = \frac{WC}{39.68 + (1.88 \text{ X BMI})} \times \frac{TG}{1.03} \times \frac{1.31}{HDL}$$

$$VAI(Female) = \frac{WC}{36.58 + (1.89 \text{ X BMI})} \times \frac{TG}{0.81} \times \frac{1.31}{HDL}$$

Where WC is waist circumference, TG is triglyceride, HDL is high density lipoprotein and BMI is body mass index

Body adiposity index was obtained using the formula proposed by Bergman et al. (2011).

$$\text{Body Adiposity Index (BAI)} = \frac{\text{Hip Circumference (cm)}}{\text{Height (m)}^{1.5}} - 18$$

Measurement of Blood Pressure

A mercury sphygmomanometer was used for measuring blood pressure. Two measurements were taken, and at least 2 minutes was allowed between readings. While the diastolic reading was taken at the level when sounds disappear (Korotkoff phase V), the systolic was taken at the level when it appears (Prisant et al., 1995). The brachial artery was the site of auscultation. Subjects were asked to refrain from smoking or ingesting caffeine for 30 minutes before measurement and the Measurement was taken after at least 5 minutes of rest (Haffner et al., 2005).

Estimation of Serum Parameters

For the estimation of serum total cholesterol(TC), triglyceride(TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and fasting blood glucose (FBG), uric acid and adiponectin, blood specimen was collected from 161 of the subjects after 10 to 12 hours of fasting via

superficial veins of the upper limb. From each selected subject, 5ml of venous blood sample was collected using a sterile 21G needle fitted with syringe. Blood collection was done at the morning hours before 8 a.m to avoid the effect of diurnal variation or circadian rhythm in the blood parameters to be measured. Standard technique of venepuncture and universal safety precaution was employed. Blood sample was transferred into a plain blood specimen bottle and allowed to clot.

. Sample was then centrifuged at 300rpm for 5 minutes after which serum was separated and immediately used for analysis. Serum glucose was measured using the enzymatic method of Trinder (1969).

Serum TC, TG and HDL concentrations were measured using enzymatic method by Wybenga et al. (1970).

LDL-cholesterol was calculated from measured values of total cholesterol, triglycerides and HDL-cholesterol according to the Friedewald's equation. (Friedewald et al., 1972)

LDL-Cholesterol =TC - (HDL-C + Triglycerides/2.2) mmol/L.

Serum uric acid concentration was measured using Caraway method (1955).

Serum adiponectin concentration was determined using the Solid - Phase ELIZA method (Pischon et al., 2003).

Statistical Analysis

The data were expressed as mean ± standard deviation. Pearson's correlation was used to determine the relationship between each adiposity measure and the metabolic parameters. Student's t test was used to compare between-group parameters of males and females. SPSS version 20 (IBM Corporation, NY) software was used for statistical analyses and $P < 0.05$ was set as level of significance.

RESULTS

A total of 465 subjects were studied, 266 males (57%) and 199 females (43%). The subjects had a mean age of 34.45 years and 32.06 years for males and females respectively. Table 1 and 2 showed descriptive statistics of age, anthropometric indices of adiposity, blood pressure and serum biomarkers metabolic syndrome of participants.

Table 2 showed correlation of anthropometric and visceral adiposity markers with MetS components in the studied population, males and females respectively. Among the anthropometric indices of adiposity in the general population, BMI showed significant positive correlation with all the serum components of MetS (r = 0.31, 0.43, 0.39 0.42) for FBG, TC, TG and LDL respectively, except HDL with significant negative correlation (r = - 0.28). Its correlation with the biomarkers of MetS was positive for serum uric acid (r = 0.31) and negative for adiponectin (r = - 0.39). However, both were statistically significant. BMI correlated positively and significantly with both SBP (r = 0.42) and DBP (r = 0.46). The Pearson coefficient (r) showed that, among the serum parameters of MetS, BMI had the strongest correlation with TC (r = 0.43) and LDL-C (r = 0.42) and weakest correlation with HDL-C. Its strength of correlation was similar for both components of BP and for SUA and Adiponectin. BMI also correlated positively with VAI (r = 0.38).

Table 1: Descriptive statistic of age, anthropometric indices of adiposity and blood pressure of participants

	Male (n=266)			Female (n= 199)		
Variables	Mean	SD	Min-max	Mean	SD	Min-max
Age	34.45	13.52	18-68	32.06	15.18	18-65
Height(cm)	169.15	6.27	142-182.3	158.53	6.83	136.9-175
Weight (Kg)	63.03	12.28	40.5-98.3	55.86	12.99	36-108.9
BMI	21.98	3.93	14.52-34.33	22.19	4.7	12.96-39.15
WC (cm)	77.28	11.17	57-111	76.02	13	51-118.5
HC (cm)	87.01	7.8	72.1-109.9	88.96	9.86	65.6-136
NC (cm)	34.99	2.29	30-42	31.58	2.46	26.5-39.5
W/H	0.89	0.08	0.71-1.11	0.85	0.11	0.65-1.25
W/Ht	0.46	0.06	0.34-0.65	0.48	0.08	0.30-0.72
BAI	21.6	3.71	13.88-33.90	26.61	4.62	15.38-45.58
DBP	82.59	12.37	54-120	84.5	12.99	60-120
SBP	128.07	20.09	90-200	130.66	21.87	95-205

BMI: body mass index, WC: waist circumference, HC: hip circumference, NC: neck circumference, W/H: waist-to-hip ratio, W/Ht: waist-to-height ratio, BAI: body adiposity index, DBP: diastolic blood pressure, SBP: systolic blood pressure

Table 2: Descriptive statistic of serum biomarkers and indices of metabolic syndrome

	Male (n=120)			Female (n= 41)		
Variables	Mean	SD	Min-max	Mean	SD	Min-max
Uric Acid	5.51	1.95	3.1-11.3	6.03	2.42	2.9-10.10
Adiponectin	23.28	5.96	7.8-33.90	22.55	7.45	14.4-33.9
FBG	84.67	24.73	53.6-187.2	100.63	34.9	54.6-176.4
TC	174.35	32.31	123.7-256.1	187.32	43.85	127.3-290.7
HDL-C	44.1	6.32	28-54.1	47.83	6.71	38.9-60.6
TG	117.18	31.76	74.3-196.5	121.83	29.25	80.4-165
LDL–C	106.81	32.44	58.14-192.82	115.12	44.05	54.36-214.46

Table 3: Correlation of anthropometric and visceral adiposity markers with MetS components in the general population

	DBP	**SBP**	**SUA**	**Adiponectin**	**FBG**	**TC**	**HDL-C**	**TGR**	**LDL-C**	**VAI**
BMI	0.468**	0.427**	0.314**	-0.397**	0.318**	0.434**	-0.287**	.396**	0.420**	0.380**
WC (cm)	0.573**	0.578**	0.540**	-0.582**	0.502**	0.626**	-0.482**	.586**	0.615**	0.644**
HC (cm)	0.208**	0.114*	0.046	-0.114	0.061	0.166*	0.004	0.138	0.141	0.199*
NC (cm)	0.303**	0.270**	0.294**	-0.364**	0.177*	0.357**	-0.502**	.399**	0.381**	0.318**
W/H	0.638**	0.739**	0.841**	-0.834**	0.760**	0.837**	-0.791**	.805**	0.845**	0.834**
W/Ht	0.561**	0.609**	0.558**	-0.593**	0.553**	0.643**	-0.442**	.602**	0.622**	0.675**
BAI	0.142**	0.139**	0.097	-0.142	0.172*	0.205**	0.058	.178*	0.164*	0.261**
VAI	0.795**	0.866**	0.886**	-0.854**	0.860**	.0901**	-0.809**	.937**	0.891**	1

BMI: body mass index, WC: waist circumference, HC: hip circumference, NC: neck circumference, W/H: waist-to-hip ratio, W/Ht: waist-to-height ratio, BAI: body adiposity index, DBP: diastolic blood pressure, SBP: systolic blood pressure, FBG: fasting blood glucose, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, TG: triglyceride, LDL-C: low density lipoprotein cholesterol, VAI: visceral adiposity index * P <0.05, ** P <0.001

Table 4: Correlation of anthropometric and visceral adiposity markers with MetS components in male participants

	DBP	SBP	SUA	Adiponectin	FBG	T C	HDL -C	TG	LDL -C	VAI
BMI	0.331**	0.356**	0.289**	-0.300**	0.247**	0.372**	-0.332**	0.379**	0.361**	0.339**
WC (cm)	0.543**	0.611**	0.552**	-0.573**	0.522**	0.590**	-0.600**	0.600**	0.587**	0.635**
HC (cm)	0.229**	0.225**	0.069	-0.129	0.071	0.133	-0.142	0.173	0.127	0.176
NC (cm)	0.402**	0.396**	0.446**	-0.474**	0.443**	0.503**	-0.489**	0.505**	0.497**	0.512**
W/H	0.657**	0.777**	0.830**	-0.802**	0.774**	0.816**	-0.823**	0.789**	0.819**	0.844**
W/HtR	0.486**	0.595**	0.573**	-0.591**	0.524**	0.615**	-0.616**	0.631**	0.609**	0.648**
BAI	0.061	0.138*	0.107	-0.158	0.068	0.181*	-0.167	0.230*	0.168	0.193*
VAI	0.777**	0.877**	0.905**	-0.870**	0.910**	0.908**	-0.917**	0.944**	0.898**	1

BMI: body mass index, WC: waist circumference, HC: hip circumference, NC: neck circumference, W/H: waist-to-hip ratio, W/Ht: waist-to-height ratio, BAI: body adiposity index, DBP: diastolic blood pressure, SBP: systolic blood pressure, FBG: fasting blood glucose, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, TG: triglyceride, LDL-C: low density lipoprotein cholesterol, VAI: visceral adiposity index * P <0.05, ** P <0.001

Compared to BMI, WC showed a stronger but similar pattern of correlation with all MetS Components. It had a strong positive and significant correlation with all the serum components of MetS. (r = 0.5, 0.62, 0.58, 0.61) for FBG, TC, TG and LDL respectively, but HDL showed a negative and significant correlation (r = - 0.48). Its correlation with the biomarkers of MetS was positive for serum uric acid (r = 0.54) and negative for adiponectin (r = - 0.58). WC correlated positively and significantly with both SBP (r = 0.57) and DBP (r = 0.57). For the serum parameters of MetS, WC also had the strongest correlation with TC while the weakest correlation was with HDL-C. Pearson's correlation coefficient of WC with BP was similar for SBP and DBP and was also similar for SUA and adiponectin. WC also had a positive correlation with VAI (r = 0.64).

BAI, HC and NC relatively showed weaker correlation with MetS components. The weakest was HC which showed very weak correlations with DBP (r = 0.20), SBP (r = 0.14) and TC (r = 0.16). No significant correlation was observed between HC and SUA, adiponectin, FBG, HDL, TG, and LDL. The correlation of HC with VAI was also weak (r = 0.19). BAI had no significant correlation with serum biomarkers and HDL. Its correlation with FBG, TC, LDL and TG were relatively weak, with its highest coefficient of correlation observed for TC (r = 0.20). Its correlation with VAI (r = 0.26) was also weak when compared with BMI and WC. NC like HC and BAI showed a weak correlation with MetS parameters, but

Table 5: Correlation of anthropometric and visceral adiposity markers with MetS components in the female participants

Variables	DBP	SBP	SUA	Adiponectin	FBG	TC	HDL-C	TG	LDL-C	VAI
BMI	0.616**	0.501**	0.328*	-0.596**	0.359*	0.508**	-0.397*	0.431**	0.509**	0.387*
WC (cm)	0.621**	0.555**	0.490**	-0.612**	0.399**	0.662**	-0.472**	0.563**	0.656**	0.611**
HC (cm)	0.177*	-0.004	-0.082	-0.059	-0.138	0.121	0.082	0.015	0.106	0.072
NC (cm)	0.458**	0.355**	0.302	-0.394*	0.177	0.534**	-0.343*	0.473**	0.521**	0.519**
W/H	0.679**	0.758**	0.872**	-0.901**	0.792**	0.902**	-.825**	0.866**	0.908**	0.875**
W/Ht	0.636**	0.624**	0.551**	-0.705**	0.499**	0.702**	-.592**	0.637**	0.704**	0.668**
BAI	0.184**	0.114	-0.116	-0.108	-0.065	0.049	-0.033	0.022	0.052	0.036
VAI	0.824**	0.823**	0.868**	-0.872**	0.761**	0.909**	-0.930**	0.974**	0.917**	1

BMI: body mass index, WC: waist circumference, HC: hip circumference, NC: neck circumference, W/H: waist-to-hip ratio, W/Ht: waist-to-height ratio, BAI: body adiposity index, DBP: diastolic blood pressure, SBP: systolic blood pressure, FBG: fasting blood glucose, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, TG: triglyceride, LDL-C: low density lipoprotein cholesterol, VAI: visceral adiposity index * P <0.05, ** P <0.001

its correlation coefficient with all the components of MetS was higher than observed for BAI and HC. WHtR correlated positively and strongly with DBP, SBP and all serum parameters except HDL and adiponectin with which it showed significant negative correlation. The correlation coefficient of WHtR with MetS Components was slightly higher than that of WC except for HDL and DBP where the correlation of WC was stronger. WHtR showed a positive and significant correlation with VAI and its strength of correlation with VAI (r = 0.67) was similar to that of WC (r = 0.64).

Putting all the anthropometric adiposity indices together, WHR showed the strongest correlation with blood pressure and serum components of MetS. Higher correlations of WHR were observed for SUA (r = 0.84), adiponectin (r = -0.83), TC (r = 0.83), LDL (r = 0.84) and VAI (r =0.83). Comparing the index of visceral adiposity with all the anthropometric indexes, higher correlation coefficients were observed between VAI and all the parameters of MetS. However, the correlation strength of WHR was close to that of VAI. In males and females, the anthropometric adiposity markers correlated with MetS components in a similar pattern of varying strength. In that, while all the indices correlated positively with DBP, SBP, FBG, SUA, TC, TG and LDL, they showed a negative correlation with adipoonectin and HDL. HC showed only a weak correlation with both SBP and DBP in males and with only DBP in females. Its correlation with DBP is stronger is stronger in males. The correlation of BMI with all MetS parameters was stronger in females. Also, in female subjects, the WC had higher correlation with TC, TG and LDL while in males, it had higher correlation with SUA, adiponectin, FBG and HDL. The correlation of WC with VAI was similar in both sexes. The correlation coefficient of NC with the MetS indicators was similar for males and females. However, it had a slightly higher correlation with the serum biomarkers in males. Also, NC had no significant correlation with FBG in female subjects. While WHR showed comparable powers of correlation with MetS in both sexes, WHtR showed higher correlation among females. In both sexes, BAI correlated weakly with some of the MetS components. Correlating only with DBP in females, in males it correlated with SBP, TC, and TG. VAI showed weak and similar correlation with BAI in both sexes.

DISCUSSION

The positive correlation between the indices of body adiposity and MetS parameters observed in this study is in keeping with many studies (Eckel et al., 2010; Okampka et al., 2016). Similarly, the correlation between body adiposity measures and SUA and their negative correlation with adiponectin as observed in this study is also in conformity with previous findings (Hotta et al., 2001; Stefan et al., 2002). The positive correlation of SUA with MetS as observed in this study agrees with previous studies (Billietet al., 2014), and is believed to have an evolutionary basis resulting from uricase mutation in order to confer a survival advantage by helping to maintain blood pressure (BP), stimulate salt-sensitivity, induce insulin resistance (IR) and obesity, thereby helping promote survival during a period of famine or stress (Johnson et al., 2008). Studies have also showed that hyperuricemia is an independent predictor of MetS (Kadiri and Salako, 1997; Billiet et al., 2014). Also, since studies have demonstrated the protective effect of adiponectin against MetS (Hotta et al., 2000; Weyer et al., 2001), it therefore means that as obtained in this study, all adverse metabolic indicators are expected to correlate inversely with adiponectin and positively with SUA.

The antagonist effect of adiponectin against MetS which may be the basis for the inverse correlation observed in this study is reported to result from its anti-atherogenic (Ouchi et al., 2001; Okamoto et al., 2002), anti-diabetic (Yamauchi et al., 2002; Stefan et al., 2003) and anti-inflammatory (Engeli et al., 2003) effects. Therefore, similar to the result obtained in this study, low plasma levels of adiponectin is reported to characterize higher measures of body adiposity and adverse metabolic parameters (Engeli et al., 2003). In this study, one of the serum components of MetS, HDL correlated negatively with body adiposity measures. This finding is prviously (Bergman et al., 2006).

Consequent to this inverse relationship, unlike other serum components, lower levels of HDL characterize obesity and MetS (Bergman et al., 2006). Also, the significant correlation between anthropometric measures of adiposity and VAI observed in this study is in line with documented reports showing positive correlation between various measures of visceral adiposity and anthropometric measures (Després et al., 2000; Lara-Castro et al., 2002).

Comparing the pattern of correlations observed in this study to those of previous studies, while close similarities were observed for some of the indices, wide variations were noted in others. These variations are not unexpected as there is currently an ongoing controversy on the adiposity measure with the highest discriminatory power for MetS because of conflicting reports from different ethnicity and populations (Tulloch-Reid et al., 2003; Shao et al., 2010). The relatively weak correlation of BMI with MetS indices and VAI when compared with indices of centripetal adiposity as found in this study is supported by many other studies (Grundy et al., 2005; Pischon et al., 2008; MacKay et al., 2009). There is increasing number of reports pointing at the probable superiority of central measures of adiposity compared to BMI (Pischon et al., 2008; MacKay et al., 2009). This is mainly because of its reported tight association with intra–abdominal visceral fat which is a critical determinant of MetS (Adiels et al., 2008; Korenblat et al., 2008). Also, the unique anatomic location of visceral adipose tissue (Kraegen et al., 1991), difference in structural and functional characteristics between visceral and subcutaneous adipocytes (Mathieu et al., 2009; Browning et al., 2010), differernce in pattern of vascularisation (Bergman et al., 2011; Bélanger et al., 2002) are additional theories that have been put forward to explain these findings of central adiposity measures correlating with MetS better than BMI. Additionally, in the case of this study which included adults of advanced age, since elderly people are more likely to be physically inactive and physical inactivity has been shown to preferentially increase visceral adipose reserve (Ross and Janiszewski, 2008) manifesting as increased central adiposity measure, this factor may further contribute to the superiority of central indices over BMI as observed in this particular study.

Contrarily, there are some studies which either showed both to be equivalent or found BMI to be superior in its discriminatory power for all or some components of MetS (Ford et al. 2003 and Wang et al. 2005).

These wide variation and conflicting reports on the comparison of generalized and central adiposity measure may suggest that there are probably population specific factors that determine the interrelationship between body adiposity measures and MetS. These factors may include race, ethnicity, diet and physical activity level. For example, in the case of race, it is documented that blacks have lower body fat content for the same adiposity measure when compared to whites (Deurenberg et al., 1998). Since adipose tissue reserve is the main consideration, this has implication on the interrelationship between adiposity and metabolic parameters and this also means that subjects belonging to different races although may have similar adiposity measures, the MetS parameters and their pattern of relationship with adiposity may differ. In case of physical activity, individuals with similar body adiposity measures but different levels of physical activity may have different metabolic profile since PA has been shown to correlate negatively with metabolic parameters independent of adiposity measures (Andersen, 2006; Butte et al., 2007). In any case, the difference between the results of this study compared to those obtained from different populations on this issue further strengthen the current recommendation that anthropometric criteria for metabolic risk assessment should be population specific (Lear et al., 2010; Katzmarzyk et al., 2011).

Interestingly, the result of this study shows that even the indices of central adiposity do not exhibit the same strength of relationship with MetS indices. WHR in both males and females had the highest correlation with all the components of MetS. This relationship was further validated by WHR showing the strongest relationship with SUA and adiponectin which are serum biomarkers that could test the validity of relationships between body adiposity measures and MetS parameters. The finding in the present study that BAI is a relatively weak adiposity tool is very similar to that demonstrated by Melmer et al. (2013) who conducted one of the first studies after the discovery of BAI.

The superiority of VAI over all the anthropometric measures obtained in this study is similar to reports from different populations (Amato et al., 2010; Amato and Giordano, 2014). However, this index, according to the present study differed from some studies in terms of its predilection for certain components of MetS. Deviating slightly from this study which shows the highest predilection of VAI for TC and TG, the study of Knowles et al. (2011) found significant association of VAI with all MetS components, but with a stronger predilection for triglyceride and HDL-C in both genders. The study of Heloisa et al. (2015) differs from the present study in that, even though VAI showed superior correlation with MetS components compared to anthropometric measures of adiposity like this study, unlike this study, the superiority of VAI did not cut across all the components of MetS because according to Heloisa et al. (2015), BMI in the general population and in females showed a higher correlation with serum glycemia. From the result of the present study showing the weakest correlation of VAI with DBP and SBP compared to other MetS parameters, it

may be speculated that the relationship between BP and visceral adiposity may be weaker compared to other MetS components. This may be due to larger number of factors that come into play in the regulation of BP compared to other MetS components, making the contribution of visceral adipose tissue deposit less in the pathogenesis of hypertension. Further, the higher mRNA concentrations for angiotensinogen reported for visceral compared to abdominal subcutaneous adipose tissue is thought to be a major pathophysiologic mechanism linking hypertension with visceral adipose tissue as well as adipocyte differentiation (Dusserre et al., 2000). This pathogenic pathway may seem to be longer than those linking visceral adiposity with serum lipids and glucose which often involves direct release of lipid products into the circulation (lipidemia) or glucose release via hepatic glycogenolysis (Matsuzawa, 2008; Browning et al., 2010).

There are speculations that the superior discriminatory ability of visceral adipose tissue over other adiposity measures may not be a unanimous contention and may not follow a uniform trend in all population suggesting that factors such as ethnicity may influence the interrelationships between visceral adipose tissue and Mets. Moreover, Goh et al. (2014) has reported that ethnicity is a principal determinant of the extent of impact of a particular adiposity measure on MetS components. This means that ethnic specific factors may either up regulate or down regulate the relationship. Overall, the superior performance of visceral adipose measure observed in this study may have its explanation rooted to the fact that visceral adipose tissue is tied to overproduction of triglyceride-rich lipoproteins and glucose, leading to the dysglyecaemic and dyslipidaemic state found in viscerally obese subjects (Adiels et al., 2008; Korenblat et al., 2008).

This study reveals that, for the Hausas of Kano, visceral adiposity index is better correlated with metabolic syndrome indices when compared to all anthropometric adiposity measures. Waist to hip ratio is superior to other anthropometric markers. Body adiposity index, neck circumference and hip circumference are weak correlates of metabolic parameters.

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Calcium-rich Diet and Vitamin D Supplementation Improve Lipid Profiles and Reduce Atherogenic Index in High Salt fed Male Wistar Rat

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Summary: To ascertain the effect of calcium rich diet and/ or vitamin D supplementation on atherogenic parameters in high salt loaded rats. Thirty male rats were randomly assigned into five groups of six rats each, namely; control; salt only; salt + Calcium; salt + Vit. D and salt + Vit. D + Calcium. High salt diet constituted 8% NaCl diet + 1% NaCl drinking water, while calcium diet was made from 2.5% $CaCl_2$ diet. Serum lipids and atherogenic indices were estimated using standard laboratory procedures. The control rats took normal rodent chow, the feeding lasted 6 weeks. Rats fed high salt diet only had significantly ($p<0.05$) reduced high density lipoprotein cholesterol levels, however this was significantly ($p<0.05$) increased upon treatment with calcium rich diet and vitamin D supplementation. The high salt groups placed on Vit. D and/or calcium diet supplementation had a significant ($p<0.05$) decrease in low density lipoproteins, total cholesterol and atherogenic indices (cardiac risk ratio, atherogenic coefficient and atherogenic index of plasma) compared to the group fed on high salt only. These results suggest the ameliorative potentials of calcium rich diets and vitamin D supplementation against atherogenic tendencies and possibly cardiovascular diseases.

Keywords*:* Calcium rich diet, vitamin D, serum lipids, atherogenic indices, high salt loading.

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INTRODUCTION

Dietary salt forms an essential part of our meal (Ha, 2004), it is composed of sodium chloride (40% sodium and 60% chloride), (Alderson, 2010). Dietary or table salt serves as food preservative, it is also a component of oral rehydration therapies and used as wound disinfectant, (Ha, 2004).

However, intake of dietary salt in excess of the body's requirement (5g/day) could be deleterious in some humans and animals. With the recently large increase in the consumption of highly salted processed food, salt intake is increasing astronomically in most countries around the world (Alderson, 2010). High salt intake is a predisposing factor to high blood pressure and mortality. High salt has been reported to cause increase in oxidative stress by induction of the release of free radicals in tissues of the kidneys, and in the arteriolar and venular walls of skeletal muscles in rats (Lenda and Boegehold, 2002). Excessive amount of salt consumption has also been shown to adversely affect the pattern and duration of sleep in experimental animals (Heydapour and Heydarpour, 2014). Previous reported has also revealed that high salt damages and destroys cardiac tissues and promotes cardiac hypertrophy and cardiovascular diseases (Conrad et al., 1996).

High salt diet is also associated with the activation of adipokines that may stimulate hepatic triacylglycerol synthesis, which in turn promote assembly and secretion of low density lipoprotein cholesterol (LDL-c), very low density lipoprotein cholesterol (VLDL-c) and reduction of high density lipoprotein cholesterol (HDL-c), (Gorter et al., 2004). In addition, high salt intake causes leptin resistance, insulin resistance and the development of obesity by stimulating endogenous fructose production and fructose metabolism into cholesterol (Lanaspa et al., 2018).

Calcium, an essential food nutrient plays vital role in signal transduction pathways. Calcium rich diet reduces incidence of hypertension (Singh et al., 1987) and high serum cholesterol (Olatunji et al., 2008). In rats, the recommended daily allowance (RDA) is 5.0 mg per kg feed (National Research Council, 1995).

Vitamin D is a secosteroids which cannot be produced by human metabolism, the source of vitamin D in human is from plant and animal products like fatty fish (tuna, mackerel, and salmon), orange, soy milk, cereals, beef liver, cheese and egg yolks. Vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol) are the most important forms of vitamin D, (Holick, 2006; Holick et al., 1971). Vitamin D is required for the intestinal absorption of calcium, magnesium and

phosphate and it enhances other biological effects in the body (Holick, 2004). Vitamin D plays a significant role in calcium homeostasis and metabolism thereby enhancing availability of calcium ions in the blood, (Wolf, 2004).

High salt intake alters atherogenic indices, which is a predisposing factor to hypertension. There is paucity in scientific literature on the role of calcium rich diet or vitamin D supplementation in ameliorating the menace of high salt loading in atherogenic indices in rats. Hence, this present study investigated the effects of calcium rich diet and / or vitamin D on atherogenic indices in salt loaded rats.

MATERIALS AND METHODS

Preparation of the experimental diets: The salt used for this study was a product of Sigma Aldrich (USA). High salt diet containing 8% sodium chloride was prepared using a standard diet containing 0.3% sodium chloride. The salt drinking water was prepared as 1% NaCl solution according to the method of Obiefuna and Obiefuna, (2001)

Calcium rich diet containing 2.5% of calcium chloride was prepared from the rat chow composed of 0.3% calcium chloride. The $CaCl_2$ used in this study was purchased from Sigma Aldrich (USA). The 2.5% calcium rich diet for the high salt fed group was prepared using the already prepared 8% sodium chloride diet (which also contained 0.3% calcium chloride) (Ladipo *et al.,* 2006). In rats, the recommended daily allowance (RDA) is 5.0 mg per kg feed (National Research Council, 1995).

Vitamin D administered was calculated thus; Vitamin D dose – 200 IU/kg i.e. 20 IU/ 100g (rat weight). 400 IU was dissolved in 5ml of olive oil (yielding 80 IU per 1ml of olive oil. It was administered orally to the rats using orogastric cannula, (Ghaly *et al*., 2019).

The standard rat chow used in this study was produced by Pfizer feeds Aba, it was purchased from a local dealer in Calabar, Nigeria.

Experimental animals: In total, 30 male albino Wistar rats weighing between 90-120g were used for this study. They were obtained from the Animal Science Department of the University of Calabar, Nigeria. They had access to their feed and water *ad-libitum.* Approval for the use of the animals was obtained from the College Ethical Committee on the use of experimental animals, the animals were housed and handled in accordance with internationally accepted principles for laboratory animal use and care as found in the European Community guidelines (EEC, 1986).

Experimental design: The 30 rats were randomly assigned into five groups of 6 rats, namely; control group that received normal rodent chow, group 2 were fed high salt diet (8% NaCl diet + 1% NaCl drinking water), group 3 took high salt diet + calcium rich diet, group 4 received high salt diet + Vitamin D and group 5 took high salt diet + calcium rich diet + Vitamin D. The feeding was done orally *ad libitum* for 6 weeks.

Thereafter, rats were sedated with 5% chloroform (Goodies *et* al., 2015) after an overnight fast. The thoracic cage was dissected to expose the heart and blood samples were collected via cardiac puncture, a modified method of Ohwada (1986). The sera obtained were used to assay for the different serum lipids.

Atherogenic indices: Atherogenic index (AI) was calculated using the formula: log (TG / HDL-c) (Takasaki, 2005; Onat *et al.,* 2010). The cardiac risk ratio (CRR) was calculated using the formula: TC / HDL-c (Ikewuchi and Ikewuchi, 2009) while the atherogenic coefficient (AC) was calculated using the formula: (TC – HDL-c) / HDL-c (Ajiboye *et al.,* 2015).

Lipid profile Analysis: Serum and tissues TC, TG and HDL-c were determined by enzymatic colorimetric method using Dialab kit, while LDL-c and VLDL-c were calculated using the formula of Friedwald *et al.,* (1972), thus:

VLDL-c in mmol/L = TG / 2.2

LDL-c in mmol/L = TC - (HDL-c + VLDL-c)

Statistical Analysis: All data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc student's Newman–Keuls test done with SPSS (17.0) for Windows, (SPSS Inc., Chicago, IL). Results obtained were presented as mean + SEM and p-value ≤ 0.05 was considered statistically significant.

RESULTS

As shown in Fig. 1, the mean values of total cholesterol (TC) in the control group was 3.91 ±0.06 mmol/L, in the group fed with high salt diet only (HS) it was 3.60 ±0.08 mmol/L, TC levels in the group fed with high salt + calcium rich diet (HSCR) was 3.29 ±0.03 mmol/L, while in the group fed with high salt + Vit. D supplementation (HSVD) it was 2.66 ±0.06 mmol/L and in the group fed with high salt + calcium rich diet + Vit. Supplementation (HSCRVD) it was 2.60 ±0.06 mmol/L. TC concentrations of HS and HSCR groups decreased significantly ($p<0.05$) compared with the control group. It was in turn significantly lower in the HSVD and HSCRVD groups were compared to HS and HSCR.

In Fig. 2, the mean values of triglyceride (TG) in the control group was 0.31 ±0.02 mmol/L, in the group fed with high salt diet only (HS) it was 0.20 ±0.01 mmol/L, TC levels in the group fed with high salt + calcium rich diet (HSCR) was 0.22 ±0.01 mmol/L, while in the group fed with high salt + Vit. D supplementation (HSVD) it was 0.15 ±0.01 mmol/L and in the group fed with high salt + calcium rich diet + Vit. Supplementation (HSCRVD) it was 0.11 ±0.01 mmol/L. TC concentrations of HS and HSCR groups decreased significantly ($p<0.05$) compared with the

control group. It was in turn significantly lower in the HSVD and HSCRVD groups were compared to HS and HSCR.

High density lipoprotein cholesterol (HDL-c) concentration of the control group was 0.98 ±0.03 mmol/L. It was significantly ($p<0.05$) lower in the HS, 0.55 ±0.04 mmol/L and HSCR, 0.77 ±0.01 mmol/L groups compared with the control group. HDL-c concentration in the HSVD, 0.79 ±0.02 mmol/L and HSCRDV, 0.83 ±0.02 mmol/L were in turn significantly ($p<0.05$) higher compared to control and HS groups (Fig. 3).

Low density lipoprotein cholesterol (LDL-c) in control, HS, HSCR, HSVD a HSCRVD groups were 2.49 ±0.03 mmol/L, 2.77 ±0.06, 2.20 ±0.01, 1.65 ±0.06 and 1.61 ±0.04 mmol/L respectively. LDL-c concentration of the HS group was significantly higher ($p<0.05$) compared to the control group but was significantly ($p<0.05$) lower in HSCR, HSVD and HSCRVD groups compared with control and HS groups, (Fig. 4).

Mean values of the very low density lipoprotein cholesterol (VLDL-c) reduced significantly ($p<0.05$) in all the test groups compared to the control (Fig. 5). The mean values of VLDL-c in control group was 0.14 ±0.01 mmol/L, in HS group it was 0.09 ±0.001, the

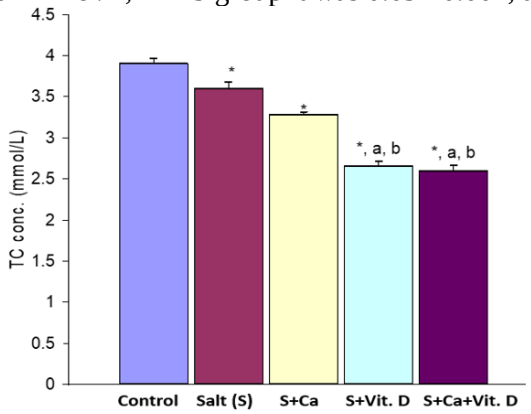

Fig. 1: Comparison of total cholesterol concentration in the different experimental groups. *=p<0.05 when compared with control, a=p<0.05 when compared with salt, b=p<0.05 when compared with Salt + Ca.

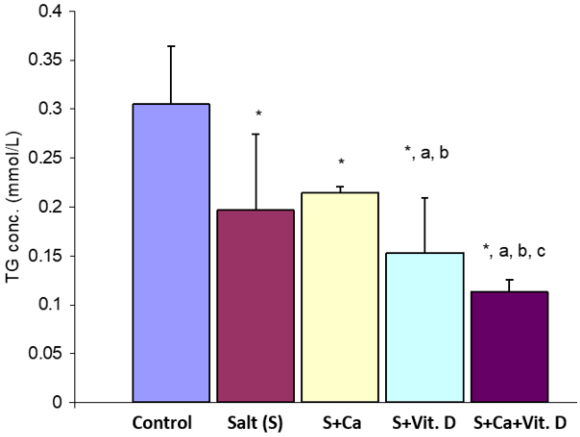

Fig. 2: Comparison of triglyceride concentrations in the different experimental groups. *=p<0.05 when compared with control, a=p<0.05 when compared with salt, b=p<0.05 when compared with Salt + Ca.

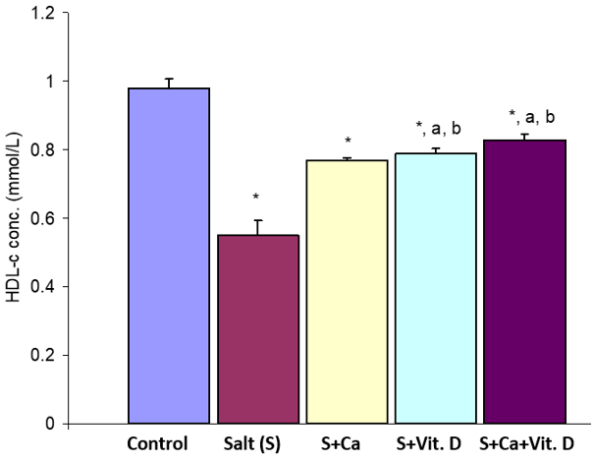

Fig. 3: Comparison of high density lipoprotein cholesterol concentration in the different experimental groups. *=p<0.05 when compared with control, a=p<0.05 when compared with salt, b=p<0.05 when compared with Salt + Ca.

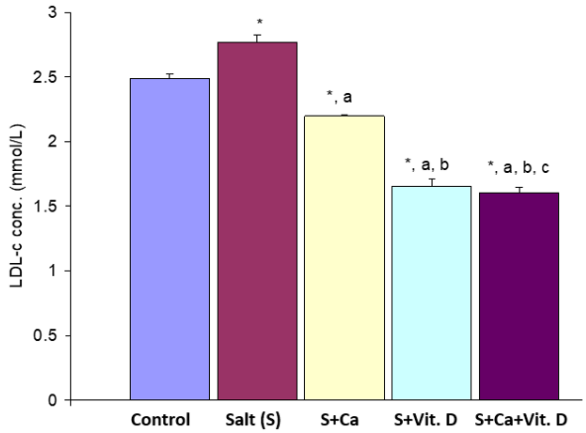

Fig. 4: Comparison of low density lipoprotein cholesterol concentration in the different experimental groups. *=p<0.05 when compared with control, a=p<0.05 when compared with salt, b=p<0.05 when compared with Salt + Ca.

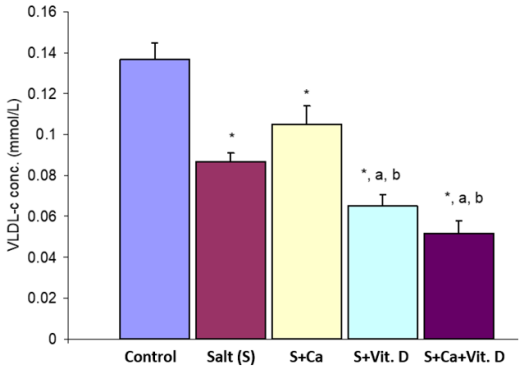

Fig. 5: Comparison of very low density lipoprotein concentrations in the different experimental groups. *=p<0.05 when compared with control, a=p<0.05 when compared with salt, b=p<0.05 when compared with Salt + Ca.

Table 1: Atherogenic indices of the different experimental groups

Groups	Cardiac risk ratio	Atherogenic coefficient	Atherogenic index
Control	4.00 ±0.07	3.00 ± 0.07	2.55 ± 0.07
Salt only	6.71 ±0.44*	5.71 ± 0.43*	5.15 ±0.37*
Salt + Ca	4.28 ±0.05*	3.28 ± 0.05*	2.86 ± 0.04*
Salt + Vit. D	3.38 ±0.09*, a, b	2.38±0.09*, a, b	2.10±0.09*, a, b
Salt + Ca + Vit D	3.14±0.06*, a, b	2.14±0.06*, a, b	1.94±0.06*, a, b

Values are presented as mean + SEM, n = 6. * = significantly different from control at $p<0.05$; a = significantly different from salt only at $p<0.05$; b = significantly different from salt + Calcium at $p<0.05$.

HSCR group recorded a mean value of 0.11 ±0.01 mmol/L, HSVD group had 0.07 ±0.01 mmol/L as their mean value while 0.05 ±0.01 mmol/L was recorded for HSCRVD group.

In Table 1, the cardiac risk ratio (CRR), atherogenic coefficient (AC) and atherogenic index (AI) increased significantly ($p<0.05$) in the HS group compared to control, whereas the values for the other treated groups (HSCR, HSVD and HSCRVD) were significantly lower compared to the HS group. HSVD and HSCRVD had significantly ($p<0.05$) lower values compared with the control groups.

DISCUSSION

In this study, results obtained indicate that calcium rich diet and vitamin D supplementation reduced the elevated triglycerides, LDL-c, and VLDL-c concentrations, but increased the low HDL-c concentration caused by high salt loading in rats. This agrees with previous studies which indicate that increased calcium from 0.9% to 2.5% abrogated altered plasma lipoprotein-cholesterol levels (Olatunji *et al.,* 2008), although not in high salt loaded rats. It has been noted that the cholesterol lowering effect of dietary calcium could be attributed to the ability of calcium to bind bile acids and saturated fatty acid in the gut and form inabsorbable chelates, therefore resulting in impaired lipid absorption (Vaskonen *et al.*, 2002).

The salt-induced increase in LDL-c (bad cholesterol), TG and VLDL-c levels as observed in this study has been reported as one of the predisposing factors to the development of atherosclerosis a major cause of hypertension and other cardiovascular diseases (Messerli *et al.*, 1997). Earlier report shows that high salt diet is associated with the activation of adipokines that may stimulate hepatic triacylglycerol synthesis, which in turn promote assembly and secretion of low density lipoprotein (LDL), very low density lipoprotein (VLDL) and reduction of high density lipoprotein (Gorter et al., 2004). In addition, high salt intake causes leptin resistance, insulin resistance and development of obesity by stimulating endogenous fructose production and fructose metabolism into cholesterol (Lanaspa et al., 2018).

