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Full Length Research Article

Midgestational Characterization of Cytokine Profiles in HIV Infected and Uninfected Black South African Pregnant Women

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Summary: Pregnancy is characterized by an anti-inflammatory milieu in the second trimester despite a pro-inflammatory response in the first and third trimesters. Nonetheless a disproportionate inflammatory response is risky in pregnancy. This retrospective study evaluated the mid-gestational expression of inflammatory and anti-inflammatory cytokines in HIV infected pregnant women at their first antenatal visit. Archived serum samples were collected from seventy (n=70) black pregnant women, attending a primary health care centre in KwaZulu-Natal, South Africa. The demographic and clinical profiles were procured from patient medical records and cytokine levels were measured in all samples. A statistically significant difference was noted for IP-10 ($p<0.05$) between the HIV positive and HIV negative groups. Likewise, a statistically significant difference was observed for IL-7 in the HIV population, when further stratified based on ART usuage. Significant correlations were noted between IL-7 and birthweight ($r=0.35$, $p<0.05$); IFN-δ and maternal age ($r=-0.27$, $p<0.05$); TNF-α and gestational age ($r=0.26$, $p<0.05$); VEGF and systolic blood pressure ($r=0.40$, $p<0.05$); IL-4 and gestational age ($r=-0.30$, $p<0.05$). A positive correlation was noted for inflammatory IL-1b with anti-inflammatory IL-5; IL-5 and FGF basic; inflammatory IL-2 with anti-inflammatory IL-5, IL-10 and FGF basic. A negative correlation was noted between the inflammatory IL-12 with anti-inflammatory IL-1ra and Il-4; and between IL-17A with IL-10. This study reveals midgestational variation in serum inflammatory and anti-inflammatory immunologic profile of pregnant women, irrespective of the use of antiretroviral therapy. This disparity in the susceptible HIV infected women will affect progression of pregnancy and encourage fetal morbidity and mortality.

Keywords*: inflammatory, anti-inflammatory, cytokines, interleukins, pregnancy*

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INTRODUCTION

During the pathogenic process of HIV replication, cytokines regulate the innate and adaptive immune response (Clerici, 2010). Moreover, they also influence reproductive function such as follicle growth, blastocyst implantation, trophoblast growth and parturition (Bowen *et al*., 2002). The physiologic control of innate immunity during pregnancy is required to support the acceptance of the fetal allograft. The switch from the T helper (Th)1 (cell-mediated response) to Th2 (humoral response) facilitate pregnancy success (Challis *et al*., 2009; Mosmann & Sad, 1996; Raphael *et al*., 2015). This feto-maternal rapport is safeguarded by the balance created by the Th1/Th2 activity in response to the placental production of cytokines (Challis *et al*., 2009; Robertson *et al*., 1994). More specifically, placental trophoblasts secrete pro-inflammatory cytokines (Szekeres-Bartho & Wegmann, 1996), whilst the anti-inflammatory cytokines are secreted by tissues (Joachim *et al*., 2003), both of which are central to trophoblast/angiogenic function (Omar *et al*., 2005). Pregnancy is thus characterized by an anti-inflammatory milieu in the second trimester despite a pro-inflammatory response in the first and third trimesters (Marie-Pierre Piccinni *et al*., 2015). Nonetheless a disproportionate inflammatory response is risky in pregnancy (Orsi & Tribe, 2008).

The Th1-response stimulates macrophages and cell mediated immunity through secretion of interleukins (IL-1, IL-2, IL-6, IL-12, IL-15, IL-18), interferons (IFN-gamma) and tumor necrosis factor alpha (TNF-α) (Challis *et al*., 2009; Marie-Pierre Piccinni *et al*., 2015). The Th2-response in contrast accelerates humoral immunity in response to secretion of IL-4, IL-5, IL-10, IL-13, and granulocyte macrophage colony stimulating factor (GM-CSF) (Challis *et al*., 2009; Marie-Pierre Piccinni *et al*., 2015). Infections or inflammatory events are known to disrupt this balance causing a dominant Th1 control, which is believed to increase the inflammatory cytokine production (Orsi & Tribe, 2008). Increased levels of serum pro-inflammatory cytokines are known to be a risk factor for preterm delivery by causing premature uterine contraction and membrane rupture (Murtha *et al*., 2007). This maternal immunomodulation is also transmuted in pregnancy pathologies such as preeclampsia and intrauterine growth restriction resulting in a higher Th1 response (Hu *et al*., 2007; Orsi & Tribe, 2008).

HIV infection prevalence rates in South African females of childbearing age, is approximately 22.8% (Statistics South Africa, 2015), with this prevalence being greater amid pregnant females (National Department of Health, 2013). With the recent rollout and increase in ART access, almost 95% of HIV-infected pregnant women receive triple drug antiretroviral therapy (ART) during pregnancy and breast feeding (United Nations Programme on HIV/AIDS, 2017). Whilst ART decreases HIV related maternal morbidity and mortality, it also demonstrates a characteristic shift from a Th1 to Th2 immune response in HIV treated pregnancies (Osakwe *et al*., 2010; Marie-Pierre Piccinni *et al*., 2015). This immune restorative effect of antiretroviral therapy normalizes the shift in normal pregnancy (Fiore *et al*., 2006). Based on the reduced morbidity and mortality observed in pregnant women on ART for prevention of mother to child transmission (PMTCT), a dual antiretroviral program was endorsed by the South African National Department of Health, as the standard of care for PMTCT regardless of the infection stage or immunological status of the pregnant women (Moodley *et al*., 2016; National Department of Health, 2008). Dual ART exposure is also linked with reduced odds for adverse birth outcomes (Moodley *et al*., 2016).

HAART-mediated endothelial dysfunction has been correlated with elevated blood pressure in HIV-positive patients (Seaberg *et al*., 2005; Stein, 2003). A recent meta-analysis revealed that systolic and diastolic blood pressure levels and hypertension risk are significantly increased in response to HAART treatment (Nduka *et al*., 2016). This corroborates the study done by Bergersen and co-workers in a Norwegian cohort of HIV-positive patients receiving HAART (Bergersen *et al*., 2003). Their study indicated that the incidence of hypertension was increased amongst those on HAART in comparison to those not on HAART (Bergersen *et al*., 2003).

At present there is conflicting data concerning BMI and weight gain amongst HIV patients on HAART (Amorosa *et al*., 2005; Crum-Cianflone *et al*., 2008; Gallant *et al*., 2004; Mallon *et al*., 2003; Sharma *et al*., 2014; Shikuma *et al*., 2004). Despite a high prevalence of overweight and obese women in their cohort of HIV infected pregnancies, Sharma *et al*., (2014) demonstrated only a small increase in BMI over the course of pregnancy (Sharma *et al*., 2014). Pregnancy is identified as an immune process and may be especially disturbed by HAART use (Sebitloane & Moodley, 2017). Limited South African studies are available that characterize the cytokine profiles during pregnancy and its link with HAART and adverse birth outcomes. This study therefore evaluated the mid-gestational serum levels of both inflammatory and anti-inflammatory cytokines in HIV+ve and HIV-ve Black South African pregnant women. We also provide an epidemiological correlation between the cytokines and BMI, BP, hemoglobin and birth weight.

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MATERIALS AND METHODS

Study population and sample collection: This was a retrospective study, which used archived serum samples collected from seventy (n=70) Black pregnant women attending a primary health care centre in KwaZulu-Natal, South Africa. The demographic and clinical profiles of the study population were obtained from patient medical records. Anemia in pregnancy, was defined as a hemoglobin level less than 11.0 g/dl (World Health Organization, 2011). Serum samples were collected at their first antenatal booking, between 10-20 weeks' gestation. Circulating cytokine levels were measured in all serum samples collected. All samples were stratified by HIV status into HIV-ve and HIV+ve normotensive pregnant groups.

Pregnant women with underlying medical conditions and those unable to provide informed consent were excluded from the study.

Ethical consideration: Ethical approval was obtained from the Durban University of Technology Institutional Research Ethics Committee (IREC 010/17) and the KZN Department of Health.

Quantification of analytes: The cytokine expression was determined using the Bio-Plex Pro Human Cytokine Group I Assays panel kit (cat no: M500KCAF0Y, Bio-Rad, USA), in accordance to the manufacturer's protocol (www.bioplex.com/bioplex). All reagents and samples were brought to room temperature before use. Coupled magnetic beads (Bio-Rad) were dispensed into each well of the assay plate, followed by washing. Standards and samples (1:4) were diluted, vortexed and incubated for 1 hour at room temperature with vigorous shaking at 850±50 rpm. Post incubation, plates were washed and detection antibodies were incubated for 30 minutes at room temperature. Streptavidin-PE (1x) was added to each well and incubated in the dark at room temperature for 10 min at 850±50rpm. Plates were washed and re-suspended in assay buffer at 850±50 rpm for 30 seconds and then read on a Bio-Plex Pro MAGPIX system. The serum values for each cytokine was generated using the Bio-Plex Manager software (version 6.1, Bio-Rad).

Data analysis: STATA (version 12, STATACORP) was used for data analysis. Data is presented as mean and standard deviation for continuous data and frequency distributions for categorical variables. The Pearson's chi-squared test was used to evaluate bivariate associations between demographic and clinical variables stratified by HIV status. HIV status was evaluated as a binary variable (HIV Positive (+) vs HIV Negative (-). Similar bivariate analysis was used to compare clinical characteristics stratified by the use of antiretroviral therapy (HAART). As the distributions of measured cytokines were not normally distributed, we performed a log-transformation to normalize data and geometric means were compared by HIV status and use of HAART. Pearson's correlation was used to assess the relationship between inflammatory and anti-inflammatory cytokines. Correlation was also used to estimate whether levels of cytokines depended on maternal epidemiological and clinical characteristics. A p-value ≤ 0.05 was considered statistically significant at a 95% confidence level.

RESULTS

Participant demographics: The demographic profile of the study population, stratified by HIV status is shown in Table 1. The mean age of the HIV+ve and HIV-ve study groups

was 26.89±5.07 and 24.54±5.02 yrs respectively. Of the total participants enrolled, 41 (59%) were HIV+ve, of which 23 (56%) received antiretroviral treatment (Table 1). A significant difference was noted for gravida between the HIV+ve and HIV-ve study groups.

Table 1:
Demographic and clinical profile of the study population (N=70)[#]

		HIV+ve (n=41)	HIV-ve (n=29)	P value
Age (years) (mean, SD)		26.89 (5.07)	24.54 (5.02)	
PMTC (n, %)		23 (56%)	n/a	
Parity (n, %)	*Nulliparous*	8 (21.05)	12 (50.00)	0.06
	Primiparous	15 (39.47)	9 (37.50)	
	Multiparous	15 (39.47)	3 (12.50)	
***Gravida** (n, %)	*primigravida*	7 (18.42)	11 (45.83)	0.05
	multigravidaє	31 (81.57)	13 (54.17)	
***Gestational age at booking** (wks, mean±SD)		15.44 (5.31)	12.22 (5.27)	0.03
BMI (kg/m^2, mean±SD)		29.58 (10.9)	26.63 (3.94)	0.34
Blood pressure (mmHg, mean±SD)	*Systolic*	108.38 (13.73)	112.13 (14.69)	0.49
	Diastolic	62.85 (9.34)	66.4 (6.31)	0.24
Hemoglobin (g/dl, mean±SD)		10.63 (3.41)	11.39 (2.10)	0.48
Birthweight (kg, mean±SD)		3.10 (0.44)	3.18 (0.65)	0.65

**p<0.05 was considered statistically significant*
[#] data was missing in certain categories

Table 2:
Clinical characteristics based on antiretroviral therapy status

HIV Treatment Regimen (n=41)			
	HAART use (n=23)	**No HAART (n=18)**	***p*-value**
Body Mass Index (kg^2, mean±SD)	29.45 (8.25)	29.67 (13.13)	0.97
Blood pressure (mmHg, mean±SD)			
Systolic	114.83 (17.66)	102.86 (6.31)	0.12
Diastolic	67.83 (8.61)	58.57 (8.16)	0.07
Haemoglobin (g/dl)	12.35 (2.15)	9.16 (3.73)	0.09
Birthweight (kg)	3.09 (0.44)	3.13 (0.48)	0.89
***p<0.05 was considered statistically significant**			

Values are expressed as mean±SD

Gestational age was significantly different (p=0.03) between the HIV+ve and -ve groups. The BMI was greater within the HIV+ve group compared to the HIV-ve group (Table 1). In contrast, both the mean systolic and diastolic blood pressure was lower in the HIV+ve group compared to the HIV-ve group. The hemoglobin levels were also higher in the HIV negative group compared with the HIV positive group. None of the participants smoked and only 1 consumed alcohol. This data was therefore excluded from further analyses.

Bivariate analyses between selected clinical factors and the use of antiretroviral therapy (ART) is shown in Table 2. No statistically significant difference was observed for any of the factors. However, of note, hemoglobin was elevated amongst those receiving dual ART compared to ARV naïve patients. Additionally, both systolic and diastolic mean blood pressures were higher amongst those on dual ART exposure in contrast to those not.

Cytokine expression: The mean± standard deviation for the measured cytokines are presented in Table 3. There were no statistically significant differences noted between the HIV-positive and HIV-negative groups for both the inflammatory and anti-inflammatory cytokines as well as chemokines when stratified by HIV status, except for inflammatory IP-10 (p<0.05). Similarly, when the HIV population was stratified based on ART usage, only the inflammatory IL-7 was statistically different (p<0.05).

Correlations between inflammatory/anti-inflammatory cytokine expressions and epidemiological factors: The Pearson correlation was used to compare associations between both the inflammatory/ anti-inflammatory cytokines with selected epidemiological factors (Table 4). Our data demonstrates significant correlations between IL-7 and birthweight (r=0.35, p<0.05); IFN-δ and maternal age (r=-0.27, p<0.05); TNF-α and gestational age (r=0.26, p<0.05); VEGF and systolic blood pressure (r=0.40, p<0.05); IL-4 and gestational age (r=-0.30, p<0.05).

Correlations between inflammatory and anti-inflammatory cytokine expressions: The Pearson correlation revealed significant associations between pro- vs anti-inflammatory cytokines (Table 5). A positive correlation was noted for inflammatory IL-1b with anti-inflammatory IL-5, IL-5 and FGF basic; inflammatory IL-2 with anti-inflammatory IL-5, IL-10 as well as FGF basic (Table 5). A negative correlation between the inflammatory IL-12 with anti-inflammatory IL-1ra and Il-4 as well as that between IL-17A with IL-10 (Table 5) was noted.

DISCUSSION

Our study evaluated the mid-gestational expression of selected inflammatory and anti-inflammatory cytokines as well as chemokines in HIV infected pregnant women at their first antenatal visit. Pregnancy in HIV-infected women, poses an additional challenge to the already interrupted immune system. In light of the pervasive burden of HIV infection and the enormous ARV rollout in SA, the immune reconstitution effects of HAART during pregnancy may also impact the expression of both the inflammatory and anti-inflammatory cytokines (Maharaj *et al*., 2017).

Our data suggests that gestational age was significantly different between the HIV+ve and -ve groups. In South Africa, antenatal care is usually initiated before 20 weeks of gestation as prescribed by the National Department of Health (National Department of Health, 2013). It is possible that the HIV positive women sought antenatal care later than the HIV negative group, reflective of possible anxiety associated with mandatory HIV testing at the first antenatal visit.

Table 3:
Cytokine profiles of study population stratified by HIV status –log transformed #

Cytokines	Total sample n = 70	Total sample		HIV +ve (n=41)	
		HIV+ve (n=41)	HIV-ve (n=29)	HAART use (n=23)	No HAART (n=18)
Inflammatory					
IL-1b	7.20 (0.54)	7.18 (0.53)	7.18 (0.54)	7.22 (0.51)	7.20 (0.63)
IL-2	7.41 (0.08)	7.42 (0.00)	7.39 (0.14)	7.42 (0.00)	7.42 (0.00)
IL-6	6.16 (0.57)	6.13 (0.57)	6.16 (0.60)	6.11 (0.51)	6.15 (0.62)
IL-7	6.44 (1.36)	6.36 (1.28)	6.41 (1.47)	**5.95 (1.13)**	**6.89 (1.33)***
IL-8	5.47 (0.49)	5.46 (0.46)	5.51 (0.53)	5.52 (0.41)	5.34 (0.55)
IL-12(p70)	9.05 (0.67)	9.05 (0.74)	9.07 (0.56)	8.92 (0.97)	9.14 (0.30)
IL-15	5.59 (0.28)	5.56 (0.35)	5.63 (0.14)	5.53 (0.41)	5.60 (0.24)
IL-17A	7.63 (2.05)	7.36 (2.12)	8.17 (1.75)	7.71 (1.98)	6.78 (2.30)
Eotaxin	4.69 (1.55)	4.70 (1.60)	4.69 (1.63)	4.56 (1.31)	4.81 (1.81)
GM-CSF	8.91 (0.93)	8.90 (0.96)	8.98 (0.84)	8.99 (0.77)	8.69 (1.22)
IFN-g	3.63 (0.38)	3.62 (0.37)	3.62 (0.44)	3.61 (0.34)	3.65 (0.38)
IP-10	1.86 (1.10)	**1.68 (1.03)**	**2.28 (1.08)***	1.77 (1.16)	1.43 (0.92)
MCP-1(MCAF)	7.21 (2.10)	7.02 (2.06)	7.61(2.18)	6.91 (2.01)	6.91 (2.11)
MIP-1a	0.64 (1.39)	0.53 (1.37)	0.91 (1.48)	0.68 (1.33)	0.25 (1.36)
PDGF-bb	1.70 (1.49)	1.60 (1.62)	1.86 (1.04)	1.66 (1.57)	1.56 (1.91)
MIP-1b	5.41 (1.71)	5.44 (1.78)	5.61 (1.68)	5.53 (1.69)	5.09 (1.88)
RANTES	2.51 (0.86)	2.54 (0.88)	2.60 (0.51)	2.51 (1.06)	2.38 (0.98)
TNF-a	5.38 (1.46)	5.36 (1.56)	5.43 (1.38)	5.21 (1.48)	5.43 (1.61)
VEGF	7.97 (1.63)	7.82 (1.61)	8.12 (1.68)	7.89 (1.70)	7.77 (1.59)
Anti-inflam					
IL-1ra	4.49 (1.24)	4.54 (1.30)	4.43 (1.27)	4.82 (1.50)	4.19 (0.85)
IL-4	6.35 (0.31)	6.34 (0.34)	6.37 (0.27)	6.35 (0.31)	6.33 (0.37)
IL-5	7.23 (1.51)	7.20 (1.44)	7.18 (1.67)	7.11 (1.51)	7.33 (1.37)
IL-10	6.50 (1.78)	6.37 (1.67)	6.20 (2.04)	5.80 (2.07)	7.32 (0.92)
FGF basic	6.16 (1.03)	6.12 (0.98)	6.16 (1.16)	6.36 (0.87)	5.88 (1.04)
G-CSF	4.34 (0.66)	4.25 (0.61)	4.47 (0.80)	4.33 (0.73)	4.18 (0.38)
Chemokines					
IL-8	5.47 (0.49)	5.46 (0.46)	5.51 (0.53)	5.52 (0.41)	5.34 (0.55)
Eotaxin	4.69 (1.55)	4.70 (1.60)	4.69 (1.63)	4.56 (1.31)	4.81 (1.81)
IP-10	1.86 (1.10)	**1.68 (1.03)**	**2.28 (1.08) ***	1.77 (1.16)	1.43 (0.92)
MCP-1(MCAF)	7.21 (2.10)	7.02 (2.06)	7.61(2.18)	6.91 (2.01)	6.91 (2.11)
MIP-1a	0.64 (1.39)	0.53 (1.37)	0.91 (1.48)	0.68 (1.33)	0.25 (1.36)
MIP-1b	5.41 (1.71)	5.44 (1.78)	5.61 (1.68)	5.53 (1.69)	5.09 (1.88)
RANTES	2.51 (0.86)	2.54 (0.88)	2.60 (0.51)	2.51 (1.06)	2.38 (0.98)

** p < 0.05 was considered statistically significant*
 # Data for cytokine levels were log transformed

This is corroborated by a recent study which indicated that both knowledge and timing of a new diagnosis of HIV infection at the first antenatal visit places women at an increased risk for the development of depression during pregnancy (Nydoo *et al*., 2017). The burden of HIV/AIDS diagnosis, combined with an increased risk for the development of depression may adversely affect both mother and child health outcomes (Iyun *et al*., 2018; Moodley *et al*., 2016; Ramirez-Avila *et al*., 2012; Rochat *et al*., 2013).

Despite the implementation of the 2014 new ARV South African guidelines as a means to improve antiretroviral treatment coverage for all HIV positive pregnant women, there are still pregnant women who are unregistered for antenatal care, with unknown HIV status (Iyun *et al*., 2018; Moodley *et al*., 2016). Earlier studies have also suggested that being younger with minimal education, single with no partner support, and low socioeconomic status as potential reasons for being unregistered for antenatal care and thus being categorized as untreated HIV infected women (Fawcus & SR, 1992).

Despite the lack of statistical significance, our study demonstrates a trend in both the systolic and diastolic blood pressure between those receiving HAART compared to those not receiving HAART. Higher blood pressure is known to be elevated in patients receiving ART as it induces vascular endothelial alterations (Chow *et al*., 2003; Nduka *et al*., 2016). A recent systematic analysis confirmed that due to data deficiency, it is unclear if the distinctive effect of ART exposure on the risk of developing hypertension is due to the regimen of the ART or duration (Nduka *et al*., 2016). It is also possible that antiretroviral drugs compromise the production of vasodilatory molecules such as nitric oxide (Nduka *et al*., 2016). However, an earlier report also suggests that these elevations in blood pressure amongst HIV infected individuals, may arise in response to an exaggerated immune response subsequent to the use of antiretroviral drugs (Bosamiya, 2011). Nonetheless, the use of ART is linked to antiretroviral-associated hypertension, a phenomenon that is rapidly gaining momentum in becoming a major healthcare burden (Nduka *et al*., 2016).

Table 4:
Pearson's Correlation between cytokine profiles and epidemiological factors

Cytokines	Age	Gestational Age (wks)	BMI (kg/m^2)	Blood pressure (mmHg)		Hemoglobin (g/dl)	Birth weight (kg)
				Systolic	Diastolic		
Inflammatory							
IL-1b	-0.11	-0.08	0.08	0.23	0.15	-0.15	0.24
IL-2	0.01	-0.19	0.01	-0.11	0.03	0.16	0.23
IL-6	-0.04	-0.06	0.29	0.26	0.34	0.03	-0.05
IL-7	-0.04	-0.07	-0.10	-0.08	-0.01	0.06	**0.35***
IL-12(p70)	0.01	0.23	0.01	-0.11	0.03	0.16	0.10
IL-15	0.05	-0.07	-0.00	-0.03	0.01	0.24	0.09
IL-17A	-0.13	-0.02	-0.01	0.05	-0.15	-0.29	0.06
GM-CSF	-0.25	0.08	-0.06	0.05	-0.20	-0.35	-0.11
IFN-g	**-0.27***	-0.14	0.24	0.35	0.23	-0.16	0.08
PDGF-bb	-0.12	0.08	-0.03	0.08	0.14	0.03	0.08
TNF-a	-0.06	**0.26***	0.18	0.09	-0.16	-0.16	-0.02
VEGF	-0.24	0.08	0.35	**0.40***	0.32	-0.01	-0.02
Anti-inflam							
IL-1ra	-0.23	-0.01	0.14	0.11	0.16	-0.17	0.08
IL-4	-0.18	**-0.30***	0.21	0.24	0.28	-0.02	0.32
IL-5	-0.10	-0.18	0.07	0.10	0.20	0.22	0.20
IL-10	0.17	0.05	-0.07	0.08	0.15	0.17	-0.00
FGF basic	-0.19	-0.18	0.11	0.14	-0.10	-0.17	-0.11
G-CSF	-0.12	-0.04	-0.07	0.20	0.11	-0.09	0.08
Chemokines							
IL-8	-0.14	-0.05	0.09	0.274	0.15	-0.15	0.08
Eotaxin	-0.08	-0.09	-0.09	-0.002	-0.13	-0.18	-0.30
IP-10	-0.22	0.12	0.07	0.103	-0.02	-0.01	0.00
MCP-1(MCAF)	0.04	-0.06	-0.12	0.255	0.07	-0.23	0.12
MIP-1a	-0.01	0.20	-0.06	-0.007	0.03	-0.02	0.29
MIP-1b	-0.15	0.19	-0.21	-0.039	0.11	-0.01	0.09
RANTES	-0.09	0.05	-0.13	-0.156	-0.14	-0.25	0.16

**p<0.05 was considered statistically significant*

Table 5:
Pearson's correlation between Inflammatory and anti-inflammatory cytokines

		Anti-inflammatory cytokines					
		IL-1ra	**IL-4**	**IL-5**	**IL-10**	**FGF basic**	**GM-CSF**
Inflammatory	**IL-1b**	**0.69****	**0.72****	**0.33***	0.04	**0.29***	0.14
	IL-2	0.17	0.19	**0.81****	**0.53****	**0.34***	0.02
	IL-6	**0.84****	**0.67****	**0.50****	0.06	**0.43****	**0.41***
	IL-7	**0.25***	**0.31***	**0.71****	**0.52****	**0.30***	0.07
	IL-12(p70)	-0.01	-0.13	0.22	0.20	0.23	0.06
	IL-15	0.21	0.17	0.18	**0.25***	0.03	0.20
	IL-17A	**0.44****	**0.49****	0.15	-0.00	**0.41****	**0.43****
	GM-CSF	**0.53****	0.23	0.20	0.08	**0.58****	1.00
	IFN-g	**0.76****	**0.71****	**0.47****	0.07	**0.49****	**0.31***
	PDGF-bb	**0.39***	**0.50****	0.04	-0.09	0.13	**0.30***
	TNF-a	0.03	-0.11	0.05	0.04	**0.27***	0.16
	VEGF	**0.45****	**0.33***	**0.25***	0.04	**0.50****	**0.32***
Chemokines	**IL-8**	**0.55****	**0.50****	**0.24***	0.07	**0.40***	**0.25***
	Eotaxin	**0.39***	**0.31***	0.23	0.03	**0.34***	0.19
	IP-10	**0.38***	0.20	0.11	-0.03	**0.52****	**0.67****
	MCP-1(MCAF)	**0.25***	**0.25***	0.07	0.07	**0.53****	**0.41***
	MIP-1a	0.16	0.17	-0.08	-0.05	-0.04	0.02
	MIP-1b	**0.32***	**0.29***	0.07	-0.09	**0.27***	**0.40***
	RANTES	**0.28***	0.20	0.04	-0.01	**0.27***	**0.42****

**p<0.05 was considered statistically significant*
*** p<0.001 was considered statistically significant*

We also demonstrate a significant correlation between vascular endothelial growth factor (VEGF) and systolic blood pressure. Vascular Endothelial Growth Factor regulates angiogenesis, maintaining vascular homeostasis and permeability (Ghazizadeh *et al*., 2017). It is also responsible in maintaining endothelial function and blood pressure by stimulating NO synthase expression and the release of vasodilatory nitric oxide and prostacyclin (Ylä-Herttuala *et al*., 2007). However, elevated VEGF concentration is reported to physiologically predispose one to a greater risk of emergent cardiovascular anomalies (Ghazizadeh *et al*., 2017). This risk is amplified as a result of its release from foam cells and macrophages which stimulates the development of atherosclerosis (Ghazizadeh *et al*., 2017), combined with reports that the HIV accessory protein *tat* mimics VEGF increasing angiogenesis, thus supporting its powerful angiogenic effects (Barillari *et al*., 1999).

The anti-inflammatory IL-4 and IL-10 cytokines are instrumental in resolving inflammation during pregnancy (Chatterjee *et al*., 2014). Our data demonstrates statistically significant positive correlations between IL-4 and several of the inflammatory cytokines measured, including IL-1b, IL-6, IL-7, IL-8, IFN-G. In addition, we demonstrate an inverse association between IL-4 and IL-12, which corroborates Ouyang *et al*., (1998), who previously suggested that the Th2 immune response is strengthened by IL-4 due to suppression of the Th1 immune activity and IL-12 signaling (Ouyang *et al*., 1998). The anti-inflammatory effects of IL-10 is achieved due to the blockage of the inflammatory effects of IL-1, IL-6, IL-12, TNF and chemokines (Saraiva & O'Garra, 2010). Our data is similar in that it also demonstrates an inverse relation between IL-10 and the chemokines IP-10, MIP-1a, MIP-1b and RANTES, endorsing the anti-inflammatory effects required for pregnancy success. Thus, balance in IL-4 and IL-10 is essential in controlling the maternal inflammatory response, thereby endorsing the crucial crosstalk between the placenta and the fetus during pregnancy (Thaxton & Sharma, 2010). In the absence of this resolution by the anti-inflammatory cytokines, various pregnancy related anomalies may prevail which can jeopardize both maternal and neonatal health (Chatterjee *et al*., 2014). Ferguson *et al*., (2014) reiterates this finding, suggesting that maternal inflammatory cytokines such IL-6 and IL-10 may be related with a greater risk of spontaneous preterm birth, and placentally mediated preterm birth respectively (Ferguson *et al*., 2014). Likewise, cytokines IL-8, IFNγ, and TNFα are also reported to be associated with preeclampsia development and intrauterine growth restriction that is coupled with deficient placental function (Raghupathy *et al*., 2012).

The global increase in HIV-infected young women is associated with disruptions in the maternal immune status which subsequently disrupts the neonatal immune status (Kasahara *et al*., 2013). Despite the obvious anti-retroviral associated reduction in perinatal transmission, antiretroviral therapy during pregnancy is associated with a greater risk of premature births (Fiore *et al*., 2006). Our data demonstrates a reduction in IL-10 levels amongst the treated HIV+ve women in contrast to untreated women. Despite the lack of statistical significance, we also demonstrate and inverse relationship between IL-10 and birthweight. More recently, it was shown that low serum levels of IL-10 between 22 to 25 weeks' gestation, may be correlated with the risk of preterm birth in primigravidae (de Brito Pereira *et al*., 2016).

We also report a statistically significant difference in IL-7 levels between the HIV+ve cohort on ARV treatment compared to those not. Birth weight was not different between the latter 2 groups, however, a statistically significant correlation between IL-7 levels and birth weight was noted. It is possible that the anti-retroviral drugs are autonomously regulating the placental cytokine production as well as tit anti-HIV effect (Faye *et al*., 2007). Interestingly, a study has linked antiretroviral therapy to lower birth weight infants regardless of gestational age, which may be linked to both maternal and fetal TNF-α production (Kasahara *et al*., 2013). In contrast, more recent studies indicate that ART reportage either as ZDV prophylaxis or triple ARV schedules, is correlated with lower probabilities for adverse birth outcomes (Moodley *et al*., 2016). However, there is greater likelihood for poor birth outcomes amongst HIV infected women who are unregistered for antenatal care and therefore untreated (Moodley *et al*., 2016). However, it is possible that factors such as socio-economic status, the initiation of ART either during pregnancy or before pregnancy as well as ART duration prior to delivery may also influence the birth outcomes (Moodley *et al*., 2016).

Christian and Porter (2014) also demonstrated elevations in both IL-6 and TNF-α throughout pregnancy and postpartum, whilst IL-8 and IL-1b declined during the early gestational period and later into pregnancy, but heightened at postpartum (Christian & Porter, 2014). Previous studies demonstrated higher Th-2 cytokine levels and a greater immunity during the course of pregnancy in the HIV-uninfected groups in contrast to the HIV-infected groups (Kolte *et al*., 2011). Moreover, increased IL-4 and IL-10 concentrations were observed in HIV-ve women in comparison to HIV+ve women (Kolte *et al*., 2011). It is possible that these increased cytokine concentrations are hormone induced since progesterone promotes the production of IL-4 and IL-5 (M-P Piccinni *et al*., 2000). However, we only report cytokine concentrations measured at one gestational point, nonetheless, our Pearson's correlation analyses demonstrate an inverse relationship between IL-1b, Il-8 and IL-4 with gestational age. Our findings corroborate that reported by Christian and co-workers, who demonstrated a U-shaped curve for IL-8 and IL-1b, suggestive of cytokine reductions during mid to late gestation in comparison to early pregnancy (Christian & Porter, 2014). This is indicative of the foundational inflammatory response observed in early pregnancy which down-regulates over time (Christian & Porter, 2014). However, our data failed to show any visible differences in the concentrations of IL-8 and IL-1b when compared between HIV status and antiretroviral treatment status.

Interestingly, we also demonstrate a significant correlation between TNF-alpha levels and gestational age, similar to that reported by Christian and Porter (2014). More recently, antiretroviral therapy correlates with a major reduction in the levels of IL-2, TNF-α and IL-6 in pregnancy (Maharaj *et al*., 2017). Despite the lack of statistical significance, our data demonstrate similar reductions for Il-6 and TNF-α amongst those on ART compared to those that aren't. Tumor necrosis factor alpha (TNF-α) is linked to the

cell mediated cytotoxic arm of the specific immune response whilst the inflammatory IL-2 controls both the cellular and humoral immune reaction (Fiore *et al*., 2006; Osakwe *et al*., 2010). We also reveal an inverse relationship between IL-2 and gestational age, albeit non-significant. Our data corroborates the reduction in IL-2 through pregnancy, as observed by Russell *et al*. (1997) (Russell *et al*., 1997). Likewise, Shimaoka *et al*., (2000) also revealed reductions in the concentrations of IL-2, IFN-g, IL-4 and IL-10 throughout pregnancy (Shimaoka *et al*., 2000). These cytokines represent the Th1 and Th2 immune response, indicative that an overall reduction in maternal immunity manifests during pregnancy.

Despite the lack of statistically significant difference, our data report the potential for the onset of anemia in the HIV-ve pregnant group. It is possible that the levels of hemoglobin may be associated with the increase in plasma volume during pregnancy. We also demonstrate a noticeable difference in hemoglobin levels between those receiving treatment in contrast to those not on treatment amongst the HIV positive group. The hemoglobin levels were higher amongst those receiving treatment, indicative of the possibility of being anemic. Our data also supports Nandlall and co-workers, who report higher predisposition to anemia development amongst HIV+ve pregnant women on antiretroviral therapy (Nandlal *et al*., 2014).

Despite the lack of a statistical significance, we demonstrate a trend in IL-17A expression between the HIV+ve and -ve cohorts, as well as treated versus untreated HIV participants. The HAART treated women had higher cytokine expressions compared to untreated women. Interleukin 17-A is pro-inflammatory in nature and exerts its effect in response to activation of the inflammatory effectors, the antimicrobial molecules defensins and mucins, the chemokines, the inflammatory IL-6 and tumor necrosis factor-α, and the anti-inflammatory granulocyte colony-stimulating factor (Onishi & Gaffen, 2010). It is possible that the restorative immune effect of ART is associated with this pro-inflammatory effect. Moreover, the proinflammatory role of IL-17A in pre-eclampsia development received authentication, based on elevated levels observed in pre-eclamptic pregnancies compared with normotensive pregnancies (Molvarec *et al*., 2015). However, a limitation of our study is that the circulating values of several cytokines in our study were below the detection limit of the assay. This may be attributed to the use of the Multiplex bead array technology, indicative that this technology may not be the ideal platform for measuring analytes with very low concentrations due to it elevated dynamic range.

In conclusion, this study demonstrates midgestational variation in serum inflammatory and anti-inflammatory immunologic profile of pregnant women, regardless of the use of antiretroviral therapy. This disparity in the vulnerable HIV infected women will affect progression of pregnancy and promote fetal morbidity and mortality.

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REFERENCES

Amorosa, V., Synnestvedt, M., Gross, R., Friedman, H., MacGregor, R. R., Gudonis, D., Frank, I., & Tebas, P. (2005). A tale of 2 epidemics: the intersection between obesity and HIV infection in Philadelphia. J Acquir Immune Defic Syndr, 39(5), 557-561.

Barillari, G., Sgadari, C., Palladino, C., Gendelman, R., Caputo, A., Morris, C. B., Nair, B. C., Markham, P., Nel, A., & Stürzl, M. (1999). Inflammatory cytokines synergize with the HIV-1 Tat protein to promote angiogenesis and Kaposi's sarcoma via induction of basic fibroblast growth factor and the αvβ3 integrin. The Journal of Immunology, 163(4), 1929-1935.

Bergersen, B. M., Sandvik, L., Dunlop, O., Birkeland, K., & Bruun, J. N. (2003). Prevalence of hypertension in HIV-positive patients on highly active retroviral therapy (HAART) compared with HAART-naive and HIV-negative controls: results from a Norwegian study of 721 patients. Eur J Clin Microbiol Infect Dis, 22(12), 731-736. doi: 10.1007/s10096-003-1034-z

Bosamiya, S. S. (2011). The immune reconstitution inflammatory syndrome. Indian J Dermatol, 56(5), 476-479. doi: 10.4103/0019-5154.87114

Bowen, J., Chamley, L., Keelan, J., & Mitchell, M. (2002). Cytokines of the placenta and extra-placental membranes: roles and regulation during human pregnancy and parturition. Placenta, 23(4), 257-273.

Challis, J. R., Lockwood, C. J., Myatt, L., Norman, J. E., Strauss III, J. F., & Petraglia, F. (2009). Inflammation and pregnancy. Reproductive sciences, 16(2), 206-215.

Chatterjee, P., Chiasson, V. L., Bounds, K. R., & Mitchell, B. M. (2014). Regulation of the Anti-Inflammatory Cytokines Interleukin-4 and Interleukin-10 during Pregnancy. Front Immunol, 5, 253. doi: 10.3389/fimmu.2014.00253

Chow, D. C., Souza, S. A., Chen, R., Richmond-Crum, S. M., Grandinetti, A., & Shikuma, C. (2003). Elevated blood pressure in HIV-infected individuals receiving highly active antiretroviral therapy. HIV clinical trials, 4(6), 411-416.

Christian, L. M., & Porter, K. (2014). Longitudinal changes in serum proinflammatory markers across pregnancy and postpartum: effects of maternal body mass index. Cytokine, 70(2), 134-140. doi: 10.1016/j.cyto.2014.06.018

Clerici, M. (2010). Beyond IL-17: new cytokines in the pathogenesis of HIV infection. Current Opinion in HIV and AIDS, 5(2), 184-188.

Crum-Cianflone, N., Tejidor, R., Medina, S., Barahona, I., & Ganesan, A. (2008). Obesity among patients with HIV: the latest epidemic. AIDS Patient Care STDS, 22(12), 925-930. doi: 10.1089/apc.2008.0082

de Brito Pereira, T. B., Thomaz, E. B. A. F., do Nascimento, F. R. F., Batista, R. L. F., Bettiol, H., de Carvalho Cavalli, R., Barbieri, M. A., & da Silva, A. A. M. (2016). Regulatory cytokine expression and preterm birth: case-control study nested in a cohort. PLoS One, 11(8), e0158380.

Fawcus, J., & SR, K. (1992). Booked and unbooked mothers delivering at Harare Maternity Hospital, Zimbabwe: a comparison of maternal characteristics and foetal outcome. Central african journal of medicine, 38(10), 402-408.

Faye, A., Pornprasert, S., Mary, J. Y., Dolcini, G., Derrien, M., Barré-Sinoussi, F., Chaouat, G., Menu, E., team, A. s., & PMTCT-PlaNet, t. H. (2007). Characterization of the main placental cytokine profiles from HIV-1-infected pregnant women treated with anti-retroviral drugs in France. Clinical & Experimental Immunology, 149(3), 430-439.

Ferguson, K. K., McElrath, T. F., Chen, Y. H., Mukherjee, B., & Meeker, J. D. (2014). Longitudinal profiling of inflammatory cytokines and C-reactive protein during uncomplicated and preterm pregnancy. American journal of reproductive immunology, 72(3), 326-336.

Fiore, S., Newell, M. L., Trabattoni, D., Thorne, C., Gray, L., Savasi, V., Tibaldi, C., Ferrazzi, E., & Clerici, M. (2006).

Antiretroviral therapy-associated modulation of Th1 and Th2 immune responses in HIV-infected pregnant women. J Reprod Immunol, 70(1-2), 143-150. doi: 10.1016/j.jri.2005.12.001

Gallant, J. E., Staszewski, S., Pozniak, A. L., DeJesus, E., Suleiman, J. M., Miller, M. D., Coakley, D. F., Lu, B., Toole, J. J., Cheng, A. K., & Study, G. (2004). Efficacy and safety of tenofovir DF vs stavudine in combination therapy in antiretroviral-naive patients: a 3-year randomized trial. JAMA, 292(2), 191-201. doi: 10.1001/jama.292.2.191

Ghazizadeh, H., Fazilati, M., Pasdar, A., Avan, A., Tayefi, M., Ghasemi, F., Mehramiz, M., Mirhafez, S. R., Ferns, G. A., Azimi-Nezhad, M., & Ghayour-Mobarhan, M. (2017). Association of a Vascular Endothelial Growth Factor genetic variant with Serum VEGF level in subjects with Metabolic Syndrome. Gene, 598, 27-31. doi: 10.1016/j.gene.2016.10.034

Hu, W., Wang, H., Wang, Z., Huang, H., & Dong, M. (2007). Elevated serum levels of interleukin-15 and interleukin-16 in preeclampsia. Journal of reproductive immunology, 73(2), 166-171.

Iyun, V., Brittain, K., Phillips, T. K., Le Roux, S., McIntyre, J. A., Zerbe, A., Petro, G., Abrams, E. J., & Myer, L. (2018). Prevalence and determinants of unplanned pregnancy in HIV-positive and HIV-negative pregnant women in Cape Town, South Africa: a cross-sectional study. BMJ open, 8(4), e019979.

Joachim, R., Zenclussen, A. C., Polgar, B., Douglas, A. J., Fest, S., Knackstedt, M., Klapp, B. F., & Arck, P. C. (2003). The progesterone derivative dydrogesterone abrogates murine stress-triggered abortion by inducing a Th2 biased local immune response. Steroids, 68(10-13), 931-940.

Kasahara, T. M., Hygino, J., Blanco, B., Xavier, L., Araújo-Lima, C. F., Guillermo, L. V., Bittencourt, V. C. B., Guimarães, V., Andrade, A. F., & Bento, C. A. (2013). The impact of maternal anti-retroviral therapy on cytokine profile in the uninfected neonates. Human immunology, 74(9), 1051-1056.

Kolte, L., Gaardbo, J. C., Karlsson, I., Sørensen, A. L., Ryder, L. P., Skogstrand, K., Ladelund, S., & Nielsen, S. D. (2011). Dysregulation of CD4+ CD25+ CD127lowFOXP3+ regulatory T cells in HIV-infected pregnant women. Blood, 117(6), 1861-1868.

Maharaj, N. R., Phulukdaree, A., Nagiah, S., Ramkaran, P., Tiloke, C., & Chuturgoon, A. A. (2017). Pro-Inflammatory Cytokine Levels in HIV Infected and Uninfected Pregnant Women with and without Preeclampsia. PLoS One, 12(1), e0170063. doi: 10.1371/journal.pone.0170063

Mallon, P. W., Miller, J., Cooper, D. A., & Carr, A. (2003). Prospective evaluation of the effects of antiretroviral therapy on body composition in HIV-1-infected men starting therapy. AIDS, 17(7), 971-979. doi: 10.1097/01.aids.0000060348.78202.74

Molvarec, A., Czegle, I., Szijarto, J., & Rigo, J., Jr. (2015). Increased circulating interleukin-17 levels in preeclampsia. J Reprod Immunol, 112, 53-57. doi: 10.1016/j.jri.2015.05.007

Moodley, T., Moodley, D., Sebitloane, M., Maharaj, N., & Sartorius, B. (2016). Improved pregnancy outcomes with increasing antiretroviral coverage in South Africa. BMC pregnancy and childbirth, 16(1), 35.

Mosmann, T. R., & Sad, S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today, 17(3), 138-146.

Murtha, A. P., Sinclair, T., Hauser, E. R., Swamy, G. K., Herbert, W. N., & Heine, R. P. (2007). Maternal serum cytokines in preterm premature rupture of membranes. Obstet Gynecol, 109(1), 121-127. doi: 10.1097/01.AOG.0000250474.35369.12

Nandlal, V., Moodley, D., Grobler, A., Bagratee, J., Maharaj, N. R., & Richardson, P. (2014). Anaemia in pregnancy is associated with advanced HIV disease. PLoS One, 9(9), e106103. doi: 10.1371/journal.pone.0106103

National Department of Health. (2008). Policy and Guidelines for the Implementation of the PMTCT Programme. South Africa

National Department of Health. (2013). The 2013 national antenatal sentinel HIV prevalence survey South Africa. South Africa: National Department of Health Pretoria.

Nduka, C. U., Stranges, S., Sarki, A. M., Kimani, P. K., & Uthman, O. A. (2016). Evidence of increased blood pressure and hypertension risk among people living with HIV on antiretroviral therapy: a systematic review with meta-analysis. J Hum Hypertens, 30(6), 355-362. doi: 10.1038/jhh.2015.97

Nydoo, P., Naicker, T., & Moodley, J. (2017). Depressive scores in newly diagnosed HIV-infected and HIV-uninfected pregnant women. S Afr J Psychiatr, 23, 1085. doi: 10.4102/sajpsychiatry.v23i0.1085

Omar, M. H., Mashita, M., Lim, P. S., & Jamil, M. (2005). Dydrogesterone in threatened abortion: pregnancy outcome. The Journal of steroid biochemistry and molecular biology, 97(5), 421-425.

Onishi, R. M., & Gaffen, S. L. (2010). Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. Immunology, 129(3), 311-321. doi: 10.1111/j.1365-2567.2009.03240.x

Orsi, N., & Tribe, R. (2008). Cytokine networks and the regulation of uterine function in pregnancy and parturition. Journal of neuroendocrinology, 20(4), 462-469.

Osakwe, C. E., Bleotu, C., Chifiriuc, M. C., Grancea, C., Oţelea, D., Paraschiv, S., Petrea, S., Dinu, M., Băicuş, C., & Streinu-Cercel, A. (2010). TH1/TH2 cytokine levels as an indicator for disease progression in human immunodeficiency virus type 1 infection and response to antiretroviral therapy. Roum Arch Microbiol Immunol, 69(1), 24-34.

Ouyang, W., Ranganath, S. H., Weindel, K., Bhattacharya, D., Murphy, T. L., Sha, W. C., & Murphy, K. M. (1998). Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity, 9(5), 745-755.

Piccinni, M.-P., Lombardelli, L., Logiodice, F., Kullolli, O., Romagnani, S., & Le Bouteiller, P. (2015). T helper cell mediated-tolerance towards fetal allograft in successful pregnancy. Clinical and Molecular Allergy, 13(1), 9.

Piccinni, M.-P., Maggi, E., & Romagnani, S. (2000). Role of hormone-controlled T-cell cytokines in the maintenance of pregnancy: Portland Press Limited.

Raghupathy, R., Al-Azemi, M., & Azizieh, F. (2012). Intrauterine growth restriction: cytokine profiles of trophoblast antigen-stimulated maternal lymphocytes. Clin Dev Immunol, 2012, 734865. doi: 10.1155/2012/734865

Ramirez-Avila, L., Regan, S., Giddy, J., Chetty, S., Ross, D., Katz, J. N., Freedberg, K. A., Walensky, R. P., Losina, E., & Bassett, I. V. (2012). Depressive symptoms and their impact on health-seeking behaviors in newly-diagnosed HIV-infected patients in Durban, South Africa. AIDS and Behavior, 16(8), 2226-2235.

Raphael, I., Nalawade, S., Eagar, T. N., & Forsthuber, T. G. (2015). T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. Cytokine, 74(1), 5-17.

Robertson, S. A., Seamark, R. F., Guilbert, L. J., & Wegmann, T. G. (1994). The role of cytokines in gestation. Critical Reviews™ in Immunology, 14(3-4).

Rochat, T. J., Tomlinson, M., Newell, M. L., & Stein, A. (2013). Detection of antenatal depression in rural HIV-affected populations with short and ultrashort versions of the Edinburgh Postnatal Depression Scale (EPDS). Arch Womens Ment Health, 16(5), 401-410. doi: 10.1007/s00737-013-0353-z

Russell, A. S., Johnston, C., Chew, C., & Maksymowych, W. P. (1997). Evidence for reduced Th1 function in normal pregnancy: a hypothesis for the remission of rheumatoid arthritis. J Rheumatol, 24(6), 1045-1050.

Saraiva, M., & O'Garra, A. (2010). The regulation of IL-10 production by immune cells. Nat Rev Immunol, 10(3), 170-181. doi: 10.1038/nri2711

Seaberg, E. C., Munoz, A., Lu, M., Detels, R., Margolick, J. B., Riddler, S. A., Williams, C. M., Phair, J. P., & Multicenter, A. C. S. (2005). Association between highly active antiretroviral

therapy and hypertension in a large cohort of men followed from 1984 to 2003. AIDS, 19(9), 953-960.

Sebitloane, H. M., & Moodley, D. (2017). The impact of highly active antiretroviral therapy on obstetric conditions: a review. European Journal of Obstetrics & Gynecology and Reproductive Biology, 210, 126-131.

Sharma, A., Bynum, S. A., Schneider, M. F., Cox, C., Tien, P. C., Hershow, R. C., Gustafson, D., & Plankey, M. W. (2014). Changes in Body Mass Index Following HAART Initiation among HIV-Infected Women in the Women's Interagency HIV Study. J AIDS Clin Res, 5. doi: 10.4172/2155-6113.1000323

Shikuma, C. M., Zackin, R., Sattler, F., Mildvan, D., Nyangweso, P., Alston, B., Evans, S., Mulligan, K., & Team, A. C. T. G. (2004). Changes in weight and lean body mass during highly active antiretroviral therapy. Clin Infect Dis, 39(8), 1223-1230. doi: 10.1086/424665

Shimaoka, Y., Hidaka, Y., Tada, H., Nakamura, T., Mitsuda, N., Morimoto, Y., Murata, Y., & Amino, N. (2000). Changes in cytokine production during and after normal pregnancy. Am J Reprod Immunol, 44(3), 143-147.

Statistics South Africa. (2015). Mid-year population estimates, 2015. Statistical release P0302.

Stein, J. H. (2003). Dyslipidemia in the era of HIV protease inhibitors. Prog Cardiovasc Dis, 45(4), 293-304. doi: 10.1053/pcad.2003.4

Szekeres-Bartho, J., & Wegmann, T. (1996). A progesterone-dependent immunomodulatory protein alters the Th1Th2 balance. Journal of reproductive immunology, 31(1-2), 81-95.

Thaxton, J. E., & Sharma, S. (2010). Interleukin-10: a multi-faceted agent of pregnancy. Am J Reprod Immunol, 63(6), 482-491. doi: 10.1111/j.1600-0897.2010.00810.x

United Nations Programme on HIV/AIDS. (2017). UNAIDS DATA 2017. Geneva.

World Health Organization. (2011). Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity: World Health Organization.

Ylä-Herttuala, S., Rissanen, T. T., Vajanto, I., & Hartikainen, J. (2007). Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. Journal of the american college of cardiology, 49(10), 1015-1026.

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Research article

Changes in Selected Renal Function Parameters of Newly Admitted COVID-19 Patients from One Infectious Diseases Center in Ibadan, Nigeria: Indication for Immunopathology

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Summary: COVID-19 caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) enters the host cells through attachment to the Angiotensin Converting Enzyme-2 receptors (ACE-2) on the host cells. Angiotensin Converting Enzyme-2 is known to affect renal functions, vasoconstriction and fluid homeostasis. Thus, the impact of SARS-CoV-2 infection on renal function parameters is worth investigating. Plasma obtained from whole blood samples collected from consecutive and newly diagnosed two hundred and two COVID-19 patients admitted for management at the Infectious Disease Center, Olodo in Ibadan the capital of Oyo State, South Western Nigeria were analysed for albumin, urea, creatinine, Na, K, Cl and HCO_3 using auto analysers. The results obtained were used to determine the frequency of COVID-19 patients with abnormal renal function parameters. It was observed that 57.1%, 37.8%, 32.7%, 28.1%, 18.7%, 17.8% and 3.4% of newly diagnosed COVID-19 patients had values of Cl, creatinine, albumin, Na, K, HCO_3 and urea respectively outside the reference ranges. While 43.3%, 4.7%, 2.5%, 2.5%, 2.0%, 1.7% and 1.0% of COVID-19 patients had values of Cl, creatinine, Na, K, albumin, Urea and HCO_3 respectively above the reference ranges. Of all admitted patients, 33.1%, 30.7%, 25.6%, 16.8%, 16.3%, 13.8% and 1.7% had creatinine, albumin, Na, HCO_3, K, Cl and urea values respectively below reference ranges. The changes in some renal function parameters of newly diagnosed COVID-19 patients portend that renal failure is possible in poorly managed COVID-19 patients and this has immunopathologic implications.
Keywords*: COVID-19, Reference Ranges, Creatinine, Electrolytes, Urea, Immunopathology*

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which causes the disease named by World Health Organisation (W.H.O) as coronavirus disease 2019 (COVID-19)is still spreading worldwide (Center for Systems Science and Engineering 2020; Chan et al., 2020). The prevalence of asymptomatic infected carriers is unknown (Flaxman et al., 2020) but symptomatic patients presents with upper and lower respiratory tract illnesses, multiple organ failure and in some cases, death (Zhou et al., 2020). A significant number of SARS-COV-2 infected patients have acute renal dysfunction which may progress to renal failure requiring dialysis (Chu et al., 2005; Nehme et al., 2015; Rodrigues et al., 2017). In previous reports of SARS and MERS-CoV infections, acute kidney injury (AKI) developed in 5–15% cases with60–90% mortality (Chu et al., 2005). Nevertheless, the incidence of acute kidney failure in SARS-CoV-2 patients is unknown and information about the clinical impact remains sparse (Chu et al., 2005; Nehme et al., 2015; Rodrigues et al., 2017). In this sense, early estimation of renal functional changes in COVID-19 patients should be taken as a priority.

Angiotensin Converting Enzyme-2 (ACE-2) receptors are found in high concentrations in the kidney, small intestine, testis, heart, and the thyroid gland and are known to bind and facilitate SARS-CoV-2 entry into the cells in patients with COVID-19 (Nehme et al., 2015; Rodrigues et al., 2017). Binding of SARS-COV-2 Spike (S) protein with ACE-2 cause reduction of membrane-bound ACE-2 and subsequently an imbalance of therenin–angiotensin system (RAS). ACE-2 is also largely expressed in the nasal and oropharyngeal epithelium (Nehme et al., 2015), where SARS-CoV-2 entrance intothe hosts also occurs. Activation of the ACE 2/Angiotensin II/Angiotensin-II Type-1 receptor axis aggravates inflammation, fibrosis, oxidative

stress, cellular growth and migration while Angiotensin II is known for its cardiovascular and renal functions, vasoconstriction and fluid homeostasis (Rodrigues et al., 2017). It was thus suggested that up-regulation of this classical renin-angiotensin system might partially be responsible for the deleterious pathophysiological derangements observed in COVID-19 (Imai et al., 2005) especially renal functions which is the rationale for this study.

Acute kidney injury (AKI) was hypothesised to contribute to COVID mortality (Cheng et al., 2020) and a previous report showed a strong association between acute elevation of creatinine and mortality in COVID-19 patients (Wang et al., 2020). Another study indicated that 3% of hospitalized patients with COVID-19 had AKI but the incidence was higher (19%) in COVID-19 patients admitted into the Intensive Care Unit (Ng et al., 2020). These observations suggested an association between COVID-19 severity and renal injury. A multicentre cohort study on hospitalized COVID-19 patients found a markedly high rate of AKI (36.6%) in COVID-19 patients. These authors also found that diabetes, hypertension, and cardiovascular disease were the co-morbid conditions closely associated with therisk of AKI (Hirsch et al., 2020). Therefore, both pre-existing conditions in a given patient population must be considered carefully when evaluating the prevalence of AKI and or its use as a predictor of clinical outcome in COVID-19. The same multi-centered study pointed out that AKI rates peaked on the first day after admission, suggesting that monitoring for AKI early in hospital admissions could greatly aid decision making.

Another study (Cheng et al., 2020) recorded baseline serum creatinine levels of patients with COVID-19 on admission and found that patients with elevated creatinine had a significantly higher rate of AKI. The authors also found that elevated creatinine at baseline correlated with a 2.5-fold higher risk of death in this patient population (13.2% vs 33.7%), suggesting it as a significant risk factor for mortality in the presence of COVID-19 infection. Apart from creatinine, several other markers of kidney injury were also associated with significantly increased risk of mortality of COVID-19 patients. Both elevated serum creatinine and serum urea nitrogen on admission were associated with significantly increased risk of mortality, but elevated serum urea nitrogen (Cheng et al., 2020) had more than twice the level of associated risk compared with AKI. Therefore, both serum creatinine and serum urea nitrogen concentrations on admission were concluded to serve as useful guidelines for assessment of disease severity and level of care for patients with COVID-19.

Previous study reported that kidney failure affects general immunity by causing intestinal barrier dysfunction, systemic inflammation and immunodeficiency (Kurts et al., 2013, Betjes, 2013; Yatim and Lakkis, 2015). It is therefore essential to manage renal dysfunctions as indirect measures to sustain immunity during SARS-COV-2 infection.

MATERIALS AND METHODS

Study site*:* The COVID-19 patients were enrolled from the Infectious Diseases Centre, Olodo, Oyo State, Nigeria.