On the contrary, HDL-c counters the adverse effect of the bad cholesterol by sequentially mopping up LDL-c from the blood, hence HDL-c it is regarded or called the "good cholesterol", this property of HDL-c is physiologically significant because it enhances good cardiovascular health (Nerses *et al.,* 2007). HDL-c also plays a vital role in 'reverse transport' of cholesterol from extra-hepatic tissues to the liver for onward excretion in the bile (Rader *et al.*, 2009). Cholesterol is also removed from macrophages in the sub-intima of vessel wall by the interaction of HDL with ABCA-1, SR-B1, or by passive diffusion process, (Ohashi *et al*., 2005). HDL-c plays another vital role preventing oxidative damage of the vascular system and other parts of the body by acting as a carrier of lipid hydro-peroxides and paraoxynase, these enzymes are involved in preventing and reversing oxidative damage (Shao *et al*., 2009).

The increase in cardiac risk ratio (CRR), atherogenic coefficient (AC) and atherogenic index (AI) observed in HS group were reversed by calcium rich diet and vitamin D supplementation. These atherogenic indices (CRR, AC and AI) are strong markers or indicators of cardiovascular risk (Emul *et al*., 2016). The ability of calcium rich diet and vitamin D supplementation to lower the levels of these indices clearly shows the hypocholesterolemic and hypolipidemic effect of dietary calcium. Vitamin D may have played a complementary role to cause this effect because one of the functions of vitamin D is the enhancement of calcium absorption in the gut.

In conclusion, calcium rich diet and vitamin D supplementation reverses increases in TG, LDL-c, VLDL-c and atherogenic indices occasioned by high salt loading. Calcium rich diet and vitamin D supplementation also improve HDL-c levels in high salt loaded rats. These show the ability of the calcium rich diet and vitamin D supplementation to minimize cardiovascular risk in the face of high salt loading in rats.

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Morinda lucida Aqueous Stem Bark Extract Ameliorates Hepato-Renal Dysfunctions in Experimental Diabetes Model

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Summary: Diabetes Mellitus (DM) is a leading pan-systemic endocrine disorder with attendant high morbidity and mortality owing to its deleterious effects on vital body organs caused by untreated chronic hyperglycemia, attendant oxidative stress and glycation processes. The present study is designed to investigate possible protective role and mechanism(s) of action of 125-500 mg/kg/day of *Morinda lucida* aqueous stem bark extract (MLASE) on renal and hepatic functions in alloxan-induced hyperglycemic rats for 8 days. Forty-two alloxan-induced hyperglycemiic male Wistar rats were randomly allotted to Groups II-VI and orally treated with 10 ml/kg/day distilled water, 5 mg/kg/day glibenclamide, 125 mg/kg MLASE, 250 mg/kg MLASE, and 500 mg/kg/day MLASE, respectively. Group I normal rats served as untreated control and were orally treated with 10 ml/kg of distilled water, all under same sham-handling. Blood samples were taken for measurement of fasting blood glucose, renal and hepatic function profile. Liver and kidney tissue samples were taken for determination of the activities of oxidative stress markers such as malondialdehyde (MDA), reduced glutathione (GSH), and glutathione peroxidase (GPx), catalase (CAT) and superoxidase dismutase (SOD). Results showed that intraperitoneal injection with 120 mg/kg of alloxan in cold 0.9% normal saline reliably and significantly induced a steadily sustained hyperglycemia which were ameliorated by short-term oral treatment with 125-500 mg/kg/day of MLASE, dose dependently, similar to that ameliorated by the standard antihyperglycemic drug, glibenclamide. Similarly, MLASE significantly mitigated against derangements in the measured renal and hepatic function parameters as well as oxidative stress induced by alloxan-induced hyperglycemia. In conclusion, results of this study showed the protective role of 125-500 mg/kg/day of MLASE in chronic hyperglycemia-associated renal and hepatic dysfunctions which was mediated via antioxidant and free radical scavenging activities of MLASE.

Keywords*:* Induced hyperglycemia, hepatic and renal function profile, oxidative stress markers, *Morinda lucida*.

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INTRODUCTION

Diabetes mellitus (DM), a pan-systemic endocrine disorder which is characterized by chronic hyperglycemia, remains the most common disorder of carbohydrate, lipid and protein metabolism resulting from insulin deficiency and/or insulin action (American Diabetes Association, 2019).

Untreated or poorly controlled DM is often associated with multi-systemic complications such as such as vasculopathy, retinopathy, nephropathy, neuropathy and cardiovascular diseases (Kaneto *et al.*, 2007; American Diabetes Association, 2019). Indeed, DM is known to be the most common single cause of kidney failure, a condition known as diabetic nephropathy which affects about 20-30 % of diabetic subjects (Mehdi and Toto, 2009; Hahr and Molitch, 2015). Diabetic nephropathy is a major complication associated with poor diabetic control and a leading cause of end stage renal failure (Fonteles *et al.*, 2007; Alicic *et al.*, 2017). Similarly, type 2 DM is now estimated to be the most common cause of liver diseases (including abnormal hepatic enzyme levels, non-alcoholic fatty liver disease (NAFLD), cirrhosis, hepatocellular carcinoma, and acute liver failure) in the United States of America (Tolman *et al.*, 2007; Blendea *et al.*, 2010). Cryptogenic cirrhosis, of which diabetes is, by far, the most common cause, has become the third leading indication for liver transplantation in the U.S. (Golabi *et al.*, 2018).

Morinda lucida Benth, belonging to the Rubiaceae family, is a medium-sized tree used as a medicinal plant in West Africa (especially in Nigeria) in the local treatment of malaria and other febrile conditions, diabetes, hypertension, cerebral congestion, dysentery, stomach ache, ulcers, leprosy, and gonorrheal (Adeneye and Agbaje, 2008; Adebayo and Krettli, 2011). Different parts of the plant have been reported

to possess antimicrobial (Fakoya *et al.*, 2014). In the same vein, different extracts of different parts of *Morinda lucida* plant have been reported to elicit antihyperglycemic activities in different experimental diabetes models (Olajide *et al.*, 1999; Adeneye and Agbaje, 2008; Odutuga *et al.*, 2010; Adeneye *et al.*, 2017). Report equally has it that *Morinda lucida* is also one of the foremost medicinal plants used in the treatment of liver diseases in the Maritime region of Togo (Kpodar *et al.*, 2016). Amongst herbalists in South West Nigeria, water decoction of the stem bark of *Morinda lucida* is used in the management of diabetes mellitus and its attendant complications. Unfortunately, despite the wide application of different decoction of *Morinda lucida* in the management of diabetes and diabetes complications, there are no scientific reports to validate or refute this folkloric use. Therefore, the present study is designed at investigating the possible short-term protective potential and mechanism(s) of 125, 250 and 500 mg/kg/day of the *Morinda lucida* aqueous stem bark extract (MLASE) on both the hepatic and renal function parameters and the oxidative stress markers in their tissues in alloxan-induced hyperglycemic rats.

MATERIALS AND METHODS

Plant sample collection and preparation

Two kilograms of fresh stem bark and some fresh leaves of *Morinda lucida* were collected from an uncultivated farmland on the outskirt of Low Cost Housing Estate, Oke-Afa, Isolo, Lagos State, Nigeria in the month of April, 2014. The harvested plant materials were processed for voucher referencing as previously described by Adeneye and Agbaje (2008). The fresh stem bark peels were gently rinsed under tap water and dried under laboratory room temperature protected from direct heat and sunlight for 3 weeks. Afterwards, the dried samples were pulverized using laboratory hammer-mill in the Department of Pharmacognosy, Faculty of Pharmacy, University of Lagos, Idi-Araba, Surulere, Lagos State, Nigeria.

Aqueous extraction

Fifty gram (50 g) of the pulverized sample was boiled in 500 ml of distilled water under continuous stirring for 1 hour after which it was filtered using a piece of clean white 2-layer cotton cloth. The filtrate was then transferred to an aerated oven already preset at 40℃ and completely dried until solid residue was left behind. The procedure was repeated two more times. The solid residue (MLASE) obtained on each extraction process was kept in a water- and air-proof container in the refrigerator maintained at -4℃ until required for experimentation.

Determination of 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of MLASE

The scavenging activities of MLASE against DPPH free radicals were estimated using the methods of Liyana-Pathiranan and Shadidi (2005). A solution of 0.135 mM DPPH (Sigma Aldrich, St. Louis, U.S.A.) in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of methanol containing 25-100 µg/ml of MLASE. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid (Sigma Chemicals Co., St. Louis, U.S.A.) equally prepared at same concentration of 25-100 µg/ml was used as reference drug. The experiment was conducted in triplicate. The ability to scavenge DPPH radical was calculated by the following equation:

DPPH radical scavenging activity (%) =

$$\frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} X100$$

where:

$Abs_{control}$ = Absorbance of DPPH radical + methanol

Abs_{sample} = Absorbance of DPPH radical + sample extract/standard

Determination of nitric oxide scavenging activities of MLASE

Nitric oxide scavenging activities of MLASE were evaluated using the methods of Sreejayan and Rao (1997). Ascorbic acid at same concentrations as that of MLASE (25-100 µg/ml) was used standard drug.

Inhibition (%) =

$$\frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} X100$$

Determination of the Ferric Reducing Antioxidant Power of MLASE

MLASE reducing power was determined according to the method described by Oyaizu (1996) using ascorbic acid as the reference drug. Briefly described, 1 ml of increasing concentrations (25, 50, 75 and 100 µg/ml) of MLASE or ascorbic acid was added to 1 ml of distilled water and mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. After incubation at 50°C for 20 mins, trichloroacetic acid (2.5 ml) was added and samples centrifuged at 3000 rpm for 10 minutes. 2.5 ml aliquot of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ and the absorbance of the resulting mixtures were measured at 700 nm. 1ml of distilled water was added to 2.5 ml sodium phosphate buffer and 2.5 ml potassium ferricyanide in a test tube as the blank. The reducing power was expressed as ascorbic acid equivalent (AAE) per dry weight of crude extract (mM/mg crude extract).

Experimental animals

Healthy 8-10 weeks male albino Wistar rats (110-150 g) used in this study were obtained from Bayo Farms, Sango-Otta, Ogun State, Nigeria, after an ethical approval for the study was obtained. The rats were housed in polypropylene cages and handled in accordance with international principles guiding the

Use and Handling of Experimental Animals (NIH publication 85-23, 1985) and Committee for the Update of Guide for the Care and Use of Laboratory Animals (2011). Rat feed (Livestock Feeds, Lagos, Nigeria) and tap water were provided *ad libitum*. The rats were maintained at an ambient temperature between 23-26 ºC, humidity of 60 ± 5%, and 12 hour day/night photoperiod.

Experimental induction of diabetes mellitus

Experimental type 1 diabetes was induced in 50 rats using the method described by Venugopal *et al*. (1998) and as modified by Iwalewa *et al.* (2008). Rats were injected with freshly prepared 120 mg/kg body weight of alloxan monohydrates dissolved in sterile cold 0.9% normal saline, given via the intraperitoneal route. The rats were then orally treated with 5% dextrose solution for the next 24 hours in order to prevent hypoglycemia which often accompanies alloxan-associated hyperinsulinemia resulting from massive pancreatic β-cells destruction (Gupta *et al.*, 1984). Fasting blood glucose levels in rats were measured on the 3rd day post-alloxan injection and treated rats with fasting blood glucose levels equal to or above 200 mg/dl were considered diabetic and used for the study.

Body weight measurement

Body weights of all rats were measured on the 1st and 8th day of study using digital Mettler weighing balance (Mettler Toledo Type BD6000, Mettler-Toledo GmbH, Greifensee, Switzerland). The weight difference on the 1st and 8th day in reference to the initial weight per group was calculated.

Experimental design and oral treatment of alloxan-induced hyperglycemic rats

Oral treatments of alloxan-induced hyperglycemic rats for 8 days were as follows:

Group I: normal control rats received 10 ml/kg and 1 ml/kg of distilled water via the oral and intraperitoneal routes, respectively

Group II: alloxan-induced hyperglycemic rats received 10 ml/kg of distilled water

Group III: alloxan-induced hyperglycemic rats orally received 5 mg/kg of glibenclamide in distilled water

Group IV: alloxan-induced hyperglycemiic rats orally treated with 125 mg/kg of MLASE in distilled water

Group V: alloxan-induced hyperglycemic rats orally treated with 250 mg/kg of MLASE in distilled water

Group VI: alloxan-induced hyperglycemic rats orally treated with 500 mg/kg of MLASE in distilled water

Blood glucose measurement

Whole fasting blood glucose (FBG) of treated rats was collected by tail tipping method and determined by the glucose oxidase method of Trinder (1969) using a One Touch Basic Blood Glucose Monitoring System® (Life Scan Inc., Milpitas, California, U.S.A.). The blood glucose monitor was calibrated and validated at the beginning of, midway into and at the end of the experiment.

Measurement of hepatic function and renal function parameters

On day 8, after an overnight fast, the final fasting blood glucose was determined before treated rats were sacrificed after light diethyl anesthesia. After anesthesia, blood samples were collected directly from the heart chamber into 10 ml plain bottles. The blood samples obtained were immediately frozen at -70 ºC and centrifuged at 3000 rpm for 20 min to separate out the serum that was then analyzed for the serum electrolytes (sodium, potassium, chloride, bicarbonates), urea and creatinine using standard diagnostic test kits (Randox Laboratories, Crumlin, U.K.) on Automated Clinical System (Sychron Clinical System®, model: CX5 PRO) (Beckman Coulter Inc., Galway, Ireland). Similarly, hepatic function as measured by serum liver enzymes (AST, ALT, ALP), proteins (total protein, albumin), lipids (TG, TC, LDL-c, HDL-c) and bilirubin (total and conjugated bilirubin) were measured using standard procedures and standard diagnostic test kits (Randox Laboratories, Crumlin, U.K.) on Automated Clinical System (Sychron Clinical System®, model: CX5 PRO) (Beckman Coulter Inc., Galway, Ireland).

Hepatic and renal tissue estimation of antioxidant and free radical scavenging activities of MLASE in alloxan-induced hyperglycemic rats

Immediately the blood samples were collected, the liver and kidneys were identified and removed and briskly rinsed in ice-cold 1.14% KCl solution in order to preserve the activities of the oxidative stress markers before there were separately homogenized in 0.1 M tris-HCl buffer of pH 7.4 to give a 10% homogenate. These homogenates were used for the appropriate oxidative stress markers estimation. Superoxide dismutase (SOD) activity in the liver and kidney tissues were determined by the method of Kakkar *et al.* (1984) while that of liver MDA, catalase (CAT) and reduced glutathione (GSH) were determined by the methods of Kumar *et al*. (2010), Sinha (1972) and Rahman *et al.* (2006), respectively. Tissue glutathione peroxidase (GPx) activity was also determined using the method of Weydett and Cullen (2010).

Statistical Analysis

Results were presented as mean ± S.E.M. for body weights and % weight changes while that of FBG and serum hepatic and renal function parameters were expressed as mean ± S.E.M. of six observations. Statistical analysis was done using two-way analysis of variance followed by post-hoc test, Student-

Newman-Keuls test, on SYSTAT 10.6. Statistical significance were considered at $p<0.05$, $p<0.001$ and $p<0.0001$.

RESULTS

Extraction of MLASE

Extraction of MLASE yielded a deep brown, sticky, solid residue which is soluble in petroleum ether, methanol, ethanol, butan-1-ol and water. The calculated yield was 12.87 ± 0.41%

DPPH free radical scavenging activities of MLASE

Using DPPH, the free radical scavenging activities of MLASE and ascorbic acid (reference drug) were observed to be dose related with MLASE at its highest dose of 100 µg/ml having the most significant ($p<0.001$) free radical scavenging activity which was comparable to that of the standard drug (ascorbic acid) (Figure 1).

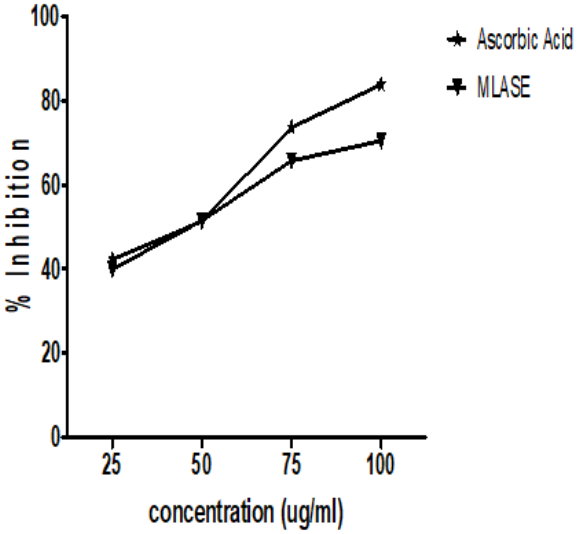

Figure 1. *In vitro* dose-related DPPH free radical scavenging activities of 25-100 µg/ml of *Morinda lucida* aqueous stem bark extract (MLASE) (test extract) and Ascorbic acid (standard drug).

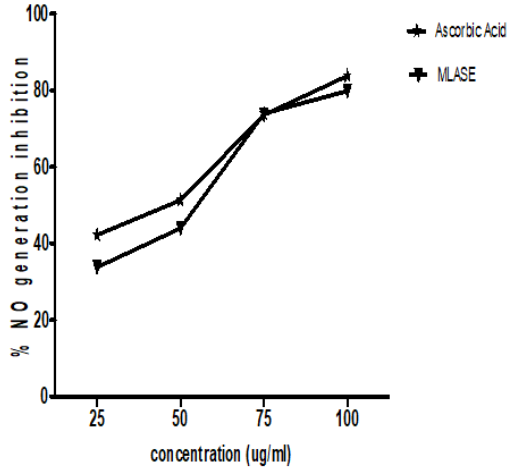

Figure 2. *In vitro* dose-related NO generation inhibitory activities of 25-100 µg/ml of *Morinda lucida* aqueous stem bark extract (MLASE) (test extract) and Ascorbic acid (standard drug)

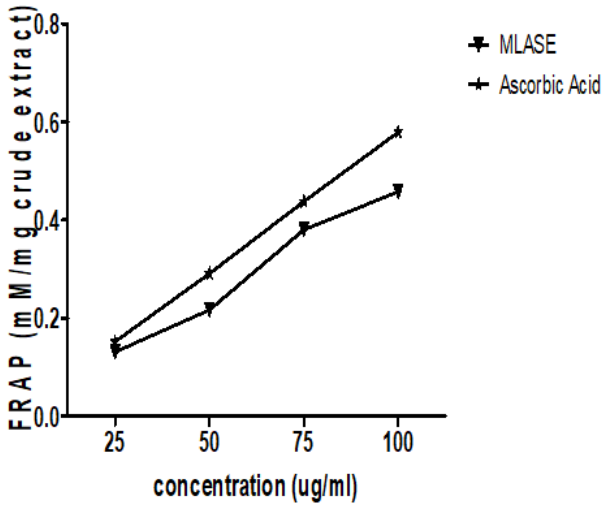

Figure 3. Ferric reducing antioxidant power of 25-100 µg/ml of *Morinda lucida* aqueous stem bark extract (MLASE) (test extract) and Ascorbic acid (standard drug)

Nitric oxide scavenging activities of MLASE

MLASE significantly ($p<0.05$) significantly inhibited the generation of nitric oxide from nitroprusside solution with the IC_{50} values of 73.98 ± 1.03 and 79.88 ± 2.00 µg/ml at 75 and 100 µg/ml, respectively which were comparative to those obtained for the standard drug (ascorbic acid) (Figure 2).

Ferric reducing antioxidant power

MLASE at concentrations (25-100 µg/ml) significantly ($p<0.05$, $p<0.01$ and $p<0.001$) inhibited the reducing power of $FeCl_3$ in a concentration-related pattern with the most significant effect recorded at the highest concentration of 100 µg/ml which favorably compared with the standard drug (ascorbic acid) at the same varying concentrations (Figure 3).

Effect of 125-500 mg/kg of MLASE on post-treatment FBG and percentage post-treatment FBG changes in alloxan-induced hyperglycemic rats

Intraperitoneal injection of 120 mg/kg of alloxan monohydrate dissolved in cold 0.9% normal saline significantly ($p<0.0001$) resulted in sustained hyperglycemia (above 200 mg/dl) by the 3rd day post-alloxan treatment (Table 1). Subsequent repeated daily oral treatments with 5 mg/kg of glibenclamide and 125, 250 and 500 mg/kg of MLASE for 8 days resulted in significant ($p<0.05$, $p<0.01$ and $p<0.001$) dose-related reductions in the FBG as well as the post-extract treatment FBG changes (Table 1).

Effect of 125-500 mg/kg of MLASE on serum renal function parameters in alloxan-induced hyperglycemic rats

Intraperitoneal injection of 120 mg/kg of alloxan monohydrate dissolved in cold 0.9% normal saline to the treated Wistar rats resulted in significant ($p<0.05$, $p<0.001$ and $p<0.0001$) increases in the serum Na^+, K^+, Cl^-, Ca^{2+}, PO_4^{2-}, uric acid, urea and creatinine when

Table 1. Effect of repeated oral treatment with 125-500 mg/kg of MLASE on the fasting blood glucose levels and percentage change in the fasting blood glucose levels in alloxan-induced hyperglycaemic rats on the 3rd day post-alloxan induction (PI) and 8th day MLASE treatment

Groups	Fasting blood glucose (FBG) levels (mg/dl) on		
	day 1	3rd day PI (with %ΔFBG)	8th day post-MLASE (with %ΔFBG)
I	51.9 ± 7.3	51.7 ± 1.6 (1.4 ± 6.2)	52.3 ± 0.9 (1.4 ± 1.9)
II	52.3 ± 1.7	228.3 ± 4.7 (336.8 ± 8.5) [c]	247.6 ± 3.0 (8.6 ± 1.4) [c]
III	59.1 ± 8.1	237.0 ± 4.8 (305.7 ± 17.2) [c]	107.1 ± 4.0 (-54.6 ± 2.2) [f]
IV	54.0 ± 5.5	231.1 ± 4.2 (331.1 ± 14.6) [c]	169.1 ± 2.2 (-26.6 ± 2.1) [e]
V	55.4 ± 10.1	228.6 ± 4.8 (323.5 ± 28.3) [c]	135.1 ± 2.52 (-40.6 ± 2.2) [f]
VI	51.1 ± 6.0	228.0 ± 5.5 (349.5 ± 10.7) [c]	101.1 ± 3.0 (-55.5 ± 2.2) [f]

[c] represents a significant increase in FBG value at $p<0.001$ when compared to FBG value on day 1 while [e] and [f] represent significant decreases in FBG values at $p<0.01$ and $p<0.001$, respectively, when compared to the 3rd day PI values and Group II values on the 8th day MLASE treatment. I = Control, II = Diabetic + 10 ml/kg of DW, III = Diabetic + 5 mg/kg of glibenclamide, IV = Diabetic + 125 mg/kg MLASE, V = Diabetic + 250 mg/kg MLASE, VI = Diabetic + 500 mg/kg MLASE

Table 2a. Effect of repeated oral treatments with 125-500 mg/kg of MLASE on serum Na^+, K^+, HCO_3^-, Cl^-, Ca^{2+} and PO_4^{2-} in alloxan-induced hyperglycaemic rats

Groups	Na^+ (mEq/L)	K^+ (mEq/L)	HCO_3^- (mEq/L)	Cl^- (mEq/L)	Ca^{2+} (mEq/L)	PO_4^{2-} (mEq/L)
I	140.1 ± 0.9	8.1 ± 1.5	20.7 ± 1.5	102.3 ± 0.8	2.2 ± 2.4	2.56 ± 0.17
II	145.4 ± 1.7[a]	17.5 ± 0.7[b]	13.1 ± 0.8[e-]	112.1 ± 0.4[c]	2.6 ± 0.1[b]	3.74 ± 0.30[a]
III	138.1 ± 1.9[e]	11.5 ± 2.9[d]	20.1 ± 1.4[b+]	100.9 ± 1.2[f]	1.9 ± 1.2[f]	2.06 ± 0.15[e]
IV	140.6 ± 0.8[d]	9.7 ± 1.4[e]	15.7 ± 1.8	103.7 ± 0.9[f]	2.3 ± 0.4[d]	3.10 ± 0.47
V	139.7 ± 1.3[d]	9.3 ± 1.1[e]	19.6 ± 1.0[b+]	103.1 ± 0.6[f]	1.8 ± 0.1[f]	2.34 ± 0.28[e]
VI	136.9 ± 0.8[e]	7.3 ± 0.7[e]	21.0 ± 0.8[c+]	99.7 ± 1.3[f]	1.6 ± 0.1[f]	2.17 ± 0.11[e]

[a, b] and [c] represent significant increases at $p<0.05$, $p<0.001$ and $p<0.0001$, respectively while [e-] represents a significant decrease at $p<0.0001$ when compared to Group I values. [e] and [f] represent significant decreases $p<0.001$ and $p<0.0001$, respectively, while [b+] and [c+] represent significant increases at $p<0.001$ and $p<0.0001$, respectively, when compared to Group II values. I = Control, II = Diabetic + 10 ml/kg of DW, III = Diabetic + 5 mg/kg of glibenclamide, IV = Diabetic + 125 mg/kg MLASE, V = Diabetic + 250 mg/kg MLASE, VI = Diabetic + 500 mg/kg MLASE

Table 2b. Effect of repeated oral treatment with 125-500 mg/kg of MLASE on serum uric acid, urea, and creatinine in alloxan-induced hyperglycaemic rats

Groups	uric acid (mg/dl)	urea (mg/dl)	creatinine (mg/dl)
I	6.2 ± 0.9	41.7 ± 1.1	1.17 ± 0.06
II	13.9 ± 0.4[b]	64.1 ± 4.8[b]	1.46 ± 0.08[a]
III	12.4 ± 1.5	47.9 ± 4.9[d]	0.70 ± 0.14[d]
IV	8.7 ± 1.1[d]	60.1 ± 5.4	1.13 ± 0.22
V	8.7 ± 1.1[d]	50.9 ± 1.8	0.97 ± 0.13
VI	6.3 ± 0.5[e]	44.1 ± 4.4[d]	0.86 ± 0.07[d]

[a] and [b] represent significant increases at $p<0.05$ and $p<0.001$, respectively when compared to Group I values while [d] and [e] represent significant decreases $p<0.05$ and $p<0.001$, respectively, when compared to Group II values. I = Control, II = Diabetic + 10 ml/kg of DW, III = Diabetic + 5 mg/kg of glibenclamide, IV = Diabetic + 125 mg/kg MLASE, V = Diabetic + 250 mg/kg MLASE, VI = Diabetic + 500 mg/kg MLASE.

compared to the values of the normal (Group I) values (Tables 2a and 2b). Alloxan treatment also resulted into a significant ($p<0.001$) decrease in the serum HCO_3^- level when compared to Group I values (Table 2a). However, with repeated daily oral treatments with 125, 250 and 500 mg/kg of MLASE for 8 days, there were significant ($p<0.05$, $p<0.001$ and $p<0.0001$) dose-related ameliorations in the serum Na^+, K^+, Cl^-, Ca^{2+}, PO_4^{2-} uric acid, urea and creatinine when compared to those values for the untreated alloxan-induced hyperglycemic rats (Tables 2a and 2b). Similarly, repeated oral treatments with 125-500 mg/kg of MLASE significantly ($p<0.001$ and

Table 3a. Effect of repeated oral treatments with 125-500 mg/kg of MLASE on serum ALB, TP, TB and CB in alloxan-induced hyperglycemic rats

Groups	ALB (mg/dl)	TP (mg/dl)	TB (mg/dl)	CB (mg/dl)
I	3.2 ± 0.1	7.1 ± 0.2	0.9 ± 0.2	0.3 ± 0.1
II	2.3 ± 0.1^{c-}	6.0 ± 0.1^{b-}	0.8 ± 0.1	0.5 ± 0.1
III	3.1 ± 0.1^{c+}	7.6 ± 0.2^{c+}	0.8 ± 0.1	0.3 ± 0.0
IV	2.8 ± 0.1^{b+}	6.3 ± 0.2	1.0 ± 0.1	0.6 ± 0.1
V	3.6 ± 0.1^{c+}	7.7 ± 0.2^{c+}	0.9 ± 0.1	0.3 ± 0.0
VI	4.2 ± 0.2^{c+}	9.2 ± 0.2^{c+}	0.8 ± 0.1	0.3 ± 0.1

$^{b-}$ and $^{c-}$ represent significant decreases at p<0.001 and p<0.0001, respectively when compared to Group I values while $^{b+}$ and $^{c+}$ represent significant increases at p<0.001 and p<0.0001, respectively, when compared to Group II values. I = Control, II = Diabetic + 10 ml/kg of DW, III = Diabetic + 5 mg/kg of glibenclamide, IV = Diabetic + 125 mg/kg MLASE, V = Diabetic + 250 mg/kg MLASE, VI = Diabetic + 500 mg/kg MLASE.

Table 3b. Effect of repeated oral treatment with 125-500 mg/kg of MLASE on serum hepatic enzymes (AST, ALT and ALP) in alloxan-induced hyperglycemic rats

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)
I	109.1 ± 9.7	33.9 ± 3.7	110.6 ± 5.8
II	137.1 ± 10.6^{a}	42.1 ± 9.5^{a}	98.0 ± 7.7
III	122.4 ± 18.3^{e}	24.1 ± 2.2^{f}	84.3 ± 10.1^{d}
IV	138.9 ± 17.8^{a}	37.7 ± 4.3^{d}	90.0 ± 12.8^{d}
V	134.6 ± 13.0^{a}	27.9 ± 6.7^{f}	86.6 ± 8.6^{d}
VI	98.6 ± 9.4^{f}	23.0 ± 3.6^{f}	79.3 ± 10.8^{e}

^{a} represents a significant increase at p<0.05 when compared to Group I values while $^{d, e}$ and ^{f} represent significant decreases p<0.05, p<0.001 and p<0.0001, respectively, when compared to Group II values. I = Control, II = Diabetic + 10 ml/kg of DW, III = Diabetic + 5 mg/kg of glibenclamide, IV = Diabetic + 125 mg/kg MLASE, V = Diabetic + 250 mg/kg MLASE, VI = Diabetic + 500 mg/kg MLASE.

Table 4. Effect of repeated oral treatments with 125-500 mg/kg of MLASE on renal tissue SOD, CAT, GSH, GPx and MDA levels in alloxan-induced hyperglycemic rats

Groups	SOD (U/mg protein)	CAT (U/mg protein)	GSH (U/mg protein)	GPx (U/mg protein)	MDA (nM/mg protein)
I	74.9 ± 4.7	8.2 ± 0.7	0.4 ± 0.0	0.3 ± 0.0	0.1 ± 0.0
II	47.5 ± 5.8^{c-}	4.6 ± 0.6^{b-}	0.3 ± 0.0^{a-}	0.8 ± 0.1^{c}	0.1 ± 0.0^{b}
III	78.0 ± 6.1^{c+}	10.8 ± 1.4^{c+}	0.6 ± 0.1^{c+}	0.4 ± 0.0^{f-}	0.0 ± 0.0^{f-}
IV	76.6 ± 3.5^{c+}	8.4 ± 0.4^{b+}	0.6 ± 0.0^{b+}	0.5 ± 0.0^{f-}	0.0 ± 0.0^{f-}
V	78.7 ± 3.2^{c+}	12.5 ± 0.4^{c+}	0.6 ± 0.0^{c+}	0.4 ± 0.1^{f-}	0.0 ± 0.0^{f-}
VI	82.9 ± 1.7^{c+}	13.7 ± 0.47^{c+}	0.8 ± 0.0^{c+}	0.4 ± 0.0^{f-}	0.0 ± 0.0^{f-}

$^{a-}$, $^{b-}$ and $^{c-}$ represent significant decreases at p<0.05, p<0.001 and p<0.0001, respectively while ^{b} and ^{c} represent significant increases at p<0.001 and p<0.0001, respectively when compared to Group I values. $^{b+}$ and $^{c+}$ represent significant increases at p<0.001 and p<0.0001, respectively, while $^{f-}$ represents a significant decrease at p<0.0001 when compared to Group II values. . I = Control, II = Diabetic + 10 ml/kg of DW, III = Diabetic + 5 mg/kg of glibenclamide, IV = Diabetic + 125 mg/kg MLASE, V = Diabetic + 250 mg/kg MLASE, VI = Diabetic + 500 mg/kg MLASE.

p<0.0001) restored the serum HCO_3^- to about the values recorded for normal (Group I) rats (Table 2a).

Effect of 125-500 mg/kg of MLASE on serum hepatic function parameters in alloxan-induced hyperglycemic rats

Intraperitoneal injection of 120 mg/kg of alloxan monohydrate dissolved in cold 0.9% normal saline resulted in significant (p<0.001 and p<0.0001) decreases in the serum ALB and TP while it did not cause significant (p>0.05) increases in the serum TB, CB, AST and ALT when compared to the untreated normal (Group I) values (Tables 3a and 3b). Conversely, alloxan injection did not cause significant (p>0.05) decreases in the serum ALP levels when compared to Group I values (Table 3b). However, with repeated daily oral treatments with 125-500 mg/kg of MLASE for 8 days, there were significant (p<0.001 and p<0.0001) dose-related increases in the serum

Table 5. Effect of repeated oral treatments with 125-500 mg/kg of MLASE on hepatic tissue SOD, CAT, GSH, GPx and MDA levels in alloxan-induced hyperglycemic rats

Groups	SOD (U/mg protein)	CAT (U/mg protein)	GSH (U/mg protein)	GPx (U/mg protein)	MDA (nM/mg protein)
I	63.2 ± 6.5	10.6 ± 1.2	0.4 ± 0.1	0.5 ± 0.0	0.1 ± 0.0
II	40.8 ± 1.4^{a-}	7.2 ± 0.4^{a-}	0.2 ± 0.0^{a-}	0.8 ± 0.1^{c}	0.4 ± 0.2^{c}
III	79.0 ± 6.3^{a+}	13.7 ± 1.8^{c+}	1.1 ± 0.2^{c+}	0.4 ± 0.1^{d-}	0.0 ± 0.0^{f-}
IV	76.6 ± 3.5^{c+}	8.4 ± 0.4^{b+}	0.6 ± 0.0^{b+}	0.5 ± 0.0^{d-}	0.0 ± 0.0^{f-}
V	78.7 ± 3.2^{c+}	12.5 ± 0.4^{c+}	0.6 ± 0.0^{c+}	0.4 ± 0.1^{d-}	0.0 ± 0.0^{f-}
VI	82.9 ± 1.7^{c+}	13.7 ± 0.47^{c+}	0.8 ± 0.0^{c+}	0.4 ± 0.0^{d-}	0.0 ± 0.0^{f-}

$^{a-}$ represents a significant decrease at $p<0.05$ while ^{c} represents a significant increase at $p<0.0001$ when compared to Group I values. $^{a+, b+}$ and $^{c+}$ represent significant increases at $p<0.05$, $p<0.001$ and $p<0.0001$, respectively, while $^{d-}$ and $^{f-}$ represent significant decreases at $p<0.05$ and $p<0.0001$, respectively, when compared to Group II values. I = Control, II = Diabetic + 10 ml/kg of DW, III = Diabetic + 5 mg/kg of glibenclamide, IV = Diabetic + 125 mg/kg MLASE, V = Diabetic + 250 mg/kg MLASE, VI = Diabetic + 500 mg/kg MLASE.

ALB and TP when compared to those recorded for the untreated hyperglycaemic (Group II) rats (Table 3a). However, the MLASE repeated oral treatment for 8 days did not cause significant ($p>0.05$) alterations in the serum TB, CB, AST, ALT and ALP levels in the Group IV-V rats when compared to the values obtained in Group II (Tables 3a and 3b).

Effect of 125-500 mg/kg of MLASE on renal tissue oxidative stress markers in alloxan-induced hyperglycemic rats

Treatment with 150 mg/kg of alloxan given intraperitoneally resulted in significant ($p<0.0001$, $p<0.001$ and $p<0.05$) reductions in the renal tissue SOD, CAT activities and GSH levels, respectively, while significantly ($p<0.0001$) enhancing the GPx and MDA activities when compared to untreated normal (Group I) (Table 4). However, oral treatments with 125-500 mg/kg/day of MLASE significantly ($p<0.001$ and $p<0.0001$) reversed these effects dose dependently with the most significant improvement recorded for the group treated with the highest dose (500 mg/kg) of MLASE when compared to untreated diabetic group (Group II) (Table 4).

Effect of 125-500 mg/kg of MLASE on hepatic tissue oxidative stress markers in alloxan-induced hyperglycemic rats

Similarly, intraperitoneal injection of 150 mg/kg of alloxan and its subsequent establishment of diabetes resulted in significant ($p<0.05$) reductions in the renal tissue SOD, CAT activities and GSH levels while significantly ($p<0.0001$ and $p<0.05$) enhancing the GPx and MDA activities, respectively when compared to untreated normal (Group I) (Table 5). Repeated oral treatments with 125-500 mg/kg/day of MLASE for 8 days significantly ($p<0.05$, $p<0.001$ and $p<0.0001$) reversed these effects dose dependently with the most significant improvement recorded for the group treated with 500 mg/kg of MLASE when compared to untreated diabetic group (Group II) (Table 5). Also, 500 mg/kg/day of MLASE offered by better ameliorative effect than that offered by the standard oral antihyperglycemic drug (10 mg/kg of glibenclamide) (Table 5).

DISCUSSION

Diabetes mellitus is a multi-systemic metabolic disorder affecting body organs such as kidney, testis, liver, heart, eyes and the brain to a varying degree depending on the severity and chronicity of the persistent hyperglycemia (Fowler, 2008; Satirapoj, 2010; Forouhi and Wareham, 2019).

The kidney is an extremely complex organ with broad ranging functions in the body, including, waste excretion, ion and water balance, maintenance of blood pressure, glucose homeostasis and generation of erythropoietin or activation of vitamin D (Forbes and Cooper, 2012; Hesaka *et al.*, 2019). With diabetes, many of these integral processes are interrupted via a combination of hemodynamic and metabolic changes; hyperglycemia also activates a series of changes leading to glomerular and tubular dysfunction and accelerates glomerular cell apoptosis (Ayodele *et al.*, 2004; Forbes and Cooper, 2012). Similarly, the liver plays an important role in the regulation of glucose, lipid and protein homeostasis, most especially, in the maintenance of blood glucose homeostasis, because it warehouses superfluous blood glucose and demobilizes same in hypoglycemic states (Indradevi *et al.*, 2012). Furthermore, the liver is the focal organ of oxidative and detoxifying processes as well as free radical reactions and the biomarkers of oxidative stress are elevated in the liver at an early stage in many diseases, including diabetes mellitus (Stadler *et al.*,

2003). In experimental diabetes, alloxan monohydrate exerts its toxic effects on kidney, liver and other organs in addition to pancreatic cells after it is accumulated intracellularly to generate cytotoxic free radicals. The pancreatic insulin insufficiency and hyperglycemia that result from pancreatic β-cell necrosis further augment renal and hepatocellular damages through reactive free radicals mediated lipid peroxidation of glomerular and hepatocellular membranes (Kume *et al.*, 2004).

In the present study, there were profound elevations in the serum levels of both hepatic and renal function parameters following the establishment of diabetes with intraperitoneal injection of alloxan monohydrate. In addition, activities of hepatic and renal tissue SOD, CAT and GSH were profoundly inhibited while those of GPx and MDA were significantly enhanced. The observations are similar to those earlier reported that oxidative stress plays a pivotal in the pathophysiology of hepatic and renal dysfunctions in the diabetic state (Albano, 2008; Kashihara *et al.*, 2010; Hahr and Molitch, 2015). Literature has it that the pathogenesis of diabetic nephropathy and hepatic dysfunction is multifactorial in which chronic hyperglycemia plays an important (Palsamy *et al.*, 2010). During diabetic milieu, supraphysiological glucose is known to result in oxidative stress from AGEs formation and the mitochondrial free radicals generation with consequent cell death, hepatic and renal dysfunctions (Forbes *et al.*, 2008). The fact that repeated oral treatments with MLASE profoundly ameliorated alterations in the hepatic and renal function and oxidative stress parameters strongly highlight the protective potential of MLASE against diabetic hepatic and renal dysfunctions. These antioxidant results of this study are strongly in consonance with the previous report of Domekouo *et al.* (2016) which reported the antioxidant properties of *Morinda lucida* aqueous stem bark extract in streptozotocin induced hyperglycemic rats orally treated with 50-500 mg/kg/day of extract for 28 days. These *in vivo* findings appeared to have been corroborated by the positive results of the *in vitro* free radicals scavenging and antioxidant studies also undertaken in the current study.

The hepatorenal protections offered by MLASE could be attributed to the presence of flavonoids, alkaloids, saponin, terpenoids, phenols, tannins and phlobatinnins in MLASE (Adeneye *et al.*, 2017) which are known to exhibit potent antioxidant activities either singly or in combination with one another as literature has shown that flavonoids, alkaloids, and other phenolic compounds to be reputably potent antioxidant phytochemicals (Rice-Evans *et al.*, 1995; Procházkova *et al.*, 2011; Orčić *et al.*, 2011). Similarly, previous other studies have attributed the antioxidant profile of *Morinda lucida* to its high phenolic contents (Ojewunmi *et al.*, 2013).

In conclusion, results of the study showed the ameliorative role of MLASE on renal and hepatic functions in the diabetic state mediated via free radical scavenging and antioxidant activities, thus, setting the scene for considering MLASE as an effective therapy in the management of not only diabetes but its associated hepatic and renal dysfunctions.

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Cognitive Dysfunction Among *Primi gravidae* Attending an *Ante Natal* Clinic in Kano, Northwest Nigeria

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Summary: Cognitive deficits among pregnant women have been reported, though the evidence is equivocal. Cognitive dysfunction during pregnancy may have negative consequences on maternal and child health. Yet, very little is known about cognitive function of pregnant women in general and *primi gravidae* in particular in the area under study. This study aimed to evaluate cognitive function among *primi gravidae*. About 120 *primi gravidae* were studied in a large urban hospital in Kano. Mini-Mental State Examination (MMSE) was used to evaluate cognitive function; socio-demographic and clinical data were obtained. Data were processed using IBM SPSS statistics version 20.0. Significant values of *P* were those < 0.05. The median age of the *primi gravidae* was 20.0 (3) years; 80 % of them had secondary school education, 88.3 % were in their third trimester of pregnancy and 42.5 % did not have any medical complaints at the time of presentation. The median MMSE score of the *primi gravidae* was 22.00 (19.0), indicating mild cognitive impairment. Majority of the women (88.3%) had either mild (58.3%) or severe (30%) cognitive dysfunction. The cognitive dysfunction was influenced by level of education (X^2 = 11.961, P = 0.003) and type of presenting complaints (X^2 = 13.514, P = 0.036). There was significant association between the mild cognitive impairment and level of education (X^2 = 11.426, P = 0.022). This study concluded that the *primi gravidae* had mild cognitive impairment; with majority (88.3%) of them having cognitive dysfunction, which was significantly associated with level of educational attainment and was influenced by the level of education and presenting complaints.

Keywords*:* cognitive impairment, cognition, *primi gravidae*, pregnancy, Kano, Nigeria.

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INTRODUCTION

Cognition (or cognitive function) is the process of consciously using one's brain. It involves higher brain functions such as learning, memory, attention, executive function, problem solving and thought. Pregnancy is a period in a female's life from conception to delivery of an offspring. Pregnancy has been associated with cognitive slips during the activities of daily living of the pregnant woman (Crawley *et al.,* 2008). Indeed, there is fairly well documented scientific evidence behind this observation. The pregnant woman comes under the influence of an array of physical, physiological, psychological changes and socio-cultural events occurring during the pregnancy; and these changes may have negative consequences on cognitive function.

Buckwalter *et al*. (1999) reported cognitive changes and related them to hormonal levels during pregnancy. Other studies also reported cognitive deficits among pregnant women (Casey *et al.*, 1999; Christensen *et al.*, 2010). Pregnant women are at risk of depression (Parcells, 2010). In fact, specific types of cognitive dysfunction due to depression in pregnancy and post-partum period have been reported (Larry, 2017). Despite this, the evidence of cognitive dysfunction among pregnant women is equivocal and little is known about how these deficits progress with time (De Groot *et al.,* 2006).

Logically, the changes occurring during pregnancy would be even more obvious in women having their pregnancy for the first time (*primi gravidae*). *Primi gravidae* reported significant sleep disruptions which was a significant predictor of their memory changes (Janes *et al.,* 2009). Parson *et al*. (2004) suggested that first-time mothers had worse cognitive deficit than women who have previously had children.

Cognitive dysfunction during pregnancy may have negative consequences on maternal and child health with consequent poor pregnancy outcome and poor health status of the offspring. Yet, very little is known about cognitive function in pregnant women in general, and *primi gravidae* in particular in the

environment under study. Based on previous reports, we hypothesize that *primi gravidae* in this environment will have significant cognitive dysfunction. It is pertinent, therefore, to study the nature and magnitude of cognitive dysfunction among *primi gravidae*. This study aimed to evaluate cognitive function among *primi gravidae* attending an antenatal clinic in Kano. Knowledge about cognitive changes during pregnancy may lead to better understanding and development of effective management strategies.

MATERIALS AND METHODS

Study setting and ethical considerations: The study area was Murtala Mohammed Specialist Hospital, a 250-bed capacity hospital located in the city of Kano, Nigeria. It offers services at all levels of healthcare and runs antenatal clinic which attends to about 1000 pregnant women per week, out of which about 60 are *primi gravidae*. Ethical approval for the study was given by the Health Research Ethics committee, Kano State Ministry of Health (Ref.: MOH/Off/797/T.I/464; dated 31/07/2017). Each participant voluntarily consented to the study and signed informed consent form before the commencement of the study. The study conformed to the provisions of the declaration of Helsinki 1995 (as reviewed in Tokyo in 2004).

Study population and sampling: The study population comprised of about 240 *primi gravidae* attending the antenatal clinic per month, out of which 120 were selected for this cross-sectional study using systematic sampling.

Eligibility and exclusion criteria: All the *primi gravidae* attending the *ante natal* clinic were eligible for inclusion in the study. However, those with on-going fever, diarrhea and respiratory tract infection, presence or history of mental illness or any chronic disease not related to pregnancy such as hypertension or diabetes were excluded from the study.

Data collection: Socio-demographic, anthropometric and clinical data were obtained during an interview and physical examination and recorded into a data capture form specially designed for this study. Mini-Mental State Examination (MMSE) was used to evaluate cognitive function. The cognitive assessment was conducted by a health personnel (rater) who was specially trained for that purpose.

Briefly, the MMSE measured orientation to time and place, immediate recall, short-term verbal memory, calculation, language, and construct ability. Each area tested has a designated point value, with the maximum possible score on the MMSE being 30/30. Levels of cognitive dysfunction were classified as: severe (0 – 17), mild (18- 23) and normal (24- 30). The MMSE was originally introduced by (Folstein *et al.*, 1975), in order to differentiate organic from functional psychiatric patients and incorporated tests which were in use prior to its publication. The standard MMSE form which is currently published by Psychological Assessment Resources is based on its original 1975 conceptualization, with minor subsequent modifications by the authors.

Statistical Analyses: Data were processed using IBM SPSS statistics version 20.0 (SPSS Inc., IL., USA). Values were summarized using frequencies (and percentages) or median (and interquartile range). Cognitive function was compared between categories of socio-demographic and clinical characteristics using Kruskal-Wallis test, while the relationship of cognitive function with these characteristics was evaluated using Chi-square test for (categorical variables) or Kendall's tau b (numerical variables). Values of $P < 0.05$ were considered significant

RESULTS

Socio-demographic characteristics of the participants: The median age of the *primi gravidae* was 20.0 (3) and 90% of them were within the age range of 16 – 25 years (Table 1). Most of them were urban-dwelling (75.8%), unemployed (68.3%) fulltime house-wives who were educated up to the secondary school level (80%), with family income above the poverty line (2 USD/day) for about 98 % of them.

Clinical characteristics of the participants: Most of the women studied were in their third trimester of pregnancy (88.3%) with singleton fetus (72.5%) and 42.5% did not have any medical complaints at the time of presentation (Table 2).

Table 1: Influence of socio- demographic characteristics on cognitive function among *primi gravidae*

Characteristics	MMSE Score	N (%)	Test of Significance
Age			
16- 25	22.00 (5.5)	108 (90)	$X^2 = 0.065$
26- 35	22.00 (7.5)	12 (10)	df = 1
36-45	0	0 (0)	$P = 0.799$
Level of Education			
Primary	20.00 (4.5)	17 (14.2)	$X^2 = 11.961$
Secondary	23.00 (5.9)	96 (80)	df = 2
Post- Secondary	26.50 (7.5)	7 (5.8)	$P = 0.003$
Occupation			
Fulltime housewives	22.00 (6.5)	82 (68.3)	$X^2 = 1.116$
Self- employed	22.50 (4.3)	34 (28.3)	df = 2
Civil Servant	23.50 (7.9)	4 (3.3)	$P = 0.572$
Income (per month)			
≤ ₦10,999 (30 USD)	0	0 (0)	$X^2 = 4.048$
₦11,000- ₦21,999	19.75	2 (1.7)	df = 2
₦22,000- ₦43,999	21.50 (6.9)	76 (63.3)	$P = 0.132$
≥ 44,000	23.00 (4.1)	42 (35)	
Area of Residence			
Urban	23.00 (5)	91 (75.8)	$X^2 = 1.118$
Semi-urban	21.25 (7.6)	24 (20)	df = 2
Rural	20.00 (4.3)	5 (4.2)	$P = 0.572$

Table 2: Influence of clinical characteristics on cognitive function among *primi gravidae*

Characteristics	MMSE Score	N (%)	Test of Significance
Weight (kg)			
40 - 49.9	23.00 (8.8)	17 (14.2)	$X^2 = 3.822$
50 - 59.9	23.00 (6.5)	55 (45.8)	df = 4
60 - 69.9	21.00 (6.5)	31 (25.8)	$P = 0.353$
70 - 79.9	23.00 (3.3)	12 (10.0)	
80 - 89.9	20.00 (7.0)	5 (4.2)	
Height (m)			
1.4 - 1.49	22.00 (7.5)	4 (3.3)	$X^2 = 0.502$
1.5 - 1.59	21.5 (4.5)	39 (32.5)	df = 2
1.6 - 1.7	23.00 (6.5)	77 (64.2)	$P = 0.302$
BMI (kg/m^2)			
< 18.5	32.00 (9.0)	7 (5.8)	$X^2 = 1.861$
18.5 - 24.9	22.00 (7.3)	90 (75.0)	df = 3
25.0 - 29.9	20.50 (3.5)	16 (13.3)	$P = 0.602$
≥ 30	22.00 (4.5)	7 (5.8)	
Gestational age			
1st trimester	21.25	2 (1.7)	$X^2 = 0.604$
2ndtrimester	23.50 (6.9)	12 (10)	df = 2
3rd trimester	22.00 (5.6)	106 (88.3)	$P = 0.739$
Number of fetuses			
Unknown	22.75 (6.6)	30 (25)	$X^2 = 3.785$
Singleton	22.00 (5.0)	87 (72.5)	df = 2
Twin	27.00	3 (2.5)	$P = 0.151$
Presenting complaints*			
Nil	23.00 (5.5)	51 (42.5)	$X^2 = 13.514$
Nausea	21.00 (8.0)	7 (5.8)	df = 6
Vomiting	18.50 (4.5)	7 (5.8)	$P = 0.036$
Fatigue	26.00 (6.5)	9 (7.5)	
Tiredness	22.00 (9.5)	12 (10)	
Others	19.50 (5.6)	6 (5)	
More than one complaint	21.25 (4.3)	28 (23)	

*Significant difference between the categories

Table 3: Relationship of cognitive function with socio-demographic and clinical characteristics of *primi gravidae*

Characteristics	Test of Relationship	
Socio-demographic	Chi-square	*P*
Age	0.397	0.820
Level of Education	11.426	0.022*
Occupation	2.979	0.561
Family Income	34.625	0.181
Area of Residence	3.931	0.415
Pregnancy		
Gestational age	4.76	0.313
Number of Fetuses	2.181	0.703
Presenting Complaints	19.056	0.087
Anthropometric	Kendall's tau-b	*P*
Weight	-0.068	0.290
Height	0.046	0.477
BMI	-0.090	0.154

*Significant difference between the categories

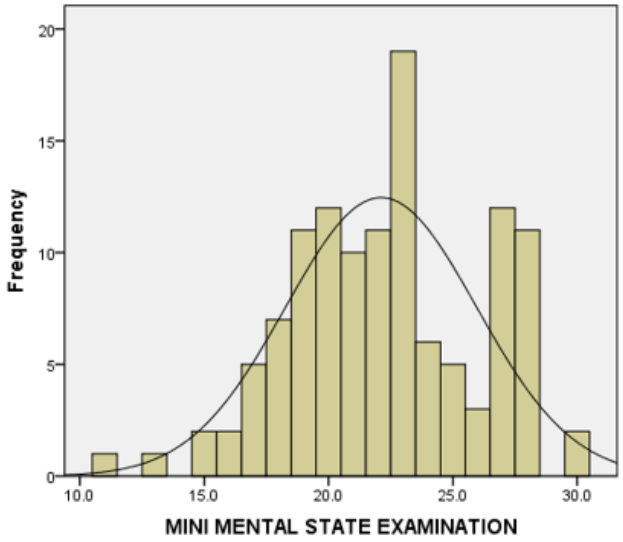

Figure 1: Frequency distribution of MMSE scores of *primi gravidae*

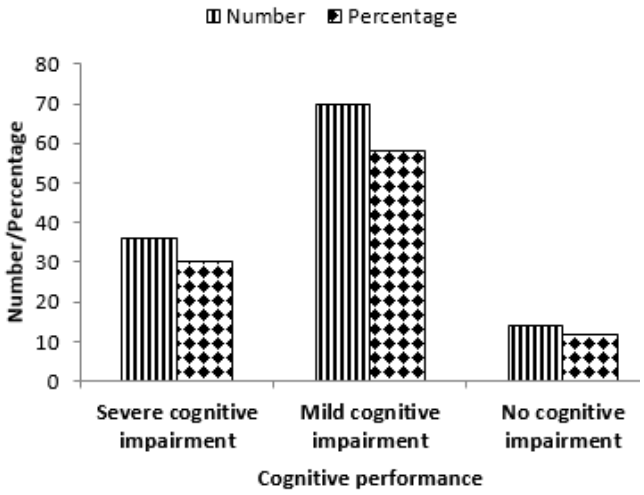

Figure 2: Distribution of cognitive function among *primi gravidae*

Cognitive function of the participants: The median MMSE score of the *primi gravidae* was 22.00 (19.0), indicating mild cognitive impairment and this variable had skewed distribution (Kalmagorov-Smirnov statistic = 0.232, $P = 0.001$) (Fig. 1). Only 11.7% of the women had normal cognitive function, while the majority of them (88.3%) had either mild (58.3%) or severe (30%) cognitive dysfunction (Fig. 2). The cognitive performance of the participants was influenced by their level of education (X^2=11.961, $P = 0.003$) and nature of presenting complaints ($X^2 = 13.514$, $P = 0.036$) with vomiting accounting for the lowest cognitive performance (Table 2). Weight, height, BMI, gestational age and number of fetuses did not significantly influence cognitive function ($P > 0.05$). There was significant association between the mild cognitive impairment and level of education ($X^2 = 11.426$, $P = 0.022$). However, there was no association between the cognitive impairment observed and any socio-demographic or clinical characteristics of the participants (Table 3).

DISCUSSION

This study assessed cognitive function among 120 *primi gravidae* attending antenatal clinic in an urban hospital. It was found that 88.3% of the *primi gravidae* had cognitive dysfunction, either mild (58.3%) or severe (30%).

Cognitive dysfunction refers to deficits in attention, verbal and non-verbal learning, short-term and working memory, visual and auditory processing, problem solving, processing speed, and motor functioning (Raymond *et al.,* 2014). The reported cognitive dysfunction could be due to stress of pregnancy and influence of hormonal changes during pregnancy as reported earlier (Jessica *et al.,* 2012). During pregnancy, levels of some steroid hormones such as estradiol and progesterone increase by up to 30- and 70-fold, respectively, in comparison to non-pregnant levels (Tulchinsky *et al.,* 1972).

During pregnancy, a considerable number of women experience some degree of cognitive changes that has come to be colloquially called pregnancy brain (Casey *et al*., 1999). The symptoms most frequently reported by women are forgetfulness and memory disturbances (Casey *et al.,* 1999), poor concentration, increased absentmindedness and difficulty reading (Parsons *et al*., 1991).

The result of this study agrees with previous reports which have shown that *primi gravidae* demonstrated impairment in selective abilities such as verbal free recall and working memory (Henry and Rendell, 2007). Evidence from prospective studies also supports the idea that changes in cognitive function occur during pregnancy, but it is unclear when the impairments occur (De Groot *et al.,* 2006). Performance on paragraph recall (Keenan *et al.,* 1998) and cognitive speed task (Chris *et al*., 2010) was worse in the third trimester. This is also in agreement with the present study since majority (88.3%) of the women studied, who also had PSS-10 score of 22 (within the range of impairment) were in their third trimester.

Previous studies conducted in the same locality with the present study have reported normal cognitive function in apparently healthy non-pregnant females and males (Yarube *et al*., 2019); and impaired cognition among diabetes mellitus (Yarube and Mukhtar, 2018; Yusuf *et al*., 2018) and post-stroke subjects (Hassan and Yarube, 2018). Contrary to the findings of this study, other studies have failed to find any differences in scores on objective measures of cognitive function during pregnancy (Christensen *et al.,* 1999; Casey, 2000; Crawley *et al.,* 2008).

In the present study, level of education of the participants showed a significant relationship with cognitive dysfunction. Moreover, the level of educational attainment had a significant influence on the cognitive performance, with those at the primary school level having the lowest performance and those at the post post-secondary level having not only the highest but also unimpaired performance. Similar findings were reported in previous studies (Yarube *et al*., 2019; Yusuf *et al*., 2018). The neurobiological mechanisms responsible for this association have not been fully elucidated. One possible explanation is that education impacts the rate at which plagues and tangles accumulate in the brain during memory formation and consolidation (Konstantinos *et al*., 2012). Education is considered to provide a cognitive and neurological reserve through neuronal changes or increased efficacy of processing networks (Stern, 1999). Individuals with higher levels of educational attainment tend to allocate more time and put forth more effort when engaging in intellectual complex activities (Parisi, 2010). As a result, the accumulated exposure to cognitively charged environments may have a direct beneficial effect on the brain structure and function, resulting in greater neurological development such as increase in synaptic density or efficient use of existing brain networks (Colcombe *et al.,* 2003; Park and Reuter-Lorenz, 2009). Higher educational attainment is associated with greater levels of cognitive performance as well as with a reduced risk of dementia (Bennett *et al.,* 2003). Educational experiences provide the foundation for continued intellectual stimulation across the life course, resulting in improved cognitive functioning (Stain *et al.,* 2002).

Presenting complaints significantly influenced cognitive dysfunction, with vomiting and fatigue accounting for the worst and best cognitive performances, respectively. Evidence of the cause of vomiting in pregnancy points to rapid changes in hormone levels (Suzanne, 2017) and emotional liability (Roger, 2017) observed during pregnancy.

Other socio-demographic and clinical characteristics were not significantly related to cognitive dysfunction of the participants. Consideration of the fact that the additional weight gain by a pregnant woman is as a result of her own weight gain and that of the conceptus (Mariana *et al.,* 2016) undermines the accuracy and utility of BMI in pregnancy. It's no surprise that no association was found between BMI and cognitive dysfunction. Also, age of the participants had no significant relationship with cognitive dysfunction. This is despite the known fact that measurable changes in cognition occur with normal aging (Daniel *et al.,* 2015). The absence of relationship might be due to the fact that 90% of the participants recruited were within one age range (of 16 – 25 years), which is also the youngest age category, leaving just 10% in one other category.

It will be interesting to find out the cognitive performance of non-pregnant women with similar age and some other characteristics as the participants of the present study, for comparison with what was reported in this study. This would have enabled us to more soundly conclude on the influence of pregnancy on

cognition. This is a limitation of this study and may be a good idea for another study.

This study concluded that the *primi gravidae* had mild cognitive impairment as evidenced by median MMSE score of 22.00 (19.0); majority (88.3%) of them had cognitive dysfunction either in the form of mild (58.3%) or severe (30%) cognitive impairment. This impairment was significantly associated with level of educational attainment and was influenced by the level of education and presenting complaints.

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Ovarian Weight, Follicle Count and Retrieved Oocyte Characteristics in West African Dwarf Goat Does Experimentally Infected with *Trypanosoma brucei*

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Summary: Trypanosomosis has been described as the single largest disease entity limiting livestock development in sub-Saharan Africa. The effects on ovarian weight, follicle count and retrieved oocyte characteristics in ten West African dwarf goat does (control=5, infected=5) experimentally infected with *Trypanosoma brucei* were investigated. The does were fed with elephant grass and supplement (15.23% CP) daily. Infected does received $4.8x10^5$ *T. brucei* intravenously and thereafter, all does were synchronized using Lutalyse®. The results showed that the differences between control and infected does for ovarian weight (0.68±0.56 g and 0.40±0.09 g) and follicle count (10.50±1.25 and 2.50±1.22), respectively were significant (P<0.05). The difference in retrieved-oocytes-count between control (30, 57.7%) and infected (22, 42.3%) does was not significant (P>0.05). The differences in proportion between control and infected does for well-formed-oocytes (90.5% and 9.5%), completely-denuded-oocytes (30.8% and 69.2%) and proportion per group of oocytes with substantial-investment-of-cumulus (63.3% and 9.1%), respectively were significant (P<0.05). The difference in extensively-denuded-oocytes between control (38.9%) and infected (61.1%) does was not significant (P>0.05). These findings suggest that experimental *Trypanosoma brucei* infection caused reduction in ovarian weight and follicle count, number of oocytes as well as proportion of well-formed oocytes that are capable of supporting embryonic development.

Keywords*: Trypanosoma brucei*, goat does, Lutalyse®, ovarian follicles, oocytes.

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INTRODUCTION

Artificial production of embryos or *in vitro* embryo production (IVEP) is an important procedure/technique capable of boosting animal production in any part of the world. While interest is growing in the technique with goat practitioners, it is a major focus in bovine industries (Camargo *et al.*, 2010). In Brazil, more than 40% of cattle embryos transferred to recipients in 2004 alone consisted of those produced through *in vitro* technique, involving *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culturing (IVC) of immature oocytes (Viana *et al.*, 2010). The oocytes are usually collected through ultrasound guided aspiration or from ovaries post-slaughter (Salamone *et al.*, 2001). IVM is a critical step to *in vitro* embryo production (IVEP/IVP) and the integrity of oocyte morphology at retrieval/collection is a limiting factor (Calder *et al.*, 2005; Pereira *et al.*, 2010). Cumulus-oocytes complexes (COC) with larger follicular diameter, several layers of unbroken cumulus investment and homogenous ooplasm possess greater potential for IVM (Crozet *et al.*, 1995; Gandolfi, 1996). One of the greatest challenges to global animal production is disease. In Africa, trypanosomosis is a major disease entity limiting livestock production and development (Swallow, 2000), with an estimated yearly loss of 4.5 billion USD (Affognon, 2007). *Trypanosoma congolense*, *T. vivax*, *T. brucei*, *T. evansi* and *T. equiperdum* are the most important trypanosomes causing disease in livestock species in Africa (Chitanga *et al.*, 2011). Losses to trypanosomosis have been associated with varying degrees of infertility among other symptoms (Faye *et al.*, 2004; Prashant *et al.*, 2005). The West African Dwarf (WAD) goat has huge economic importance and potential relevance to reproductive biotechnology in Nigeria. This study investigated the effects of experimental *Trypanosoma brucei* infection on ovarian weight, follicle count and retrieved oocyte characteristics in WAD goat does.

MATERIALS AND METHODS

The study was carried out at the small ruminant Unit of the Department of Theriogenology, University of Ibadan, Ibadan, Nigeria. The Unit houses a little over 100 goats, the WAD being the most predominant breed.

Ten (10) adult WAD goat does weighing between 16.0 kg and 17.5 kg were used in the study. The does

were randomly but equally divided into groups 'A' (control) and 'B' (infected). Each group was kept in a separate pen. The does were fed with Elephant grass in the mornings and commercial feedstuff containing 15.23% Crude Protein (CP) at the rate of 0.25kg/head in the afternoons. Freshwater as well as salt licks were provided *ad libitum* throughout the study. None of the animals was however allowed to graze.

Trypanosoma brucei (Kaura strain) was obtained from passaged rats from the Department of Veterinary Pathology, University of Ibadan. The parasite was originally obtained from the Nigerian Institute for Trypanosomasis Research (NITR), Vom, Plateau State, Nigeria.

Parasitaemia in albino rats was evaluated at $8.4x10^7$ trypanosomes per millilitre by haemocytometric method (Lumsden *et al.,* 1979) using blood obtained from the tail vein of the rats. Further dilution of blood was made with normal saline to obtain an inoculation dose of 4.8×10^5 trypanosomes/ 0.5ml diluted blood per doe (Leigh and Fayemi, 2010). This dose was used to infect each doe via the jugular vein.

Traces of sodium, calcium, potassium and magnesium salts along with Hepes were dissolved in Tridest water at 7.3-7.4 alkalinity and 282 mOsm and kept as stock media. To 25 ml of the stock media, 250 microliters of sodium pyruvate and gentamicin were added along with 0.075 g of bovine serum albumin to make the ready-to-use COCs media at PH 7.3-7.5 (Lonergan *et al.,* 2003; Viana and Camargo, 2007).

Both groups of does were synchronized using an earlier described method (Leigh *et al.,* 2010). Briefly, this involved the intramuscular administration of a double injection, seven days apart, of 5 mg prostaglandin F_2 alpha-Lutalyse$^{(R)}$ (Pharmacia & Upjohn Co. NY). Twelve days prior to treatment with Lutalyse$^{(R)}$ in both groups, does in group 'B' were administered 4.8×10^5 trypanosomes/ 0.5ml diluted blood.

The does (Groups 'A' and 'B') were euthanized on day 23 post Lutalyse$^{(R)}$ administration such that they would be around the first oestrus following the synchronized oestrus. Following laparoscopy, ovaries were severed from the broad ligament with the aid of a sharp knife and transported to the laboratory in labelled Thermo flasks containing phosphate buffered saline- PBS (without calcium and magnesium substituted with 1 ml Gentamycin/litre) at 38.5^0C. The weights of the ovaries were determined immediately post-harvest with the aid of a digital Top Load electronic balance (Lark Inc., Denver). At the laboratory, the ovaries were washed twice in PBS at 38.5^0C. All follicles >2 mm were counted with the aid of a magnifying glass following which follicular fluid was aspirated into 2 ml COCs media in an 18G x 1½ inch needle on plastic disposable syringe. The needle was inserted into the ovarian parenchyma underneath each follicle and by sucking back the air around it, follicular fluid containing oocytes was drained. The content of the syringe was slowly emptied into sterile centrifugation tube and placed in a warm water bath at 38.5^0C for 10 minutes. Afterwards, the top 75% of the follicular fluid was carefully decanted. With the aid of a 20 µl Gilson's pipette set at 10 µl, the oocytes were picked up from the bottom of the centrifugation tubes and washed in a petri dish containing about 2000 µl COCs media. The characteristics of retrieved oocytes were then identified under low magnification microscopy based on investment of cumulus layers (Gandolfi, 1996).

Statistical Analysis

Data were subjected to Student t- statistic (GraphPad, 2000). Values of $P \leq 0.05$ were considered significant.

RESULTS

The results are presented in Tables 1 and 2 as well as Plates 1 and 2.

Ovarian weights and number of follicles counted

Table 1 shows the differences in ovarian weights, number of follicles and number of oocytes between control and infected does. The differences between control and infected does for mean ovarian weights (0.68 ± 0.56 and 0.40 ± 0.09) g as well as number of follicles >2 mm (10.50 ± 1.25 and 2.50 ± 1.22), respectively were significant ($P<0.05$).

Retrieved oocytes and their characteristics

Table 2 shows the differences in the characteristics of cumulus-oocyte complexes retrieved from the ovaries. Among the 21 well-formed oocytes, 19 (90.5%) were obtained from control does while 2 (9.5%) were obtained from infected does ($P<0.05$). Among

Table 1: Ovarian weight and number of follicles (Mean±S.D) counted in WAD goat does experimentally infected with *T. brucei*.

Parameter (s)	Control	Infected
Weight of ovary (g)	0.68 ± 0.56	0.40 ± 0.09*
Number of follicles visible on ovaries (i.e. follicle count)	10.50 ± 1.25	2.50 ± 1.22*

*P < 0.05.

Table 2: Characteristics of oocytes retrieved from the ovaries of WAD goat does infected with *T. brucei.*

Characteristics of oocytes	Control	Infected
Well-formed oocytes (fair/substantial investment of cumulus cells - SIC)	19 (90.5%)	2 (9.5%) *
Oocytes with extensively denuded cumulus cell layers (ED)	7 (38.9%)	11 (61.1%)
Completely denuded oocytes (CD)	4 (30.8%)	9 (69.2%) *
Total	30 (57.7%)	22 (42.3%)
Proportion of SIC/group	63.3%	9.1%

*P < 0.05.

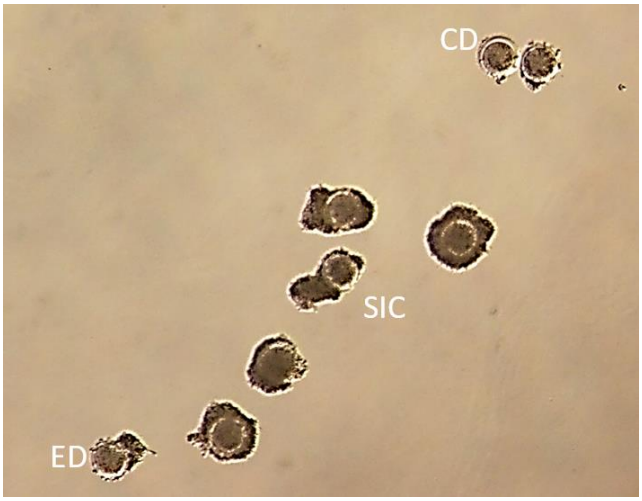

Plate 1: Cumulus oocyte complexes from control does suspended in selection media showing 1 completely denuded (CD), 2 extensively denuded (ED) and 5 with substantial investment of cumulus cells (SIC). (x100).

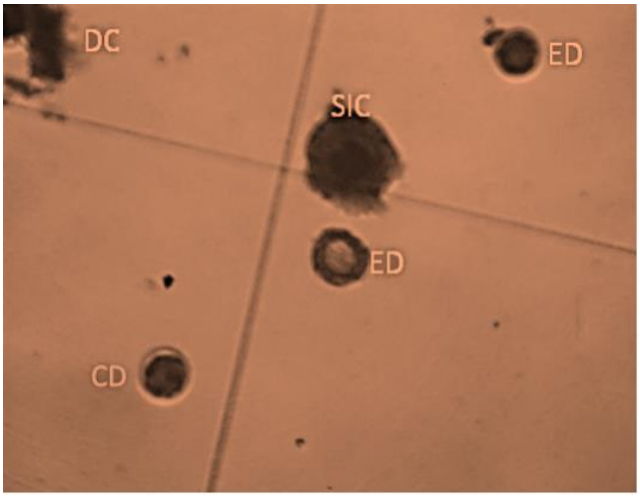

Plate 2: Cumulus oocyte complexes from infected does suspended in selection media showing 1 completely denuded oocyte (CD), 2 extensively denuded (ED), 1 with substantial investment of cumulus cells (SIC) and detached cumulus cells (DC) (x150).

extensively denuded oocytes, the difference between control (7, 38.9%) and infected (11, 61.1%) was not significant (P>0.05). For completely denuded oocytes, the difference between control (4, 30.8%) and infected (9, 69.2%) was significant (P<0.05). The difference between total number of oocytes counted: control (30, 57.7%) and infected (22, 42.3%), was not significant (P>0.05). The difference in the proportions per group, of oocytes with substantial investment of cumulus cells between control (63.3%) and infected (9.1%) does was however significant (P<0.05). Plates 1 and 2 shows the characteristics of retrieved oocytes from the ovaries of control and infected does, respectively. Plate 1 shows 1 completely denuded oocytes (CD), 2 extensively denuded oocytes (ED) and 5 oocytes with substantial investment of cumulus cells (SIC) while Plate 2 shows 1 CD, 2 ED and 1 SIC and some detached cumulus (DC) cells.

DISCUSSION

The mean ovarian weight of control does compare well with those reported for other goat breeds (Islam *et al.*, 2007; Haque *et al.*, 2016) whereas that of infected does was lower (P<0.05). The reduction in ovarian weight as shown in Table 1 may suggest concomitant reduction in physiological activities within the ovary and may be responsible for the observed reduction in numbers of ovarian follicles (P<0.05) as well as oocytes (comparative). Folliculogenesis and oocyte production are components of fertility in the female animal. A reduction in these, therefore, is suggestive of some level of infertility caused by experimental infection with *T. brucei*. An earlier study (Rodriguez *et al.*, 2013) reported key ovarian disorders such as follicular degeneration and reduced weight in goats experimentally infected with *Tyrpanosoma vivax*, similar to findings of the present study. According to these authors, the ovarian findings appeared to be involved in the aetiology of anestrus/infertility observed in the goats. Earlier, Wittmaack *et al.*, (1994) also observed that dams with poorly formed follicles often have poor oocyte recovery and cleavage in in vitro fertilization cycles and are therefore of reduced value for embryo production.

The characteristics of retrieved oocytes in the present study further clarified the infertility caused by *T. brucei*, in that <1 out of 10 well-formed oocytes, and >60% of extensively and/or completely denuded oocytes were obtained from infected does. Similarly, the proportion of well-formed oocytes to retrieved oocytes for infected does was lower (P<0.05) compared to that in control does as shown in Table 2 as well as Plates 1 and 2. Although, it is not known whether the low proportion of developmentally viable oocytes will be sufficient to achieve reproduction still, the finding suggest that experimental *T. brucei* infection led to an increase in the proportion of developmentally non-viable oocytes as well as reduction in the proportion of viable oocytes. This finding is perhaps worrisome due to the nature of the difference (P>0.05) in total retrieved oocytes between control and infected goats. Even though, the result showed a comparative reduction in retrieved oocytes of infected goats, the proportion of that, that could support embryo generation, as assessed by morphological characteristics, describes the toll of *T. brucei* on the reproductive usefulness of infected animals. Since the goal of *in vitro* embryo production is to generate numerous oocytes that are developmentally viable, infected animals may not be selected for the purpose since they are less likely to produce oocytes capable of supporting embryonic development (Crozet *et al.*, 1995). The infection may therefore lead to rejection of animals by *in vitro* embryo production industries. These findings add to existing literature on symptoms of infertility such as

anoestrus (El-Hassan *et al.*, 2005) and hypogonadism (Petzke *et al.*, 1996) that had been reported in trypanosomosis.

It is concluded that experimental *T. brucei* infection led to reduced ovarian weight, follicle count and proportion of developmentally viable cumulus oocyte complexes in West African dwarf goat does.

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Cytoarchitecture of the Hippocampal Formation in the African Giant Rat (*Cricetomys gambianus*, Waterhouse)

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Summary: The African Giant Rat, AGR is an indigenous nocturnal rodent noted for its unique olfactory and cognitive abilities. They have been deployed more recently in the detection of landmines and diagnosis of tuberculosis – two scourges that have had a tremendous negative impact on the African landscape. This olfactory-aided cognition has been linked to the hippocampus. While the anatomical infrastructure of the olfactory bulb of the AGR has been elucidated, little is known about the adaptive cytoarchitecture of the AGR hippocampal formation. This study describes the histological features, including subfields and stratifications of the AGR hippocampus using Nissl and Golgi stains. The basic cytoarchitecture of the AGR hippocampus observed in this study, with respect to stratification, subfields and cell types, is similar to those reported in the laboratory rats. Cell types identified in the AGR hippocampus include pyramidal cells, granule cells and mossy cells with mossy fibers and Schaffer collaterals also delineated. Hippocampal proper subfields CA1 to CA4 were identified. CA3 pyramidal neurons formed a well-defined cell layer starting in between the upper and lower ends of the dentate gyrus and had larger, more distinct pyramidal cells and higher cell layer thickness (240.0±6.0 µm) relative to subfields CA1 (87.0±2.0 µm) and CA2 (109.0±4.20 µm) with significant statistical differences at $p<0.001$. The detailed, delicate arrangement of various cell types and subfields, intricate wiring with synapses and laminar organization of the hippocampal formation noticed in the AGR strongly supports the canonical trisynaptic circuitry of the hippocampus. It will however be necessary to carry out densitometric studies and detailed neurochemical profiling of the AGR hippocampus to fully elucidate the functional leverage of this unique rodent. We, therefore, suggest the suitability of this rodent as a model for olfaction-linked memory studies.

Keywords*:* African giant rat, brain, cornu ammonis, dentate gyrus, hippocampus, histology.

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INTRODUCTION

The African Giant Rat (AGR) also called Sniff rat, is a large nocturnal rodent with very poor eyesight and so it depends on its keen sense of smell (Kalan, 2014). This adaptive feature has further been buttressed by possessing relatively large olfactory bulbs (Nzalak *et al.,* 2005; Olude *et al.,* 2014a; 2014b). In addition, this unique African rodent is also noted for its high cognitive abilities (Olude *et al.,* 2014a). These two attributes (olfaction and cognition) have been positively explored by a registered Belgian non-governmental organization, APOPO, in the detection of landmines and diagnosis of tuberculosis – two scourges that have had a tremendous negative impact on the African landscape (Weetjens *et al.,* 2009; Mahoney *et al.,* 2012; Carrington, 2014; Poling *et al.,* 2015). This olfactory-aided cognition has been linked to the hippocampus (Vanderwolf, 1992; Herz and Engen, 1996). The hippocampal formation, a prominent C-shaped structure on the floor of the temporal horn of the lateral ventricle, consists of the hippocampal proper, dentate gyrus, subiculum and entorhinal area (Kunzle and Radtke-Schuller, 2001; Schultz and Engelhardt, 2014). Histologically, it is partitioned into region and sub-fields according to the neuronal cell body location, cell body shape and size, proximal terminations, complex spines, distal branching characteristics as well as afferent and efferent projections (Turner *et al.,* 1998).

Vanderwolf (1992) demonstrated an increased hippocampal activity, specifically at the hilus of the dentate gyrus, following an olfactory stimulus in rats and suggested sniffing as an olfactory input to the dentate gyrus of the hippocampus. Interestingly, he observed that these heightened hippocampal activities were not elicited by visual, auditory, or somatosensory

inputs and is not related to motor activity. Thus, olfaction seems to be the sensory modality that is physically closest to the limbic system of which hippocampus and the amygdala are a part (Herz and Engen, 1996). The direct connection established by the olfactory bulb and piriform/olfactory cortex on these two structures sustains the unique ability of odours to activate emotions and memory (Mouly and Sullivan, 2010). While the anatomical infrastructure of the olfactory bulb of the AGR has been elucidated by Olude et al., (2014a), little is known about the adaptive cytoarchitecture of the AGR hippocampal formation as it relates to the transfer of procedural learning into declarative long term memory. This work thus reports preliminary findings in the cytoarchitecture of the AGR hippocampal formation, as part of an ongoing work aimed at providing probable evidence-based explanation for its cognitive capacities. This study describes the histological features, including subfields and stratifications of the AGR hippocampus using Nissl and Golgi stains.

MATERIALS AND METHODS

Experimental Animals, Perfusion and Brain Harvest

A total number of 5 adult male AGRs acquired from the wild by local hunters in Southwestern Nigeria were purchased from a local market for the purpose of this experiment. They were observed physically to exclude any physical deformities that may interfere with the study; stabilized for 48 hours and fed *ad libitum*. The body weights of the AGR were obtained using a dial spring scale (CAMRY® J1610494297). Animals were anaesthesized with Ketamine (50mg/kg) and Xylazine (5mg/kg), perfused with 4% paraformaldehyde transcardially and sacrificed. Their brains were carefully harvested from the cranium with the use of a bone nipper as described by Olude *et al.*, (2014a). Harvested brains were subsequently post-fixed in 4% paraformaldehyde for 48 hours. Coronal sections through the optic chiasma at the temporal lobes were made for all brain samples and were embedded in paraffin blocks for Nissl (Cresyl Violet) staining to illustrate the neuronal somata and cytoarchitecture of the hippocampus. Golgi Silver impregnation stain was used for highlighting individual neuronal morphology, as well as their axonal and dendritic arborizations.