Study Population: Ethical approval (reference number: UI/EC/20/0283) was obtained from UI/UCH Ethic Committee. A total of 202 COVID-19 patients (not in severe or critical stage) were consecutively enrolled into this study. The study participants were COVID-19 cases certified by real-time reverse-transcriptase polymerase-chain reaction (RT-PCR) assay using nasal and pharyngeal swab specimens following W.H.O guideline (W.H.O, 2020). The participants also exhibited signs and symptoms of COVID-19 such as fever, cough, anosmia, agenuia and headache. All subjects with co-morbidities (sickle cell anaemia, peptic ulcer, hypertension and diabetes mellitus) or on compulsory medication were excluded from this study.

Sample collection: Two millilitres of 10 ml venous blood obtained from the SARS-CoV-2 infected patients at the point of admission to the center was used for this study. The blood samples were dispensed into lithium heparin containing sample bottles.

Plasma analyses: Plasma obtained from whole blood sample was analysed for albumin, urea, creatinine, Na, K, Cl and HCO_3 using auto-analysers (Erba Mannheim XL-200, Germany). The auto-analyser was calibrated daily and the standard samples were included during all analyses. Reference values for clinical chemistry were used to classify the results as within or outside the normal reference range (Bugdayci et al., 2015).

Result presentation: The results obtained from these analyses were used to determine the frequency of COVID-19 patients having values of blood parameters below, within and above reference ranges. Data were represented as frequencies and percentages.

RESULTS

The prevalence of newly-diagnosed COVID-19 patients with abnormal renal function parameters is shown in Table 1. 57.1%, 37.8%, 32.7%, 28.1%, 18.7%, 17.8% and 3.4% COVID-19 patients had Cl, creatinine, albumin, Na, K, HCO_3 and urea respectively outside the reference ranges. As also shown in Table 1, 43.3%, 4.7%, 2.5%, 2.5%, 2.0%, 1.7% and 1.0% of COVID-19 patients had Cl, creatinine, Na, K, albumin, Urea and HCO_3 respectively above the reference ranges while 33.1%, 30.7%, 25.6%, 16.8%, 16.3%, 13.8% and 1.7% of the patients had creatinine, albumin, Na, HCO_3, K, Cl and urea respectively below reference ranges.

DISCUSSION

Reference values provide a basis for interpretation of laboratory data. In clinical practice, it is a usual practice to compare patient's result with the corresponding reference interval, which is bounded by a pair of reference limits (Bugdayciet al., 2015).

Between 3.4% to 51.7% newly diagnosed COVID-19 patients recruited for this study had one or more deranged values of renal function parameters on admission. Previous reasons for the involvement of COVID-19 in renal abnormalities were based on high ACE-2 expression in the kidneys (Li et al., 2020), direct cytopathic effects on kidney tissue by SARS-CoV-2 especially the podocytes and proximal straight tubule cells (Pan et al., 2020), cytokine storm-mediated kidney damage (Jin et al., 2020) and formation of immune complexes (Tisoncik et al., 2012).

Table 1:
Plasma electrolyte and urea levels of newly-diagnosed COVID-19 patients with abnormal renal function parameters

Variables (Reference Ranges)	Within Reference Ranges	Abnormal Reference Ranges		
		Below	Above	Total
Albumin (3.5-5g/dL)	136 (67.3)	62(30.7)	4 (2.0)	66 (32.7)
Urea (15-45mg/dL)	169 (96.6)	3(1.7)	3 (1.7)	6 (3.4)
Creatinine (0.5-1.5mg/dL)	107 (62.2)	57(33.1)	8 (4.7)	65 (37.8)
Na^+ (0.5-1.5mg/dL)	146 (71.9)	52(25.6)	5 (2.5)	57 (28.1)
K^+ (3.5 -5mmmol/L)	165 (81.3)	33(16.2)	5 (2.5)	38 (18.7)
Cl^- (95-100MEq/L)	87 (42.9)	28(13.8)	88(43.3)	116(57.1)
HCO_3(20-30mmol/L)	166 (82.2)	34(16.8)	2 (1.0)	36 (17.8)

Percentages in parentheses

Understanding renal functions during COVID-19 may give part of pathophysiologic clue to SARS-CoV-2 infection. SARS-CoV-2 ribonucleic acid (RNA) was shown to bind ACE-2 of proximal tubule of COVID-19 infected patient (Rodrigues et al., 2017). After binding and endocytosis, surface ACE-2 is down-regulated resulting in angiotensin-2 accumulation (Kai and Kai, 2020). Angiotensin-2 facilitates sodium reabsorption by stimulating sodium-hydrogen exchange in the proximal convoluted tubule of the kidney (Imai et al., 2005). Increased renal sodium re-absorption is accompanied by increased renal chloride reabsorption and increased potassium excretion, potentially resulting in hyperchloremia (as recorded in 13.8% of the present COVID-19 patients) and hypokalemia (as recorded in 16.2% of present COVID-19 patients). Chen et al. (2020) reported a high prevalence of hypokalaemia in individuals with COVID-19. Increased plasma angiotensin-2 concentration has been described in patients with COVID-19, possibly acting as mediator of acute lung injury in SARS-CoV animal models (Liu et al., 2020). Another contributor to hypokalemia and other electrolyte imbalance in some COVID-19 patients might be gastrointestinal losses, with diarrhea and nausea (Pan et al., 2020). Hypomagnesaemia which is common during COVID-19 is known to affect the performance of the sodium–potassium ATPase pump, the intracellular potassium concentration decreases following hypomagnesaemia (Imai *et al.,* 2005). As a result of hypomagnesemia, the renal outer medullary potassium channel causes a decrease in potassium by increasing the distal secretion of potassium and increasing distal sodium delivery (Huang et al., 2020).

A syndrome of inappropriate secretion of antidiuretic hormone and manifestations of hyponatremia has been reported in COVID-19 patients (Sever et al., 2020, Zhang et al., 2020, Habib et al., 2020). Hypernatremia was observed in 2.5% of COVID-19 patients in the present study. Hypernatremia is usually caused by either decreased total body water or by an inappropriately high sodium input. Hypernatremia in the present COVID-19 patients might be due to dehydration. Creatinine is produced by muscle metabolism and intake of meat diet. Kidneys filter creatinine from blood, thus improper functioning of kidneys may lead an increased level of blood creatinine (Wang et al., 2020). The study by Li et al (2020) in COVID-19 showed that 63% of the patients that exhibited proteinuria had elevated level of plasma creatinine and blood urea nitrogen. In the present study, 33.1% COVID-19 patients with creatinine levels below reference range might be due to reduced protein intake.

Hypoalbuminemia in severe COVID-19 has been reported (Zhang et al., 2020, Xie et al., 2020, Feng et al., 2020 and Guan et al., 2020) and lower albumin levels on admission was shown to predict the outcome of COVID-19. This result is consistent with a previous study associating hypoalbuminemia with severity of Acute Respiratory Disease Syndrome (Hoeboer et al., 2020) or acute kidney injury (Wang et al., 20202). A meta-analysis showed that about 80.4% of patients with abnormal liver function in COVID-19 had hypoalbuminemia, which was associated with prognosis and outcome (Wu et al 2020). In the present study, 30.7% COVID-19 patients had hypoalbuminemia. Albumin is synthesized in the liver with a serum half-life of approximately 21 days (Wu et al., 2020). Hypoalbuminemia was seen predominantly in severe COVID-19 cases (Zhang et al., 2020). compared with mild cases (Arinola et al., 2020b). Hypoalbuminemia observed in 30.7% COVID-19 patients considered for this study might not be as a result of liver dysfunction because the onset of symptoms in COVID-19 is generally 4–5 days after infection, although it can be as late as 14 days (Huang et ala., 2020; Zhang et al., 2020) which is far shorter than the half-life of serum albumin.

Systemic inflammation is common in COVID-19 (Qin et al., 2020., Arinola et al., 2020a., Arinola et al., 2021) and inflammation has been shown to cause the movement of serum albumin into interstitial space due to increased capillary permeability and increased volume distribution of albumin (Soeters et al., 2019). Thus, our study strongly implies that hypoalbuminemia might due to the systemic inflammation in some of the COVID-19 patients. Therapeutic efficacy of albumin in sepsis and cirrhosis demonstrated that it had a modulatory effect on inflammation and oxidative stress in addition to the plasma volume expansion (Bo et al., 2016, He et al., 2019). Also, a meta-analysis reported that albumin treatment improved oxygenation in acute respiratory diseases syndrome (Uhlig et al., 2014). Since there is no specific treatment for the systemic inflammation in COVID-19, an albumin treatment with low side-effect might be a potential approach. However, the efficacy and safety of albumin in COVID-19 requires further studies.

A close association have been established between immunosuppression and abnormal renal functions (Kurt et al., 2013, Tecklenbourg et al., 2018 and Huang et al., 2020), thus taking results of the present study into account, the

authors hypothesized the involvement of abnormal renal functions in the immunopathology of severe SARS-COV-2.

In conclusion, our study reports occurrence of abnormal certain renal function parameters in newly diagnosed COVID-19 patients and this may cause deranged immunity in poorly managed COVID-19 patients. Physicians should closely monitor the renal functions (especially chloride and creatinine) of COVID-19 patients during admission, so that appropriate renal replacement therapies can be administered without delay. Determination of renal function parameters in larger number of newly diagnosed COVID-19 patients and COVID-19 patients at discharge is desirable.

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REFERENCES

Arinola OG (2020a). Immune Responses During Human Coronavirus Infection: *Suggestions For Future Studies:* Nigerian Journal of Physiological Sciences. 35: 20-25.

Arinola OG, Fabian Victory Edem, Temitope Oluwagbenga Alonge. (2021). Respiratory Burst Functions in COVID-19 Nigerian Patients. Journal of Basic and Applied Research in Biomedicine. 7(1): 24-28.

Arinola OG, VF. Edem and Alonge T.O. (2020b). Levels of Plasma C-reactive protein, Albumin and Pre-Albumin in COVID-19 Nigerian Patients. Annals of Medical Research. In Press.

Atlas of tissue renin-angiotensin-aldosterone system in human: A transcriptomic meta-analysis. Sci. Rep. 5, 10.1038/srep10035

Betjes MG (2013). Immune cell dysfunction and inflammation in end-stage renal disease. Nat Rev Nephrol. 9: 255– 265.

Bohl DD, Shen MR, Kayupov E, Cvetanovich GL, Della Valle CJ. Is hypoalbuminemia associated with septic failure and acute infection after revision total joint arthroplasty? A study of 4517 patients from the National Surgical Quality Improvement Program. J Arthroplasty. 2016;31(5):963-967.

Bugdayci G, Oguzman H, Arattan HY, Sasmaz G (2015). The use of reference change values in clinical laboratories. Clin Lab. 2015;61(3-4):251-7.

Center for Systems Science and Engineering (2020). Coronavirus COVID-19 global cases. https://www.gisanddata.maps.arcgis.com/apps/opsdashboard/index.html#/bda7594740fd40299423467b48e9ecf6.

Cha RH, Yang SH, Moon KC et al (2016). A case report of a middle east respiratory syndrome survivor with kidney biopsy results. J Korean Med Sci 31(4):635–640.

Chan JF, Yuan S, Kok KH et al (2020). A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. Lancet 395(10223):514–523.

Cheng Y, Luo R, Wang K, et al (2020). Kidney disease is associated with in-hospital death of patients with COVID-19. Kidney Int. 97: 829-838.

Chernyak, B.V., Popova, E.N., Prikhodko, A.S. et al (2020). COVID-19 and Oxidative Stress. Biochemistry Moscow85: 1543–1553.

Chu KH, Tsang WK, Tang CS et al (2005) Acute renal impairment in coronavirus-associated severe acute respiratory syndrome. Kidney Int 67(2):698–705.

Cizmecioglu A, Cizmecioglu H, Goktepe M, Emsen A, Korkmaz C,Tasbent F, Colkesen F, Artac H (2020). Apoptosis-Induced T Cell Lymphopenia Is Related to COVID-19 Severity . J Med Virol.

Jun Jie Ng,a, Yang Luo, Kaiyi Phua, and Andrew M.T.L. Choonga (2020): Acute kidney injury in hospitalized patients with coronavirus disease 2019 (COVID-19): A meta-analysis. J Infect. 2020;81:647-679.

Feng G, Zheng KI, Yan QQ, et al. (2020). COVID-19 and liver dysfunction: current insights and emergent therapeutic strategies. J Clin Transl Hepatol. 8(1):1-7

Flaxman S, Mishra S, Gandy A et al (2020). Report 13: estimating the number of infections and the impact of nonpharmaceutical interventions on COVID-19 in 11 European countries. Imperial College, London. https://doi.org/10.25561/77731

Guan WJ, Zhong NS. (2020). Clinical characteristics of COVID-19 in China. N Engl J Med. 382.

Habib M.B., Sardar S., Sajid J. (2020). Acute symptomatic hyponatremia in the setting of SIADH as an isolated presentation of COVID-19. cases. ID Cases. 21 doi: 10.1016/j.idcr.2020.e00859.

Hato T, Dagher PC (2015). How the innate immune system senses trouble and causes trouble. Clin J Am SocNephrol. 10: 1459–1469.

He Y, Xiao J, Shi Z, He J, Li T. (2019). Supplementation of enteral nutritional powder decreases surgical site infection, prosthetic joint infection, and readmission after hip arthroplasty in geriatric femoral neck fracture with hypoalbuminemia. J Orthop Surg. 14(1):292.

Hirsch JS, Ng JH, Ross DW, et al. (2020). Acute kidney injury in patients hospitalizedwith COVID-19. Kidney International. 98: 209–218

Hoeboer SH, Oudemans-van Straaten HM, Groeneveld AB. (2015). Albumin rather than C-reactive protein may be valuable in predicting and monitoring the severity and course of acute respiratory distress syndrome in critically ill patients with or at risk for the syndrome after new onset fever. BMC Pulm Med. 15:22.

Huang C. -L., Kuo E. (2007). Mechanism of hypokalemia in magnesium deficiency. J Am Soc Nephrol. 2007;18:2649–2652

Huang, C., Wang, Y., Li, X., et al. (2020) Clinical Features of Patients Infected with 2019 Novel Coronavirus in Wuhan, China. Lancet. 20: 30183-30185.

Imai Y., Kuba K., Rao S., Huan Y., Guo F., Guan B. et al (2005). Angiotensin- converting enzyme 2 protects from severe acute lung failure. Nature 436, 112–116 10.1038/nature03712

Jin Y, Yang H, Ji W et al (2020). Virology, epidemiology, pathogenesis, and control of COVID-19. Viruses 12(4):372.

Kai H, Kai M. Interactions of coronaviruses with ACE2, angiotensin II, and RAS inhibitors—lessons from available evidence and insights into COVID-19. Hypertens Res. 2020; 43:648–654

Kurts C, Panzer U, Anders HJ, Rees AJ. (2013). The immune system and kidney disease: basic concepts and clinical implications. Nat Rev Immunol. 13: 738– 753.

Li Z, Wu M et al (2020) Caution on kidney dysfunctions of COVID-19 patients. medRxiv. https://doi.org/10.1101/2020.02.08.20021212.

Liu Y, Yang Y, Zhang C, Huang F, Wang F, Yuan J, et al. (2020). Clinical and biochemical indexes from 2019-nCoV infected patients linked to viral loads and lung injury. Sci China Life Sci. 63(3):364–74.

Mustafa MI, Abdelrahman H. Abdelmoneim, Eiman M. Mahmoud, Abdelrafie M. Makhawi (2020): Cytokine Storm in COVID-19 Patients, Its Impact on Organs and Potential Treatment by QTY Code-Designed Detergent-Free Chemokine Receptors Mediators of Inflammation, 2020 (8198963). https://doi.org/10.1155/2020/8198963

Ng JJ, Luo Y, Phua K, et al (2020). Acute kidney injury in hospitalized patients with novel coronavirus in Wuhan, China. Lancet 395(10223):497–506.

Northwell Nephrology COVID-19 Research Consortium (2020). Acute kidney injury in patients hospitalized with COVID-19. Kidney Int. 98: 209-218.

Pan L, Mu M, Yang P, Sun Y, Yan J, Li P, et al. (2020). Clinical characteristics of COVID-19 patients with digestive symptoms in Hubei, China: a descriptive, cross-sectional, multicenter study. American Journal of Gastroenterology. 115: 766–773

Pan X-W, Da Xu, Zhang H, Zhou W, Wang L-H, Cui X-G (2020). Identification of a potential mechanism of acute kidney injury during the COVID-19 outbreak: a study based on single-cell transcriptome analysis. Intensive Care Med. 46(6):1114-1116.

Qin C, Zhou L, Hu Z, et al. (2020). Dysregulation of immune response in patients with COVID-19 in Wuhan, China. Clin Infect Dis. 10.1093/cid/ciaa248

Rodrigues Prestes T., Rocha N., Miranda A., Teixeira A. and Simoes-e-Silva A. (2017). The Anti-Inflammatory Potential of ACE2/Angiotensin-(1-7)/Mas Receptor Axis: Evidence from Basic and Clinical Research. Curr. Drug Targets 18, 10.2174/1389450117666160727142401

Saad MA, Alfishawy M, Nassar M, Mohamed M, Esene IN, Elbendary A (2020). Covid-19 and Autoimmune Diseases: A Systematic Review of Reported Cases. CurrRheumatol Rev..doi: 10.2174/1573397116666201029155856. Epub ahead of print. PMID: 33121413

Sever M.Y., Uzun N., Ceritli S., Mutlu H., Bayramoğlu A. (2020). Hyponatremia in COVID-19 patient using angiotensin type 1 receptor (AT1R) blocker and diuretic: a case report. J Res Clin Med. 8:22.

Soeters PB, Wolfe RR, Shenkin A. (20219). ypoalbuminemia: Pathogenesis and Clinical Significance. JPEN J Parenter Enteral Nutr. 43(2):181-193.

Soos TJ, Sims TN, Barisoni L et al (2020). CX3CR1+ interstitial dendritic cells form a contiguous network throughout the entire kidney. Kidney Int. 70: 591– 596.

Su H, Yang M, Wan C et al (2020). Renal histopathological analysis of 26 postmortem findings of patients with COVID-19 in China. Kidney Int. https://doi.org/10.1016/j.kint.2020.04.003.

Tecklenborg J , Clayton D, Siebert S, Coley S.M. (2018). The role of the immune system in kidney disease. Clinical and Experimental Immunology. 192(2):142-150.

Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. (2012). Into the eye of the cytokine storm. Microbiol Mol. Biol Rev 76(1):16–32.

Uhlig C, Silva PL, Deckert S, Schmitt J, de Abreu MG. (2014). Albumin versus crystalloid solutions in patients with the acute respiratory distress syndrome: a systematic review and meta-analysis. Crit Care. 18(1): R10.

Wang B, Li D, Cheng B, Ying B, Gong Y. (2020). The neutrophil percentage-to-albumin ratio is associated with all-cause mortality in critically ill patients with acute kidney injury. BioMed Res Int. 2020:5687672-5687679.

Wang D, Hu B, Hu C, et al (2020). Clinical characteristics of 138 hospitalized patients with 2019 novel Coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323: 1061- 1069.

World Health Organization. (2020). Laboratory testing for coronavirus disease (COVID-19) in suspected human cases: interim guidance, 19 March 2020. World Health Organization. https://apps.who.int/iris/handle/10665/331501.

Wu Y, Li H, Xu X, Zheng K, Qi X, Guo X. (2020). Clinical features and outcome of treatment for novel coronavirus pneumonia: a meta-analysis. Zhonghua gan zang bing za zhi.28(3):240-246.

Xie H, Zhao J, Lian N, Lin S, Xie Q, Zhuo H. (2020). Clinical characteristics of Non-ICU hospitalized patients with coronavirus disease 2019 and liver injury: a retrospective study. Liver Int. 2020. 10.1111/liv.14449

Yang XH, Sun RH, Chen DC (2020). Diagnosis and treatment of COVID-19: Acute kidney injury cannot be ignored] Zhonghua Yi Xue Za Zhi. 100(16):1205–1208

Yatim KM, Lakkis FG (2015). A brief journey through the immune system. Clin J Am SocNephrol. 10: 1274–12 81.

Zhang W., Lu S., Zhang M., Zheng H., Huang Y., Chen S. (2020). Correlation between hyponatremia and the severity of coronavirus disease 2019] Zhonghua Wei Zhong bing Ji jiu Yi Xue. 32:774–778.

Zhang Y, Zheng L, Liu L, Zhao M, Xiao J, Zhao Q.(2020). Liver impairment in COVID-19 patients: a retrospective analysis of 115 cases from a single center in Wuhan city, China. Liver Int. liv.14455 10.1111/liv.14455

Zhou F, Yu T, Du R et al (2020). Clinical course and risk factors for mortality of adult in patients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet 395(10229):1054–1062. https://doi.org/10.1016/S0140-6736(20)30566-3.

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Full Length Research Article

Knowledge, Attitude, Practice and Stigma Related to COVID-19: A 2020 Survey in North-Central Nigeria

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Summary: Pandemics have claimed an estimated 414 million lives from 165 AD to present, with COVID-19 pandemic killing close to 2 million people. The best counter for pandemics has been the use of vaccines, but before it is widely available, the best strategy is to avoid being infected. COVID-19 pandemic was met by behaviours and attitudes ranging from unbelief to fear of dying and stigmatisation of those who have contracted the virus or recovered from the disease. This study aims to investigate the knowledge, attitudes, practices (KAP), fear and stigma of the populace towards COVID-19 from state to state of north central Nigeria. This research was a cross-sectional study carried out from April to October 2020. Using stratified sampling, the population was divided into different strata based on sex, ethnicity, level of education, occupation and marital status. Data was collected through a structured questionnaire distributed to 650 individuals. Respondents who participated were 591 (males n= 335 and females n= 256), aged 18-60 years (mean age 30.25 ± 10.45 years, range 18-60). Data were analysed using SPSS 25.0 for Windows version 26.0 (IBM Corporation, Armonk, NY, USA). Significant level was pegged at $P<0.05$, and all statistical tests were two-tailed. The results show that 98.3% of participants believe that COVID-19 disease exists. On the practice, 74.7% wear face masks, 81.5% avoid crowded places, 73.7% practice social distancing and 85.1 follow the WHO-hand-washing technique as measures to curb the spread of the disease. About 60.5% of the participants believe that lockdown is an effective measure to reduce transmission risk. 55.6% will stigmatise those who just recovered from the disease, 75.3% are afraid to visit high-risk areas as part of the protective measures, 12% believe that every infected person will die. More males (28.3%) than females (17.6%) believe that taking herbs can cure the disease ($\chi^2 = 9.32$, df= 1, $P<0.01$). All government and nongovernmental organizations must develop more awareness programs to win the battle against COVID-19 disease as the second wave is emerging.

Keywords*: COVID-19, Knowledge, Attitude, Practice, Stigma, North-Central Nigeria*

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INTRODUCTION

Many pandemics have punctuated humanity's history from the Antoine Plague to the present coronavirus pandemic (COVID-19). The COVID-19 has been the most debilitating pandemic due to its rapid spread across the world due to higher global mobility of human populations due to economic activities and lifestyle (Akin and Gozel, 2020; Morens *et al*., 2020).

COVID-19 disease is an infectious disease caused by a novel virus (Severe acute respiratory syndrome coronavirus 2, SARS-CoV-2) first reported in December 2019 in Wuhan China. On 7th of January 2020, the CDC identified the novel Coronavirus and was named 2019-nCOV by WHO. It is now commonly known as COVID-19 (WHO 2020a; Santarpia *et al.*, 2020; Kannan *et al*., 2020; Sun *et al*., 2020). In Africa, WHO reported the first case in Egypt on the 14th of February, 2020. The second on Africa's continent was in Algeria on the 25th of February, 2020 (Afro News 2020; Moses *et al.*, 2020). In Nigeria, the first case was reported on 27th February 2020, in Lagos State by Nigerian Center for Disease Control (NCDC), making it the first case in Sub-Saharan Africa. COVID-19 reached Africa through travellers returning from hotspots in Asia, Europe and the United States of America. Nigeria was not an exception as the first case was brought into Nigeria by an Italian on a business trip to Lagos (NCDC, 2020a).

On the 10th of March 2020, the NCDC announced first local case, and from then onwards cases began to rise, and the Nigerian government shut down schools on 23rd of March 2020. The government finally declared a total lockdown, and further restricted interstate travel (Ajide *et al*., 2020; Ogoina, 2020)

Respiratory droplets, direct contact with persons infected, and indirect contact with contaminated surfaces and objects in the immediate environment have been

described as the mode of transmission of this disease (WHO, 2020a; Arinola 2020). Some studies have been able to show the survival rate of SARS-COV-2 on different surfaces and in aerosol. The rooms of COVID-19 patients have shown varying levels of environmental contamination (Van Doremalen *et al.*, 2020; Chen *et al.*, 2020; Santarpia *et al.*, 2020). There are variations in the manifestation of the symptoms of SARS-CoV-2; some infected persons do not show symptoms for up to 48 hours, this accounts for the widespread of the disease (Al-Tawfiq *et al.*, 2020; Rothe *et al.*, 2020). It has been reported that about 12.6% of reported cases were from presymptomatic transmission (Du *et al.*, 2020).

COVID-19 was met by all kinds of behaviours and attitudes ranging from unbelief to fear of dying due to lack of vaccines for the disease, leading to many deaths even in the most advanced climes. This development has triggered many investigators to evaluate and report peoples KAP towards COVID-19 (Alobuia *et al*., 2020; Azlan *et al*., 2020; Hossain *et al*., 2020; Adesegun *et al*., 2020; Abdel *et al*., 2020; Olaimat *et al*., 2020; Al-Dossary *et al*., 2020; Bates *et al*., 2020; Jones *et al*., 2020).

There have been tremendous efforts by biopharma like Pfizer/BioNTech, Mordena, SinoFam, Oxford University/AstraZeneca, Novavax, CanSino Biologics, Merk, Wuhan Institute of Biological Products, Sinovac, Symvivo Corporation etc. to develop COVID-19 vaccines. They have reached different levels of clinical trials with up to 96% success (Koirala *et al*., 2020; Haque and Pant, 2020; Jeyanathan *et al*., 2020; Wang, Hu, Hu, *et al*., 2020; Peiris and Leung, 2020, Tripathi *et al.*, 2020, WHO, 2020b). The COVID-19 vaccine is the most rapidly developed in humanity's history, and this is not unconnected with the advanced technologies powering the biotech companies (Koirala *et al*., 2020; EBioMedicine, 2020; Wang, Peng, Xu, *et al*., 2020).

Presently, the statistics reported for Nigeria by the Nigerian Center for Disease Control (NCDC) as at 09/12/2020 stated confirmed cases (84,815), active cases (12,194), discharged (71,375) with fatalities (1,264), which is approximately 1.70% mortalities (www.covid19.ncdc.gov.ng, accessed 28/12/2020). Global statistics for COVID-19 infections are reported to be 81,146,264 confirmed cases, 1,771,161 deaths, with the United States having the highest infections as 19,248,978 334,361 deaths (www.conavirus.jhu.edu, accessed 28/12/2020).

This study sets out to investigate the knowledge, attitude and practices (KAP) towards COVD-19 in different states of Nigeria as it impacts the lives of the people from state to state. Also, we investigated the fear and stigma of the populace towards the disease.

MATERIALS AND METHODS

This study was cross-sectional conducted between April and October of 2020 in four states (Benue, Kaduna, Nasarawa, Plateau) and the Federal Capital Territory, Abuja. Due to COVID-19 retrictions in movement at the time of data collections, data was collected by using the convienience sampling method, through a structured questionnaire distributed to 650 individuals. Respondents who participated were 591 (males n= 335 and females n= 256) and aged 18-60 years (mean age 30.25 ± 10.45 years, range 18-60) drawn from five states in the north-central region of Nigeria. Only participants who gave their informed consent were included in the study, adults ≥ 18 years of age ≤ 60 years of age were included in the study. Participants from different ethnic backgrounds but who are residents in the study states were all included in the study. Interpreter was used for participants that cannot read English. The study was approved by the Bingham University Research and Ethics Committee (BHU/REC/20/H001).

Questionnaire: The study used a structured questionnaire to collect data from the participants. The questionnaire consisted of two sections. A general section concerned with self-reported biodata of participants and their families. The other section tested for the knowledge, attitude and practice (KAP). This section contained 27 questions, which 14 assessed knowledge, six assessed attitudes, stigma and fear, while the remaining seven assessed practices.

Statistical Analysis: Descriptive statistics and percentages were used to calculate for sociodemographic variables. A regression and correlation model was used to assess the relationship between variables. Chi-square test was used to test for the association between the variables. Multiple binary regression was used to investigate the association between KAP with sociodemographic variables by using the odd ratio (OR) and corresponding 95% confidence interval. Data were analysed using SPSS 25.0 for Windows version 26.0 (IBM Corporation, Armonk, NY, USA). Significant level was pegged at $P<0.05$, and all statistical tests were two-tailed.

RESULTS

Demographic characteristics of participants: As shown in Table 1, 335 (56.7%) of the participants are male, and 256 (43.3%) are female, out of which 14.6% are Hausa's, 12.7% are Igbos, 12.5% are Yoruba's, while the other minority tribe make up the remaining 58.9%.

Table 1:
Demographic characteristics of participants

Characteristics		Frequency	Percentage (%)
Sex	Males	335	56.7
	Females	256	43.3
Ethnic group	Hausa	98	16.4
	Igbo	76	12.7
	Yoruba	75	12.5
	Others	349	58.4
Educational attainment	No formal education	8	1.4
	Primary	12	2
	Secondary	115	19.6
	Tertiary	452	77
Occupation	Professionals	70	13.8
	Civil servants	88	17.3
	Trading	46	9.1
	Artisans	57	11.2
	Farmers	7	1.4
	Applicants	240	47.2
Marital status	Married	229	38.5
	Single	360	60.5
	Divorced	6	1

Values are expressed in frequencies and percentages except otherwise stated

Table 2:
Assessment of Knowledge and fear of the participants towards COVID-19 according to gender

		Female	Male	Overall
Knowledge	Covid-19 is caused by a Virus.	236 (92.9)	313 (94.3)	549 (93.7)
	Do you believe it exists?	238 (99.2)	295 (97.7)	533 (98.3)
What are the Symptoms of Covid-19?	Fever	157 (61.3)	220 (65.6)	377 (63.7)
	Coughing	179 (69.9)	226 (67.4)	405 (68.5)
	Difficulty breathing	204 (79.6)	239 (71.3)	443 (74.9)
	Sore throat	116 (45.3)	174 (51.9)	290 (49)
What is the mode of transmission?	Eating with an infected person	87 (33.9)	122 (47.6)	209 (35.3)
	Touching an infected person	198 (77.3)	269 (80.2)	467 (79)
	Contact with an animal	39 (15.2)	59 (17.6)	98 (16.5)
How can you protect yourself from Covid-19?	Avoid crowd	172 (67.1)	217 (64.5)	389 (65.8)
	Face mask	168 (65.6)	212 (64.7)	380 (64.2)
	Hand sanitizer	162 (63.2)	203 (61)	365 (61.7)
	Social distancing	193 (75.3)	256 (76.4)	449 (75.9)
Are you afraid to visit high-risk areas as a protective measure?		185 (74.3)	252 (76.1)	437 (75.3)
Is there a cure for Covid-19?		52 (20.4)	113 (33.9)	165 (28.1)
Can children get infected?		223 (87.1)	297 (89.2)	520 (88.3)
Should an infected person be isolated for 14 days		240 (95.2)	302 (91.2)	542 (93)
Is isolation and treatment of infected person an effective way to reduce the spread of the disease?		227 (90.4)	291 (87.7)	518 (88.9)
Can the Coronavirus survive in cold weather?		166 (65.6)	234 (70.7)	400 (68.5)
Can Coronavirus survive in hot weather?		95 (37.8)	123 (36.9)	218 (37.3)
What is your source of information on your knowledge of Coronavirus?	Social media	119 (46.4)	147 (43.8)	266 (45)
	Internet	134 (52.3)	185 (55.2)	319 (53.9)
	Television	150 (58.5)	189 (56.4)	339 (57.3)
	Radio	84 (32.8)	109 (32.8)	193 (32.6)
	Other sources	8 (3.1)	15 (4.4)	23 (3.89)

Table 3:
Knowledge of the participants towards COVID-19 according to the level of education

	Knowledge	None	Primary	Secondary	Tertiary	Total
	Covid-19 is caused by a Virus.	7 (87.5)	11 (100)	99 (86.8)	430 (95.6)	530 (98.3)
	Do you believe it exists?	5 (83.3)	12 (100)	100 (97.1)	413 (98.8)	530 (98.3)
What are the Symptoms of Covid-19?	Fever	2 (25)	5 (41.6)	66 (57.3)	301 (66.5)	374 (63.7)
	Cough	5 (62.5)	6 (50)	70 (60.8)	324 (71.6)	405 (68.9)
	Difficulty breathing	4 (50)	7 (58.3)	73 (63.4)	362 (80)	446 (75.9)
	Sore throat	2 (25)	4 (33)	43 (37.3)	242 (53.5)	291 (49.5)
What is the mode of transmission?	Eating with an infected person	4 (50)	6 (50)	39 (33.9)	158 (34.9)	207 (35.2)
	Touching an infected person	6 (75)	8 (66.6)	84 (73)	368 (81.4)	466 (79.3)
	Contact with an animal	-	-	29 (25.2)	67 (14.8)	96 (16.9)
How can you protect yourself from Covid-19?	Avoid crowd	6 (75)	6 (50)	65 (56.5)	309 (68.3)	386 (65.7)
	Face mask	3 (37.5)	6 (50)	63 (54.7)	309 (68.3)	381 (64.9)
	Hand sanitizer	1 (12.5)	7 (58.3)	55 (47.8)	301 (66.5)	364 (62)
	Social distancing	3 (37.5)	6 (50)	74 (64.3)	366 (80.9)	449 (76.4)
Are you afraid to visit high-risk areas as a protective measure?		6 (75)	10 (90.9)	88 (77.9)	330 (74.2)	434 (75.2)
	Is there a cure for Covid-19?	4 (50)	6 (50)	38 (33)	118 (26.3)	166 (28.4)
	Can children get infected?	7 (87.5)	10 (83.3)	97 (85.1)	400 (88.9)	514 (88)
	Should an infected person be isolated for 14 days	6 (75)	12 (100)	102 (91.1)	418 (93.7)	538 (93.1)
Is isolation and treatment of infected person an effective way to reduce the spread of the disease?		7 (87.5)	11 (91.7)	99 (86.1)	399 (89.7)	516 (89)
Can the Coronavirus survive in cold weather?		7 (87.5)	8 (66.7)	73 (64)	307 (68.7)	395 (68)
Can Coronavirus survive in hot weather?		4 (50)	3 (25)	43 (37.7)	164 (36.8)	214 (36.9)
What is your source of information on your knowledge of Coronavirus?	Social media	4 (50)	2 (16.6)	39 (33.9)	218 (48.2)	263 (44.8)
	Internet	3 (37.5)	2 (16.6)	43 (37.3)	269 (59.5)	317 (54)
	Television	2 (25)	6 (50)	57 (49.5)	275 (46.8)	340 (57.9)
	Radio	2 (25)	2 (16.6)	42 (36.5)	151 (16.3)	197 (33.5)
	Other sources	-	1 (8.3)	6 (5.2)	16 (3.5)	23 (3.9)

1.4% of the population have never had any formal education, 2.0% have primary school, 19.6% have a secondary school certificate, and 77% have tertiary education, other demographic characteristics studied are also presented.

Assessment of Knowledge and fear of the participants towards COVID-19 according to gender: On the knowledge of COVID-19 as caused by a virus, 93.7% of the participants were correct on the cause of COVID-19. 63.7% know that fever was associated, 68.5% agreed that coughing was associated, 74.9% responded that there is difficulty breathing in the last stages, but only 49% associated sore throat with COVID-19 as presented in Table 2. On the fear of contracting the disease, 35.3% express fear in eating with infected persons, 79.0% are afraid to touch infected persons, while 75.3% are afraid to visit high-risk areas. Almost all the respondents (93.0%) believed that infected persons should be isolated for 14 days. (Table 2). In Table 3, most tertiary education participants are confident that COVID-19 is not the same as malaria.

Attitude and stigma of participants towards COVID-19 according to gender and Level of education: More male (28.3%) than female (17.6%) believe that taking herbs and malarial drugs can treat COVID-19, while only 60.5% of participants believe that lockdown is an effective measure to curb the spread of the disease. Participants with tertiary education believe in self-protection against the disease, while only 44.4% of the participants will go close to someone who just recovered from the disease (see Tables 4 and 5).

Practice of the participants against COVID-19 according to gender and level of education: 81.5% of participants avoid crowded places, 74.7% wear face mask, more female (86.3%) than male (84.0%) follow the WHO hand-washing technique guideline. Also, more female (77.3%) than male (69.2%) strictly obeys or follow government lockdown orders, while more male (76.3) than female (70.2%) practice social distancing where ever they go. Table 7 shows that those with some form of tertiary education (82.9%) avoid crowded places and rightly wear their face mask more than those with primary and secondary education (Tables 6 and 7).

Multiple Binary logistic regression on factors associated with practice and attitude towards COVID-19: This table shows the logistics regression, odds ratios and confidence intervals of the variables associated with practice and attitudes towards COVID-19. Three questions (Do you avoid crowded places?, Do you always wear face masks when leaving home? and Do you strictly obey or follow the lockdown orders?), were found to be statistically significantly associated with practices toward COVID-19 with particular reference to males. The attitude expressed by participants were statistically significantly associated with those who have a tertiary level of education (Table 8).

Table 4:
Attitude and stigma of participants towards COVID-19 according to gender

Attitude	Female	Male	Overall
Can Covid-19 be treated?			
Herbs	45 (17.6)	95 (28.3)	140 (23.6)
Taking malarial drugs	23 (8.9)	63 (18.8)	86 (14.6)
Not sure	188 (73.4)	203 (60.5)	391 (66.1)
Can government measures reduce the spread of Covid-19?	189 (75)	254 (76.3)	443 (75.7)
Do you do self-protection against Covid-19	228 (89.8)	296 (89.4)	524 (89.6)
Will you go close to someone who just recovered from COVID-19	95 (37.8)	161 (49.5)	256 (44.4)
Will every infected person die	31 (12.1)	40 (11.9)	71 (12)
Is lockdown an effective measure for the spread of the disease	153 (61.2)	199 (59.9)	352 (60.5)

Table 5:
Attitude and stigma of participants towards COVID-19 according to the level of education

Attitude	None	Primary	Secondary	Tertiary	Total
Can Covid-19 be treated?					
Herbs	2 (22.2)	4 (28.6)	25 (21.2)	105 (22.3)	136 (22.3)
Taking malarial drugs	2 (22.2)	2 (14.3)	17 (14.4)	62 (13.2)	83 (13.6)
Not sure	5 (55.6)	8 (57.1)	76 (64.4)	303 (64.5)	392 (64.2)
Can government measures reduce the spread of Covid-19?	6 (75)	11 (91.7)	77 (68.1)	343 (76.6)	437 (75.2)
Do you do self-protection against Covid-19	6 (75)	11 (91.7)	94 (82.5)	411 (91.9)	522 (89.8)
Will you go close to someone who just recovered from COVID-19	4 (50)	5 (41.7)	43 (38.4)	201 (45.6)	253 (44.2)
Will every infected person die	1 (14.3)	2 (16.7)	21 (18.8)	45 (10.1)	69 (12)
Is lockdown an effective measure for the spread of the disease	4 (50)	9 (75)	71 (62.3)	265 (59.7)	349 (60.4)

Table 6:
Practice of the participants against COVID-19 according to gender

Practice	Female	Male	Overall
Do you avoid crowded places?	220 (85.9)	261 (78.1)	481 (81.5)
Do you always wear face masks when leaving home?	201 (78.5)	239 (71.8)	440 (74.7)
Do you wear your mask in the correct way?	218 (85.5)	278 (83.5)	496 (84.4)
Do you dispose of your mask or wash them thoroughly after use?	217 (87.5)	277 (84.7)	494 (85.9)
Do you follow the WHO-hand-washing technique?	221 (86.3)	281 (84.1)	502 (85.1)
Do you practice social distancing wherever you go?	179 (70.2)	255 (76.3)	434 (73.7)
Do you strictly obey or follow the lockdown orders?	198 (77.3)	231 (69.2)	429 (72.7)

Table 7:
Practice of the participants against COVID-19 according to the level of education

Practice	None	Primary	Secondary	Tertiary	Total
Do you avoid crowded places?	5 (62.5)	9 (75)	89 (77.4)	374 (82.9)	477 (81.4)
Do you always wear face masks when leaving home?	6 (75)	11 (91.7)	86 (74.8)	333 (74)	436 (74.5)
Do you wear your mask in the correct way?	6 (75)	10 (83.3)	90 (78.3)	384 (85.5)	490 (83.9)
Do you dispose of your mask or wash them thoroughly after use?	7 (87.5)	10 (90.9)	96 (86.5)	379 (85.7)	492 (86)
Do you follow the WHO-hand-washing technique?	7 (87.5)	12 (100)	92 (80)	385 (85.4)	496 (84.6)
Do you practice social distancing wherever you go?	6 (75)	12 (100)	86 (74.8)	325 (72.1)	429 (73.2)
Do you strictly obey or follow the lockdown orders?	6 (75)	10 (83.3)	88 (76.5)	318 (70.5)	422 (72)

Table 8:
Multiple Binary logistic regression on factors associated with practice and attitude towards COVID-19

Questions	OR (SE)	95% CI
Practice		
Do you avoid crowded places?		
Sex (male)	0.58 (0.22) *P* = **0.014**	(0.37-0.89)
Level of education (tertiary education)	1.49 (0.24) *P* = 0.093	(0.94-2.37)
Do you always wear face masks when leaving home?		
Sex (male)	0.68 (0.19) *P* = **0.048**	(0.47-1.00)
Level of education (tertiary education)	0.87 (0.23) *P* = 0.541	(0.56-1.36)
Do you wear your mask in the correct way?		
Sex (male)	0.85 (0.23) *P* = 0.477	(0.54-1.33)
Level of education (tertiary education)	1.55 (0.25) *P* = 0.079	(0.95-2.51)
Do you dispose of your mask or wash them thoroughly after use?		
Sex (male)	0.86 (0.23) *P* = 0.499	(0.55-1.34)
Level of education (tertiary education)	1.01 (0.27) *P* = 0.968	(0.60-1.70)
Do you follow the WHO-hand-washing technique?		
Sex (male)	0.82 (0.24) *P* = 0.410	(0.52-1.31)
Level of education (tertiary education)	1.24 (0.26) *P* = 0.406	(0.75-2.07)
Do you practice social distancing wherever you go?		
Sex (male)	1.37 (0.19) *P* = 0.092	(0.95-1.98)
Level of education (tertiary education)	0.76 (0.23) *P* = 0.239	(0.49-1.20)
Do you strictly obey or follow the lockdown orders?		
Sex (male)	0.65 (0.19) *P* = **0.024**	(0.45-0.95)
Level of education (tertiary education)	0.71 (0.23) *P* = 0.131	(0.45-1.11)
Attitude		
Can government measures reduce the spread of Covid-19?		
Sex (male)	1.11 (0.19) *P* = 0.580	(0.76-1.62)
Level of education (tertiary education)	1.37 (0.22) *P* = 0.145	(0.90-2.10)
Do you do self-protection against Covid-19?		
Sex (male)	0.93 (0.26) *P* = 0.789	(0.56-1.56)
Level of education (tertiary education)	2.17 (0.28) *P* <**0.005**	(1.26-3.74)
Will you go close to someone who just recovered from Covid-19?		
Sex (male)	1.57 (0.17) *P* = **0.008**	(1.13-2.19)
Level of education (tertiary education)	1.28 (0.20) *P* = 0.221	(0.86-1.89)
Will every infected person die?		
Sex (male)	0.98 (0.26) *P* = 0.950	(0.60-1.62)
Level of education (tertiary education)	0.51 (0.28) *P* = **0.015**	(0.30-0.88)
Is lockdown an effective measure for the spread of the disease?		
Sex (male)	0.99 (0.17) *P* = 0.929	(0.71-1.37)
Level of education (tertiary education)	0.86 (0.20) *P* = 0.456	(0.58-1.28)

Significant associations are expressed in boldface. For the level of education, tertiary education was used as the reference whereas, male was the reference for sex. 95% CI: 95% confidence interval

DISCUSSION

This study sets out to evaluate KAP towards COVID-19 amongst Nigerians resident in some north-central states, including the Federal Capital Territory. There have been several campaigns and health communication to enlighten people about COVID-19 and the preventive measures to stop person-to-person transmission of the virus. Some of these measures include the use of nose mask/face shield, regular washing of the hand and use of hand sanitisers (WHO 2020c, 2020d), maintenance of physical distance as well as sites where updates on recommendations and advice on COVID-19 is found (Paakkari and Okan, 2020). These measures are well recognised to prevent transmission of the virus (WHO 2020c, 2020d).

A face mask may reduce disease transmission by decreasing droplet spread from infected or asymptomatic persons, which are infected people before the symptoms

(Olaimat *et al*., 2020). Our study shows that most respondents know that face mask is a measure to protect yourself from COVID-19, some indicated that using hand sanitisers can protect you from contracting the virus. Still, more of the participants reported that social distance is an effective measure to reduce the disease's spread. Despite the knowledge of about 57.3% through television, 53.9% through the internet, 45% through social media and 32.6% through the radio about these guidelines and information, there are still mixed feelings among Nigerians. At the same time, some expressed fear of contracting the disease, and others felt the government had been too hard on the measures. On the 31st of August, 2020, the government had to appeal to its citizens that COVID-19 guidelines are not designed to oppress them but help prevent the transmission of the disease and that wearing face masks is less cumbersome than being on ventilators.

On respecting government measures, most of the respondents believe that government measures can reduce the spread of COVID-19. About 60.5% believe that lockdown is an effective measure to curb the spread of the disease. This contrasts with a study among Nepalese residents where almost all participants (96.4%) believe that lockdown is the best way to reduce transmission of COVID-19 (Asraf *et al.,* 2020). On the practice and obedience to government measures to prevent the transmission, some people complained that wearing a face mask is uncomfortable, following the WHO-hand-washing technique is cumbersome. Practising social distancing is only possible in government-owned facilities, schools, hospitals, and banks, but not in public market places, on the streets, and even in the villages where law enforcement agencies' presence is reduced.

If the battle against COVID-19 is to be won, better practice against the disease requires each citizen's sense of responsibility, be it in cities, urban areas, or villages. In most instances, more females than males practice the COVID-19 guidelines, and this is in line with the study of Olaimat *et al.* (2020) who reported that more females than males show significant higher practice scores toward COVID-19 among University students in Jordan. This is also connected to the fact that females are generally more careful in doing things, and they will also have to be there to help the children follow the COVID-19 guidelines.

Those with tertiary education correctly wear their mask more than others, while those in the primary and secondary education level obey the government lockdown orders more. The rapid development of COVID-19 into a pandemic has created awareness for people to gain and apply health information, and change their behaviour rapidly (Paakkari and Okan, 2020). Our study shows that most people know that watching television, listening to the radio or even using social media are good sources of information about the disease. All the participants know that COVID-19 exists, it is caused by a virus, and some of the early symptoms are fever, coughing and difficulty in breathing. Most of the respondents are also knowledgeable that you can contract the disease by touching an infected person. That 14 days isolation period for the infected person is of paramount importance.

On the disease's fear and stigma, most participants are afraid to visit high-risk areas as part of the protective measures. A good number of the participants reported that they would avoid someone who just recovered from the disease. More awareness is required because this means that people who just recovered from the illness are being stigmatised. Because of the stigmatisation, people with COVID-19 disease are afraid to discuss how they contracted the disease and advocate for the government guidelines to help curb the disease's spread.

Medicinal herbs have been used in the treatment and management of human diseases, because it is relatively safer, more affordable, and sometimes offers better therapeutic value than synthetic drugs, even though scientists advocate for proper toxicological studies (Danborno *et al.,* 2019). COVID-19 disease is not an exception. Scientists are also advocating medicinal herbs for its treatment (Luo *et al.,* 2020; Owoyele *et al.,* 2020; Yang *et al.,* 2020). In this study, more males than females believe that taking herbs can cure the disease, even though those with a primary education level are the ones who believe it the most.

In conclusion, pandemics like COVID-19 must be managed with caution to reduce fatalities. To achieve this, the people need to be correctly educated and be directed by the appropriate authorities such as Primary health Centers (PHC), hospitals, schools and even health care workers to help in containing the spread of the disease. This requires a serious campaign to reorientate the populace. This can be achieved through vigorous programmes to educate the citizens through the mainstream and social media, churches and mosques. The second wave of COVID-19 has been reported to be more dangerous and deadly than earlier strain; therefore, there is urgent need to enforce the COVID-19 guidelines and protocols strictly to contain the fatalities that may arise.

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REFERENCES

Abdel Wahed, W.Y., Hefzy, E.M., Ahmed, M.I. & Hamed, N.S. (2020). Assessment of knowledge, attitudes, and perception of health care workers regarding COVID-19, A cross-sectional study from Egypt. *Journal of Community Health* 45:1242–1251. https://doi.org/10.1007/s10900-020-00882-0

Adesegun, O.A., Binuyo, T., Adeyemi, O., Ehioghae, O., Rabor, D.F., Amusan, O., Akinboboye, O., Duke, O.F., Olafimihan, A.G., Ajose, O., Idowu, A.O. & Abiodun, O (2020). The COVID-19 crisis in sub-Saharan Africa: Knowledge, attitudes, and practices of the Nigerian Public. *Am. J. Trop. Med. Hyg*., 103(5), 1997–2004. doi:10.4269/ajtmh.20-0461

Afro News (2020). Afro.who.int/news/second-covid-19-cases-confirmed-africa.

Ajide, K.B., Ibrahima, R.L. & Alimi, O.Y. (2020). Estimating the impacts of lockdown on Covid-19 cases in Nigeria. *Transportation Research Interdisciplinary Perspectives* 7 http://dx.doi.org/10.1016/j.trip.2020.100217

Akin, L. & Gözel, M.G. (2020). Understanding dynamics of pandemics. *Turkish Journal of Medical Sciences,* 50: 515-519. doi:10.3906/sag-2004-133.

Al-Dossary, R., Alamri, M., Albaqawi, H., Al Hosis, K., Aljeldah, M., Aljohan, M., Aljohani, K., Almadani, N., Alrasheadi, B., Falatah, R. & Almazan, J. (2020). Awareness, attitudes, prevention, and perceptions of COVID-19 outbreak among nurses in Saudi Arabia. *International Journal of Environmental Research and Public Health*, 17, 8269; doi:10.3390/ijerph17218269

Alobuia, W.M., Dalva-Baird, N.P., Forrester, J.D., Bendavid, E. & Bhattacharya, J. Electron Kebebew. Racial disparities in knowledge, attitudes and practices related to COVID-19 in the USA. *J Public Health (Oxf)*. 2020 Aug 18;42(3):470-478. doi:10.1093/pubmed/fdaa069.

Al-Tawfiq, J.A. (2020). Asymptomatic coronavirus infection: MERS-CoV and SARS-CoV-2 (COVID-19). *Travel Medicine and Infectious Disease,* 35:101608 https://doi.org/10.1016/j.tmaid.2020.101608

Arinola, O.G. (2020). Immune responses during human Coronavirus infection: Suggestions for future studies. *Niger. J. Physiol. Sci.* 35: 20-25.

Asraf, H., Garima, T., Singh, B.M., Ram, R. & Tript, R.R. (2020). Knowledge, attitudes and Practices towards COVID-19 among Nepalese Residents: A quick online cross-sectional survey. *Asian Journal of Medical Sciences*; 11(3): 6-11.

Azlan, A.A., Hamzah, M.R., Sern, T.J., Ayub, S.H. & Mohamad, E. (2020). Public knowledge, attitudes and practices towards COVID-19: A cross-sectional study in Malaysia. *PLoS ONE* 15(5): e0233668. https://doi.org/10.1371/journal.pone.0233668

Bates, B.R., Moncayo, A.L., Costales, J.A., Herrera-Cespedes, C.A. & Grijalva, M.J. (2020). Knowledge, attitudes, and practices towards covid-19 among Ecuadorians during the outbreak: An online cross-sectional survey. *Journal of Community Health*, 45:1158–1167. https://doi.org/10.1007/s10900-020-00916-7

Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F. & Yang, H. (2020). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet*; 395: 507-513.

Du, Z., Xu, X., Wu, Y., Wang, L., Cowling, B.J. & Meyers, L.A. (2020). Serial interval of COVID-19 among publicly reported confirmed cases. *Emerging Infectious Diseases;* 26(6): 1341-1343.

Danborno, A.M., Tarfa, F.D., Toryila, J.E., Awheela, E.U. & Shekarau, V.T (2019). The Effects of *Jatropha tanjorensis* Aqueous Extract on Haematological Parameters in Wistar Rats. *Journal of African Association of Physiological Sciences,* 7(2): 133-137.

EBioMedicine. (2020). Race for a COVID-19 vaccine. *EBioMedicine*, 55:102817. https://doi.org/10.1016/j.ebiom.2020.10281710

Haque, A. & Pant, A. B. (2020). Efforts at COVID-19 vaccine development: Challenges and successes. *Vaccines*, 8, 739; doi:10.3390/vaccines8040739

Hossain, M.A., Jahid, M.I.K, Hossain, K.M.A., Walton, L.M., Uddin, Z., Haque, M.O, *et al.* (2020). Knowledge, attitudes, and fear of COVID-19 during the Rapid Rise Period in Bangladesh. *PLoS ONE*, 15(9): e0239646. https://doi.org/10.1371/journal.pone.0239646

Jeyanathan, M., Afkhami, S., Smaill, F. et al. (2020). Immunological considerations for COVID-19 vaccine strategies. *Nature Review Immunology*, 20, 615–632. https://doi.org/10.1038/s41577-020-00434-6

Jones, J., Sullivan, P.S., Sanchez, T.H., Guest, J.L., Hall, E.W., Luisi, N., Zlotorzynska, M., Wilde G., Bradley, H., Siegler, A.J. (2020). Similarities and differences in COVID-19 awareness, concern, and symptoms by race and ethnicity in the United States: Cross-sectional survey. *Journal of Medical Internet Research*, 22(7):e20001). doi: 10.2196/20001

Kannan, S., Ali, P.S.S., Sheeza, A., Hemalath, K (2020). COVID-19 (Novel Coronavirus 2019)-recent trends. *European Review for Medical and Pharmacological Sciences*, 24: 2006-2011.

Koirala, A., Joo Y. J. & A. Khatami, A. et al., (2020). Vaccines for COVID-19: The current state of play. *Paediatric Respiratory Reviews*, https://doi.org/10.1016/j.prrv.2020.06.010

Luo, H., Tang, Q. L., Shang, Y., Liang, S.B., Yang, M., Robinson, N., & Liu, J.P. (2020). Can Chinese medicine be used for prevention of coronavirus disease 2019 (COVID). A review of historical classics, research evidence and current prevention programs. *Chinese Journal of Integrative Medicine,* 26(4): 243-250.

Morens, D.M., Daszak, P., Markel, H., Taubenberger, J.K. (2020). Pandemic COVID-19 joins history's pandemic legion. *mBio* 11: e00812-20. https://doi.org/10.1128/mBio.00812-20.

Moses, M.O., Emikpe, A.O., Moses, M.K., & Emikpe, B. O. (2020). Combating COVID-19 lockdown inactivity in the African population: Use of cultural practices and One Health Approach. *Niger. J. Physiol. Sci.* 35: 4-9.

Nigerian Center for Disease Control (NCDC) (2020a). Ncdc.gov.ng/news/227/first-case-of-corona-virus-disease-comfirmed-in-nigeria.

Nigeria Centre for Disease Control (NCDC), (2020b). Confirmed COVID-19 cases by state. Available at. https://covid19.ncdc.gov.ng/.

Ogoina, D. (2020). COVID-19 and the rest of us. *Niger Delta Medical Journal*, 4:6–8.

Olaimat, A.N., Aolymat, I., Elsahoryi, N., Shahbaz, H.M. & Holley, R.A. (2020). Attitudes, anxiety, and behavioral practices regarding COVID-19 among University Students in Jordan: A cross-sectional study. *American Journal of Tropical Medicine and Hygiene*, 103(3): 1177–1183. doi:10.4269/ajtmh.20-0418

Owoyele, B.V., Bakare, A.O. & Ologe, M.O. (2020). Bromelain: A Review of its Potential as a Therapy for the Management of COVID-19. *Niger. J. Physiol. Sci.* 35: 10-19.

Paakkari, L. & Okan, O. (2020). COVID-19: health literacy is an underestimated problem. *The Lancet Public Health*, 5(5): E249-E250.

Peiris, M. & Leung, G. M. (2020) What can we expect from first-generation COVID-19 vaccines? *The Lancet*, 396: 7. https://doi.org/10.1016/S0140-6736(20)31976-0

Rothe, C., Schunk, M., Sothmann, P., Bretzel, G., Froeschl, G., Wallrauch, C., *et al*., (2020). Transmission of 2019-nCoV infection from an asymptomatic contact in

Germany. *New England Journal of Medicine.* https://doi.org/10.1056/NEJMc2001468.

Santarpia, J.L., Rivera, D.N., Herrera, V.L., Morwitzer, M.J., Creager, H.M., Santarpia, G.W. *et al*., (2020). Aerosol and surface contamination of SARS-CoV-2 observed in quarantine and isolation care. *Scientific Reports*. 10,12732. Doi/10.1038/69286-3.

Sun, P., Lu, X., Xu, C., Sun, W. & Pan, B. (2020). Understanding of COVID-19 based on current evidence. *Journal of Medical Virology,* 92:548–551. https://doi.org/10.1002/jmv.25722

Tripathi, D., Yi, G. & Vankayalapati. R. (2020). COVID-19: An update on vaccine development. *Indian Journal of Rheumatology,* 15:70-2.