Histological Staining

Nissl (Cresyl Violet)

Brain sections were mounted on frosted glass slides and were baked in a pre-heated oven for 20-30mintues prior to staining. The slides were initially deparaffinized for 5 minutes each in Xylene twice and then in equal parts of xylene and absolute alcohol. They were subsequently hydrated in descending grades of alcohol (100%, 90%, 70% and 50%) and immersed in Cresyl violet solution (2.50g Cresyl violet in 500 mls distilled water) for 8 minutes, rinsed in distilled water for one minute. Sections were then dehydrated through ascending grades of alcohol (70% Ethanol; 95% Ethanol + glacial acetic acid until satisfactory differentiation was observed; 95% Ethanol; 100% Ethanol). Stained slides were then coverslipped with DPX mountant

Golgi Stain

The tissue blocks were placed in the fixation solution (60mls of 3% potassium bichromate in 20mls of 10% formalin) for 24 hours after which they were transferred into the 3% potassium bichromate for 7 days in the dark. The solution was replaced with a fresh one each day. After the 7 days, the tissue blocks were transferred into 2% silver nitrate solution for 3 days at room temperature in the dark (filter paper was used to absorb excess solution on the blocks before putting into silver nitrate solution). The sections were then cut at 60µm thick into distilled water, mounted on glass slides and air dried for 10 minutes. Sections were dehydrated through 95% and 100% alcohol then cleared in xylene and coverslipped with Entellan (Sigma Aldrich).

Photomicrography, Image Analysis, Quantification and Data Analysis

All stained sections were viewed under the light microscope (Olympus® CX21FS1). Images of several fields of each stained tissue sections and the observed histological features of the hippocampal formation including subfields, stratification and cytoarchitecture were captured in a clock-wise manner and then, stitched together to produce a single photomicrograph of each hippocampus using AmScope® Digital Camera Software. All images were processed and labelled with Coreldraw version X7. Heat map of the dentate gyrus was generated using an image processing application (Picassa Version 3.9.0) to further highlight dendritic arborizations of the dentate granule neurons in the molecular layer of the dentate gyrus and outline the dentate somata in the granule cell layer. Average pyramidal cell layer heights of the hippocampal subfields (CA1, CA2 and CA3) were quantified by randomly measuring seven different points along each subfields using Image J 1.46r application package (1.60_20, NIH, USA).

Statistical Analyses

Data generated were expressed as Mean±SEM, and analyzed for significant difference ($p<0.05$) with one-way ANOVA using Graphpad Prism® version 4.0.

RESULTS

The term “hippocampal formation” which is a complex of the dentate gyrus, the hippocampus proper (CA1 – CA4), the subiculum and the entorhinal cortex were observed in all coronal sections of the AGR brains stained by the Nissl technique (Figure 1a).

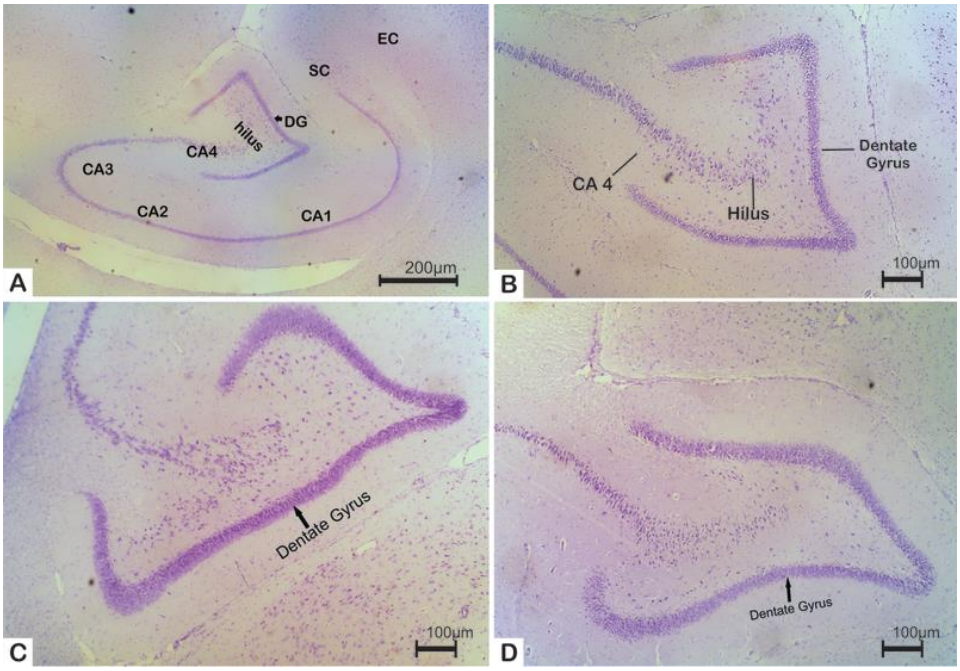

Figure 1: Nissl stained sections of the AGR hippocampus **[a]** showing various subfields (CA1-CA4) of the hippocampus proper, the dentate gyrus (DG), subiculum (SC) and entorhinal cortex (EC). Morphological plasticity was noticed in dentate gyrus presenting with three different shapes: **[b]** angled C-shaped dentate gyrus **[c]** wedge-shaped dentate gyrus and **[d]** V-shaped dentate gyrus.

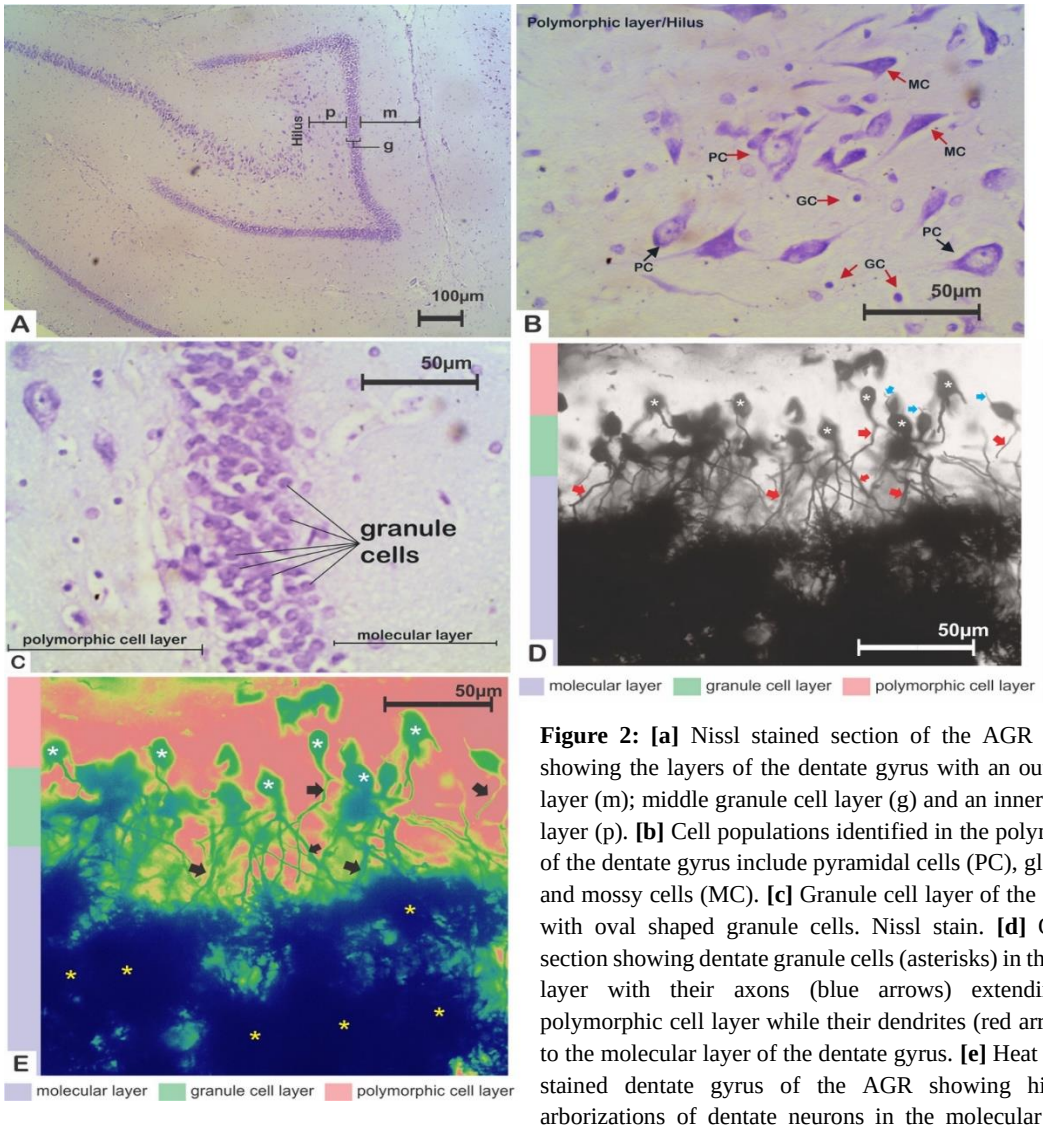

Figure 2: [a] Nissl stained section of the AGR hippocampus showing the layers of the dentate gyrus with an outer molecular layer (m); middle granule cell layer (g) and an inner polymorphic layer (p). **[b]** Cell populations identified in the polymorphic layer of the dentate gyrus include pyramidal cells (PC), glial cells (GC) and mossy cells (MC). **[c]** Granule cell layer of the dentate gyrus with oval shaped granule cells. Nissl stain. **[d]** Golgi stained section showing dentate granule cells (asterisks) in the granule cell layer with their axons (blue arrows) extending into the polymorphic cell layer while their dendrites (red arrows) extends to the molecular layer of the dentate gyrus. **[e]** Heat map of Golgi stained dentate gyrus of the AGR showing high dendritic arborizations of dentate neurons in the molecular layer of the dentate gyrus and outline of dentate somata in the granule cell layer.

Dentate Gyrus

The dentate gyrus is seen as a separate structure, it appeared as V-shaped in some sections, wedge-shaped and 'angled' C-shape in others with its concave part opened towards the hippocampus proper (Figures 1b, c and d). It is made up of the fascia dentate and the hilus. The dentate gyrus of the AGR was noted to be three-layered namely: the inner multiform/polymorphic layer, the middle granule cell layer and the outer molecular layer (Figure 2a).

The multiform or polymorphic layer is the innermost layer containing polymorphic nervous cells. It is continued into the hilus, after the granule cell layer of the dentate gyrus. Cell populations identified include the hilar mossy cells, glial cells and pyramidal cells (Figure 2b).

The granule cell layer of the dentate gyrus is the middle layer that contains oval shaped dentate granule cells with Nissl stain (Figure 2c). Mossy fibers (axonal projections of the granule cells) were projected through the polymorphic cell layer and hilus of the dentate gyrus to the hippocampus proper. The dendrites of these dentate granule cells were observed to be directed towards the molecular layer of the dentate gyrus (Figure 2d and 2e).

The molecular layer of the dentate gyrus of the AGR with Nissl stains revealed scanty cellular populations relative to its neuropil (Figure 2c) and also contains a synapse of dendrites. This was further corroborated by the Golgi stains showing majorly spiny dendrites of granule cells of the dentate gyrus (Figure 2d and 2e).

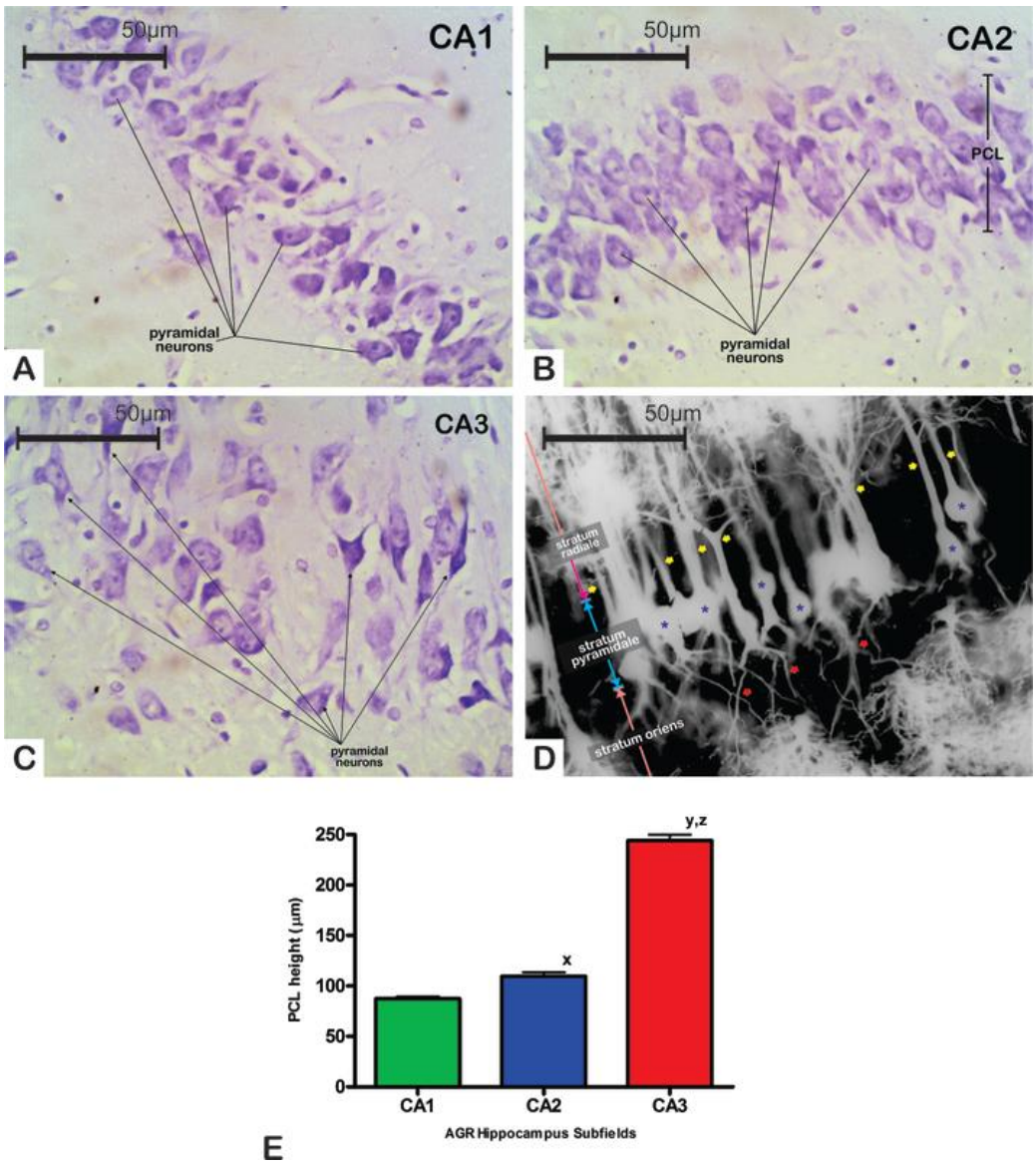

Figure 3: Nissl stained sections of the AGR hippocampal proper subfields showing **[a]** the pyramidal cell layer of CA1 subfield with less densely packed pyramidal neurons of approximately 3-4 cell layer thick; **[b]** the pyramidal cell layer (PCL) containing tightly stacked pyramidal neurons of the CA2 subfield and **[c]** abundant and relatively larger CA3 pyramidal neurons with intense cytoplasmic stain compared to CA1 and CA2 subfields. **[d]** Golgi stained section of CA3 subfield showing somata pyramidal cells (blue asterisks) with their axons (Schaffer collaterals; yellow arrows) and dendrites (red arrows) extending into the stratum radiale and stratum oriens respectively (image inverted). **[e]** Pyramidal cell layer (PCL) height of the different AGR hippocampal proper subfields. x = *statistically significant difference between CA1 and CA2; y & z = statistically significant differences between CA1 & CA3, and CA2 & CA3 respectively at p value < 0.01.*

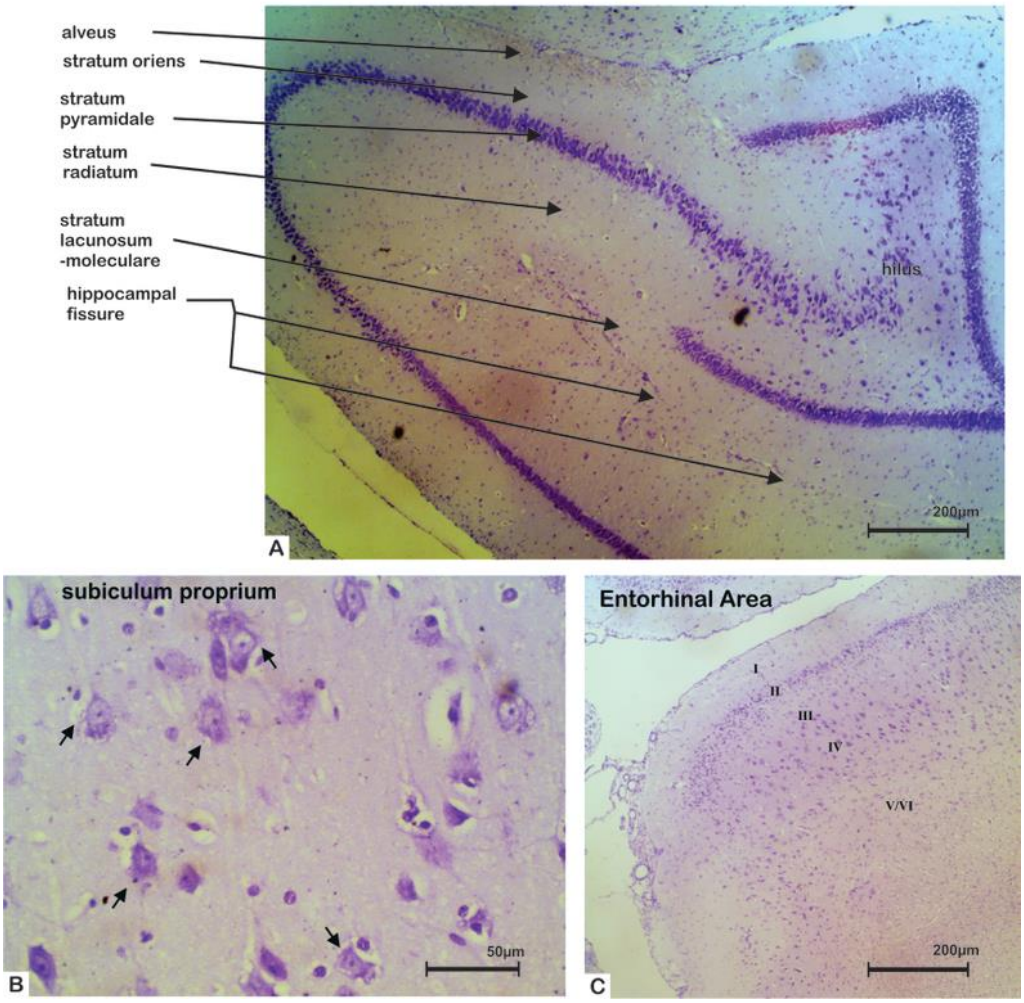

Figure 4: Nissl stained section of the AGR hippocampus showing **[a]:** the layers of the hippocampus proper, **[b]:** loosely packed deep layer pyramidal cells in the region of the subiculum proprium and, **[c]:** the entorhinal area of the hippocampal formation with the six layers (I-VI) of the entorhinal cortex

Hippocampus Proper

The hippocampus proper of the AGR had four subfields namely: Cornu Ammonis 1 (CA1); Cornu Ammonis 2 (CA2); Cornu Ammonis 3 (CA3) and Cornu Ammonis 4 (CA4) (Figures 1a and 3).

Subfield CA1: It is the first region of the hippocampal circuit that yields significant output pathway. Cells of the pyramidal cell layer of this subfield were observed to be less densely packed. Higher magnification of the pyramidal cell layer reveals pyramidal neurons of approximately 3-4 cell layer thick with an average thickness of 87.0±2.0µm (Figure 3a). This subfield continues into the CA2 of the hippocampal proper (Figure 1a).

Subfield CA2: This is a narrow subfield appearing transitional between subfields CA1 and CA3 with an average pyramidal cell layer thickness of 109.0±4.20µm (Figures 1a and 3b).

Subfield CA3: CA3 extends from the CA4 and continues with CA2. CA3 pyramidal neurons form a well-defined cell layer starting in between the upper and lower ends of the dentate gyrus (Figures 1a). This subfield revealed larger, more distinct pyramidal cells and higher cell layer thickness (240.0±6.0µm) relative to the remaining subfields with significant statistical differences at $p<0.001$ (Figures 3c - e). Schaffer collaterals were delineated as axonal extensions of the CA3 pyramidal cells extending into the stratum radiale (Figure 3d).

Subfield CA4: CA4 subfield is a direct continuation of the hilus of the dentate gyrus and thus presents similar histological features.

Stratification of the Hippocampus proper

Five layers of the hippocampus proper were outlined with the Nissl and Golgi stained sections. They include from without inwards: alveus, stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunosum-moleculare (Figures 3d and 4a).

Subiculum and Entorhinal Area

The subiculum, a component of the hippocampal formation, was observed as a continuation of the CA1 subfield and contained loosely packed deep layer of pyramidal cells (Figures 1a and 4b). The subiculum was noted to connect the hippocampal proper to the entorhinal cortex. The entorhinal cortex was

delineated as a six-layered cortical area based on the varying size of the pyramidal cells (Figure 4c).

DISCUSSION

The hippocampus is an organized laminar structure which receives sensory inputs mainly from the entorhinal cortex (Karger and Basel, 2014). The hippocampal formation is thought to play a role in memory, spatial navigation and control of attention (Andersen *et al.*, 2007). The hippocampal formation is generally accepted to consist of the dentate gyrus, hippocampus proper, subiculum and entorhinal cortex (Amaral and Lavenex, 2006). All these structures were identified in the AGR hippocampal formation from this study.

The basic cytoarchitecture of the AGR hippocampus observed in this study, with respect to stratification, subfields and cell types, is similar to those reported in the laboratory rats (Westrum and Blackstad, 1962; Seress and Ribak, 1990; Caeser and Aersten, 1991; Falougy *et al.*, 2008; Hussein and George, 2009). Cell types identified in the AGR hippocampus include pyramidal cells, granule cells and mossy cells. Pyramidal cells which are the principal cells of the hippocampus proper were found in the pyramidal cell layer extending their dendrites to the stratum oriens and axons (Schaffer collaterals) into the stratum radiatum. This is consistent with observations in the rat hippocampus (Hussein and George, 2009). The oval-shaped granule dentate cells of the AGR hippocampus were found in the distinct granule cell layer of the dentate gyrus where they send their dendrites to the molecular layer of the dentate gyrus and synapse with afferent inputs from the entorhinal cortex (Hargreaves, 2007). Mossy cells were found at the hilus/polymorphic layer of the dentate gyrus, where they have been recognized as the second principal glutamatergic (excitatory) cells of the dentate gyrus after the granule dentate cells. They are thought to have intrinsic and circuitry properties that make them suitable to activate granule dentate cells (Scharfman and Myers, 2013).

The hippocampus proper of rats as documented by Falougy *et al*, (2008) is subdivided into four regions (CA1-CA4) according to density, size and branching of the axons and dendrites of the pyramidal cells. Similarly, these four regions of the hippocampus proper were distinguished in the Nissl stained sections of the AGR brains.

The features of the hippocampal stratification of the AGR observed in this work using the Nissl and Golgi stains based on cell body location, cell body shape and size, proximal terminations, distal branching characteristics were consistent with reports for the laboratory rats (Turner *et al*, 1998). The stratum pyramidale, a layer of the principal excitatory neurons of the hippocampus proper, was delineated across all subfields. Here, the pyramidal cells extend their axons to the stratum radiatum – the Schaffer collaterals. The pyramidal layer expands significantly along its course from CA1 – CA3 and become sparse as it enters the CA4 and hilus area of the dentate gyrus. The observed significant difference in the average heights of the CA subfields with maximum values favouring CA3 is deemed necessary as CA3 has been noted to be a major recipient of sensory inputs from the entorhinal cortex via perforant pathway through the dentate gyrus (Hargreaves, 2007; Knierim and Neunuebel, 2016).

The granule cell layer of the dentate gyrus has been well established as one of the sites for adult neurogenesis in the AGR (Olude *et al.*, 2014b). This noted neural plasticity may account for the different shapes of the dentate gyrus seen in this study. Veena *et al.*, (2011) posited that several factors such as stress, acetycholine and dopamine levels play significant roles in the regulation of adult neurogenesis. For example, stress was stated to decrease neurogenesis while enriched environment and exercise increases neurogenesis with associated improvement in memory functioning and enhanced synaptic plasticity.

The detailed, delicate arrangement of cell types and subfields, intricate wiring with synapses and laminar organization of the hippocampal formation noticed in the AGR strongly supports the canonical trisynaptic circuitry of the hippocampus as described by Witter (1989) and Knierim and Neunuebel, (2016). Firstly, afferent inputs from the entorhinal cortex projects via axons of the perforant path into granule cells of the dentate gyrus. Secondly, the granule dentate cells project via mossy fibers into the pyramidal CA3 cells. Lastly, the latter project into the pyramidal cells of CA1 by means of the Schaffer collateral system. While the subiculum serves as the main hippocampal output portal, the entorhinal cortex forms the major link between the hippocampal formation and the neocortex (Ding, 2013). It has been well established that olfactory information is transmitted from the primary olfactory cortex to several other parts of the forebrain, including the hippocampus (Biella and de Curtis, 2000). Interestingly, Price (2009) remarked there is an especially strong olfactory input to the hippocampus of rats because more than half of their entorhinal cortex receives olfactory inputs. These olfactory inputs on getting to the entorhinal cortex project into the hippocampus proper via the trisynaptic circuit for memory consolidation. This pathway probably alludes to the connexion in the unique neurobehavioural attributes of this rodent: olfaction and memory.

For this study, two staining techniques (Nissl stain and Golgi impregnation) were deployed to elucidate the histology of the AGR hippocampus. Although traditional stain such as Nissl stain (Cresyl violet) gives an indication of the size and packing density of the cells and cell layers, it cannot distinguish between the various "protoplasmic processes", or trace their full extent. However, Golgi impregnation offers the

advantage of identifying the shape of the cell, and therefore its orientation, and of course the full extent of the dentritic arborization (Cimino, 2000). It will however be necessary to carry out densitometric studies and detailed neurochemical profiling of the AGR hippocampus to fully elucidate the functional leverage of this unique rodent.

The previous and widely accepted hypothesis that hippocampus is widely involved in olfaction was negated by a series of studies that could not find direct projections from the hippocampus to the olfactory bulb (Finger, 2001). However, much work done later confirmed the projections of the olfactory bulb to the ventral part of the entorhinal cortex, the CA1 field, to the main olfactory bulb (Van Groen and Wyss, 1990, Biella and de Curtis, 2000). It has now been noted that the hippocampus belongs to a larger learning system in the brain (Horel, 1994). Therefore, ideas of the hippocampus have developed into ideas of the hippocampal system (Petri and Mishkin, 1994) which is believed to play some roles in learning and memory process. Thus, AGR may serve as a suitable indigenous model for researches in olfaction-linked memory pathologies.

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Pulmonary Responses Following Quercetin Administration in Rats After Intratracheal Instillation of Amiodarone

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Summary: Amiodarone, a drug that treats arrhythmias induces pulmonary toxicity through interplay between oxidative stress and inflammation. Quercetin, a flavonoid widely occurring in natural products possesses antioxidant and anti-inflammatory properties. The aim of the present study was to evaluate the effects of quercetin on pulmonary responses in rats after amiodarone intra-tracheal instillation. Eighteen female Wistar rats (150-250 g) were randomly assigned into three groups of six animals each namely; control, amiodarone (AMI) and amiodarone + Quercetin (AMI + Quercetin) groups. AMI group received 2 intra-tracheal instillations of amiodarone (6.25mg/kg in 0.3ml of water) on days 0 and 2 and 0.4ml of 2% DMSO (Dimethyl sulfoxide) orally from day 0 for 3 weeks. AMI + Quercetin group was administered 2 intratracheal instillations of amiodarone on days 0 and 2 and 20mg/kg body weight of quercetin in 2% DMSO from day 0 for 3 weeks. Thereafter, the animals were sacrificed and bronchoalveolar lavage fluid (BALF) was collected to determine total cell polymorphonuclear (PMN) cell and macrophage counts. Inflammation of the lung tissues was also assessed. Macrophage count of AMI + Quercetin group was significantly lowered ($p<0.01$) compared to AMI group. Inflammation rate of the AMI + Quercetin group was significantly reduced compared to AMI group ($p<0.01$). Quercetin treatment markedly suppressed amiodarone induced toxicity in the pulmonary tissues.

Keywords*:* Amiodarone, Quercetin, Pulmonary inflammation, Bronchoalveolar lavage fluid, Intra-tracheal instillation

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INTRODUCTION

Amiodarone, an iodine–containing compound is a potent anti-arrhythmic agent widely used in the treatment of supra-ventricular and ventricular arrhythmias (Nacca et al., 2012) with a possibility of playing a role in post-myocardial infarction mortality reduction (Chen and Hedges, 2003). It has numerous well-described biochemical and electrophysiological effects (Vasallo and Trohman, 2007; Zimetbaum, 2007). Amiodarone has prophylactically been used in the peri-operative period in patients undergoing thoracic surgery including coronary revascularization and valve replacement (Aasbo et al., 2005).

Despite its vital role in relieving cardiac related problems, it is however associated with variety of adverse effects involving many different organs and system (Wolkove and Baltzan, 2009; Al-shammari et al., 2016). It tends to accumulate in several organs, including the lungs (Wolkove and Baltzan, 2009). Of all the adverse effects of amiodarone, the most serious, feared and potentially life threatening is Amiodarone-induced Pulmonary toxicity (APT) (Nacca et al., 2012; Wolkove and Baltzan, 2009; Al-shammari et al., 2016).

Amiodarone and its metabolites produce lung damage directly by a cytotoxic effect and also indirectly by an immunological reaction (Martin and Rosenow, 1988). The active principal metabolite, desethylamiodarone, penetrates tissues and accumulates therein, thus providing a sustained source of release (Nacca et al., 2012). Direct damage to cells occurs through the production of toxic O2 radicals (Wolkove and Baltzan, 2009). The indirect effect is supported by the report of the presence of cytotoxic T cells in BALF from patients diagnosed with APT (Jessurum and Crijins, 1997). APT is partly characterized as oedema, phospholipidosis, inflammation and thickening of the alveolar septa, intra-alveolar inflammation, as well as lung infection (pneumonia), as well as pulmonary fibrosis (Mason, 1987; Martin and Rosenow, 1988). Other, more localized forms of pulmonary toxicity may occur, including pleural disease, migratory infiltrates and single or multiple nodules (Wolkove and Baltzan, 2009).

Intra-tracheal instillation is a technique of introducing substances directly into the trachea. It is widely used in assessing respiratory toxicity of a substance as an alternative to inhalation in animal

testing (Driscoll et al., 2005). In the present study, intra-tracheal administration of amiodarone was employed to induce pulmonary toxicity.

Quercetin, a bioflavonoid widely distributed in nature and found in many grains, leaves, fruits and vegetables (Shaik et al., 2006) is used as ingredient in supplements or foods (Formica and Regelson, 1995). Studies have reported that quercetin inhibits the oxidation of other molecules and therefore serves as an anti-oxidant (Williams et al., 2004; Russo et al., 2014). It contains polyphenolic chemical structure which halts oxidation of free radicals that mediate oxidative chain reactions (Murakami et al., 2008). Also, quercetin is reported to possess anti-inflammatory properties (Shaik et al., 2006). It inhibits the growth of certain malignant cells in vitro, and histamine and most cyclin-dependent kinases and also displays unique anti-cancer properties (Shaik et al., 2006; Russo et al., 2014). A study reported that quercetin is a non-specific protein kinase enzyme inhibitor (Russo et al., 2014) that activates or inhibits the activities of a number of proteins (Feitelson et al., 2015) and blocks substances involved in allergies by inhibiting mast cell secretion.

In spite of enormous information as regards the induction of pulmonary fibrosis by amiodarone, the management of this lung disorder is frequently difficult and unfortunately, therapeutic approaches to inhibit the development of amiodarone pulmonary toxicity are scanty (Punithavathi et al., 2003). As a natural therapy with numerous beneficial effects including anti-inflammatory and anti-oxidant effect, it is conceivable that quercetin can influence pulmonary toxicity. Hence, the present study was carried out to evaluate effects of quercetin on pulmonary responses in the lungs of rats after intra-tracheal administration of Amiodarone.

MATERIALS AND METHODS

Chemicals: Amiodarone, ketamine hydrochloride, dimethyl sulfoxide (DMSO) and quercetin were purchased from Medreich plc, UK, Kwality Pharmaceuticals Pvt, India, Guanghua Chemical, China and Sigma, USA respectively.

Animals: Eighteen female Wistar rats weighing between 150-250 g were used for this study. They were procured from the Animal house of the Department of Physiology, College of Medical Sciences, University of Calabar, Nigeria. The animals were kept in well ventilated cages at room temperature 25+2^{o}C and exposed to a normal 12/12 hours light/dark cycle. They had access to rodent chow and clean drinking water ad libitum. All procedures involving animal handling followed the National Institutes of Health guide for the care and use of Laboratory animals (NIH publications No. 8023, revised 1978) and approved by the Faculty of Basic Medical Sciences Animal Ethics Committee.

Experimental Protocol: The animals were weight matched into three groups: Control (CTL), Amiodarone (AMI) and Amiodarone plus quercetin (AMI + Quercetin) groups. The CTL group received 0.3 ml of distilled water (vehicle) by intra-tracheal instillation on days 0 and 2 and 0.4 ml of 2% DMSO orally from day 0 for 3 weeks. The AMI group received 2 intra-tracheal instillations of amiodarone (6.25 mg/kg in 0.3 ml of water) on days 0 and 2 and 0.4 ml of 2% DMSO orally from day 0 for 3 weeks. The AMI + Q group was treated with 2 intra-tracheal instillations of amiodarone on days 0 and 2 and 20mg/kg body weight of quercetin in 2% DMSO from day 0 for 3 weeks. Drugs and vehicle dosages were based on previous studies (Hamid-Reza et al., 2016; Ujah et al., 2016).

At the end of the study period, the animals were sacrificed under deep anaesthesia by ingestion of pentobarbital and dissected to remove the lungs. BALF was collected by inserting a cannula into the right lung via the respiratory tract, with the left main bronchus clamped, and pouring in a physiological saline (5 to 10 mL each). Fifty ml of BALF per group was centrifuged (1500 rpm; 10 min) to separate the cellular components. After 1 mL of buffer was added and stirred, the fluid was examined for macrophages and neutrophils (cell/μL) with an automatic blood cell counter (Celltac MEK 5204 Nihon Koden, Tokyo). The results were expressed as the total number of cells in the BALF.

Also, from the samples of the BALF collected, smears were made and stained. The slides were then observed by an optical microscope at ×400 magnification; the alveolar macrophages (AM) and PMN in the BALF were identified by their shape. The number of PMN in the BALF was obtained by multiplying the percentage of PMN by the total number of cells (Okada et al., 2016).

Histopathology of the Lung: The left lung of each rat (the clamped side in the BALF collection) was fixed with 10% buffered formalin. Paraffin sections of the left lung (3 μm thickness) were stained with hematoxylin eosin (HE).

Point count method for HE staining: This method was adapted by Okada et al., (2016). Lung tissue specimens are stained. After staining, digital images of each lung section were photographed under light microscopy at x100 magnification. In order to eliminate measurer bias, a third person randomly assigned numbers from 1 to 90 to microscope photographs per time point and returned them to the measurer. The images were chiefly focused on the alveoli. Photographs obtained were returned to the original order through the use of conversion table. Place over each image on a computer screen was a 300-point grid. Pulmonary inflammation at each time point was examined using the point counting method (PCM) (Ogami et al., 2004). The

accumulation of macrophages and PMN was counted mainly as inflammatory change. The rate of points of inflammation was calculated as shown; (I=X/300)

I = inflammation rate,
X = the number of inflammation points among 300 points.

Statistical Analyses: All data were analyzed by one-way analysis of variance followed by post hoc student Tukey test using Graph pad prism version 7.01. Values are presented as mean ± S.E.M. Significance was accepted at P< 0.05.

RESULTS

The total cell count of both the AMI and AMI + Quercetin groups was not significantly different compared to the control. However, the value for the AMI + Quercetin group was significantly lower (p<0.01) compared to AMI group (Figure 1). The poly morphonuclear cell count of both the AMI and AMI + Quercetin groups showed no significant difference compared to the control (Figure 2).

Figure 3 showed that the macrophage count in the AMI group was significantly higher (p<0.001) compared to control. The value for AMI + Quercetin group was significantly lower (p<0.01) compared to AMI group. The HE-stained images of the lung tissues following quercetin administration after amiodarone intra-tracheal instillation are shown in Figure 4. In Figure 4A, the lung tissue section shows prominent alveoli spaces with intact linings. The intervening inter-alveoli septa are thin. Numerous collapsed alveoli spaces and widened inter-alveoli septa consisting of mononuclear inflammatory cells and proliferative pneumocytes were observed in the AMI group (Figure 4B). There is marked inflammation around the terminal bronchioles. In the AMI + Quercetin group, the inter-alveoli septa were observed to be mildly thickened. Also a few of the alveoli spaces are dilated with intact epithelial cells (Figure 4C).

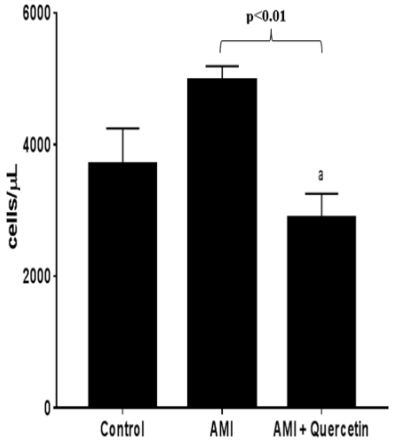

Fig.1: Total cell count in control, AMI and AMI + Quercetin experimental groups. Values are expressed as Mean ± SEM, n=6, a=p<0.01 vs AMI

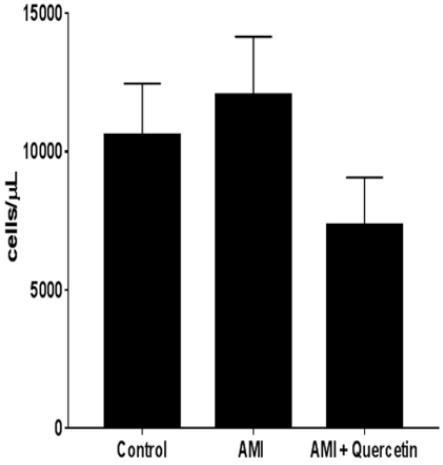

Fig.2: Polymorphonuclear cell count in control, AMI and AMI+ Quercetin experimental groups. Values are expressed as Mean + SEM, n=6

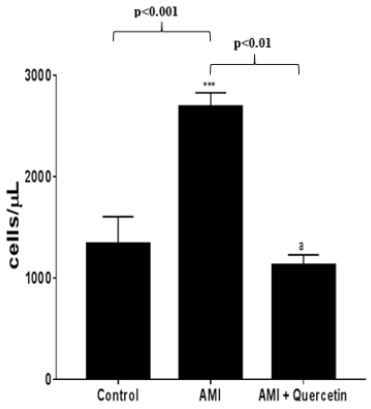

Fig. 3: Macrophage count in control, AMI and AMI + Quercetin experimental groups. Values are expressed as Mean ± SEM, n= 6. ***= p<0.001 vs control, a= p<0.01 vs AMI

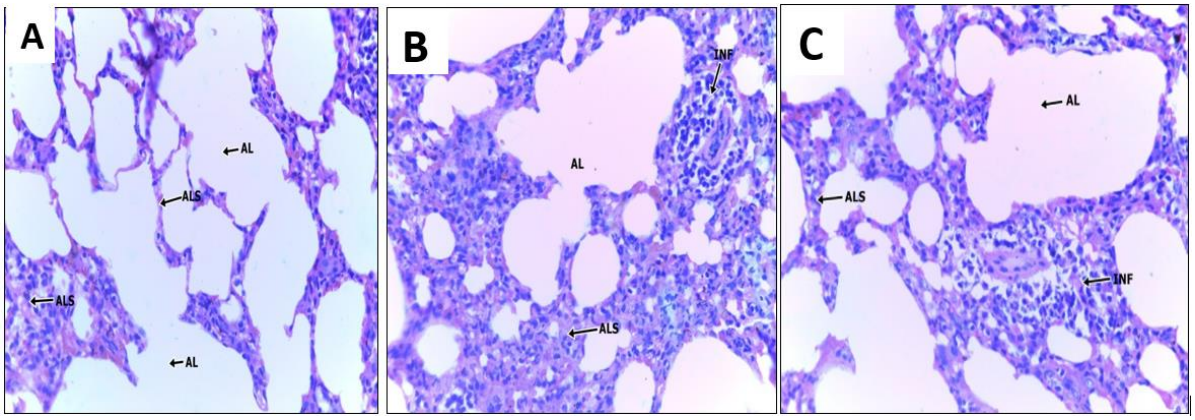

Fig.4: Lung sections(x400) with HE stain at 3 days for the (A) Control: section of lung tissues show prominent alveoli spaces (AL) with intact linings consisting of prominent pneumocytes. The alveoli spaces are dilated and empty of secretion and the intervening inter-alveoli septa (ALS) are thin. (B) AMI: section of lung tissues shows numerous collapsed alveoli spaces (AS) and widen inter-alveoli septa (ALS) consisting of mononuclear inflammatory cells and proliferating pneumocytes. There is marked inflammation around the terminal bronchioles bronchioles. Few of the alveoli spaces are dilated, lined by flattened intact epithelial cells and empty (C) AMI + Quercetin groups: section of lung tissues shows numerous collapsed alveoli spaces separated by mildly thickened inter-alveoli septa consisting of mild mononuclear inflammatory cells and proliferating pneumocytes. Few of the alveoli spaces are dilated, lined by flattened intact epithelial cells and empty.

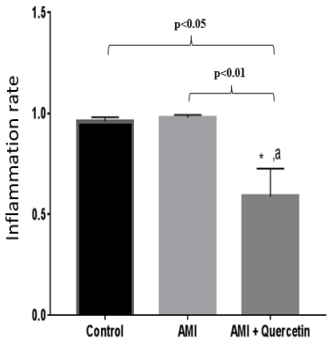

Fig.5: Inflammation rate in control, AMI and AMI + Quercetin experimental groups. Values are expressed in Mean ± SEM, n=6. *=p<0.05 vs control, a=p<0.01 vs AMI.

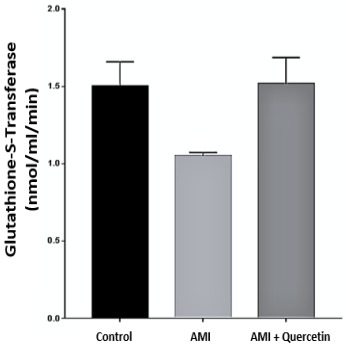

Fig. 6: Glutathione-S-Transferase levels in control, AMI and AMI + Quercetin experimental groups. Values are expressed in Mean ± SEM, n=6.

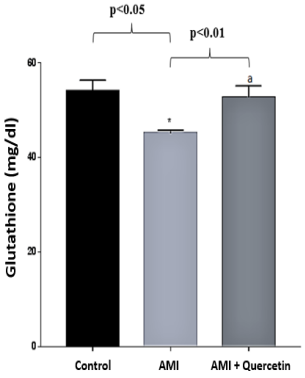

Fig. 7: Glutathione levels in control, AMI and AMI + Quercetin experimental groups. Values are expressed as Mean ± SEM, n=6. *=p<0.05 vs control, a=p<0.01 vs AMI.

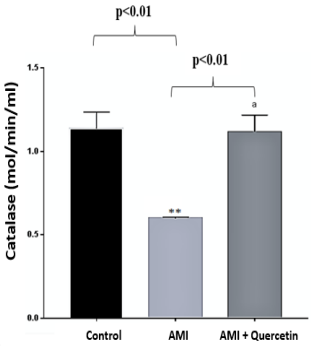

Fig. 8: Catalase levels in control, AMI and AMI + Quercetin experimental groups. Values are expressed in Mean ± SEM, n=6. **=p<0.01 vs control, a=p<0.01 vs AMI.

The inflammation rate of the AMI group was not significantly different compared to control, whereas the value for AMI + Quercetin group was significantly lower compared to both control and AMI groups ($p<0.05$ and $p<0.001$ respectively) (Figure 5).

The Glutathione-S-Transferase levels of both the AMI and AMI + Quercetin groups were not significantly different from the control, although that of AMI + Quercetin group appeared to be slightly higher compared to the AMI group (Figure 6).

While the Glutathione level of the mice in the AMI group was significantly lower compared to control ($p<0.05$), the value for AMI + Quercetin group was observed to be significantly increased compared to the AMI group ($p<0.01$) (Figure 7).

The Catalase level of AMI group was observed to be significantly lower compared to the control ($p<0.01$), whereas that of AMI + Quercetin group was higher compared to AMI group with a significance of $p<0.01$ (Figure 8).

DISCUSSION

In the present study, total cell count was observed to be higher in amiodarone administered rats. This is also in line with the report of with the report of Punithavathi et al. (2003). This increase might have been as a result of the reaction of the body to the drug, a response to fight off any infection. Quercetin treatment resulted in marked reduction in total cell count. This report is consistent with that of Liu et al., (2013), who reported a reduction in total cell count following quercetin administration.

PMN, the first line of defence against invading pathogens was observed to have a slightly increased count following amiodarone intra-tracheal instillation. Quercetin was observed to lower the PMN cell count of the animals. Quercetin might have lowered the PMN by inhibiting several factors activated under inflammatory conditions (Gonzalez et al., 2011). This is consistent with the report of Nikfarjam et al. (2017), who stated a reduction of PMN, occurred as a result of the reduction of nitric oxide (NO), tumour necrosis factor α (TNF- α) and myeloperoxidase (MPO).

Macrophage count of the amiodarone only treated group was significantly higher than the control. Our data is consistent with that of the study conducted by El-Mohandes et al., (2017). These macrophages appear to play vital roles in amiodarone induced pulmonary fibrosis (El-Mohandes et al. 2017). This cellular response is indicative of the development of a drug-induced phospholipidosis (Reasor et al., 1988).

The HE stained images of the lung tissues of the AMI group showed many collapsed alveoli spaces and widened inter-alveoli septa. Also, there was marked pneumonitis and vacuolization of the bronchial epithelial cell. This is supported by the report of Wolkove and Baltzan (2009). This hyperplasia of the pneumocytes and alveolar septa widening may be as a result of the toxic O_2 radical production, which damage the cells directly and also promote the accumulation of phospholipids in tissues (Jessurum and Crijins, 1997; Halliwell et al., 1997). This amiodarone-induced phospholipidosis triggered the formation of lip-laden macrophages as observed in the present study.

Administration of quercetin reduced thickening of the inter-alveoli septa. The antioxidant activity of quercetin as observed in the present study may be attributed to many factors including free radical scavenging, protection against lipid oxidation or up-regulation of antioxidant enzymes (Liu et al., 2013). Heijnen et al. (2002), reported that structural features allowed quercetin to donate hydrogen to scavenge free radicals and increase the stability of flavonoid radicals.

Inflammation, the most common appearance of tissue pathology, is involved in the pathogenesis of many disease conditions including APT. The data obtained from our study showed suppression of the inflammatory response (triggered by amiodarone instillation) of the lung tissues following quercetin administration. This result is consistent with the report of Nikfarjam et al. (2017). Quercetin has been reported to suppress inflammation by reducing TNF- α and NO synthase production in obese Zucker rats.

The anti-inflammatory effects of quercetin as observed in the present study may be attributed to the interaction between oxidative stress and inflammation (Boots et al., 2008). Reactive oxygen species (ROS) scavenging by quercetin not only prevents oxidative stress, but also mitigates inflammation. This is because ROS are not only involved in the occurrence of oxidative stress but likewise in the promotion of inflammatory processes (Madamanchi et al., 2005). They also induce production of TNF- α, which also trigger inflammation (Rahman, 2002).

Study by Nair et al. (2006) reported that quercetin inhibited TNF- α production and gene expression through nuclear factor (NF)-KB modulation. Hence, it is likely that suppressed inflammatory responses observed in this study may be due to inhibition of TNF- α production by quercetin.

The significant increase of the levels of superoxide dismutases including catalase and glutathione following quercetin administration further highlights or serves as pointers to the cytoprotective tendencies of quercetin. This is because these enzymes help in protecting cells from oxidative damage by reactive oxygen species (ROS). This is supported by Pritchard et al., (2014) and Kerksick and Willoughby (2005). In conclusion, we suggest that, intra-tracheal instillation of amiodarone triggered a cascade of immunological responses in the lung tissues of rats. These responses which are deleterious and indicative of pulmonary damage, were ameliorated following quercetin administration.

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Fatty Acids in Some Cooking Oils as Agents of Hormonal Manipulation in a Rat Model of Benign Prostate Cancer

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Summary: Anti-androgenic substances, mainly prostate 5α-reductase inhibitors, used in the treatment of benign prostatic hyperplasia (BPH) have been associated with side effects in man and animals. To reduce these side effects as well as suppress BPH development, the management of the condition has come to include dietary interventions. This study investigated the effect of some cooking oils on testosterone-induced hyperplasia of the prostate in rats. Male Sprague-dawley rats were distributed into eighteen groups (n=6) as A-R. A negative control group was injected subcutaneously with soya oil; while prostatic hyperplasia was induced subcutaneously in groups B-R with 3mg/kg testosterone daily for 14days. Group B was the positive control (BPH group) while groups C-R were also administered orally 800mg/kg of coconut, castor, canola, cottonseed, pomegranate, blackseed, sheabutter, olive oil, codliver, sardine, palm, repeatedly heated palm (RHPO), vegetable, repeatedly-heated vegetable (RHVO), sesame, and groundnut oils respectively, daily, for 14 days. Blood sample was drawn via retro-orbital sinus for the estimation of serum testosterone(T) and dihydrotestosterone (DHT) level and rats were thereafter euthanized to obtain the prostates for T and DHT determination as well as tissue weights. Data are mean ± SEM, compared by ANOVA. The oils significantly reduced the increase in prostate weight (PW) to body weight (BW) ratio induced by testosterone. Apart from the fact that all the oils reduced the PW:BW ratio, the blackseed, sheabutter, sardine, vegetable and groundnut oils suppressed the DHT level in the serum, while pomegranate, olive, RHPO reduced DHT level in the prostate compared to the BPH rats. This study suggests that blackseed, sheabutter, sardine, vegetable, groundnut, pomegranate, olive, and RHPO oils could inhibit testosterone-induced hyperplasia of the prostate and therefore may be beneficial in the management of BPH.

Keywords*:* benign prostatic hyperplasia, cooking oils, fatty acids, rat, dihydrotestosterone, testosterone

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INTRODUCTION

Benign prostatic hyperplasia (BPH) is an enlargement of the prostate gland from progressive hyperplasia or abnormal growth of cells of glandular epithelial and stromal cells. It is associated with impairment in urinary function, as such it is a common urogenital disorder that affects up to 85% of men over 50 years of age (Arruzazabala *et al.*, 2007). The inability to delay urination, incomplete emptying of the bladder, bladder outlet obstruction, bloody urination, nocturia, as well as dysuria, are common symptoms of BPH (Barkin, 2011). Although BPH is not a known risk factor for prostate cancer, it may increase the chance of its occurrence (Chang *et al.*, 2012).

The hormones, testosterone (T) and dihydrotestosterone (DHT) have key roles to play in the development and growth of the entire male genital tract, as such they stimulate differentiation of the prostate gland (Andriole *et al.*, 2004). The adrenal glands and testes synthesize testosterone, and the enzyme prostatic 5α-reductase type 2, converts it to DHT (Carson and Rittmaster, 2003). DHT then binds to the androgen receptor (AR), which is transported to the nucleus, where it regulates genes important for prostate growth and differentiation (Mizokami *et al.*, 2009). When aging sets in, the production and accumulation of DHT in the prostate increases, thus encouraging cell growth and causing hyperplasia (Carson and Rittmaster, 2003, Arruzazabala *et al.*, 2007). Risk factors such as race, ethnicity and family history of prostate cancer occurrence play important roles in the development of BPH. In addition, environmental factors like diet also play a role in prostate cancer incidence. The increase in prostate disorders because of dietary changes has been demonstrated in both human and animal studies (Rohrmann *et al.*, 2007, Torricelli *et al.*, 2013). Drugs, such as Finasteride® developed with a focus to

reducing DHT level in BPH patients have been associated with side effects such as nasal congestion, decreased libido, erectile dysfunction and so on. Thus, to reduce these side effects as well as suppress BPH development, the management of the condition has come to include dietary interventions as well as natural materials such nutraceutical preparations which include pumpkin seed oil (Gossell-Williams *et al.*, 2006), coconut oil (Arruzazabala *et al.*, 2007) as well as herbal extracts mainly saw palmetto lipid extracts (SPLC) (Arruzazabala *et al.*, 2007).

Over the last few decades, there has been a growing public concern about the significant interplay between health, food and nutrition. Fat is an essential macronutrient of the human diet and vegetable oils represent a more highly consumed fat. The effects of high-fat diet mainly in fatty acids have been the emphasis of several dietary guidelines targeting the reduction of some diseases and especially cancer prevention. In this investigation, we employed a BPH model (testosterone propionate (TP)-induced BPH) to examine whether oral dosing with coconut, castor, canola, cottonseed, pomegranate seed, blackseed, sheabutter, olive, cod-liver, sardine, palm, repeatedly-heated palm, vegetable, repeatedly-heated vegetable, sesame and groundnut oils could prevent prostatic hyperplasia induced by testosterone in rats.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats, 12weeks old and weighing 350-370g, were obtained from the Animal house of the College of Medicine, University of Lagos and adapted to laboratory conditions (25 ± 3°C), relative humidity 61± 3%, light/dark cycles of (12 h) for 7 days. Free access to feed (rodent chow was from Centre Point Agro and Livestock Raw Materials Depot, Lagos) and water *ad libitum* were provided. Animal handling and experimental protocols were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Oils

Extra-virgin coconut oil (Andalucia, Spain), castor oil (KTC Limited, UK), canola oil (ConAgra Foods, USA), cottonseed oil (Shamad Concept, Nigeria), pomegranate oil (Hemani, Pakistan), blackseed oil (Hemani, Pakistan), sheabutter (bought in a market in Lagos, Nigeria), olive oil (ICEA, Bologna), codliver oil (Olive Healthcare, India), sardine (UNIMER, Morrocco),palm oil (Farmland produce, Nigeria), repeatedly-heated palm oil (palm oil was used for frying beef and plantain 3 consecutive times at 180^{o}C), vegetable oil (Laziz vegetable oil, Apple & Pears Ltd. Nigeria), repeatedly-heated vegetable oil (vegetable oil was used for frying beef and plantain 3 consecutive times at 180^{o}C), sesame oil (OPW Ingredients GmBH, Germany),groundnut oil (bought from a market in Nigeria) were used for the study.

Administration and dosage of oils

Testosterone propionate (Cuban Medical Pharmaceutical Industry, Cuba), was diluted in soya oil and injected subcutaneously at 3mg/kg, daily for 14 days as described previously (Arruzazabala *et al.*, 2004; Noa *et al.*, 2005). Oils were diluted in soya oil and administered orally at 800mg/kg as this dose showed better inhibition against BPH model in rats (Arruzazabala *et al.*, 2007). The rats were randomly distributed into eighteen groups (6 rats each): the negative control (Group A), received daily subcutaneous injection of soya oil (vehicle) and seventeen groups received daily subcutaneous injection of testosterone at 3mg/kg dosage. Group B was the positive control (BPH) while the other sixteen groups received subcutaneous injection of testosterone propionate plus oral administration of 800mg/kg of the oils as follows: Group C : coconut oil, Group D: castor oil, Group E: canola oil, Group F: cottonseed oil, Group G: pomegranate seed oil, Group H: blackseed oil, Group I: sheabutter, Group J: olive oil, Group K: codliver oil, Group L: sardine oil, Group M: palm oil, Group N: repeatedly-heated palm oil (RHPO), Group O: vegetable oil, Group P: repeatedly-heated vegetable oil, Group Q: sesame oil, Group R: groundnut oil. The treatments were administered for 14 days.

Body and prostate weight

The animals were weighed at the start of the experiment, the day before administration of oils and weekly thereafter. At the end of the experiment, animals were euthanized with CO_2 anaesthesia, and blood samples were collected and centrifuged at 5000 revs for 10 minutes while the prostates were carefully recovered and weighed using a digital weighing scale (Scout Pro, Ohaus Corporation, USA) immediately and frozen for further studies. The prostate weight (PW) and prostate weight to body weight ratios (PW/BW) were recorded.

Percentage inhibition was calculated as follows: **100-[(TG-NC)/(PC-NC) x 100]**, where PC, NC, and TG were the values of the positive control, negative control, and treated groups, respectively.

Measurement of DHT and T levels in the serum and prostate

Prostate tissue was homogenized in lysis buffer containing protease inhibitors (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.1% SDS, 1 mM EGTA, 100 μg/mL PMSF, 10 μg/mL pepstatin A, and 100 μM Na3VO3). The homogenates were centrifuged at 5000 revs for 10 mins at 4°C, and the protein concentrations in the supernatant fractions were determined using Bradford reagent (Bio-Rad, Hercules, CA). Enzyme-linked immunosorbent assays

(ELISAs) were performed according to the manufacturer's instructions. DHT and testosterone levels in the serum and prostates were measured with ELISA kits (Sigma, USA).

Statistical Analysis

Data are expressed as mean ± standard error of mean (SEM) and were analyzed with Graphpad software version 3.05(San Diego, California, U.S.A.) using one-way ANOVA for multiple comparisons between groups, followed by the student-Newman-Keuls test, differences between groups were considered significant at $P<0.0001$.

RESULTS

Effect of cooking oils on prostate weight and PW/BW ratio

Table 1 summarizes the effects of the oils on testosterone -induced prostate enlargement. PW and PW/BW ratio increased in the positive control compared with negative control group. The cooking oils: coconut (C) , castor (D), canola (E), cottonseed (F), pomegranate (G), blackseed (H), sheabutter (I), olive (J), codliver (K), sardine (L), palm (M), RHPO (N) vegetable (O), RHVO (P), sesame (Q) and groundnut (R) when compared with the positive control significantly decreased the PW gain induced with testosterone by 65.2%, 60.6%, 63.6%, 72.3%, 68.2%, 65.2%, 71.2%, 65.2%, 62.1%, 72.7%, 48.5%, 59.1%, 50.0%, 50.0%, 48.5%, 66.7% respectively. Although the percentage inhibition achieved with palm oil (M) and sesame (Q) were lower compared with those achieved by the other oils. The increase in PW/BW ratio which was as a result of the testosterone-induced prostate enlargement was also decreased by the oils as follows: coconut (C) 68.6% , castor (D) 54.9%, canola (E)52.5%, cottonseed (F)59.7%, pomegranate (G)60.41%, blackseed (H)53.93%, sheabutter (I)65.8%, olive (J)59.1%, codliver (K)56.3%, sardine (L)56.7%, palm (M)60.1%, RHPO (N)62.1%, vegetable (O)54.6%, RHVO (P)55.9%, sesame (Q)54.3% and groundnut (R) 62.5%.

Effect of cooking oils on testosterone and DHT levels in the serum

Castor oil, and sheabutter induced a decrease in the testosterone serum level compared to the BPH (positive control) group, while the vegetable oil had an increased level compared to the BPH group. The vegetable, sardine, palm oil, RHVO, RHPO, coconut, canola, cottonseed, sesame, codliver, groundnut, blackseed, olive, pomegranate, sheabutter and castor oil levels were significantly increased compared with the negative control. Group D, the castor oil induced a significant decrease compared to vegetable, sardine, palm, RHVO, RHPO, coconut and canola oils while sheabutter group induced a significant decrease compared to the vegetable, sardine, palm, RHVO, RHPO, and coconut oils. The pomegranate seed oil also induced a significant decrease compared to the vegetable, sardine and palm oils, while olive oil induced a significant decrease compared to vegetable and sardine oils. Blackseed oil induced a significant decrease compared to vegetable and sardine oils. Groundnut, codliver, cottonseed, canola, coconut, and RHPO oils all induced significant decreases compared to the vegetable oil. Sesame oil induced a significant decrease compared to the sardine oil (Fig 1).

Table 1: Effect of cooking oils on prostate enlargement in rats treated with testosterone.

Groups	PW (g)	%Inhibition	PW/BW ($x10^{-3}$)	%inhibition
A	0.34±0.03	-	1.40±0.01	-
B	1.00±0.03#	-	4.33±0.05	-
C	0.57±0.01*	65.2	2.32±0.02*	68.6
D	0.60±0.02*	60.6	2.72±0.01*	54.9
E	0.58±0.03*	63.6	2.79±0.02*	52.5
F	0.49±0.02*	72.3	2.58±0.01*	59.7
G	0.55±0.05*	68.2	2.56±0.03*	60.4
H	0.57±0.02*	65.2	2.75±0.01*	53.9
I	0.53±0.06*	71.2	2.40±0.01*	65.8
J	0.57±0.06*	65.2	2.60±0.01*	59.1
K	0.59±0.02*	62.1	2.68±0.01*	56.3
L	0.52±0.06*	72.7	2.67±0.01*	56.7
M	0.68±0.04*	48.5	2.57±0.01*	60.1
N	0.61±0.07*	59.1	2.51±0.01*	62.1
O	0.67±0.06*	50.0	2.73±0.01*	54.6
P	0.67±0.06*	50.0	2.69±0.01*	55.9
Q	0.68±0.05*	48.5	2.74±0.02*	54.3
R	0.56±0.07*	66.7	2.50±0.01*	62.5

PW, prostate weight; BW, body weight; Positive control (BPH), received administration of testosterone (s.c) injection Negative control= Group A, Positive control (BPH) = Group B, BPH +coconut oil= Group C, BPH + castor oil =Group D, BPH +canola oil = Group E, BPH +cottonseed oil = Group F, BPH +pomegranate seed oil = Group G, BPH +blackseed oil=Group H, BPH +shea butter = Group I, BPH +olive oil = Group J, BPH +cod liver oil =Group K, BPH + sardine oil =Group L , BPH + palm oil =Group M, BPH + RHPO = Group N, BPH +vegetable oil = Group O, BPH +RHVO = Group P, BPH +sesame oil = Group Q, BPH +groundnut oil= Group R.

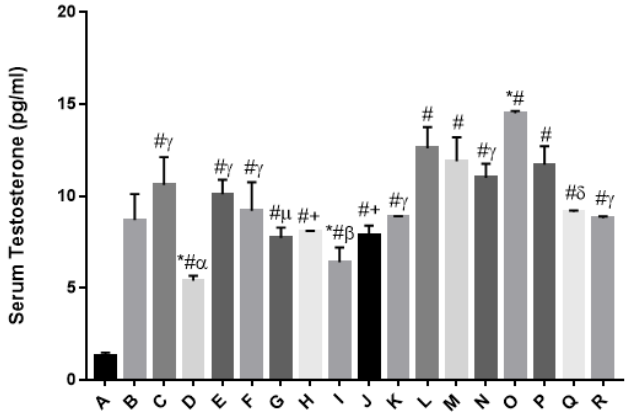

Fig.1 Effect of cooking oils on serum testosterone levels. Values are expressed as mean ± SEM., n=6/group. *P< 0.0001 vs BPH, #P< 0.0001 vs negative control, αP< 0.0001 vs C,E,L,M,N,O,P, βP< 0.0001 vs C,L,M,N,O,P, μP< 0.0001 vs L,M,O, +P< 0.0001vs L,O, δP< 0.0001 vs L, γP< 0.0001 vs O. Negative control= Group A, Positive control (BPH) = Group B, BPH +coconut oil= Group C, BPH + castor oil =Group D, BPH +canola oil = Group E, BPH +cottonseed oil = Group F, BPH +pomegranate seed oil = Group G, BPH +blackseed oil=Group H, BPH +shea butter = Group I, BPH +olive oil = Group J, BPH +cod liver oil =Group K, BPH + sardine oil =Group L , BPH + palm oil =Group M, BPH + RHPO = Group N, BPH +vegetable oil = Group O, BPH +RHVO = Group P, BPH +sesame oil = Group Q, BPH +groundnut oil= Group R.

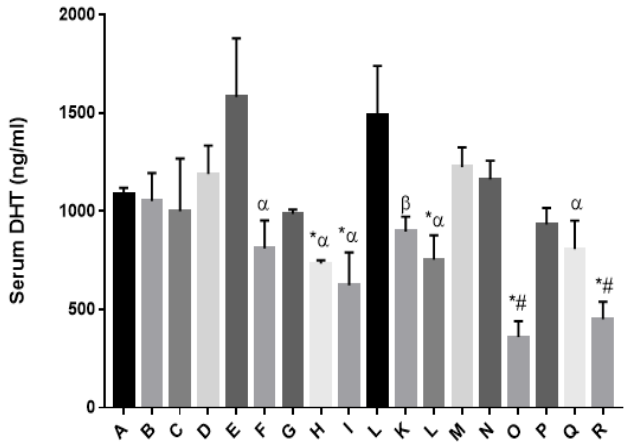

Fig 2 Effect of cooking oils on serum DHT levels. Values are expressed as mean ± SEM., n=6/group. *P< 0.0001 vs BPH, #P< 0.0001 vs D, E,J,M,N αP< 0.0001 vs E,J, βP< 0.0001 vs E. Negative control= Group A, Positive control (BPH) = Group B, BPH +coconut oil= Group C, BPH + castor oil =Group D, BPH +canola oil = Group E, BPH +cottonseed oil = Group F, BPH +pomegranate seed oil = Group G, BPH +blackseed oil=Group H, BPH +shea butter = Group I, BPH +olive oil = Group J, BPH +cod liver oil =Group K, BPH + sardine oil =Group L , BPH + palm oil =Group M, BPH + RHPO = Group N, BPH +vegetable oil = Group O, BPH +RHVO = Group P, BPH +sesame oil = Group Q, BPH +groundnut oil= Group R.

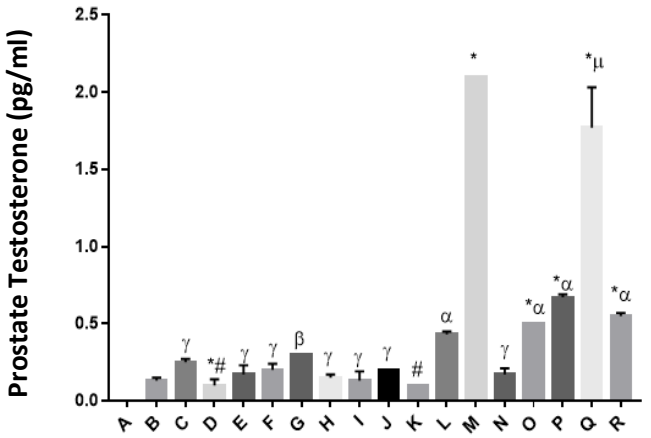

Fig 3 Effect of cooking oils on prostate testosterone levels. Values are expressed as mean ± SEM., n=6/group. *P< 0.0001 vs BPH, #P< 0.0001 vs L,M,O,P,Q,R, γP< 0.0001 vs M,O,P,Q,R, αP< 0.0001 vs M,Q, βP< 0.0001 vs M,P,Q,R, μ P< 0.0001 vs M. Negative control= Group A, Positive control (BPH) = Group B, BPH +coconut oil= Group C, BPH + castor oil =Group D, BPH +canola oil = Group E, BPH +cottonseed oil = Group F, BPH +pomegranate seed oil = Group G, BPH +blackseed oil=Group H, BPH +shea butter = Group I, BPH +olive oil = Group J, BPH +cod liver oil =Group K, BPH + sardine oil =Group L , BPH + palm oil =Group M, BPH + RHPO = Group N, BPH +vegetable oil = Group O, BPH +RHVO = Group P, BPH +sesame oil = Group Q, BPH +groundnut oil= Group R

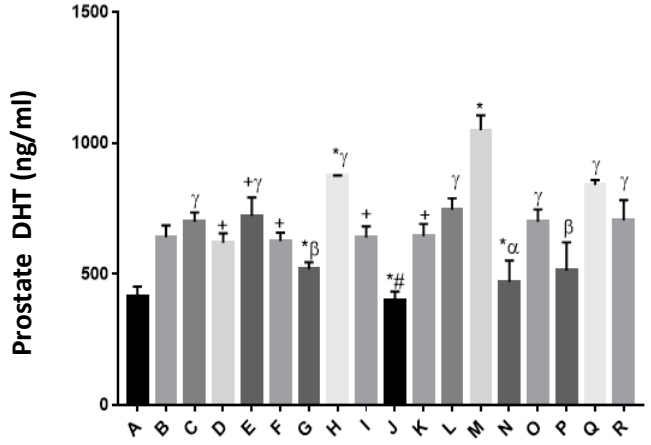

Fig 4 Effect of cooking oils on prostate DHT levels. Values are expressed as mean ± SEM., n=6/group. *P< 0.0001 vs BPH, #P< 0.0001 vs C, D,E,F,H,I,K,L,M,O,Q,R, α P< 0.0001 vs C,E,H,L,M,O,Q,R, βP< 0.0001 vs H,L,M,Q, + P< 0.0001 vs M, H, γ P< 0.0001 vs M. Negative control= Group A, Positive control (BPH) = Group B, BPH +coconut oil= Group C, BPH + castor oil =Group D, BPH +canola oil = Group E, BPH +cottonseed oil = Group F, BPH +pomegranate seed oil = Group G, BPH +blackseed oil=Group H, BPH +shea butter = Group I, BPH +olive oil = Group J, BPH +cod liver oil =Group K, BPH + sardine oil =Group L , BPH + palm oil =Group M, BPH + RHPO = Group N, BPH +vegetable oil = Group O, BPH +RHVO = Group P, BPH +sesame oil = Group Q, BPH +groundnut oil= Group R

The blackseed, sheabutter, sardine, vegetable and groundnut oils induced a significant decrease in the DHT serum level in the rats compared to the BPH group. The vegetable oil induced a significant decrease compared to the canola, olive, palm, castor, RHPO oils. Groundnut oil induced a significant decrease compared to canola, olive, palm, castor and RHPO oils. Sheabutter, blackseed, sardine, seseame and cottonseed oils induced significant decrease compared to canola and olive oils, while codliver oil induced a significant decrease compared to canola oil only.

Effect of cooking oils on testosterone and DHT levels in the prostate

The castor oil induced a significant decrease in the testosterone prostate homogenate level compared to the BPH group while palm, vegetable, RHVO, sesame, and groundnut oils had increased levels compared to the BPH group. The castor oil and codliver oil induced

significant decrease compared to palm, sesame, RHVO, groundnut, vegetable and sardine oils. Sheabutter, canola, RHPO, cottonseed, olive, coconut and blackseed oils induced significant decrease compared to palm, sesame, RHVO, groundnut and vegetable oils. Sardine, vegetable, groundnut, RHVO oils induced significant decrease compared to palm and sesame oils. Pomegranate seed oil induced a significant decrease compared to palm, sesame, RHVO, groundnut oils while sesame oil induced a significant decrease compared to palm oil only (Fig 3).

The pomegranate seed oil, olive oil, RHPO, induced significant decrease in the DHT prostate homogenate oil compared to the BPH group while palm and blackseed oils induced an increase compared to the BPH group. Differences within the groups are as follows: Olive oil induced a significant decrease compared to palm, blackseed, sesame, sardine, canola, groundnut, vegetable, coconut, codliver, sheabutter, cottonseed, and castor oils. RHPO induced a significant decrease compared to palm, blackseed, sesame, sardine, canola, groundnut, vegetable and coconut oils. RHVO and pomegranate oils induced significant decrease compared to palm, blackseed, sesame, and sardine oils. Castor, cottonseed, sheabutter and cod liver oils induced significant decrease compared to palm and blackseed oils. Coconut, vegetable, groundnut, canola, sardine, seseame and blackseed oils induced significant decrease compared to palm oil.

DISCUSSION

This study revealed that the oils administered orally at 800mg/kg for 14days significantly inhibited the increased prostate weight (PW) and the prostate to body weight (PW/BW) ratio induced by testosterone in the rats. Studies have shown that PW gain induced by testosterone in rats is accompanied by histological changes indicative of prostatic hyperplasia (PH), and that treatments that prevented the increase in PW also lowered histological scores of PH (Mitra *et al.*, 1999, Noa *et al.*, 2005). Thus, the effects of these oils on PW increase and increase in PW/BW can be explained as preventive effects on testosterone -induced PH in rats. The inhibitory effect of coconut oil on PW (65.2%), and PW/BW ratio (68.6%) found in this study is consistent with the report of Arruzazabala *et al.*, (2007) who stated that a significant inhibition in the increase in PW (82.0%) and PW/BW ratio (74.3%) respectively also at a dose of 800mg/kg administered orally for 14 days was achieved. The effects of coconut oil are consistent with its high lauric and myristic acids. It is worthy to note that the oils used in this study contains oleic, linoleic, myristic or lauric acids and at varying appreciable concentrations, these fatty acids had been documented to inhibit 5α-reductase enzyme which converts testosterone into dihydrotestosterone thus their preventive effects (Gossell-Williams *et al.*,2006, Raynaud *et al.*, 2002, Tsai *et al.*,2006). Lauric and oleic acids have been discovered to inhibit the activity of both 5α-reductase isoforms. Several substances that have been implicated to inhibit BPH have fatty acid components. Saw palmetto lipid extracts (SPLE) have been shown to possess lauric, oleic and myristic acids (Arruzazabala *et al.*, 2011). D-004, a lipid extract of royal palm (*Roystonea regia*) fruit has been shown to contain a mixture of free fatty acids, and oleic, lauric, palmitic and myristic acids were its major components (Arruzazabala *et al.*, 2011).

The presence of excess polar compounds in repeatedly heated frying oil has been associated with increased risk of developing hypertension (Azman *et al.*, 2012). This is because the consumption of repeatedly used frying oil might increase the risk of developing atherosclerosis (Williams *et al.*, 1999). The consumption of repeatedly-heated cooking oil has also been associated with increased total serum and low-density lipoprotein (LDL) level. In another study, repeatedly heated oil increased lipid peroxidation and LDL in ovariectomized female rats suggesting that repeatedly- heated oil may contribute to the pathogenesis of atherosclerosis in post-menopausal women (Siti *et al.*, 2008). The fatty acid components present in the palm oil were also present in the RHPO even though it was re-heated three times. Palm oil contains a high percentage of oleic and a little linoleic acid. In this study however, both repeatedly-heated palm and vegetable oils ameliorated the effect of BPH in the rats. This is because of the presence of oleic and linoleic acids in the oils and these acids have been shown to reduce the activity of BPH. Pomegranate seed oil also contains a little percentage of oleic acid and linoleic acid but a large percentage of punicic acid (PA) which has been implicated to possess anticancer attributes. In a study PA inhibited breast cancer cell proliferation through its lipid properties and by affecting the protein kinase C pathway (Grossmann *et al.*, 2010). PA has also been found to inhibit both proliferation of cell lines and secretion of proinflammatory cytokines (Rocha *et al.*, 2012). PA decreased tumour activity in mice skin cancer cells (Hora *et al.*, 2003). Anticancer effects of PA have also been studied in prostate cancers. PA inhibits the expression of prostate specific antigen and steroid 5α-reductase type and dihydrotestosterone-induced androgen receptor nuclear accumulation. Studies also showed that PA stimulated DNA fragmentation and internal apoptotic activity through caspase-dependent pathway (Gasmi and Sanderson, 2010). This study has been able to relate with the recently renewed drive to identify natural remedies such as pomegranate plant to fight prostate cancer (Wang and Martins-Green, 2014).

Androgenic hormones, testosterone (T) and dihydrotestosterone (DHT) play crucial roles in the aetiology of BPH (Carson and Rittmaster, 2003). BPH

is caused by DHT, a metabolite obtained from the conversion of T by 5α-reductase (Mc Connell *et al.*, 1992). Subsequently, inhibitors of 5α-reductase which block production of DHT ultimately slow down the development of BPH. The two main classes of drugs used as BPH treatments are inhibitors of α1-adrenoceptor inhibitors, which inhibit smooth muscle cell contraction (Furuya *et al.*, 1982), and inhibitors of type II 5α-reductase, an enzyme responsible for the conversion of testosterone to the more potent androgen dihydrotestosterone (DHT) (Griffiths and Denis, 2000). Steroid 5α-reductase converts testosterone to DHT, an active form of androgen, in the prostate. Increased production of DHT results in the development of prostatic hyperplasia (Pais, 2010). DHT has 10 times higher affinity for the androgen receptor than testosterone as such DHT easily binds to androgen receptor, which stimulates the transcription of growth factors that are mitogenic for the epithelial and stromal cells for prostate (Carson and Rittmaster, 2003). The importance of DHT in prostatic hyperplasia was demonstrated by previous studies in which an inhibitor of 5α-reductase was administered to experimental animals with BPH (Roehrborn, 2011). These findings agree with our results. In this study, blackseed, sheabutter, sardine, vegetable and groundnut oils reduced the DHT level in the serum, while pomegranate, olive, RHPO reduced the DHT level in the prostate compared to the BPH rats. These results thus suggest that blackseed, sheabutter, sardine, vegetable, groundnut, pomegranate, olive, and RHPO oils inhibited the development of BPH via downregulation of DHT.

The testosterone serum level in the castor oil and sheabutter groups were decreased compared to the BPH group while the castor oil group also had a decreased prostate testosterone level compared to the BPH group. The palm, vegetable, RHVO, sesame, and groundnut groups had the testosterone prostate levels increased compared to BPH group while the vegetable oil group increased the testosterone level compared to the BPH group. To the best of our knowledge, no study on fatty acid based dietary intervention or natural materials such as pumpkin seed oil (Gossell-Williams *et al.*, 2006), coconut oil (Arruzazabala *et al.*, 2007) as well as herbal extracts mainly saw palmetto lipid extracts (SPLC) (Arruzazabala *et al.*, 2007) have investigated the effect of DHT and T levels of such substances in experimental BPH, although some studies have shown an increase in T level of some drugs like Finasteride®. According to Pais, (2010) and Roehrborn, (2011), the administration of Finasteride® showed that the testosterone level was increased compared with that of BPH animals and in these studies, it was due to the inhibition of the transformation of testosterone to DHT. Unlike Finasteride®, sheabutter significantly reduced the concentrations of both testosterone and DHT compared with the BPH treatment group. It is suggested that the inhibitory effect of sheabutter likely occurs at the time of the development of BPH (Park 2016).

In this study the pomegranate seed oil significantly reduced the testosterone-induced prostate enlargement as well as the increase in PW and PW/BW ratios by 68.2%; 60.4% respectively as well as reducing the DHT prostate level. The anticarcinogenic properties of pomegranate seed oil could be because of its anticarcinogenic activities and inhibition of prostaglandin synthesis. Dietary pomegranate seed oil has been observed to diminish the growth of human prostate cancer LNCaP and DU145 cells to significantly reduce the invasiveness of the PC-3 cell line (Albrecht *et al.*, 2004). The anticancer activity of punicic acid (PA) is mediated by decreasing cell migration and CXCL12 chemotaxis, increasing cell adhesion as well as inhibiting epithelial-mesenchymal transition and inhibiting angiogenesis and proliferation (Wang *et al.*, 2012). The antimestastatic effect of PA occur via targeting hyaluronan signalling pathways in prostate cancer cells, in addition, PA possibly inhibits cytokine and chemokine pathways as well (Wang *et al.*,2011).

Blackseed, shea butter, sardine, vegetable, groundnut, pomegranate, olive, and RHPO oils administered orally at 800mg/kg appeared to be effective in reducing established prostate hyperplasia.