Van Doremalen, N., Bushmaker, T. & Morrris, H.D. (2020). Aerosol and surface stability of SARS-CoV-1. *New England Journal of Medicine*, 382: 1564-1567.

Wang, D., Hu, B., Hu, C., Zhu, F., Liu, X., Zhang, J., Wang, B. *et al*., (2020). Clinical characteristics of 138 hospitalised patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA* 323(11): 1061-1069.

Wang, J., Peng, Y., Xu, H., Cui, Z., & Williams III, R.O. (2020). The COVID-19 Vaccine Race: Challenges and Opportunities in Vaccine Formulation. *AAPS Pharm SciTech*, 21: 225. DOI: 10.1208/s12249-020-01744-7.

World Health Organization (WHO). (2020a). 2019-nCoV outbreak is an emergency of international concern. http://www.euro.who.int/en/health-topics/emergencies/pages/news/news/ 2020/01/2019-ncov-outbreak-is-an-emergency-ofinternational-concern.

World Health Organization (WHO). (2020b). Draft landscape of COVID-19 candidate vaccines. Sept 9, 2020. https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines (accessed December 11, 2020).

World Health Organization (2020c). Questions and answers on coronaviruses (COVID-19). https://www.who.int/news-room/q-a-detail/q-a-coronaviruses

World Health Organization (2020d). Coronavirus disease 2019 (COVID-19) Situation Report – 51. https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200311-sitrep-51-covid-19.pdf

Yang, Y., Islam, M.S., Wang, J., Li, Y., & Chen, X. (2020). Traditional Chinese medicine in the treatment of patients infected with 2019-new Coronavirus (SARS-COV-2): A review and perspective. *International Journal of Biological Sciences*, 16(10): 1708-1717.

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Full Length Research Article

Mesowear Pattern of the Fourth Upper Premolar in Tropical Raccoons (*Procyon cancrivorus)* From Three Nigerian Ecologic Zones: Intra-specific Dietary Resource Partitioning

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Summary: Tooth-wear signatures obtained from maxillary carnassial fourth premolar teeth of raccoons in three ecologic regions in Nigeria testified to segregations in diet of the species with more abrasive diet in specimens from coastal south-western areas compared to more vegetal diet content of those from middle belt and northern areas. Endoloph assessments showed sexually dimorphic mesowear signals between and within locations suggestive that males are more exposed to dental wears compared to females; Male and female specimens from rainforest zone had 40.2% and 34.2% respectively, Sudan Savanna zone had 46.8% and 40.6% for females and males while 67.6% and 44.3% for Sahel zone specimens in similar order. We investigated dietary resource use for sustained survivability within limits of interspecific spatial overlaps using seasonal rainfall indices between two years. There was 86% per high dental occlusal surface relief in the specimens from the savannas while 32% per low relief was observed in South-Western badgers teeth samples. This study observed a change in habitat use as a predisposing factor to sub-regional dental wear differences among age groups as well as sexes of species from three geographic climatic areas. The richness of the eco-habitat/life expectancy found in the rain forest can be ascribed to diet availability which is reduced in the savanna areas. The study suggests minimal change in habitat use as a predisposing factor in sub-regional species dental relief differences observed among age groups and sexes of the species from three geographic climatic areas and also represents quality of the eco-habitats.

Keywords*: Procyon cancrivorus, premolar, dental wears, mesowear*

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INTRODUCTION

The knowledge of range distribution, diet and spatial interaction of tropical raccoons, *Procyon cancrivorus* in Nigeria with respect to geographic and climatic variations has not been documented beyond primary records in the South-Western regions compared to the *Procyon oideslotor* or the common raccoons (Happolds and Happolds, 1987; Mackwell, Simon-Miller *et al*, 2013). A fauna qualitative and quantitative accessibility difference exists within the three ecological zones and impact on diet composition (Drygala and Zoller, 2013). Dietary components in raccoons consists of 40% invertebrates, 33% plant materials and 27% vertebrates (Zeveloff, 2002), this proportion alters with season and availability (Kitchen *et al*, 1999; Feldhamer *et al*, 2003) and are dependent on ocean tides, temperature deviations, precipitations and intercontinental discontinuity shifts especially in competitive or tolerant interactions and colonization of spatial territories (Pergams and Lawler, 2009) occasioned by climate dynamics (National Meteorological Agency, 2010). Lower coastal temperatures, longer period of rain and thick under vegetation in coastal and mangrove areas; characterizes the South-Western rain forest and favorable for richer fauna while the Semi-Savanna and arid Savanna zones are synonymous with a higher and sustained temperature deviations coupled with thin sparse vegetation supporting fewer prey species (NIMET, 2010; Rivals, Solounias and Mihlbachler, 2007). The three ecological zones evaluated for the diet partitioning in Nigeria are separately characterized by varied precipitation and consequent fauna and flora types (Happolds and Happolds, 1987). Rainfall in the rainforest zone averages 2500mm but 250-750mm in the Sahel zone (NMET, 2010). Vegetation varies with soil structure, available water and photoperiodicity (Happolds and Happolds, 1987; Calandra *et al*, 2016). Dental-based method as suggested by Fortelius and Solounias (2000), Kaiser (2002) and Franz-Odendaal and Kaiser (2003) allows for dietary reconstructions through the use of mesowear method on dental structures alone and has been employed in comparison-inference based differences in food availability and habitat structure (Schulz *et al*, 2007). This study captions age class identity diet type by mesowear equilibrium and age segregations in relation to fauna quality (ecologic zones). Age segregations is also assessed relative to locality climate, and further evaluate sexual segregations in food type selection, availability and spatial interaction using mesowear equilibrium; a method which assess dental occlusal relief relative parameters in inference of diet types. The resolution of accuracy of this method increases with

sample size and has been speculated to find utility in demographic studies and determination of fauna index (Popowics, 2003). This species' peculiar behavior in different ecologic environment variations might be a substrate in conservation efforts on the coastal extant species (International Union of Conservation of Nature, IUCN, 2012). We therefore hypothesize that:

- The mesowear equilibrium should reflect either differential food availability to different age classes or ontogenetic gradients in tooth wear and morphology
- Age segregation should be related to environmental conditions and dietary signals should therefore reflect abiotic habitat parameters such as the local climate and geomorphology.
- The mesowear equilibrium should resolve proposed sexual segregation in forage selection and availability

A combination of the machinery of principal components, cluster analysis and correlations was utilized in the discrimination of dietary traits evidenced from the dental profiles exhibited by this species from three ecological zones in Nigeria (Happolds and Happolds, 1987).

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MATERIALS AND METHODS

Sampling (ethics, sites, technique and time): Ethical approval for the study was given by the University of Ibadan Animal Care, Use and Research Committee (UI-ACUREC/App/2015/055). For the purpose of this investigation, 376 maxillary dentitions were obtained from wild-captured *Procyon cancrivorus* species from three locations in Nigeria: South West rain forest (Oyo state), the middle belt semi-savanna (Benue state) and Savanna (Adamawa state) eco-environmental zones respectively (Happolds and Happolds, 1987; Winkler *et al*, 2016). The specimens represented an aggregate of museum skulls and currently captured forty animals. Samples from the climes had an average of a year difference in date of collection while climatic data remains valid.

Samples distribution and animal aging: One hundred and eighty-eight dentulous maxilla comprising of 27 females (54 P^4) and 33 males (66 P^4), 30 females (60 P^4) and males (60 P^4) apiece, and 35 females (70 P^4) and 33 males (66 P^4) were used for the study. This comprised of 120 P^4 from the rain forest, semi-savanna (120 P^4) and savanna zones (136 P^4) respectively. They all contributed 376 upper 4^{th} premolars (Females =184, Males=192). Age determination was according to Grau *et al*, 1970; Samaranch and Gonzalez, 2000; Androukaki *et al*, 2002. The age classes 1-9 were recognized but animals under 2years (age class 1-6) were ignored for attrition-abrasion equilibrium stability while age class 7-9 were utilized for the study.

Images: Images of the 4^{th} upper premolar were taken with a digital camera CANON EOS 1200D with EFS 18-55mm and stabilizer equipped with HAMA tripod (Figure 1A-C). Digital images were taken with a DIN 6cm, focal length of 5.6cm, ISO speed 200, sensitivity of 1/500 and a ruler scale. Linear evaluations of occlusal surface, cusp depths and height changes were analyzed with Motic ® Images 2.01.

Mesowear method and modifications: Mesowear method measures attrition/abrasion equilibrium in tooth wear and has been used extensively in quantifying herbivores and ungulates dental wears. It provides an average wear signal over a considerable period of time (Calandra *et al*, 2016; Kaiser, 2002). Lingual/mesial surface images of maxillary 4^{th} premolar (P^4) were taken for lingual mesial aberrations (Ellis *et al*, 2008; Ulbricht *et al*, 2015), occlusal surface relief (OSR) and cusps shape (Fortelius and Solounias, 2000). Polysiloxane putty was employed in molding tooth surface layer after cleaning with hydrogen peroxide 6.6% w/v. The sharper lingual surface cusps of P^4 were scored relative to the deepest valley between adjacent cusps. The surface relief was classified as either low (l) or high (h) (limit set arbitrarily at 0.15mm based on the average of total sample range) by the relief profiles observed in samples from the three locations in a co-relative and subjective manner and represented in our analyses as percentages of sample population (per high= percentage high, %h) and (per low= percentage low, %l).

The degree of cusp facet development and distinctness forms the basis of a second mesowear variable as characterized by sharpness (s, terminates in a sharp point with no beveling on both mesial and distal phase facets), roundness (r, with distinctly rounded apex and facets are observable on the lower slopes) or bluntness (b, no observable facets altogether). These variables are also represented as percentages of morbid populations (Pers (%s) = percentage sharp, Perr (%r) = percentage round and Perb (%b) = percentage blunt) (Table 2).

Principal component analysis (%h, %s, %r and %b factors), hierarchical clustering based on Euclidean distance using paired group matrix analysis (UPGMA) algorithm was used in ordering the cluster trees as a reflection of dietary classifications according to ecological areas. Surface analysis was also done using PAST (Paleontological statistical package) 3.01 versions (Hammer *et al*, 2001).

RESULTS

The age class distribution considered for the purpose of this survey was 7-9years (Androukaki et. al., 2002). Mean individual age in CpRF (*P. cancrivorus* from the rain forest zone), CpSS (*P. cancrivorus* from Sudan Savanna zone) and CpS (*P. cancrivorus* from Sahel Savanna zone) were 12.2, 13.3 and 16.3 years respectively. Two sample tests showed males (9 years) were significantly ($p<0.01$) older than females (8 years) in CpRF whereas there were no significances ($p<0.5$) in the other groups (Table 1).

Table 1:
Sample distribution and structure in *P. cancrivorus* taken from three geographical areas. SID: sample identification, (CpRF: *P. cancrivorus* from the rain forest zone, CpSS: *P. cancrivorus* from Sudan Savanna zone and CpS: *P. cancrivorus* from Sahel Savanna zone), ♂=female, ♀= male, SD= standard deviation

SID	Sex symbol	Number of specimen	Age distribution	±SD
CpRF	♂	127	8.02	1.06
CpRF	♀	133	8.78	0.99
CpSS	♂	130	8.81	1.94
CpSS	♀	130	9.00	1.00
CpS	♂	135	7.54	0.89
CpS	♀	133	8.80	1.77

Table 2:
Mesowear datasets of *P. cancrivorus* from the rainfall ecological zone (CpRF) Sudan (CpSS) and Sahel (CpS) Savanna zones. Numbers 7-9 denote age classes considered. (SID: sample identification)

SID	%h	%s	%r	%b	Number	p value
CpRF(mean)	36.20	10.10	68.60	23.00	160	<0.001
CpSS(mean)	42.80	16.80	33.20	50.00	160	<0.001
CpS (mean)	54.20	46.60	22.50	30.90	168	<0.001
CpRF♂	34.20	8.90	65.10	21.00	127	<0.001
CpRF♀	40.20	11.90	71.80	25.00	133	<0.001
CpSS ♂	46.04	29.76	31.24	39.00	130	<0.003
CpSS ♀	40.89	10.07	62.70	26.60	130	<0.006
CpS ♂	67.60	58.20	19.78	22.02	135	<0.003
CpS ♀	44.30	30.10	18.67	51.23	133	<0.006
CpRF7	14.20	7.20	47.00	45.80	75	<0.030
CpRF8	48.80	13.00	86.00	1.00	62	<0.070
CpRF9	2.70	0	80.00	20.00	23	<0.020
CpSS7	23.40	13.70	71.40	14.90	32	n.s
CpSS8	22.10	22.30	45.20	32.50	64	<0.4
CpSS8	11.00	19.10	20.00	61.90	64	n.s
CpS7	38.30	31.10	61.90	7.10	48	<0.973
CpS8	45.00	35.20	32.00	32.80	39	<0.232
CpS9	11.00	0	72.60	27.40	81	<0.239

The first two principal components factors from age and sex based segregation analysis (Figure 1D and 2A) represents a minimum of 52% of the total variance. Resolution of discrimination in dietary habits is made obvious by Principal Component Analysis (PCA) and clustering analysis (Figure 2C and D). The pattern demonstrated by the cluster tree (Figure 2D) is used in circumscriptions of lines in the PCA plot (Figure 2C) within the spectrum of dental signatures expressed by all considered groups in making inferences on predominant dietary type expositions in all groups. Figure 2C showed that the average mesowear signal of both raccoon populations (RF and SS mean) was dominated with abrasion compared to the CpS (attrition dominated).

Table 3 demonstrates the sexual dimorphism in the different geographical locations assessed in this study. There were significant dimorphic scores between the females and males of CpSS as well as CpS. Figure 3A differentiates the species along ecologic and sex biases; whereas the species from the rain forest zones (both males and females) (the leader and first joiners) were more significantly affected and followed by the Sudan savanna males. Sahel savanna females remained the least joiners. Loading plot of occurrences among the parameters evaluated demonstrated percentage sharp (%s) recorded as the highest incident while the percentage round (%r) had the least scores.

Table 3:
Scores in both sexes across geographical locations (sexually significant dimorphic scores are bold)

Specimen	PC 1	PC 2	PC 3	PC 4
CpRF♂	-29.20	2.29	-5.36	0.71
CpRF♀	-30.05	5.43	4.38	1.49
CpSS♂	**14.51**	**-8.18**	-0.32	0.42
CpSS♀	**-23.79**	**0.74**	1.02	-2.60
CpS♂	**42.93**	**21.58**	-0.20	-0.11
CpS♀	**25.60**	**-21.86**	0.48	0.09

(Principal component (PC) CpRF: P. cancrivorus from the rain forest zone; CpSS: P. cancrivorus from Sudan Savanna zone; CpS: P. cancrivorus from Sahel Savanna zone; ♂=female; ♀= male)

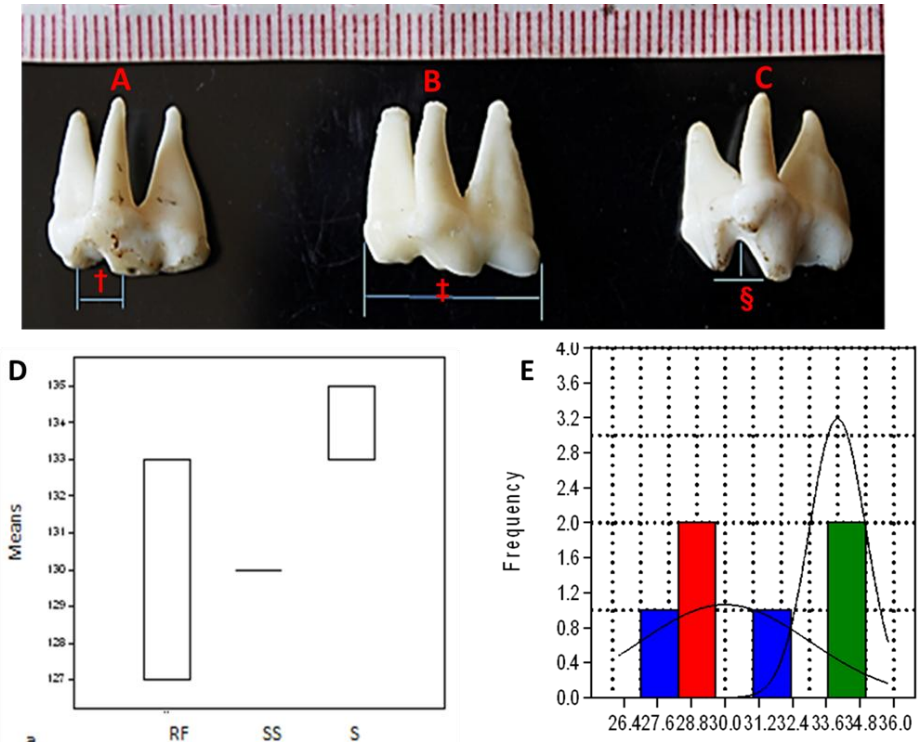
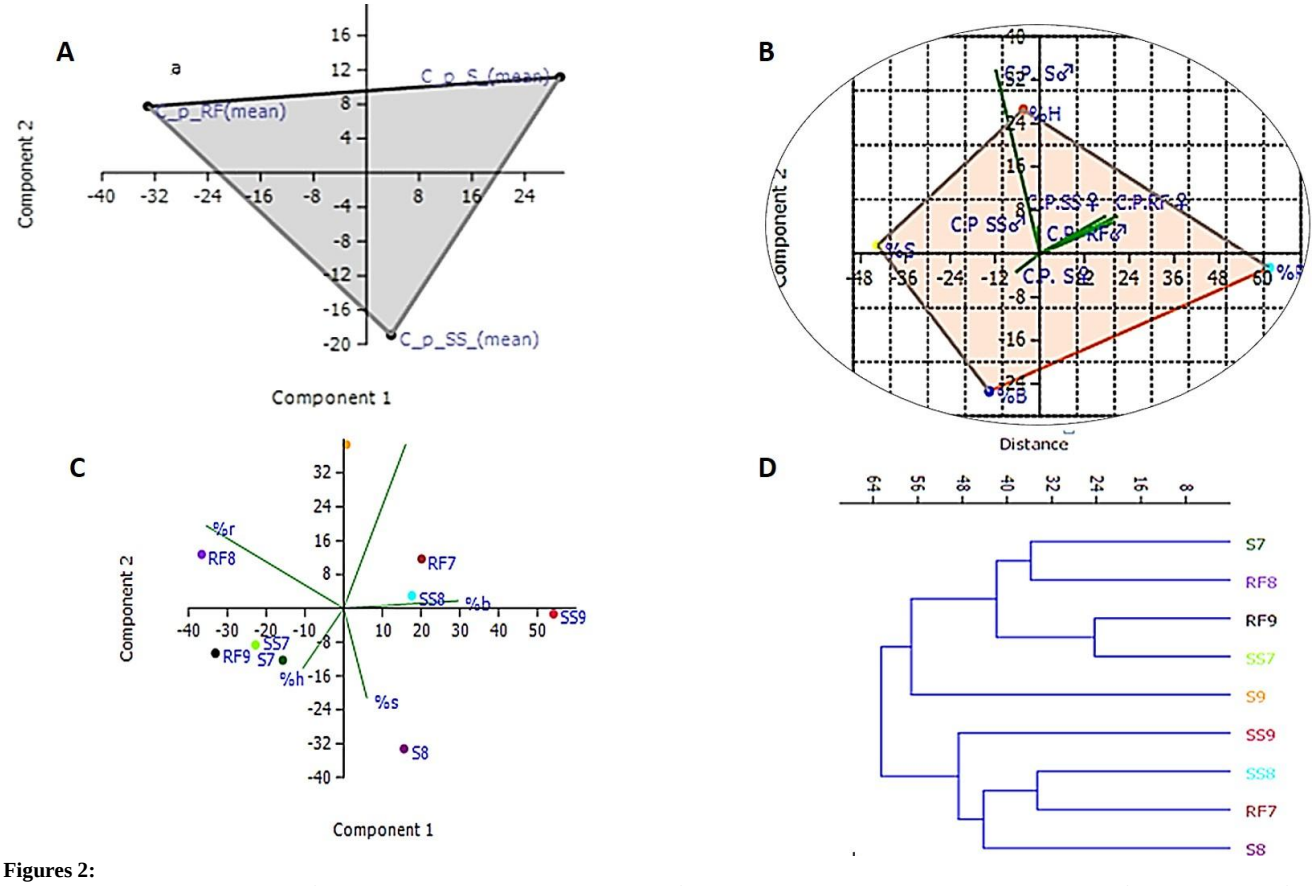


Figure 1:
Sample pictures of upper 4th premolar (P^4) teeth showing lingual surface projection in the three ecological zones (A) rain forest zone (B) Sudan Savanna zone (C) Sahel Savanna zone (†: distance between adjacent cusps; ‡ : width of the premolar tooth mesiodistally; §: depth between cusps); (D) Bar chart of means and confidence interval or quartiles box plot for the three locations and (E) histogram plot with normal fit of sex frequency distribution representing specific age class. RF (rainfall zone samples); SS (Sudan Savanna samples); S (Sahel savanna samples).



Figures 2:
(A). Principal component analysis of ecologic zone sample population means of percentage occlusal wear, (B) Sexual segregation of samples analysis from the three zones; PC = 81.81%, PC 2 = 16.93% of the total variance respectively; females of CpS had a highest %s and %h whereas CpRF and CpSS males are intermediate for %r while CpSS females and CpS males are lowest for %b PCA with Biplot of the samples from the three regions evaluated (CpRF: *P. cancrivorus* from the rain forest zone, CpSS: *P. cancrivorus* from Sudan Savanna zone, CpS: *P. cancrivorus* from Sahel Savanna zone, ♂=female, ♀= male; %s, %h, %r, %b: percentage sharp, high, round and blunt respectively); (C) Principal component analysis PC1 = 2.60%, PC2 = 27.03% of the total variance respectively (scatter plot of sample population from ecologic zones and parameters occurrence distribution with convex hulls; biplot), (D) hierarchical cluster tree based on mesowear variables %h, %s, %r and %b depending on age group segregation, rounded rectangles age group diet with higher animal tissue percentage content revealed lowest %h while rectangles showed the age group with lowest %s and highest %r (RF: Rain Forest zone; SS: Sudan Savanna zone; S: Sahel Savanna zone; 7,8,9: age classes considered; ♂=female; ♀= male; %s, %h, %r, %b: percentage sharp, high, round and blunt respectively).

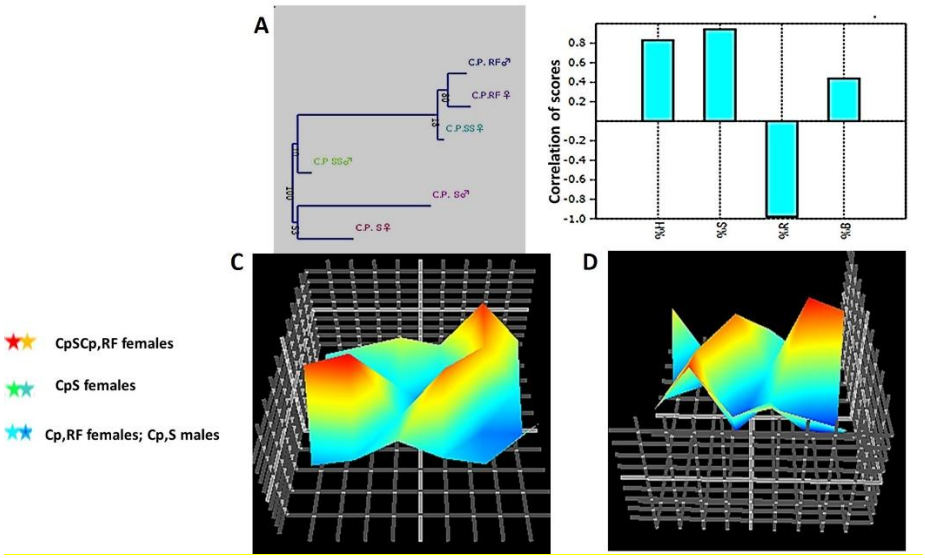


Figure 3:
(A) closest neighbor joining (NJ) bootstrapped (n=1000) based on ecologic relationship depending on sex group segregation. The first leader and joiner are CpRF and CpSS males, but Cp SS females possess the shortest branch length (B) Loading plot of correlation scores of evaluated variables %s, %h, %r, and %b. (CpRF: *P. cancrivorus* from the rain forest zone, CpSS: *P. cancrivorus* from Sudan Savanna zone and CpS: *P. cancrivorus* from Sahel Savanna zone), ♂=female, ♀= male; %s, %h, %r, %b: percentage sharp, high, round and blunt respectively); (C&D): Gender-based 4th premolar surface plot showing CpRF (Females), CpRF (Males), CpSS (Females), CpSS (Males), CpS (Females) and CpS (Males) samples versus %h, %s, %r and %b values at 0º Azimuth, 40º elevations and 180º Azimuth and 40º elevations respectively based on mesowear variables from the geographic location. Star colors show samples with highest and lowest values for the parameters in the order. (CpRF: *P. cancrivorus* from the rain forest zone, CpSS: *P. cancrivorus* from Sudan Savanna zone, CpS: *P. cancrivorus* from Sahel Savanna zone, ♂=female, ♀= male; %s, %h, %r, %b: percentage sharp, high, round and blunt respectively)

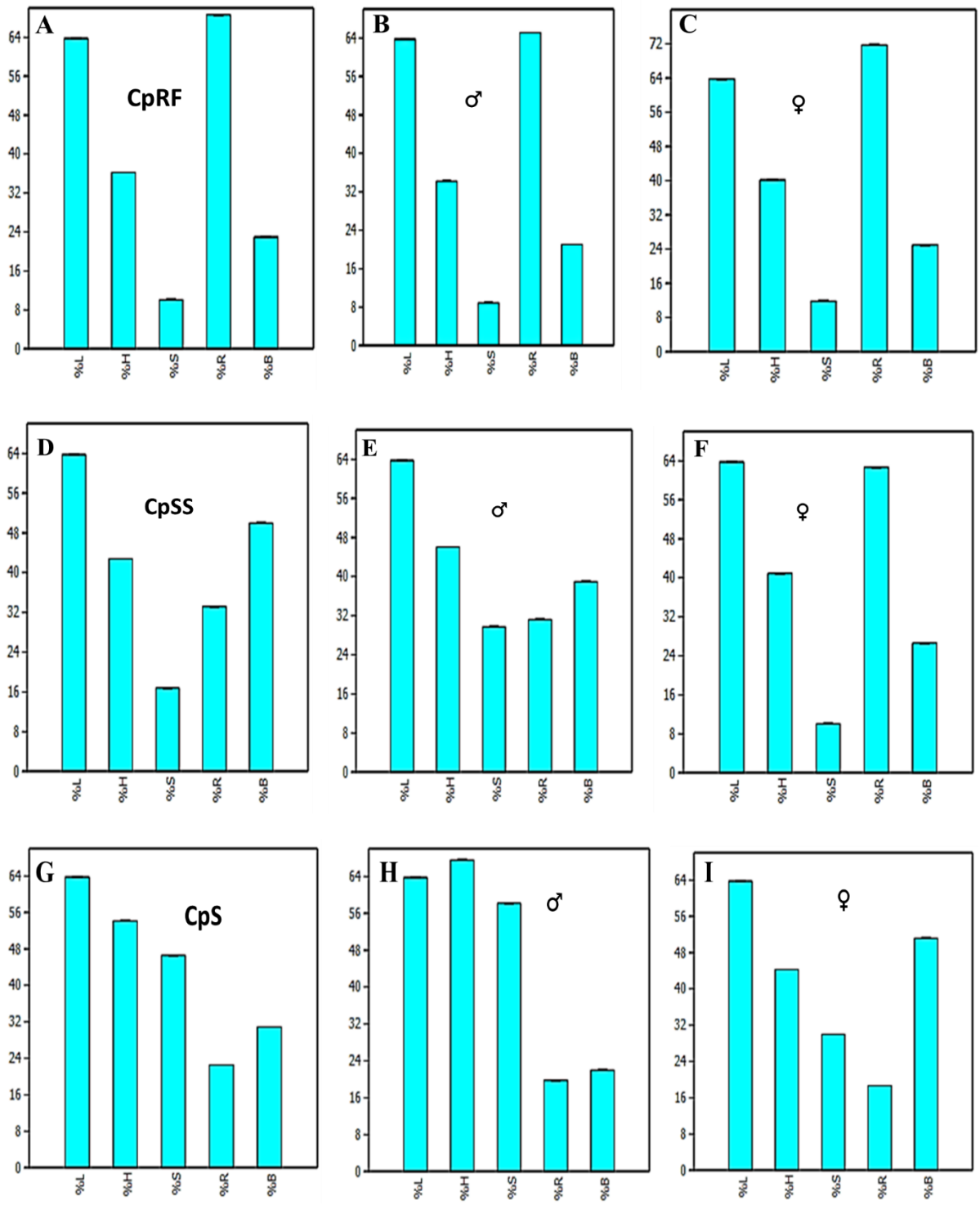


Figure 4
(A-I): Bar charts showing frequencies of cusp shape and occlusal relief parameters (%L, %H, %S, %R, %B: percentage low, high, sharp, round and blunt respectively; ♂=female, ♀= male)

Sexual segregation in both sexes from the geographical habitats of the species was significant as the three groups plots far from the population means (Figure 2A) with males (♀) differing generally more widely from the population means than females (♂) (Figure 2B). Female specimen samples from the rain forest zones demonstrated lower % R (percentage round) cusps (65.1%) compared to the males (71.8%) and a %B of 21 and 25% respectively in similar order (Table 2). Gender-based 4thpremolar surface plot showing CpRF (Females), CpRF (Males), CpSS (Females), CpSS (Males), CpS (Females) and CpS (Males) samples versus %h, %s, %r and %b values at 0º Azimuth, 40º elevations and 180º Azimuth and 40º elevations respectively based on mesowear variables from the geographic location (Figure 3C and D).

The trend (lower occlusal relief in females) is similar to occlusal surface relief results (Figure 4B and C) for %h observed to be 40% in males and 34% in females. Figure 4E

and F demonstrates a reversal of the trend in CpSS males shown to have lower occlusal surface relief of 48% and 40% for ♂ perh. Females and males had 31.24%, 62.7% perr, and then 39% and 26.6% perb respectively. In CpS, a slight significance ($p<0.003$) 19.78% and 18.67% perr exists in ♂ and ♀, then 22.02% and 51.23% perb in a similar order. The ♂ had a lower relief 67% compared to (44%) perh in ♀ (Figure 4H and I). The mesowear signatures observed in both CpSS8 and S8 were constant with advancing age (Figure 1D) while CpRF9, SS7 and S7 were observed to be closely linked with similar pattern in an overlap of age group and eco-geographic habitat axis. The results also revealed that older age brackets compared to the younger ones exposed more abrasions in all the groups except in the CpS9 group.

DISCUSSION

We have been able to ascertain that there are no shifts in the dental signatures irrespective of age. The three ecological zones evaluated for the diet partitioning in *P. cancrivorus* in Nigeria are separately characterized by varied precipitation, fauna and flora qualities (Happolds and Happolds, 1987). Rainfall in the rainforest zone averages 2500mm but 250-750mm in the Sahel zone [8]. Vegetation varies with soil structure, available water and photoperiodicity (Happolds and Happolds, 1987; Rivals *et al*, 2007). On the contrary, population differences in age classes may not be satisfactorily elucidated by the mesowear method, we observe a drift in mesowear equilibrium in dental signatures towards abrasion dominance with increasing age in the studied dental specimens from the rain forest, Sudan and Sahel savanna zones but in varying degrees which is consistent with the observations of Fortelius and Solounias, (2000) and Calandra *et al* (2016). A closer phenetic tree branch relationship was observed between RF9 and SS7 age groups, whereas S9 showed the farthest tree branch to the former. The means of the zonal group has been initially corroborated (Rivals *et al*, 2007) where sample population means showed varied patterns of occlusal relief with highest frequencies of percentage height and lowest percentage sharp occurring in *P. cancrivorus* from the rain forest zone. However, the opposite occurred in species from the Sahel with lowest percentage height and highest percentage sharp means which is in consonant with studies on the vertebrate feeding long-nose mongoose (*Xeno galenaso*) and the flat headed Cusimanse (*Crossachus platycephalus*) (Gilchrist *et al*, 2009). The above observation therefore satisfied our first hypothesis that the mesowear equilibrium should reflect either differential food availability to different age classes or ontogenetic gradients in tooth wear and morphology.

Age segregation in relation to environmental conditions was confirmed by the results of the investigation in terms of climatic, geomorphology with mean individual age in CpRF, CpSS and CpS which is an obvious evidence of relative longevity (*ceteris paribus*) in the latter of about three years older at death compared to others from rain forest and Sudan savannah zones.

We hypothesized that available diet type to the Sahel population of the species is more heterogeneous in composition (Rivals *et al*, 2007; Calandra *et al*, 2016) with less abrasive diet when compared to those from rain forest and Sudan Savanna areas, this therefore demonstrate the divergent fauna structure in the three habitats. The species from Sudan savanna area demonstrated intermediate dietary behavior between the other two, which impacted on the mesowear principal component analysis (PCA) revealing a negative percentage high in SS7 (positive percentage blunt) with major displacement on principal component 1 axis. Environmental factors which are likely to favor or disfavor availability of preferred food type in this habitat area is shown by percentage round in RF8 and percentage sharp in S8 which are more related to principal component 2 axis in distribution. These include flood and precipitation around the plateau where vegetation and climate types favor better food availability of varieties (Happolds and Happolds, 1987; NIMET, 2010; Winkler *et al*, 2016) or drought with concomitant increased forest fire incidences (jeopardizing food availability) scattered around areas in this location; thus establishing our second hypothesis. Our findings also revealed that a closer relationship exists between RF9 and SS7 male age groups while RF and S females were distinctly dissimilar. Limited resources in this location due to reduced/absent coastal and mangrove biomes, soil type as well as waning rainfall levels with increasing altitude above sea level northwards may have accounted for these results (Ita, 1994; Mackwell *et al*, 2013). A more anthropogenous habitat with relatively more abundant lower animal food resource characterization (Drygala and Zoller, 2013; Calandra *et al*, 2016) compared to a mosaic structure with multilayered dense forests allows the interpretation of abrasive-dominated mesowear dental signatures of the southern rainforest zone in our dataset to reflect a differential in food type availability (Schulz *et al*, 2007; Hirasawa and Kanda, 2006; Ulbricht *et al*, 2015) in the three habitats considered. This is also consistent with a similar investigation in other invasive species (Winkler *et al*, 2016). Patterns in forest fragmentation (not included in this study) due to structure differentiates the species in habitat utility (Ward and Wurster-Hill, 1989; Winkler *et al*, 2016) and its attendant migrations is evident in the level of occlusal surface wear in the teeth structure.

Both sexes revealed more intra-specific territory influenced diet character differences than geographic placement which suggests females are more exposed to wears due to competitive feeding (Winkler *et al*, 2016) due to nursing needs and urbanization of their ranges, leading to contraction and overlaps of spatial territories. They exhibit more reluctance to increase home range areas and are spatially restricted to fewer food resource choices (IUCN, 2012). Since niche partitioning is a mechanism for achieving co-existence of multiple species in a similar habitat (Okabe & Agetsuma, 2007) with spatial dietary resource overlaps, as demonstrated in Caiman alligator, *Melanosuchus niger* (Foth, Bona and Desojo, 2015) in relation to diet-partitioning. The raccoon dog (*Nyctereutes procyonoides*) and the red fox (*Vulpes vulpes*) (Drygala and Zoller, 2013) are comparably similar-body-sized mammals found in the same eco-environment. The percentage sharp frequencies were least and similar in CpRF and CpSS males respectively. The principal component scores between males and females in rain forest specimen are not significantly different when viewed against scores of specimens from other climes. Highest sex specific significant scores observed in CpS females inferred to

belong to a diet of least fracture content thus validating our third hypothesis. Interactions between species and environment represented by fauna (quality and quantity), forest types and water source proximity is a regular trigon whose relative proximity impacts on food type consumption as well as dental occlusal surface topography,

On the basis of the factors considered above, we therefore postulate that the possibility of an early compromised dental prey handling ability exist more in the rain forest zone group compared to other zones resulting in dietary imbalance and a reduced competitive ability especially in a competitive-tolerant spatial eco-environment

REFERENCES

Androukaki, E.E., Fataea, L., Hart, A., Osterhaus, E., Tounta, S., Kotomatas, E. 2002. Growth and development of Mediterranean Monk Seal pups during rehabilitation. The Monachus Guardian (5)1 http://www*Monachus-guardian.org*

Calandra, I. & GaëlleL abonne, E.S., Kaiser, T.M., Montuire, S. 2016. Tooth wear as a means to quantify intra-specific variations in diet and chewing movements *Scientific Reports* 6:Article number: 34037 2016 doi:10.1038/srep3403

Drygala, F., Zoller, H. 2013. Spatial use and interaction of the invasive raccoon dog and the native red fox in Central Europe: competition or coexistence? European Journal of Wildlife Research. Doi:10.1007/s10344-013-0722-y

Ellis, J.L., Thomason, J.J., Kebreab, E., France, J. 2008. Calibration of estimated biting forces in domestic canids; comparison of post-mortem and in-vivo measurements J Anat. 212:769-780

Feldhamer, G., Thompson, B., Chapman, J. 2003. Wild mammals of North America. Baltimore and London: The John Hopkins University press. pp.117.

Fortelius, M., Solounias, N. 2000. Functional characterization of ungulate molars using the abrasion-attrition wear gradient: A new method for reconstructing palaeodiets.–Novitates 3301:1-36.

Foth, C., Bona, P., Desojo, J.B. 2015. Intraspecific variation in the skull morphology of the black caiman *Melanosuchus niger* (Alligatoridae, Caimaninae). Acta Zoologica (Stockholm) 96:1–13

Franz-Odendaal, T.A., Kaiser, T.M. 2003. Differential mesowear in the maxillary and mandibular cheek dentition of some ruminants (Artiodactyla) – Ann. Zool. Fennici 40: 395-410.

Gilchrist, J.S., Jennings, A.P., Veron, G., Cavallini, P. 2009. The Herpestidae: The Handbook of the Mammals of the World. Carnivora Herpestidae Eds: Wilson, D.E. & Mittermeier R.A. Lynx Edicions. Barcelona. pp.263

Grau G., Glen A., Sanderson C., Rogers J.P. 1970. Age Determination of Raccoons. The Journal of Wildlife Management. 34(2):364-372.

Hammer, Ø., Harper, D.A.T., Ryan, P.D. 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica 4(1): Oral Sci. 20:17-31. http://folk. uio.no/ohammer/past

Happolds, D.C.D., Happolds, E. 1987. *Mammals of Nigeria.* Clarendon Press, Oxford. pp.122-144.

Hirasawa, M.E., Kanda, S.T. 2006. Seasonal food habitats of the raccoon dog at a western suburb of Tokyo. Mammal Study. 31:9-14.

International Union of Conservation of Nature, IUCN. 2012. List of threatened species Issue 10

Ita, E.O. 1994. Aquatic Plants and Wetland Wildlife Resources of Nigeria. CIFA Occasional Paper 21 Rome, FAO. pp.52

Kaiser, T.M. 2002. Modellierung Ernahrungsbezogener Attritions-Abrasions-Gradient tender Dentition neogener und pleistozäner Equiden (Perissodactyla) mitbesonderer Berücksichtigungder Basisgruppe der eurasischen Radiation. - Habilitationsschrift, Zoologisches Institutund Museum, Universität Greifswald. Pensoft Publishers. pp.69

Kitchen, AM., Gese, M., Eric, M., Schauster, ER. 1999. Resource partitioning between coyotes and swift foxes: space, time, and diet Can. J. Zool. 77:1645-1656

Mackwell, S.J., Simon-Miller, A.A., Harder, J.W., Bullock M.A. 2013. Comparative Climatology of Terrestrial Planets. University of Arizona Press, Tucson In collaboration with Lunar and Planetary Institute Houston

Nigerian Meteorological Agency (NIMET) 2010. Nigeria Climate Review Bulletin 10: 15-20

Okabe, F., Agetsuma, N. 2007. Habitat use by introduced raccoons and native raccoon dogs in a deciduous forest of Japan. Journal of Mammalogy. 88(4):1090-1097

Pergams O.R.W., Lawler J.J. 2009. Recent and Widespread Rapid Morphological Change in Rodents PLoS ONE 4(7): e6452. doi:10.1371/ journal.pone.0006452

Popowics E.T. 2003. Post canine dental form in the Mustelidae and Viveridae. J Morphol. 256:322-341

Rivals, F., Solounias, N., Mihlbachler, M.C. 2007. Evidence for geographic variation in the diets of late Pleistocene and early Holocene *Bison* in North America, and differences from the diets of recent *Bison* Quartenary Research. 68(3):338–346

Samaranch, R., Gonzalez, L.M. 2000. Changes in morphology with age in the medittareanean monk seal (*Monachus monachus*) Mar mamm. Sci. 16:141-157.

Schulz, E., Kaiser, T.M., Stubbe, A., Stubbe, M., Samjaa, R., Batsajchan, N., Wussow, J. 2007. Comparative demography and dietary resource partitioning of two wild ranging Asiatic equid populations Erforsch. Biol. Res. 10:77-90

Ulbricht A., Christian Maul L.C., Schulz, E. 2015. Can mesowear analysis be applied to small mammals? A pilot-study on leporines and murines. 80(1):14–20

Ward, O.G., Wurster-Hill, D.H. 1989. Ecological studies of Japanese raccoon dogs, Nyctereutes procyonoides viverrinus. Journal of Mammalogy. 70:330-334.

Winkler, D.E., Andrianasolo, T.H., Andriamandimbiarisoa, L., Ganzhorn, J.U., Rakotondranary, S.J., Kaiser, T.M., Schulz-Kornas, E. 2016. Tooth wear patterns in black rats (*rattusrattus*) of Madagascar differ more in relation to human impact than to differences in natural habitats. Ecology and Evolution. 6(7):2205–2215 doi:

Zeveloff, S.I. 2002. Raccoons: A Natural History. Washington, D.C.: Smithsonian Books. ISBN978-1-58834-033-7 p.113-4

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Full Length Research Article

Dysthyroidism Induces Hepatorenal Injury by Modulating HSP70/HSP90 and VEGF Signaling in Male Wistar Rats

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Summary: Thyroid hormones have been shown to promote the generation of reactive oxygen species (ROS), consumption of antioxidants, and induction of oxidative stress, which triggers the release of heat shock proteins (HSPs) and VEGF-dependent angiogenesis. The present study investigated the effect of altered thyroid states, hypothyroidism and hyperthyroidism, on hepatic and renal functions, oxidative stress biomarkers, and hepatorenal expressions of HSP70, HSP90, and VEGF. Male Wistar rats were randomized into vehicle-treated control, carbimazole-induced hypothyroidism, or levothyroxine-induced hyperthyroidism. Altered thyroid states caused impaired hepatic and renal functions accompanied by elevated malondialdehyde and reduced glutathione content and superoxide dismutase and catalase activities in the hepatic and renal tissues. These derangements were associated with down-regulation of hepatic and renal HSP70 and HSP90 and upregulation of hepatic and renal VEGF expression. Findings of histopathological examinations of the hepatic and renal tissues align with the biochemical derangements observed. This study reveals that dysthyroidism impairs hepatorenal function via induction of oxidative stress and modulation of HSP70/HSP90/VEGF signaling.

Keywords*: Hypothyroidism; hyperthyroidism; HSP70; HSP90; VEGF; oxidative stress*

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INTRODUCTION

Thyroid hormones (THs) are endocrine regulators of cellular activities such as thermoregulation and metabolism (Mariani and Berns, 2012; Ajayi *et al*. 2018a; Ajayi *et al*. 2018b). THs regulate cellular basal metabolism and oxidative processes (Klein and Danzi, 2007) by accelerating basal cellular metabolism, hence increasing metabolic reactions (Ajayi *et al*. 2017a). Also, they influence the electron transport chain, thus increasing oxygen consumption (Weetman *et al*. 1992). This promotes the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Weetman *et al*. 1992), consumption of antioxidants (Mano *et al*. 1995; Ajayi *et al*. 2017b), and induction of oxidative stress (Ajayi *et al*. 2017b; Guerrero *et al*. 1999; Akhigbe and Ajayi, 2021). The intracellular level of triiodothyronine (T3) determines the biological activities of THs. T3, produced via outer-ring 5-deiodination of thyroxine (T4) (Pavelka, 2014), binds with nuclear receptors of THs with a higher affinity than T4 (Asmaa *et al*. 2016). The liver and kidney are the main sites of the conversion of T4 to T3 (Brown *et al*. 2013) and are also affected by altered thyroid states (Ajayi *et al*. 2018b; Ajayi and Akhigbe, 2012; Ajayi *et al*. 2019). Although dysthyroidism has been reported to be an endocrine disruptor causing derangement of hormonal milieu and alteration in reproductive organ cytoarchitecture (Ajayi *et al*. 2013), most of the damages caused by dysthyroidism have been linked with oxidative stress (Ajayi *et al*. 2018a; Ajayi *et al*. 2018b; Ajayi *et al*. 2017a; Ajayi *et al*. 2017b; Pavelka, 2014).

Oxidative stress occurs when ROS generation exceeds cellular antioxidant buffering capacity (Akhigbe *et al*. 2021). This results in lipid peroxidation, protein denaturation, and oxidative DNA damage of the affected cell (Akhigbe and Ajayi 2021; Akhigbe *et al*. 2021; Akhigbe *et al*. 2020; Saka *et al*. 2020; Ige and Akhigbe, 2013). Besides the antioxidant system, the cells protect themselves by up-regulating the expression of heat shock proteins, HSPs, in response to ROS (Ikwegbue *et al*. 2018). HSPs response is primarily regulated through heat shock factor-1 (HSF-1), although post-transcriptional mRNA stabilization contributes some measure of regulation (Kaarniranta *et al*.

1998). HSPs synthesis is initiated in response to triggers such as physical, metabolic, and oxidative stress (Kaarniranta *et al*. 1998; Oosten-Hawle *et al*. 2013; Oksala *et al*. 2007). Subsequently, HSPs confer cytoprotection via chaperoning activities, including polypeptide folding, assembling, and translocation of organelles across membranes, conducting repairs, and degradation of irreparable peptides (Shiber and Ravid, 2014; Mayer and Bukau, 2005), and prevention of ROS-induced DNA fragmentation (Jacquier-Sarlin *et al*.1994). Hence, the antioxidant system works in synergy with HSPs to either prevent or neutralize the cellular impacts of ROS (Trott *et al*. 2008; Wu *et al*. 2015).

Despite the deleterious effects of oxidative stress on tissues, many compelling studies have established the positive role of oxidative stress in angiogenesis. Angiogenesis, either physiological or pathological, is activated by a rise in tissue demand for oxygen and nutrients, leading to hypoxia/re-oxygenation cycle and promotion of ROS generation (Kim and Byzova, 2013). Studies have shown that hydrogen peroxide induces vascular endothelial growth factor (VEGF) in vascular smooth muscle cells and endothelial cells, thereby enhancing angiogenic responses (Reuf *et al*. 1997; Chua *et al*. 1998). In addition, studies have revealed that ROS-mediated angiogenesis is linked with VEGF expression (Reuf *et al*. 1997; Li *et al*. 2010; Lu *et al*. 1998). ROS influence VEGF-stimulated VEGF receptor (VEGFR) 2 dimerization and autophosphorylation, required for VEGFR2 activation and angiogenesis (Ushio-Fukai *et al*. 2002; Colavitti *et al*. 2002). Hence, ROS has been established to promote angiogenic responses via modulation of VEGF/VEGF2 signaling.

Although we have previously demonstrated that dysthyroidism, hypothyroidism and hyperthyroidism, results in disruption of hepatic and renal cytoarchitecture, which is accompanied by impairment of hepatic and renal function (Ajayi *et al*. 2018b; Ajayi and Akhigbe, 2012; Ajayi *et al*. 2019), the roles of HSPs and VEGF in altered thyroid state-induced hepatorenal injury are unknown. Thus, the present study explores the role of HSPs and VEGF in dysthyroidism-induced hepatorenal injury.

MATERIALS AND METHODS

Ethical approval: The study was approved by the Ministry of Health Research Ethics Committee, Oyo State, Nigeria (reference number: AD13/479/460).

Experimental Animals: Male Wistar rats of comparable weight (230±20g) were used in this study. Animals were obtained from the Institute of Advanced Medical Research and Training, University College Hospital, Ibadan Nigeria. The animals were kept in wired mesh cages and acclimatized for two weeks before the commencement of the experiment. Animals had unrestricted access to standard rat pellet and water. The study was approved by the Ministry of Health Research Ethics Committee, Oyo State, Nigeria (reference number: AD13/479/460) and carried out following the Guide for the Care and Use of Laboratory Animals of the National Academy of Science (NAS), published by the National Institute of Health.

Experimental design: The rats were randomly allotted to three groups (n = 6): Control, Carbimazole-induced hypothyroid state, and Levothyroxine-induced hyperthyroid state. The control animals were administered 1 ml of distilled water as a vehicle. In contrast, carbimazole-treated animals received 20 mg/kg BW of carbimazole and the Levothyroxine-treated animals received 50µg/kg BW of levothyroxine. All treatments were via gavage and once daily for 35 consecutive days as previously reported (Ajayi *et al*. 2018a; Ajayi *et al*. 2018b; Ajayi *et al*. 2017a; Ajayi *et al*. 2017b; Ajayi and Akhigbe, 2012; Ajayi *et al*. 2019; Ajayi *et al*. 2013).

Sample collection: The Wistar rats were humanely culled under anaesthesia by administering 40 mg/kg of 5% ketamine and 4 mg/kg of 2% xylazine intraperitoneally (Ajayi and Akhigbe 2020). Blood samples were obtained through cardiac puncture into lithium-heparinized sample bottles and centrifuged at 3000 g for 5 minutes to obtain the serum. The liver and kidneys of each rat were excised, separated from surrounding structures, blotted, and weighed immediately. A weighed section of each organ was homogenized in an appropriate volume of cold Phosphate Buffer Solution. The homogenates were centrifuged at 12000 g for 15 minutes. The supernatant was separated into sample tubes, frozen overnight to maximize the release of the enzymes in the tissue.

Determination of hepatic and renal functions: Activities of hepatic aspartate aminotransferase (AST) (Agappe, India), alanine aminotransferase (ALT) (Randox Laboratory Ltd., Antrim, UK), and alkaline phosphatase (ALP) (Teco Diagnostics, USA) were used as indices of hepatic function and determined spectrophotometrically as previously documented (Saka *et al*. 2011; Hamed *et al*. 2021). Serum concentrations of creatinine and urea were determined using colorimetric methods (Randox Laboratory, Antrim, UK) (Saka *et al*. 2011; Hamed *et al*. 2021).

Determination of hepatic and renal redox markers: Colorimetry was used to determine the hepatic and renal levels of malondialdehyde (MDA) (Ajayi and Akhigbe 2020, Adegunlola *et al*. 2012), reduced glutathione (GSH) (Ajayi and Akhigbe 2020), and activities of superoxide dismutase (SOD) (Ajayi and Akhigbe 2020; Hamed *et al*. 2021) and catalase (Ajayi and Akhigbe 2020; Saka *et al*. 2011).

Determination of hepatic and renal HSP 70, HSP 90, and VEGF: Hepatic and renal concentrations of HSP70, HSP90, and VEGF were measured using ELISA kits (Elabscience, Biotechnology Co., Ltd, USA) per the manufacturer's guidelines.

Histopathological examinations: The harvested hepatic and renal tissues were fixed in 10% formalin immediately. The specimens were dehydrated in alcohol, cleared of xylene and embedded in paraffin. Seral sections were cut, stained, and examined under light microscope. Photomicrographs were taken at 400 x magnification.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism (Versions 5). One-way analysis of variance (ANOVA)

followed by Tukey's posthoc test was used to compare the mean values across and between the groups. Values are presented as mean ± SD. *P* values < 0.05 was considered statistically significant.

RESULTS

Effects of dysthyroidism on hepatic and renal functions: Carbimazole-induced hypothyroidism caused a significant decrease in the activities of hepatic AST, ALT and ALP compared to the control and levothyroxine-induced hyperthyroidism (Figure 1). However, the activities of hepatic AST, ALT, and ALP were comparable between the control and hyperthyroid rats (Figure 1). Although serum creatinine concentration was similar across the groups, the serum urea concentration increased significantly in the hypothyroid and hyperthyroid rats (Figure 2).

Effects of dysthyroidism on markers of oxidative stress: There was a significant rise in the hepatic and renal concentrations of MDA in dysthyroid rats compared to the control group. The rise in hepatorenal MDA was significantly more prominent in hyperthyroid rats than hypothyroid rats (Figure 3A and 3B). Also, carbimazole-induced hypothyroidism and levothyroxine-induced hyperthyroidism caused a significant reduction in the hepatic and renal levels of GSH (Figure 3C and 3D). The activity of hepatic and renal SOD was significantly reduced in hypothyroid and hyperthyroid groups compared with the control. However, the decline observed in SOD activity was more pronounced in the hyperthyroid rats (Figure 3E and 3F). Similarly, hepatic and renal catalase activity was significantly suppressed in carbimazole-induced hypothyroidism and levothyroxine-induced hyperthyroidism compared with the control. This alteration was also observed to be more prominent in the hyperthyroid animals than the hypothyroid animals (Figure 3G and 3H).

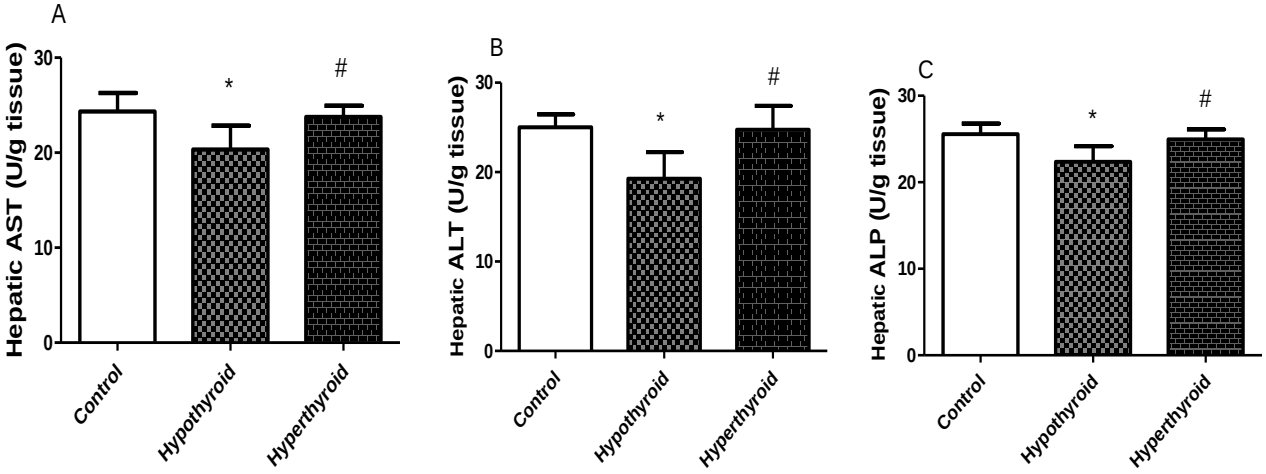

Figure 1:
Effect of hyperthyroidism and hypothyroidism on hepatic marker enzymes; aspartate amino transaminase (AST) (A), alanine transaminase (ALT) (B), and alanine phosphatase (ALP) (C). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean ± SD of 5 replicates per group. *p<0.05 vs control, #p<0.05 vs hypothyroid.

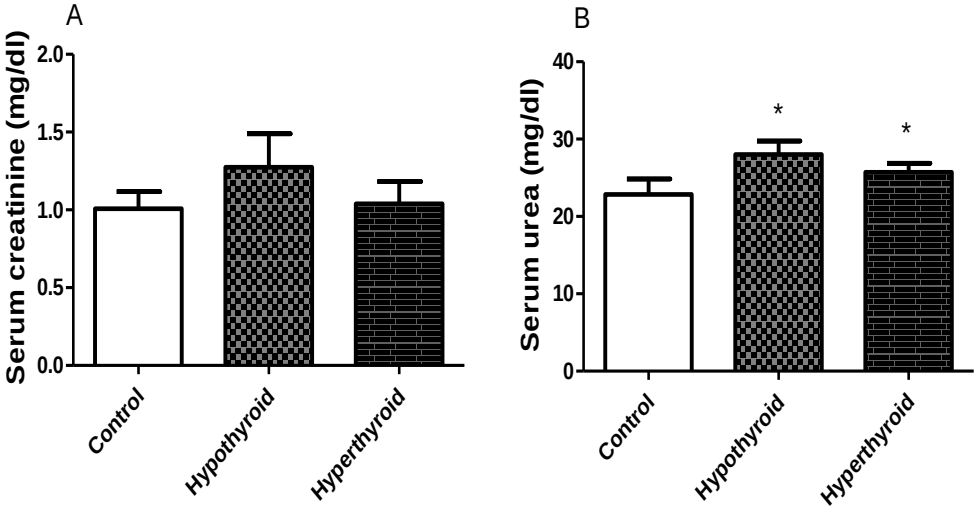

Figure 2:
Effect of hyperthyroidism and hypothyroidism on renal function markers; serum creatinine (A), and serum urea (B). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean ± SD of 5 replicates per group. *p<0.05 vs control.

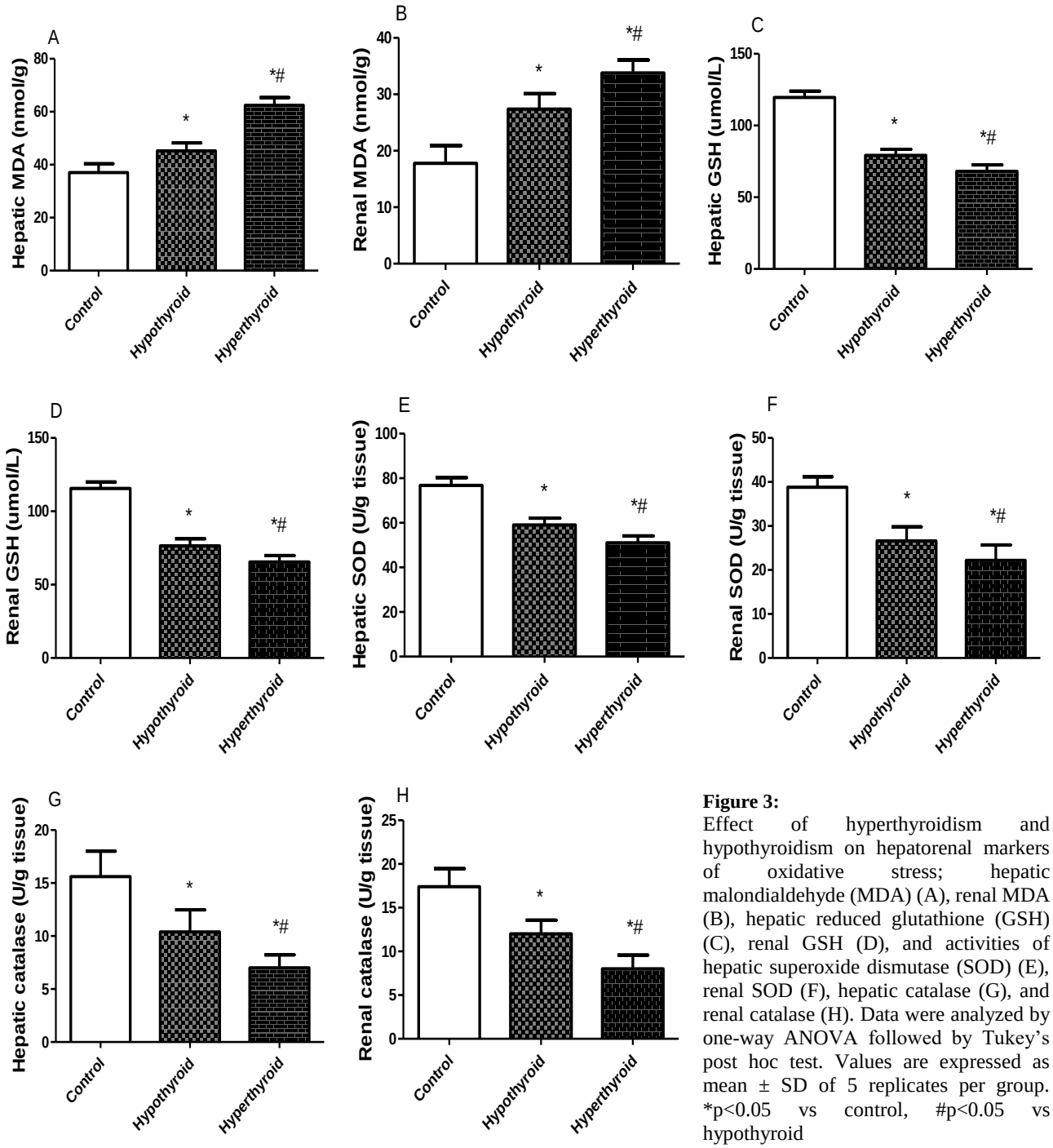

Figure 3:
Effect of hyperthyroidism and hypothyroidism on hepatorenal markers of oxidative stress; hepatic malondialdehyde (MDA) (A), renal MDA (B), hepatic reduced glutathione (GSH) (C), renal GSH (D), and activities of hepatic superoxide dismutase (SOD) (E), renal SOD (F), hepatic catalase (G), and renal catalase (H). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean ± SD of 5 replicates per group. *p<0.05 vs control, #p<0.05 vs hypothyroid

Effects of dysthyroidism on HSPs: Hepatic HSP70 was comparable between the hypothyroid and hyperthyroid rats but significantly lower in hypothyroid and hyperthyroid rats when compared with the control (Figure 4A). In addition, carbimazole-induced hypothyroidism and levothyroxine-induced hyperthyroidism led to a significant reduction in renal HSP70. The observed reduction in renal HSP70 in dysthyroid states was significantly more prominent in the hyperthyroid rats (Figure 4B). There was a significant reduction in hepatic HSP90 in the dysthyroid rats when compared with the control; however, hepatic HSP90 was significantly higher in levothyroxine-induced hyperthyroidism than in carbimazole-induced hypothyroidism (Figure 4C). There was reduced expression of HSP90 in the renal tissues of dysthyroid animals when compared with the control. The observed reduction in renal HSP90 was similar between the hypothyroid and hyperthyroid rats (Figure 4D).

Effects of dysthyroidism on VEGF: Figure 5 depicts the effects of carbimazole-induced hypothyroidism and levothyroxine-induced hyperthyroidism on hepatic and renal VEGF. There was a significant rise in hepatic VEGF level in carbimazole-induced hypothyroidism and levothyroxine-induced hyperthyroidism compared with the control animals (Figure 5A). Compared with hypothyroid rats, hyperthyroid animals showed significantly higher hepatic VEGF levels (Figure 5A). Renal VEGF level was comparable in the hypothyroid and hyperthyroid animals, whereas renal VEGF level was significantly higher in hypothyroid and hyperthyroid animals when compared with the control group (Figure 5B).

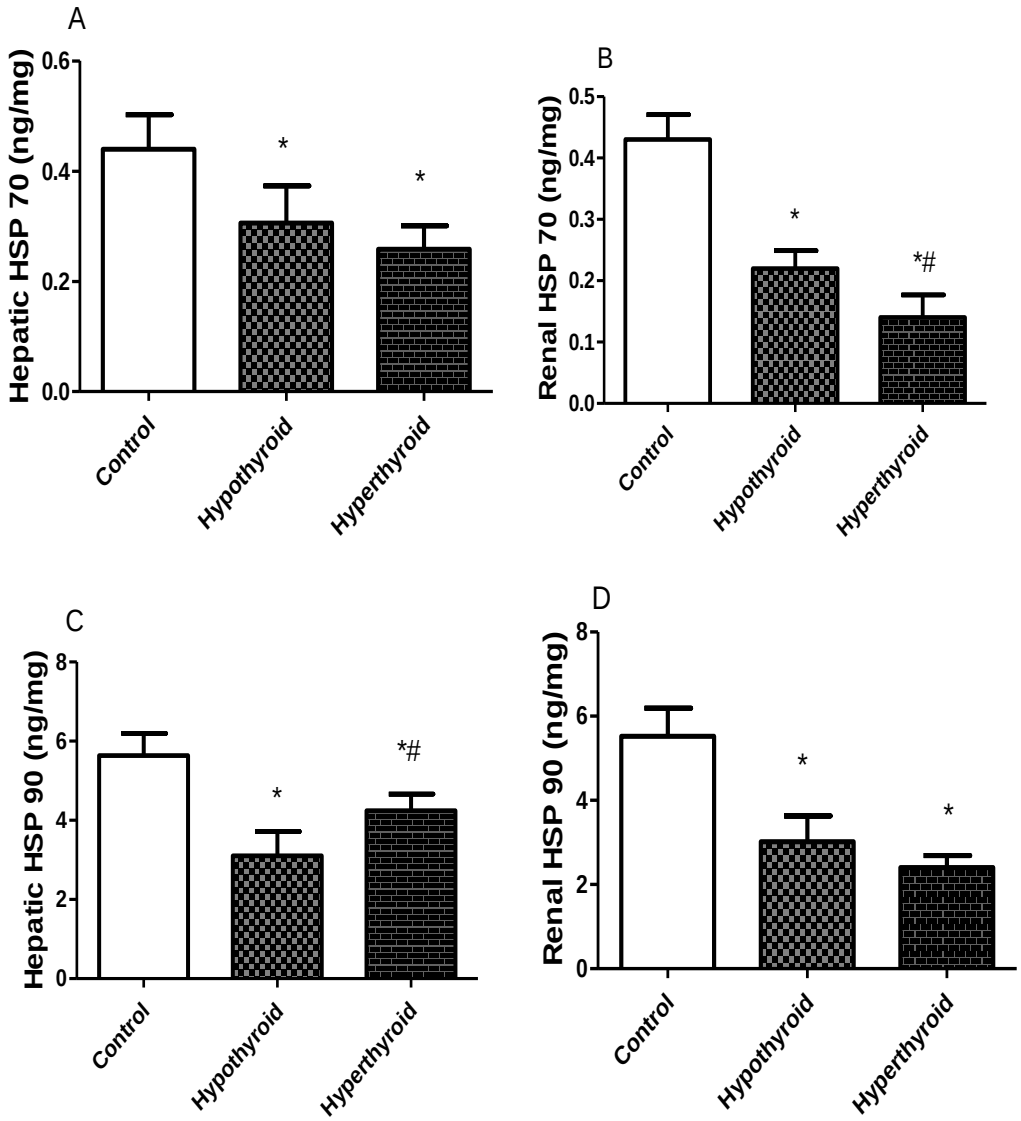

Figure 4:
Effect of hyperthyroidism and hypothyroidism on heat shock proteins (HSP); hepatic HSP 70 (A), renal HSP 70 (B), hepatic HSP 90 (C), and renal HSP 90. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean ± SD of 5 replicates per group. *p<0.05 vs control, #p<0.05 vs hypothyroid.

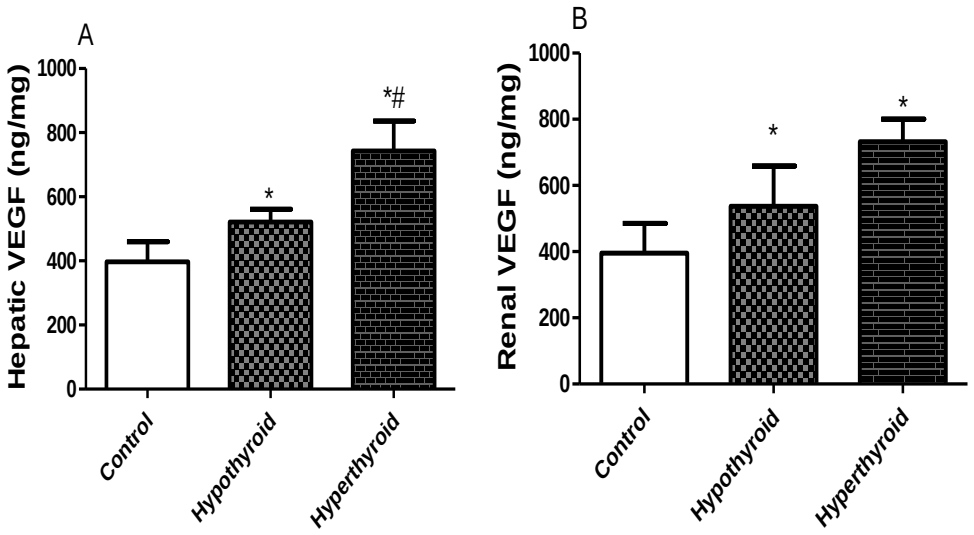

Figure 5:
Effect of hyperthyroidism and hypothyroidism on vascular endothelial growth factor (VEGF) in the liver (A) and kidney (B). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean ± SD of 5 replicates per group. *p<0.05 vs control, #p<0.05 vs hypothyroid.

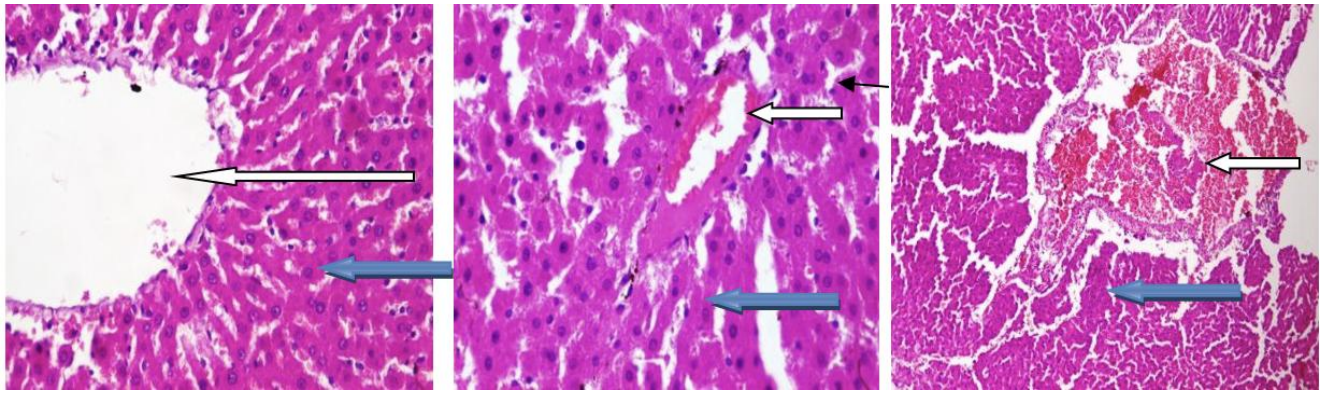

Plate 1

Photomicrograph of the liver section stained by Haematoxylin and Eosin. The control rats show normal central venules without congestion (white arrow). The morphology of the hepatocytes appear normal (blue arrow) and the sinusoids also appear normal without infiltration of inflammatory cell (slender arrow). The hypothyroid and hyperthyroid rats show central venules with mild congestion (white arrow). The morphology of the hepatocytes appear normal (blue arrow) and the sinusoids appear normal (slender arrow). Image is at 400 x magnification.

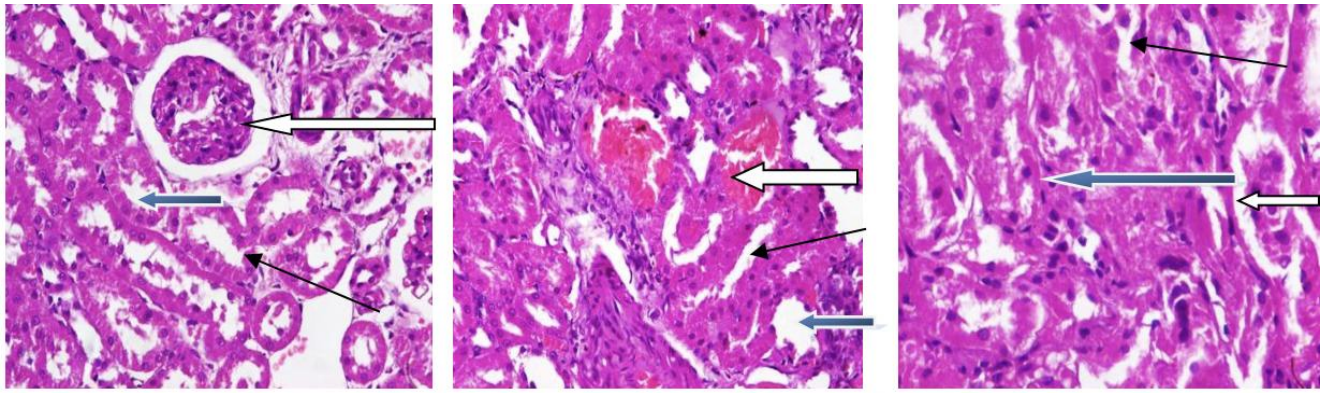

Plate 2:

Photomicrograph of the kidney section stained by Haematoxylin and Eosin. The kidneys of the control rats show normal architecture. The renal cortex show normal glomeruli with normal mesengial cells and capsular spaces (white arrow). The renal tubules including distal convoluted tubules and proximal convoluted tubules appear normal (blue arrow), and the interstitial spaces appear normal (slender arrow). The hypothyroid rats show moderate architecture. The renal cortex show normal glomeruli with normal mesengial cells and capsular spaces (white arrow). The renal tubules appear normal (blue arrow), and the interstitial spaces show mild vascular congestion with mild perivascular infiltration of inflammatory cells (slender arrow). The hyperthyroid rats show renal cortex with normal glomeruli with normal mesengial cells and capsular spaces (white arrow). The renal tubules show severe desquamation and severe tubular necrosis (blue arrow), and the interstitial spaces appear normal (slender arrow). Image is at 400 x magnification.

Effects of dysthyroidism on hepatorenal cytoarchitecture: The control rats showed presevred hepatic cytoarchitecture with normal central venules and no congestion (Plate 1). The morphology of the hepatocytes appeared normal and the sinusoids also appeared normal without infiltration of inflammatory cells. The hypothyroid and hyperthyroid rats showed central venules with mild congestion. The morphology of the hepatocytes appeared normal and the sinusoids appear normal. In addition, the renal tissue of the control rats showed normal architecture. The renal cortex showed normal glomeruli with normal mesengial cells and capsular spaces.

The renal tubules including distal convoluted tubules and proximal convoluted tubules appeared normal, and the interstitial spaces also appeared normal. The hypothyroid rats showed moderate architecture. The renal cortex showed normal glomeruli with normal mesengial cells and capsular spaces. The renal tubules appeared normal, and the interstitial spaces showed mild vascular congestion with mild perivascular infiltration of inflammatory cells. The hyperthyroid rats showed renal cortex with normal glomeruli and normal mesengial cells and capsular spaces. The renal tubules showed severe desquamation and severe tubular necrosis, and the interstitial spaces appeared normal (Plate 2).

DISCUSSION

In the current study, we explored the effects of altered thyroid states on hepatorenal function. We further determined the effects of dysthyroidism on hepatic and renal HSP and VEGF. It was hypothesized that the effects of dysthyroidism on hepatorenal function would be via HSP70/HSP90/VEGF-dependent pathway. Although we did not present the data on thyroid function test (thyroid stimulating hormone, TSH, T4 and T3) in the present report,

earlier studies in our laboratory have consistently established that 20 mg/kg of carbimazole and 50μg/kg of levothyroxine induce hypothyroidism and hyperthyroidism respectively in a rat model (Ajayi *et al*. 2018a; Ajayi *et al*. 2018b; Ajayi *et al*. 2017a; Ajayi *et al*. 2017b; Ajayi and Akhigbe, 2012; Ajayi *et al*. 2019; Ajayi *et al*. 2013). Our present data confirm that hypothyroidism and hyperthyroidism cause hepatorenal dysfunction. They also demonstrate that dysthyroidism induces oxidative stress in hepatorenal tissues evident by elevated hepatic and renal MDA and reduced GSH level and suppressed activities of SOD and catalase. These effects were accompanied by downregulation of hepatorenal HSP70 and HSP90 and upregulation of VEGF expression in the hepatic and renal tissues. These data suggest that hypothyroidism and hyperthyroidism induce hepatorenal dysfunction via oxidative stress and modulation of HSP70/HSP90/VEGF signalling.

As expected, the findings in the present study revealed that hypothyroidisim and hyperthyroidism caused hepatorenal dysfunction evident by significant alterations in marker enzymes of hepatic function and serum urea. This aligns with previous findings (Ajayi *et al*. 2018a; Ajayi *et al*. 2018b; Ajayi and Akhigbe 2012; Ajayi *et al*. 2019; Ellervik *et al*. 2019; Basu *et al*. 2012; Capasso *et al*.1999; Kim *et al*. 2020; Sequeira *et al*. 2011; Arora *et al*.2009), demonstrating hepatorenal dysfunction in dysthyroidism. In a human study, Ellervik *et al.* (2019) revealed that hypothyroidism reduced glomerular filtration rate (eGFRcrea) and increased incident chronic kidney injury. Similarly, Capasso *et al.* (1999) demonstrated that dysthyroidism led to perturbation of glomerular filtration rate and renal plasma flow through modification of proximal tubular sodium transport on Na/K-ATPase. Also, Arora *et al.* (2009) documented that hypothyroidism induces hepatorenal dysfunction by elevating hepatic marker enzyme activities and increasing serum creatinine and uric acid levels.

Although compelling shreds of evidence show that dysthyroidism induces hepatorenal injury, the associated mechanisms are yet to be fully explored. The finding that dysthyroidism is accompanied by oxidative hepatorenal damage is thus noteworthy. The lipid peroxidation induced by altered thyroid states is associated with a decline in the level of GSH in hepatorenal tissues and reduced activities of SOD and catalase in hepatic and renal tissues. This suggests the loss of the protective ability of SOD and catalase as enzymatic antioxidants in hepatorenal tissues in altered thyroid states. ROS act as signaling molecules at physiological concentrations and mediate a wide range of physiological processes, including homeostasis maintenance (Akhigbe and Ajayi, 2021). However, excessive ROS production is a key player in the initiation, progression and clinical outcomes of oxidative stress (Usman *et al*. 2019), resulting in pathological states such as organ dysfunction. The oxidation of the polyunsaturated fatty acid content of the membrane phospholipids (lipid peroxidation) in the cell membrane and membrane of cellular organelles triggers conformational changes that impair membrane and organelle functions (Akhigbe and Ajayi, 2021; Usman *et al*. 2019). This process leads to organ toxicity (Akhigbe and Ajayi, 2021; Awasthi *et al*. 2004) and cell death via the generation of MDA (Akhigbe and Ajayi, 2021). The observation of a rise in hepatic and renal MDA concentrations could infer that dysthyroidism triggers hepatorenal lipid peroxidation and possible cell death. The observed suppression of hepatic and renal activities of SOD and catalase provides further evidence that dysthyroidism enhances ROS generation (Weetman *et al.* 1992), antioxidants consumption (Mano *et al*. 1995; Ajayi *et al*. 2017b), and induction of oxidative stress (Ajayi *et al*. 2017b; Guerrero *et al*. 1999; Akhigbe and Ajayi, 2021).

HSPs are important sensors of cellular redox change and confer cellular protection in synergy with antioxidants. These sensors are triggered in response to oxidative stress (Kalmar and Greensmith, 2009) and act as molecular chaperones, promoting folding and inhibiting protein aggregation or targeting improperly folded proteins to specific degradative pathways (Oosten-Hawle *et al*. 2013; Kalmar *et al*. 2009). More so, HSP70 has been established to exert anti-inflammatory actions via prevention of the activation of NF-kB, cyclo-oxygenase 2 (COX-2), and nitric oxide synthase (NOS) (Kalmar and Greensmith, 2009). In addition, HSP70 and HSP90 inhibit apoptotic cascade by preventing the activation of caspase-3 by apoptosis protease activating factor-1 (Apaf-1) (Kalmar and Greensmith, 2009). Hence, the effects of HSPs go beyond the maintenance of protein folding-competent states. Kalmar and Greensmith (Kalmar and Greensmith, 2009) earlier suggested that most of the tissue damage that occurs due to oxidative stress occurs after the actual insult. Thus, it is plausible to infer that the observed low levels of HSP70 and HSP90 accompanied by a rise in MDA and reduced SOD and catalase activities in hepatic and renal tissues in dysthyroidism is a result of excessive accumulation of ROS and suppression of antioxidant with possible prevention of heat shock factor-1 (HSF-1), which is responsible for the biosynthesis and release of the cytoprotective HSPs. This possibly led to cell death and hepatorenal dysfunction observed. The present study found that dysthyroidism induces oxidative stress and suppression of HSPs in agreement with our previous study that demonstrated that dysthyroidism activates TNF-dependent inflammation and oxidative stress in rat cardiac tissue (Ajayi *et al*. 2017b), which could imply the loss of cytoprotective ability of HSPs in altered thyroid states. The observed distortion in the hepatorenal cytoarchitecture in dysthyroid animals could be explained by the loss of the protective effects of HSPs and resultant oxidative stress.

Earlier studies have reported that VEGF could be constitutively expressed or upregulated by oxidative stress (Klettner and Roider, 2009; Nagineni *et al*. 2003; Treins *et al*. 2001). VEGF is the primary physiological growth factor in angiogenesis. It has been reported to play a central role in the female reproductive cycle and wound healing (Klettner and Roider, 2009), maintenance of existing vasculature by inhibiting apoptosis of the endothelial cells (El-Remessy *et al*. 2004), and conferring neuroprotection in the eye (Zachary, 2004). Thus, the increase in hepatic and renal levels of VEGF observed in this study suggests that dysthyroidism causes pathological angiogenesis. A previous study has reported that inhibition of JNK promotes oxidative stress-dependent secretion of VEGF, while inhibition of p38 and Erk diminishes VEGF expression (Klettner and Roider, 2009). This could suggest that inhibition of JNK and activation of p38/Erk could cause

upregulation of VEGF expression. Taken together, therefore, our finding in this study that dysthyroidism leads to enhanced expression of VEGF in hepatic and renal tissues is noteworthy since it could infer that dysthyroidism could upregulate hepatorenal VEGF expression via modulation of mitogen-activated protein kinase (MARK) signaling (which includes JNK, p38, and Erk).

In conclusion, the current study demonstrates that hypothyroidism and hyperthyroidism cause hepatic and renal dysfunction and hepatorenal oxidative stress. These events are associated with suppression of HSP70 and HSP90 and upregulation of VEGF expressions in hepatic and renal tissues. These findings have far-reaching imports and could suggest alternative pathways for dysthyroidism-induced hepatorenal dysfunction. This study shows that modulation of HSP70/HSP90/VEGF signaling mediates dysthyroidism-induced oxidative hepatorenal damage.

Authors' contributions

Conceptualization and study design: AFA, REA, OSO
Experimentation: LOM, AG, OAI, ATI
Statistical analysis: AFA, REA
Writing of first draft: REA
Revision of the first draft: All authors
Approval for submission and publication: All authors

REFERENCES

Adegunlola, J.G., Afolabi, O.K., Akhigbe, R.E., Adegunlola, G.A., Adewumi, O.M., Oyeyipo, I.P. (2012). Lipid peroxidation in brain tissue following administration of low and high doses of arsenite and L-ascorbate in Wistar strain rats. Toxicol Int. 19:47–50. https://doi.org/10.4103/0971-6580.94516

Ajayi A.F, Akhigbe R.E, Ajayi L.O. (2019). Lipid peroxidation and enzymatic antioxidant activities in the kidneys of experimental dysthyroid rabbits: an assessment of renal redox state. Int J Infect Trop Dis. 5:16-23.

Ajayi A.F, Akhigbe R.E, Ajayi LO. (2018b). Influence of thyroid dysfunction on Urea/ Creatinine ratio: possible role of TNF-α and IL-6. Int J Med Biomed Res. 7(3):94-102.

Ajayi AF and Akhigbe RE. (2020). In vivo exposure to codeine induces reproductive toxicity: role of HER2 and p53/Bcl-2 signaling pathway. Heliyon 2020; 6: e05589.

Ajayi AF, Adelakun AA, Akhigbe RE. (2017a). Gastric mucosa damage and impairment of secondary immune response in dysthyroidism is associated with TNF-α expression. Int J Biol Med Res. 8 (3): 6063-6069.

Ajayi AF, Akhigbe RE, Ajayi LO, Adeleye GS, and Adebayo-Gege GI. (2018a). Serum and gastric tissue electrolyte levels in carbimazole-treated and levothyroxine-treated male New Zealand white rabbits. World Journal of Pharmacy and Pharmaceutical Sciences. 7 (10): 142-155.

Ajayi AF, Akhigbe RE, Ajayi LO. (2013). Hypothalamic-pituitary-ovarian axis in thyroid dysfunction. West Indian Med J. 62 (9): 835-838.

Ajayi AF, Akhigbe RE, Ajayi LO. (2017b). Activation of Cardiac TNF-α In Altered Thyroid State-Induced Cardiometabolic Disorder. J Cardiovasc Dis¬ease Res. 8(4):151-6.

Ajayi AF, Akhigbe RE. (2012). Implication of altered thyroid state on liver function. Thyroid Res Pract. 9:84-7.

Akhigbe RE and Ajayi AF. (2021). The impact of reactive oxygen species in the development of cardiometabolic disorders: a review. Lipids in Health and Disease. 20:23.

Akhigbe RE, Ajayi LO, Adelakun AA, Olorunnisola OS, Ajayi AF. (2020). Codeine-induced hepatic injury is via oxido-inflammatory damage and caspase-3-mediated apoptosis. Molecular Biology Reports. https://doi.org/10.1007/s11033-020-05983-6

Akhigbe RE, Ajayi LO, Ajayi AF. (2021). Codeine exerts cardiorenal injury via upregulation of adenine deaminase/xanthine oxidase and caspase 3 signaling. Life Sciences. 273: 118717.

Arora S, Chawla R, Tayal D, Gupta VK, Sohi JS, Mallika V. (2009). Biochemical markers of liver and kidney function are influenced by thyroid function- a case-controlled follow up study in Indian hypothyroid subjects. Indian Journal of Clinical Biochemistry. 24 (4): 370-374.

Asmaa M.S. Gomaa, Ebtihal A. Abd El-Aziz. (2016). Omega-3 fatty acids decreases oxidative stress, tumor necrosis factor-alpha, and interleukin-1 beta in hyperthyroidism-induced hepatic dysfunction rat model. Pathophysiology. 23: 295–301.

Awasthi Y.C. (2004). Regulation of 4-hydroxynonenal-mediated signaling by glutathione S-transferases. Free Radical Biology and Medicine. 37(5): 607–619.

Basu G, Mohapatra A. (2012). Interactions between thyroid disorders and kidney disease. Indian J Endocr Metab.16:204-13.

Brown A.R., Simmen R.C., Simmen F.A. (2013). The role of thyroid hormone signaling in the prevention of digestive system cancers. Int. J. Mol. Sci. 14:16240–16257.

Capasso G, De Tommaso G, Pica A, Anastasio P, Capasso J, Kinne R, De Santo NG. (1999). Effects of thyroid hormones on heart and kidney functions. Miner Electrolyte Metab. 25: 56-64.

Chua CC, Hamdy RC, Chua BH. (1998). Upregulation of vascular endothelial growth factor by H2O2 in rat heart endothelial cells. Free Radic Biol Med. 25(8):891-897.

Colavitti R, Pani G, Bedogni B, Anzevino R, Borrello S, Waltenberger J, Galeotti T. Reactive oxygen species as downstream mediators of angiogenic signaling by vascular endothelial growth factor receptor-2/KDR. J Biol Chem. 277(5):3101-3108.

Ellervik C, MoraS, Ridker PM, Chasman DI. (2019). Hypothyroidism and kidney function-a Mendelian randomization study. Thyroid. Doi: 10.1089/thy.2019.0167

El-Remessy AB, Bartoli M, Platt DH, Fulton D, Caldwell RB. (2004). Oxidative stress inactivates VEGF survival signaling in rential endothelial cells via PI 3-kinase tyrosine nitration. J Cell Sci. 118:243–252, doi:10.1242/jcs.01612

Guerrero A, Pamplona R, Portero-Otín M, Barja G, López-Torres M. (1999). Effect of thyroid status on lipid composition and peroxidation in the mouse liver. Free Radical Biology and Medicine. 26(1-2):73–80.

Hamed MA, Aremu GO, and Akhigbe RE. (2021). Concomitant administration of HAART aggravates anti-Koch-induced oxidative hepatorenal damage via dysregulation of glutathione and elevation of uric acid production. Biomedicine & Pharmacotherapy. 137:111309

Ige SF and Akhigbe RE. (2013). Common onion (Allium cepa) extract reverse cadmium-induced organ toxicity and dyslipidaemia via redox alteration in rats. Pathophysiology. 20:269-274.

Ikwegbue PC, Masamba P, Oyinloye BE, Kappo AP. (2018). Roles of heat shock proteins in apoptosis, oxidative stress, human inflammatory diseases, and cancer. Pharmaceuticals. 11, 2; doi:10.3390/ph11010002.

Jacquier-Sarlin, M.R.; Fuller, K.; Dinh-Xuan, A.T.; Richard, M.J.; Polla, B.S. (1994). Protective effects of HSP70 in inflammation. Cell. Mol. Life Sci. 50, 1031–1038.

Kaarniranta K, Elo M, Sironen R, Lammi MJ, Goldring MB, Eriksson JE, Sistonen L, Helminen HJ. (1998). HSP 70 accumulation in chrondrocytic cells exposed to high continuous hydrostatic pressure coincides with mRNA stabilization rather than transcriptional activation. Proceedings of the National Academy of Sciences of the United States of America. 95 (5): 2319-2324.

Kalmar B, Greensmith L. (2009). Induction of heat shock proteins for protection against oxidative stress. Advanced Drug Delivery Reviews. 61(4): 310–318. http://dx.doi.org/10.1016/j.addr.2009.02.00319248813.

Kim SH, Min HK, Lee SW. (2020). Relationship between thyroid and kidney function: analysis from the Korea National Health and Nutrition Examination Survey between 2013 and 2015. Kidney and Blood Pressure Research. doi: 10.1159/000507290.

Kim Y and Byzova TV. (2014). Oxidative stress in angiogenesis and vascular disease. Blood. 123: 625-631. doi:10.1182/blood-2013-09-512749

Klein I, Danzi S. (2007). Thyroid disease and the heart. Circulation. 116: 1725-1735.

Klettner A and Roider J. (2009). Constitutive and oxidative-stress-induced expression of VEGF in the RPE are differently regulated by different Mitogen-activated protein kinases. Graefes Arch Clin Exp Ophthalmol. 247:1487–1492. Doi: 10.1007/s00417-009-1139-x

Li J, Wang JJ, Yu Q, Chen K, Mahadev K, Zhang SX. (2010). Inhibition of reactive oxygen species by Lovastatin downregulates vascular endothelial growth factor expression and ameliorates blood retinal barrier breakdown in db/db mice: role of NADPH oxidase 4. Diabetes. 59(6): 1528-1538.

Lu M, Kuroki M, Amano S. (1998). Advanced glycation end products increase retinal vascular endothelial growth factor expression. J Clin Invest. 101(6):1219-1224.

Mano T, Sinohara R, Sawai Y. (1995). Effects of thyroid hormone on coenzyme Q and other free radical scavengers in rat heart muscle. J Endocrinol. 145: 131-136.

Mariani LH, Berns JS. (2012). The renal manifestations of thyroid disease. J Am Soc Nephrol. 23(1): 22–6.

Mayer, M.P.; Bukau, B. (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. Cell. Mol. Life Sci. 62, 670.

Nagineni CN, Nagineni S, Samuel W, Pardhasaradhi K, Wiggert B, Detrick B, Hooks JJ. (2003). TGF-ß induces expression of VEGF in human RPE cells: involvement of MAPK. J Cell Physiol. 197:453–462, doi:10.1002/jcp.10378

Oksala NK, Lappalainen J, Laaksonen DE, Khanna S, Kaarniranta K, Sen CK, Atalay M. (2007). Alpha-lipoic acid modulates heat shock factor-1 expression in streptozotocin-induced diabetic rat kidney. Antioxidants & Redox Signaling. 9 (4): 497-506.

Oosten-Hawle PV, Porter RS, Morimoto RI. (2013). Regulation of organismal proteostasis by transcellular chaperone signaling. Cell. 153 (6): 1366-1378.

Pavelka S. (2014). Development of radiometric assays for quantification of enzyme activities of the key enzymes of thyroid hormones metabolism. Physiol. Res. 63:S133–140.

Ruef J, Hu ZY, Yin LY, *et al*. (1997). Induction of vascular endothelial growth factor in balloon injured baboon arteries. A novel role for reactive oxygen species in atherosclerosis. Circ Res. 81(1):24-33.

Saka WA, Akhigbe RE, Ishola OS, Ashamu EA, Olayemi OT, Adeleke GE. (2011). Hepatotherapeutic effect of Aloe vera in alcohol-induced hepatic damage, Pak. J. Biol. Sci. 14: 742–746.

Saka WA, Ayoade TE, Akhigbe TM, Akhigbe RE. (2020). Moringa oleifera seed oil partially abrogates 2,3-dichlorovinyl dimethyl phosphate (Dichlrovos)-induced cardiac injury in rats: evidence for the role of oxidative stress. J Basic Clin Physiol Pharmacol. 20190313. https://doi.org/10.1515/jbcpp-2019-0313.

Sequeira E, Wanyonyi S, Dodia R. (2011). Severe propylthiouracil-induced hepatotoxicity in pregnancy managed successfully by liver transplantation: A case report. Journal of Medical Case Reports. 5:461.

Shiber, A.; Ravid, T. (2014). Chaperoning proteins for destruction: Diverse roles of HSP70 chaperones and their co-chaperones in targeting misfolded proteins to the proteasome. Biomolecules. 4: 704–724.

Treins C, Giorgetti-Peraldi S, Murdaca J, Van Obberghen E. (2001). Regulation of VEGF expression by advanced glycation End products. J Biol Chem. 276:43836–43841, doi:10.1074/jbc.M106534200

Trott, A.;West, J.D.; Klai´c, L.;Westerheide, S.D.; Silverman, R.B.; Morimoto, R.I.; Morano, K.A. (2008). Activation of heat shock and antioxidant responses by the natural product celastrol: Transcriptional signatures of a thiol-targeted molecule. Mol. Biol. Cell. 19, 1104–1112.

Ushio-Fukai M, Tang Y, Fukai T, *et al* (2002). Novel role of gp91(phox)-containing NAD(P)H oxidase in vascular endothelial growth factor-induced signaling and angiogenesis. Circ Res. 91(12):1160-1167.

Usman TO and Olatunji LA. (2019). Late gestationaltestosterone exposure causes glucose deregulation and elevated cardiac VCAM-1 and DPP-4 activity in rats, Archives of Physiology and Biochemistry. DOI: 10.1080/13813455.2019.1650068

Weetman AP, Tandon N, Morgan BP. (1992). Antithyroid drugs and release of inflammatory mediators by complement-attacked thyroid cells. Lancet. 12; 340(8820): 633-6.

Wu, C.-W.; Biggar, K.K.; Zhang, J.; Tessier, S.N.; Pifferi, F.; Perret, M.; Storey, K.B. (2015). Induction of antioxidant and heat shock protein responses during torpor in the gray mouse lemur, Microcebus murinus. Genom. Proteom. Bioinform. 13, 119–126.

Zachary I. (2004). Neuroprotective role of VEGF: signalling mechanisms, biological function, and therapeutic potential. Neurosignals. 14:207–221, doi:10.1159/000088637..

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Full Length Research Article

Therapeutic Potential of Hesperidin in Parkinson's Disease with Dementia: Inhibition of Alpha Synuclein and Amyloid Beta in *Drosophila melanogaster*

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Summary: Neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD) share similar cellular and molecular mechanisms such as protein aggregation and inclusion body formation. Thus, we evaluated the action of hesperidin on α-synuclein and amyloid-β-induced neurodegeneration in *Drosophila melanogaster*. To model PD and Alzheimer's disease (AD), the bipartite system of GAL4 transcriptional activator was placed under a cell-type specific promoter; embryonic lethal abnormal visual system-GAL4 (ELAV-GAL4; pan-neuronally) or dopa decarboxylase (Ddc-GAL4; dopaminergic neurons) for the expression of amyloid-beta (Aβ42) or α-synuclein (α-syn), respectively, under the control of the upstream activating sequence (UAS) in Drosophila melanogaster.. Flies were either grown on food media supplemented with or without hesperidin (HSD) (1, 5, or 10 mM). Behavioral assays were carried to investigate the effect of treatment on fecundity, larval motility, climbing ability, and lifespan. UAS-$A\beta_{42}$>Elav-GAL_4 or UAS-α-synuclein>DDC-GAL_4 caused significant decrease in fecundity, larva motility, survival rate, and climbing activities in flies showing neurodegenerative phenotype. However, supplementation of flies' media with hesperidin (1, 5 and 10 mM) showed a dose-dependent increase in the number of flies' egg-laying ability, larva motility and adult climbing activity in comparison with flies grown on food media only. Conversely, supplementation of fly feed with HSD caused no significant change in lifespan. Findings from this experiment showed that hesperidin could be a potential neuroprotective agent in the amelioration of PD and AD pathogenesis.

Keywords: Alzheimer's disease; amyloid-beta; alpha-synuclein; Drosophila melanogaster; negative geotaxis assay; Parkinson's disease

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INTRODUCTION

Neurodegenerative diseases are known as a group of neurological disorders characterized by progressive loss of brain and spinal cord cells, motor response impairment (ataxia) and sensory dysfunction (dementia) (Jeong, 2017, Minter et al., 2016, Wyss-Coray, 2016). The etiology of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (AD) are multifactorial including genetic mutation, environmental factors and brain aging which is the most important cause of neurodegenerative diseases, cellular and molecular factors such as potent production of oxidative stress, mutation in mitochondrial DNA, inflammatory responses, defective regulation of apoptosis etc. also play significant role in the pathogenesis of neurodegenerative diseases (Bredesen et al., 2006; Minter et al., 2016). AD and PD have been reported to be the leading causes of all reported cases of neurodegenerative disease accounting for over 10% causes of neurological disorders and over 60% causes of dementia in Nigeria (Adeloye et al., 2019, WHO, 2019). Most common features of AD and PD are misfolding and aggregation of amyloid-β42 and α-synuclein proteins into seeds which further distort similar proteins causing them to aggregate and produce pathogenic assemblies (Jucker and Walker, 2013).

Hesperidin (C28H34O15) is a flavanone glycoside, richly found in the citrus fruits such as lemon, sweet orange (*Citrus sinensis*), and grapefruits. Its antioxidant and neuroprotective actions have been reported (Kesh et al., 2021). We have also reported the potential of hesperidin in the enhancement of antioxidant defense, cholinergic/BDNF signaling and spatial learning in mice (Ishola et al., 2019). Due to the strong neuroprotective, memory enhancing and antioxidant effects of hesperidin (Ishola et al., 2019; Kesh et al., 2021).

The fruitfly Drosophila melanogaster is widely used to model neurodegenerative disease evidence in its potential in the identification of genes that are required to maintain the structural integrity of the brain, defined by recessive mutations that cause adult onset neurodegeneration (Lessing and Bonini, 2009). Moreover, flies genetics are instrumental in the analysis of neurodegenerative disease with better understanding of AD and PD. In addition, *Drosophila* has a short lifecycle of ~10 days to the adult with complex central nervous system with neurons and glia cells protected by a blood-brain barrier, and shares striking similarities with the vertebrate brain. The smaller size of the *Drosophila* genome (1.2×10^8 base pairs) compared to human genome

(3.3*10^9 base pairs) and smaller number of genes in *Drosophila* (~14,000 *vs*. ~20,000-25,000 protein-encoding genes) have an important implication. In *Drosophila*, the reduced genome complexity allows easier interpretation of loss-of-function studies. The UAS-GAL4 system is an efficient bipartite approach in the activation of gene expression in *Drosophila* (Duffy, 2002). It is widely used to drive gene expression in a multitude of cell- and tissue-specific patterns. The UAS, together with a specific gene of interest, is kept in one fly lines, and GAL4 with tissue-specific promoter is kept in another. When flies of these lines are crossed, the GAL4 protein will activate the UAS-linked gene in specific tissue. One advantages of this system is that toxic genes will only be expressed when bound to the GAL4 protein. This allows flies carrying the inactivated form of a toxic protein to survive normally. Thus, this study sought to evaluate the potential neuroprotective effect of hesperidin on neurodegenerative disease in *Drosophila melanogaster*.

MATERIALS AND METHODS

Hesperidin, malt-agar (Sigma Aldrich, St. Loius MO, USA), diethyl-ether (GuangdonngGuangbua Sci. Tech CO. Ltd. China), sugar, corn flour (Latyf food and beverages Ventures LTD, Ogun state, Nigeria), yeast (STK Industries Ltd, China), agar (Himedia Laboratories Pvt. Ltd, Mumbai, India), methyl-p-hydroxy benzoate, propanoic acid (LOBA Chemie Laboratory Reagents & fine Chemicals, Mumbai, India), orthophosphoric acid (Thermo Fischer Scientific, Mumbai, India), phosphate buffered saline (Gibco Technologies, USA).

***Drosophila Melanogaster* Fly Stock and Culture:** *Drosophila melanogaster* strains; UAS-Syn/Cyo, UAS-Aβ42/TM3, Elav-GAL4/FM, Ddc-GAL4/TM3, Canton-Special (CS), and Wild type (W1118), were kind gifts from Dr. Rakesh Mishra Drosophila Laboratory, Centre of Cellular & Molecular Biology, Hydrabad, India, for the purpose of this study. The flies were maintained at an optimal temperature of 23ºC (±2ºC) and 60% humidity. Flies were allowed to develop on a standard diet (31.5% Sugar, 29.7% Corn flour, 9.5% Yeast,7.1% Agar, 24.6% Malt, 0.045% Methyl-p-hydroxyBenzoic, 0.045% Propanoic acid and 0.01% Orthophosphoric Acid) in 50 ml plastic vials (15-20 flies per vial) and cultured under 12:12 hours day/night cycle. Flies were transferred every 4 days into another vial containing the standard diet (Ishola et al., 2021).

Collection of Female Virgins and Crossing: Virgin female flies were collected within 6 hours of eclosion at 23ºC (±2ºC) after confirming the presence of meconium at the upper quadrant of the ventro-lateral part of abdomen under a stereomicroscope (AmScope, MO, Nigeria). Ten virgin female flies each expressing Ddc-GAL4/TM3 and Elav-GAL4/FM were crossed with males carrying UAS-Syn/Cyo and UAS-Aβ42/TM3, respectively. Similarly, ten virgin female flies each expressing Ddc-GAL4/TM3 and Elav-GAL4/FM were crossed with males of W1118 as negative control.

Fecundity Assay: Ten female virgins and 5 male Canton-special (CS) strains were crossed and placed on the appropriate diets for fecundity assay. The numbers of eggs laid were counted at intervals of 24 hours for 3 days with the use of a stereomicroscope. This assay was carried out on three separate vials per experimental diets. Simultaneously, total numbers of dark pupa and freshly eclosed flies were recorded (Chattopadhyay et al., 2015).

Larval Motility Assay: At day 6 after crossing, larvae were assessed for larva motility assay. Viable (second instar) larvae were carefully extracted from the fly vials, washed in phosphate buffered saline and transferred onto 15 cm diameter petri dish, half-filled with freshly prepared, solidified 2% agarose solution. The petri dish was placed over a graph paper with a $0.1cm^2$ grid. The larvae were allowed to acclimatize for 10 sec, after which the numbers of grid lines crossed in 60 sec were counted and recorded (Nichols et al., 2012).

Negative Geotaxis Assay: Three separate vials containing 20 adult flies per group were used for the negative geotaxis assay (climbing assay) at the end of the experimental treatment days. The flies were anaesthetized and placed at the bottom of a clean measuring cylinder 15 cm tall. After recovery time of 15 mins, the measuring cylinder housing the flies was gently tapped to allow the flies settle at the bottom, and then the numbers of flies that crossed an height of 8cm within 8 sec was recorded. This was repeated periodically for 28 days at interval of 7day for each group (Poetini et al., 2018b, Shaltiel-Karyo et al., 2012).

Longevity Assay: Twenty (20) male/female flies per experimental groups were placed on standard fly diet at regulated temperature (18ºC - 23ºC). The flies were counted every 4 days and transferred to new diet vials. At each time point, the numbers of dead flies were counted until the entire flies were dead (Chattopadhyay et al., 2015).

Statistical Analysis

Data analyses were performed using GraphPad Prism software version 7 (GraphPad Software, Inc, CA, USA). Results were expressed as mean±standard deviation and analyzed using one or two-way analysis of variance (ANOVA) followed by Turkey's *Post hoc* multiple comparison tests.

RESULTS

Larva Motility Assay: First filial generation (F1) Larvae harvested from UAS-Aβ > elav-Gal4 or UAS-syn< DDC-Gal4 placed on the standard diet showed no significant change in locomotion activities (number of lines crossed) compared to W1118>elav-Gal4 (Fig. 1) or W1118 > DDC-Gal4, respectively (Fig. 2) (normal control). However, UAS-Aβ> elav-Gal4 [$F = 6.726$; $P= 0.002$] and UAS-syn>DDC-Gal4 [$F = 7.507$; $P< 0.05$] produced significant increase in locomotion activities of F1 larvae harvested from diet containing 5 or 10 mM of hesperidin compared to those placed on standard diet, respectively (Fig. 1 and 2). Conversely, F1 larvae harvested from 1mM of hesperidin diet showed no significant changes in locomotion activities compared to larvae of W1118>elav-Gal4 or W1118>DDC-Gal4.

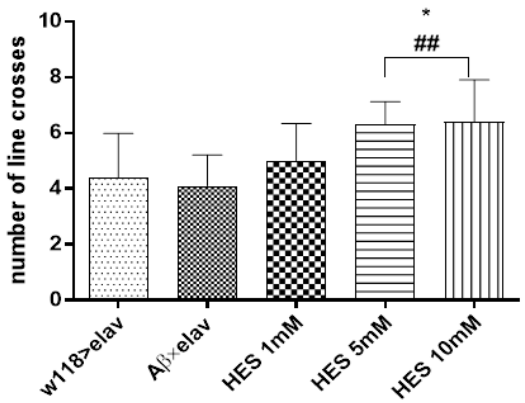

Figure 1:
Effect of hesperidin on F1 larva motility (number of line crosses in UAS-Aβ>elav-Gal4). (*****P < 0.05 compared to W1118> elav-Gal4, #P < 0.05, ##P < 0.01 compared to UAS-Aβ < elav-Gal4)

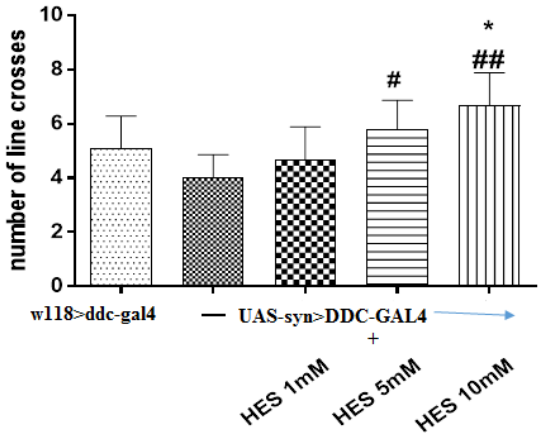

Figure 2:
Effect of hesperidin on UAS-syn < DDC-Gal4 F1 larvae motility (number of lines crossed). (***:** P < 0.05 compared to W1118 < DDC-Gal4, **#:** P < 0.05, **##:** P < 0.01 compared to UAS-syn < DDC)

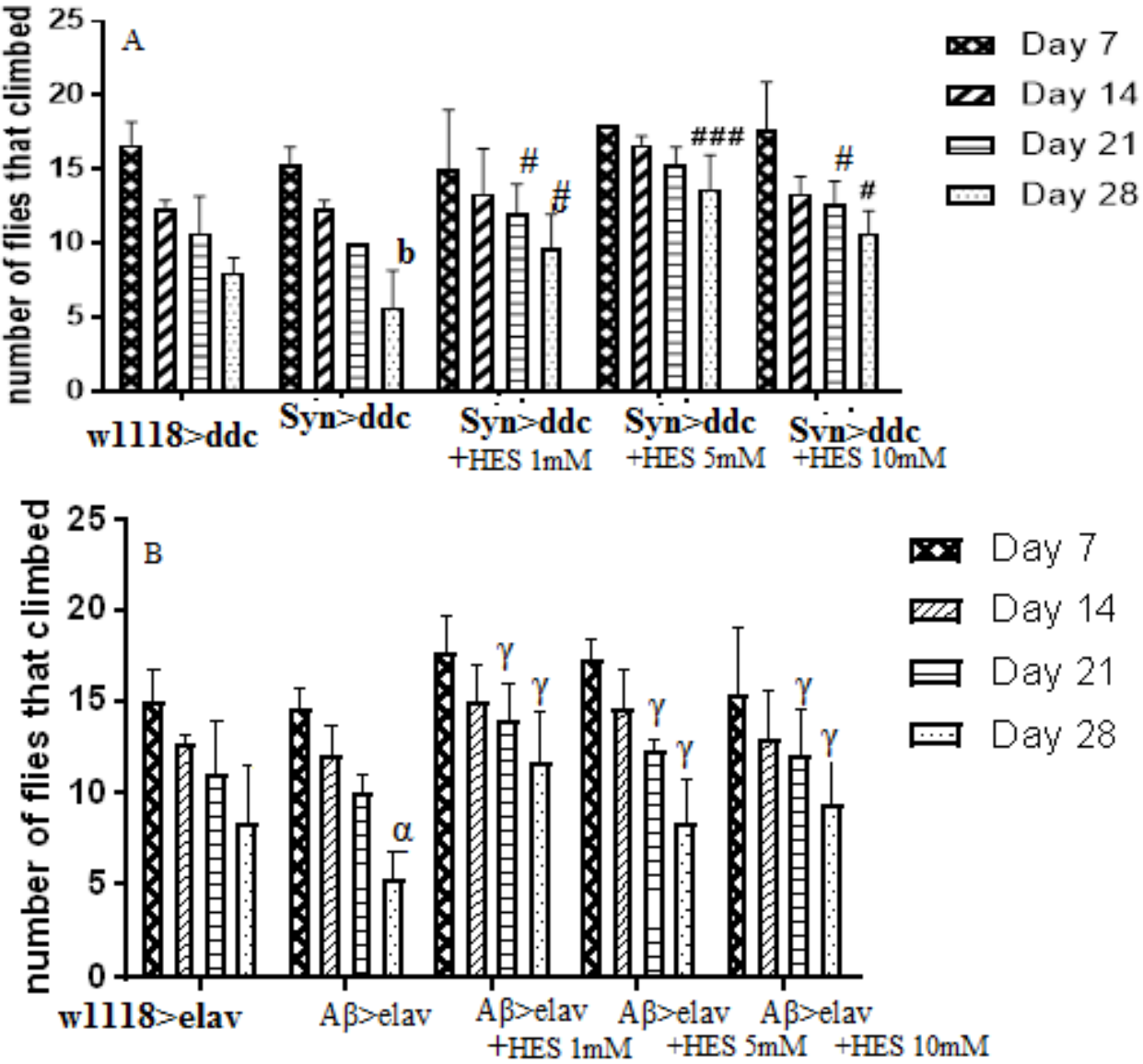

Figure 3A-B:
αEffect of hesperidin on climbing ability in (a) syn>ddc and (b) Aβ>elav adult flies. $^{b}p<0.01$ versus w1118>ddc; $^{\#}P< 0.05$,$^{\#\#\#}p<0.001$ versus syn>ddc; $^{\alpha}p<0.01$ versus w1118>elav; $^{\gamma}p<0.001$ versus Aβ>elav, statistical level of significance analysis by two way ANOVA followed by Tukey *post hoc* test.

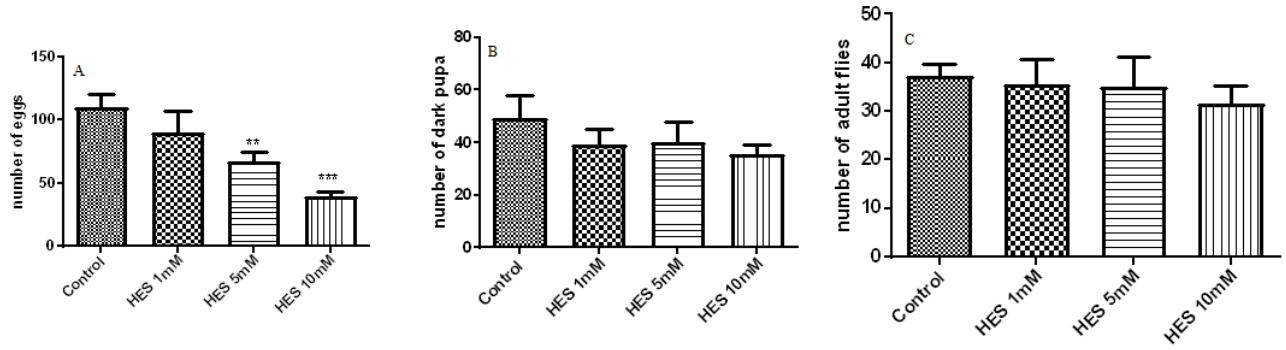

Figure 4a-c:
Effect of hesperidin on fecundity parameters in Cs flies (a) numbers of eggs laid, (b) number of dark pupa and (c) number of adult flies. Statistical level of significance analysis by one way ANOVA followed by Tukey post hoc test. ****P*<0.01, *****P*< 0.001 compared to control)

Climbing Assay

Effect of hesperidin on negative geotaxis in synuclein expressing flies: Figure 3a shows time course decrease in climbing activity across treatment groups. Syn>DDC showed significant decrease in negative geotaxis with peak deficit on day 28 when compared with w1118>ddc. α-syn aggregation in dopamine neuron induced motor deficit were significantly reversed in Syn>DDC flies placed on hesperidin 1, 5 and 10mM supplemented media. Post hoc analysis showed significant increase in number of flies that crossed the 8 cm mark at days 21 and 28. Post hoc analysis showed that adult flies expressing $A\beta_{42}$ pan-neuronally (elav) displayed significant time course decrease in climbing activity when compared with w1118>elav (Fig. 3b). however, neuronal aggregation of $A\beta_{42}$-induced motor deficits were reversed by supplementation of flies media with HES (1, 5 or 10mM).

Effect of hesperidin treatment on fecundity wild flies: Supplementation of flies' media with HES (5 or 10mM) caused statistically significant decrease in the numbers of eggs laid by CS flies when compared with control on standard diet (Fig. 4a). However, despite the decrease in number of eggs laid, we observed no significant change in number of dark pupa (Fig. 4b) and enclosed flies (Fig. 4c) when compared to the standard diet.