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Haemogram and Serum Biochemical Values of Four Indigenous Species of Monkeys in South West Nigeria

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Summary: Haematological and serum biochemical values are useful guides and biomarkers in health and diseases for reaching a diagnosis, estimating disease prognosis and monitoring treatment progress, in mammals. Reference ranges for some parameters differ among species of mammals and between sexes within a species. There is dearth of information on standard reference value for blood parameters for Nigerian indigenous monkeys. Whole blood and serum samples obtained from 50 apparently healthy adult monkeys in both captivity and from the wild in southwest Nigeria were subjected to haematology and serum biochemistry to obtain preliminary reference values for haematological and serum biochemical analytes for *Cercocebus sebaeus* (Green monkey), *Cercopithecus mona* (Mona monkey), *Erythrocebus patas* (Patas monkey) and *Papio anubis* (Anubis baboon). Numerical data were summarized as mean and standard deviation and subjected to statistical analysis; Student t test and analysis of variance, to compare values of blood parameters obtained between species and gender. A p-value less than 0.05 was considered significant. The hematocrit of male animals were significantly higher than that of females (P=0.01) in all the 4 species studied but there was no significant difference in other blood parameters such as total white blood cell and the differential counts, platelet count, serum aspartate transferase, alanine transferase, alkaline phosphatase, total plasma protein, albumin, and globulin concentrations between the sexes. Generally, there was no significant difference between total white blood cell and the differential counts, hematocrit, red cell count, haemoglobin concentration, platelet count, serum aspartate transferase, alanine transferase, alkaline phosphatase, total plasma protein, albumin, and globulin concentrations among the monkey species.

Keywords*:* Haematology, Monkeys, Reference data, Serum biochemistry.

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INTRODUCTION

Haematological and serum biochemical parameters are useful indicators of health and ill-health in humans and animals (Milner et al., 2003; Bhatia et al., 2004). They are useful guides for reaching a diagnosis, estimating disease prognosis and monitoring treatment progress (Bhatia et al., 2004; Abro et al., 2009). When non-human primates (NHPs) are used in biomedical research, normal reference values for haematological and serum biochemical parameters are often needed for comparison with experimental outcomes (McPherson, 2013). Reference value range for blood parameters often differ between species of animals and significant differences may be noticed between sexes and age groups within a species (Xie et al, 2013; Castro et al, 2016). Diet and environmental influences also affect the normal blood parameter values in NHPs (McPherson, 2013). The green monkey has been used extensively for biomedical research and therefore has received enormous attention, including basic reference value, unlike other breeds of indigenous monkeys in Nigeria with limited data (reference value to biomarker of health) but also receive a wide use in research (Kagira et al., 2007; Chichester et al., 2015; Castrol et al, 2016). There are currently no standard reference values for blood parameters in Nigerian indigenous monkeys (Mona monkey, Patas monkey, Anubis baboon, etc). Hence, as part of a region wide serosurveillance for antibodies to zoonotic diseases among NHPs (August 2015 to February 2017), whole blood and serum samples obtained from 50 apparently healthy adult (>5years old) monkeys in southwest

Nigeria were subjected to haematology and serum biochemistry to present preliminary reference data for haematological and serum biochemical analytes for 4 indigenous species of monkeys.

MATERIALS AND METHODS

Study sites and animal sampling

A total of 50 whole blood and serum samples were obtained from adult (>5years) individuals belonging to 4 species of captive and wild NHPs in 5 locations within southwest Nigeria: Zoological garden, University of Ibadan, Oyo State (7.4434°N, 3.8956°E); Biological garden, University of Ilorin, Kwara State (8.4817°N, 4.6382°E); Agodi gardens, Ibadan, Oyo State (7.4069°N, 3.8994°E); Osun Osogbo sacred groove, Osun State (7.7592°N, 4.5569°E); and University of Lagos, Akoka campus (6.5155°N, 3.3878°E). Domestic (pet) monkeys in Ibadan, Oyo State, whose owners consented to the sampling of their animals, were also enrolled in the study. Table 1 shows the distribution of sampled animals among the various study sites. The four Mona monkeys (*Cercopithecus mona*) from Osun Osogbo sacred groove and one out of the four Mona monkeys from University of Lagos Akoka campus were trapped directly from the wild. Other types of monkeys sampled were Green monkeys (*Cercocebus sebaeus*), Patas monkeys (*Erythrocebus patas*), and Anubis baboons (*Papio anubis*). Age estimation of the animals was based on curator records in the host facility and date of acquisition of pet animals. Attempts were made at sampling as many NHPs as permitted by curators at the various locations. All animals were apparently healthy at time of sampling and they showed no signs of illness up to three months post sampling. Caged monkeys were darted at close range (1-5m) with a blow pipe delivering anaesthetic (ketamine hydrochloride) at 10mg/kg body weight of the monkey while tame animals were injected by hand. Locally fabricated, self-triggering traps (approximate size= 2m x 1m x 1m made of aluminum wire netting attached to a wooden frame), with guillotine-type trap door were used to trap free-ranging monkeys.Wild monkeys were first habituated for 2 weeks by daily placement of suitable food items around the trapping sites. Phlebotomy was via cephalic or tibial venipuncture of sedated animals. Five millilitres (5mls) of blood was collected, 1ml into heparinized tubes; the remaining into sterile plain tubes for serum separation. Samples were conveyed on ice packs to the Clinical pathology laboratory, Faculty of Veterinary Medicine, University of Ibadan where haematology and serum biochemical analyses were done immediately. Ethical approval for capture and sampling of the monkeys was obtained from the University of Ibadan, Animal Care and Use Research Ethics Committee (UI ACUREC/App/2015/054).

Haematology and serum biochemistry

Haematology: To determine the packed cell volume (PCV) capillary tubes were filled with blood until three-quarter full. One end was then sealed with plasticine. The capillary tubes were spun in a micro haematocrit centrifuge (Hawksley and Sons, London) for five minutes at 1200×g and the PCV values were determined using the Hawksley haematocrit reader. Haemoglobin concentration was determined using the Sahli's method. Total red blood cell (RBC), total white blood cell (WBC) and platelet counts were determined manually using a Neubaeur haematocytometer according to Weiss and Tvedten (2004). Mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were determined using specific formulae (Weiss and Tvedten, 2004). The differential WBC count was determined by identifying 100 WBCs on a blood smear to determine the percentage of each type of WBC. Total plasma protein concentration was determined using a refractometer.

Serum biochemistry: Blood was collected in plain bottles for serum preparation. Serum samples were collected by centrifugation of blood at 224g for 10 minutes. The concentrations of albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, blood urea nitrogen, creatinine, cholesterol and glucose were determined by spectrophotometry using respective analytic kits supplied by Randox (Randox, USA). Kits were used according to manufacturer's directions. Serum globulin concentration was calculated as the difference between total serum protein and albumin concentrations

Statistical Analysis: Statistical analysis was done using SPSS 20® statistical computer software. Numerical data were summarized as mean and standard deviation of the parameters measured. Analytical statistics; Student t test and Analysis of variance, were used to compare values of blood parameters obtained between species and gender. Statistical significance was determined at 95% confidence interval.

RESULTS

Values of the haematological and serum biochemical parameters measured in 50 adult (>5years old) monkeys belonging to 4 indigenous species of both sexes are summarized in Table 2. The mean values of red cell parameters; PCV(%)/RBC($x10^6$/µl)/HB(g/dl)

Table 1: Sampling scheme for monkey species obtained in south west Nigeria

Sampling location	*Cercopithecus mona*	*Cercocebus Sebaeus*	*Erythrocebus patas*	*Papio anubis*	Total
1	2(50%) *	8(100%)	7(100%)	5(100%)	22
2	1(100%)	4(57%)	4(100%)	AV	9
3	4(100%)	NA	NA	2(100%)	6
4	4(6.7%)	NA	NA	NA	4
5	NA	2(100%)	1(100%)	1(100%)	4
6	AV	1(?)	2(?)	2(?)	5
Total	11	15	14	10	50

*Sampling proportions are in parentheses (i.e. the number of individuals sampled out of the total number of individuals of the species present in the facility); ? =unknown sampling proportion; NA= species not available at location;AV= species available at location but not sampled. 1=Zoological garden, University of Ibadan, Oyo State, 2=Biological garden, University of Ilorin, Kwara State, 3=University of Lagos, Lagos State, 4=Osun-Osogbo sacred groove, Osun State, 5=Agodi gardens, Ibadan, Oyo State, 6=Pet monkeys within Ibadan, Oyo State

Table 2: Haemogram/serum biochemical values of four species of adult (>5years) monkeys in south west Nigeria

Parameters	*Cercopithecus mona*		*Cercocebus sebaeus*		*Erythrocebus patas*		*Papio anubis*	
	Female (N=7)	Male (N=4)	Female (N=8)	Male (N=7)	Female (N=10)	Male (N=4)	Female (N=3)	Male (N=7)
Packed cell volume (%)	39±2*	40±3	36±4	45±4	38±3	45±4	35±3	38±8
Haemoglobin (g/dl)	11.4±4.3	13.3±1.2	11.8±1.4	14.8±1.4	12.8±1.2	14.9±1.4	11.3±1.3	12.7±2.7
Red blood cells (x10^6/ µl)	6.70±0.59	6.92±0.64	5.80±0.90	7.59±0.79	6.34±0.60	7.39±0.67	5.63±0.57	6.38±1.34
MCV (fl)	58.2±2.2	57.8±0.0	62.1±2.8	59.3±0.9	59.9±1.0	60.9±0.1	62.2±1.0	59.6±0.1
MCHC (g/dl)	29.2±9.6	33.3±0.5	32.8±0.3	32.9±0.2	33.7±0.6	33.1±0.2	32.3±0.9	33.4±0.1
Platelet counts (x10^3/µl)	41.49±27.42	19.03±6.58	20.78±10.62	39.67±37.52	40.90±34.48	29.45±33.83	17.93±7.91	17.73±5.76
Total white cell counts/µl	7650±3660	8725±5060	6650±1858	5793±2206	5190±1502	7238±3141	5400±1146	6143±1739
Lymphocytes (%)	49±21	56±7	63±7	64±8	57±6	63±8	61±10	58±6
Neutrophils (%)	40±18	34±6	29±7	32±6	36±9	31±11	30±7	33±4
Monocytes (%)	4±1	5±2	3±2	4±2	3±1	3±1	3±1	4±1
Eosinophils (%)	7±3	6±5	3±2	2±1	3±2	4±3	6±3	5±2
Total protein (g/dl)	8.3±0.6	8.0±0. 6	7.2±1.2	7.9±0.5	7.2±1.3	8.1±0.8	7.6±1.6	7.6±0.9
Albumin (g/dl)	3.5±0.2	3.3±0.6	2.9±0.5	3.1±0.4	2.8±0.8	3.5±0.4	3.4±0.6	3.0±0.4
Globulin (g/dl)	4.8±0.5	4.8±0.1	4.3±1.0	4.8±0.2	4.4±0.7	4.6±0.5	4.2±1.0	4.6±0.7
A-G Ratio	0.74±0.08	0.65±0.07	0.69±0.19	0.64±0.09	0.65±0.18	0.70±0.08	0.80±0.01	0.66±0.09
AST (IU/L)	21±4	23±4	26±6	22±5	20±3	22±5	24±5	23±4
ALT (IU/L)	28±6	32±4	32±5	30±6	27±4	32±3	32±5	32±3
ALP (IU/L)	224±77	228±91	236±67	241±58	209±48	212±70	209±70	166±55
Blood urea nitrogen (mg/dl)	12±1	12±1	11±1	11±1	11±1	11±1	12±1	11±1
Creatinine (mg/dl)	0.8±0.2	0.7±0.2	0.8±0.2	0.8±0.1	0.7±0.1	0.8±0.2	0.8±0.2	0.7±0.1
Glucose (mg/dl)	106±11	108±10	92±13	82±9	106±25	95±12	93±19	104±11
Cholesterol (mg/dl)	105±18	111±19	90±24	90±21	94±18	114±13	107±17	95±8

*All values are expressed as mean ± standard deviation.Note: PCV=Packed cell volume; RBC=Red blood cells; MCV=Mean corpuscular haemoglobin; MCHC= Mean corpuscular haemoglobin concentration; A-G Ratio=albumin-globulin ratio AST= Aspartate transferase, ALT= Alanine transferase, ALP= Alkaline phosphatase.

Table 3: A comparison of blood parameters between male and female monkeys irrespective of species differences.

Blood parameters	**Sex**	
	Male(N=22)	**Female(N=28)**
Packed cell volume (%)	41±6	37±4*
Haemoglobin(g/dl)	13.9±2.1	12.2±2.4*
Red blood cells(x10^6/µl)	7.0±1.0	6.2±0.8*
White blood cells(x10^3/µl)	6.7±2.9	6.2±2.4
Platelets(x10^3/µl)	27.1±2.6	32.8±2.7
Lymphocytes (%)	60.4±7.2	57.3±12.6
Neutrophils (%)	32.3±6.2	34.2±11.7
Monocytes (%)	3.7±1.5	3.3±1.6
Eosinophils (%)	4.1±2.8	4.4±3.1
Total protein (%)	7.8±0.7	7.5±1.2
Albumin(g/dl)	3.2±0.4	3.1±0.7
Globulin(g/dl)	4.7±0.4	4.4±0.7
A-G Ratio	0.66±0.08	0.70±0.16
AST (IU/L)	22.6±4.1	22.2±5.0
ALT(IU/L)	31.4±4.4	29.1±5.5
ALP(IU/L)	209.5±68.7	220.4±61.3
Blood urea nitrogen(g/dl)	11.2±0.8	11.2±1.0
Creatinine(g/dl)	0.77±0.14	0.76±0.16
Glucose(g/dl)	97.8±12.8	121±14.8
Cholesterol(g/dl)	99.7±18.1	97.1±19.8

*Values of corresponding parameter is significantly higher in males than in females at p≤0.05

in the males (41±6/7.0±1.0 /13.9±2.1), were significantly higher (p=0.01/ p=0.02/p=0.05) than in the females (37±4/6.2±0.8/12.0±2.4) irrespective of the species (Table 3). BUN had significant (p=0.02) variability between species with Anubis baboon and Mona monkey having a significantly higher values when compared with that from either Green or Patas monkey.

DISCUSSION

All blood parameters measured in this study appear to be similar in value across the species except for eosinophils, with relatively fewer numbers and wider ranges, which showed significant differences attributable to outlier effects. The consistently higher erythrocytic parameters in adult male monkeys relative to values in adult females is similar to findings in the Rhesus monkeys (*Mucaca mulatta*) where PCV(%)/RBC(x106/µl)/HB(g/dl) values in adult males (41±4/5.5±0.4/13.2±1.3) were found to be significantly higher (p<0.01) than in adult

females(39±3/5.1±0.39/12.3±1.3) (Stanley and Cramer, 1966); Cynomolgus monkeys (*Mucaca fascicularis*) where respective values in adult males (46±3/5.9±0.3/13.9±0.9) were consistently higher than in adult the adult females (44±3/5.6±0.4/13.5±0.6) (Park et al., 2016); and in Chimpanzees where the values in adult males (47±5/5.5±0.7/15.4±1.5) were also consistently higher than in adult the adult females (42±7/5.1±0.9/13.6±2.1) (Howell et al 2003). This form of sexual dimorphism which appears to be common manifestation in all mammals has been attributed to polymorphisms of the erythropoietin (EPO) gene and its receptor (EPOR) (Zeng et al., 2001).

The white cell parameters showed no significant difference in males and females in all monkey species included in this study in agreement with findings in Cynomolgus monkeys (Park et al., 2016), but this is contrary to studies in other primates, such as the Rhesus monkey, in which adult males were found to have higher total white cell and lymphocyte counts but a lower neutrophil count than in adult females (Stanley and Cramer, 1966). Generally, there appears to be no uniform trend in sexual differences of white blood parameters in primates. For example, Howell et al. (2003) found that white cell parameters in male chimpanzees were not significantly different from that of the females whereas Herndon and Tigges (2001) found that total WBC and lymphocyte counts in female chimpanzees were significantly higher than in the males. Haematological values reported here for the Patas monkey (*Erythrocebus patas*) were very similar to those reported by Calle and Joslin (2014), from Composite Med ARKS Records comprising of not less than 400 individuals. Similar values were also reported for the African green monkey (Sato et al, 2005; Liddie et al, 2010) but literature is sparse for values for other monkeys sampled in this present study.

There was no significant difference between any of the serum biochemical parameters measured in male and female animals irrespective of species. The serum biochemical values obtained in this study fall within the range of values obtained in other studies on old world monkeys which typically have a relatively large standard deviation (Calle and Joslin, 2014; Castro et al., 2016). However, sexual differences were found in those studies. For example, ALT and Creatinine values for adult male obtained in the green monkey by Castro et al., (2016) was significantly higher than that of adult females while ALB and Cholesterol levels in females were higher than that of males. Inter species variations in the titre of serum biochemical parameters was generally minimal with BUN being the only parameter having significant (p=0.02) variability between species.

Since all animals were anesthetized for collection and examination, the effects of ketamine must be taken into consideration. Ketamine has been reported to reduce leukocyte and red blood cell counts as well as reduce corpuscular haemoglobin and haematocrit in rhesus monkeys (*Macaca mulatta*) when blood parameters were compared between manually restrained and ketamine immobilized subjects (Swindle et al, 2002). This should therefore be taken into consideration when interpreting haematology results of monkeys using tables provided in this study.

The limited sample size did not allow for more robust comparisons of parameters across species and habitats. Despite this limitation, preliminary findings such as influence of gender on hematologic values was revealed and implies that gender peculiarities should be always be considered when evaluating haematological and serum biochemistry values for health and disease in monkey. Values reported in this study can serve as guide to developing more elaborate blood parameter reference ranges for Nigerian indigenous monkeys in the future; considering influences such as age, gender, species and captivity on physiologic status of non-human primates.

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Ameliorative Effects of Hydromethanolic Extract of *Citrullus lanatus* (Watermelon) Rind on Semen Parameters, Reproductive Hormones and Testicular Oxidative Status Following Nicotine Administration in Male Wistar Rats

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Summary: The present study examines the possible ameliorative effects of the hydromethanolic extract of *Citrullus lanatus* rind (HECL) on some reproductive function and oxidative indices of the testes in male Wistar rats following administration of nicotine. Twenty male rats were assigned into four groups: Group A to D of five rats each. Group A served as control and received 2ml/kg body weight of 10% extract vehicle; Group B received 1mg/kg body weight of nicotine; Group C were co-administered 1mg/kg body weight nicotine and 500 mg/kg body weight of HECL and Group D received only 500mg/kg body weight of HECL. The drugs and extracts were administered orally to the rats for 42days; blood samples were collected by direct cardiac puncture for determination of serum concentrations of testosterone, Follicle Stimulating Hormone and Luteinizing Hormone. The testes were also harvested for determination of semen parameters: motility, morphology, viability and count and testicular tissue processed for superoxide dismutase and malondialdehyde concentration. Compared to Group A control rats, administration of HECL significantly increased sperm count and reproductive hormone concentrations amongst Group B rats ($p<0.05$). Treatment with nicotine caused a significant reduction in the levels of all reproductive hormones with significant diminution of some sperm parameters: motility, morphology and viability; and decrease in superoxide dismutase and increase in malondialdehyde concentration amongst Group B rats compared to Group A control rats ($p<0.05$). Co-administration of HECL with nicotine to Group C rats apparently reversed the effects of nicotine resulting in significant increases in sperm count and the reproductive hormones concentration as compared to Group A control rats ($p<0.05$). Amongst Group D rats, the extract also caused a significant increase in superoxide dismutase concentration and a significant decrease in malondialdehyde concentration compared with the Group A control rats ($p<0.05$). The findings suggest that the hydromethanolic extract of *Citrullus lanatus* rind possibly ameliorates the deleterious effects of nicotine on some reproductive indices in male Wistar rats.

Keywords*: Citrullus lanatus*; nicotine, superoxide dismutase, lipid peroxidation, semen; reproductive hormones.

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INTRODUCTION

Although, nicotine is a naturally occurring alkaloid found in a wide variety of plants (Doolittle et al., 1995) the principal source of human nicotine exposure is through tobacco use, nicotine replacement therapy such as transdermal nicotine patches and nicotine containing gum (Heisheman et al., 1994). Nicotine can cross biological membranes including the blood brain barrier and once absorbed, is extensively metabolized by the liver to a number of major and minor metabolites (Snyder et al., 1993; Cashman et al., 1992; Neurath, 1994; Crooks and Godin, 1988; Godin and Crooks,1986; Booth and Boyland, 1971; Kyerematen et al., 1990). Nicotine is considered as the primary chemical in tobacco responsible for engendering tobacco use and dependence (Di Chiara, 2000, Harvey et al., 2004). Several adverse effects of nicotine leading to various diseases and pathological conditions in humans have been described (Hammer and Mitchell, 1979, Christensen et al., 1984, Wilkins et al, 1982, Pullan et al.,1994). Nicotine along with cotinine (a nicotine metabolite with a longer half-life) adversely affects spermatogenesis, epididymal sperm count, spermatocyte motility and fertilizing potential (Aydos et al., 2001). Oral administration of nicotine in male rats have been associated with testicular degeneration and disorganization of the cytoarchitecture, decreased serum testosterone, reduced semen characteristics and impaired fertility (Oyeyipo et al., 2010; Oyeyipo et al., 2011). In female rats, nicotine administration have been reported to significantly reduce the weights of visceral organs

including the ovary, kidney, pituitary and uterus while increasing the weights of the heart and liver with appearance of cartilaginous cells in the heart and deposition of adipose around the portal veins: associated necrosis, congestion, fibrosis, follicular and endometrial degeneration were also observed in the brain, pituitary, kidney, ovary and uterus respectively (Iranloye and Bolarinwa, 2005).

Citrullus lanatus (watermelon) is one of the major under-utilized fruits grown in the warmer part of the world. The juice or pulp from watermelon is consumed by humans, while the rind and seeds are major solid wastes (Ahmed,1996; Lewinsohn et al., 2005; Bawa and Bains, 1977). The rind is utilized for products such as pickles and preserves, as well as for extraction of pectin (Leong and Shui, 2002; Gyamfi et al.,1999). The therapeutic effect of watermelon has been reported and has been ascribed to its composition of a number of antioxidant compounds; amongst them, citrulline and lycopene have been demonstrated to play prominent roles (Melo et al., 2006; Minotti and Aust, 1987). The rind has recently been evaluated as a wheat flour substitute for cake making (Al-Sayeed and Ahmed, 2013) and is recommended in both alcoholic poisoning and diabetes (Duke and Ayensu 1985; Jiyun et. al., 2011). Furthermore, the rind has been shown to contain alkaloids, saponin, cardiac glycosides, flavonoids, phenol, moisture, lipid, protein, fiber and carbohydrates (Erukainure et. al., 2010; Erhirhie and Ekene, 2013). The possible ameliorative effects of the methanolic extract the rind of Citrullus lanatus on some semen parameters and reproductive hormones following lead acetate induced toxicity (Kolawole et al., 2014), following aspirin induced gastric ulceration (Kolawole et al., 2016a) and in alloxan induced diabetes (Kolawole et al., 2016b) in male Wistar rats has recently been reported from our laboratory.

As part of a series of studies exploring the potential beneficial effects of the rind of Citrullus lanatus the present report describes the possible ameliorative effects of the hydromethanolic extract of the rind on some semen parameters, reproductive hormones and indices of testicular oxidative status following the administration of nicotine using male Wistar rat as models.

MATERIALS AND METHODS

Plant material and preparation of extracts: Fresh plant and fruits of watermelon were obtained from the community market in Elele, Rivers State, Nigeria. The plant materials were identified and authenticated by Dr. Chimezie Ekeke of the Department of Plant Science and Biotechnology, University of Port Harcourt, Nigeria. Voucher specimens were also deposited with the herbarium number UPH/V/1214. The rinds were peeled off from the whole fruit washed thoroughly, sun-dried and milled into a fine powder. The method of extraction employed is percolation (Adesanya *et. al.*, 2011). 24g of the powdered sample was soaked in a beaker containing 100ml of 98% methanol for a period of 48 hours and then filtered with a Whatman No. 1 filter paper size. The volume of filtrate obtained was 150ml before concentration; the filtrate was subsequently concentrated using a rotary evaporator. The weight of residue obtained was 8.5g.

Determination of median lethal dose (LD_{50}): Acute toxicity study (LD_{50}) was determined using the method described by Lorke 1983. The (LD_{50}) of the extract was found to be greater than 2000mg/kg body weight.

Nicotine preparation: Nicotine hydrogen tartrate with product number 36733-1G (99% nicotine); was obtained from Sigma Chemical Corporation (Sigma Aldrich, St. Louis, Mo, USA). Nicotine stock solution was prepared at concentration of 1mg/ml and stored in foil-wrapped glass bottle 4ºC for no longer than ten days before use.

Experimental procedure: Twenty male Wistar rats were used for this study. The rats weighed between 170g and 200g; were divided into four groups: Groups A to D of 5 rats each. The rats in each group were placed in separate cages in the Animal House of Madonna University, Nigeria under natural day and night cycles. The rats had free access to normal rat chow and tap water *ad libitum*. They were allowed two weeks of acclimatization to their environment and subsequently treated as follows:

Group A: Control group. Rats in this group were given 2ml/kg body weight 10% of extract vehicle.

Group B: Nicotine only group. Rats in this group were given 1mg/kg body weight of nicotine as described by Oyeyipo *et. al*., 2010.

Group C: Nicotine + extract group. Rats in this group were co-administered 1mg/kg body weight of nicotine and 500mg/kg of hydromethanolic extract of *Citrullus lanatus* rind.

Group D: Extract only group. Rats in this group were given 500mg/kg body weight of hydromethanolic extract of *Citrullus lanatus* rind.

The hydromethanolic extract of *Citrullus lanatus* rind, nicotine and extract vehicles were administered to the rats daily using a gastric cannula. All the rats were treated for a total of 42 days. On day 43, blood samples were collected from chloroform anesthetized rats through direct cardiac puncture. The blood samples were placed in heparinized sample bottles, centrifuged at 1500rpm for 5 minutes and plasma obtained for assay of testosterone, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). The animals were then sacrificed and the testes immediately harvested for determination of semen parameters: motility, morphology, viability and count. The testes were then homogenized and the homogenate used for the assay of superoxide dismutase (SOD) and

malondialdehyde (MDA) concentration. All animal experiments were conducted in accordance with the National Institute of Health Guide for Care and Use of Laboratory animals (Pub No. 85-23 revised 1985) (NRC, 1985).

Determination of plasma concentrations of the reproductive hormones: The plasma concentrations of testosterone, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) were determined by the enzyme-linked immunosorbent assay (ELISA) technique using commercially available kits. The hormonal kits used for the assay was obtained from Monobind Inc. Lake Forest, CA, USA. Samples were run in the same assay to avoid inter-assay variations and determined on the same day of collection of blood samples.

Determination of semen parameters:

Motility: This was as described previously by Kaur and Bansal, 2004. Briefly, the caudal epididymis was identified and its content carefully expressed into 1ml of normal saline at room temperature. One drop of the semen suspension was charged into a Makler counting chamber and the number of motile and non-motile spermatocytes counted in ten random fields. The number of motile spermatocytes was then expressed as a percentage of the total number of the counted spermatocytes (Mahaneem *et al.*, 2011).

Sperm morphology: This was determined by smearing a drop of the stained semen suspension obtained during determination of sperm count on a glass slide; the smear was allowed to dry and subsequently examined under the light microscope at X400 magnification. For each sample, 200 spermatocytes were carefully observed and the percentage of total abnormalities of the spermatocyte head and total abnormalities of the spermatocyte tails were determined as described by Narayana *et al*., 2005.

Sperm viability: To determine viability, fluid from the caudal epididymis was carefully dropped on a slide and mixed with a drop of 0.5% eosin solution. After 2 minutes, the slide was examined under a light microscope at X40 magnification. The percentage of viable (unstained) and non-viable spermatocytes (stained red) was determined as described by Cheesbrough (2006).

Sperm count: This was determined as described earlier by Narayana *et al*., (2005) with minor modifications. Briefly, the caudal epididymis was carefully separated from the testis and minced in 2ml of normal saline followed by filtration through a nylon mesh. The suspension was then stained with 2% eosin in normal saline. The spermatocytes heads were counted using a Neubauer haematocytometer. Chamber counts for the sperm head in eight chambers (except the central chamber) were averaged and expressed as the number of sperm per caudal epididymis (Mahaneem *et al*., 2011).

Determination of testicular superoxide dismutase (SOD) and malondialdehyde (MDA) activity: The levels of SOD activity was determined by the method of Misra and Fridovich (1972). This involves inhibition of epinephrine autoxidation in an alkaline medium at 480nm in a UV vial spectrophotometer. For the determination of specific activity of SOD in homogenate sample of testes tissue, the rate of autoxidation of epinephrine was noted at 30 seconds' intervals in all groups. The enzyme activity was expressed in arbitrary units considering inhibition of autoxidation, as 1 unit of SOD specific activity. The determination of malondialdehyde (an index of lipid peroxidation) activity was as previously described (Kolawole *et al.,* 2016). Briefly, 2ml of thiobarbituric acid (TBA) reagent and 1ml of trichloro acetic acid (TCA) were mixed with 2ml of homogenate of testes tissue. The mixture was heated at 60°C for 20 minutes and then cooled before centrifugation at 400rpm for 10 minutes. The absorbance of the obtained supernatant was read at a wavelength 540nm.

Statistical Analyses: Significant differences were determined using the one-way analysis of variance (ANOVA) followed by the LSD post hoc tests. A p value <0.05 was considered statistically significant. The results are presented in Tables 1 and Figures 1, 2, 3 and 4. All data were expressed as mean ± standard error of mean (SEM).

RESULTS

Table 1 shows the effects of hydromethanolic extract of *Citrullus lanatus* rind on some semen parameters of male Wistar rats when administered alone (Group D) and when co-administered with nicotine (Group C). It also shows the values of the semen parameters in Group A control and in rats administered nicotine only (Group B). Results obtained indicate that administration of nicotine alone in Group B rats generally caused a diminution of sperm quality with significant reductions in percentages of motile and viable cells and corresponding significant increases in percentages sluggish cells, cells with tail defects, non-viable cells as compared to Group A control rats ($p<0.05$). Reductions in sperm counts were however, not significant ($p>0.05$). Compared to Group A control rats, co-administration of nicotine with the hydromethanolic extract of the rind of Citrullus lanatus in Group C rats generally caused an improvement in all the sperm parameters; however, significant differences were seen only for sperm count, viable cells and cells with tail defects ($p<0.05$) when compared to Group A control rats. However, despite the significant improvement in sperm counts, there

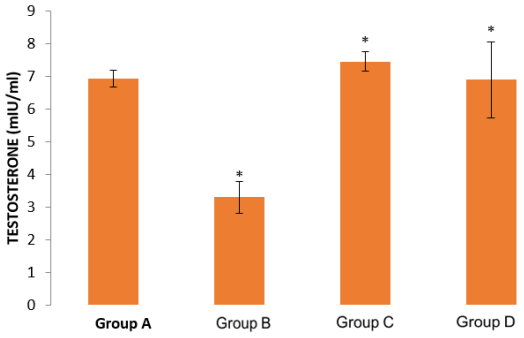

Fig. 1: Effect of the hydromethanolic extract of the rind of *Citrullus lanatus* on serum testosterone concentration in male Wistar rats following nicotine administration.

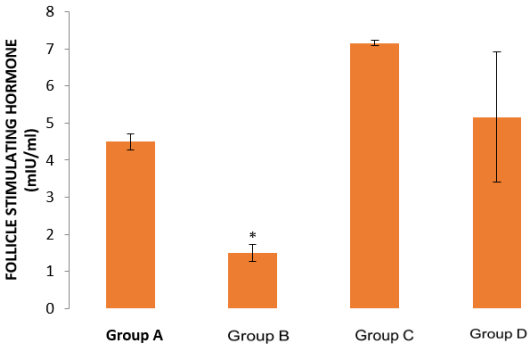

Fig. 2: Effect of the hydromethanolic extract of the rind of Citrullus lanatus on Follicle Stimulating Hormone concentration in male Wistar rats following nicotine administration.

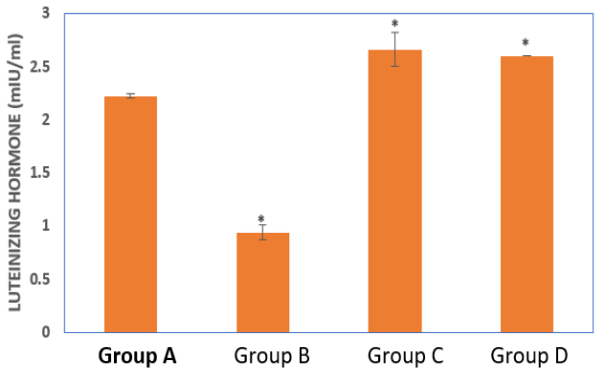

Fig. 3: Effect of the hydromethanolic extract of the rind of *Citrullus lanatus* on Luteinizing Hormone concentration in male Wistar rats following nicotine administration.

were significantly higher cells with tail defects and significantly lower percentage viable cells amongst Group C rats compared to Group A control rats ($p<0.05$). Group D rats were found to have significantly lower values of cells percentage motile cells, higher value of percentage with tail defects, non-viable cells and sperm counts as compared to Group A control rats. Values of these parameters are as shown in the Table 1.

Figures 1, 2 and 3 are results of plasma concentration of testosterone, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) respectively for all groups. Generally, administration of nicotine to rats in Group B caused a significant reduction in the value of each hormone compared to Group A control rats ($p<0.05$). However, administration of the hydromethanolic extracts of the rind of Citrullus lanatus caused significant increases in the values of each of the hormone as seen amongst rats in Group D ($p<0.05$).

Similarly, co-administration of both nicotine and the hydromethanolic extract of the rind of Citrullus lanatus to rats in Group C caused significant increase in the plasma levels of all the reproductive hormones compared to Group A control rats ($p<0.05$). The pattern of changes in values of reproductive hormones are as shown in Figures 1, 2 and 3.

Figure 4 shows the results of assay of superoxide dismutase and malondialdehyde in testes tissue for all Groups of rats. Administration of nicotine caused a significant reduction in the value of superoxide dismutase in Group B rats as compared to Group A control rats ($p<0.05$). However, administration of the hydromethanolic extracts of the rind of Citrullus lanatus to rats in Group D caused a significant increase in the concentration of superoxide dismutase ($p<0.05$). Similarly, co-administration of nicotine and the hydromethanolic extract of the rind of Citrullus lanatus to rats in Group C caused significant increase in the superoxide dismutase concentration as compared to Group A control rats ($p<0.05$). Administration of nicotine only to rats in Group B caused a significant increase in the levels of malondialdehyde compared to Group A control rats ($p<0.05$). Although, administration of the hydromethanolic extracts of the rind of Citrullus lanatus to rats in Group D caused significant decreases in the malondialdehyde concentration ($p<0.05$); co-administration of both nicotine and the hydromethanolic extract of the rind of Citrullus lanatus to rats in Group C similarly caused a significant decrease in the malondialdehyde levels, as compared to Group A control rats ($p<0.05$); however, the decrease was less than that observed for Group D rats.

Table 1: Effect of hydromethanolic extract of the rind of *Citrullus lanatus* extract on some semen parameters of male albino Wistar rats following nicotine administration

Groups	Sperm Motility (%)			Abnormal sperm morphology (%)		Sperm viability (%)		Sperm count (%)
	Actively motile	Motile	Sluggish	Head defect	Tail defect	Viable	Non-viable	
A	74.00±2.44	87.00±1.22	13.00±1.22	1.20±0.49	1.80±0.09	90.6±0.97	9.40±2.19	18.20±1.95
B	27.00±1.22*	50.00±0.00*	23.00±1.22*	0.60±0.20	2.20±0.01*	60.00±1.00*	40.00±2.24*	12.20±1.71
C	58.00±4.89	67.00±4.41	9.20±0.49	1.40±0.21	4.20±0.73*	84.40±0.98*	15.60±2.19	28.20±0.20*
D	53.00±12.00*	60.00±12.2*	7.00±2.00	1.80±0.48	4.40±0.24*	95.60±0.24	4.40±0.54*	32.40±5.14*

*All values=Mean ± SEM; * significantly different from values of Group A control at p<0.05*

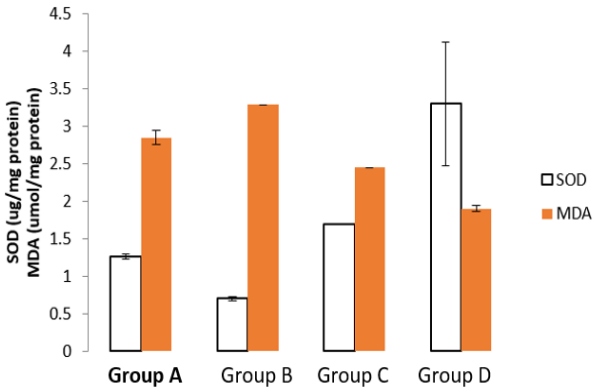

Fig. 4: Effect of the hydromethanolic extract of the rind of Citrullus lanatus on testicular tissue super oxide dismutase and lipid peroxidation activities in male Wistar rats following nicotine administration.

DISCUSSION

Expectedly, the observed diminution of semen parameters and reduced reproductive hormone concentrations following the administration nicotine seen in the present study is consistent with most of the previous reports (Yamamoto et. al., 1998; Aruldhas et. al., 2005; Seema et.al., 2007; Jana et. al., 2010; Oyeyipo et. al., 2011; Oyeyipo et. al., 2013). Several reasons have been advanced for these detrimental effects of nicotine on reproductive function. For instance, increased production of reactive oxygen species (ROS) especially testicular (cellular) hydrogen peroxide and hydroxyl radicals (Aruldhas et. al., 2005; Bandopadhyay et. al., 2008) may account for these effects of nicotine on semen parameters: Increased free radicals and ROS has been reported to cause lipid peroxidation of spermatozoa membrane with possible destruction of sperm DNA (Anto et. al., 2016). Further, the observed reduction in testosterone, FSH and LH concentrations could also contribute: testosterone the principal androgen plays an important role in several aspects of sexual maturation including behavior, spermatogenesis, differentiation and maintenance of the accessory sex organs (Ojeda and Urbanski, 1994). The synthesis and release of androgens is dependent on the pituitary gonadotropins: FSH and LH. Both gonadotropins are essential for testicular functions including spermatogenesis. LH is the main tropic regulator of Leydig cell function without which androgen production is impaired. The reduced levels of these gonadotropins could also contribute to the observed adverse effects of nicotine seen in the present study. Oyeyipo et al. (2010) further ascribed the decrease in serum testosterone level of rats treated with nicotine to disruption of testicular cytoarchitecture consequently adversely affecting Leydig cell number leading to decreased serum testosterone secretion. The results obtained from this study is consistent with the report of Oyeyipo et al., 2013 of a significant decrease in serum testosterone level in rats treated with varying doses of nicotine. Perhaps, the reduced gonadotropin hormone secretion seen in the present study following nicotine administration may be from a possible depressive effect on the hypothalamic neural mechanisms essential for the release of Gonadotropin Releasing Hormone (GnRH) (Reddy et al., 1995; Didia et. al., 2000). This eventually could lead to disturbances in the secretion of pituitary gonadotropins essential for both spermatogenesis and steroidogenesis (Aydos et.al.,2001). Co-administration of nicotine and the hydromethanolic extract of the rind of Citrullus lanatus caused some improvements in the semen parameters with increases in testosterone and gonadotropins secretions. The ameliorative effect of the extract may possibly be due to its content of phenols and flavonoid which serve as potent anti-oxidants (Eruikainure et al., 2010). The rind has been shown to be rich in some antioxidant vitamins especially tocopherol and ascorbic acid (Edwards et al., 2003; Johnson et al., 2013).