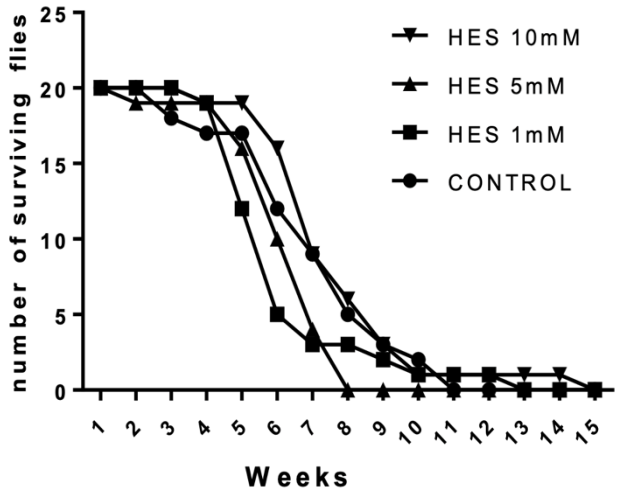

Figure 5:
Effect of hesperidin on Cs lifespan. Values are expressed as mean±SD. Statistical level of significance analysis by two way ANOVA followed by Dunnett's multiple comparison tests; p>0.05.

Lifespan Assay: The supplementation of fly media with hesperidin failed to confer significant change in lifespan in Cs flies (Fig. 5). No significant change in number of surviving flies when compared with the control on standard diet. Interestingly, flies cultured on HES 10mM media showed slight increase longevity when compared with control at the end of the 15th week.

DISCUSSION

Findings from this study showed that the UAS-GAL4 bipartite system increased the expression of α-syn in dopamine neuron and $A\beta_{42}$ pan-neuronally leading to motor deficits as observed in climbing assay which were ameliorated by hesperidin supplementation. Conversely, the expression of α-syn in dopamine neuron and $A\beta_{42}$ pan-neuronally did not affect larva motility. Moreso, hesperidin did not produce adverse effect on fecundity parameters in the present study.

The incidence of neurodegenerative disorders due to aging has been on the increase in Nigeria and Africa, with Parkinson's and Alzheimer's disease reported as the commonest form of neurodegenerative disorders in Nigeria and Sub-Saharan Africa (Lekoubou et al., 2014, Olayinka and Mbuyi, 2014). The advents of synthetic therapy including levodopa and acetylcholinesterase inhibitors have revolutionized the treatment of PD and AD, respectively but not without adverse events after prolonged use such as dyskinesia and failure to modify the aetiopathogenesis. Moreover, incidences of neuropsychiatric side effect associated with these therapies have also been on the increase, thus, limiting their use (Smith et al., 2012; Hashimoto et al., 2000; dos Santos Moraes et al., 2006).

Findings from our study showed that the expression of α-synuclein in dopamine neurons using UAS-GAL4 system might have contributed to the reduction in larva motility and progressive deterioration in negative geotaxis performance of the adult flies. Interestingly, supplementation of flies' media with hesperidin did not affect fecundity but improved spontaneous motor activities in both the larva and adult flies. The ability of hesperidin to reduce the expression of α--synuclein and Leucine-rich repeat kinase 2 (LRRK2) enzymes as well as its potential ability to inhibit the depletion of dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) have been reported as possible mechanism for

the improved motor functions (Antunes et al., 2014; Santos et al., 2016; Hajialyani et al., 2019). Similar to PD, incidence of motor dysfunction have been strongly associated with elderly people living with AD (Härlein et al., 2009; Buchman and Bennett, 2011). Accumulations of amyloid beta in the extracellular matrix in the neurons of the cortical region of the brain have been linked with the incidence of motor dysfunction and cognition impairment associated with AD (Pedrinolla et al., 2018, Reitz and Mayeux, 2014, Albers et al., 2015). It is worthy of note that in the present study, accumulation of amyloid beta protein pan-neuronal induced motor dysfunction in the larva but not in the adult flies. This is in agreement with report that flies expressing the mutation of human APP gene alone showed no statistically significant difference in motor dysfunction (Mhatre et al., 2014, Chakraborty et al., 2011; Holschneider et al., 2011, Shinotoh et al., 1999, Zhou et al., 2016)

Reduction in female fecundity and male fertility have been associated with accumulation of amyloid beta and alpha synuclein (Oriá et al., 2020, Naghavi, 2018). Hence, adjuvant for the management of these neurodegenerative disorders should have the potency of improving fecundity. In this study, hesperidin maintained the metamorphosis of dark pupa into adult flies. Scavenging of reactive oxygen species (ROS) by hesperidin have been highlighted as a possible mechanism for the ameliorative function in both Drosophila and rodent models of neurodegenerative diseases (Poetini et al., 2018a, Jayapalan et al., 2020). Also, its activities in suppressing various inflammatory signaling markers including glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule 1 (Iba-1), NF-kB and TNF-α and improvement of sexual hormones (FSH and LH) by enhancing the pituitary gland of the CNS have been described as potential mechanism for enhancing fecundity and fertility in neurodegenerative disorders (Justin-Thenmozhi et al., 2018; Hozayen, 2012).

Reductions in life span due to neuronal death and loss of systemic coordination by the brain have been strongly associated with neurodegenerative disorders including AD and PD. Cellular lipid peroxidation have been strongly linked as possible mechanism encouraging cell death in neurodegenerative disorders. Over the decade, improvements in cellular function and protection by the use of hesperidin, an antioxidant and anti-inflammatory phytochemical present in citrus, have been linked to its ability to inhibit accumulation of lipid peroxides in the extracellular matrix of neurons (Abolaji et al., 2017; Arumugam et al., 2018; Jayapalan et al., 2020). This could be the possible mechanism employed by hesperidin in the present study towards enhancing life span of CS flies.

In conclusion, findings of this study suggest that hesperidin, a bioflavonoid, could be a potential natural product for improved treatment of neurodegenerative diseases evidenced in its ability to improve lifespan and motor activity.

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REFERENCES

Abolaji, A. O., Babalola, O. V., Adegoke, A. K. & Farombi, E. O. (2017). Hesperidin, a citrus bioflavonoid, alleviates trichloroethylene-induced oxidative stress in Drosophila melanogaster. *Environmental toxicology and pharmacology,* 55**,** 202-207.

Adeloye, D., Auta, A., Ezejimofor, M., Oyedokun, A., Harhay, M. O., Rudan, I. & Chan, K. Y. (2019): Prevalence of dementia in Nigeria: a systematic review of the evidence. *Journal of global health reports,* 3.

Albers, M. W., Gilmore, G. C., Kaye, J., Murphy, C., Wingfield, A., Bennett, D. A., Boxer, A. L., Buchman, A. S., Cruickshanks, K. J. & Devanand, D. P. (2015): At the interface of sensory and motor dysfunctions and Alzheimer's disease. *Alzheimer's & Dementia,* 11**,** 70-98.

Antunes, M. S., Goes, A. T., Boeira, S. P., Prigol, M. & Jesse, C. R. (2014): Protective effect of hesperidin in a model of Parkinson's disease induced by 6-hydroxydopamine in aged mice. *Nutrition,* 30**,** 1415-1422.

Arumugam, M., Jayapalan, J. J., Abdul-Rahman, P. S., Hashim, O. H. & Subramanian, P. (2018): Effect of hesperidin on the temporal regulation of redox homeostasis in clock mutant (Cryb) of Drosophila melanogaster. *Biological Rhythm Research,* 49**,** 93-102.

Bredesen, D. E., Rao, R. V. & Mehlen, P. (2006): Cell death in the nervous system. *Nature,* 443**,** 796-802.

Buchman, A. S. & Bennett, D. A. (2011): Loss of motor function in preclinical Alzheimer's disease. *Expert review of neurotherapeutics,* 11**,** 665-676.

Chakraborty, R., Vepuri, V., Mhatre, S. D., Paddock, B. E., Miller, S., Michelson, S. J., Delvadia, R., Desai, A., Vinokur, M. & Melicharek, D. J. (2011): Characterization of a Drosophila Alzheimer's disease model: pharmacological rescue of cognitive defects. *PloS one,* 6**,** e20799.

Chattopadhyay, D., James, J., Roy, D., Sen, S., Chatterjee, R. & Thirumurugan, K. (2015): Effect of semolina-jaggery diet on survival and development of Drosophila melanogaster. *Fly,* 9**,** 16-21.

Davis, R. C., Marsden, I. T., Maloney, M. T., Minamide, L. S., Podlisny, M., Selkoe, D. J. & Bamburg, J. R. (2011): Amyloid beta dimers/trimers potently induce cofilin-actin rods that are inhibited by maintaining cofilin-phosphorylation. *Molecular neurodegeneration,* 6**,** 10.

Dos Santos Moraes, W. A., Poyares, D. R., Guilleminault, C., Ramos, L. R., Bertolucci, P. H. F. & Tufik, S. (2006): The effect of donepezil on sleep and REM sleep EEG in patients with Alzheimer disease: a double-blind placebo-controlled study. *Sleep,* 29**,** 199-205.

Duffy, J. B. (2002): GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *genesis,* 34**,** 1-15.

Hajialyani, M., Hosein Farzaei, M., Echeverría, J., Nabavi, S. M., Uriarte, E. & Sobarzo-Sánchez, E. (2019): Hesperidin as a neuroprotective agent: a review of animal and clinical evidence. *Molecules,* 24**,** 648.

Härlein, J., Dassen, T., Halfens, R. J. & Heinze, C. (2009): Fall risk factors in older people with dementia or cognitive impairment: a systematic review. *Journal of advanced nursing,* 65**,** 922-933.

Hashimoto, M., Imamura, T., Tanimukai, S., Kazui, H. & Mori, E. (2000): Urinary incontinence: an unrecognised adverse effect with donepezil. *The Lancet,* 356**,** 568.

Holschneider, D. P., Guo, Y., Roch, M., Norman, K. M. & Scremin, O. U. (2011): Acetylcholinesterase inhibition and locomotor function after motor-sensory cortex impact injury. *Journal of Neurotrauma,* 28**,** 1909-1919.

Hozayen, W. G. (2012) Effect of hesperidin and rutin on doxorubicin induced testicular toxicity in male rats. *Int J Food Nutr Sci,* 1**,** 31-42.

Irizarry, M. C., Growdon, W., Gomez-Isla, T., Newell, K., George, J. M., Clayton, D. F. & Hyman, B. T. (1998): Nigral and cortical Lewy bodies and dystrophic nigral neurites in Parkinson's disease and cortical Lewy body disease contain α-

synuclein immunoreactivity. *Journal of Neuropathology & Experimental Neurology*, 57**,** 334-337.

Ishola I.O., Jacinta Aa, Adeyemi O.O.. (2019) Cortico-hippocampal memory enhancing activity of hesperetin on scopolamine-induced amnesia in mice: role of antioxidant defense system, cholinergic neurotransmission and expression of BDNF. Metab Brain Dis.;34(4):979-989.

Ishola I.O., Badru A, Ofi Eo, Akinleye Mo, Adeyemi O.O. (2021): Ameliorative influence of atorvastatin in transgenic *Drosophila Melanogaster* model of neurodegenerative diseases *Nigerian Journal of Pharmacy 55 (1) 40-45*

Jayapalan, J. J., Subramanian, P., Kani, A., Hiji, J., Najjar, S. G., Abdul-Rahman, P. S. & Hashim, O. H. (2020): Hesperidin modulates the rhythmic proteomic profiling in Drosophila melanogaster under oxidative stress. *Archives of Insect Biochemistry and Physiology***,** e21738.

Jeong, S. (2017):. Molecular and cellular basis of neurodegeneration in Alzheimer's disease. *Molecules and cells*, 40**,** 613.

Jucker M, Walker L.C. (2013): Self-propagation of pathogenic protein aggregates in neurodegenerative diseases. Nature; 501(7465):45-51.

Justin-Thenmozhi, A., Bharathi, M. D., Kiruthika, R., Manivasagam, T., Borah, A. & Essa, M. M. (2018): Attenuation of aluminum chloride-induced neuroinflammation and caspase activation through the AKT/GSK-3β pathway by hesperidin in wistar rats. *Neurotoxicity research*, 34**,** 463-476.

Kesh S, Kannan Rr, Sivaji K, Balakrishnan A. (2021): Hesperidin downregulates kinases lrrk2 and gsk3β in a 6-OHDA induced Parkinson's disease model. Neurosci Lett. 2021;740:135426.

Lekoubou, A., Echouffo-Tcheugui, J. B. & Kengne, A. P. (2014): Epidemiology of neurodegenerative diseases in sub-Saharan Africa: a systematic review. *BMC public health*, 14**,** 653.

Mhatre, S. D., Satyasi, V., Killen, M., Paddock, B. E., Moir, R. D., Saunders, A. J. & Marenda, D. R. (2014): Synaptic abnormalities in a Drosophila model of Alzheimer's disease. *Disease models & mechanisms*, 7**,** 373-385.

Minter, M. R., Taylor, J. M. & Crack, P. J. (2016): The contribution of neuroinflammation to amyloid toxicity in Alzheimer's disease. *Journal of neurochemistry*, 136**,** 457-474.

Naghavi, M. H. (2018): " APP" reciating the complexity of HIV-induced neurodegenerative diseases. *PLoS pathogens*, 14**,** e1007309.

Nichols, C. D., Becnel, J. & Pandey, U. B. (2012): Methods to assay Drosophila behavior. *JoVE (Journal of Visualized Experiments)***,** e3795.

Olayinka, O. O. & Mbuyi, N. N. (2014): Epidemiology of dementia among the elderly in Sub-Saharan Africa. *International Journal of Alzheimer's disease,* 2014.

Oriá, R. B., De Almeida, J. Z., Moreira, C. N., Guerrant, R. L. & Figueiredo, J. R. (2020): Apolipoprotein E Effects on Mammalian Ovarian Steroidogenesis and Human Fertility. *Trends in Endocrinology & Metabolism*.

Pedrinolla, A., Venturelli, M., Fonte, C., Munari, D., Benetti, M. V., Rudi, D., Tamburin, S., Muti, E., Zanolla, L. & Smania, N. (2018): Exercise training on locomotion in patients with Alzheimer's disease: a feasibility study. *Journal of Alzheimer's Disease*, 61**,** 1599-1609.

Poetini, M., Araujo, S., Trindade, D. P. M., Bortolotto, V., Meichtry, L., Polet, D. A. F., Jesse, C., Kunz, S. & Prigol, M. (2018a): Hesperidin attenuates iron-induced oxidative damage and dopamine depletion in Drosophila melanogaster model of Parkinson's disease. *Chemico-biological interactions*, 279**,** 177.

Poetini, M. R., Araujo, S. M., De Paula, M. T., Bortolotto, V. C., Meichtry, L. B., De Almeida, F. P., Jesse, C. R., Kunz, S. N. & Prigol, M. (2018b): Hesperidin attenuates iron-induced oxidative damage and dopamine depletion in Drosophila melanogaster model of Parkinson's disease. *Chemico-Biological Interactions*, 279**,** 177-186.

Reitz, C. & Mayeux, R. (2014): Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers. *Biochemical pharmacology*, 88**,** 640-651.

Santos, G., Giraldez-Alvarez, L. D., Ávila-Rodriguez, M., Capani, F., Galembeck, E., Neto, A. G., Barreto, G. E. & Andrade, B. (2016): SUR1 receptor interaction with hesperidin and linarin predicts possible mechanisms of action of Valeriana officinalis in Parkinson. *Frontiers in aging neuroscience*, 8**,** 97.

Shaltiel-Karyo, R., Davidi, D., Menuchin, Y., Frenkel-Pinter, M., Marcus-Kalish, M., Ringo, J., Gazit, E. & Segal, D. (2012): A novel, sensitive assay for behavioral defects in Parkinson's disease model Drosophila. *Parkinson's disease,* 2012.

Shinotoh, H., Namba, H., Yamaguchi, M., Fukushi, K., Nagatsuka, S. I., Iyo, M., Asahina, M., Hattori, T., Tanada, S. & Irie, T. (1999): Positron emission tomographic measurement of acetylcholinesterase activity reveals differential loss of ascending cholinergic systems in Parkinson's disease and progressive supranuclear palsy. *Annals of neurology*, 46**,** 62-69.

Smith, G. A., Heuer, A., Dunnett, S. B. & Lane, E. L. (2012): Unilateral nigrostriatal 6-hydroxydopamine lesions in mice II: predicting l-DOPA-induced dyskinesia. *Behavioural brain research*, 226**,** 281-292.

Wenk, G. L. 2003. Neuropathologic changes in Alzheimer's disease. *The Journal of Clinical Psychiatry*.

W.H.O 2019. Dementia fact sheet. . May 14, 2019. ed.: World Health Organisation.

Wirdefeldt, K., Adami, H.-O., Cole, P., Trichopoulos, D. & Mandel, J. (2011):. Epidemiology and etiology of Parkinson's disease: a review of the evidence. *European Journal Of Epidemiology*, 26**,** 1.

Wyss-Coray, T. (2016): Ageing, neurodegeneration and brain rejuvenation. *Nature*, 539**,** 180-186.

Zhou, L., Tan, S., Shan, Y.-L., Wang, Y.-G., Cai, W., Huang, X.-H., Liao, X.-Y., Li, H.-Y., Zhang, L. & Zhang, B.-J. (2016): Baicalein improves behavioral dysfunction induced by Alzheimer's disease in rats. *Neuropsychiatric Disease and Treatment*,12**,** 3145.

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Full Length Research Article

Maternal exposure to Bonny Light Crude Oil Altered Reproductive indices in Male and Female offspring of Wistar rats

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Summary: In this study, the effects of maternal exposure to Bonny Light Crude Oil (BLCO) on reproductive functions of the offspring was investigated in Wistar rats. Ten pregnant rats were divided into two groups (n=5). Group 1 served as the control, it was administered 0.75ml/Kg bwt/day normal saline and Group 2 was administered 0.75ml/Kg bwt/day BLCO. Serum hormonal profile, sperm indices, estrous cycle length and pubertal timing were assessed as measures of reproductive function. Tissue Malondialdehyde, Catalase and SOD activities were assessed as indices of oxidative stress. Results obtained showed that BLCO significantly ($p<0.05$) reduced birth weight, anogenital distance (AGD) at birth, sperm count, motility and normal morphology, serum testosterone, testicular and epididymal SOD and catalase activities in the male offsprings. However, days of preputial separation, relative weight of testis and epididymis, testicular and epididymal MDA were significantly ($p<0.05$) raised by gestational exposure to BLCO. In the female offspring, birth weight, AGD at birth, relative weight of ovaries and uterus, SOD, catalase activities, serum LH were significantly reduced by BLCO exposure during gestation. Moreover, uterine and testicular MDA, serum estradiol and FSH were significantly increased by BLCO treatment during gestation. In conclusion, maternal exposure to BLCO during gestation may alter reproductive indices in the offspring and increased occurrence of oxidative stress in reproductive structures in male and female offspring of Wistar rats.

Keywords*: Bonny Light Crude Oil, Reproductive function, offspring, gestation, oxidative stress*

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INTRODUCTION

Infertility is a clinical problem that affect people medically, and also affects the family stability. According to Giwa-Osagie (2003) about 10-25% of couples in African countries are sub-fertile and male factor infertility accounts for 30-40% of these cases. Recent studies have shown that reproductive dysfunction can be epigenetically induced due to exposure to environmental agent such as heavy metals and other environmental pollutants including crude oil (Ola-Mudathir *et al*., 2008; Fischer *et al*., 2013). Exposure of humans to disruptive chemicals appears to be related to various reproductive health problems such as decreased fertility, menstrual disorders, impaired spermatogenesis, cryptorchidism, hypospadias, low birth weight, structural and functional birth defects and postnatal developmental defects (Kumar, 2008). Research from a wide range of scientific disciplines have shown that the reproductive performance of animals at adult life is determined, in part, by a variety of endocrine disruptors acting at different stages of development from fetal to early neonatal life (Jeje and Raji, 2017). These effects are probably mediated through changes in the hypothalamic-pituitary-gonadal axis but the physiological system that is affected depends on the stage of development at which the influence is applied (Stewart *et al*., 2001).

Bonny Light Crude Oil (BLCO) is characterized by low Sulphur content and low corrosive property (Fischer *et al*., 2013). It was first associated with Bonny Island area of southern edge of Rivers State in the Niger Delta region of Nigeria. In Nigeria, frequent oil spills resulting from pipeline vandalism, theft and poor maintenance are major source of environmental pollution. When crude oil or other petroleum products leak into the environment, the different compounds evaporate into the air and are absorbed by the soil and water, crop or fish consumed by humans. Previous studies have suggested that BLCO is a potent reproductive toxicant and an anti-androgenic agent (Orisakwe *et al*., 2004; Fischer *et al*., 2013). In addition, BLCO induced alterations in liver mitochondria DNA concentration and increased the binding of nickel to chromatin proteins in guinea pig (Oruambo *et al*., 2007). Therefore, crude oil and its constituent hydrocarbons have been suggested to be responsible for at least some component of infertility/ sub - fertility in the Nigerian population. This study is aimed at investigating the effect of maternal exposure to BLCO during gestation on reproductive function of the offspring in Wistar rats.

MATERIALS AND METHODS

Animals: Adult male and female Wistar rats (10 weeks old, weight 180-200g and 150-170g for male and female respectively) obtained from the Department of Physiology Animal House, Federal University of Technology, Akure, Nigeria were used. These animals were housed in cages in the Department of Physiology Animal House and had access to food (Ladokun Feeds Limited, Ibadan, Nigeria) and water for the entire duration of the study *ad libitum*. The females were nulliparous and the males used for the mating were

proven male breeder. The animals were kept under standard laboratory condition. Animals were allowed to acclimatize for 2 weeks to the laboratory conditions. The study was conducted in accordance with the International Ethical Norms on Animal Care and Use as contained in NIH publication/80-23, revised in 2010.

Bonny Light Crude Oil (BLCO): BLCO was obtained from the Nigerian National Petroleum Corporation (NNPC) Warri, Nigeria**.** A daily oral dose 0.75ml of BLCO/Kg bwt/day was administered to the treatment group.

Experimental Protocol: In total, 10 female Wistar rats (10 weeks; 150–170 g) with normal estrous cycle were used. The estrous cycle of the rats was monitored daily according to the method described by Marcondes *et al*. (2002). Rats in proestrous were mated with proven male breeder at a ratio of 1:1 overnight and the presence of sperm in their vaginal or copulatory plug in the next morning marked gestational day (GD 1). After pregnancy had been confirmed, animals were randomly assigned into two groups of five animals each and treated accordingly during gestation. Administration was carried out through oral gavage between 8 am and 10 am daily at gestation days 1-21. Group 1 was administered 0.75ml of distilled water/kg bwt/day (control). Group 2 was administered 0.75ml of BLCO/Kg bwt/day. The litter size was standardized to six pups per litter. After delivery, the following parameters were measured: body weight at birth, postnatal day (PND) 21 (at end of weaning) and PND 90 (at adulthood). Malondialdehyde (MDA) levels, catalase and superoxide dismutase (SOD) activities in the homogenate of the testis and epididymis (Male)/Ovary and uterus (Female) were also assessed at PND 90. Serum testosterone, LH and FSH levels as well as the sperm indices (Motility, Morphology, and Counts) were assessed in the male offspring. Serum estradiol, LH and FSH, estrous cycle length and frequency were assessed in female offspring. Pubertal timing was also assessed in both male and female offsprings.

Serum and Tissue Collection: Blood samples were collected from the orbital sinus of male and female offsprings under Sodium thiopentone anaesthesia (50 mg/kg, i.p.) at PND 90 days into polythene tubes and allowed to clot for 1 h. The blood samples were then centrifuged at 3000 rpm for 10 min. Serum was aspirated and stored at 4°C.

After blood sample collection, the rats were carefully sacrificed by cervical dislocation. During dissection, the testes and epididymis in male/ovary and uterus in females were carefully collected and rinsed in ice-cold 1.15% KCl solution. Dry weights of the tissues were recorded. They were thereafter placed in 0.1 M potassium phosphate buffer pH 6.5 and homogenized using a homogenizer. The homogenate was centrifuged in a cold centrifuge at 10,000 rpm for 10 min. The supernatant was removed and stored in a refrigerator (at about 4◦C) for analysis of oxidative stress. Biochemical analysis was done within 48 hrs of sample collection.

Biochemical Assays

Determination of Oxidative Stress (Lipid Peroxidation Assessment): Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation. This was carried out according to the methods described by Buege and Aust (1978).

Determination of Catalase Activity: Catalase activity was determined according to the method of Claiborne (1985). The method is based on the loss of absorbance observed at 240 nm as catalase splits hydrogen peroxide. Despite the fact that hydrogen peroxide has no absorbance maximum at this wavelength, its absorbance correlates well enough with concentration to allow its use for a quantitative assay. An extinction coefficient of 0.0436 mM-1cm-1 was used.

Determination of Superoxide Dismutase (SOD) Activity.: SOD activity was evaluated according to methods of Misra and Fridovich (1972). The ability of superoxide dismutase to inhibit the autooxidation of adrenaline at pH 10.2 makes this reaction a basis for the SOD assay. Superoxide anion (O_2) generated by the xanthine oxidase reaction is known to cause the oxidation of adrenaline to adrenochrome. The yield of adrenochrome produced per superoxide anion increased with increasing pH and also with increasing concentration of adrenaline. These led to the proposal that autooxidation of adrenaline proceeds by at least two distinct pathways, one of which is a free radical chain reaction involving superoxide radicals, and hence could be inhibited by SOD.

Determination of Tissue Protein Level: Protein estimation was done by method of Lowry *et al*. (1951). The Folin-Ciocalteau reagent was used in the quantification of proteins by Lowry. In its simplest form, the reagent detects tyrosine residues due to their phenolic nature. The reaction of a protein in solution with the Folin reagent occurs in two stages: Reaction with Cu^{++} in alkaline medium and reduction of the phosphomolybdic-phosphotungstic reagent by the Cu^{++-} protein complex. The reduced complex gives a blue solution with an absorption in the red portion of the visible spectrum (600–800 nm).

Determination of anogenital distance: Anogenital distance (AGD) at birth was determined by using a digital Vernier calliper to measure the distance between the posterior base of the sex papilla and the anterior anus at PND 1.

Detecting Testes Descent: The rats were studied daily and the days testes descent was noticed and recorded (Ostby and Gray, 2004).

Detecting Puberty in Male and Female Offsprings: To detect the periputial separation (PPS), male rats were checked daily beginning after testis descent was noticed in the rat to ensure no rats have periputial separation. This was done by applying gentle pressure to the prepuce to retract the prepuce and expose the glans penis. PPS is complete when the entire perimeter of the prepuce can be retracted evenly around the base of the glans penis (Ostby and Gray, 2004). The day of vaginal opening was recorded and taken as the onset of puberty in female (Ostby and Gray, 2004).

Determination of Sperm Indices: Sperm analysis was done by microscopy as previously described (Raji and

Bolarinwa, 1997; Raji *et al*., 2003). Epididymal spermatozoa were obtained by mincing the epididymis with anatomical scissors in 5ml of pre-warmed physiological saline and incubated for 2 min. An aliquot of this solution was placed in improved Neubauer counting hemocytometer and motile sperm were counted by using microscope at 400× magnification. Non-motile sperm numbers were first determined, followed by counting of total sperm. Sperm motility was expressed as a percentage of motile sperm of the total sperm counted. Percentage of morphologically abnormal spermatozoa was determined by preparing two slides with Hemaoxylin and Eosin stains for morphological examination of live–dead ratio. A total of 400 sperm cells were counted on each slide under light microscope at 400× magnifications. Sperms with abnormal head and/or tail were considered abnormal. Sperm motility, viability and count were done immediately and quickly. A sperm viability test was done using eosin/negrosin stain (containing 1 g of Eosin and 4 g of Negrosin in 100 ml phosphate buffer). A drop of the epididymal fluid was placed on the slide and two drops of the stain was added. A thick smear was made from this and dried. After this, the slide was studied under light microscope using 40x objective lens. The unstained spermatic cells were considered as live sperms while the stained ones was considered as dead sperm. A minimum of 100 spermatic cells (both stained and unstained) was counted and an average was taken for the percentage live sperm.

Determination of Estrous cycle

Vagina cytology: Using Marcondes technique (Marcondes *et al*., 2002), about 0.1ml of 0.9% normal saline solution was gently introduced 2-3 times into the vagina of the rat to produce a vaginal lavage. The pipette was withdrawn and its content was smeared on a microscope slide and viewed using x40 magnification lens of the microscope. Estrous cycle lasted about 4-5days extending from the day of proestrus characterized by the presence of nucleated vaginal epithelial cells followed by the estrus phase presenting cornified vaginal mucosa cells, metestrus, was a combination of the cells and the diestrus phase, characterized by the presence of leukocytes in the vaginal smear.

At the end of the experiments animals of control and BLCO treated groups were euthanised.

Statistical analysis: Data were expressed as means ± S.E.M. Statistical comparisons were performed using independent student t-test. Differences between the treatment groups with a P-value < 0.05 were considered significant. Data were analyzed with the use of GraphPad Prism software version 8.02® (LA Jolla, CA, USA).

RESULTS

Effects of maternal exposure to BLCO during gestation on birth weight anogenital distance, testis descent and preputial separation/vaginal opening of the offspring of Wistar rats: There was a significant reduction (P<0.05) in birth weight and anogenital distance (AGD) in the male and female offspring of rats exposed to BLCO during gestation (Table 1) when compared with the control. There was also a significant increase (P<0.05) in the days of testis descent and preputial separation in the male offspring as well as vaginal opening in the offspring of rats exposed to BLCO during gestation relative to the control (Table 2).

Table 1:
The morphometric indices of the male offspring following maternal treatment with BLCO during gestation

Group/	Birth weight (g)	AGD at Birth (cm)	Preputial separation (days)	Testis descent (days)
Control	7.78 ±0.03	4.21 ±0.01	53.00 ±0.78	17.00 ±1.21
Treatment (BLCO)	4.98 ±0.00*	3.31 ±0.03*	65.60 ±0.40*	23.00 ±1.01*

**the mean is statistically significant in the treatment group when compared with the control*

Table 2:
The morphometric indices of the female offspring following maternal treatment with BLCO during gestation

Group/	Birth weight (g)	AGD at Birth (cm)	Vaginal opening	Estrous cycle length (days)
Control	7.58 ±0.32	2.34 ±0.01	47.00± 0.575	4.700 ± 0.03
Treatment (BLCO)	5.72 ±0.31*	1.82 ±0.01*	55.60 ±0.43*	4.50 ±0.04

*the mean is statistically significant in the treatment group when compared with the control

Effects of maternal exposure to BLCO during gestation on relative testicular and epididymal weight of the Male offspring of Wistar rats: Maternal exposure to BLCO during gestation significantly raised (P<0.05) the relative testis, epididymal, ovary and uterine weight in the offspring of Wistar rats when compared with the control (Figure 1 and 2).

Effects of maternal exposure to BLCO during gestation on sperm indices (count, motility and morphology) of the male offspring of Wistar rats: There was a significant reduction in sperm count and sperm motility of the male offspring of Wistar rats exposed to BLCO during gestation. However, percentage abnormal morphology sperm cells was significantly raised in the group exposed to BLCO during gestation when compared with the control group (Figure 2, 3 and 4).

Effects of maternal exposure to BLCO during gestation on length of estrous cycle of the female offspring of Wistar rats: There was no significant difference in the overall length of the cycle, length of proestrus and estrous phase in the female offspring of the BLCO treated rats and control. However, the length of metestrous was raised while that of diestrous was reduced (p<0.05).

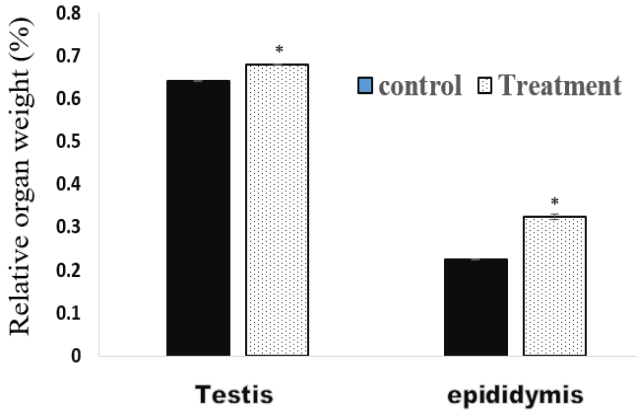

Figure 1:
Relative organ weight of the male offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

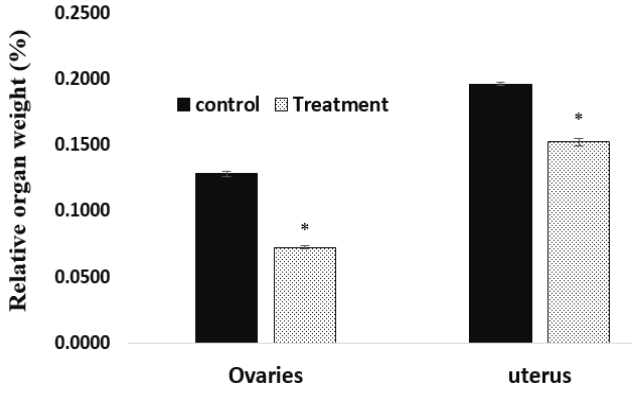

Figure 2:
Relative organ weight of the female offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

Effects of maternal exposure to BLCO during gestation on serum hormonal profile of the Male (FSH, LH and Testosterone) and female (estradiol, FSH and LH) offsprings of Wistar rats: Serum testosterone, was significantly reduced in the male offspring of BLCO group at 12 weeks of postnatal life relative to the control. However, FSH level was significantly raised ($p<0.05$) in the male offspring of BLCO treated group when compared with the control. In the female offspring, there was a significant increase in the serum estradiol and FSH level. Meanwhile serum LH level was significantly reduced ($p<0.05$) at 12 weeks of postnatal life in the female offspring of BLCO group when compared with the control.

Table 3:
The sperm indices of the male offspring following maternal treatment with BLCO during gestation

Group/	Sperm count (10^6/ml)	Sperm Motility (%)	Sperm Morphology (Abnormal) (%)
Control	185.10 ±4.31	84.00 ±2.12	47.00 ±0.70
Treatment (BLCO)	174.20 ±2.90*	62.00 ±1.930*	67.40 ±0.61*

**Mean is statistically significant in the treatment group when compared with the control*

Table 4:
The estrous cycle length of the female offspring following maternal treatment with BLCO during gestation

Group/	Proestrous (%)	Estrous (%)	Diestrous (%)	Metestrous (%)
Control	13.93 ± 1.21	14.42 ± 0.04	53.20 ± 0.24	17.90 ±0.09
Treatment (BLCO)	13.00 ±0.92	14.50 ±0.36	48.40± 0.29*	24.50± 0.72*

*the mean is statistically significant in the treatment group when compared with the control

Effects of maternal exposure to BLCO during gestation on indices of oxidative stress in the Male (testes and epididymis) and female (ovaries and uterus) offspring of Wistar rats: The level of malondialdehyde (MDA) was significantly raised in the ovaries and uterus of the BLCO treated female offsprings of Wistar rats as compared to the control. Catalase activity was significantly decreased in the ovaries and uterus of the BLCO treated female offspring of wistar rats relative to the control. The level of superoxide dismutase (SOD) was significantly lower in the ovaries and uterus of the BLCO treated female offsprings of Wistar rats relative to the control. The testicular and epididymal MDA was significantly raised in the male offsprings. The SOD and Catalase activities were significantly reduced in the testis and epididymis of the male offsprings exposed to BLCO during gestation.

Table 5:
The serum hormonal profile of offspring following maternal treatment with BLCO during gestation

	Male			Female		
Group/	Testosterone (nmol/l)	FSH (mlU/ml)	LH (mlU/ml)	Estradiol (pg/ml)	FSH (mlU/ml)	LH (mlU/ml)
Control	0.69± 0.02	0.35± 0.002	0.26± 0.00	15.55±0.39	0.59± 0.05	0.52± 0.31
Treatment (BLCO)	0.57± 0.01*	0.51± 0.01*	0.25± 0.00	17.92±0.37*	0.92± 0.03*	0.09± 0.00*

*the mean is statistically significant in the treatment group when compared with the control

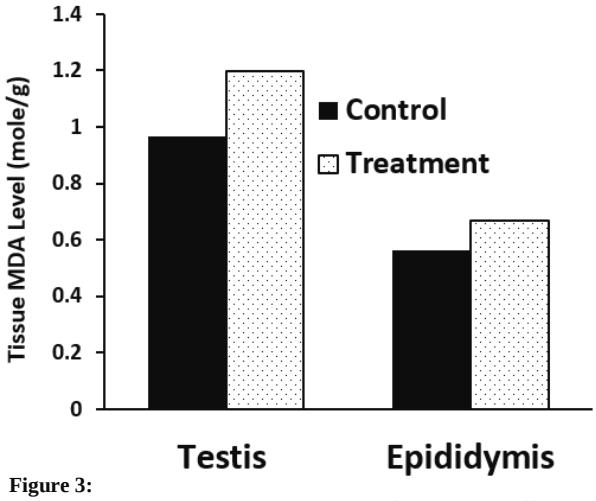

Figure 3:
Tissue Malondialdehyde (MDA) level of the male offsprings following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

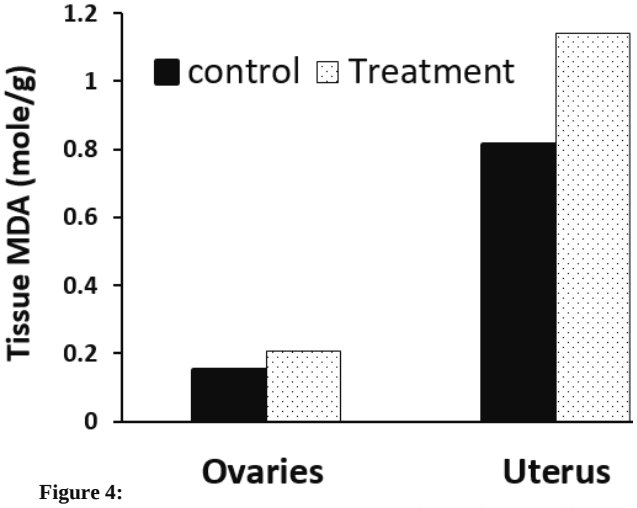

Figure 4:
Tissue Malondialdehyde (MDA) level of the female offspring following maternal exposure to BLCO during gestation in wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

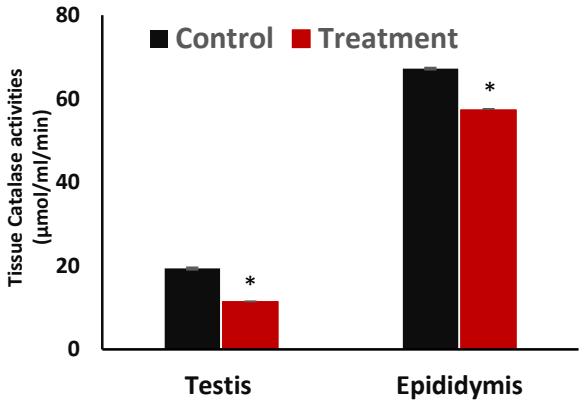

Figure 5:
Tissue Catalase activities of the male offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

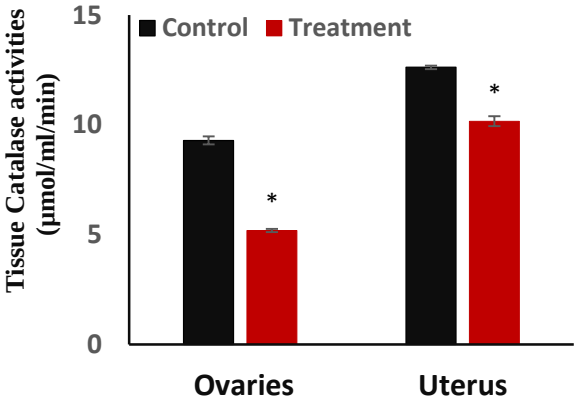

Figure 6:
Tissue Catalase activities of the female offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats.
**Mean is statistically significant in the treatment group when compared with the control*

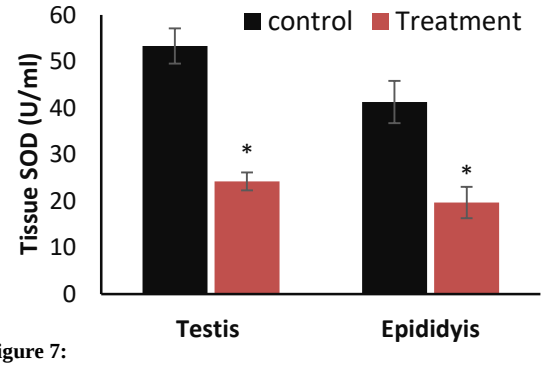

Figure 7:
Tissue Superoxide Dismutase (SOD) activities of the male offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

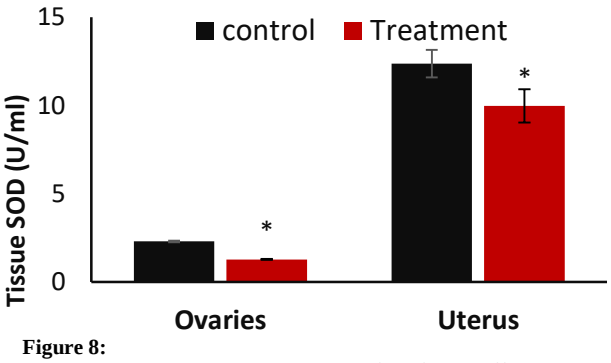

Figure 8:
Tissue Superoxide Dismutase activities of the female offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

DISCUSSION

Several environmental pollutants have been reported to interfere with the endocrine system. Many of these endocrine disruptors are released into the environment due to several human activities such as industrial and manufacturing activities, mining and oil exploration activities (Darbre, 2018). In this study, exposure to BLCO during gestation significantly reduced birth weight, relative ovarian weight and uterine weight of the BLCO treated

female offspring of Wistar rats. The decrease in the F1 offspring body weight and relative organ weight may be a pointer to reduction in the growth and development of reproductive organs (Tutian *et al*., 2008). Barker's hypothesis postulates that an infant's birth weight (BW) is influenced by the intrauterine environment, and an adverse intrauterine environment altered the expression of certain genes that control the development and function of organs and tissues (De Boo & Harding, 2006). In agreement with this observation BLCO and its constituent hydrocarbon (such as Polycyclic Aromatic Hydrocarbon (PAF) have been previously reported to induce developmental malformation in the offsprings (Feuston and Hamilton, 1997; Fischer *et al*., 2007).

Disorders in sex steroid balance during fetal development generally affects the reproductive system development (Sharpe, 2001). Reduction in proportion of androgen to estrogen level and exposure to other anti-androgenic agents induced gross alteration in male reproductive structures and functions from fetal life (Sharpe, 2001; Rivas *et al*., 2002; Welsh *et al*., 2008). In agreement with this observation, sperm indices, anogenital distance at birth and serum testosterone level were reduced in the male offspring following maternal treatment with BLCO during gestation in this study. In addition, pubertal timing, testis descent was also delayed. These indices are generally known to be androgen dependent (Ostby and Gray, 2004; Jeje and Raji, 2017). These suggest that BLCO exposure during gestation may affect the androgen production and or released from the testis. If the alteration in testosterone level is centrally mediated required further assessment. We observed that serum LH level was not significantly influenced by the low testosterone in the male offspring. Normally, testosterone released is regulated through a negative feedback mechanism that release LH from the anterior pituitary gland (Aron *et al*., 2007). Disruption in the negative feedback mechanism could therefore affect reproductive functions (Aron *et al*., 2007).

Irregularity in estrous cycle was observed in the female offspring of treated rat's, however, there was no statistical difference in the length of estrous cycle of treated rat F1 offspring and the control F1 offspring. This is in agreement with Raji and Hart (2012), who also reported no significant difference in length of estrous cycle following pre and post treatment with BLCO at different doses in female Wistar rats. This indicates that BLCO sub-lethal administration might not interfere in oestrous cycle and ovarian cycle activities of not just the rats exposed but also in the F1 female offspring. Conversely, there were changes at each phase of the cycle, length of estrus and proestrus were insignificantly different but there was a significant increase in metestrus phase and a significant decrease in diestrus phase.

The increase in metestrus phase indicates the availability of matured Graafian follicles and maturation of secondary follicles, suggesting that ovulation was inhibited since length of estrus phase was not significantly different (Shrestha *et al*., 2010). Diestrus is characterized by the elevation in circulating progesterone from the corpus luteum that rises shortly after ovulation and it persist until luteolysis occurs because at the diestrus phase, the corpus luteum now actively secretes progesterone. Therefore, a shorter diestrus length in the treated rats offspring may further worsen distorted ovarian cycle, consequently reducing oocyte number that may ultimately reduce fertility and number of viable offspring (Raji, and Hart., 2012).

Reports from several studies have implicated oxidative stress in the pathogenesis of infertility (Ola-Mudathir *et al*., 2008). Oxidative stress is linked with high level of reactive oxygen species which results in lipid peroxidation of the spermatozoa outer membrane. This results in loss of motility (Urata *et al*., 2001), decrease sperm-oocyte fusion capacity and increased cell destruction due to chromatin damage (Aitken, 1994; Aitken and Krausz, 2001). Therefore, for the protection of reproductive system from oxidative stress, the testis, epididymis and spermatozoa have the capacity to produce antioxidant enzymes such as catalase, SOD, glutathione reductase (Tramer *et al*., 1998; Zubkova and Robaire; 2004). The result from this study suggests a significant reduction in the antioxidant enzyme SOD and catalase with an increased in the level of by-product of lipid peroxidation (MDA) in the reproductive structures of male and female offsprings. This suggests the possibility of an increase exposure to oxidative damage in the reproductive structures following maternal exposure to BLCO during gestation.

In conclusion, maternal exposure to bonny light crude oil during gestation may induced alterations in reproductive functions in the male and female offspring. In addition, the reproductive structures may be more susceptible to oxidative stress.

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REFERENCES

Aitken R.J. (1994). A free radical theory of male infertility. Reprod Fertil Dev 6: 19–23.

Aitken R.J and Krausz C. (2001). Oxidative stress, DNA damage and the Y chromosome. Reproduction. 122: 497–506.

Aron D.C., Finding J.W. and Tyrrell J.B. (2007). Glucocorticoids and adrenal androgen;Greenspan‟s Basic and Clinical Endocrinology (David and Shoback) 8th edition McGraw Hill pp 246-295

Buege J.A and Aust S.D. (1978). Microsomal lipid peroxidation. Methods in enzymology 52, 302-310, 1978.

Darbre PD. Overview of air pollution and endocrine disorders (2018). Int J Gen Med. 2018 May 23;11:191-207. doi: 10.2147/IJGM.S102230.

De Boo, H.A, & Harding, J.E. (2006). The developmental origins of adult disease (Baker) hypothesis. Aust N Z J Obstet Gynaecol, 46(1), 4-14.

Federica Tramer, Francesca Rocco, Fulvio Micali, Gabriella Sandri, Enrico Panfili. (1998). Antioxidant systems in rat epididymal spermatozoa. Biology of reproduction 59 (4), 753-758, 1998.

Fischer CJ, Saha A, Cairns BR. (2007) Kinetic model for the ATP-dependent translocation of Saccharomyces cerevisiae RSC along double-stranded DNA. Biochemistry 46(43):12416-26

Fischer, V.A., Fischer, C.E., Akpaso, O., Ashiru, O.A. (2013). Hormonal Assays Following Oral Administration of Bonny Light Crude Oil on Male Wistar Rats. Journal of Experimental and Clinical Anatomy; Vol. 12(2): 53-56.

Giwa-Osagie O.O. (2003). Nigeria has twelve million infertile persons. Pharma News 25:48-49.

Jeje SO, Ola-Mudathir FK, Raji Y (2017). Experimental maternal treatment with dexamethasone during lactation induces neonatal testicular and epididymal oxidative stress;

Implications for early postnatal exposure. Pathophysiology. 2017 Dec; 24(4):261-265.

Jeje S.O and Raji Y (2017). Maternal treatment with dexamethasone during gestation alters sexual development markers in the f1 and f2 male offspring of Wistar rats. Journal of Developmental Origin of Health and Diseases. 8(1): 101-112. doi: https://doi.org/10.1017/S2040174416000453

Kenichiro Urata, Hisashi Narahara, Yuichiro Tanaka, Toru Egashira, Fusako Takayama, Isao Miyakawa. (2001). Effect of endotoxin-induced reactive oxygen species on sperm motility. Fertility and sterility 76 (1), 163-166, 2001

Kumar S. (2008). Is environmental exposure associated with reproductive health impairments? J. Turk Ger Gynecol Assoc 9: 60-69.

Lowry O.H, Rosebrough N.J, Farr A.L, Randall R.J (1951). Protein measurement with the Folin phenol reagent. J Biol Chem. 1951 Nov;193(1):265-75. PMID: 14907713.

Marcondes, F., Bianchi, F., & Tanno, A. (2002). Determination of the estrous phase of rats: some helpful considerations. Brazilian Journal of Biology, 62(4a),609-614.

Misra H.P and Fridovich I. (1972). The purification and properties of superoxide dismutase from Neurospora crass. Journal of Biological Chemistry 247 (11), 3410-3414, 1972.

Ola-Mudathir K.F, Suru S.M, Fafunso M.A, Obioha U.E, Faremi T.Y. 2008. Protective roles of onion and garlic extracts on cadmium-induced changes in sperm characteristics and testicular oxidative damage in rats. Food Chem Toxicol 46: 3604– 3611.

Omayone T.P, Salami A.T, Olopade J.O, Olaleye S.B. Attenuation of ischemia-reperfusion-induced gastric ulcer by low-dose vanadium in male Wistar rats. Life Sci. 2020 Oct 15;259:118272. doi: 10.1016/j.lfs.2020.118272. Epub 2020 Aug 12. PMID: 32800836.

Orisakwe O.E., Akumka D.D., Njan A.A., Afonne J.O. (2004). Testicular toxicity of Nigeria bonny light crude oil in male albino rats. Reprod Toxicol 18:439-442.

Oruambo I.F., Jones A.B. (2007). Alterations in the concentrations of liver mitochondrial DNA: Cytoplasmic total hydrocarbon and calcium in guinea pigs after treatment with Nigerian light crude oil. Int J Environ Res Public Health 4:23-27.

Ostby J.S and Gray E Jr. Transgenerational (in utero and lactational) exposure to

investigate the effects of endocrine disrupting compounds in rats. Curr Protoc Toxicol. 2004; 16, 16.8.

Raji Y, Bolarinwa AF. Antifertility activity of Quassia amara in male rats - in vivo study. Life Sci. 1997;61(11):1067-74. doi: 10.1016/s0024-3205(97)00615-2. PMID: 9307052.

Raji Y, Udoh U.S, Mewoyeka O.O, Ononye F.C, Bolari nwa A.F. (2003). Implication of reproductive endocrine malfunction in male antifertility efficacy of Azadirachta indica extract in rats. African journal of medicine and medical sciences 32 (2), 159-165, 2003.

Raji Y, Hart VO. Influence of prolonged exposure to Nigerian Bonny light crude oil on fertility indices in rats. Niger J Physiol Sci. 2012 Jun 7;27(1):55-63. PMID: 23235309.

Rivas A., Fisher J.S., Mckinnell, Atanassova N. and Sharpe (2002). Induction of

reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbesterol: evidence for importanceof the androgen –estrogen balance. Endocrinology. 143: 4797-4808

Sharp R.M. (2001). Hormones and testis development and the possible effects of environmental chemicals. Toxicol. Lett. 120: 221-232

Sherry H Stewart, Matt G Kushner. (2001). Introduction to the special issue on "Anxiety Sensitivity and Addictive Behaviors". Addictive Behaviors 26 (6), 775-785, 2001

Shrestha, J., Shanbhag, T., Shenoy, S., Amuthan, S., Prabhu, K., Sharma, S., … Kafle, S. (2010). Antiovulatory and abortificient effects of Araca catechu (betel nut) in female rats. India J. Pharmacol(42), 306-311.

Tramer F., Rocco F., Micali F., Sandri G. and Panfili E.(1998). Antioxidant systems in rat epididymal spermatozoa. Biol Reprod. 59:753–758.

Tutian, Z., Fazelipur, S., & Shadkhast, M. (2008). Study of histologic and histometric change on ovarian cortex after administration of morphine in mice. Iranian Journal of Veterinary Medicine, 4(4), 90-6.

Welsh M., Saunders P.T., Fsiken M., Scott H.M., Hutchison G.R., Smith L.B. and Sharpe R.M. (2008). Identification in rats of aprogramming window for reproductive tract masculinisation, distruption of which leads to hypospadias and cryptochidism. J. Clin . Invest. 118: 1479-1490

Zubkova E.V. and Robaine.B. (2004). Effects of Glutathione Depletion on Antioxidant

Enzymes in the Epididymis, Seminal Vescicle and Liver and on Sperm motility in

Ageing Brown Norway Rats. Biology of Reproduction 71: 1002-1008.

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Full Length Research Article

Selected Antihypertensive Agents and their Fixed-Dose Combinations Effectively Ameliorate Trastuzumab-Mediated Cardiac Dysfunctions in Rats

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Summary: This study evaluates the therapeutic potentials of selected antihypertensive drugs [valsartan (VAL), amlodipine (ADP), lisinopril (LSP) and their fixed-dose combinations [(amlodipine + lisinopril) (ADP + LSP) and (valsartan + lisinopril) (VAL + LSP)] in ameliorating trastuzumab (*TZM*)-induced cardiovascular dysfunctions in experimental rats. In-bred female Wistar rats were randomly allotted into 10 groups of 6 rats per group. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial blood pressure (MAP) as well as electrocardiogram (ECG) of the treated rats were measured using non-invasive procedures on days 1 and 7 of the experiment, following which the treated rats were sacrificed under light inhaled diethyl ether and histopathological evaluation of all treated hearts was done. Results showed that repeated *TZM* treatment profoundly ($p<0.05$) raised SBP, DBP and MAP values from 115.0 ± 17.1 mmHg, 85.1 ± 15.1 mmHg and 94.7 ± 15.5 mmHg, respectively on day 1 to 127.7 ± 27.8 mmHg, 87.4 ± 27.3 mmHg and 100.5 ± 26.4 mmHg, respectively, on day 7. Oral pretreatments with VAL, ADP, LSP and their fixed-dose combinations profoundly ($p<0.05$) attenuated increases in the SBP, DBP and MAP values with the most significant attenuation mediated by the fixed-dose VAL + LSP combination at the SBP, DBP and MAP values of 103.8 ± 20.6 mmHg, 65.5 ± 18.8 mmHg, and 77.9 ± 18.7 mmHg, respectively. *TZM* treatment also profoundly ($p<0.05$) prolonged the QT and corrected QT intervals from 85.0 ± 11.5 ms and 161.6 ± 20.3 ms, respectively, on day 1 to 110.2 ± 21.5 ms and 226.5 ± 41.5 ms, respectively, on day 7. However, these QT and corrected QT interval prolongations were effectively and profoundly attenuated by oral pretreatments with VAL, ADP, LSP and their fixed-dose combinations. In addition, *TZM* cardiotoxicity was characterized by marked vascular and cardiomyocyte congestion and coronary artery microthrombi formation. However, these histopathological changes were reversed with oral pretreatments with ADP, LSP, VAL and fixed-dosed [(ADP + LSP) and (VAL + LSP)] combinations although fixed-dose VAL + LSP was associated with histopathological lesions of coronary arterial wall cartilaginous metaplasia. Overall, this study revealed the promising therapeutic potentials of VAL, ADP, LSP and their fixed-dose combinations as repurposed drugs for the prevention of *TZM*-mediated cardiac dysfunctions.

Keywords*: Valsartan, Amlodipine, Lisinopril, Trastuzumab cardiotoxicity, Blood Pressure parameters, ECG, Histopathology*

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INTRODUCTION

Cancer remains the second topmost major non-communicable disease worldwide, with estimated 18.1 million sufferers globally and an estimated 9.6 million deaths in 2018 resulting from complications of the disease (Siegel *et al.*, 2020). It is projected that by 2040, the global burden of cancer will grow to 27.5 million new cancer cases and 16.3 million cancer deaths as a result of population growth and ageing population (Rahib *et al.*, 2020). In Nigeria, an estimated yearly 10,000 cancer deaths and annual 250,000 new cases were reported (Akinde *et al.*, 2015). However, one of the novel targeted therapies in clinical cancer management involves the use of monoclonal antibody cytotoxics such as trastuzumab, pertuzumab, bevacizumab, margetuximab, atezolizumab, *etc.* (Costa and Czerniecki, 2020).

Trastuzumab (*TZM*) is a DNA-derived, human epidermal growth factor receptor type 2 (HER2) targeted recombinant monoclonal antibody directed against loco-regional and metastatic breast cancer (Piotrowsk *et al.*, 2011; Porta *et al.*, 2015, Fang *et al.*, 2020), gastric cancers (Lameire, 2015; Porta *et al.*, 2015), gastro-esophageal adenocarcinoma (Blackwell *et al.*, 2010; Poon *et al.*, 2013) and salivary duct carcinoma (Gibo *et al.*, 2019). *TZM* is reported to be clinically effective either as a monotherapy or in combination with other agents including the anthracyclines (ElZarrad *et al.*, 2013).

Undoubtedly, the clinical use of *TZM* has resulted in significant improvement in the prognosis of patients with advanced HER2-overtlyexpressing breast and gastric cancers but this use has reportedly been associated with cumulative but reversible off-target cancer therapy-related cardiac dysfunction (CTRCD) (Fang *et al.*, 2020; Brown *et al.*, 2020), either on acute or long-term use (Klein and Dybdal, 2003; Matos *et al.*, 2013; Hidalgo *et al.*, 2013; Mohan *et al.*, 2018). *TZM* is known to de-express myocardial genes, decrease left ventricular function and induce cardiomyocytes ultrastructural changes (ElZarrad *et al.*, 2013). *TZM* has also been reported to increase myocardial oxidative and nitrative stress and activates apoptotic pathways, resulting in profound elevations in the serum troponin-I and cardiac myosin light chain 1 (cMLC1) levels (ElZarrad *et al.*, 2013). Other reported *TZM*'s myocardial cytotoxicity include: disruption of signal transduction pathways, DNA disrepair, decreased angiogenesis, cell cycle disruption, and activation of antibody-dependent cellular cytotoxicity (Spector and Blackwell, 2009; Poon *et al.*, 2013, Lameire, 2015). *TZM* binds to the extracellular membrane domain of HER2 to inhibit proliferation and survival of HER2-dependent tumors after reversing the phenotype of HER2/neu expressing tumor cells (Drebin *et al.*, 1984; Mandaliya *et al.*, 2015). Clinically, acute *TZM* cardiotoxicity may manifest as myocardial dysfunction, ischemia, hypotension, hypertension, edema, prolonged QT-interval, arrhythmias and thromboembolism (Jones *et al.*, 2009; Alghafar *et al.*, 2020) while its long-term manifestations include progressive decline in left ventricular ejection fraction (LVEF) with subsequent left ventricle dysfunction, congestive cardiac failure, left bundle branch block (LBBB), and negative T-waves on ECG (Piotrowski *et al.*, 2012).

There are independent scientific reports on the therapeutic potentials of some of the known classes of antihypertensive agents in mitigating anthracycline- and trastuzumab-induced cardiotoxicities (Akolkar *et al.*, 2015; Rygiel, 2016; Wittayanukorn *et al.*, 2018; Blanter and Frishman, 2019; Brown *et al.*, 2020). These classes of antihypertensive agents include angiotensin converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs) and beta(β)$_1$-adrenoceptor antagonists (Rygiel, 2016; Gujral *et al.*, 2018; Sharma *et al.*, 2018; Ma *et al.*, 2019; Guglin *et al.*, 2019). ACEIs and ARBs are notable antihypertensive and anti-cardiac failure agents that mediate their pharmacological action by regulating the renin-angiotensin-aldosterone system (RAAS)-dependent blood pressure homeostasis (Guo *et al.*, 2020). In heart failure patients, these agents have been reported to prevent and at times reverse left ventricular hypertrophy, hypertensive cardiomyopathy and heart failure, preserving cardiac function, and improving recovery prognosis (Gilbert, 1995; Gregory, 2001; Ruggenenti *et al.*, 2008; Goda and Masuyama, 2014; Messerli *et al.*, 2017). Similarly, other few small-scale randomized controlled trials (RCTs) have also reported the chemotherapeutic/chemopreventive potentials of ACEIs/ARBs may be in CTRCD (Pinter *et al.*, 2018; Blanter and Frishman, 2019). Unfortunately, results from these clinical studies have remained largely inconsistent (Boekhout *et al.*, 2016; Janbabai *et al.*, 2017; Gupta *et al.*, 2018; Guglin *et al.*, 2019). Thus, casting doubts on the efficacies of these therapeutic agents in *TZM* cardiotoxicity. Recently, the ameliorating effects of VAL, ADP, LSP and their fixed-dosed combinations in TZM-induced cardiotoxic rats that were mediated via reduced caspase-3 and caspase-9 expression and enhanced antioxidant mechanisms were reported (Olorundare *et al.*, 2021). This study, therefore, is a further study designed at evaluating the therapeutic potential of amlodipine (an angio-selective calcium channel blocker), lisinopril (a competitive angiotensin converting enzyme inhibitor), valsartan (angiotensin II receptor blocker) and their fixed-dose combinations in acute *TZM*-induced cardiotoxicity in Wistar rats outside their approved clinical use as antihypertensive and anti-cardiac failure regimen, thus, re-purposed. In doing this, effects of the oral pretreatments with these drugs and their fixed dose combinations on blood pressure parameters (systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure), electrocardiogram (P- wave duration, heart rate, QRS duration, QT segment, corrected QT segment, and R wave amplitude) and cardiac muscle histopathological endpoints were evaluated in *TZM*-induced cardiotoxicity.

MATERIALS AND METHODS

Drugs and Chemicals: Drugs used include amlodipine besylate (Pfizer Norvasc™ 5 mg, R-Pharm Germany GmbH, Heinrich-Mack-Str. 35, 89257 Illertissen, Germany), lisinopril dihydrate (Zestril™ 5mg, AstraZeneca Pharnaceutical Co., Ltd, Wuxi, Jiangsu, People's Republic of China), valsartan (Diovan® 160, Norvatis Pharma AG, Basel, Switzerland), xylazine, ketamine (Bayer, Germany). Chemicals such as hydrochloric acid (HCl), thiobarbituric acid (TBA), 1,2-dichloro-4-nitrobenzene, trichloroacetic acid (TCA), sodium hydroxide, xylenol orange (XO), potassium hydroxide, reduced glutathione (GSH), and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. All other chemicals were of analytical grade and were purchased from ThermoFisher Scientific, Cambridge, Massachusetts, U.S.A.

Experimental Animal Care: After an ethical approval (UERC Approval number: UERC/ASN/2020/2027) was obtained from the University of Ilorin Ethical Review Committee for Postgraduate Research, young adult female Wistar Albino rats (aged 8-12 weeks old and body weight: 170-190 g) were procured from the Animal House of the Lagos State University College of Medicine, Ikeja, Lagos State, Nigeria. The procured rats were handled in accordance with international principles guiding the Use and Handling of Experimental Animals as provided by the

National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011). The rats were maintained on standard rat chow and potable water which were made available *ad libitum* and standard laboratory conditions (room temperature: 23-26 ^{o}C, relative humidity: 55 ± 5%, and controlled photoperiod of 12 hours light/12 hours dark periodicity).

Body weight Measurement: The body weights of rats were taken on days 1 and 7 of the experiment using a digital rodent weighing scale (®Virgo Electronic Compact Scale, New Delhi, India). The weight values obtained were expressed in grams (g).

Experimental Induction of TZM-induced cardiotoxicity and drug treatments of rats: *TZM*-induced cardiotoxicity was achieved via repeated intraperitoneal injections of 2.25 mg/kg of *TZM* as well as their oral pretreatments with VAL, ADP, LSP and their fixed-dosed combinations were as previously described by Olorundare *et al.* (2021).

Electrocardiography Measurement in treated rats: Electrocardiography was carried out using a 6-lead computer ECG machine using modified method of Adedapo *et al.* (2016). Briefly described, rats were placed on right lateral recumbency on an insulated board under light sedation with a proportional combination of ketamine (Ketavet®, 100 mg/ml, Pfizer, Berlin, Germany) and xylazine (Rompun® 2%, 20 mg/ml, Bayer, Leverkusen, Germany) (100 mg/kg ketamine + 5 mg/kg xylazine) administered intramuscularly to aid stabilization (Albrecht *et al.*, 2014). Skin fur was shaved to improve contact between the ECG pad and the skin as well as electrode gel was used to improve contact between the rat skin and ECG electrodes. The ECG's electrodes which were six in number were placed on both fore limbs, both hind limbs and chest of the treated rats. The electrodes were then connected to the ECG machine using color-coded cables while the ECG recording was done in a calm and quiet environment to avoid recording interference. The machine was calibrated and preset at 10 m/mV and 50 mm/s paper speed. From the standard lead-II tracings, ECG parameters such as heart rate, p-amplitude, PR-duration, R-amplitude, QRS complex, as well as QT/QTc parameters were evaluated. The corrected QT(QTc) was calculated using Bazett's formula:

$$QTc = \frac{\text{QT interval in seconds}}{\sqrt{\text{cardiac cycle in seconds}}} = \frac{QT}{\sqrt{RR}}$$

(Phan *et al.*, 2016)

Electrocardiography measurements were recorded on days 1 and 7 of the experiment.

Blood Pressure Measurement in treated rats: Blood pressure parameters such as systolic blood pressure, diastolic blood pressure and mean arterial pressure were measured in conscious but slight sedated rats by tail cuff plethysmography using CODA™ Non-invasive Computerized Blood Pressure Acquisition System (Kent Scientific, Torrington, Connecticut, USA). This non-invasive rat computerized blood pressure measurement was done as described by Gangwar *et al.* (2014) and Jayeola *et al.* (2020). For each rat, a total of nine readings were taken in the quiescent state after the animals had been well acclimatized to the procedure. Blood pressure parameter measurements were recorded on days 1 and 7 of the experiment. The Mean Arterial blood pressure (MAP) was calculated as:

Mean Arterial Blood Pressure (MAP) = {diastolic blood pressure + ($^{1}/_{3}$) × pulse pressure}, where pulse pressure (PP) = {systolic blood pressure (SBP) minus diastolic blood pressure (DBP)}

Harvesting and weighing selected vital organs: The hearts of treated rats were identified, freed of adjoining adventitia, dissected out *en bloc* and weighed on a digital weighing scale.

Histopathological evaluation of the heart tissues: The preparation of the heart tissues and their histopathologic evaluation were done using procedures earlier described by Olorundare *et al.* (2020).

Data Analysis: Data were presented as mean ± S.D. of six observations for the body weight parameters while data for the blood pressure and ECG measurements were expressed as mean ± S.E.M. of nine observations. Statistical analysis was done using One-way ANOVA followed by post-hoc Turkey's *post hoc* test, on GraphPad Prism Version 5. Statistical significance were considered at $p<0.05$, $p<0.001$, and $p<0.0001$.

Table 1.
Group treatment of rats

Groups	Treatments
Group I	Oral 10 ml/kg/day of sterile water. + 1 ml/kg/day of sterile water given *i.p.* for 7 days
Group II	Oral 5 mg/kg/day of valsartan in sterile water + 1 ml/kg/day of sterile water given *i.p.* for 7 days
Group III	Oral 0.25 mg/kg/day of amlodipine in sterile water + 1 ml/kg of sterile water given *i.p.* for 7 days
Group IV	Oral 0.035 mg/kg/day of lisinopril in sterile water + 1 ml/kg of sterile water given *i.p.* for 7 days
Group V	Oral 10 ml/kg/day of sterile water + 2.25 mg/kg/day of trastuzumab given *i.p.* for 7 days
Group VI	Oral 5 mg/kg/day of valsartan in sterile water + 2.25 mg/kg of trastuzumab given *i.p.* for 7 days
Group VII	Oral 0.25 mg/kg/day of amlodipine in sterile water + 2.25 mg/kg/day of trastuzumab given *i.p.* for 7 days
Group VIII	Oral 0.035 mg/kg/day of lisinopril in sterile water + 2.25 mg/kg/day of trastuzumab given *i.p.* for 7 days
Group IX	Oral 0.25 mg/kg/day of amlodipine + Oral 0.035 mg/kg/day of lisinopril *p.o.* + 2.25 mg/kg/day of trastuzumab given *i.p.* for 7 days
Group X	Oral 5 mg/kg/day of valsartan + Oral 0.035 mg/kg/day of lisinopril dissolved in sterile water + 2.25 mg/kg/day of trastuzumab given *i.p.* for 7 days

RESULTS

Effect of valsartan (VAL), amlodipine (ADP), lisinopril (LSP) and their fixed-dose combinations on body weights and body weight changes (% Δbwt.) *TZM*-treated rats: Table 2 shows the effect of repeated daily intraperitoneal injection with 2.25 mg/kg of *TZM* and their oral pretreatments with VAL, ADP, LSP and the fixed-dose combinations of ADP + LSP and VAL + LSP, respectively, on the average body weight and % Δbwt. on days 1 and 7. Repeated oral pretreatments with ADP and LSP to normal rats resulted in profound ($p<0.001$) reductions in % Δbwt. when compared to Groups I. Similarly, *i.p. TZM* treatment and oral pretreatments with ADP, LSP, VAL and their combinations caused similar significant ($p<0.001$) weight reduction in *TZM*-intoxicated rats when compared to untreated normal (Group I) rats (Table 2).

Table 2.
Effect of repeated oral treatment with valsartan, amlodipine, lisinopril and their fixed-dose combinations on the body weight and percentage body weight changes (% Δbwt.) of *TZM*-treated rats

Group	Day 1 body wt. (g)	Day 7 body wt. (g)	Δbwt.
I	208.60 ± 32.22	223.20 ± 35.12	06.95 ± 05.03
II	203.00 ± 17.43	216.70 ± 18.64	06.79 ± 03.83
III	200.60 ± 28.18	207.10 ± 30.61	03.25±02.76^{b-}
IV	206.10 ± 20.88	208.80 ± 18.82	02.12±01.91^{b-}
V	194.90 ± 11.56	195.30 ± 16.28	00.47±10.60^{b-}
VI	201.40 ± 16.36	204.40 ± 16.00	01.57±03.85^{b-}
VII	202.80 ± 15.36	209.30 ± 19.05	03.13±02.99^{b-}
VIII	204.60 ± 13.59	210.80 ± 06.87	02.98±03.70^{b-}
IX	207.00 ± 10.24	201.80 ± 13.34	01.80±03.17^{b-}
X	194.80 ± 20.82	198.90 ± 24.05	02.08±03.63^{b-}

$^{b-}$ represents a significant decrease at $p<0.001$ when compared to untreated normal control (Group I) and valsartan-treated rats (Group II).
Group I - 10 ml/kg/day sterile water given p.o. + 1 ml/kg/day sterile water given i.p.; Group II - 5 mg/kg/day valsartan given p.o. + 1 ml/kg/day sterile water given i.p. ; Group III - 0.25 mg/kg/day amlodipine given p.o. + 1 ml/kg sterile water given i.p. ; Group IV - 0.035 mg/kg/day lisinopril given p.o. + 1 ml/kg sterile water given i.p.; Group V - 10 ml/kg/day sterile water given p.o. + 2.25 mg/kg/day TZM given i.p.; Group VI - 5 mg/kg/day valsartan given p.o. + 2.25 mg/kg TZM given i.p.; Group VII - 0.25 mg/kg/day amlodipine given p.o. + 2.25 mg/kg/day TZM given i.p; Group VIII - 0.035 mg/kg/day lisinopril given p.o. + 2.25 mg/kg/day TZM given i.p.; Group IX - 0.25 mg/kg/day amlodipine + 0.035 mg/kg/day lisinopril given p.o. + 2.25 mg/kg/day TZM given i.p.; Group X - 5 mg/kg/day valsartan + 0.035 mg/kg/day lisinopril given p.o. + 2.25 mg/kg/day TZM given i.p

Effect of valsartan (VAL), amlodipine (ADP), lisinopril (LSP) and their fixed-dose combinations on SBP, DBP and MAP of *TZM*-intoxicated rats: The baseline blood pressure parameters (SBP, DBP and MAP) of treated rats on day 1 of treatment were not significantly different from one group to another (Table 3). With repeated *i.p. TZM* treatment, there were profound ($p<0.05$) increases in the SBP, DBP and MAP from 115.0±17.1 mmHg, 85.1±15.1 mmHg and 94.7±15.5 mmHg, respectively, on day 1 (Table 3) to 127.7±27.8 mmHg, 87.4±27.3 mmHg and 100.5±26.4 mmHg, respectively, on day 7, respectively in the untreated *TZM* intoxicated (Group V) rats on day 7 of treatment (Table 4). However, with repeated oral pre-treatments with VAL, ADP, LSP and their fixed-dose combinations, increases in these parameters were significantly ($p<0.05$) attenuated with the most significant attenuation offered by the fixed-dose VAL + LSP combination (Table 4).

Effect of valsartan (VAL), amlodipine (ADP), lisinopril (LSP) and their fixed-dose combinations on ECG parameters on days 1 and 7 of treatment: Table 5 shows the baseline ECG parameters (P wave duration, HR, QRS duration, QT interval, corrected QT interval and R-wave interval) of treated on day 1. Following repeated *i.p. TZM* injection to treated rats for 7 days, the QT intervals and corrected QT intervals were significantly ($p<0.05$) increased from 85.0 ± 11.5 ms and 161.6 ± 20.3 ms, respectively, on day 1 (Table 5), to 110.2 ± 21.5 ms and 226.5 ± 41.5 ms, respectively, in untreated *TZM* intoxicated (Group V) rats (Table 6 and Figure 1E) when compared to 89.5 ± 16.0 ms and 167.5 ± 48.3 ms, respectively, in untreated control (Group I) values (Table 6 and Figure 1A). However, with repeated oral pretreatments with VAL, ADP, LSP and their fixed-dose combinations, prolongation in the QT and corrected QT intervals were significantly ($p<0.05$) attenuated (Table 6, Figures 1B-1D, 1F-1I) with the most profound attenuation ($p<0.05$ and $p<0.001$) offered by VAL+LSP fixed-dose combination (Table 6, Figures 1J).

Table 3.
Baseline SBP, DBP and MAP before *TZM*-intoxication and oral pretreatments with valsartan, amlodipine, lisinopril and their fixed-dose combinations in allotted groups of Wistar rats on day 1

Treatment Groups	Systolic Blood Pressure (SBP) (mmHg)	Diastolic Blood Pressure (DBP) (mmHg)	Mean Arterial Pressure (MAP) (mmHg)
I	108.3 ± 21.1	73.9 ± 18.5	85.0 ± 18.0
II	112.0 ± 22.0	76.4 ± 19.6	88.0 ± 19.2
III	106.8 ± 26.7	76.1 ± 29.8	86.0 ± 28.2
IV	107.9 ± 20.6	74.2 ± 18.6	85.1 ± 18.6
V	115.0 ± 17.1	85.1 ± 15.1	94.7 ± 15.5
VI	116.7 ± 23.8	82.6 ± 23.0	93.7 ± 22.7
VII	100.9 ± 19.7	67.1 ± 16.0	76.3 ± 17.4
VIII	102.3 ± 15.4	71.8 ± 16.9	81.7 ± 15.4
IX	112.2 ± 23.3	79.9 ± 22.5	90.3 ± 22.0
X	115.3 ± 29.1	79.4 ± 25.3	90.9 ± 25.8

Table 4.
Effect of oral pretreatment of valsartan, amlodipine, lisinopril and their oral fixed-dose combinations in on SBP, DBP and MAP in *TZM*-intoxicated rats on day 7

Treatment Groups	Systolic Blood Pressure (SBP) (mmHg)	Diastolic Blood Pressure (DBP) (mmHg)	Mean Arterial Pressure (MAP) (mmHg)
I	109.1 ± 22.7	69.6 ± 20.5	82.5 ± 20.7
II	122.3 ± 35.6	85.5 ± 30.7	97.5 ± 31.9
III	115.5 ± 19.0*$^{-}$	83.7 ± 15.0	93.9 ± 15.7
IV	107.6 ± 19.6*$^{-}$	72.0 ± 21.7*$^{-}$	83.6±20.3*$^{-}$
V	127.7 ± 27.8*	87.4 ± 27.3*	100.5 ± 26.4*
VI	108.6 ± 27.9*$^{-}$	69.2 ± 18.3*$^{-}$	82.1 ± 20.1*$^{-}$
VII	115.8 ± 30.0*$^{-}$	74.9 ± 26.4*$^{-}$	88.2 ± 26.9*$^{-}$
VIII	137.7±26.9*	92.3 ± 24.5*	107.0 ± 23.7*
IX	113.6 ± 22.7*$^{-}$	69.1 ± 18.7*$^{-}$	83.6 ± 18.7*$^{-}$
X	103.8 ± 20.6	65.5 ± 18.8	77.9 ± 18.7

** represents a significant increase at $p<0.05$ when compared to untreated normal (Group I) values while*
**$^{-}$ represents a significant decrease at $p<0.05$ when compared to untreated TZM-intoxicated (Group V) values*

Table 5.
Baseline ECG parameters before *TZM*-intoxication and oral pretreatments with valsartan, amlodipine, lisinopril and their fixed-dose combinations in allotted groups of Wistar rats on day 1.

Treatment Groups	P-duration (ms)	HR (/min)	QRS duration (ms)	QT segment (ms)	QT segment (corrected) (ms)	R amplitude (mV)
I	16.8±2.8	221.0±34.7	16.6±2.1	94.6±14.4	181.2±33.0	0.47±0.08
II	22.5±2.9	225.0±42.3	16.2±1.7	76.8±18.0	147.3±33.7	0.42±0.09
III	21.2±3.3	240.±27.5	13.8±1.1	63.2±6.9	125.4±12.6	0.31±0.06
IV	18.0±3.5	215.8±22.3	13.7±2.2	83.8±8.4	158.7±23.5	0.55±0.15
V	17.6±4.0	220.2±32.6	15.4±3.8	85.0±11.5	161.6±20.3	0.52±0.20
VI	21.0±5.1	216.4±12.3	14.4±2.7	96.8±11.3	182.8±19.1	0.38±0.12
VII	25.6±3.6	247.8±26.1	15.0±0.7	89.4±13.4	179.8±17.8	0.48±0.11
VIII	21.5±3.8	205.7±25.9	15.2±2.6	97.7±11.7	179.0±14.8	0.51±0.18
IX	21.2±2.9	221.7±15.9	14.7±0.8	100.2±17.4	191.5 ±31.9	0.49±0.16
X	20.4±3.0	238.0±28.4	13.8±1.3	84.0±14.3	165.8±24.3	0.44±0.08

Table 6.
Effect of valsartan, amlodipine, lisinopril and their fixed-dose combinations on the ECG parameters in *TZM*-intoxicated rats on day 7

Treatment Groups	P-duration (ms)	HR (/min)	QRS duration (ms)	QT segment (ms)	QT segment (corrected) (ms)	R amplitude (mV)
I	18.0±3.0	259.5±11.8	14.2±2.4	89.5±16.0	167.5±48.3^{**-}	0.39±0.13
II	18.2±6.3	217.5±29.0^{*-}	13.3±2.6	70.5±23.1^{*-}	150.8±27.9^{*-}	0.42±0.14
III	17.5±3.7	243.7±12.6	14.0±1.5	91.2±15.3^{*-}	190.0±19.1	0.47±0.04
IV	20.0±3.4	257.3±17.5	15.2±3.7	93.0±15.78^{*-}	191.8±29.5^{*-}	0.50±0.08
V	20.0±2.5	255.7±12.4	13.5±3.0	110.2±21.5*	226.5±41.5*	0.47±0.08
VI	21.0±2.5	250.4± 15.9	14.6±2.2	75.6±4.0^{*-}	153.8±8.3^{*-}	0.40±0.16
VII	18.8±6.1	221.6±10.6^{*-}	14.6±0.5	82.0±13.2^{*-}	157.6±28.4^{*-}	0.58±0.12
VIII	16.8±4.0	219.6±37.7^{*-}	16.0±1.2	65.8±11.8^{**-}	129.0±25.9^{***-}	0.46±0.14
IX	15.3±6.1	252.0±28.6	16.3±0.6	79.3±8.1^{*-}	161.0 ±7.8^{*-}	0.39±0.07
X	16.7±2.3	222.3±13.5^{*-}	14.3±1.0	72.8±9.2^{*-}	139.5±19.0^{**-}	0.41±0.12

* *represents a significant increase at p<0.05 when compared to untreated normal control (Group I) values while* $^{*-}$ *,* $^{**-}$ *and* $^{***-}$ *represent significant decrease at p<0.05, p<0.001 and p<0.0001, respectively, when compared to untreated TZM-intoxicated (Group V) values.*

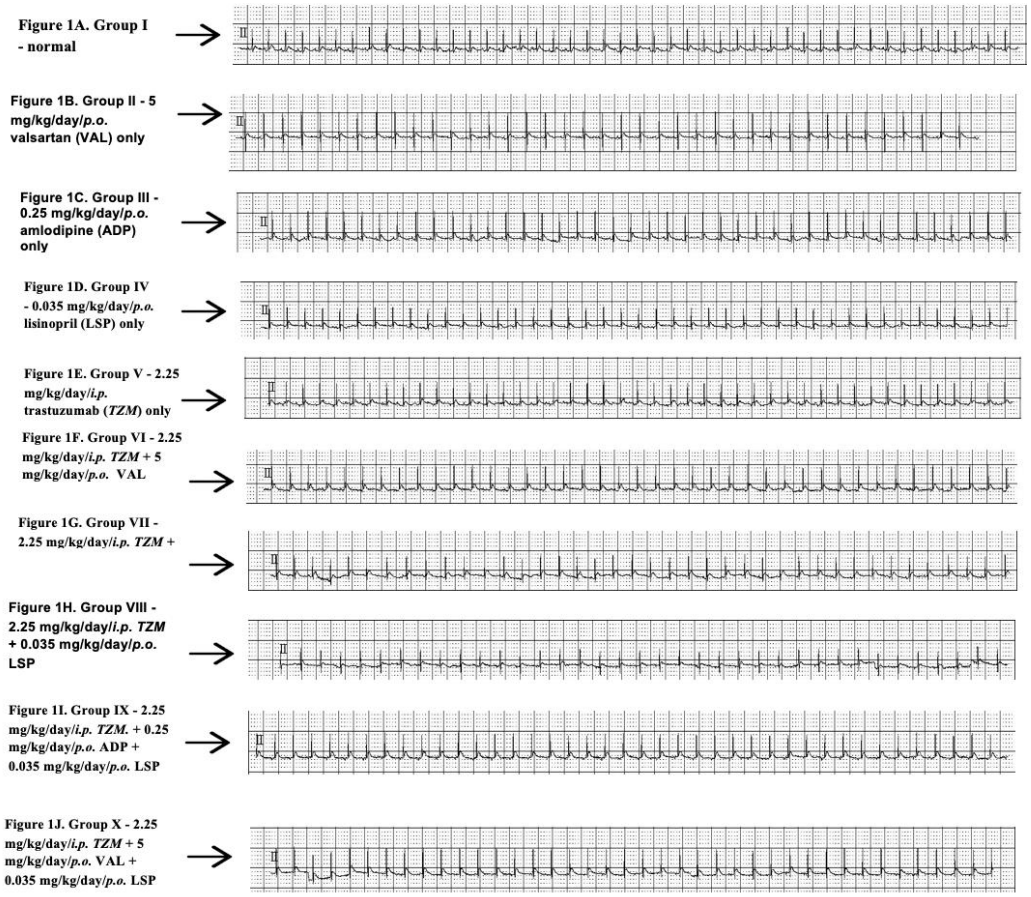

Figure 1.
ECG tracings showing effects of amlodipine (ADP), valsartan (VAL), lisinopril (LSP) and their fixed-dose combinations on the P wave duration, HR, QRS duration, QT interval, corrected QT interval and R-wave interval in *TZM*-intoxicated rats. *TZM* intoxication was characterized by prolonged QT intervals (Figure 1E) which were profoundly attenuated by oral pretreatments with VAL, ADP and LSP (Figures 1B-1D, respectively) and their fixed dose combinations (Figures 1F-J) with the ECG parameters returning to about normal (Figure 1A).

DISCUSSION

HER-2 targeted agents such as trastuzumab, pertuzumab, lapatinib and other congeners, remain the gold standard treatment strategy for both early and metastatic HER2-positive breast cancers, which are marked by the overexpressed HER2 genes (Sendur *et al.*, 2013; Perez *et al.*, 2019). The antibody targeted therapy, *TZM*, is notoriously reputed for its non-dose dependent, cumulative but reversible off-target cancer therapy-related cardiac dysfunction, especially on prolonged use. Trastuzumab-induced cardiotoxicity is believed to be the result of attenuated cardiac HER2-mediated signaling, ultimately resulting in decreased cardiomyocyte functionality with HER2 functioning as a compensatory mechanism against anthracycline-related cardiac stress. This is believed to be mediated via two processes namely: an increased activation of HER2-HER4-mediated cardiomyocyte survival pathways and cardiac dysfunction through inhibition of the HER2-HER4-mediated signaling (Perik *et al.*, 2007). Thus, *TZM* causes type II chemotherapy related cardiotoxicity mediated partly through ErbB2 pathway, which is dose independent, largely reversible and does not produce ultrastructural changes on histological examination (Tsang and Moe, 2007; Hamed *et al.*, 2016).

Previous studies have reported the protective role that some classes of antihypertensive agents play in ameliorating *TZM*- and anthracycline-induced cardiotoxicity (Rygiel, 2016; Brown *et al.*, 2020). These classes include cardioselective β_1-blockers (e.g. bisoprolol, carvedilol, metoprolol, *etc*) (Gulati *et al.*, 2016; Pituskin *et al.*, 2017; Gujral *et al.*, 2018; Guglin *et al.*, 2019; Brown *et al.*, 2020), calcium channel blockers (e.g. amlodipine), angiotensin converting enzyme inhibitors (e.g. lisinopril, enalapril, *etc*) (Vaduganathan *et al.*, 2019; Guglin *et al.*, 2019; Brown *et al.*, 2020) and angiotensin receptor blockers (e.g. losartan, valsartan, telmisartan, candesartan, *etc*) (Gulati *et al.*, 2016; Gujral *et al.*, 2018; Boekhout *et al.*, 2016), although there have been conflicting results with differing magnitudes of therapeutic benefits of these antihypertensive classes of drugs (Gujral *et al.*, 2018). Similarly, ACEIs and β-blockers have been reported to be effective at improving LVEF in non-ischemic cardiomyopathy, including chemotherapy-mediated LV dysfunction, especially with prolonged trastuzumab use (Vejpongsa and Yeh, 2014; Leong *et al.*, 2019). In view of these drawbacks, the present study was aimed at evaluating the therapeutic potential of amlodipine, lisinopril, valsartan, individually as well as their fixed-dose combinations in ameliorating *TZM*-associated cardiotoxicity in experimental rats using reliable cardiovascular parameters like blood pressure parameters (systolic blood pressure, diastolic blood pressure, mean arterial blood pressure and heart rate), ECG parameters as well as histopathological endpoints.

TZM is reported to cause profound acute coronary syndrome, left ventricular systolic dysfunction (LVSD) (manifesting as LVEF reductions) (Hildago *et al.*, 2013; Florido *et al.*, 2017, Tahir *et al.*, 2019; Chen *et al.*, 2020), cardiac failure (Florido *et al.*, 2017) systemic hypertension (Hildago *et al.*, 2013; Chen *et al.*, 2020) and most recently pulmonary arterial hypertension (PAH) following its prolonged use (Kowalczyk *et al.*, 2017; Umoru *et al.*, 2020) although other school of thought believes that the pre-existing hypertension, diabetes mellitus and obesity/hyperlipidemia as well as advanced age are known major risk factors for *TZM* cardiotoxicity (Jawa *et al.*, 2016; Eiger *et al.*, 2020).

Electrocardiogram (ECG) remains effective screening tool and gold standard for the diagnosis of LVSD (Olesen *et al.*, 2015; Boonman-de Winter *et al.*, 2015) and may help determine the underlying cause of LVSD (Khunti *et al.*, 2004; Davenport *et al*., 2006). However, LVSD is often characterized by five major EGC correlates which are abnormal Q-waves, atrial fibrillation, ventricular racing, left bundle branch block and prolonged QRS duration (Olesen and Andersen, 2016). Similarly, ECG manifestations of *TZM* cardiotoxicity include abnormal P wave, anterolateral T wave inversions (otherwise known as negative T wave), and prolonged QT segment (indicating bundle branch block) (Piotrowski *et al.*, 2012; Hildago *et al.*, 2013). Therefore, the fact that in this study, repeated *TZM* treatment was associated with elevated blood pressure parameters such as SBP, DBP and MAP suggested that *TZM* cardiotoxicity was fully established. Similarly, *TZM* intoxication was associated with prolonged QT segment suggesting the establishment of bundle branch block. Hence, this result is in complete agreement with earlier reports that prolonged *TZM* use could cause bundle branch block (Piotrowski *et al.*, 2012; Tahir *et al.*, 2019). The fact that oral pretreatments with VAL, ADP, LSP and their fixed-dose combination effectively attenuated increases in the measured blood pressure parameters and QT duration are strong indications of the therapeutic potential of these drugs in ameliorating *TZM*-mediated systemic hypertension. Although, the exact mechanism(s) of ameliorating *TZM*-mediated cardiotoxicity were not investigated in this study but could related to heart remodeling, injury reperfusion and cardiomyocytes anti-apoptosis activities of these drugs, especially ACEI and ARBs, which have previously been reported (Iqbal *et al*., 2008; Zablocki and Sadoshima 2013; Akolkar *et al.*, 2015). On molecular basis, angiotensin II is an effective downregulator of the actions of the NRG-1/ErbB system (Lemmens *et al.*, 2006; Vermeulen *et al.*, 2017), and strongly suggesting that the beneficial role of ACE inhibition may be related to this effect (Munster *et al.*, 2019). Thus, lisinopril, could be mediating its cardioprotective mechanism via the heart remodeling pathway, most likely through reduced caspase-3 and caspase-9 production and increased antioxidant mechanisms, which were the mechanisms through which VAL, ADP, LSP and their fixed-dose combinations mediated their mitigating effect in *TZM*-induced cardiotoxicity (Olorundare *et al.*, 2021).

TZM is known to cause related cardiac dysfunction without corresponding histoarchitectural distortion of the myocytes (Jones *et al.*, 2009) although *TZM* was recently reported to induce severe vascular congestion and associated microthrombi formation without attendant significant alterations in the myocyte histoarchitecture (Olorundare *et al.*, 2020) in treated experimental rats which the present study is in tandem with. However, the fact that these histopathological changes were profoundly improved by ADP, VAL and fixed-dose (ADP + VAL) combination

pretreatments, strongly suggest the cardioprotective potential of these drugs. Another important finding of this study is the histological finding of coronary artery cartilaginous metaplasia which was a prominent cardiac histopathological lesion found in rat hearts on LSP- and fixed-dose VAL + LSP combination pretreatments. Vascular cartilaginous/osseous metaplasia, which classically features the presence of arterial chondrocytes expressing type II collagen, is known to be part of the progression of mineralization or atherosclerotic lesion (Qiao *et al.*, 1995; Wallin *et al.*, 2001; Fitzpatrick *et al.*, 2003; Nguyen *et al.*, 2012). It also provides evidence of cardiac extracellular matrix remodeling for post-infarcted heart and may constitute a supplemental factor for heart failure when it calcifies (Manole *et al.*, 2019; Carreon *et al.*, 2020). Thus, cartilaginous metaplasia is seen as a potential pathway for artery wall calcification associated with the atherosclerotic plaque (Qiao *et al.*, 1995). Thus, the marked presence of coronary artery cartilaginous metaplasia as seen in the fixed-dose valsartan-lisinopril (VAL + LSP) combination-pretreated heart is suggestive of the either vascular remodeling of the *TZM*-mediated endothelial injury or coronary artery atheromatous plaque formation. However, the former appears to be more likely as the histological finding of coronary artery recanalization was observed with the drug combination pretreatment.

Overall, findings of this study highlight the promising therapeutic potentials of the antihypertensives – amlodipine, lisinopril, valsartan and their fixed-dose combinations as repurposed therapeutics in the management of *TZM*-induced cardiac dysfunctions.

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REFERENCES

Adedapo, A., Babarinsa, O., Oyagbemi, A., *et al.* (2016). Cardiotoxicity study of the aqueous extract of corn silk in rats. *Macedonian Veterinary Review* 39 (1): 43-49.

Akinde, O.R., Phillips, A.A., Oguntunde, O.A., *et al.* (2015). Cancer mortality pattern in Lagos University Teaching Hospital, Lagos, Nigeria. *Journal of Cancer Epidemiology* 2015: Article ID 842032; https://doi.org/10.1155/2015/842032

Akolkar, G., Bhullar, N., Bews, H., *et al.* (2015). The role of renin angiotensin system antagonists in the prevention of doxorubicin and trastuzumab induced cardiotoxicity. *Cardiovascular Ultrasound* 13: 18.

Albrecht, M., Henke, J., Tacke, S., *et al.* (2014). Effects of isoflurane, ketamine-xylazine and a combination of medetomidine, midazolam and fentanyl on physiological variables continuously measured by telemetry in Wistar rats. *BMC Veterinary Research* 10: 198.

Alghafar, D.A., Younos, I., Baimani, K.A., *et al.* (2020). Trastuzumab cardiotoxicity in HER2-positive breast cancer patients in tertiary health care center, Sultanate of Oman. *Journal of Oncology and Pharmacy Practice* 27: 1078155220919888

Blackwell, K.L., Burstein, H.J., Storniolo, A.M., *et al.* (2010) Randomized study of lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. *Journal of Clinical Oncology* 28: 1124-1130.

Blanter, J.B., Frishman, W.H. (2019). The preventive role of angiotensin converting enzyme inhibitors/angiotensin-II receptor blockers and beta-adrenergic blockers in anthracycline- and trastuzumab induced cardiotoxicity. *Cardiology Reviews* 27(5): 256259;

Boekhout, A.H., Gietema, J.A., Milojkovic Kerklaan, B., *et al.* (2016). Angiotensin II-receptor inhibition with candesartan to prevent trastuzumab-related cardiotoxic effects in patients with early breast cancer: a randomized clinical trial. *JAMA Oncology* 2(8): 1030-1037;

Boonman-de Winter, L.J.M., Rutten, F.H., Cramer, M.J., *et al.* (2015). Efficiently screening heart failure in patients with type 2 diabetes. *European Journal of Heart Failure* 17: 187-195.

Brown, S-A., Okwuosa, T.M., Barac, A., *et al.* (2020). The role of angiotensin converting enzyme inhibitors and β-blockers in primary prevention of cardiac dysfunction in breast cancer patients. *JAHA* 2020: 9;

Carreon, C.K., Sanders, S.P., Ashworth, M.T. (2020). Chondroid and osseous metaplasia of the central fibrous body in adolescent hearts with mutations in *TNNI3* and *TNNT2* genes. *Pediatric and Development Pathology*;

Chen, M.F., Manson, D.K., Yuan, A., *et al.* (2020). Trastuzumab-induced cardiotoxicity and hypertension among racially and ethnically diverse patients with HER2-positive early-stage breast cancer. *Journal of Clinical Oncology* 38(15Suppl.): e24092;

Costa, R.L.B., Czerniecki, B.J. (2020). Clinical development of immunotherapies for $HER2^+$ breast cancer: a review of HER2-directed monoclonal antibodies and beyond. *Npj Breast Cancer* 6: 10 (2020)

Drebin, J.A., Stern, D.F., Link, V.C., *et al.* (1984). Monoclonal antibodies identify a cell-surface antigen associated with an activated cellular oncogene. *Nature* 312(5994): 545-548

Eiger, D., Franzoi, M.A., Pondé, N., *et al.* (2020). Cardiotoxicity of trastuzumab given for 12

months compared to shorter treatment periods: a systematic review and meta-analysis of six clinical trials. *ESMO Open* 5: e000659.

ElZarrad, M.K., Mukhopadhyay, P., Mohan, N., *et al.* (2013) Trastuzumab alters the expression of genes essential for cardiac function and induces ultrastructural changes of cardiomyocytes in mice. *PLoS One* 8(11): e79543;

Fang, K., Zhang, Y., Liu, W., *et al.* (2020). Effects of angiotensin-converting enzyme inhibitor/angiotensin receptor blocker use on cancer therapy-related cardiac dysfunction: a meta-analysis of randomized controlled trials. *Heart Failure Reviews*

Fitzpatrick, L.A., Turner, R.T., Ritman, E.R. (2003). Endochondral bone formation in the heart: A possible mechanism of coronary calcification. *Endocrinology* 144(6): 2214-2219

Florido, R., Smith, K.L., Cuomo, K.K., *et al.* (2017). Cardiotoxicity from human epidermal growth factor receptor-2 (HER2) targeted therapies. *JAHA* 6(9):

Gangwar, A., Kumar, P., Rawat, A., *et al.* (2014). Noninvasive measurement of systolic blood pressure in rats: A novel technique. *Indian Journal of Pharmacology* 46(3): 351-352.

Gibo, T., Sekiguchi, N., Gomi, D., *et al.* (2019). Targeted therapy with trastuzumab for epidermal growth factor receptor 2 (HER2)-positive advanced salivary duct carcinoma: A case report. *Molecular & Clinical Oncology* 11: 111-115.

Gilbert, B.W. (1992). Inhibitors and regression of left ventricular hypertrophy. *Clinical Cardiology* 15(10): 711-714.

Goda, A., Masuyama, T. (2014). Left ventricular hypertrophy as a target of treatment in patients with hypertension. *Circulation Journal* 78(11): 2633-2634.

Gregory, Y.H. (2001). Regression of left ventricular hypertrophy and improved prognosis: Some hope now….or hype? *Circulation* 104: 1582-1584.

Guglin, M., Krischer, J., Tamura, R., *et al.* (2019). Randomized trial of lisinopril *vs.* carvedilol to prevent trastuzumab cardiotoxicity in patients with breast cancer. *Journal of American College of Cardiology* 73(22): 2859-2868.

Gujral, D.M., Lloyd, G., Bhattacharyya, S. (2018). Effect of prophylactic beta-blocker or ACE inhibitor on cardiac dysfunction & heart failure during anthracycline chemotherapy ± trastuzumab. *Breast* 37: 64-71.

Guo, S., Tse, G., Liu, T. (2020). Protective strategies to prevent trastuzumab-induced cardiotoxicity. *The Lancet* 395(10223): 491-492.

Hamed, R.H., Salim, K., Alzahrani, A., *et al.* (2016). Cardiotoxicity of the adjuvant trastuzumab in a Saudi population: clinical experience of a single institution. *Forum of Clinical Oncology* 7(2): 1-6.

Hidalgo, S., Albright, C.A., Wells, G.L. (2013) A case of trastuzumab-associated cardiomyopathy presenting as an acute coronary syndrome: Acute trastuzumab cardiotoxicity. *Case Reports in Cardiology* 2013: 473979.

Iqbal, M., Dubey, K., Anwer, T., *et al.* (2008). Protective effects of telmisartan against acute doxorubicin-induced cardiotoxicity in rats. *Pharmacological Report* 60: 382-390.

Janbabai, G., Nabati, M., Faghihinia, M., *et al.* (2017). Effect of enalapril on preventing anthracycline-induced cardiomyopathy. *Cardiovascular Toxicology* 17(2): 130-139;

Jawa, Z., Perez, R.M., Garlie, L., *et al.* (2016). Risk factors of trastuzumab-induced cardiotoxicity in breast cancer: A meta-analysis. *Medicine* 95(44):e5195.

Jayeola, O.C., Oyagbemi, A.A., Okunlola, I.O., *et al.* (2020). Effect of cocoa powder on hypertension and antioxidant status in uninephrectomized hypertensive rats. *Veterinary World* 13(4): 695-705.

Jones, A.L., Barlow, M., Barrett-Lee, P.J., *et al.* (2009). Management of cardiac health in trastuzumab-treated patients with breast cancer: updated United Kingdom National Cancer Research Institute recommendations for monitoring. *British Journal of Cancer* 100: 684-692.

Klein, P.M., Dybdal, N. (2003). Trastuzumab and cardiac dysfunction: update on preclinical studies. *Seminars in Oncology* 30(5 Suppl. 16): 49-53.

Kowalczyk, L., Bartsch, R., Singer, C.F., *et al.* (2017). Adverse events of trastuzumab emtansine (T-DM1) in the treatment of HER2-positive breast cancer patients. *Breast Cancer* 12(6): 401-408.

Lameire, N. (2014). Nephrotoxicity of recent anti-cancer agents. Clinical Kidney Journal 7(1): 11-22.

Lemmens, K., Segers, V.F., Demolder, M., *et al.* (2006). Role of neuregulin-1/ErbB2 signalling
in endothelium-cardiomyocyte cross-talk. *Journal of Biological Chemistry* 281: 19469-19477.

Leong, D.P., Cosman, T., Alhussein, M.M., *et al.* (2019). Safety of continuing trastuzumab despite mild cardiotoxicity: A Phase I trial. *JACC: Cardiology* 1(1): 1-10.

Ma, Y-X., Bai, F., Qin, F., *et al.* (2019). Beta-blockers for the primary prevention of anthracycline-induced cardiotoxicity: a meta-analysis of randomized controlled trials. *BMC Pharmacology and Toxicology* 20: 18;

Mandaliya, H., Baghi, P., Prawira, A., *et al.* (2015). A rare case of paclitaxel and/or trastuzumab induced acute hepatic necrosis. *Case Reports in Oncological Medicine* 2015: Article 825603;

Manole, C.G., Marinescu, B.G., Marta, D. (2019). Areas of cartilaginous and osseous metaplasia
after experimental myocardial infarction in rats. *The Anatomical Record (Hoboken)* 302(6): 947-953.

Matos, L., Nzikou, J.M., Matouba, E., *et al.* (2009) Studies of *Irvingia gabonensis* seed kernel: oil technological applications. *Pakistani Journal of Nutrition* 8: 151-157.

Messerli, F.H., Rimoldi, S.F., Bangalore, S. (2017). The transition from hypertension to heart failure: contemporary update. *JACC: Heart Failure* 5(8): 543-553.

Mohan, N., Jiang, J., Dokmanovic, M., *et al.* (2018). Trastuzumab-mediated cardiotoxicity: current understanding, challenges, and frontiers. *Antibody Therapy* 1(1): 13-17.

Munster, P., Krischer, J., Tamura, R., *et al.* (2019). A randomized community-based trial of an angiotensin converting enzyme inhibitor, lisinopril or a beta blocker, carvedilol for the prevention of cardiotoxicity in patients with early stage HER2-positive breast cancer receiving adjuvant trastuzumab. *Cancer Research* 79(4 Suppl): Abstract GS5-01; https://doi.org/10.1158-7445.SABCS18-GS501

National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011) Guide for the Care and Use of Laboratory Animals. The National Academies Press, Washington D.C., U.S.A.

Nguyen, N., Naik, V., Speer, M.Y. (2012). Diabetes mellitus accelerates cartilaginous metaplasia and calcification in atherosclerotic vessels of LDL mutant mice. *Cardiovascular Pathology* 22(2): 167-175.

Olesen, L.L., Andersen, A. (2016). ECG as a first step in the detection of left ventricular systolic dysfunction in the elderly. *ESC Heart Failure* 3(1): 44-52.

Olesen, L.L., Andersen, A., Thaulow, S. (2015). Hand-held echocardiography is useful for diagnosis of left systolic dysfunction in an elderly population. *Danish Medical Journal* 62: A5100.

Olorundare, O., Adeneye, A., Akinsola, A., *et al.* (2020). African vegetables (*Clerodendrum volubile* leaf and *Irvingia gabonensis* seed extracts) effectively mitigates trastuzumab-induced cardiotoxicity in Wistar rats. *Oxidative Medicine and Cellular Longevity* 2020: Article ID 9535426.

Olorundare, O.E., Adeneye, A.A., Akinsola, A.O., *et al.* (2021). Therapeutic potentials of selected antihypertensive agents and their fixed-dose combinations against trastuzumab-mediated cardiotoxicity. *Frontiers in Pharmacology* (In Press).

Perez, I.E., Alam, S.T., Hernandez, G.A., *et al.* (2019). Cancer therapy-related cardiac dysfunction: An overview for the clinician. *Clinical Medicine Insights: Cardiology* 13: 1-11.

Perik, P.J., de Korte, M.A., van Veldhuisen, D.J., *et al.* (2007). Cardiotoxicity associated with the use of trastuzumab in breast cancer patients. *Expert Review of Anticancer Therapy* 7(12): 1763-1771.

Phan, D.Q., Silka, M.J., Lan, Y-T., *et al.* (2015). Comparison of formulas for calculation of the corrected QT interval in infants and young children. *Journal of Pediatrics* 166(4): 960-964.e2;

Pinter, M., Kwanten, W.J., Jain, R.K. (2018). Renin-angiotensin system inhibitors to mitigate cancer treatment-related adverse events. *Clinical Cancer Research* 24(16): 3803-3812.

Piotrowski, G., Gawor, R., Stasiak, A., *et al.* (2012). Cardiac complications associated with trastuzumab in the setting of adjuvant chemotherapy for breast cancer overexpressing human epidermal growth factor receptor type 2 - a prospective study. *Archives of Medical Sciences* 8(2): 227-235.

Pituskin, E., Mackey, J.R., Koshman, S., *et al.* (2017). Multidisciplinary approach to novel
therapies in cardio-oncology research (MANTICORE 101-breast): a randomized trial for the prevention of trastuzumab-associated cardiotoxicity. *Journal of Clinical Oncology* 35: 870-877.

Poon, K.A., Flagella, K., Beyer, J., *et al.* (2013). Preclinical safety profile of trastuzumab emtansine (T-DM1): Mechanism of action of its cytotoxic component retained with improved tolerability. *Toxicology and Applied Pharmacology* 273:298-313.

Porta, C., Cosmai, L., Gallieni, M., *et al.* (2015) Renal effects of targeted anticancer therapies. *Nature Reviews Nephrology* 11: 354-370.

Qiao, J-H., Fishbein, M.C., Demer, L.L., *et al.* (1995). Genetic determination of cartilaginous

metaplasia in mouse aorta. *Arteriosclerosis, Thrombosis, and Vascular Biology* 15: 2265-2272.

Rahib, L., Wehner, M., Matrisian, L.M., *et al*. (2020). Pojection of cancer incidence and death to 2040 in the US: Impact of cancer screening and a changing demographic. *Journal of Clinical Oncology* 38(15_suppl.): 1566.

Ruggenenti, P., Iliev, I., Costa, G.M., *et al.* (2008). Preventing left ventricular hypertrophy by

ACE inhibition in hypertensive patients with type 2 diabetes: A prespecified analysis of the Bergamo nephrologic diabetes complications trial (BENEDICT). *Diabetes Care* 31(8): 1629-1634.

Rygiel, K. (2016). Benefits of antihypertensive medications for anthracycline- and trastuzumab-induced cardiotoxicity in patients with breast cancer: Insights from recent clinical trials. *Indian Journal of Pharmacology* 48(5): 490-497;

Sendur, M.A., Aksoy, S., Altundag, K. (2013). Cardiotoxicity of novel HER2-targeted therapies. *Current Medical Research and Opinion* 29: 1015-1024.

Sharma, P., Hakimian, S., Camacho, J., *et al.* (2018). Prevention of Chemo-induced cardiotoxicity with beta-blockers. *Journal of the American College of Cardiology* 71(11 Suppl): A1803.

Siegel, R.L., Miller, K.D., Jemal, A. (2020). Cancer Statistics, 2020. *CA Cancer Journal for Clinicians* 70: 7-30.

Spector, N.L., Blackwell, K.L. (2009) Understanding the mechanisms behind trastuzumab therapy for human epidermal growth factor receptor 2-positive breast cancer. *Journal of Clinical Oncology* 27: 5838-5847.

Tahir, H., Bardia, N., Bath, K., *et al.* (2019). Trastuzumab-induced cardiomyopathy and intermittent left bundle branch block. *Cardiology Research* 10(4): 230-235.

Tsang, W., Moe, G. (2007). Trastuzumab and heart failure. *Cardiology Rounds* 8: 1-6.

Umoru, G., Taitano, M., Beshay, S., *et al.* (2020). Pulmonary arterial hypertension in breast cancer patients on HER2-targeted therapy: a review of FDA Adverse Events Reporting System data. *ERJ Open Research* 6: 00199-2020;

Vaduganathan, M., Hirji, S.A., Qamar, A., *et al.* (2019). Efficacy of neurohormonal therapies in preventing cardiotoxicity in patients with cancer undergoing chemotherapy. *JACC Cardio-Oncology* 1: 54-65.

Vejpongsa, P., Yeh, E.T.H. (2014). Prevention of anthracycline-induced cardiotoxicity: challenges and opportunities. *Journal of American College of Cardiology* 64: 938-945.

Vermeulen, Z., Hervent, A-S., Dugaucquier, L., *et al.* (2017). Inhibitory actions of the NRG-

1/ErbB4 pathway in macrophages during fibrosis in the heart, skin, and lung. *Integrative Cardiovascular Physiology and Pathophysiology* 313(5): H934-H945.

Wallin, R., Wajih, N., Greenwood, G.T., *et al.* (2001). Arterial calcification: A review of mechanisms, animal models, and the prospects for therapy. *Medicinal Research Reviews* 21(4): 274-301.

Wittayanukorn, S., Qian, J., Westrick, S.C., *et al.* (2018). Prevention of trastuzumab and anthracycline-induced cardiotoxicity using Angiotensin-converting Enzyme inhibitors or β-blockers in older adults with breast cancer. *American Journal of Clinical Oncology* 41(9): 909-918.

Zablocki, D., Sadoshima, J. (2013). Angiotensin II and oxidative stress in the failing heart. *Antioxidants & Redox Signaling* 19(10): 1095-1109.

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Full Length Research Article

Kolaviron Protects Rats from Cognitive Decline Induced by Lipopolysaccharide in Wistar Rat

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Summary: Kolaviron **(**Kol-v) is a mixture of bioflavonoid from the seed of *Garcinia kola*, and has been previously shown to inhibit neuro-inflammation in Lipopolysaccharide (LPS)-activated BV-2 microglia. In this study, we investigated neuroprotective effects of Kol-v in LPS-induced memory impairment in Wistar rat. Wistar rats (225-250) g were used in this study. Memory impairment was induced with the systematic administration of 250µg/mg LPS. The effect of Kol-v on cognition and learning processes were assessed using the behavioral responses in the Morris water maze model; Effects of LPS on bodily activities were assessed by biochemical assays before and after treatment. Intra-peritoneal administration of LPS reduced the core body temperature, cognitive and locomotor process. Kol-v ameliorated the effect LPS on the core body temperature by restoring it back to normal, and significantly improved the cognitive and learning processes. Kol-v significantly increased the level of SOD and CAT and reduction in the levels of NO, GSH, and MDA. Kolaviron showed significant anti-inflammatory potentials through its protection against cognitive decline and oxidative properties induced by lipopolysaccharide in the laboratory Wistar rat.

Keywords: *Cognition, Kolaviron, Lipopolysaccharide, Memory Impairment, Neuro-inflammation, Oxidative stress*

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INTRODUCTION

Cognition involves processes in the brain that includes the ability to learn, remember, and make judgments. When cognition is impaired, it generates great impact on an individual's overall health and well-being. Cognitive decline ranges from mild cognitive impairment to dementia, a form of diminution in capabilities severe enough to meddle with daily life activity. Several pathophysiological mechanisms have been suggested to contribute to neuronal damage in many neurodegenerative disorders. One of the most investigated mechanisms in these conditions is glia-dependent neuro-inflammatory mechanisms, which also include astrocytes, the complement system, as well as cytokines and chemokines (Van *et al*., 2016). Cognitive impairment leads to trouble in remembering, learning new things, concentrating, or making decisions that affect everyday life which leads to reduction in quality of life and is related to neurodegenerative disorders. However, there is a correlation between these diseases and oxidative stress. Recent studies have also established the genetic risk factors in the role of inflammation in memory and learning deficits, since disorders like Alzheimer's disease are connected with increased levels of pro-inflammatory cytokines combined with decreased levels of anti-inflammatory cytokines (Barrientos *et al.*, 2009).

Oxidative stress is a pathophysiological mechanism that is closely associated with neurodegenerative disorders. It can be defined as an imbalance between reactive oxygen (ROS) and nitrogen species (RNS) and attenuated antioxidant defenses. The increased oxidative activities such as generation of superoxide anion ($O2^-$) and hydrogen peroxide (H_2O_2), and subsequently reactive oxygen species (ROS) can result in neuronal oxidative damage (Sorce *et al.*, 2017).

Kol-v is a mixture of three compounds - *Garcinia biflavonoid GB1, GB2 and Kola flavanone* (Iwu *et al*, 1990). Bi-flavonoids are the most abundant compounds in *Garcinia kola* a popular West African edible seed, while the kola flavones are the major components of Kol-v. *Garcinia biflavonoids* have been attributed to their ability to scavenge free radicals, induce detoxification, inhibit stress response proteins and interfere with DNA binding activities of some transcription factors. A number of studies have reported that Kol-v exhibited antioxidant anti-inflammatory activity *in vivo* and *in vitro* (Abarikwu 2014; Farombi *et al.*; 2009 Olaleye *et al*., 2010).

Indications about the ability of Kol-v to protect neurons from damage were reported in studies involving neurotoxicity induced with environmental chemicals including atrazine (Abarikwu *et al.*, 2011), vanadium (Igado *et al.*, 2012) and sodium azide (Olajide *et al.*, 2015). It has been reported earlier that Kol-v inhibits neuroinflammation and microglia-mediated neurotoxicity through mechanisms involving Nrf2/ARE antioxidant pathway (Onasanwo *et al.*, 2016). The anti-inflammatory and anti-oxidative potentials of Kol-v are yet to be properly explored in laboratory rodents induced with lipopolysaccharide. This study was

designed to investigate the impact of Kol-v on lipopolysaccharide-induced cognitive decline and oxidative stress in Wistar rat.

Plate 1:
Garcinia kola in its Pod (bitter kola, a family of *Guttiferae*) from Ife (Adebimpe-John E.O Original 2014)

MATERIALS AND METHODS

Extraction of Kolaviron: Kolaviron was extracted from the seeds of *Garcinia kola* using a widely reported protocol (Iwu 1985) and earlier described (Onasanwo *et al.*, 2016). Briefly, *Garcinia kola* nuts were purchased from local market in Ile-Ife, Nigeria. The seeds were peeled and air-dried in the laboratory. 8.2kg air dried seeds were ground into powdered form. The powdered seeds were extracted with n-hexane, in a soxhlet extractor. The de- fatted; dried mass was repacked and then extracted with methanol in a soxhlet extractor. The extract was concentrated and was diluted to twice its volume in distilled water and partitioned with chloroform. The concentrated chloroform fraction gave a yellow-brown solid known as Kolaviron, which was allowed to dry in oven (40˚C), and ground to powdered form. Kolaviron was suspended in corn oil for *in vivo* experiments. The rationale for the doses of Kolaviron used was in relation to the least intake of 3 seeds of the raw consumption of Garcinia kola daily. In comparison, all doses of the extracts used in this study were expressed in terms of the dried sample.