Expectedly, testicular malondialdehyde concentration was also increased following administration of nicotine: this finding is consistent with previous reports (Husain et al., 2001; Yao et al., 2006). An elevated malondialdehyde concentration is direct evidence of toxic processes induced by free radicals (Sieja and Talerczyk, 2004). Enhanced level of tissue lipid peroxides in nicotine treated rats has been shown to be accompanied by a significant decrease in the levels of ascorbic acid, tocopherol, glutathione, glutathione peroxidase, superoxide dismutase and catalase (Kalpana et al., 2004). In the present study, the increase in testicular tissue malondialdehyde levels indicates a nicotine induced oxidative stress; suggesting an increased generation of free radicals in testicular tissue following exposure to nicotine. Increased production of free radicals or decreased function of the defense system play an important role in nicotine toxicity (Peltola et al.,1994). Hydrogen peroxide is not a particularly reactive product, but it may be reduced to the highly reactive metabolites: hydroxyl radicals or single oxygen (Peltola et al., 1992). Lipid peroxidation as evaluated by malondialdehyde value significantly increases during accumulation of hydrogen peroxide in a concentration-dependent manner (Garcia et al., 2005). Nicotine apparently may potentiate this oxidative stress in the testes of experimental animals. In the present study, lipid peroxidation in nicotine treated rats was accompanied by depletion of the concentration of antioxidant enzyme superoxide dismutase. Superoxide dismutase rapidly converts superoxide anion to less toxic hydrogen peroxide. The principal mechanism of hydrogen peroxide toxicity is thought to involve the generation of highly reactive hydroxyl ion radical through its interaction with Fe2+ in the Fenton reaction (Sewerynek et al., 1995). Presumably, the increase

superoxide dismutase concentration found in the present study could cause rapid conversion of hydrogen peroxide to water thus preventing hydrogen peroxide accumulation and availability to promote a shift towards lipid peroxide production. The significantly increased superoxide dismutase concentration in nicotine treated rats caused by the extract could be attributed to its phytochemical constituents especially flavonoids that have ability to act as antioxidants (Eruikainure et al., 2010). The flavones and catechins are perhaps some of the most powerful flavonoids for protecting the body against damage by reactive oxygen species (Sodipo et al., 2000). Antioxidant activity of any food source significantly increases with the presence of a high concentration of total phenol and flavonoid (Jayaprakasha et al., 2001). Therefore, the high phenolic and flavonoid contents of the watermelon rinds strongly suggest a high antioxidant potential. Another mechanism by which the extract could enhance superoxide dismutase concentration may be due to the presence of vitamins especially ascorbic acid and tocopherol; both vitamins possess antioxidant properties (Traber and Stevens, 2011). Ascorbic acid has been demonstrated to protect against some of the deleterious effects of nicotine in the lungs of experimental rat models (Maritz and van Wyk 1997); and have been reported to be significantly high in the pulp, seed and rind of Citrullus lanatus (Johnson et al., 2013). Low dietary intake of antioxidant vitamins such as ascorbic acid and tocopherol increases the risk of disease, whereas high dietary intake is apparently protective in function (Neunteufl et al., 2000). Furthermore, tocopherol is an effective antioxidant converting superoxide dismutase and lipid peroxy radicals to less reactive forms (Valk and Hornstra, 2000).

In conclusion, the present study reports that administration of the hydromethanolic extract of the rind of Citrullus lanatus fairly ameliorates the deleterious effects of nicotine administration on testicular and reproductive function in male Wistar rats. The beneficial effects of the extract could be attributable to its constituents. Our results are preliminary and could benefit from further studies.

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Reproductive Indices and Oxidative Stress Biomarkers of Male Wistar Rats Prenatally Exposed to Cigarette Smoke

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Summary: The negative influence of cigarette smoking on developing fetus is well documented but reports of prenatal cigarette smoking on male reproductive hormones are controversial. However, shortened anogenital distance (AGD) has been established to be an indicator of potential male infertility. We therefore investigated the effects of prenatal exposure to passive cigarette smoke on AGD, reproductive hormones and oxidative stress biomarkers of Wistar rats. Female rats were randomly divided into two groups (n=5) and cohabited with male. Group 1 was exposed to smoke from an idling cigarette from day 1 of gestation till parturition, while Group 2 served as control (no-exposure). Morphometric variables of the litters were recorded on postnatal day 1 (PND1) and at 6th week postnatal life. The male offspring were then sacrificed by cervical dislocation. Testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were analysed using ELISA. Serum levels of Catalase, sodium dismutase (SOD), malondialdehyde (MDA), lipid profile and liver function biomarkers were examined spectrophotometrically. On PND1, crown rump length and total body length of rats prenatally exposed to cigarette smoke were significantly shorter. Significantly shorter AGD and crown rump length were also observed at 6th week. Testosterone, LH and FSH were not significantly affected. Cigarette smoke exposure significantly decreased Catalase and SOD while MDA increased. Liver function biomarkers, HDL and LDL were not affected but serum levels of total cholesterol and triglyceride significantly increased. The observed decline in AGD and precipitation of oxidative stress by intrauterine cigarette smoke exposure may predispose to male infertility at adulthood.

Keywords*:* Prenatal, Cigarette smoke, Anogenital distance, Sex hormones, Oxidative stress.

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INTRODUCTION

Cigarette smoke is a leading cause of avoidable death and it is a big menace to public health in the world today. According to the World Health Organization (2015), more than 1 billion people around the world smoke and about 6 million people die each year from tobacco-related illness. More than 5 million of those deaths are the result of direct tobacco use while more than 600, 000 are the result of non-smokers being exposed to second-hand smoke. That is about one person dying every six seconds. The most common reason for maintaining smoking behavior is due to nicotine addiction (Buczkowski *et al.*, 2014).

There are various documented effects of active and passive smoking on pregnancy which includes intra-uterine growth retardation, sudden infant death syndrome, neuro-developmental and behavioural problems (Wickstrom, 2007). About 15–25% of women smoke while pregnant (Coleman, 2004). Prenatal cigarette smoking is associated with a high frequency of complications during pregnancy. These include preterm birth, spontaneous abortions, premature rupture of membranes, placenta previa, ectopic pregnancies and abruptio placentae (Eastham and Gosakan, 2010). It has been established that active smoking during pregnancy induces early morphological changes of the placenta, resulting in a reduced volume of maternal intervillous space and a reduced volume and surface area of fetal capillaries (Hofhuis *et al.*, 2003). These morphological changes results in a decrease in the diffusion coefficient of oxygen across the placenta, and the outcome seem to be all-or-none, and not dose dependent effect. The fetus of pregnant smokers therefore suffers from chronic hypoxic stress. These factors may be responsible for alteration of weight, length and head circumference neonate (Deng *et al.*, 2013).

Cigarette smoke affects various physiological processes including secretion of pituitary, thyroid, adrenal and sex hormones in humans. These are mediated chiefly through pharmacological actions of nicotine and as a consequence of smoking-induced stress (Kapoor and Jones, 2005). Cigarette smoke is believed to affect testosterone levels in males and it is likely due to alterations in globulin-binding affinity

rather than a direct effect of nicotine on testosterone. It has also been established to have an effect on serum progesterone and have anti-estrogenic effect in women, which may be probably due to changes in hepatic estrogen metabolism induced by smoking. Some estrogen-dependent physiological processes such as the menstrual cycle are affected as it causes an increase in menstruation length. This leads to an increased risk of anovulation which increases with smoking intensity. These decreases fertility capability and decreases age of menopause (Kapoor and Jones, 2005).

The intra uterine development duration is a critical period for human being, as any adverse prenatal exposure and condition may affect normal growth, development and physiology of the fetus in the intra uterine life as well as postnatal health and behavior. Nicotine is generally fat soluble and of small molecular size. These characteristics help it to cross the placenta and concentrate the fetal blood and amniotic fluid. Also, it is detectable in breast milk during lactation (Lambers and Clark, 1996). Prenatal exposure to cigarette smoke can affect the fetus in utero and also after birth. The health risks include pregnancy complication and premature birth. Maternal cigarette smoking is associated with elevated prevalence of low birth weight (Suzuki et al., 2008). Older smoker women are more susceptible to the effects of maternal smoking (Zheng et al., 2016). Infants of maternal smokers weigh on average 200 g less those born by none smoker women. Also, these infants are up to three times more likely to die of sudden infant death syndrome (Bajanowski et al., 2008).

Awobajo et al (2015) reported significant reduction of sex hormones of pregnant rats exposed to cigarette smoke and the decline in hormones increased with progression of pregnancy. We reported earlier that prenatal exposure to passive cigarette smoke caused a decrease in fasting glucose level and increased the serum nitric oxide of experimental rats (Obembe et al., 2010). Since nitric oxide is an important physiological signaling molecule and its availability is a marker of oxidative stress, we undertook this study to examine the effects of prenatal exposure to passive cigarette smoke in Wistar rats on oxidative stress biomarkers. The effects on sex hormones and anogenital distance were also examined.

MATERIALS AND METHODS

Experimental animals

Wistar rats (20 male and 10 female) were obtained from and kept in well aerated cages with solid floors covered with wood shavings in the Animal House of the College of Health Sciences, Osun State University, Osogbo with a constant 12 hour light 12 hour dark cycle. Nulliparous female rats (180 – 200 g) aged 14 – 16 weeks were used for this study, while the male rats (220 – 240 g) were of proven fertility. The rats were fed with standard pellets purchased from Ladokun livestock feeds, Ibadan, which contained 21% protein, 35% fat, 30% carbohydrate, 0.8% phosphorus and 0.8% calcium and had access to water *ad libitum*. All procedures in this study conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding principles in the care and Use of animals (2002) as amended and were approved by the Research Ethics Committee of the College of Health Sciences, Osun State University, Osogbo Nigeria.

Female rats were allowed to acclimatize for 10 days and thereafter randomly assigned to two groups (1 and 2, n =5). Group 1 was exposed to passive smoke from an idling cigarette in an exposure chamber throughout duration of pregnancy while Group 2 served as the control (no cigarette smoke exposure during pregnancy).

Mating

Ovulation was induced by administration of a single oral dose of Stilboestrol (0.042 mg/kg bw), an orally active synthethic oestrogen (Obembe *et al*., 2010). Thereafter, the female rats were cohabited with male rats at the ratio of 1:2 (Obembe *et al*., 2012). The presence of sperm plug in vaginal of the female rats confirmed mating, and was recorded as the first day of pregnancy. Pregnancy was obvious on day 14 of pregnancy and each pregnant rat was isolated in an exclusive cage.

Cigarette Smoke Exposure

Female group 1 rats were exposed to cigarette smoke from an idling cigarette in an exposure chamber at 9.00 am, over a period of 30 minutes every day from the day 1 to day 21 of gestation. Three cigarette sticks was used consecutively for each animal group per exposure (30 mins), per day (Obembe *et al*., 2010). London king size (menthol) cigarettes produced by London tobacco company was used. Each cigarette stick contains 1.2 mg nicotine and 14.9 mg tar.

Measurement of Morphometric Variables

On postnatal day 1, pups were weighed using a digital weighing scale (EasyWay Medical, England) and morphometric variables were taken by a digital Vernier caliper (Mitutoyo, Kawasaki, Japan). The morphometric variables measured were the anogenital distance (AGD), head diameter, abdominal diameter, crown rump length and total body length. These measurements were taken by two different trained technologists who were blinded to the study design so as to avoid bias. Mean values of their records were taken**.** On PND 21, litter size of dams was standardized, so as to eliminate concerns of disparity in nutrient and milk availability to pups. Measurements were repeated at the 6th week of postnatal life (PND 42) and then sacrificed.

Animal Sacrifice

On PND 42, the dams were discarded while the male offspring were sacrificed after overnight fast by cervical dislocation under sodium pentobarbital (30 mg/kg i.p) anesthesia and blood was obtained by cardiac puncture (Institutional Animal Care and Use Committee, 2013). Blood was centrifuged at 3000 rpm for 5 minutes, the clear supernatant, that is, the serum was obtained and was stored at -20 ˚C. The reproductive organs (testis, epididymis, seminal vesicle, prostate gland) and visceral organs of the male litters were dissected and weighed (Obembe *et al*., 2014).

Hormone and Oxidative Stress Assay

One male offspring was randomly selected amongst the pups from each dam. Serum obtained from these male offspring was used for hormonal analysis. Another set of male pups were randomly selected as above from each dam and serum obtained from these was assayed for oxidative stress biomarkers. Testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were assayed using enzyme-linked immunoassay method (ELISA). Oxidative stress was assayed spectrophotometrically as follows - malondialdehyde (MDA) according to Uchiyama and Mihara (1978), superoxide dismutase (SOD) according to Sun and Zigman (1978) and Catalase level was done as described by Aebi (1984).

Serum Lipid Profile and Liver function Biomarkers

Total cholesterol, triglyceride, high-density lipoproteins (HDL) and low-density lipoprotein (LDL) in serum obtained were determined by enzymatic colorimetric method as described by Rifai *et al* (1999). The determination was based on the formation of colour after enzymatic hydrolysis and oxidation. The indicator quinoneimine used was formed from H_20_2 and 4-amino-antipyrine in presence of phenol. All biochemical parameters were assayed kits obtained from Diasys Diagnostic systems (Istanbul, Turkey) on a Statfax Diasys 1904 plus Biochemical Analyzer.

The serum levels of alkaline phosphatase (ALP), alanine aminotrasferase (ALT) and aspartate aminotransferase (AST) were assayed by the method of Moss and Henderson (1999), using the respective available kits.

Statistical Analysis

Data were expressed as Mean ± Standard Error of Mean (SEM). Sample size for each treatment group is stated in respective table or figures. Data obtained were analyzed using Student's t-test for comparison between means of the two groups with similar sample size and one way analysis of variance (ANOVA) using SPSS version 16 (SPSS Inc., Chicago, IL) for comparing means of unequal sample size. $P<0.05$ was considered as significant.

RESULTS

Exposure of pregnant dams to cigarette smoke had no significant effect on litter size. Dams exposed to passive cigarette smoke during pregnancy had a litter size of (7 ± 0.7) while the control dams had a litter size of (7.2 ± 0.7). Prenatal exposure to passive cigarette smoke significantly decreased crown rump length and total body length but had no effect on total body weight, head diameter and abdominal diameter on postnatal day 1 (Table 1). However, a significant decrease in total body weight and crown rump length was observed at 6th week postnatal life. Head diameter, abdominal diameter and total body length were not affected (Table 1).

Table 1: Morphometric variables of offspring at postnatal day 1 and at 6th week postnatal life.

	Treated (PND1, n = 35)	**Control (PND1, n = 36)**	**Treated (6th wk, n = 27)**	**Control (6th wk, n = 27)**
Litter Size	7.0 ± 0.7	7.2 ± 0.7	5.4 ± 0.24	5.4 ± 0.24
Total Body Weight (g)	5.13 ± 0.12	5.65 ± 0.13	75.33 ± 3.45*	91.07 ± 2.26
Head diameter (mm)	11.05 ± 0.30	11.15 ± 0.13	18.90 ± 0.28	18.68 ± 0.30
Abdominal Diameter (mm)	12.83 ± 0.37	13.72 ± 0.29	23.66 ± 0.45	24.14 ± 0.25
Crown Rump Length (mm)	41.73 ± 0.66*	47.68 ± 0.76	131.87 ± 2.83*	142.45 ± 1.32
Total Body Length (mm)	54.00 ± 1.02*	61.35 ± 0.95	240.87 ± 3.84	247.19 ± 2.66

Values are Mean ± SEM, *P<0.05.

Table 2: Anogenital distance of the male offspring at PND1 and 6th week postnatal life

	Treated (mm)	**Control (mm)**
PND1	3.31 ± 0.21	3.48 ± 0.14
6th week	22.29 ± 0.51*	28.14 ± 0.57

Values are Mean ± SEM, *P<0.05.

Intrauterine cigarette smoke exposure also had no significant effect on anogenital distance on postnatal day 1, but after 6 weeks of postnatal life, male offspring of pregnant smoker rats had a significantly shorter anogenital distance (Table 2). At 6th week, a significant decrease in total body weight and absolute organ weights of heart and kidney were observed, while absolute weights of the lungs, liver and spleen were not affected (Table 3). Also, the absolute weights of testis, epididymis and seminal vesicles of male offspring of the pregnant smoker rats were significantly lower than offspring of the control. Weight of prostate gland was not affected. However,

Table 3: Total body weight and visceral organ weights of male offspring at 6th week postnatal day

	Treated	Control
Total body weight (g)	73.09 ± 3.01*	99.68 ± 4.78
Lungs (g)	0.79 ± 0.06 (1.09± 0.08)*	0.84 ± 0.02 (0.85 ± 0.03)
Liver (g)	2.89 ± 0.16 (3.95 ± 0.18)	3.46 ± 0.54 (3.98 ± 0.11)
Heart (g)	0.33 ± 0.02* (0.46 ± 0.02)	0.55 ± 0.19 (0.40 ± 0.02)
Kidney (g)	0.37 ± 0.02* (0.50 ± 0.02)	0.46 ± 0.02 (0.47 ± 0.02)
Spleen (g)	0.39 ± 0.03 (0.53 ± 0.04)	0.55 ± 0.19 (0.54 ± 0.17)
Testis (g)	0.32 ± 0.03* (0.45 ± 0.05)	0.56 ± 0.05 (0.55 ± 0.00)
Epididymis (g)	0.06 ± 0.01* (0.09 ± 0.01)	0.11 ± 0.01 (0.11 ± 0.01)
Seminal Vesicle (g)	0.05 ± 0.01* (0.08 ± 0.01)	0.11 ± 0.02 (0.11 ± 0.01)
Prostate Gland (g)	0.04 ± 0.01 (0.06 ± 0.01)	0.06 ± 0.01 (0.07 ± 0.01)

Values are Mean ± SEM, n=13. Absolute organ weights, (% Relative organ weights). *P<0.05.

Table 4: Serum Lipid Profile of male offspring of pregnant smoker rats

	Treated	Control
Total Cholesterol (mmol/l)	2.9±0.03*	2.22±0.16
Triglyceride (mmol/l)	1.62±0.08*	1±0.14
Low density lipoprotein (mmol/l)	1.38±0.12	1.4±0.18
High density lipoprotein (mmol/l)	0.74±0.02	0.72±0.02

Values are Mean ± SEM, n = 5. *P < 0.05.

Table 5: Liver function biomarkers of male offspring of pregnant smoker rats

	Treated	Control
Total bilirubin (µmol/l)	1.76±0.09	1.34±0.10
Conjugated bilirubin (µmol/l)	0.32 ± 0.03	0.26±0.05
Albumin (g/l)	23.20 ± 2.15	20.00±1.14
ALT (U/L)	13.60 ±1.72	9.60 ± 0.92
AST (U/L)	19.80 ±1.02	17.80 ± 0.86
ALP (U/L)	87.80 ±3.49	90.20 ± 3.72

Values are Mean±SEM, n=5 (ALT= Alanine aminotransferase, AST=Aspartate aminotransferase, ALP=Alkaline phosphatase) * P < 0.05

the percentage relative organ weight of the lungs of male offspring of pregnant rats prenatally exposed to cigarette smoke was significantly higher than the control (Table 3). The relative weight of other visceral organs – liver, heart, kidney, spleen and reproductive organs- testis, epididymis, seminal vesicles, prostate gland were not statistically affected.

Testosterone, LH and FSH levels of the offspring of pregnant smoker rats were not significantly different from control (Fig 1). A significant decrease in serum sodium dismutase and catalase levels and a significant

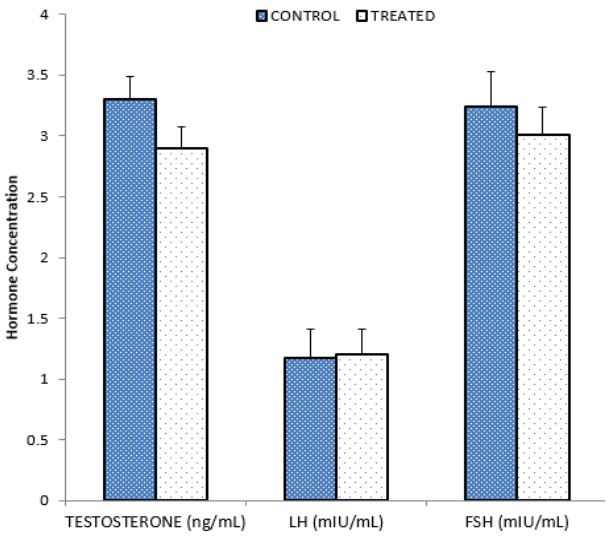

Figure 1: Reproductive hormones of male offspring at postnatal week 6. Values are expressed as Mean±SEM, n=5.

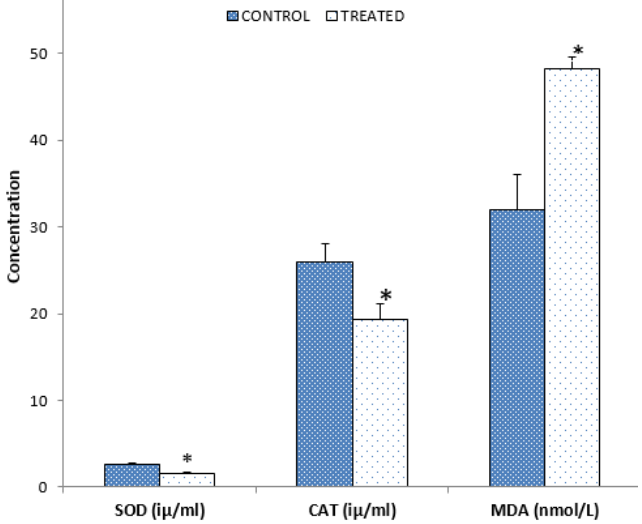

Figure 2: Oxidative stress biomarkers of the male offspring after six weeks. SOD – superoxide dismutase, CAT – Catalase, MDA – Malondialdehyde. Values are expressed as Mean ± SEM, n=5. *P<0.05.

increase in malondialdehyde levels were however observed (Fig 2). Also, serum total cholesterol and triglyceride levels were significantly higher in offspring of treated rats. However, HDL, LDL, conjugated and total bilirubin, albumin, ALT, AST and ALP were not affected (Table 4 and 5).

DISCUSSION

Cigarette smoke has been established to have several adverse effects on reproductive health of humans, though it is still unclear if these detrimental effects are entirely due to nicotine alone. In women, cigarette smoke has been reported to have anti estrogenic effects, probably due to changes in hepatic oestrogen metabolism induced by smoking. These anti estrogenic effects cause problems with the menstrual cycle, anovulation, early menopause, problems with the

placenta and adverse effects on fetal growth and development (Windham *et al*., 2005).

Several studies have reported low birth weight as an effect of intrauterine cigarette smoke. However, the degree of low birth weight associated with gestational cigarette smoking is dependent on the exposure duration and sticks of cigarette used (Omotoso, Adeyemi, 2014). The lack of effect of prenatal cigarette smoke on birth weight on postnatal day 1 observed in this study could therefore be ascribed to the comparatively low exposure duration of 30 minutes only. However, at the end of 6th week (PND 42), the body weight of offspring of smoker rats was significantly lower than the corresponding control (Table 1).

Prenatal cigarette smoke had no effect on head diameter and abdominal length of pups, both at postnatal day 1 and at six weeks after birth. Menounou (2011) reported that measuring neonatal head size can serve as a surrogate measurement of brain size and brain growth. Also, Meldere *et al* (2015) reported that measuring neonatal abdominal size serves as an important diagnostic tool in evaluating diseases of the abdominal cavity most especially necrotizing enterocolitis. These could be inferred to mean that intrauterine cigarette smoke at dose administered had no effect on brain size, brain growth nor induce development of abnormalities of abdominal structures. However, crown rump length and total body length of treated rats were significantly reduced on PND 1 and crown rump length was also shorter than control rats after six weeks of postnatal life. Dwivedi and Verma (2015) reported that length at birth may have an effect on the future height of a baby. The shorter crown rump length at 6th week therefore means that prenatal exposure to cigarette might affect height of the neonate in later life.

Swan *et al* (2005) reported a shorter AGD in the male infants of women exposed to increasing levels of known endocrine disruptors, suggesting an impairment of *in utero* male genital development. Furthermore, Einsenberg *et al* (2011) correlated AGD with a male human's fertility potential and reported a significantly lower sperm density, motile sperm count and sperm motility in men with shorter AGD. Also, males with short AGD were reported to have seven times the chance of being sub-fertile than those with a longer AGD as it is linked to both semen volume and sperm count and may give rise to conditions like cryptorchidism, hypospadiasis and testicular tumors in adulthood (Hsieh *et al.*, 2008). Shorter male AGD observed in this study at 6th week postnatal life therefore indicates that prenatal exposure to cigarette smoke predisposes to male infertility. The potential male infertility observed as short AGD in this study was not accompanied by hormonal derangement as no significant effect was observed on the testosterone, LH and FSH. This corroborates the work of Parra *et al* (2016) who reported lack of correlation between decline in AGD and testosterone level of undergraduate Spanish men. Also Eisenberg *et al* (2011) earlier reported lack of association between serum levels of male sex hormones (testosterone, LH and FSH) and shorter AGD and penile length observed in infertile men of stratified race. They noted that though AGD was significantly shorter in the infertile men, no significant difference was observed in FSH, LH and testosterone when compared with fertile men.

The reproductive functions are controlled by complex interactions between the reproductive organs and sex hormones. These interactions are however important for processes such as spermatogenesis, development of sex and accessory sex organs, sexual performance and male fertility in general. Though prenatal smoking reduced absolute testicular weight, it caused an apparent decline in serum levels of testosterone and FSH, however this was statistically insignificant. The decline in testicular weight may therefore not be solely caused by decreased testosterone level. The apparent decline in testosterone appeared to be transiently compensated for by an apparent negative feedback increase in LH.

The weight of reproductive organs usually provides a useful reproductive risk assessment in experimental studies (Raji *et al*., 2005). Prenatal cigarette smoke exposure reduced the absolute weight of the testis, epididymis and seminal vesicle. This further buttress the risk to fertility capability of male offspring of pregnant smokers. However, no effect was observed on prostate gland weight. The absolute weights of the lung, liver and spleen were also not affected but it significantly reduced weight of heart and kidney. The reduction in heart weight is consistent with the research carried out by Omotoso and Adeyemi (2014) who reported that intra uterine cigarette smoke exposure resulted in significant decline in the heart weight of juvenile rats, coupled with

alterations in the cardiac muscle which could affect cardiac functions and lead to cardiac disorder. These changes were suggested to be due to apoptosis or premature exit of cardiomyocytes from cell cycle, as occurs in hypoxia to compensate for reduced oxygenation. The reduction in kidney weight is not surprising as maternal smoke exposure leads to developmental abnormalities in the kidney in early life and functional deterioration in adulthood though the mechanism underlying this is still unclear as reported by Al-Odat *et al* (2014). The observed increase in relative lung weight of treated rats may be due to emphysema or otherwise. Future studies will include histopathological examination of the lungs amongst others in other to account for this. Metabolism may not have been affected, as no significant effect was observed on liver function biomarkers.

Cigarette smoke contains a wide range of xenobiotics, including oxidants and oxygen free radicals such as superoxide radicals, nitric oxide and hydroquinones that can increase lipid peroxidation and promote oxidative damage. Its toxicity is enhanced by the stimulation of reactive oxygen species production by neutrophil. Cigarette smoke has been calculated to contain 10^{17} oxidant molecules per puff, of which 10^{14} are reactive oxygen species and some of these have been documented to cross the placental barrier (Church and Pryor, 1985; Pryor and Stone, 1993; Perera *et al*., 2004). These reactive oxygen species or oxidants are cleared off by antioxidants such as catalases and superoxide dismutases. Oxidative stress results from the imbalance between the generation of reactive oxygen species and the antioxidant defense system in favor of the former. Prenatal exposure to cigarette smoke decreased the serum catalase activity and sodium dismutase (SOD) activity thereby promoting oxidative stress (Fig 4). This result correlates with reports of Kar *et al* (2008) on smoking induced oxidative stress in the serum and neutrophil. Catalase acts as a preventive antioxidant and SOD is a chain breaking antioxidant and they both play key role in the protection against the injurious effects of lipid peroxidation (Dinvoko-Kotsova, 2002). Where SOD stops its function, catalase exerts its function. The primary role of catalase is to scavenge hydrogen peroxide (H_2O_2) that has been generated by free radicals or by SOD in removal of superoxide anions and convert it to water (Ribbiere *et al*., 1992). In this study, the decrease in catalase activity is suggested to be due to excess H_2O_2 produced by smoking or SOD inhibition.

Due to oxidative stress, the ROS causes a progressive damage to lipid macromolecules in a process called lipid peroxidation. Peroxidation of membrane of lipids leads to loss of membrane fluidity and elasticity, impaired cellular functioning and even cell rupture. Malonialdehyde (MDA) is the terminal product of lipid peroxidation and serves as its index. This biomarker of oxidative stress was significantly increased ($p<0.05$) due to prenatal cigarette smoke exposure. Lipid peroxidation can indirectly reflect the status of the metabolism of free radicals, the degree to which the cells are attacked by free radicals and the degree to which lipid is peroxidated (Mirela *et al*., 2012). The lower levels of catalase and SOD antioxidants and higher levels of MDA due to prenatal cigarette smoke signifies increased oxidative stress and an increased risk of the development of chronic diseases. Kummerow *et al* (2000) demonstrated that circulating levels of lipid oxidation products correlated with the degree of coronary artery stenosis. In several studies, lipid oxidation was linked to cardiac disease and atherosclerosis, the primary cause of heart disease. Detrimental effects on offspring vascular function were also demonstrated in 4 week old rats exposed to sidestream smoke *in utero,* and they had increased aortic ring sensitivity to phenylephrine-induced vasoconstriction and reduced maximum endothelium-dependent acetylcholine-induced relaxation (Sheung *et al*., 2009).

Increased total cholesterol and triglyceride levels of offspring of pregnant smokers further corroborates a correlation between maternal smoking and offspring dyslipidemia. This reflects proatherogenic phenotype that may culminate in initiation and progression of atherosclerotic plaques, and therefore greater tendency towards increased risk for atherosclerosis and other cardiovascular diseases (Sheung *et al*., 2009; Glass, 2001).

In conclusion, prenatal exposure to passive cigarette smoke induces oxidative damage that may predispose to male infertility at adulthood as evidenced by congenitally short AGD. Therefore, induction of free radicals by constituents of cigarette smoke may probably explain the mechanism by which it causes deleterious toxicity to reproductive health of the fetus. Offspring of pregnant smokers are also predisposed to

increased risk of cardiovascular diseases as a result of the smoke induced dyslipidemia. Summing up, these findings further reiterate the call for cessation of cigarette smoking during pregnancy.

Disclosure

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D-dimer as a Predictor of Altered Coagulation in HIV Patients in Nigeria

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Summary: Recent medical advances have improved the quality of life and correspondingly reduced the morbidity and mortality associated with HIV infection. However increased life expectancy has led to a relative rise in comorbidities and complications such as alterations in coagulation systems. This study is aimed at the evaluation of D-dimer level as a predictor of thromboembolic risk in HIV patients. A total of 152 HIV positive and negative subjects and control respectively attending the PEPFAR clinic UCH in Ibadan were recruited both for a questionnaire-based survey and a coagulation profile screening. Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT), D-dimer level the viral load indices of the HIV patients and their CD4 counts were also evaluated. In the subjects, the D-dimer level was significantly higher (193.6 ± 177.00ng/ml) than the controls (118.10 ± 140.58ng/ml) while a significantly lower APTT was also reported (36.22 ± 4.05 seconds) compared to the controls (41.14 ± 8.87 seconds). An evaluation of the coagulation profile in the Highly Active Antiretroviral Therapy (HAART) naive and experienced group revealed only a significant difference (417.4 ± 162.0 versus 268.2 ± 193.5; p value 0.000) in the CD4 counts whilst no significant changes in the coagulation profile. In our study, a higher predisposition to a hypercoagulable state presenting as a short APTT was observed. This finding along with the higher D-dimer level underscores the relevance of the evaluation of this biomarker as an important predictor of thromboembolic event risk.

Keywords*:* HIV, thrombotic event, D-dimer, altered coagulation profile, CD4 count, APTT, PT

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INTRODUCTION

The impact of recent medical advances in HIV patients' health care, early screening and improved health promotion has resulted in a higher survival rate and chronicity of HIV and AIDS infection which has heralded the emergence of previously unknown complications (Antiretroviral Therapy Cohort Collaboration, 2017; Katz and Maughan-Brown, 2017). A notable example of this complication associated with the emergence of the era of Highly Active Antiretroviral Therapy (HAART) is a corresponding increase in reports of thrombotic events in HIV infected patients, a phenomenon previously uncommon among such patients (Copur *et al.*, 2002; Jacobson, Dezube and Aboulafia, 2004). A recent study has also suggested a ten-fold higher prevalence of thrombotic events in this group than in the general population while autopsy studies have revealed significantly higher rates of undiagnosed thromboembolism among AIDS patients (Jacobson, Dezube and Aboulafia, 2004; Freiberg *et al.*, 2016). In most of the patients in these case reports, there were no obvious predisposing risk factors such as surgery, trauma, stasis, nephrotic syndrome, pregnancy or other medical conditions commonly associated with thrombus formation (Heit, 2010).

A possible explanation for this high thrombotic event prevalence has been adduced to the reports of a preferential predisposition of HIV and AIDS patient to a hypercoagulable state. This has been associated with the detection and presence of various abnormalities of clotting related factors such as antiphospholipid antibodies, lupus anticoagulants, protein S & C deficiencies, heparin cofactor II, antithrombin III and elevated von-Willebrand by various authors (Saif, 2000; Jacobson, Dezube and Aboulafia, 2004; Borges *et al.*, 2014).

HIV infection has also been associated with the comorbidities of other medical conditions (such as malignancy, inflammatory and autoimmune disorders) which also increase patients' predisposition to thrombosis. An interesting perspective from recent medical literatures has been the report of deep venous thrombosis, portal vein thrombosis and pulmonary embolism among previously healthy HIV infected

patients recently started on antiviral drugs such as protease inhibitor therapy (Saif, 2000).

The recent growing chronicity of HIV and AIDS infection with resultant chronic inflammation directly and indirectly associated with comorbidities along with the activated coagulation events (both HIV infection features) have been associated with thrombotic events and mortality risks (Neuhaus *et al.*, 2010; Borges *et al.*, 2014). The combined impact of these two features (chronic inflammation and activated coagulation) has thus over the years led to a more extensive mortality predicting biomarkers studies (such as the Strategies for Management of Antiretroviral Therapy (SMART) study). D-dimer was found to be the most predictive biomarker (El-Sadr *et al.*, 2006; Borges *et al.*, 2014).

D-dimer is a specific degradation product of the fibrinolysis of cross-linked fibrin, hence recurrent coagulation and fibrinolysis cycle characteristic of thrombosis and thromboembolic disorders is associated with elevated D-dimer levels. D-dimer therefore serves as an important sensitive biomarker of endogenous fibrinolysis and a detection of an elevated level could be associated with thromboembolic disorder in patients (Funderburg *et al.*, 2010; Heit, 2010; Riley *et al.*, 2016). Aside the role in hypercoagulable state, D-dimer is also an important biomarker of inflammatory changes thus broadening the relevance as an important biomarker of AIDS and non-AIDS events associated with thrombotic events and mortality in HIV and AIDS patients (Freiberg *et al.*, 2016). Various studies on the significant role of D-dimer in the overall pathophysiology of HIV and AIDS morbidity and mortality has also revealed the correlation of d-dimer with endothelial dysfunction, microbial translocation and HIV viral load (Baker, Quick and Russ, 2010; Funderburg *et al.*, 2010).

This study is therefore aimed at the determination of the D-dimer level in adult HIV patients (treatment naïve or on antiretroviral therapy) as a predictor of thromboembolic disease risk and development. The research finding will be significant as the basis for the exclusion of patients not at risk of thromboembolic disorder and for whom urgent additional investigations for DVT or other thromboembolic disorders would be unnecessary.

MATERIALS AND METHODS

This was a case-control, questionnaire driven, interviewer administered, cross-sectional study, where one hundred and fifty-two (152) subjects both HIV and apparently healthy patients were recruited. Ninety-six were all HIV patients attending the PEPFAR Clinic in UCH Ibadan from whom blood samples were taken after responding to the consent form/ questionnaire. The control group comprised fifty-six (56) apparently healthy blood donor individuals who met the study inclusion criteria, gave consent, and tested negative for HIV. Patients with HIV who responded to the questionnaire and consented in writing were also included in the study 1as the cases. Patients on therapeutic dose of anticoagulation within 48 hours prior to sampling, pregnant women, those on hormonal contraceptive, cigarette smokers and non-consenting subjects were excluded.

Data and Sample Collection

The items on the interviewer administered questionnaire included respondents' socio-demographic data, HIV and drug history. Venous blood sample (4.5mls) was collected for D-dimer assay, PT and APTT into 0.5ml of 0.1 molar trisodium citrate, at a ratio of 9:1.

The blood sample was centrifuged at 2000g for 15 minutes to ensure a platelet poor plasma (PPP). PT and aPTT were performed on the freshly prepared citrated PPP according to Essien (1978) & Matchet and Ingrams' (1965) method. The remaining PPP sample was rapidly stored for 4 weeks at -20^{o}C in a single freeze-store cycle for pooled D-Dimer assay thus affording optimal assay response. The D-dimer level was quantified using Biopool TintElize® (Trinity Biotech Plc, Ireland) enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instruction. The enzyme immunoassay operates on a double-antibody principle using microtiter plate coated with MA-8D3 monoclonal antibody against D-dimer for the quantitative detection of the D-dimer level.

For the analysis, the D-dimer level for the patients was delineated using the manufacturers normal range (39±12ng/ml) into 3 ranges viz <39, 39 – 130 and >130 ng/ml which was used to categorize the patients based on their D-dimer level as below normal, normal and above normal respectively.

Statistical Analysis

The collected data were entered into Microsoft Excel for collation and proper screening for error. The data were then appropriately coded and statistically analyzed using IBM Statistical Package for Social Sciences (version 24) (IBM Corporation, 2016). Numerical data and continuous variables were summarized descriptively as means and standard deviation while strengths of association were compared using students' t-test. Correlation was determined using Pearsons' correlation coefficient. Categorical variables were expressed as percentages and strength of association was determined using Chi square test. Strength of association and comparison of differences was considered significant at probability level $p < 0.05$.

RESULTS

A total of 152 subjects were recruited into this study comprising of 96 HIV positive subjects (cases) and 56 HIV negative subjects (controls). Among the 96 cases,

33 (34.4%) were males, 63 (65.6%) were females giving a male to female ratio of 1: 1.9 and mean age of 39.02±0.09 years. The cases were consenting attendees of the PEPFAR Clinic University College Hospital (UCH), Ibadan. The controls were made up of 56 apparently healthy and fit blood donors at the UCH. The male to female ratio of the control group was 1.2 :1 while the mean age was 32.2±1.2 years. There was no significant difference in the proportion of the male and female participants, among the cases and controls (p>0.05) but there was a significant difference in the mean ages of the cases and controls. The peak age group was 35 – 45 years with 40 participants (41.7) followed by the 25- 35 group with 35 participants (36.5%).

Majority of the cases were married (68.8%), while 15.6% and 11.5% were singles and widows respectively. Among the controls 48.2% were married while 50% were singles, no widow, widower or separated participants were recruited for the control.

Among the HIV positive and controls, the Yorubas constituted the largest ethnic group 83.3% and 83.9% respectively. There was a significantly lower proportion of cases (2/96; 2.1%) compared to the controls (8/56; 14.3%) with D dimer level below normal level (39–130 ng/ml) (p = 0.001)). Only 46.9% (45/95) of cases but as many as 60.7% of controls (34/56) had D dimer level within normal limits, the difference was significant p = 0.000. The proportion of cases 51% (49/96) who had abnormal D dimer > 130ng/ml was significantly different from 25 % of controls (14) p = 0.001.

As shown in Table 4, an analysis of the quintile distribution of the 45 cases with normal D-dimer level revealed a total of 20 cases in the 3rd to 5th quintile while 20 cases were in the 1st and 2nd quintile. Using an arbitrary high level of 250 ng/ml by the authors, the probability of exceptionally raised D-dimer level > 250ng/ml among HIV patients was significantly higher (OR= 4.6 95%; CI= 1.33- 16.4; p= 0.010) than that of controls.

Table 1: Distribution of Patients and Controls based on the gender and marital status

Demographic Characteristics		Patients	Control	Total
Gender	Female	63/96 (65.6%)	38/56 (67.9%)	101/152 (66.4%)
	Male	33/96 (34.4%)	18/56 (32.1%)	51/152 (33.6%)
Marital Status	Married	66/96 (68.8%)	27/56 (48.2%)	93/152 (61.18%)
	Single	15/96 (15.6%)	28/56 (50%)	43/152 (28.29%)
	Widow	11/96 (11.5%)	-	11/152 (7.24%)
	Widower	1/96 (1.0%)	-	1/152 (0.66%)
	Separated	1/96 (1.0%)	-	1/152 (0.66%)
	Divorced	2/96 (2.1%)	1/56 (1.8%)	3/152 (1.97%)

Table 2: Comparison of Mean D-dimer level, PT and APTT of Patients and Controls

Coagulation Profile	Respondent Category	N	Mean±SD	T-test	P-value	Remark
D-dimer (ng/ml)	Patients	96	193.6±177.00			
	Control	56	118.10±140.58	2.73	0.02	Sig
PT (seconds)	Patients	96	13.46±1.60			
	Control	56	13.29±1.45	0.663	0.66	Not Sig
APTT (seconds)	Patients	96	36.22±4.05			
	Control	56	41.14±8.11	- 4.07	0.000	Sig

Table 3: Cross-tabulation of patients and controls with below normal, normal and above normal D-dimer levels

D-dimer levels		Category of Respondent		Total (n=152)	P-value
Category	(ng/ml)	Patients (n = 96)	Control (n=56)		
Below Normal	<39	2/96 (2.1%)	8/56 (14.3%)	10/152 (6.6%)	0.001
Normal	39 – 130	45/95 (46.9%)	34/56 (60.7%)	79/152 (52%)	
Above Normal	>130	49/96 (51%)	14/56 (25%)	63/152 (41.4%)	

Pearson Chi-Square test value=15.095; degree of freedom = 2

Table 4: Quintile distribution of cases with D-dimer levels within normal range

Quintile	D-dimer (ng/ml)	Cases with normal D-dimer level
1st Quintile	39 – 53.4	20 (44.44%)
2nd Quintile	53.5 – 75	
3rd Quintile	76 – 94.2	25 (55.56%)
4th Quintile	94.3 – 113.2	
5th Quintile	>113.2	

The mean CD4 count of the patients was 296.4 cells/ml (range 11 – 966 cells /ml; median 253 cells/mm^3) while the mean viral load was 9.6×10^5 copies /ml (range = 6×10^1 - 5.9×10^6; median = 1.9×10^3 copies/ml).