Animals: Wistar rats (225–250g) were used in these experiments, and were acclimatized for two weeks. After acclimatization of two weeks the animals were randomly divided into six study groups of (6) rats per group. The animals were fed with standard rat pellet, and were given water liberally. Animals were housed in clean plastic cages under natural light and dark cycle, and at room temperature. The negative control group received corn-oil only, positive control group were induced with LPS and received nothing, while other groups were treated with varying concentration of Kolaviron (50mg/kg, 100mg/kg, 200mg/kg) and Sulindac sulfide 100ug/kg respectively. Animals were taken care of according to the rules and guidelines of the National Institute of Health (NIH) for laboratory animal care and use. The proposal and use was approved by the University of Ibadan Animal Ethics Review Committee (Number UI-ACUREC/17/0024).

Body temperature: Four days prior to the experiment, the rat's temperature was taken at days 1, 3, 5, 7 and four hours after injection of either LPS or saline and on days 9 and 11 before sacrificing them. Body temperature was recorded using a rectal digital thermometer probe (WPI, Sarasota FL) at the same time each day (8:00 -9:00 hours) to minimize variation due to circadian rhythm

Behavioral test (Morris Water Maze Model): The Morris water maze test is used to assess spatial memory and non-spatial discrimination learning, and was conducted as described by (Morris, 1984). Rats were administered 50mg/kg, 100mg/kg and 200mg/kg Kol-v followed by LPS (250 µg/kg; i.p.) for 7 days. Animals in the vehicle control group received corn oil, while animals in the LPS control group did not receive any treatment. On the 4th day of treatment, learning and memory were evaluated using the Morris water maze test. The water maze consisted of a circular pool of water (150 cm in diameter) with a circular platform of 10 cm diameter and 1 cm below the water surface. The animals had two acquisition trials per day for 3 days during which they were placed in water and expected to find the hidden escape platform. This was taken as a measure of learning ability of the rats. On the last day, the platform was removed and the length of time the rats spent in the quadrant and distance travelled before getting to the region of the platform was recorded.

Preparation of Brain Tissues for Biochemical Assays: The animals were sacrificed firmly through cervical dislocation and the brains were immediately removed while the animal was still alive, washed in potassium chloride buffer and left at 4°C for 30 min. Thereafter, the whole brain was weighed and blotted with 10 % w/v phosphate buffer (0.1 M, pH 7.4), followed by homogenization to obtain the post mitochondrial fraction. Protein concentrations of various samples were determined using the Lowry method (Lowry *et al*., 1951) using bovine serum albumin as a standard. A breakdown product of lipid peroxidation thiobarbituric acid reactive substances (TBARS) was assayed as malondialdehyde (MDA) and measured according to the method of (Beuge *et al*., 1978). The concentration of the reduced glutathione (GSH) in the brain post mitochondrial supernatant was estimated using the method of (Jollow *et al*.,1974) and the levels of total SOD activity in the tissues were determined by the method of (Misra *et al*.,1972). Nitric oxide level was determined using the Griess method (Griess *et al*.,1879) while the catalase activity was determined by the method of Clairborne (Clairborne., 1995).

Statistical Analysis: Data are presented as mean ± S.E.M. Statistical analysis was done using one-way analysis of variance (ANOVA) test, followed by Student Newman-Keuls test for multiple comparisons. The level of significant difference between the groups was evaluated at $p< 0.05$. All statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Effects of Kolaviron on lipopolysaccharide-induced changes in body weight: The weight changes of animals

were monitored during the seven days of pre-treatment and post-treatment with fourteen days of pre-post treatment. At the beginning of the treatment, a significant weight loss that is reflected by the LPS injection was observed in all the groups (Figure 1a, b and c) however, the animals seemed to have recovered from the LPS treatment and showed weight gain with the vehicle treated animals. Kolaviron at the higher dose (200mg/kg) showed an increased weight with the vehicle animals, supplementation with Kolaviron restored the relative body weight.

Effects of Kolaviron on lipopolysaccharide-induced changes in the body temperature: There was a marked drop in temperature at day 1and at day 7, four hours after LPS injection (Fig 1d,e and f), it showed that the LPS treatment induced a significant reduction of body temperature after the first dose of LPS On subsequent treatment, there was a significant increase in the body temperature after few days of LPS induction p value < 0.0045(A), the animals showed a difference in their core body temperature compared to the vehicle group. However, the result was significantly different at p < 0.0001.

Effects of Kolaviron on learning and memory tasks in rats: The results of the acquisition training (Figure2a, 3a, 4a and 2b, 3b, 4b; invisible platform trial) of six trials (two times per day) for 3 days to measure the escape latency and path-length showed a gradual reduction in percentage time along the days between the groups during the training trial.

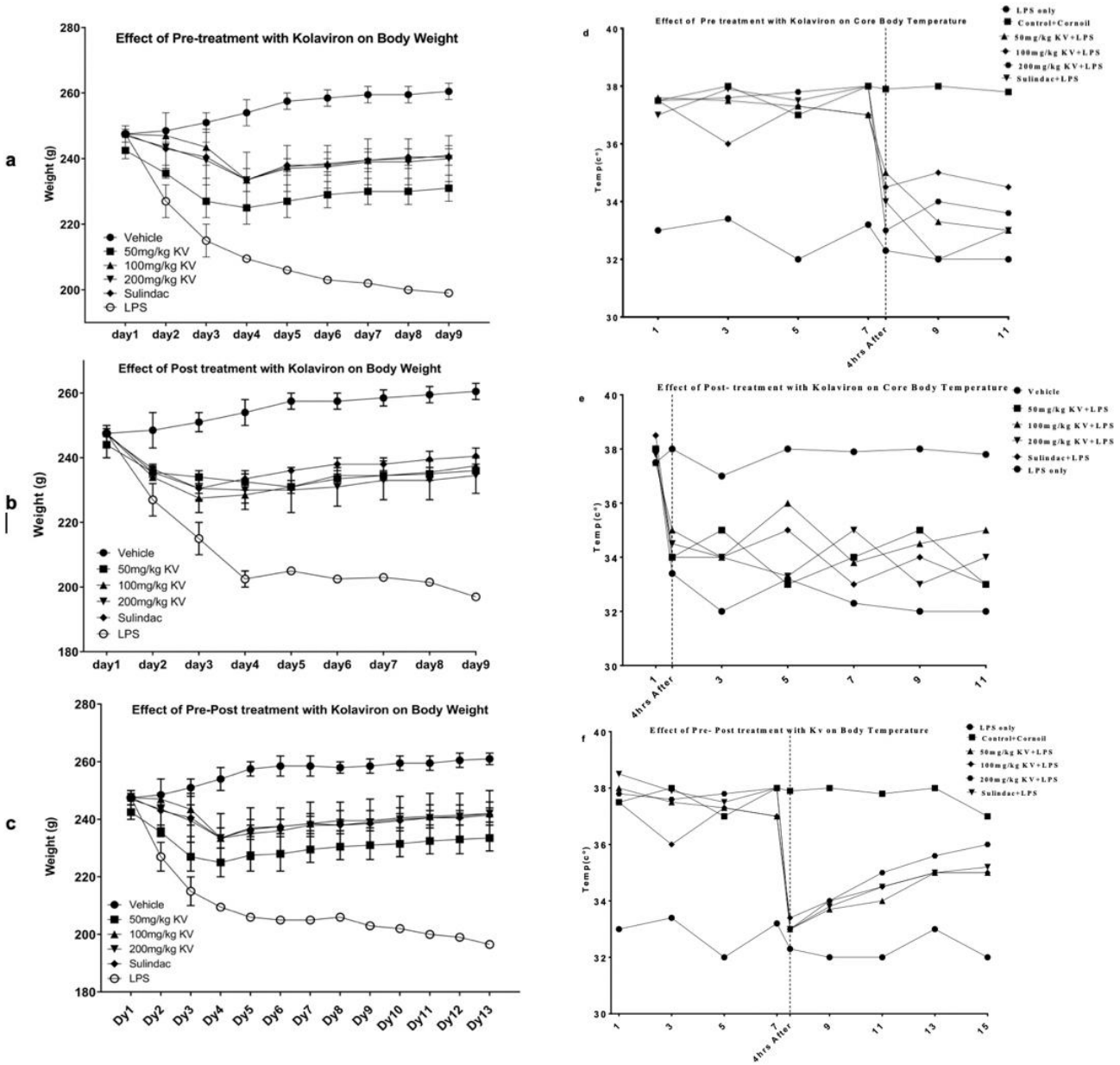

Figure 1:
Effects of Kol-v on single dose of LPS (250µg/kg on the body weight and core body temperature (a)Pre-treatment (b) Post-treatment and (c) Pre-Post-treatment. Following treatment with Kol-v (50mg/kg, 100mg/kg and 200mg/kg) for 7days and 14 days. Each value is mean ± S.EM. *Significantly different from vehicle (negative control group). Significance was verified with one-way ANOVA test, followed by Student Newman-Keuls (SNK) test for multiple comparisons. P < 0.001; N = 6

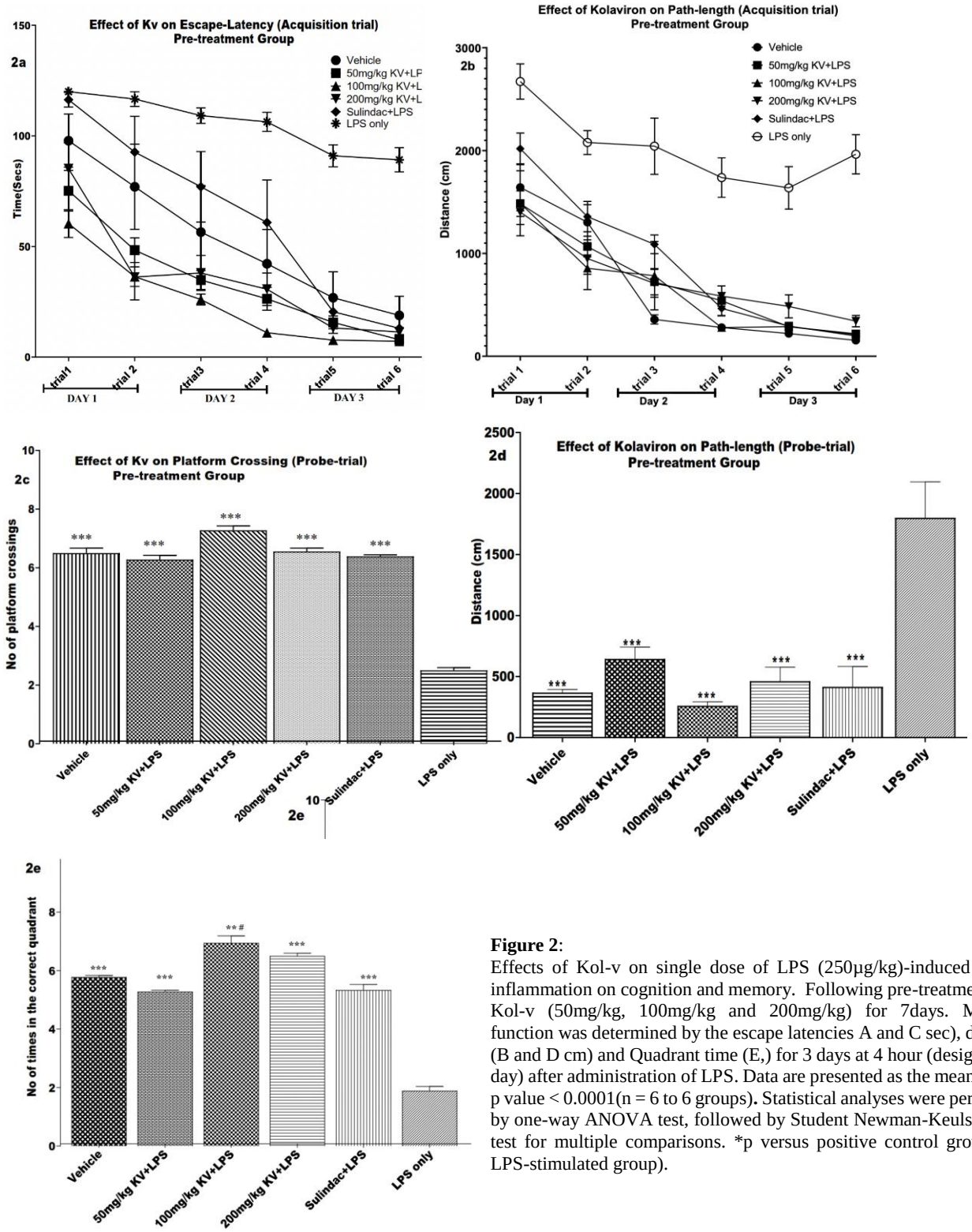

Figure 2:
Effects of Kol-v on single dose of LPS (250µg/kg)-induced neuro-inflammation on cognition and memory. Following pre-treatment with Kol-v (50mg/kg, 100mg/kg and 200mg/kg) for 7days. Memory function was determined by the escape latencies A and C sec), distance (B and D cm) and Quadrant time (E,) for 3 days at 4 hour (designated 1 day) after administration of LPS. Data are presented as the mean ± S.D. p value < 0.0001(n = 6 to 6 groups)**.** Statistical analyses were performed by one-way ANOVA test, followed by Student Newman-Keuls (SNK) test for multiple comparisons. *p versus positive control group (the LPS-stimulated group).

The results were averaged across two trials per day for 3days. The results of the probe trial (Figure 2c, 3c, 4c; non-visible platform trial) LPS treated group (positive control) showed an increase in time to locate the platform when compared to other groups that has been pre-treated with Sulindac sulfide (100µg/kg,) and Kol-v (50mg/kg, 100mg/kg and 200mg/kg) they took a shorter time to locate the platform along the experimental days. The LPS-treated rat travelled a longer distance to reach the platform while the other groups travelled a relative short distance to reach the platform (Figure2d, 3d, 4d).The number of time they entered into the quadrant (quadrant time) was reduced in the LPS group compared to other groups with higher frequency of entering the quadrant. The effect Kol-v was seen to be effective at 100mg/kg for Pre-treatment, 50mg/kg for Post-treatment and 200mg/kg for Pre-Post treatment (Figure 2e, 3e, 4e).It is considered that these differences between the groups reflected the differences in timing of cognitive decline.

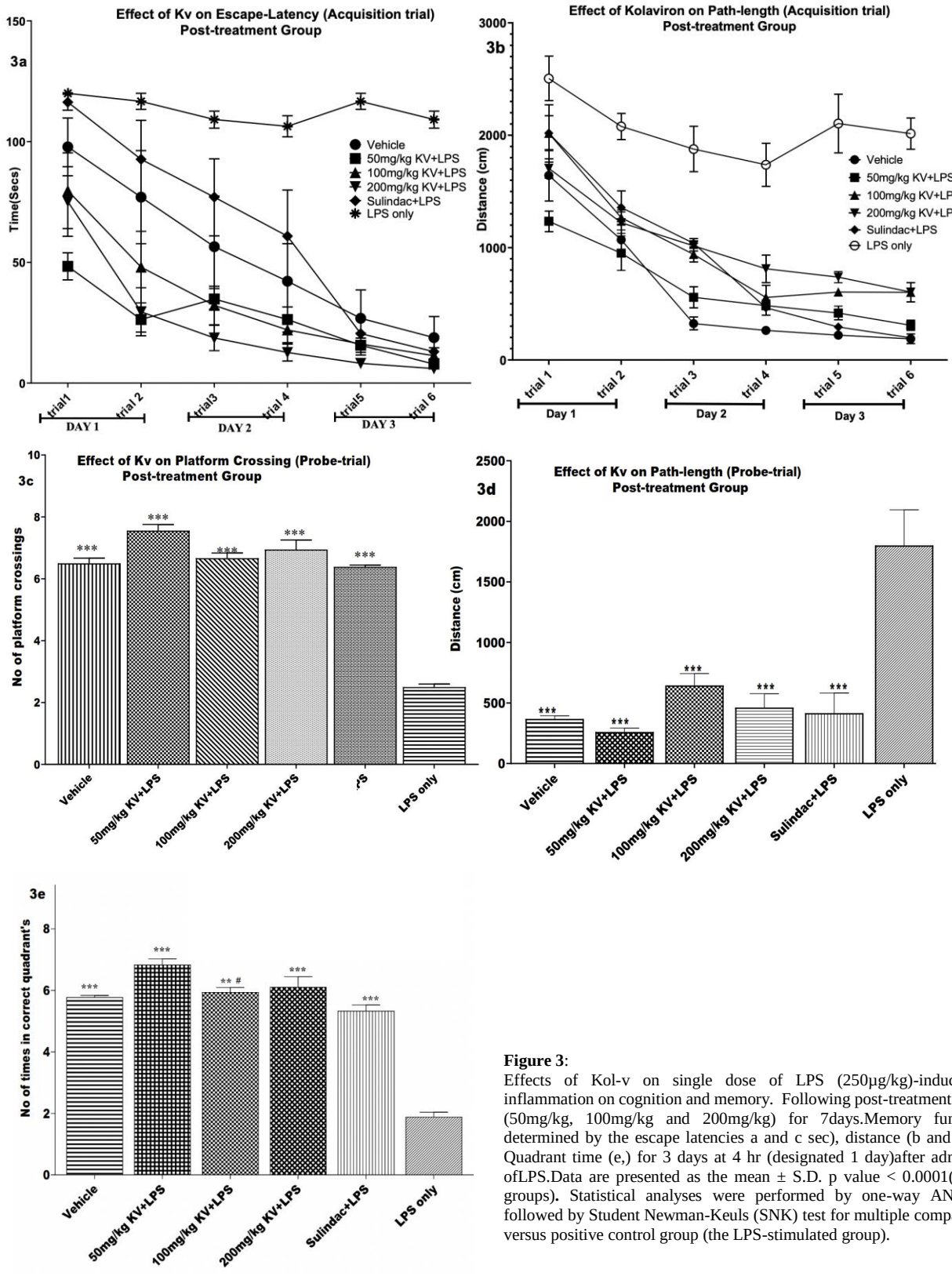

Figure 3:
Effects of Kol-v on single dose of LPS (250µg/kg)-induced neuro-inflammation on cognition and memory. Following post-treatment with Kol-v (50mg/kg, 100mg/kg and 200mg/kg) for 7days.Memory function was determined by the escape latencies a and c sec), distance (b and d cm) and Quadrant time (e,) for 3 days at 4 hr (designated 1 day)after administration ofLPS.Data are presented as the mean ± S.D. p value < 0.0001(n = 6 to 6 groups). Statistical analyses were performed by one-way ANOVA test, followed by Student Newman-Keuls (SNK) test for multiple comparisons. * p versus positive control group (the LPS-stimulated group).

Effects of Kolaviron on the MDA Level in cognitive decline induced by LPS: The effect of pre-treatment, post-treatment and pre-post treatment with varying concentration of Kol-v (50mg/kg, 100mg/kg, 200mg/kg) and Sulindac sulfide (100µg/kg, orally) on the MDA level was examined as shown in (Figure 5a, b and c).

Effects of Kolaviron on the GSH Level in cognitive decline induced by LPS: We examined the effect of pre-post treatment with varying concentration of Kol-v (50mg/kg, 100mg/kg, 200mg/kg) and Sulindac sulfide (100µg/kg, orally) on the GSH level. As shown in (Figure 6a, b and c).

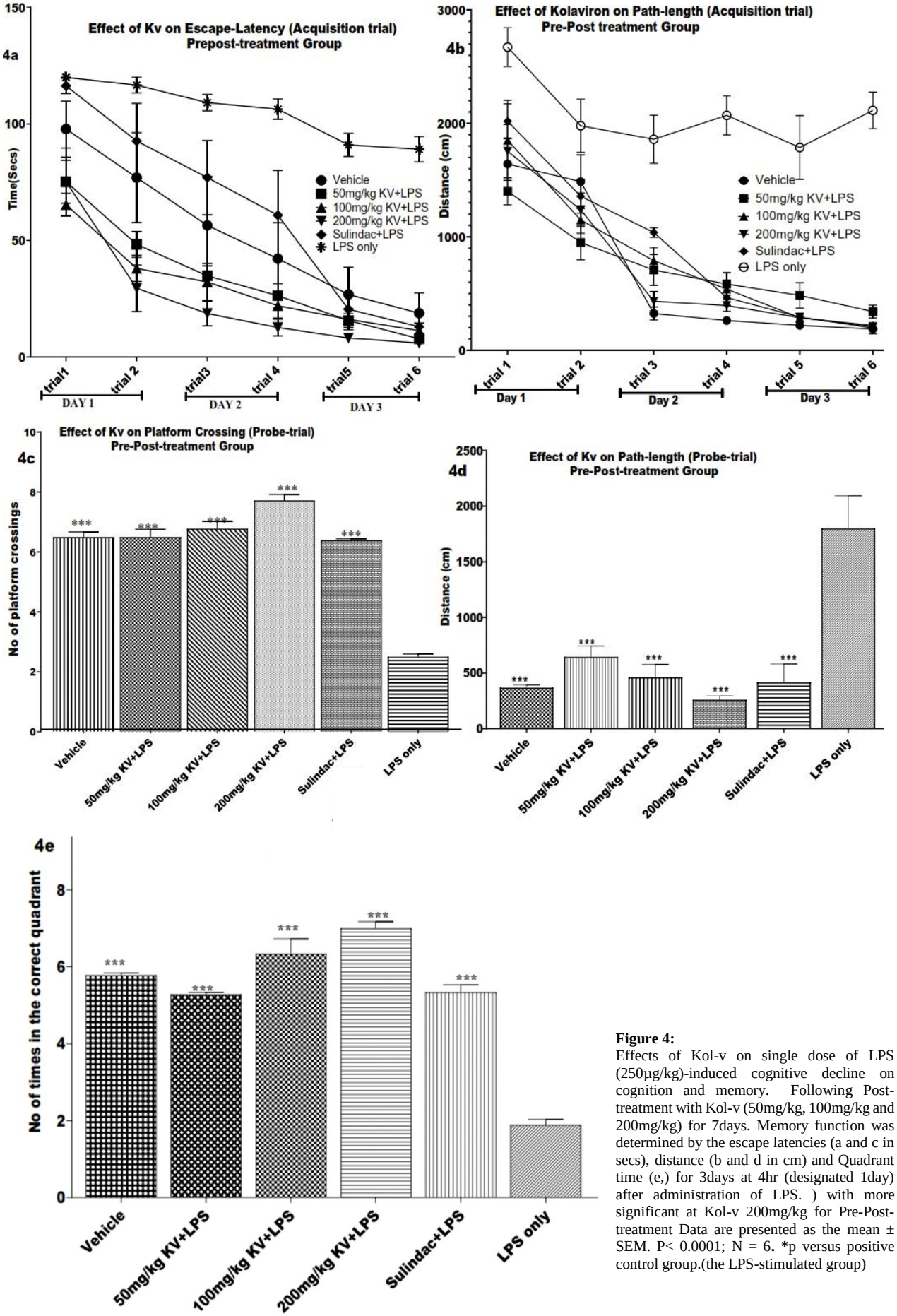

Figure 4:
Effects of Kol-v on single dose of LPS (250µg/kg)-induced cognitive decline on cognition and memory. Following Post-treatment with Kol-v (50mg/kg, 100mg/kg and 200mg/kg) for 7days. Memory function was determined by the escape latencies (a and c in secs), distance (b and d in cm) and Quadrant time (e,) for 3days at 4hr (designated 1day) after administration of LPS.) with more significant at Kol-v 200mg/kg for Pre-Post-treatment Data are presented as the mean ± SEM. P< 0.0001; N = 6**.** *p versus positive control group.(the LPS-stimulated group)

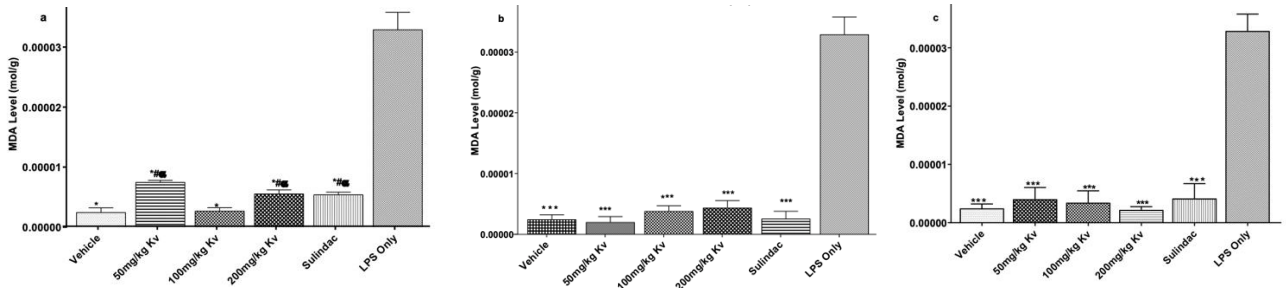

Figure 5:
Effects of Kol-v on single dose of LPS (250µg/kg)-induced cognitive decline on MDA analysis .This result shows the lipid peroxide levels were high in the case of negative control animals (LPS group) which were significantly lowered during the administration of varying concentration of Kolaviron especially at 100mg/kg for Pre-treatment, 50mg/kg for Post-treatment and 200mg/kg for Pre-Post treatment. *****p versus positive control group (the LPS-stimulated group) #p versus negative group (the Vehicle Corn oil only), **ᵅ**p versus 100mg/kg Kol-v. Values are mean ± SEM P< 0.0001; N = 6.

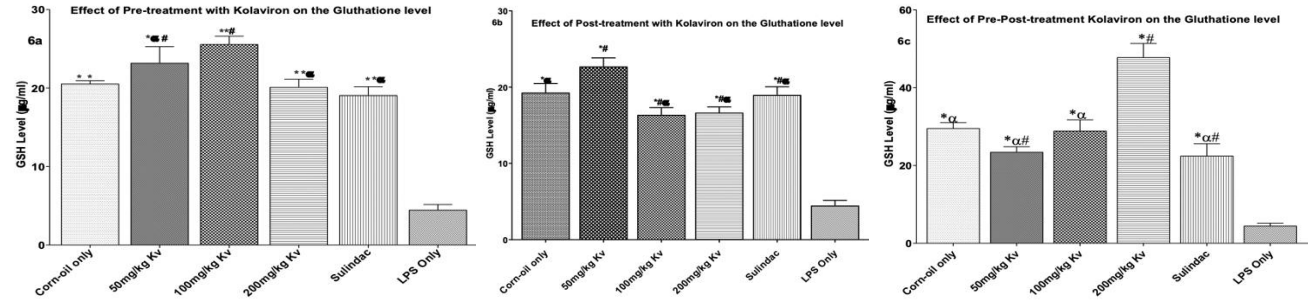

Figure 6:
Effects of Kol-v on single dose of LPS (250µg/kg)-induced cognitive decline on GSH level. This results shows that cognitive decline was stimulated by a significant reduction in the level of the reduced glutathione in the case of negative control animals (LPS group) which were significantly increased by the administration of pre-treatment with Kol-v especially at 100mg/kg for Pre-treatment,50mg/kg for Post-treatment and 200mg/kg for Pre-Post treatment. *****p versus positive control group (the LPS-stimulated group) #p versus negative group (the Vehicle Corn oil only), **ᵅ**p versus 50mg/kg,100mg/kg and 200mg/kg Kol-v respectively. Values are mean ± SEM P< 0.0001; N = 6.

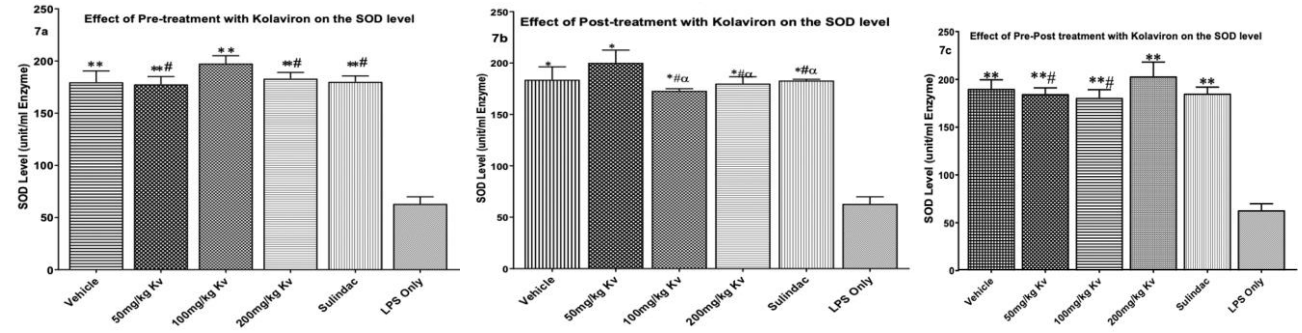

Figure 7:
Effects of Kol-v on single dose of LPS (250µg/kg)-induced cognitive decline on SOD analysis This result shows the SOD level was low in the case of negative control animals (LPS group) which were significantly increased after post-treatment with Kol-v especially at 100mg/kg for Pre-treatment, 50mg/kg for Post-treatment and 200mg/kg for Pre-Post treatment*****p versus positive control group (the LPS-stimulated group) #p versus negative group (the Vehicle Corn oil only), **ᵅ**p versus 100mg/kg Kol-v. Values are mean ± SEM P < 0.0001; N = 6.

Effects of Kolaviron on the Nitric-oxide (NO) Level in cognitive decline induced by LPS*:* The interest in measuring Nitric-Oxide level in biological tissues and fluids remains strong as it is an inflammatory marker. As shown in (Figure 8a, b and c).

Effects of Kolaviron on the Catalase (CAT) Level in cognitive decline induced by LPS*:* The catalase activity on the effect of treatment with varying concentration of Kolaviron (50mg/kg, 100mg/kg, 200mg/kg) and Sulindac sulfide (100µg/kg, orally) on cognitive decline induced by LPS was examined. As shown in (Figure 9a, b and c).

DISCUSSION

Intra-peritoneal lipopolysaccharide (LPS) treatment has been connected with cognitive deficits in rodents, and this has be observed in learning and memory tasks in the Morris water maze (Arai*et al*., 2001; Rosi *et al*. 2006S).

In this study, rats were administered with LPS (250 µg/kg) and were subsequently tested in the Morris water maze, the memory enhances effect and anti-inflammatory properties of Kol-v, a defatted seed extract of *Garcinia kola*, was investigated. We have shown here that Kolaviron exhibited a high level of anti-oxidative properties and very strong anti-inflammatory activities when compared to a standard reference drug, Sulindac sulfide. Kol-v also been interlaced in the neuroprotective role against gamma-radiation-induced brain injury (Adaramoye, 2010), 3-nitro- propionic and methamphetamine-induced neurotoxicity (Nwoha *et al*., 2007; Ijomone *et al*., 2012).

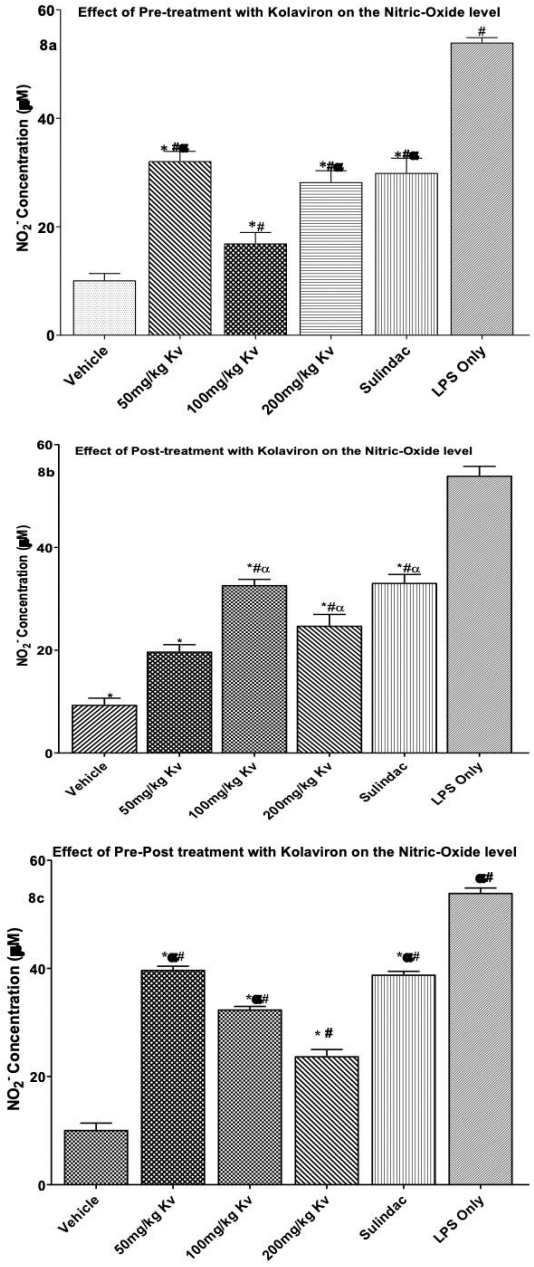

Figure 8:
Effects of Kol-v on single dose of LPS (250µg/kg)-induced cognitive decline on NO_2 analysis the level of the nitric oxide was high in the case of negative control animals (LPS group) which were significantly reduced by the administration of post-treatment with varying concentration of Kolaviron (50mg/kg, 100mg/kg and 200mg/kg) with more significant at Kol-v 100mg/kg for Pre-treatment, 50mg/kg for Post-treatment and 200mg/kg for Pre-Post treatment. *****p versus positive control group (the LPS-stimulated group) **#**p versus negative group (the Vehicle Corn oil only), **α**p versus 50mg/kg, 100mg/kg and 200mg/kg Kol-v respectively. Values are mean ± SEM P< 0.0001; N = 6.

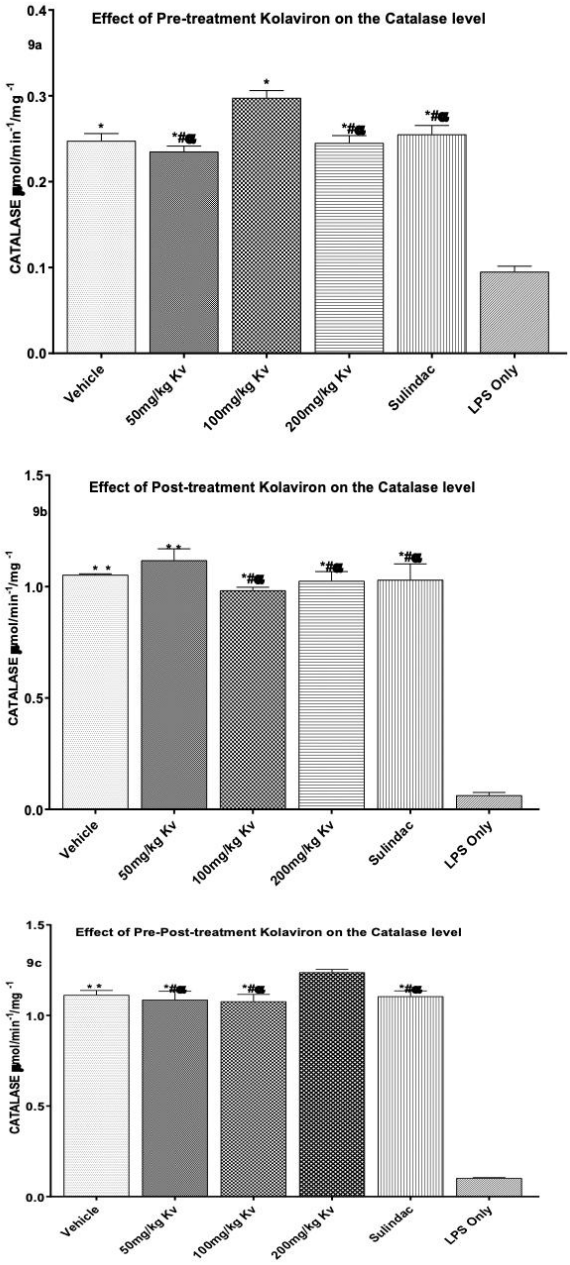

Figure 9:
Effects of Kol-v on single dose of LPS (250µg/kg)-induced cognitive decline on SOD analysis, the Catalase level was reduced in the LPS group which were significantly increased by the post-treatment with Kolaviron (50mg/kg, 100mg/kg and 200mg/kg) with more significant at Kol-v 100mg/kg for Pre-treatment, 50mg/kg for Post-treatment and 200mg/kg for Pre-Post treatment. . *****p versus positive control group (the LPS-stimulated group) **#**p versus negative group (the Vehicle Corn oil only), **α**p versus 50mg/kg, 100mg/kg and 200mg/kg Kol-v respectively. Values are mean ± SEM P < 0.0001; N = 6

Learning and memory tasks was assessed in the test trial (acquisition training), while the long-term memory was assessed in probe trial (non-visible platform trial) In these experiments, Kol-v improved cognitive and learning processes, as shown by a decrease in escape latency in the Morris water maze during training and increase in time spent in quadrant during retrieval. The effect of Kol-v was seen to be effective at 100mg/kg for pre-treatment, 50mg/kg for post-treatment and 200mg/kg for pre- and post-treatment. As reported that probe trials may be more accurately described as extinction procedures (Takeda *et al*., 2004; Lattal *et al*., 2003).

There was a marked drop in temperature four hours after LPS injection, this strong link aligns the fact that hypothermic animal have higher neurological deficits and cognitive decline. However the hypothermia observed in the present study may be considered as an adaptive thermoregulatory or a survival response to a systemic inflammation which is correlated with sepsis or stroke. These suggest that body temperature is a prognostic for cognitive decline such as Alzheimer's and most neurological disease. Furthermore, LPS produced a marked reduction in body weight, noticeable at the first time point of 4 hours. The reduction in body weight is a sign of

inflammatory disease because LPS acts as the hypothalamic center for energy homeostasis (Cia *et al*., 2009). This effect is in agreement with previous reports, showing that relatively high doses of LPS induced weight loss (Kwang *et al*., 2013; Aubert*et al*., 2005; Lugarini*et al*., 2002; Plata-Salaman *et al*., 1993).

The brain is particularly susceptible to oxidative stress, which is explained by its relatively low levels of antioxidants, high concentration of polyunsaturated fatty acids, along with an increased oxygen demand of the brain. Oxidative stress is considered as a baleful condition for normal brain functioning since the brain uses reactive species, which differ chemically for signal transmission. Oxidative stress has been thought to be one of the major processes in development of a wide range of diseases including Alzheimer's disease (Yirmiya *et al*., 2001; Christen *et al*., 2000) and neuro-degeneration in motor neuron diseases (Nunomura *et al*., 2006). Several antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione S-transferase protect DNA from oxidative stress. Antioxidants are also being investigated as possible treatments for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Khan*et al*., 2010; Di Matteo*et al*., 2003). The present results showed changes in biomarkers of oxidative damage, resulting in a higher oxidative imbalance when induced with LPS.

Higher levels of MDA was seen in the LPS group compared to Kol-v treated groups, which is a major product of the reactive species attack on polyunsaturated fatty acids, and is widely used as a biomarker of lipid peroxidation. It is one of the distinctive features of neuro-degeneration because the brain has high lipid content, after the adipose tissue, thus, elevated serum levels of lipid peroxidation products have been often reported in brain disorders. MDA is a reliable marker for determining oxidative stress in clinical situations and due to MDA's high reactivity and toxicity; treatment with varying Kol-v, reduced the level of lipid peroxides indicating the effective anti-oxidative properties of Kol-v in the moderation of tissue damage in the LPS-stimulated rats at varying concentration. We observed that animals treated with varying concentration of Kol-v especially 100mg/kg for Pre-treatment, 50mg/kg for Post-treatment and 200mg/kg for Pre-Post treatment decreased the lipid peroxidation leading to an increase in GSH, since GSH plays a vital in the role of cellular oxidant defense and is indispensable to demobilize lipid peroxidation (Fulvio *et al*., 2020).

The antioxidant glutathione (GSH) is essential for the cellular detoxification of reactive oxygen species in brain cells. An impaired GSH system in the brain has been intertwine with the oxidative stress occurring in neurological diseases. Recent data demonstrate that besides intracellular functions GSH has also important extracellular functions in brain. The tripeptide glutathione and its related enzymes partake in conserving oxidant homeostasis in aerobic cells. Various biomolecules with redox dependent activity are postulated in the neuronal plasticity events that have a role in learning and memory functions. The up-keep of normal glutathione level is essential for acquisition, but not consolidation, of spatial memory. Glutathione unavailability leads to failures in hippocampal synaptic plasticity mechanisms, which are conceivably related to spatial memory deficit. After LPS administration, there was a decrease in glutathione level that was seen in the LPS-untreated group and Kol-v treated group, this was reflected during the acquisition and probe trial. Cognitive decline have been considered to be an indicator of increased oxidative stress. Kolaviron was more significant at 100mg/kg for Pre-treatment, 50mg/kg for Post-treatment and 200mg/kg for Pre-Post treatment in elevating the GSH level in the experimental rats. It was observed that there was increase in expression of this antioxidant enzyme in cognitive declined rats, which implies that Kol-v has a potential oxidative property for defense activated with the capability to generate detoxification through enhanced scavenging of oxy-radicals and GSH plays an important role in cognition and memory enhancement.

Catalase (CAT) is a heme protein which catalyzes the reduction of hydrogen peroxides and protects the tissues from hydroxyl radicals. Catalase is one of the essential antioxidant enzymes that play a vital role by breaking down hydrogen peroxide and maintaining the cellular redox homeostasis. Catalase has a prime role in regulating the cellular level of hydrogen peroxide and its hydrogen peroxide catabolism protects the cells from oxidative assault (Habib *et al*., 2010). Catalase is a key enzyme which uses hydrogen peroxide, a non-radical ROS, as its substrate. This enzyme is responsible for negating by decomposing hydrogen peroxide, to maintain an optimum level of the molecule in the cell which is also essential for cellular signaling processes. A deficiency in CAT has been associated with many diseases such as diabetes mellitus, cardiovascular diseases, hypertension, anemia, Alzheimer's disease, bipolar disorder, and schizophrenia (Al-Abrash *et al*., 2000). Catalase enzyme was implicated in mutagenesis and inflammation conditions as well as during the suppression which are all known to be associated with oxidative stress conditions. There was decrease in the CAT level after LPS injection which we observed during the learning and memory tasks affecting cognition as CAT is known to play an important role in learning and tolerance to oxidative stress as an adaptive response. There is a closed link between catalase metabolism and oxidant homeostasis, which is expressed during the catalytic decomposition of hydrogen peroxide H_20_2 to water and oxygen which is formed as a by-product of numerous oxidases and SOD reactions.

Superoxide Dismutase (SOD) is the only antioxidant enzyme that salvages the superoxide anion by converting this free radical to oxygen and hydrogen peroxide, thus preventing peroxynitrite production and further damage. Free radicals are strongly associated with many pathological processes in the body. Due to this scavenging ability, SOD has acquired significant attention for therapeutic use. Superoxide dismutase is extensively researched, and used in anti-inflammatory, antitumor, radiation protection, and antisenility applications (Luisa *et al*., 2002). The physiological importance of SOD is illustrated by the severe pathologies evident in mice genetically engineered to lack these enzymes (Lob *et al*., 2002). Mice lacking SOD die several days after birth, amid massive oxidative stress (Seguí *et al*., 2004). Treatment with Kol-v with more significant at 100mg/kg for Pre-treatment, 50mg/kg for Post-treatment and 200mg/kg for Pre-Post treatment

significantly reduced the metabolism of oxidative stress by increasing SOD and CAT level in the LPS induced rats treated with Kol-v.

Nitric oxide signaling in the brain has been reported to regulate different processes (long-term potentiation and depression, LTP and LTD) controlling rhythmic activity, involvement in learning and memory mechanisms through mediation of specific forms of LTP in the cerebellum (Jacoby *et al*., 2001), hippocampus (Garthwaite *et al*., 2008) and neo-cortex, and LTD in the cerebellum. These findings provide strong support to the concept that NO plays a vital role in both learning process and memory of the learnt task (Choopani *et al*., 2008). Since NO is known to relax blood vessels and to increase blood supply to the brain, we accessed the action of NO role in inducing neuronal activity. In this study, a decrease in NO synthesis following an inhibition of NOS activity after LPS injection resulted in vasoconstriction and a decrease in perfusion into the brain. The sympathetic nervous system initially responds to hypothermia by triggering peripheral vasoconstriction, hypertension, tachycardia, and increased cardiac output, justifying the reduction in body temperature after LPS injection when we accessed it. This effect of NOS inhibitors can be proposed for an impairment of learning and memory processes in animals. We established evidence for the involvement of NO in learning and memory processes, the experimental findings demonstrated synthesis of NO and the neuronal action of NO during the period when the animals were trained to learn, and then to remember during the probe trial in the Morris Water Maze. The cognitive effects of LPS increased NO concentration while Kol-v was able to ameliorate its effects by decreasing NO concentration. This is because an increased synthesis of NO has been found to produce neurotoxicity due to accumulation of its toxic metabolite, peroxynitrite which can be a further source of hydroxyl radicals (Massaad 2011). These findings provide strong support to the concept that NO plays a vital role in both learning process and memory of the learnt task; which indicates the anti-inflammatory properties of Kol-v as earlier suggested (Olaleye *et al*., 2010).

In conclusion, we can establish a relationship between the progress of cognitive decline and increased oxidative stress with lipopolysaccharide. Kolaviron showed significant anti-inflammatory potentials through its protection against cognitive decline and oxidative properties, induced by lipopolysaccharide in the laboratory Wistar rat. Also, Kolaviron showed protection against oxidative stress in the laboratory Wistar rat.

REFERENCES

Abarikwu, S. O. (2014). Kolaviron, a natural flavonoid from the seeds of Garcinia kola, reduces LPS-induced inflammation in macrophages by combined inhibition of IL-6 secretion, and inflammatory transcription factors, ERK1/2, NF-?B, p38, Akt, p-c-JUN and JNK. Biochem Biophys Acta. 1840: 2373 - 2381.

Abarikwu, S. O., Farombi, E. O. and Pant, A. B. (2011). Biflavanone-kolaviron protects human dopaminergic SH-SY5Y cells against atrazine induced toxic insult. ToxicolIn Vitro. 25: 848 - 858.

Adaramoye, O. A. (2010). Protective Effect of Kolaviron, a Biflavonoid from Garcinia kola Seeds, in Brain of Wistar Albino Rats Exposed to Gamma-Radiation. Biological & Pharmaceutical Bulletin, 33: 260 - 266.

Arai, K., Matsuki, N., Ikegaya, Y. and Nishiyama, N. (2001). Deterioration of spatial learning performances in lipopolysaccharide treated mice, Jpn J Pharmacol. 87: 195 - 201.

Aubert, A. and Dantzer, R. (2005). The taste of sickness: lipopolysaccharide-induced finickiness in rats. Physiol Behav. 84: 437–444.

Barrientos, R. M., Frank, M. G., Hein, A. M. *et al.,* (2009). "Time course of hippocampal IL-1$\beta$and memory consolidation impairments in aging rats following peripheral infection," Brain, Behavior, and Immunity. 23(1): 46 – 54.

Beuge, J. A. and Aust, S. D. (1978). Microsomal Lipid peroxidation. Mehod Enzymol. 30: 302-310.

Bluthe, R. M., Castanon, N., Pousset, F., *et al*. (1999). Central injection of IL-10 antagonizes the behavioral effects of lipopolysaccharide in rats. Psychoneuroendocrinology; 24: 301–311.

Choopani, S., Moosavi, M., and Naghdi, N. (2008). Involvement of nitric oxide in insulin-induced memory impairment. Peptides. 29: 898–903. [PubMed] [Google Scholar]

Christen, Y. (2000). Oxidative stress and Alzheimer disease, Am J Clin Nutr.71(2): 621S–629S.

Cia, G. and Freeman, G. J. (2009) The CD160, BTLA, LIGHT/HVEM pathway: a bidirectional switch regulating T-cells activation. Immunol Rev. 229: 244-58.

Clairborne, A. (1995). Catalase Activity. In handbook of Methods for Oxygen Radical Research. A .R Greewald Editors. CRC Boca Raton, Florida, USA 237-242.

Das, S., Vasisht, S., Snehlata, R., Das, N. and Srivastava, L. M. (2000). Correlation between total antioxidant status and lipid peroxidation in hypercholesterolemia, Curr Sci. 78: 486-487.

Di Matteo, V. and Esposito, E. (2003). Biochemical and therapeutic effects of antioxidants in the treatment of Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, Curr Drug Targets CNS NeurolDisord 2(2) 95–107.

Farokhnia, M., ShafieeSabet, M., Iranpour, N., Gougol, A., Yekehtaz, H., Alimardani, R., Farsad, F., Kamalipour, M., Akhondzadeh, S. (2014). Comparing the efficacy and safety of Crocus sativus L. with memantine in patients with moderate to severe Alzheimer's disease: a double-blind randomized clinical trial. Hum Psychopharmacol. 29: 351-359.

Farombi, E. O., Shrotriya, S. and Surh, Y. J. (2009). Kol aviron inhibits dimethyl nitrosamine-induced liver injury by suppressing COX-2 and iNOS expression via NF-kappa B and AP-1. Life Sci. 84: 149-155.

Fulvio, U. and Matilde, M. (2020). Lipid peroxidation and ferroptosis: The role of GSH and GPx4, Free Radical Biology and Medicine, Volume 152, , Pages 175-185, ISSN 0891-5849,https://doi.org/10.1016/j.freeradbiomed.2020.02.027.

Garthwaite, J.,(2008)**.** Concepts of neural nitric oxide-mediated transmission.*Eur J Neurosci*.; 27 2783-802.

Griess, P. (1879) Bemerkungen zu der abhandlung der H.H. Weselsky und Benedikt Uebereinige azoverbindungen." *Chem. Ber.* **12**, 426–8.

Hiner, A., Raven, E., Thorneley, R., García-Cánovas, F. and Rodríguez-López, J. (2002). "Mechanisms of compound I formation in heme peroxidases". J Inorg Biochem. 91 (1): 27–34.

Hrupka, B. J. and Langhans, W.(2001). A role for serotonin in lipopolysaccharide-induced anorexia in rats.PharmacolBiochemBehav.; 68 355–362.

Igado, O. O., Olopade, J. O., Adesida, A., Aina, O. O., Farombi, E. O. (2012). Morphological and biochemical investigation into the possible neuroprotective effects of kolaviron (*Garcinia kola* bioflavonoid) on the brains of rats exposed to vanadium. Drug Chem Toxicol. 35: 371-380.

Ijomone, O. M., Nwoha, P. U., Olaibi, O. K., Obi, A. U. and Alese, M. O. (2012). Neuroprotective Effects of Kolaviron, a Biflavonoid Complex of Garcinia kola, on Rats Hippocampus

against Methamphetamine-Induced Neurotoxicity. Macedonian Journal of Medical Sciences, 5: 10-16.

Iwu, M. M. (1985). Antihepatoxic constituents of Garcinia kola seeds. Experientia 41: 699-700.

Jacoby, S., Sims, R. E. and Hartell, N. A. (2001). Nitric oxide is required for the induction and heterosynaptic spread of long-term potentiation in rat cerebellar slices. J Physiol. 535: 825-839.

Jollow, D. J., Mitchell, J. R., Zampaglione, N. and Gillette, J. R. (1974). Bromobenzene induced liver necrosis protective role of glutathione and evidence for 3,4, Bromobenzene oxide as the hepatoxic metabolite. Pharmacology 135: 151-169.

Lattal, K. M., Mullen, M. T. and Abel, T. (2003). Extinction, renewal, and spontaneous recovery of a spatial preference in the water maze. BehavNeurosci. 117(5): 1017-1028.

Lee, Y, Kim MS, Lee J2017.Neuroprotective strategies to prevent and treat Parkinson's disease based on its pathophysiological mechanism. Arch Pharm Res. Oct; 40(10):1117-1128.

Lee, J., Torosyan, N. and Silverman, D. H. (2017). Examining the impact of grape consumption on brain metabolism and cognitive function in patients with mild decline in cognition: a double-blinded placebo controlled pilot study. Exp. Gerontol. 87: 121-128.

Lob, H. E., Marvar. P. J., Guzik, T.J., Sharma, S., McCann, L. A., Weyand, C, Gordon, F. J. and Harrison, D. G. (2010). "Induction of hypertension and peripheral inflammation by reduction of extracellular superoxide dismutase in the central nervous system". Hypertension. 55 (2): 277–83, 6p following 283. doi:10.1161/HYPERTENSIONAHA.109.142646. PMC 2813894. PMID 20008675.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L and Randall, R.J. (1951). Protein measurement with folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lugarini, F., Hrupka, B. J., Schwartz, G. J., Plata-Salaman, C. R. and Langhans, W. (2002). A role for cyclooxygenase 2 in lipopolysaccharide-induced anorexia in rats. Am J PhysiolRegulIntegr Comp Physiol. 283: R862–R868.

Mao, X. Y., Jin, M. Z., Chen, J. F., Zhou, H. H., Jin, WL. (2018). Live or let die: Neuroprotective and anti-cancer effects of nutraceutical antioxidants. Pharmacol Ther. 183: 137-151.

Misra, H. P., and Fridovish, I. (1972). The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. J Biol. Chem. 247: 3170-3175

Morris, R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. Journal of Neuroscience Methods, 11: 47-60.

Nunomura, A., Castellan, R., Zhu, X., Moreira, P., Perry G. and Smith, M. (2006). Involvement of oxidative stress in Alzheimer disease, J NeuropatholExp Neurol. 65(7): 631–641.

Nwoha, P. U., Ojo, B., Ajayi, S. A., Ofusori, D. A., Oluwayinka, O. P., Odukoya, S. A. and Falana, B. A. (2007). Garcinia kola Diet Provides Slight Protection to Mice Hippocampal Neurons against Neuro-Toxins. J. Environ Neurosci. Biomed. 1: 125-136.

Olajide, O. J., Enaibe, B. U., Bankole, O. O., Akinola, O. B., Laoye, B. J., Ogundele OM (2015) Kolaviron was protective against sodium azide (NaN3) induced oxidative stress in the prefrontal cortex. Metab Brain Dis. 31(1): 25-35.

Olaleye, S. B., Onasanwo, S. A., Ige, A. O., Wu, K. K. and Cho, C. H. (2010) Anti-inflammatory activities of a kolaviron-inhibition of nitric oxide, prostaglandin E2 and tumor necrosis factor-alpha production in activated macrophage-like cell line. Afr J Med Med Sci. Suppl 39: 41-46.

Onasanwo, S. A., Velagapudi, R., El-Bakoush, A. and Olajide, O. A. (2016). Inhibition of neuroinflammation in BV2 microglia by the biflavonoidkolaviron is dependent on the Nrf2/ARE antioxidant protective mechanism. Mol Cell Biochem. 414(1-2): 23-36.

Palumbo, M. L., Fosser, N. S., Rios, H., Zorrilla Zubilette MA, Guelman, L. R., *et al*. (2007). Loss of hippocampal neuronal nitric oxide synthase contributes to the stress-related deficit in learning and memory..,J Neurochem. 102: 261–74. [PubMed] [Google Scholar]

Plata-Salaman, C. R. and Borkoski, J. P. (1993). Centrally administered bacterial lipopolysaccharide depresses feeding in rats. PharmacolBiochem Behav. 46: 787–791.

Rao, A. and Balachandran, B. (2002). Role of oxidative stress and antioxidants in neurodegenerative diseases, Nutr Neurosci. 5 (5): 291–309.

Rosi, S., Vazdarjanova, A., Ramirez-Amaya, V., Workey, P. F., Barnes, C. A. and Wenk, G. L. (2006). Memantine protects against LPS-induced neuroinflammation, restores behaviourally-induced gene expression and spatial learning in the rat. Neurosci. 142: 1303-1315.

Seguí, J., Gironella, M., Sans, M., Granell, S., Gil, F., Gimeno, M., Coronel, P., Piqué, J. M. and Panés, J. (2004). "Superoxide dismutase ameliorates TNBS-induced colitis by reducing oxidative stress, adhesion molecule expression, and leukocyte recruitment into the inflamed intestine". Journal of Leukocyte Biology. 76 (3): 537–44. doi:10.1189/jlb.0304196. PMID 15197232. S2CID 15028921.

Sorce, S., Stocker, R., Seredenina, T,, Holmdahl, R., Aguzzi, A., Chio, A., Depaulis, A., Heitz, F., Olofsson, P., Olsson, T., Duveau, V., Sanoudou, D., Skosgater, S., Vlahou, A., Wasquel, D., Krause, K. H., Jaquet, V. (2017). NADPH oxidases as drug targets and biomarkers in neurodegenerative diseases: What is the evidence? Free RadicBiol Med. 112: 387-396.

Takeda, K. and Akira, S. (2004). TLR signaling pathways. Seminars in Immun. 16: 3-9.

Ullah, F., Liang, A., Rangel, A., Gyengesi, E., Niedermayer, G., Münch, G. (2017). High bioavailability curcumin: an anti-inflammatory and neurosupportive bioactive nutrient for neurodegenerative diseases characterized by chronic neuroinflammation. Arch Toxicol. 91: 1623-1634.

Van Eldik, L. J., Carrillo, M. C., Cole, P. E., Feuerbach, D., Greenberg, B. D., Hendrix, J. A., Kennedy, M., Kozauer, N., Margolin, R. A., Molinuevo, J. L., *et al*. (2016). The roles of inflammation and immune mechanisms in Alzheimer's disease.Alzheimer's Dement. Transl. Res. Clin. Interv. 2: 99–109.

Yirmiya, R., Pollak, Y., Barak, O., Avitsur, R., Ovadia, H., Bette, M., Weihe, E. and Weidenfeld, J. (2001). Effects of antidepressant drugs on the behavioral and physiological responses to Lipopolysaccharide (LPS) in rodents, Neuro-psychopharmacology. 24: 531–544.