There was no significant difference (p = 0.211) between the mean PT of patients on HAART (13.50 seconds) and those not on treatment (13 seconds). The

Table 5: Effect of HAART on cases: D-dimer level, coagulation profile (PT and APTT), and CD4 Count.

Profile	n	HAART Treatment Group	Mean ± SD	Median (Range)	P – value
D – dimer level	84	Treatment	186.8 ± 160.7	135.0 (20.0 – 893.0)	0.85
	12	No Treatment	191.5 ± 202.9	140.0 (40 – 930)	
PT (seconds)	84	Treatment	13.5 ± 1.6	13.0 (10.0 – 21.0)	0.21
	12	No Treatment	13.2 ± 1.7	13.0 (11.0 – 19.0)	
APTT (seconds)	84	Treatment	35.9 ± 3.9	35.0 (23.0 – 50.0)	0.22
	12	No Treatment	37.5 ± 4.1	37.0 (32.0 – 47.0)	
CD4 Counts (cells/mm^3)	84	Treatment	268.2 ± 193.5	238.0 (11.0 – 976.0)	0.00
	12	No Treatment	417.4 ± 162.0	338.0 (115.0 – 614.0)	

Where n is number of patients

Table 7: Comparison of the effect of PI-based HAART and other HAART on patients' coagulation profile and HIV Indices.

Profile	Treatment Groups	n	Mean±SD	P – value
D – dimer level	PI-based HAART	10	173.3 ± 141.1	0.70
	Other HAART	68	195.6 ± 175.4	
PT (seconds)	PI-based HAART	10	13.2 ± 1.5	0.54
	Other HAART	68	13.5 ± 1.6	
APTT (seconds)	PI-based HAART	10	37.4 ± 4.1	0.19
	Other HAART	68	35.6 ± 3.9	
CD4 Counts (cells/mm^3)	PI-based HAART	10	256 ± 151.4	0.78
	Other HAART	68	275 ± 201.4	
Viral Load (10^5 Copies/ml)	PI-based HAART	10	1.02 ± 1.98	0.98
	Other HAART	68	1.06 ± 5.01	

PI = Protease Inhibitors; n = number of cases

Table 8: Correlation of D-dimer level with coagulation profile,
CD4 count and viral load

Variables	Coefficient of correlation (r)	P – value
PT	- 0.011	0.310
APTT	- 0.209	0.010
CD4 Count	- 0.082	0.426
Viral Load	0.011	0.927

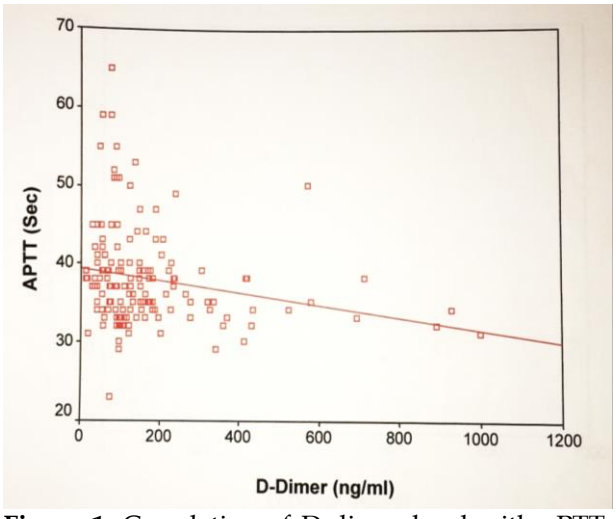

Figure 1: Correlation of D-dimer level with aPTT in HIV patients

difference between the mean aPTT of HAART patient and HAART naïve patients was also insignificant (p = 0.0216). There was no significant difference in the D dimer level of HAART experienced patients and HAART naïve patients; mean 186ng/ml and 191ng/ml respectively p = 0.85 (Table 5).

The HIV cases on HAART were further clustered based on their treatment into those on Protease Inhibitor (PI) based HAART and non-PI treatments. The PI-based treatment group (10/84) were significantly fewer than the non-PI HAART group (68/84). The value for 6 PI based patients were not available. No significant difference was however recorded in the mean level of the coagulation profile (aPTT, PT), D-dimer level, CD 4 count and the patient viral loads.

An evaluation of the relationship between the D-dimer level and the PT of patients revealed an insignificant (p = 0.310) inverse and poor correlation (r = -0.083). As shown in Fig 1., there was a significant weak inverse correlation between the D-dimer level and the APTT level in the patients (r = - 0.209; p = 0.010). The correlation of the D-dimer level with the coagulation profile, CD4 count and viral load are as summarized in Table 8. There was no significant correlation between the D-dimer and viral load of patients (r = 0.011; p = 0.927) while the weak inverse correlation between the D-dimer level and CD4 count (r= - 0.082; p = 0.426) was also not significant.

DISCUSSION

Thrombosis continues to be a progressively emerging complication of HIV and AIDS whose other well-known complications and comorbidities remain challenges to the physicians. The existing confirmatory tests and strategies such as Venography, Duplex ultrasound or Impedance Plethysmography (IPG) are either too expensive, invasive or not readily available during emergencies in some settings. This study was conducted with a view to utilizing D dimer as a screening test. The findings from this study highlights the relevance of D-dimer testing as a potential biomarker of interest in overcoming these shortcomings as the assay can be completed within 10mins with a negative predictive value of as high as 99.6% (Wells et al., 2003). Consequent of this, costs and lives can be saved when early and accurate diagnosis is made.

Our study population had nearly twice as many women as men infected with HIV receiving one form of therapy or the other. A higher prevalence of HIV in women than men has been described by several authors in different places reported a prevalence of 1:1.1 male/female ratio in the general population in Ibadan (Olaleye, 2004) while in Kenya, 23% of women aged 15-19 years were HIV infected compared with 3.5% of men of the same age group (a male to female ratio of 1:6.6) (Nkengasong, 2004). The predominant ethnicity in our study could be ascribed to mirror the tribal mix in the general population of the study.

The higher population of married people in the patient population of our present study is similar to previous reports. This is prime in the attribution of "marriage as a risk factor for HIV infection'' though other reports have reported a higher prevalence of HIV among the young unmarried population due to their predisposition for various risky behaviours (Glynn *et al.*, 2001; Mkandawire-Valhmu *et al.*, 2013; Bekker *et al.*, 2015).

Due to the ensuing improved life expectancy and chronicity of HIV and AIDS and emergence of HAART, there has been increased incidence of altered coagulation in HIV patients. There is a relative dearth of information and available literature on the association of the coagulation profile with HAART regimen.

In our study, APTT in HIV subjects was short but no significant difference was seen in the PT. APTT below the normal implies a hypercoagulable state which predisposes to thrombotic events due to the increased activation of the intrinsic pathways and corresponding reduction in activated protein C effect (Copur *et al.*, 2002; Jacobson, Dezube and Aboulafia, 2004; Tripodi *et al.*, 2004; Zakai *et al.*, 2008). Our finding while not consistent with reports by other authors in which a prolonged PT and APTT were reported among HIV patients (Omoregie *et al.*, 2009; MO and Sylvester N, 2016) underscores the wide variance in the reports of the comparison of coagulation profile in HIV patients and non-HIV patients (Tene *et al.*, 2014; Ephraim *et al.*, 2018).

Furthermore, in our study a significantly higher D-dimer level was reported in the HIV subjects compared to the controls. D-dimer is a specific fibrin degradation product (FDP) formed solely by plasmin degradation of cross-linked fibrin (and not intact fibrinogen). This elevated D-dimer level can be ascribed to an increased reactive fibrinolysis consequent to a hypercoagulable state and thrombotic events. The higher level of D-dimer reported in this study is also consistent with previous reports and further underscores the elevated risks of hypercoagulable state and thrombotic events in HIV patients (Saif, 2000; Jacobson, Dezube and Aboulafia, 2004). The preponderance of hypercoagulable state and other haemostatic disturbances in HIV patients have been associated with abnormalities in various coagulation factors (and the pathways), the potential role of HIV and AIDS comorbidities and the role of antiretroviral drug use (Saif, 2000; Jacobson, Dezube and Aboulafia, 2004; Thulasi Raman *et al.*, 2016). Our study revealed a 4.65 higher risk of an elevated D-dimer level in patients with a likelihood of higher risk of thrombosis. (Cushman, 2007; Tripodi, 2011).

Despite the significance of D-dimer as a predictor of thrombotic event, an elevated value has low specificity for any type of thrombosis as raised level is found in many clinical conditions (including DVT, PE, malignancy, pregnancy, post-surgery, sickle cell crisis and DIC) (Jacobson, Dezube and Aboulafia, 2004; Cushman, 2007; Heit, 2010; Piel, Steinberg and Rees, 2017). It is however a very sensitive marker of increased thrombosis and reactive fibrinolysis which can be used to predict risks of thrombosis (Wells *et al.*, 1995; Cushman, 2007). In addition, D-dimer level can be used as a first line approach in the evaluation of thrombotic risk and to safely identify those with elevated count for further confirmatory tests such as Duplex US or venography. The predictive potential of the D-dimer evaluation (being more convenient, less expensive and much less invasive) would be crucial in reducing the diagnostic time and cost of evaluation of thrombotic complications in HIV patients (both ART naïve and active users) and for further monitoring of those at risk of future thrombotic event.

The D-dimer levels in our treatment experienced and HAART naïve groups despite being lower in them was not significantly different as opposed to the CD4 counts (an important immunological factor in the evaluation and diagnosis of HIV and AIDS and infection severity) which was significantly lower in the treatment experienced group (Thulasi Raman *et al.*, 2016).

There is a wide variance in the relationship between the coagulation profile and HIV status in previous studies. In our study, a significant weak negative correlation was observed between the APTT and D-dimer level while no significant correlation was found between PT, the viral load and CD 4 count. These findings while in contrast with the correlation between PT and D-dimer as previously reported (Omoregie *et al.*, 2009) highlights the significance of APTT and D-dimer as important coagulation profile predictors in HIV patients. A positive D-dimer indicates there is a tendency to significant blood clot formation and its breakdown in the body. It also reflects the presence of abnormally high level of fibrin degradation products e.g. Disseminated Intravascular Coagulation (DIC).

Protease inhibitors (an important class of antiretroviral drugs) have been linked by previous authors with thrombotic complications in HIV and AIDS patients (Henry *et al.*, 1998; Saif, 2000). The mechanism of these thrombotic events while remaining unclear has been proposed to be due to the drug impact on lipid and glucose metabolism. The resultant effect of these metabolic changes have thus been associated with hypercholesterolaemia and endothelial dysfunction which have been suggested as potential contributing factor responsible for the thrombotic complications (Shankar S S and Dube, 2004; Andrade and Cotter, 2006; Thulasi Raman *et al.*, 2016). In our study, the subjects on PI did not appear to be more hypercoagulable judging by their coagulation profile compared to those on other HAART types. In another study, Saif et al 2000,, a mean CD4 count of 166cells/ml was correlated with an increased predictive risk of thrombotic complication due to the severity of the infections in these patients. (Saif, 2000; Saif, Bona and Greenberg, 2001; Jacobson, Dezube and Aboulafia, 2004). The high CD4 count reported in our study compared to this previous study could then be attributed as a potential factor responsible for the absence of a significant hypercoagulable state.

D-dimer is a specific FDP that is formed only by the plasmin degradation of cross-linked fibrin and not by plasmin degradation of intact fibrinogen. D-dimer are unique in that they are the breakdown products of a fibrin mesh that has been stabilized by factor XIII. Thus the presence of D-dimer indicates the fibrin has been formed and degraded (MGH Pathology service, 2006). D-dimer and FDP can be positive with DIC or thrombosis, including DVT, PE and myocardinal infaction. They also may be positive in liver disease due to decreased hepatic clearance. They can also become elevated postoperatively and in eclampsia, sickle cell crises, cancer patients and other conditions which are prethrombotic (MGH Pathology service, 2006). The results of this study highlights the elevated D-dimer level in HIV patients with potential consequences of altered coagulation parameters. We therefore recommend routine screening and monitoring of HIV patients for deranged coagulation profile using the baseline D-dimer values to predict the at-risk subjects with a view to promptly diagnose and treat thrombotic events at the earliest suspicion.

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Raffia hookeri Ethanolic Pulp Extract Ameliorated Neuronal Damage and Brain Oxidative Stress Following Mechanical-Induced Traumatic Brain Injury in Rats

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Summary: Traumatic brain injury (TBI) is a complex process resulting into structural brain damage and functional deficits as a result of an external mechanical force. This study aimed to investigate the possible ameliorative effect of *Raphia hookeri* ethanol extract (RHEE) on induced acute traumatic brain injury in rats. The choice of the plant was based on its reported anti-oxidative property. Thirty-six female Wistar rats were divided into six groups of six animals each. I: CONTROL - distilled water orally; II: RHEE - 100 mg/kg RHEE; III: Sharp trauma brain injury (STBI); IV: STBI+RHEE; V: Blunt trauma brain injury (BTBI); VI: BTBI+RHEE. Brain injury was inflicted using modified weight drop technique on experimental day 1 while RHEE was given orally by gavage for 7 days post-injury. Blood was collected serially 24hrs, 72hrs and 7 days post-trauma for full blood count and differentials of the white blood cells. On day nine, rats were euthanized and brain harvested for biochemical and histological analyses. Trauma significantly ($p<0.05$) reduced the relative brain weight of rats compared with the control. Lymphocyte count increased while neutrophils reduced in all traumatized rats compared with control group. Both BTBI and STBI significantly ($p<0.05$) elevated MDA and significantly ($p<0.05$) reduced the level of GSH, the activities of SOD and CAT enzymes compared with control group. Histologically, the extent of haemorrhage into the subarachnoid and brain parenchyma in STBI and BTBI groups was reduced in the BTBI+RHEE and STBI+RHEE groups. Administration of RHEE reduced oxidative damage and ameliorated neuronal damage in sharp and blunt brain injuries.

Keywords*:* Raphia palm fruit, induced brain injury, oxidative stress, white blood cells, cerebral cortex, haemorrhage.

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INTRODUCTION

Traumatic brain injury (TBI) is damage to the brain resulting from an external mechanical force leading to temporary or permanent impairment of cognitive, physical and psychosocial functions (Maas et al., 2008; Gaetz, 2004). Traumatic brain injury can be classified as primary which occurs immediately after trauma, and secondary which may appear several hours or even days later (Ozdemir et al., 2005). The structural damage and functional deficits following TBI are due to both primary and secondary injury mechanisms (Davis, 2000). The primary injury is characterized by the immediate mechanical disruption of brain tissue at the time of exposure to the external force and the release of the chemical mediators which act on the neighbouring cells thus promoting further cell loss (Yamaura et al., 2002; Park et al., 2004). This further cell loss and other pathological mechanisms of metabolic, cellular and molecular events such as excitotoxicity, ionic imbalances, inflammatory response and oxidative stress which evolve over minutes to months after the primary injury is referred to as the secondary injury (Lenzlinger et al., 2001; Arundine and Tymianski, 2004; Marklund et al., 2006; Bramlett and Dietrich, 2007; Guimaraes et al., 2009). Although the interplay of three major deleterious pathways namely: glutamate excitotoxicity, Ca^{2+} overload, and oxidative stress is believed to be responsible for the damage and neuronal death following TBI (Algattas and Huang, 2014), we examined the effects of only the latter in this study. The effective prevention of the variety of pathophysiological processes such as anti-inflammatory and anti-oxidative strategies is one of the key factors for improving the prognosis of TBI patients (Stelmasiak et al., 2000; Zhen-Guo et al., 2013). The devastating consequences of TBI and its pathophysiology require an effective treatment and this requires the exploration of plant products with antioxidant capability since plants are easily accessible, less toxic and less expensive and one of such is *Raphia hookeri*.

Raphia hookeri is a member of Palmaceae family that grows in the eastern and western parts of Nigeria. Its fruit is cone-shaped with an outer layer of rhomboid-triangular and overlapping reddish brown scales, a middle yellow, mealy, oil-bearing mesocarp and inner single hard nut (Mbaka et al., 2013). It has

been demonstrated to have medicinal and therapeutic properties warranting its use in herbal medicine in the treatment of various illnesses (Ogbuagu, 2008). Its seed reportedly attenuated hyperglycaemia and ameliorated dyslipidaemia (Mbaka et al., 2012) and has also been shown to effectively attenuate hyperplasia and reduced the size of enlarged prostate gland that was exogenously induced *via* its anti-oxidative activity (Mbaka et al., 2013). An earlier investigation showed that the pulp contains high concentrations of vitamins A and E, niacin, alkaloid, saponins, flavonoids and phenols all of which might enhance its antioxidant potentials (Edem et al., 1984).

The effect of brain injury will depend on the part of brain that is injured since different parts of the brain controls different functions although there is an overall synergy of the activities. In this study, we injured the frontal motor cortex of rats and thereafter observing the effect of *Raphia hookeri* ethanolic extract (RHEE) on the consequences of this induced injury in rat brain biochemically and histologically as well as on the blood profile.

This study is aimed at investigating the potential ameliorative effects of RHEE on the extent of induced acute traumatic brain injury in the frontal motor cortex of female Wistar rats and thus answer the research question of whether RHEE can protect rat brain from induced TBI.

MATERIALS AND METHODS

Plant extract processing

Raphia hookeri fruit was obtained from the swamps of Oke Odan, Apete, Ibadan, Oyo State and authenticated at the Forestry Research Institute of Nigeria (FRIN), Jericho, Oyo State, Nigeria with reference number FHI/110540. The hard, tough and scaly exocarp of the fruits were removed and discarded and the soft, mealy mesocarp (pulp) scraped from the seeds. The pulp of *R. hookeri* fruits was air-dried and grinded into powdery form for phytochemical screening and extraction at the Department of Pharmaceutical Chemistry, University of Ibadan, Nigeria. About 980 g of the grounded plant material was transferred into a glass container and 7 litres of absolute ethanol added, stirred at 2 hours intervals for 5 minutes hours and allowed to stand for 72hrs. The mixture was filtered in muslin bag followed by Whatman filter paper and the filtrate was concentrated using rotary evaporator set at 40^{o}C. The final aqueous extract of 79.24 g gave a percentage yield of 8.1% and was termed *Raffia hookeri* ethanolic extract (RHEE).

Animals and animal ethics

Thirty-six adult female Wistar rats weighing between 150-200 g were obtained from the College of Medicine Animal House, University of Ibadan and were randomly assigned into control and experimental groups. Thereafter, the rats were allowed 9 days to acclimatize to the naturally illuminated animal house of the Department of Physiology, University of Ibadan, with access to feed and water *ad libitum*. The animals were housed in transparent plastic cages with wood shavings as bedding. All of the animals received humane care according to the conditions stated in the 'Ethics Guiding the Care and Use of Laboratory Animals' published by the US Department of Health and Human Services, Washington (PHS, 1996).

Experimental design and animal treatment

The rats were randomized into six groups of six animals each as follows:

I: CONTROL administered distilled water.
II: Administered 100 mg/kg RHEE.
III: Sharp traumatic brain injury (STBI) induced.
IV: STBI induced plus 100 mg/ kg RHEE.
V: Blunt traumatic brain injury (BTBI) induced.
VI: BTBI induced plus 100mg/kg RHEE

Trauma was inflicted on day 1 of experiment and RHEE administered daily for 7 days by oral gavage according to the method of Mbaka *et al. (*2012).

Induction of Traumatic Brain Injury

After anaesthesia using 100 mg/kg body weight of Ketamine chloride injection, the hair on the animal's head was shaved off to expose the target area and methylated spirit used to clean the shaved area for antisepsis. Animals were then placed on the levered table of the modified weight drop device shown in Figure 1. A spot of injury was located, 2.5 mm posterior and 2.5 mm lateral to the bregma (Feeney et al., 1981). A round metallic ball fashioned to the end of Steinmann's pin was used for blunt type of TBI. Another Steinmann pin was fashioned to induce sharp type of TBI. The levered table was adjusted to accommodate 2.5 mm traumatic distance for each animal. A metallic object of 425 g was dropped at a uniform height of 5 cm on the skull to induce both blunt and sharp brain injury. Topical application of cotton wool soaked with methylated spirit on the bleeding spot prevented skin contamination. Finally, each animal was removed from the levered table and gently placed in the cage to recover.

TBI Inducing Apparatus (Modified Weight – Drop Device)

The weight-drop technique of Farran et al., (2014) was modified. Briefly, a retort stand held two clamps at a distance of 5cm interval, the upper clamp held a wooden block called "stopper" used to regulate the traumatic distance by stopping the Steinmann's pin (Figure 1). The lower clamp held a wooden block called "Guide" which was used to guide the Steinman's pin pathway over the rat's skull (this replaced the "Guide tube" of Farran et al.,2014). A levered table was made from a modified car jack with

a flat board on it on which the sedated animal was laid in a prone position to replace the plastic case used by Farran et al., (2014).

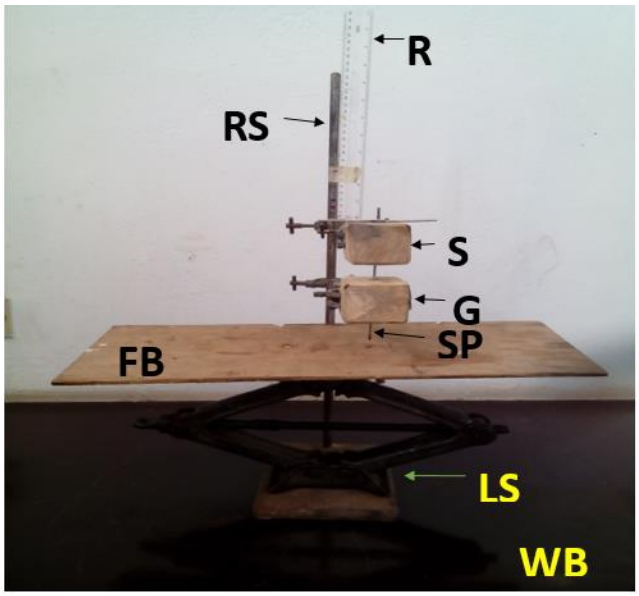

Fig. 1: Picture of the "weight drop" apparatus modified after Farran et al., (2014). R - Ruler, RS - Retort Stand, LS - Lever Stand, WB - Work Bench, FB - Flat Board, SP - Steinmann's Pin, G - Guide, S – Stopper.

Haematological Test

Blood samples were collected from the periorbital space of each rat into the heparinized bottle for estimating total white blood cell count, and the differential cell count on days 1, day 3 and day 7 post-traumatic injury. The blood was analysed using an Auto-haematological Analyser Machine at the Haematology Laboratory of the Department of Veterinary Medicine, University of Ibadan.

Animal sacrifice and biochemical assays

On day nine of the experiment, rats were euthanized with Ketamine overdose. Brain samples were collected, rinsed, weighed and divided into two halves. One half of the brain was preserved in 10% formalin for histological study while the other half of the brain for biochemical study was preserved in phosphate buffered solution PBS at pH 7.4 according to the method of Owoeye and Salami (2015). This part was were homogenized and homogenates centrifuged with cold centrifuge (4 °C at 12,000 rpm for 10 – 15 minutes). The supernatant was collected for the estimation of Malondialdehyde (MDA), Reduced Glutathione (GSH), Superoxide dismutase (SOD), Catalase (CAT). The level of MDA determined lipid peroxidation according to the method described by Varshney and Kale (1990), whereas GSH was determined at 412 nm in a colorimeter using the method described by Beutler *et al.*, (1963). The SOD activity was determined by the method described by Del-Maestro et al. (1983), while CAT activity was measured spectrophotometrically at 570 nm by the method of Sinha (1972). The fixed brain tissues were processed at the Histology Laboratory, Department of Anatomy University of Ibadan, Nigeria. Rats' brain specimens were processed through the stages of fixation, dehydration, clearing, infiltration, embedding and thereafter sectioned at 6 µm thickness with a Rotary Microtome (Leica RM2125 RTS, Germany). The ribbons were stained with haematoxylin and eosin and cresyl violet according to the method of Bancroft and Gamble (2008) to demonstrate general histology of the brain and possible microscopic alterations.

Statistical analysis

All the data were expressed as the mean ± Standard Deviation. Data was analyzed using Student's t-test and one-way ANOVA using Graph pad Prism (Version 7.00). Confidence interval was calculated at 95% and level of significance set at 5%.

RESULTS

Phytochemistry

The phytochemical screening of RHEE showed the presence of alkaloids, flavonoids, terpenoids, saponins, anthraquinones and tannins, while cardiac glycosides was absent.

General observations

***Body and brain weight changes*:** As shown in Table 1, brain trauma caused a significant ($p<0.05$) reduction of percentage weight differences in all the groups compared with the control. Similarly, the relative brain weight was significantly lower ($p<0.05$) in all the traumatized rats when compared with the control with the exception of BTBI group (Fig. 2).

Table 1: Body weight changes of the animals

Groups	Initial weight (g)	Final weight (g)	Weight difference (g)	% Weight difference
CONTROL	168.5±8.01	184.0±9.01	15.5±0.50	9±0.15
RHEE	167.0±5.87	169.0±6.63	2.0±0.01	1±0.01*
STBI	224.7±15.06	227.2±15.57	2±0.01	1±0.01*
STBI+RHEE	229.2±16.46	223.5±12.20	-5.7±0.21	-2±0.01*$^{\#}$
BTBI	185.2±8.51	192.2±13.55	7±0.23	4±0.02*
BTBI+RHEE	249.7±18.48	244.5±18.96	-5.2±0.21	-2±0.01$^{\#}$

RHEE, *Raffia hookeri* ethanolic extract; STBI, Sharp Traumatic Brain injury; STBI+RHEE, Sharp Traumatic Brain Injury +RHEE; BTBI, Blunt Traumatic Brain Injury; BTBI+RHEE, Blunt Traumatic Brain Injury±RHEE. Data are expressed as mean ± S.D. for 6 rats per group. *P<0.05 versus CONTROL, *$^{\#}$P< 0.05 versus STBI, $^{\#}$ P< 0.05 versus BTBI.

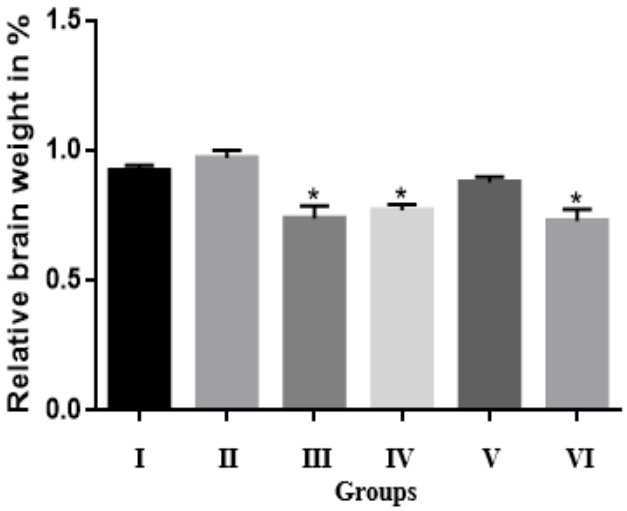

Figure 2: Relative weight of the brain of rats in I= Control; II=RHEE; III=STBI; IV=STBI+RHEE; V= BTBI; VI= BTBI+RHEE. *P<0.05 versus CONTROL.

Table 2(A): Effect of treatments on the total white blood cells of rats on Days 1, 3 and 7 post-trauma

Groups	Total White Blood Cell count (10^3/µL)		
	Day 1	Day 3	Day 7
I	4575±1312	4565±1302	4573±1222
II	3775±528	3765±524	3774±523
III	3525±493.7	6883±2289*	4508±565.2
IV	2967±375.1*	6075±1778#*	4892±572.2#*
V	4100±1305	5392±951.5#*	3608±385.2#*
VI	3117±531.7*	5875±1188#*	4708±900.8#*

I= Control; II=RHEE; III=STBI; IV=STBI+RHEE; V= BTBI; VI= BTBI+RHEE. *P<0.05 versus CONTROL; # P< 0.05 versus BTBI or STBI, *# P<0.05 versus value for previous day.

Table 2(B): Effect of treatments on the Neutrophil of rats on Days 1, 3 and 7 post-trauma.

Groups	Neutrophil Count (%)		
	Day 1	Day 3	Day 7
I	41.17±14.5	41.83±17.6	39.33±16.2
II	30±12.3	31.83±4.6	31.83±4.6
III	29.67±4.6*	27.5±5.4*	32.83±5.9
IV	26.17±2.8*	28.83±5.1*	29.67±5.1*
V	25.33±4.1*	31.83±5.0*	29.83±10.1*
VI	28.83±5.5*	27.33±4.8*	31.33±8.9*

I= Control; II=RHEE; III=STBI; IV=STBI+RHEE; V= BTBI; VI= BTBI+RHEE*P<0.05 versus CONTROL.

Table 2(C): Effect of treatments on the Lymphocytes of rats on Days 1, 3 and 7 post-trauma.

Groups	Lymphocytes counts (%)		
	Day 1	Day 3	Day 7
I	62±4.7	61.67±10.4	63.5±7.0
II	67.83±12.8	64.5±4.2	64.5±4.2
III	66.83±4.4	68.5±6.0	63.5±5.2
IV	70±2.6*	67±4.6	67.17±6.3
V	70±4.7*	63.5±4.6	67.17±11.5
VI	67.5±5.6	69.5±4.4	65.5±10.4

I= Control; II=RHEE; III=STBI; IV=STBI+RHEE; V= BTBI; VI= BTBI+RHEE. *P<0.05 versus CONTROL.

Table 3 **The effects of RHEE on the antioxidant defense mechanisms in brain of rats**

Groups	MDA (µmoles/mg protein)	SOD (µUnit/mol)	GSH (µg/ml)	CAT (µmol/min^{-1} mg^{-1})
I	1.71±0.1	3.73±0.6	59.50±4.7	6.22±0.8
II	1.91±0.1	2.73±0.4	60.01±5.2	5.01±0.7
III	6.22±0.8*	1.51±0.1*	28.41±3.3*	1.81±0.0*
IV	2.53±0.4#	0.81±0.0#	37.31±2.9*	4.13±0.9#
V	4.31±0.8*	2.2±0.1*	39.20±2.9*	1.92±0.2*
VI	1.87±0.1##	2.0±0.1*	48.41±3.2*	2.92±0.3*

I=Control; II=RHEE; III=STBI; IV=STBI+RHEE; V= BTBI; VI= BTBI+RHEE. Data are expressed as mean ± S.D. for 6 rats per group. *P<0.05 versus CONTROL, #P< 0.05 versus STBI, ## P< 0.05 versus BTBI.

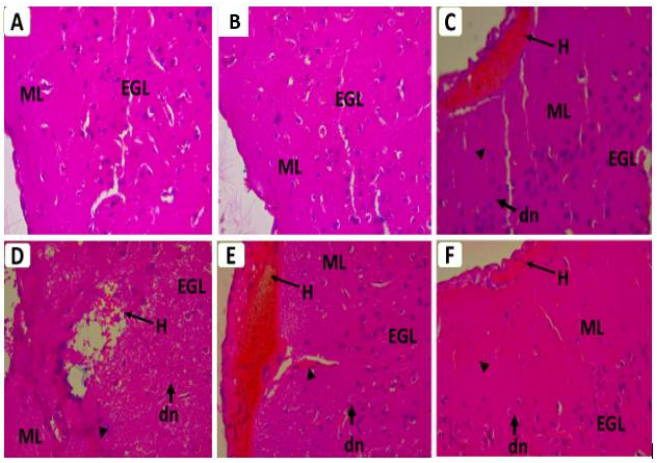

Figure 3: Representative stained sections of the cerebral cortex of rats in CONTROL(A), RHEE (B), STBI (C), STBI+RHEE (D), BTBI (E) and, BTBI+RHEE (F). Portions of the molecular and external granular layers of the cortex show evidences of haemorrhage (H). Blood extravasation into the parenchyma of the cortex is depicted with arrowheads. Normal neurons indicated as "n", while degenerated cortical neurons (dn) are deep to areas of haemorrhage. ML, Molecular layer; EGL, External Granular Layer**.** H&E stain, x400.

The effects of RHEE on the antioxidant defense mechanisms in brain of rats

Table 3 shows the effects of RHEE on the antioxidant defense system and biomarkers of oxidative stress in brain of trauma-treated rats. BTBI caused a significant (0.05) elevation in the level of malondialdehyde (MDA), and reduction in glutathione (GSH) level. However, it caused a reduction the level of glutathione and activities of SOD and CAT when compared with the control. The co-administration of RHEE ameliorated the perturbations in these parameters by restoring them to near control levels as shown in the table.

Histological evaluation of normal and traumatized cerebral cortex of rats.

Figure 3 depicts our findings of the traumatized and non-traumatized cerebral cortices of the experimental rats. Portions of the molecular (ML) and external granular layers (EGL) of the cortex show evidence of haemorrhage (H) as shown in Figures 3C, 3D, 3E, and 3F. Blood extravasation into the parenchyma of the

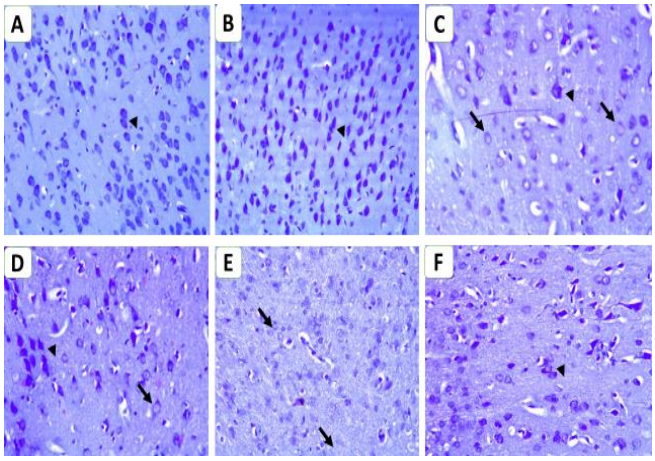

Figure 4: Representative stained sections of the cerebral cortex of rats in CONTROL(A), RHEE (B), STBI (C), STBI+RHEE (D), BTBI (E) and, BTBI+RHEE (F). Portions of the external granular layers of the cortex deep to injured part to show neuronal response to injury. Normal staining of cortical neurons in groups (arrowheads) while pale staining neurons (arrows) are prominent in STBI and BTBI groups. ML, Molecular layer; EGL, External Granular Layer**.** Cresyl violet stain, x400.

cortex is denoted with arrowheads. Normal neurons indicated as "n", while degenerated cortical neurons (dn) are deep to areas of haemorrhage. The cresyl violet stain (Figure 4) demonstrated that degenerated cortical neurons deep to the injured part of the brain were more pronounced as pale staining cells in the STBI and BTBI compared with STBI+RHEE and BTBI+RHEE groups respectively.

DISCUSSION

The present investigation provided biochemical, haematological and histological data which suggested that post-traumatic treatment of rats with *Raffia hookeri* ethanolic extract (RHEE) mitigated the adverse effects of sharp and blunt trauma in experimental rats. The RHEE prevented trauma-induced oxidative stress and cerebral cortical neuronal death, additionally, it augmented brain antioxidant defense mechanisms and ameliorated neuron damage in traumatized rats.

The reduction in the body weight of rats co-treated with RHEE after trauma (STBI+RHEE and BTBI+RHEE) might be due to the effect of the trauma resulting in reduction in activity and poor feeding due to loss of appetite. The reduction in the relative brain weight in all traumatized rats might also be secondary to the trauma.

The haematological analysis was used to measure inflammatory cell response in TBI. It has been reported that CNS expression of pro-inflammatory cytokines and complement components leads to recruitment of peripheral inflammatory cells (neutrophils and monocytes ⁄macrophages) across the blood brain barrier and enhancement of the established neuro-inflammation (Scholz et al., 2007; Szmydynger-Chodobska et al., 2012). Studies showed that after focal TBI, peripheral inflammatory cells increase in the CNS, neutrophils in particular arrive within one hour of TBI and reaches its peak by third day, and then disappears or decreases rapidly with time (Kriz, 2006; Yilmaz and Granger, 2008; de Rivero Vaccari et al., 2009). Although the total white cell count peaked on day3, the neutrophils count was not elevated whereas the lymphocyte count was raised contrary to the report of Weckbach at al. (2012) who reported that lymphocytes did not play a major role in TBI pathogenesis.

The presence of flavonoids and tannins in RHEE suggest that it might have antioxidant potential (Ayoola et al., 2008), since they are phenolic compounds which act as primary antioxidants or free radical scavengers (Dada et al., 2017). The elevated level of MDA and reduction of GSH level indicated oxidative stress in both STBI and BTBI rats which was supported by previous findings that oxidative stress occurs in traumatic brain injury (TBI) ((Inci *et al.*, 1998; Webster et al., 2015). Oxidative damage has been associated as one of the principal factors accompanying the secondary injury mechanisms and changes that worsens the outlook of TBI (Ozdemir et al., 2005). Brain 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).

Animals in groups post-treated with RHEE had their MDA levels reduced while the level of GSH was elevated suggesting antioxidant activity of RHEE to neutralize or mitigate the oxidative damage of both sharp and blunt trauma on the rat brain. Similarly, the activities of SOD and CAT that were reduced by TBI was elevated by post-trauma treatment with RHEE. The results suggested that RHEE demonstrated ameliorative effect against lipid peroxidation probably due to the high antioxidant activity associated with its high phenolic content (Dada et al., 2017). The extract also enhanced the up-regulation of the activity of CAT. Treatment with STBI+RHEE did not SOD activity an enzyme required in the conversion of O^{2-} to the less reactive H_2O_2 and O_2 (Warner et al., 2004). Catalase (CAT) is important in helping the body to eliminate the H_2O_2 which is a by-product of O^{2-} metabolism thus enhancing antioxidant defense system (Warner et al., 2004; Adedara et al., 2018). Hence, RHEE demonstrated ameliorative effect on oxidative stress in both sharp and blunt TBI.

Haemorrhage in the motor cortex of the brain as demonstrated in the STBI and BTBI groups simulated cerebrovascular haemorrahagic injury which would affect motor coordination of the animal since corticospinal and corticobulbar tracts emanate from this area (Crossman and Neary, 2015). These are the tracts that modulate the voluntary skilled motor activities *via* the cranial and spinal nerves as well as

the cerebellum. The reduction in the size of the haemorrhage as shown in the slides of the STBI+RHEE and BTBI+RHEE groups when compared with those of STBI and BTBI respectively suggested that co-treatment with RHEE ameliorated the vascular damage done by the induced trauma. Literature reports have linked free radicals to the death of neurons and endothelial cells, as well as the altered contractile response of cerebral vessels as in subarachnoid haemorrhage (Ayer and Zhang, 2008). This was shown by the reduction in GSH level and antioxidant activities of SOD and CAT in the STBI and BTBI, whereas the increase in these parameters in the STBI+RHEE and BTBI+RHEE groups suggest modulation and minimizing of the vascular injury via antioxidative intervention by RHEE which might have led to the reduction of the vascular injury (among other probable factors), and amelioration of degenerated neurons in the histology.

Taken together, both sharp and blunt traumatic brain injury caused traumatic injury demonstrated by alterations in the body and brain weight, haematological, oxidative and histological parameters. Post-traumatic administration of RHEE ameliorated these changes possibly through its antioxidant property. This suggests that RHEE could be further investigated for possible identification of promising therapeutic agents against TBI effects.

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