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Full Length Research Article

Effect of Co-administration of Glibenclamide and Aqueous Calyx Extract of *Hibiscus Sabdariffa* on Oxidative Stress Markers in Streptozotocin-Induced Diabetic Rats

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Summary: The hyperglycaemia of diabetes mellitus (DM) induces oxidative stress which damages the tissues. Glibenclamide, an oral hypoglycaemic drug used in the treatment of DM has associated side effects. Natural products are considered safe in the treatment of chronic diseases. *Hibiscus sabdariffa* (HS) is a plant that has demonstrated antidiabetic activity. We aimed to determine the potential benefits of co-administration of HS and glibenclamide in ameliorating oxidative stress in streptozotocin (STZ)-induced diabetic rats. A total of 25 male albino Wistar rats were divided randomly into five groups: control (Non-DM), diabetic (DM), diabetic treated with 600µg/kg BW of glibenclamide (DM + GLIB), diabetic treated with 500mg/kg BW of HS (DM + HS), diabetic treated with both 600µg/kg BW of glibenclamide and 500mg/kg BW of HS (DM + GLIB + HS). The interventions were administered for a period of 28 days. The Non-DM rats were significantly heavier ($p<0.01$) compared to rats in the other treatment groups. Glibenclamide or HS alone and in combination, significantly lowered ($p < 0.001$) the final fasting blood glucose concentration of the rats in the respective treatment groups. HS and a combination of HS+ GLIB resulted in increased ($p<0.05$) serum activity of catalase, glutathione peroxidase and superoxide dismutase compared to the DM untreated rats. The serum level of malondialdehyde was significantly lowered ($p=0.000$) in rats that received a combination of HS + GLIB compared to the DM untreated rats. Co-administration of HS + GLIB showed beneficial regeneration of islet-cells in the pancreas. Co-administration of HS + GLIB appears to be more beneficial in the treatment of DM and associated oxidative stress than when given as single agents. Thus, a case for their incorporation as a combined therapy for DM should be considered.

Keywords: *Diabetes; Glibenclamide; Hibiscus sabdariffa; Streptozotocin; Rats*

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INTRODUCTION

The prevalence of diabetes mellitus (DM) is on the increase globally (Forouhi and Wareham, 2019), affecting about 451 million people in 2017 and estimated to reach 693 million by 2045 (Cho *et al*., 2018). The number of people living with DM and its sequelae in Nigeria as at 2017 was about 1.7 million (Cho *et al*., 2018). DM is characterized by defects in insulin secretion and/or insulin action leading to impairment of glucose, lipid and protein metabolism (Chandra *et al*., 2019). Consequently, hyperglycaemia results in the blood and other non-insulin dependent tissues such as the pancreas and brain (Piero *et al*., 2015; Glovaci *et al*., 2019).

Hyperglycaemia favours the development of oxidative stress via glucose autoxidation, glycation reactions with proteins and lipoproteins or when glucose enters the polyol pathway and gets converted to sorbitol (Oguntibeju, 2019). Hyperglycaemia, therefore, increases the concentration of free radicals in the body and overwhelm the capacity of the antioxidant enzymes to maintain them within normal physiological ranges and thus increasing oxidative stress (Tsuruta *et al*., 2010; Sharma and Kar, 2014). Oxidative stress has been implicated in several studies as an important player in the onset and advancement of DM (Oguntibeju, 2019; Badhwar *et al*., 2020).

Glibenclamide is one of the most frequently prescribed oral hypoglycaemic agents (Zhang *et al*., 2017) that stimulates insulin secretion from the existing β cells of the pancreas and reduces hepatic gluconeogenesis resulting in reduced blood glucose (Bolanle *et al*., 2018). However, the use of glibenclamide is limited due to prolonged hypoglycaemia (Heller and Novodvorsky, 2019), high secondary failure rate and other adverse events such as vomiting, weight gain, transient leucopoenia and purpura among others (Gopalakrishna *et al*., 2017).

Studies using both clinical and experimental models have demonstrated the benefits of antioxidants in ameliorating oxidative stress in tissues (Rivera-Barahona *et al*., 2017; Mehta *et al*., 2018). Compounds isolated from natural sources, such as plants are loaded with antioxidants that are more effective and inexpensive compared to conventional therapy in management of some diseases

(Mehta *et al*., 2018). Therefore, antioxidants or nutrients with high antioxidant capacity may offer additional health benefits with possibility of limiting the progression of DM and its associated complications (Fenercioglu *et al*., 2010). *Hibiscus sabdariffa* (HS) is a plant of the Malvaceae family (Zhen *et al*., 2016). It is used in preparation of local non-alcoholic cold or hot drinks. In Nigeria, the calyces of HS are processed into a beverage called "sobo" or "zobo" (Mojiminiyi *et al*., 2012). HS is loaded with antioxidants such as anthocyanins which are thought to be the primary way by which the plant exerts its biological activities (Nguyen *et al*., 2020). HS aqueous extract was shown to prevent hyperglycaemia, hyperlipidaemia and oxidative stress (Peng *et al*., 2011).

Considering the antioxidant property of HS and its previously demonstrated antihyperglycaemic effects, we hypothesised that a combination of HS and glibenclamide could be more effective in ameliorating oxidative stress than glibenclamide alone in streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Study location: The study was conducted at the Animal house of the Usmanu Danfodiyo University Teaching Hospital complex, Sokoto and laboratories in the Department of Chemical Pathology, School of Medical Laboratory Science, Usmanu Danfodiyo University, Sokoto.

Ethical approval and compliance with ARRIVE guidelines: The protocols used in this study were approved by the Animal Research Ethics Committee of Usmanu Danfodiyo University, Sokoto, Nigeria and complied with international ethical guidelines and standards for the care and use of laboratory animals. The Animal Research: Reporting of *In vivo* Experiments (ARRIVE) guidelines have been used in writing up this manuscript.

Reagents: All reagents for the study were of good analytical grade. Accu check glucometer strips were from Roche, Germany. Biuret kit for protein estimation was from Prestige Diagnostics, UK. All the antioxidant enzymes (Glutathione peroxidase, Superoxide Dismutase and Catalase) and Malondialdehyde were assayed using the Laboratory kits from Cayman (USA).

Plant material: Dried calyces of HS were purchased from central market, Sokoto (coordinates: 13º05'N 05º15'E) and taken to Botany Department of Usmanu Danfodiyo University for authentication. A voucher number UDUH/ANS/0219 was collected and the plant specimen deposited at the herbarium of the same department of the institution.

Preparation of plant extract: The calyces were dried and pulverised with mortar and pestle. About 210 g of powder was extracted in 1400 ml of distilled water at 95ºC for two hours (Lin *et al*., 2007). The extracted powder was filtered (Whatman no. 1 filter paper) and concentrated at a temperature of 40ºC (Ibrahim *et al*., 2017) using hot air ovum (Hospibrand, USA). The residual powder extracts were recovered and preserved at 4ºc till use (Ali *et al*., 2003; Lin *et al*., 2007), and a yield of 55% was obtained.

Animals and Experimental Design: A total of 25 male adult Wistar albino rats aged 10-12 weeks (150- 200g) were purchased from the animal house, Usmanu Danfodiyo University, Sokoto. The rats were kept in standard cages in groups of five with 12-h light/dark cycles (lights on at 07.00 hours) at an ambient temperature. The rats had access to balanced and standard rats' pellets (Vital feeds, UAC, Jos, Nigeria) and plain tap water *ad libitum*.

The rats were allowed to acclimatize to the animal room conditions for five days prior to the induction of diabetes mellitus. All animal experimental protocols were conducted in compliance with animal care standards outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Experimental design

The rats were randomly allotted to groups (n=5 per group) and treated as follows:

Group 1: Non- diabetic control rats, administered distilled water (0.5ml) orally

Group 2: Diabetic control rats administered distilled water (0.5ml) orally

Group 3: Diabetic rats, administered glibenclamide 600µg/kg body weight

Group 4: Diabetic rats, administered HS extract 500mg/kg body weight (Ibrahim *et al*., 2019).

Group 5: Diabetic rats, treated with HS (500mg/body weight) and glibenclamide 600µg/body weight (Erejuwa *et al*., 2010).

The distilled water, glibenclamide alone and glibenclamide with HS were administered once daily via oral gavage for four weeks post induction of diabetes. Fasting blood glucose of the rats was measured weekly.

Induction of Diabetes: DM was induced by intraperitoneal administration of Streptozotocin (STZ) (60 mg/kg body weight dissolved in 0.1M citrate buffer, pH 4.5) to rats following 12 hours fasting. Another group of rats were injected with citrate buffer alone without STZ. This group served as control. Three days after STZ injection, diabetes was established and measurement of blood glucose was made on blood obtained via a prick to the tail vein of the rats using lancets and a calibrated Accu- check glucometer (Roche, Germany). Rats with blood glucose concentrations of ≥12 mmol/L were considered diabetic (Mardiah *et al*., 2014).

Sample preparation: At the end of the treatment period, rats were fasted for 12hrs and anaesthetised using light ether soaked with cotton wool enclosed in plastic container. About 5ml of blood was collected through cardiac puncture into lithium heparin bottles for biochemical analysis.

Biochemical analysis

Estimation of serum glucose and protein concentration: Plasma glucose concentration was determined using a calibrated glucometer (Accu-check, Roche, Germany) that utilises the glucose oxidase method (Dickson *et al*., 2019). Plasma protein estimation was done using the biuret methods as described by Gornall *et al*. (1949).

Estimation of Lipid peroxidation: Lipid peroxidation assay was done using methods described by Niehaus and Samuelson (1968).

Estimation of antioxidant enzymes activities: Estimation of Glutathione peroxidase, Superoxide dismutase, and Catalase was done using Laboratory kits from Cayman (USA).

Histology of the pancreas: The pancreas was rapidly excised and fixed in 10% formalin. Histological analysis of the pancreas was done by using haematoxylin and eosin. The organ was brought out of fixative and examined macroscopically on a cutting bench. The pancreas was grossed and placed in a pre-labelled cassette. The tissues were dehydrated, cleared and impregnated using automatic tissue processor (Leica TPO102 model, China), after which they were embedded using embedding machine (Leica EG1 160 model, China). Section of the embedded tissue blocks were cut at 3µm using rotary microtome (Leica RM212 RT, China) and then floated out on labelled glass slides. The cut sections were allowed to dry on hot plates for 15 minutes and stained in haematoxylin and eosin stains. Stained sections were examined microscopically using x 10 and x 40 objective lenses. Photomicrographs of the pancreatic tissue sections of all the intervention rats were taken using an eye-piece-mounted camera and presented alongside with the control sections.

Statistical analysis

Data were analysed using the statistical software GraphPad Prism Version 7.0 (GraphPad Software Inc., San Diego, CA, USA) and expressed as means ± standard error of the mean. A one-way analysis of variance (ANOVA) was used to analyse the data, followed by a Tukey-Kramer *post hoc* test. The level of significance was set at $p \leq 0.05$.

RESULTS

Effect of HS aqueous calyx extract and glibenclamide on the initial and final body weights of streptozotocin-induced diabetic rats: The initial and final body weights of streptozotocin-induced diabetic rats that were treated with either an aqueous calyx extract of HS, glibenclamide or both are presented as Figure 1. Except for the DM vs DM + HS groups that were statistically different ($p = 0.023$, ANOVA), the initial body weights of the rats were similar ($p > 0.05$, ANOVA) across the other treatment groups. The final body weights of the rats in the Non-DM group were significantly higher than those of the untreated DM ($p = 0.000$, ANOVA), DM + GLIB + HS ($p = 0.000$, ANOVA) and DM + HS ($p = 0.011$, ANOVA) treatment groups. Diabetic rats treated with glibenclamide (DM + GLIB) were significantly heavier ($p = 0.010$, ANOVA) than their counterparts that were treated with both glibenclamide and HS.

Effect of HS aqueous calyx extract and glibenclamide on the baseline and final fasting blood glucose concentration of streptozotocin-induced diabetic rats: Table 1 shows the mean baseline (FBG1) and final (FBG 2) fasting blood glucose concentration of streptozotocin-induced diabetic rats that were treated with either an aqueous calyx extract of HS, glibenclamide or both. The initial post-induction fasting blood glucose concentration of non-diabetic rats was significantly lower ($p < 0.001$, ANOVA) than that of the rats in the other treatment groups. Administration of glibenclamide alone and HS alone and in combination significantly lowered ($p < 0.001$, ANOVA) the final fasting blood glucose concentration of the rats in the other treatment groups.

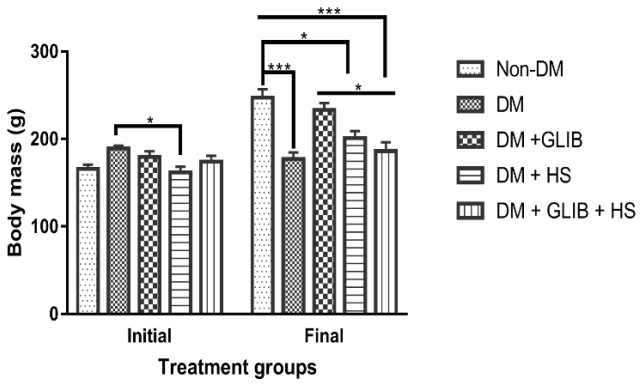

Figure 1:

Initial and final body weights of streptozotocin-induced diabetic rats

* = means are significantly different at $p < 0.05$.

*** = means are significantly different at $p < 0.001$.

Initial= initial body mass, Final= final body mass, Non-DM= administered with distilled water only, DM= administered streptozotocin 60mg/kg body weight, DM + GLIB= administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight, DM + HS= administered streptozotocin 60mg/kg body weight + HS 500mg/kg body weight, DM + GLIB + HS =administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight + HS 500mg/kg body weight. Data expressed as mean ±SEM, n= 5 per group.

Table 1:

Initial and final fasting blood glucose concentration of streptozotocin-induced diabetic rats

Treatment groups	FBG1 (mmol/L)	FBG2 (mmol/L)
Non-DM	4.32 ± 0.32^{a}	4.68 ± 0.24^{a}
DM	27.14 ± 2.99^{b}	24.00 ± 2.08^{c}
DM + GLIB	19.70 ± 2.48^{b}	8.70 ± 1.14^{b}
DM + HS	22.10 ± 3.74^{b}	9.38 ± 0.95^{b}
DM + GLIB + HS	25.60 ± 2.29^{b}	6.58 ± 0.72^{ab}

a,b,c = means with different superscripts are significantly different at $p \leq 0.005$ across columns. FBG1= baseline fasting blood glucose concentration, FBG2= final fasting blood glucose concentration. Non-DM= administered with distilled water only, DM= administered streptozotocin 60mg/kg body weight, DM + GLIB= administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight, DM + HS= administered streptozotocin 60mg/kg body weight + HS 500mg/kg body weight, DM + GLIB + HS =administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight + HS 500mg/kg body weight. Data expressed as mean ±SEM, n= 5 per group

Effects of HS aqueous calyx extract and glibenclamide on serum activities of catalase, glutathione peroxidase and superoxide dismutase: The serum activities of catalase, glutathione peroxidase, superoxide dismutase in streptozotocin induced diabetic rats that were treated with either an aqueous calyx extract of HS, glibenclamide or both are presented in Table 2.

The serum activity of catalase of Non-DM rats was significantly higher compared to the DM (p = 0.000, ANOVA) and DM + GLIB (p=0.006, ANOVA) respectively. However, the activity of catalase in DM + HS (p=0.021, ANOVA) and DM + GLIB + HS (p=0.001, ANOVA) were significantly higher compared to DM untreated rats.

Similarly, the serum activity of glutathione peroxidase was significantly higher in Non-DM compared to other treatment groups; DM (p=0.000, ANOVA), DM+ GLIB (p=0.000, ANOVA), DM+ HS (p=0.000), but the DM+ GLIB +HS group was significantly higher (p=0.011, ANOVA) compared to DM, DM +G:LIB and DM + HS
The serum activity of Superoxide dismutase of Non-DM was significantly higher when compared to DM (p=0.000, ANOVA) and DM + GLIB (p=0.000, ANOVA). However, the activity of superoxide dismutase was significantly higher in DM + GLIB + HS (p= 0.006, ANOVA) compared to DM.

Table 2:
Serum activities of catalase, glutathione peroxidase and superoxide dismutase

Treatment groups	CAT (nmol/mg)	GPx (nmol/mg)	SOD (nmol/mg)
Non-DM	0.55 ± 0.02^{a}	1.5 ± 0.12^{a}	8.26 ± 0.23^{a}
DM	0.19 ± 0.03^{b}	0.60 ± 0.03^{b}	3.19 ± 0.14^{be}
DM + GLIB	0.32 ± 0.06^{bc}	0.78 ± 0.06^{bd}	3.97 ± 0.21^{bc}
DM + HS	0.39 ± 0.04^{ac}	0.98 ± 0.03^{cd}	4.40 ± 0.86^{cd}
DM + GLIB + HS	0.46 ± 0.02^{ac}	1.15 ± 0.07^{cd}	4.03 ± 1.96^{bc}

a,b,c,d,e= means with different superscripts are statistically different at $p \leq 0.05$.
CAT= catalase, GPx= glutathione peroxidase, SOD= superoxide dismutase. Non-DM= administered with distilled water only, DM= administered streptozotocin 60mg/kg body weight, DM + GLIB= administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight, DM + HS= administered streptozotocin 60mg/kg body weight + HS 500mg/kg body weight, DM + GLIB + HS =administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight + HS 500mg/kg body weight. Data expressed as mean ±SEM, n= 5 per group.

Effects of combination of HS aqueous calyx extract and glibenclamide on serum level of malondialdehyde: The serum levels of malondialdehyde in streptozotocin induced diabetic rats that were treated with either an aqueous calyx extract of HS, glibenclamide or both are presented in Figure 2.

The serum activity of malondialdehyde in Non-DM was significantly lower when compared with the DM (p= 0.000, ANOVA) and DM + GLIB (p=0.000, ANOVA). However, the malondialdehyde activity was significantly lower in DM + HS (p=0.014, ANOVA) and DM + GLIB +HS (p= 0.000, ANOVA) when compared with the DM.

Effects of HS aqueous calyx extract and glibenclamide on the pancreatic histology of streptozotocin induced diabetic rats: Figure 3 is a micrograph of pancreatic histology showing various degree of endocrine gland (beta cells) destruction on different group of diabetic rats induced by streptozotocin. The **slide A** is a Non-DM group, which displays normal pancreatic histology with intact endocrine and exocrine glands. **Slide B** represents the DM group which was administered (60mg/kg body weight) of streptozotocin. The histology shows destruction of islet cells as a result of fibrosis of the endocrine gland**. Slide C** was administered streptozotocin also shows destruction of islet cells while **slide D** and **E** show signs of destroyed and regenerating islet cells

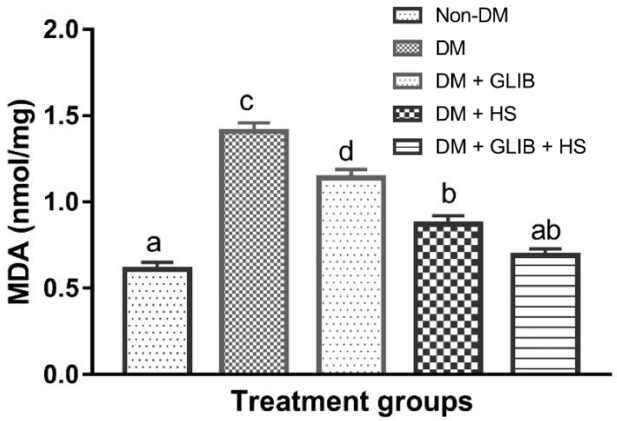

Figure 2:
Serum levels of malondialdehyde (MDA)
a, b, c, d = means various levels of malondialdehyde across the rats group induced with streptozotocin treated with either aqueous extract of HS, glibenclamide or both, MDA = malondialdehyde. Non-DM= administered with distilled water only, DM= administered streptozotocin 60mg/kg body weight, DM + GLIB= administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight, DM + HS= administered streptozotocin 60mg/kg body weight + HS 500mg/kg body weight, DM + GLIB + HS =administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight + HS 500mg/kg body weight. Data expressed as mean ±SEM, n= 5 per group.

DISCUSSION

The study was designed to investigate the effects of HS and/or glibenclamide on STZ-induced diabetes and its complications in a rat model. Upon induction of diabetes using STZ, the fasting blood glucose (FBG1) levels of the rats were all significantly elevated compared to the untreated rats. Streptozotocin is a specific cytotoxic drug that destroys the pancreatic β-cells, there by denying secretion and regulatory action of insulin (Al-Nahdi *et al*., 2019). The diabetic effect results in production of reactive oxygen species (ROS). Excessive ROS attacks protein, lipids, cellular membrane; and organs like pancreas, kidney and liver (Bathina *et al*., 2016).

Hypoglycaemic drugs are either too expensive or have undesirable side effects including haematological, neurological (e.g. coma) and disturbances of liver and kidney functions (Grieb, 2016). Controlling diabetes without any side effects is still a growing challenge in the health sector. Thus, the quest for effective, safer and affordable antidiabetic natural products like plant extracts (Saad *et al*., 2015).

In the current study, the untreated diabetic rats had significantly lower final body weight compared to their non-diabetic counterparts (control). The administration of HS and/or GLIB on the other hand improved significantly the body weights of the diabetic rats.

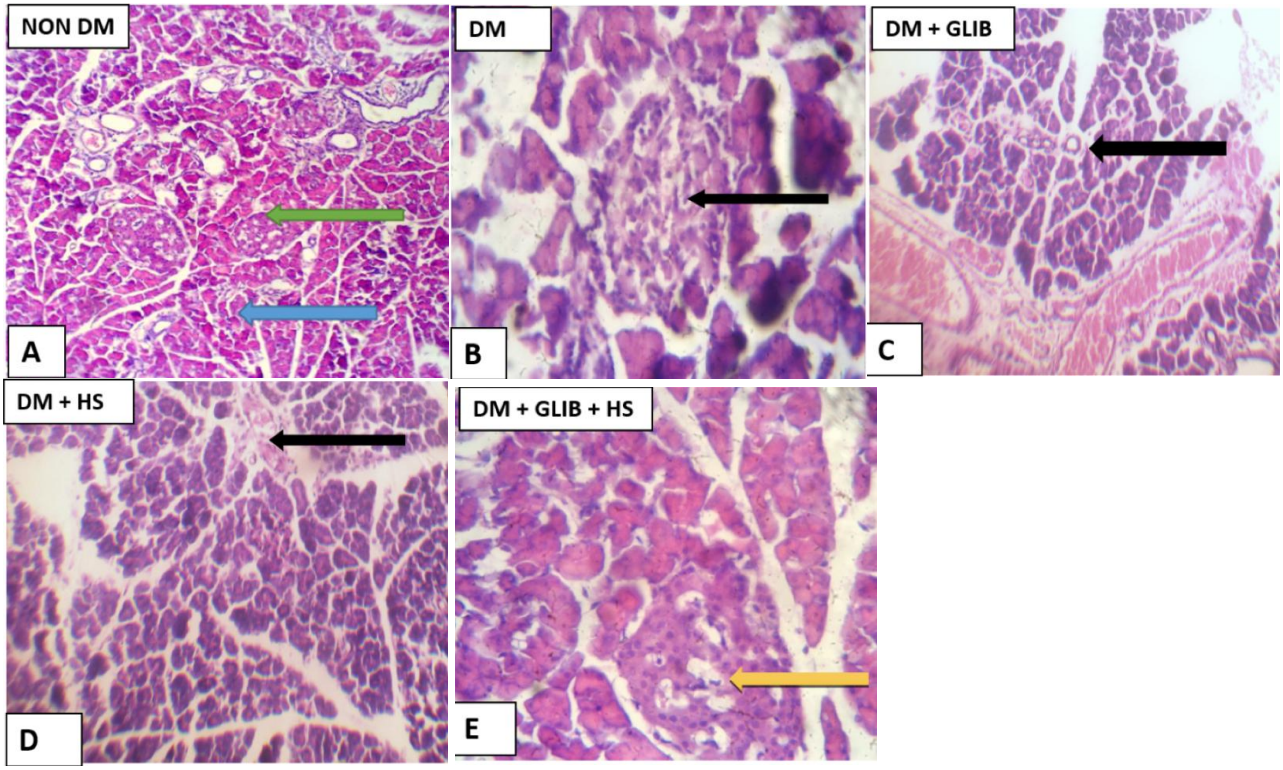

Figure 3:
Micrographs of pancreas histology (H and E staining, x 400) of streptozotocin-induced diabetic rats.
The green arrow points to normal endocrine gland, the blue arrow points to normal exocrine gland, the black arrow points to destroyed endocrine gland and yellow arrow shows regenerating islet cells. A= Non-DM; administered with distilled water only, B= DM; administered streptozotocin 60mg/kg body weight, C=DM + GLIB; administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight, D= DM + HS; administered streptozotocin 60mg/kg body weight + HS 500mg/kg body weight, E= DM + GLIB + HS; administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight + HS 500mg/kg body weight. tissue section = 3-5µm.

The significant reduction in body weight of the diabetic rats was probably as a result of impaired glucose metabolism due to absent or insufficient insulin. In diabetes, lack of insulin activates sensitive lipase which acts on adipose tissues, causing its break down and thus leading to rapid weight loss as observed in DM untreated rats (Wu and Yan, 2015).

Previous studies found similar observation of weight loss in diabetic animal models (Videla *et al*., 2009; Saad *et al*., 2015; Nafizah *et al*., 2017). The improved weight gain may be attributed to the anti-diabetic effect of both HS and glibenclamide, through improvement in glucose utilisation and limiting the breakdown of adipose tissue in rats (Adisakwattana *et al*., 2012). It is important to mention that the administration of GLIB alone to diabetic rats was more effective in improving the body weight of the rats than that of HS alone or HS and glibenclamide combined. Normal weight is an indicator of good diabetic control and glibenclamide is known to cause weight gain (Kasolo *et al*., 2019). Thus, with regards to body weight, glibenclamide may be more beneficial than HS in diabetic rats.

Following the induction of diabetes, the fasting blood glucose concentrations of the rats were significantly elevated compared to the untreated control rats. However, when the rats were treated with either an aqueous extract of HS, glibenclamide or a combination of both HS and glibenclamide, the blood glucose concentration reduced to levels almost similar to that of the control rats. A combination of HS and glibenclamide was more effective in lowering the blood glucose concentration compared to when they were administered independently. HS has been shown to possess anti-diabetic properties in previous studies. For example, when HS extract was administered to diabetic rats at 200mg/kg body weight, it led to a drastic reduction in serum glucose of rats (Peng *et al*., 2011). HS has also been shown to inhibit the enzyme pancreatic α- amylase leading to a slowing down of digestion of carbohydrates to more absorbable forms of monosaccharides (Adisakwattana *et al*., 2012). Huang *et al*. (2009) have also previously demonstrated that an HS extract suppressed the high glucose stimulated cell proliferation and migration in a dose dependent manner. Additionally, HS contains phytochemicals such as, saponins, glycosides and flavonoids which are believed to have hypoglycaemic effects, thus reducing hyperglycaemia (Idris *et al*., 2012).
In this interventional study, the activities of the antioxidant enzymes assayed (catalase, superoxide dismutase and glutathione peroxidase) were significantly reduced in DM untreated rats. However, treatment with HS and/ or glibenclamide improved the activities of the enzymes in diabetic rats. The findings in this study corroborate those of other studies that also found reduced serum activities of antioxidant enzymes in diabetes (Szkudelski, 2001; Sepici-Dincel *et al*., 2007; Singh *et al*., 2017).

Under physiological conditions, free radicals generated are scavenged by antioxidant enzymes like SOD, CAT and GPx, thus, preventing the development of oxidative stress (Saad *et al*., 2015). But in diabetes and its complications,

there is an increased generation of superoxide, hydrogen peroxide anion and lipid peroxide radicals (Gaya *et al*., 2009; Guardiola and Mach, 2014). Thus, causing reduction of antioxidant enzymes due to their increased utilisation to combat the oxidative stress.

CAT is an antioxidant that breaks down hydrogen peroxide (H_2O_2) into H_2O and O_2. The reduced activity of CAT observed in the diabetic rats might be a consequence of elevated superoxide radical (O^{2-}) since increased O^{2-} is known to inactivate catalase enzyme (Kono and Fridovich, 1983; Erejuwa *et al*., 2010).

GPx is an antioxidant enzyme involved in the detoxification of hydrogen and lipid peroxides and acts as a peroxynitrite reductase (Yun *et al*., 2019). The activity of GPx in the plasma that was initially reduced in the diabetic rats was improved with treatment with HS and/ or glibenclamide. However, the activity was more improved in the rats that were administered with both HS and glibenclamide. Generally, antioxidants protect tissues against oxidative damage (Costantini, 2019). Several studies have reported on the antioxidant potentials of HS (Olaleye and Rocha, 2008; Mossalam *et al*., 2011; Villasante *et al*., 2019). HS is thought to exert this antioxidant activity by scavenging free radicals and reactive oxygen species, inhibition of xanthine oxidase activity and prevention of cell damage via lipid peroxidation (Farombi and Fakoya, 2005; Shalgum *et al*., 2019).

SOD metabolizes O_2^- to H_2O_2 (Chung, 2017). Since the non-diabetic rats had normal SOD activity, the reduced SOD activity in the serum of diabetic rats might indicate high levels of O_2^- being generated as a result of chronic hyperglycaemia. Reduced SOD in the serum of STZ induced diabetic rats has also been reported by Qazi and Molvi (2018).

In our study, the levels of lipid peroxidation as indicated by malondialdehyde (MDA) in untreated diabetic rats was significantly higher than the other treatment groups. However, though treatment with both HS alone and glibenclamide alone reduced the level of peroxidation, the combination of HS and glibenclamide together was more beneficial in this regard. This finding indicates that GLIB alone might not offer protection against lipid peroxidative damage but requires multi-therapy approach for effective results. HS extracts have previously been reported to inhibit the formation of malondialdehyde (Usoh *et al*., 2005) and formation of thiobarbituric reactive substances (Hirunpanich *et al*., 2005). Therefore, HS may potentially be useful in alleviating the lipid peroxidation associated with diabetes.

In the current study, untreated diabetes resulted in fibrosis around the endocrine gland on the very few glands which signify cell injury as a result of cytotoxic effects of administered STZ. STZ is a glucose analogue that is selectively accumulated in pancreatic beta-cells via a GLUT 2 glucose transporter in the plasma membrane (Wu and Yan, 2015).

The pancreatic tissues of the rats treated with HS alone and those treated with a combined HS and glibenclamide showed signs of regeneration of islet cells probably as a result of antioxidant property of aqueous extract of HS. Hyperglycaemia is associated with pancreatic β-cell damage due to its toxic effects (Erejuwa *et al*., 2010). Interestingly, a recent study has demonstrated that treatment with HS in a type 1 diabetes rodent model improved the volume of the pancreatic islets and the numerical density of the β-cells depleted by STZ (Adeyemi and Adewole, 2019). Glibenclamide has also been shown to protect pancreatic cells damage through antioxidant mechanisms especially when it is administered in combination with natural polyphenols (Erejuwa *et al*., 2010). It is therefore safe to assert that HS and glibenclamide when combined may produce a more portent protection in the pancreatic cells against the oxidative damage effects of diabetes.

In conclusion, co-administration of glibenclamide and HS aqueous extract may be more beneficial in alleviating the oxidative stress generated in STZ-induced diabetes mellitus in a rodent model than when they are administered as single agents. Thus, complimentary therapy with glibenclamide and HS should be considered in the management of diabetes.

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REFERENCES

Adeyemi, D. O. and Adewole, O. S. (2019). Hibiscus sabdariffa renews pancreatic β-cells in experimental type 1 diabetic model rats. *Morphologie*, **103,** 80-93.

Adisakwattana, S., Ruengsamran, T., Kampa, P. and Sompong, W. (2012). In vitro inhibitory effects of plant-based foods and their combinations on intestinal alpha-glucosidase and pancreatic alpha-amylase. *BMC Complementary and Alternative Medicine,* **12,** 110.

Al-Nahdi, A. M. T., John, A. and Raza, H. (2019). Streptozotocin-induced molecular and metabolic targets in pancreatic beta-cell toxicity. *Hamdan Medical Journal,* **12,** 65-72.

Ali, B. H., Mousa, H. M. and El-Mougy, S. (2003). The effect of a water extract and anthocyanins of *Hibiscus sabdariffa* L. on paracetamol-induced hepatoxicity in rats. *Phytotherapy Research,* **17,** 56-59.

Badhwar, R., Kaur, G., Popli, H., Yadav, D. and Buttar, H. S. 2020. Pathophysiology of Obesity-Related Non-communicable Chronic Diseases and Advancements in Preventive Strategies. *In:* Tappia P., R. B., Dhalla N. (ed.) *Pathophysiology of Obesity-Induced Health Complications. Advances in Biochemistry in Health and Disease.* Springer, Cham.

Bathina, S., Srinivas, N. and Das, U. N. (2016). BDNF protects pancreatic β cells (RIN5F) against cytotoxic action of alloxan, streptozotocin, doxorubicin and benzo (a) pyrene in vitro. *Metabolism,* **65,** 667-684.

Bolanle, I. O., Omogbai, E. K. I. and Bafor, E. E. (2018). Effects of amlodipine and valsartan on glibenclamide-treated streptozotocin-induced diabetic rats. *Biomedicine & Pharmacotherapy,* **106,** 566-574.

Chandra, K., Singh, P., Dwivedi, S. and Jain, S. (2019). Diabetes Mellitus and Oxidative Stress: A Co-relative and Therapeutic Approach. *Journal of Clinical & Diagnostic Research,* **13,** BE07-BE12.

Cho, N., Shaw, J., Karuranga, S., Huang, Y., Da Rocha Fernandes, J., Ohlrogge, A. and Malanda, B. (2018). IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Research and Clinical Practice,* **138,** 271-281.

Chung, W.-H. (2017). Unraveling new functions of superoxide dismutase using yeast model system: Beyond its conventional role in superoxide radical scavenging. *Journal of Microbiology,* **55,** 409-416.

Costantini, D. (2019). Understanding diversity in oxidative status and oxidative stress: the opportunities and challenges ahead. *Journal of Experimental Biology,* **222,** jeb194688; doi: 10.1242/jeb.194688.

Dickson, L. M., Buchmann, E. J., Van Rensburg, C. J. and Norris, S. A. (2019). The impact of differences in plasma glucose between glucose oxidase and hexokinase methods on estimated gestational diabetes mellitus prevalence. *Scientific Reports,* **9,** 1-7.

Erejuwa, O. O., Sulaiman, S. A., Wahab, M. S., Salam, S. K., Salleh, M. S. and Gurtu, S. (2010). Antioxidant protective effect of glibenclamide and metformin in combination with honey in pancreas of streptozotocin-induced diabetic rats. *International Journal of Molecular Sciences,* **11,** 2056-2066.

Farombi, E. O. and Fakoya, A. (2005). Free radical scavenging and antigenotoxic activities of natural phenolic compounds in dried flowers of *Hibiscus sabdariffa* L. *Molecular Nutrition and Food Research,* **49,** 1120-1128.

Fenercioglu, A. K., Saler, T., Genc, E., Sabuncu, H. and Altuntas, Y. (2010). The effects of polyphenol-containing antioxidants on oxidative stress and lipid peroxidation in Type 2 diabetes mellitus without complications. *Journal of Endocrinological Investigation,* **33,** 118-124.

Forouhi, N. G. and Wareham, N. J. (2019). Epidemiology of diabetes. *Medicine,* **47,** 22-27.

Gaya, I., Mohammad, O., Suleiman, A., Maje, M. and Adekunle, A. (2009). Toxicological and lactogenic studies on the seeds of *Hibiscus sabdariffa* linn (Malvaceae) extract on serum prolactin levels of albino wistar rats. *The Internet Journal of Endocrinology,* **5**.

Glovaci, D., Fan, W. and Wong, N. D. (2019). Epidemiology of diabetes mellitus and cardiovascular disease. *Current Cardiology Reports,* **21,** https://doi.org/10.1007/s11886-019-1107-y.

Gopalakrishna, R. N., Bannimath, G. and Huded, S. P. (2017). Herb-drug Interaction: Effect of Poly-Herbal Formulation on Glibenclamide Therapy in Patients with Type-2 Diabetes Mellitus. *Pharmaceutical Methods,* **8,** 62-70.

Gornall, A. G., Bardawill, C. J. and David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *Journal of Biological Chemistry,* **177,** 751-766.

Grieb, P. (2016). Intracerebroventricular streptozotocin injections as a model of Alzheimer's disease: in search of a relevant mechanism. *Molecular Neurobiology,* **53,** 1741-1752.

Guardiola, S. and Mach, N. (2014). Therapeutic potential of Hibiscus sabdariffa: a review of the scientific evidence. *Endocrinología y Nutrición (English Edition),* **61,** 274-295.

Heller, S. and Novodvorsky, P. (2019). Hypoglycaemia in diabetes. *Medicine,* **47,** 52-58.

Hirunpanich, V., Utaipat, A., Morales, N. P., Bunyapraphatsara, N., Sato, H., Herunsalee, A. and Suthisisang, C. (2005). Antioxidant effects of aqueous extracts from dried calyx of *Hibiscus sabdariffa* Linn.(Roselle) in vitro using rat low-density lipoprotein (LDL). *Biological and Pharmaceutical Bulletin,* **28,** 481-484.

Huang, C.-N., Chan, K.-C., Lin, W.-T., Su, S.-L., Wang, C.-J. and Peng, C.-H. (2009). *Hibiscus sabdariffa* Inhibits Vascular Smooth Muscle Cell Proliferation and Migration Induced by High Glucose—A Mechanism Involves Connective Tissue Growth Factor Signals. *Journal of Agricultural and Food Chemistry,* **57,** 3073-3079.

Ibrahim, K. G., Chivandi, E., Mojiminiyi, F. B. O. and Erlwanger, K. H. (2017). The response of male and female rats to a high-fructose diet during adolescence following early administration of *Hibiscus sabdariffa* aqueous calyx extracts. *Journal of Developmental Origins of Health and Disease,* **8,** 628-637.

Ibrahim, K. G., Chivandi, E., Nkomozepi, P., Matumba, M. G., Mukwevho, E. and Erlwanger, K. H. (2019). The long-term protective effects of neonatal administration of curcumin against nonalcoholic steatohepatitis in high-fructose-fed adolescent rats. *Physiological Reports,* **7,** e14032.

Idris, M. H. M., Budin, S. B., Osman, M. and Mohamed, J. (2012). Protective role of *Hibiscus sabdariffa* calyx extract against streptozotocin induced sperm damage in diabetic rats. *Excli Journal,* **11,** 659-669.

Kasolo, J. N., Namaganda, A., Nfambi, J., Kimuli, I., Muwonge, H. and Okullo, I. (2019). Comparison of the Hyperglycemic Control of M. oleifera Leaves Aqueous Extract and Glibenclamide Tablets in Alloxan Monohydrate Induced Diabetic Rats. *Asian Journal of Research in Medical and Pharmaceutical Sciences,* **7,** 1-9.

Kono, Y. and Fridovich, I. (1983). Inhibition and reactivation of Mn-catalase. Implications for valence changes at the active site manganese. *Journal of Biological Chemistry,* **258,** 13646-13648.

Lin, T.-L., Lin, H.-H., Chen, C.-C., Lin, M.-C., Chou, M.-C. and Wang, C.-J. (2007). *Hibiscus sabdariffa* extract reduces serum cholesterol in men and women. *Nutrition Research,* **27,** 140-145.

Mardiah, Z. F., Prangdimurti, E. and Damanik, R. (2014). The effect of roselle extract (Hibiscus sabdariffa Linn.) on blood glucose level and total antioxidant level on diabetic rat induced by streptozotocin. *IOSR Journal of Pharmacy,* **4,** 8-16.

Mehta, J., Rayalam, S. and Wang, X. (2018). Cytoprotective effects of natural compounds against oxidative stress. *Antioxidants,* **7,** 147; https://doi.org/10.3390/antiox7100147.

Mojiminiyi, F. B. O., Audu, Z., Etuk, E. U. and Ajagbonna, O. P. (2012). Attenuation of salt-induced hypertension by aqueous calyx extract of *Hibiscus Sabdariffa*. *Nigerian Journal of Physiological Sciences,* 195-200.

Mossalam, H. H., Abd-El Aty, O. A., Morgan, E. N., Youssaf, S. M. and Mackawy, A. M. H. (2011). Biochemical and Ultra Structure Studies of the Antioxidant Effect of Aqueous Extract of *Hibiscus Sabdariffa* on the Nephrotoxicity Induced by Organophosphorous Pesticide (Malathion) on the Adult Albino Rats. *Journal of American Science,* **7,** 407-421.

Nafizah, A. H. N., Budin, S. B., Zaryantey, A. H., Mariati, A. R., Santhana, R. L., Osman, M., Hanis, M. I. M. and Jamaludin, M. (2017). Aqueous calyxes extract of Roselle or Hibiscus sabdariffa Linn supplementation improves liver morphology in streptozotocin induced diabetic rats. *Arab Journal of Gastroenterology,* **18,** 13-20.

Nguyen, Q. D., Pham, T. N., Binh, M. L. T., Thuan, M., Van, N. T. T., Lam, T. D. and Nguyen, P. T. N. Effects of Extraction Conditions on Antioxidant Activities of Roselle (Hibiscus sabdariffa L.) Extracts. Materials Science Forum, 2020. Trans Tech Publ, 201-206.

Niehaus, W. G. and Samuelson, B. A. (1968). Raid method for the estimation of malondialdehyde. *European Journal of Biochemistry,* **6,** 126-130.

Oguntibeju, O. O. (2019). Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links. *International Journal of Physiology, Pathophysiology and Pharmacology,* **11,** 45-63.

Olaleye, M. T. and Rocha, B. (2008). Acetaminophen-induced liver damage in mice: effects of some medicinal plants on the oxidative defense system. *Experimental and Toxicologic Pathology,* **59,** 319-327.

Peng, C.-H., Chyau, C.-C., Chan, K.-C., Chan, T.-H., Wang, C.-J. and Huang, C.-N. (2011). *Hibiscus sabdariffa* Polyphenolic Extract Inhibits Hyperglycemia, Hyperlipidemia, and Glycation-Oxidative Stress while Improving Insulin Resistance. *Journal of Agricultural and Food Chemistry,* **59,** 9901-9909.

Piero, M., Nzaro, G. and Njagi, J. (2015). Diabetes mellitus-a devastating metabolic disorder. *Asian Journal of Biomedical*

and Pharmaceutical Sciences, **5,** 1-7; DOI: 10.15272/ajbps.v4i40.645.

Qazi, M. A. and Molvi, K. I. (2018). Free radicals and their management. *American Journal of Pharmacy and Health Research,* **6,** 1-10.

Rivera-Barahona, A., Alonso-Barroso, E., Pérez, B., Murphy, M. P., Richard, E. and Desviat, L. R. (2017). Treatment with antioxidants ameliorates oxidative damage in a mouse model of propionic acidemia. *Molecular Genetics and Metabolism,* **122,** 43-50.

Saad, E. A., Hassanien, M. M., El-Hagrasy, M. A. and Radwan, K. H. (2015). Antidiabetic, hypolipidemic and antioxidant activities and protective effects of Punica granatum peels powder against pancreatic and hepatic tissues injuries in streptozotocin induced IDDM in rats. *International Journal of Pharmacy and Pharmaceutical Sciences,* **7,** 397-402.

Sepici-Dincel, A., Açıkgöz, Ş., Cevik, C., Sengelen, M. and Yeşilada, E. (2007). Effects of in vivo antioxidant enzyme activities of myrtle oil in normoglycaemic and alloxan diabetic rabbits. *Journal of Ethnopharmacology,* **110,** 498-503.

Shalgum, A., Govindarajulu, M., Majrashi, M., Ramesh, S., Collier, W. E., Griffin, G., Amin, R., Bradford, C., Moore, T. and Dhanasekaran, M. (2019). Neuroprotective effects of Hibiscus Sabdariffa against hydrogen peroxide-induced toxicity. *Journal of Herbal Medicine,* **17,** 100253; https://doi.org/10.1016/j.hermed.2018.100253.

Sharma, N. and Kar, A. (2014). Combined effects of Gymnema sylvestre and glibenclamide on alloxan induced- diabetic mice. *International Journal of Applied Pharmacology,* **6,** 11-14.

Singh, P., Khan, M. and Hailemariam, H. (2017). Nutritional and health importance of Hibiscus sabdariffa: a review and indication for research needs. *Journal of Nutrition, Health and Food Engineering,* **6,** 00212.

Szkudelski, T. (2001). The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiological Research,* **50,** 537-546.

Tsuruta, R., Fujita, M., Ono, T., Koda, Y., Koga, Y., Yamamoto, T., Nanba, M., Shitara, M., Kasaoka, S. and Maruyama, I. (2010). Hyperglycemia enhances excessive superoxide anion radical generation, oxidative stress, early inflammation, and endothelial injury in forebrain ischemia/reperfusion rats. *Brain Research,* **1309,** 155-163.

Usoh, I., Akpan, E., Etim, E. and Farombi, E. (2005). Antioxidant actions of dried flower extracts of *Hibiscus sabdariffa* L. on sodium arsenite-induced oxidative stress in rats. *Pakistan Journal of Nutrition,* **4,** 135-141.

Videla, L. A., Tapia, G., Rodrigo, R., Pettinelli, P., Haim, D., Santibañez, C., Araya, A. V., Smok, G., Csendes, A. and Gutierrez, L. (2009). Liver NF-κB and AP-1 DNA binding in obese patients. *Obesity,* **17,** 973-979.

Villasante, J., Girbal, M., Metón, I. and Almajano, M. P. (2019). Effects of pecan nut (Carya illinoiensis) and roselle flower (Hibiscus sabdariffa) as antioxidant and antimicrobial agents for sardines (Sardina pilchardus). *Molecules*, **24,** 85;

Wu, J. and Yan, L.-J. (2015). Streptozotocin-induced type 1 diabetes in rodents as a model for studying mitochondrial mechanisms of diabetic β cell glucotoxicity. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy,* **8,** 181-188.

Yun, J.-W., Zhao, Z., Yan, X., Vatamaniuk, M. Z. and Lei, X. G. (2019). Glutathione peroxidase-1 inhibits transcription of regenerating islet-derived protein-2 in pancreatic islets. *Free Radical Biology and Medicine,* **134,** 385-393.

Zhang, G., Lin, X., Zhang, S., Xiu, H., Pan, C. and Cui, W. (2017). A protective role of glibenclamide in inflammation-associated injury. *Mediators of Inflammation,* **2017,** Article ID 3578702; https://doi.org/10.1155/2017/3578702.

Zhen, J., Villani, T. S., Guo, Y., Qi, Y., Chin, K., Pan, M.-H., Ho, C.-T., Simon, J. E. and Wu, Q. (2016). Phytochemistry, antioxidant capacity, total phenolic content and anti-inflammatory activity of Hibiscus sabdariffa leaves. *Food Chemistry,* **190,** 673-680.

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Full Length Research Article

Serum P53 Protein Level and Some Haematologic Parameters among Women of Reproductive Age Living with HIV Infection

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Summary: Human immunodeficiency virus (HIV) infection remains a health challenge in Nigeria, and women of reproductive age are disproportionately infected. P53 protein, D-dimer, serum ferritin, CD4 cell count, haemoglobin concentration and haematocrit levels were measured among non-pregnant women of reproductive age living with HIV infection in order to assess the impact of HIV infection on maternal health. A hundred and sixty-two subjects categorised into three groups of 54 persons each involving; newly diagnosed, subjects on highly active antiretroviral therapy (HAART) and apparently healthy control subjects were recruited. Blood samples were analyzed for haemoglobin concentration, haematocrit, CD4 cell count, serum ferritin, D-dimer and p53 protein levels by standard methods. The CD4 cell count, serum p53 protein, and Hb Conc. were significantly lower, while serum ferritin was higher in the newly diagnosed group (p=0.001), followed by the group on HAART (p=0.001) compared to the controls. D-dimer level was significantly lower in the control group (2899.11±670.73pg/ml) than both newly diagnosed (4842.44±489.40pg/ml) and HAART (4660.31±519.83pg/ml) groups, while significant decrease in haematocrit was observed between the newly diagnosed group (0.336±0.07l/l) as against both treated (0.378±0.04l/l) and control (0.362±0.02l/l) groups. D-dimer correlated negatively with serum p53 protein level among the newly diagnosed subjects and with Hb Conc. among subjects undergoing treatment. The study concludes that women of reproductive age living with HIV infection showed higher D-dimer and lower tumour suppression protein levels as well as anaemia and reduced immune response. The newly diagnosed subjects were more affected.

Keywords*: HIV infection, anaemia, tumour suppression, activated coagulation*

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INTRODUCTION

Several decades after the emergence of human immunodeficiency virus (HIV) infection, developing countries such as Nigeria still struggle from the health burden of HIV infection. From timely detection of HIV infection in the population to effective management of diagnosed cases, efforts to bring the scourge under control are ongoing in the country. Although the Nigerian national epidemiological data on HIV infection among persons aged 15-49 years of age shows a prevalence of 1.4%, the South-South geopolitical zone of the country has the highest prevalence of 3.1% in the country (, United Nations Programme on HIV/AIDS, 2019). It is imperative that apart from the immediate concern of curbing spread and limiting prevalence, infected persons also require adequate care especially as it relates to the common complications of the infection. With HIV infection still a public health challenge in Nigeria, there is need to continue investigations in this direction.

Reports from previous studies within the studied population indicate a disproportionate age and gender distribution within the socio-demographic characteristics of persons living with HIV infection (Okafor *et al*., 2016; Akwiwu *et al*., 2017; Okafor *et al*., 2019). Women of child-bearing age constitute a vulnerable group among adults infected with the virus. Added to this unfortunate situation, substantial proportions of HIV infection in children from Calabar, Nigeria have been observed among those delivered by traditional birth attendants. A trend with implications for the eventual effective control of HIV infection in the locality as the risk of mother to child transmission could be heightened under these circumstances (Fedral Ministry of Health, 2010; World Health Organization, 2010; Ugochi *et al*., 2018). There is need for effective detection and management of people living with HIV infection, particularly women of reproductive age in order to break the transmission chain to the next generation. This concern has necessitated the present study on the possibility as well as magnitude of haemostatic and tumour suppression involvements in HIV infection as it affects women of reproductive age.

The immune deficiency that results from HIV infection is a consequence of the viral attack on the helper T-lymphocytes responsible for effective immune response. Reduction in CD4 cell count has been identified as a marker for severity of infection in HIV, and in its subsequent progression to acquired immune deficiency syndrome. Being the primary target in the viral invasion of host immunity, decline in CD4 cell count remains a relevant parameter in the assessment of the severity of immunosuppression. The CD4 cell count has also been relied on for its prognostic value as consistent decline has been associated with severity of anaemia, activated coagulation, increased risk of opportunistic infections and neoplasms (Biggar *et al*., 2007; Cummins and Bradley,

2010; Riedel *et al*., 2013; Coghill *et al*., 2015; Panwar *et al*., 2016). In fact, impaired immunity and the development of concurrent cancers culminate into a cycle that mediates HIV-related morbidity and mortality. It is thought that the pathogenesis of cancer derives from two basic mechanisms, which include the switching on of oncogenes and the suppression of suppressor genes. The tumour suppressor p53 gene and its protein take part in the early detection and deletion of tumour clones. Consequent upon this involvement, changes in the expression of p53 gene and its protein accompany associated conditions and contribute to HIV-associated mortality (Yoon *et al*., 2015; Yang *et al*., 2016).

Derangement in blood cell lineages have been among the haematologic complications of HIV infection. Particularly of interest with regards to disease progression is the occurrence of anaemia in association with HIV infection (Sullivan *et al*., 1998; Gedefaw *et al*., 2013). Anaemia has remained one of the major concerns in HIV infection especially as it is also a public health challenge in the general populations of developing countries such as Nigeria. Previous studies on the nature of this anaemia have so far revealed a derangement in the utilization of iron even in the face of excess available iron (Okafor *et al*., 2016; Akwiwu *et al*., 2017). Anaemia of chronic infections is usually accompanied with a finding of iron imbalance. A situation that is attributed to the host body's defensive mechanism as body iron is sequestered from the infective agent. In fact, serum ferritin is reported to associate with immune deficiency and heightened inflammation in HIV infection (Walsh *et al*., 2010; Ogbe *et al*., 2012; Lopez-Calderon *et al*., 2015). In view of the aforementioned concerns, this study aimed at assessing possible coagulation dysfunction, tumour suppression disturbance, immunosuppression and anaemia in women of reproductive age.

MATERIALS AND METHODS

Subjects: This study was carried out among non-pregnant women (between the ages of 21 and 43 years) living with HIV infection who were accessing healthcare for HIV diagnosis and management at University of Calabar Teaching Hospital, Calabar, Nigeria. Among the 108 HIV-infected subjects, 54 persons were newly diagnosed and were yet to be placed on highly active antiretroviral therapy (HAART). Another 54 subjects were already undergoing treatment, while an equal number of age-matched HIV sero-negative apparently healthy women were recruited as controls.

Ethical consideration: Ethical approval was obtained from the Health Research and Ethics Committee of University of Calabar Teaching Hospital. Informed consent was obtained from each participant enrolled in the research and confidentiality was maintained.

Methods: Blood samples were collected into ethylene diamine tetra-acetic acid (at a concentration of 2 mg/mL of blood) and plain bottles.

Haemoglobin concentration and haematocrit were measured by automation using Sysmex KX-21N (Japan). The sysmex Kx-21N performs blood cells count by direct current detection method. Blood cells suspended in the diluted sample passing through the aperture, cause direct current resistance to change between the electrodes. As direct current resistance changes, the blood cell size is detected as electric pulses.

The CD4+ cell count was analyzed using PartecCyflow Counter (Germany). When passing through a flow cuvette, cells are individually illuminated by light spot of a laser lamp. The scatter intensity is a measure of cell size and morphology. Additionally, due to the excitation, the dye molecules emit fluorescence of characteristic colour. This fluorescence light is separated into colour range by means of optical filters. The intensity of each colour range is analyzed for each single cell.

Serum ferritin was assayed using ELISA kit from BioCheck, Inc South San Francisco, USA. The ferritin quantitative test is based on a solid phase enzyme linked immunosorbent assay (ELISA). The assay system utilizes one rabbit anti-ferritin anti-body for solid phase (microtiter wells) immobilization and a mouse monoclonal anti-ferritin anti-body in the anti-body-enzyme (horseradish peroxide) conjugate solution. The concentration of ferritin is directly proportional to the color intensity of the developed solution. D-dimer and p53 protein were assayed using ELISA kits from Bioassay Technology Laboratory, China. For D-dimer assay, sample is added to wells pre-coated with D2D monoclonal antibody. After incubation a biotin-conjugated anti-human D2D antibody is washed away during a washing step. Following streptavidin-HRP and substrate solution additions, intensity of developed colour is measured as absorbance. Likewise, for P53 assay, sample is added to the wells pre-coated with P53/TP53 antibody. After incubation. unbound biotin conjugated anti-human P53/TP53 antibody is washed away during a washing step while the bound portion is estimated after colour development.

Statistical analysis: Statistical analysis was performed on Microsoft Excel (MS office 2010) for windows and SPSS version 20.0. One-way analysis of variance and Pearson's correlation were used in this study. Results were expressed as mean ± standard deviation. The level of significance was set at $P \leq 0.05$.

RESULTS

Table 1 is the demographic representation of the study participants. These participants were literate and mostly married women of reproductive age. Table 2 captures variations in the mean values of CD4 cell count, D-dimer, serum p53 protein and ferritin levels as well as haemoglobin concentration (Hb Conc.) and haematocrit (HCT). The CD4 cell count, serum p53 protein, and Hb Conc. were significantly lower while serum ferritin was higher among the newly diagnosed group followed by the group on treatment compared to control group. For the D-dimer, both groups living with HIV infection had significantly higher mean values compared to the control group, while significant reduction in haematocrit was observed between the newly diagnosed against both treated and control groups.

Table 1.
Socio-demographic characteristics of study participants

Characteristics		Control subjects n =54 (100%)	Newly diagnosed subjects n =54 (100%)	Subjects on HAART n =54(100%)
Age range (years)	21-30	26 (48)	28 (52)	23 (43)
	31-40	18 (33)	19 (35)	17 (31)
	>40	10 (19)	7 (13)	14 (26)
Educational level	Secondary level	12 (22)	11 (20)	7 (13)
	Tertiary level	42 (78)	43 (80)	47 (87)
Marital status	Single	0 (0)	6(11)	3 (6)
	Married	54 (100)	48 (89)	51(94)
HIV status	Positive	0 (0)	54 (100)	54 (100)
	Negative	54 (100)	0 (0)	0 (0)
Treatment (HAART)	Commenced	0 (0)	0 (0)	54 (100)
	Yet to commence	0 (0)	54 (100)	0 (0)
	Not applicable	54 (100)	0 (0)	0 (0)

Table 2.
Mean values of the studied parameters among the participants

Parameters	Control subjects n=54	Newly diagnosed subjects n=54	Subjects on HAART n=54	p-Value
CD4 (cells/ml)	908.07±220.22 [a]	339.83±125.24 [a]	640.35±i60.31 [a]	0.001
D-Dimer (pg/ml)	2899.11±670.73 [b]	4842.44±489.40	4660.31±519.83	0.001
P53 (ng/l)	2041.93±482.77 [a]	1478.72±312.34 [a]	1834.28±400.61 [a]	0.001
Serum Ferritin (µg/l)	66.94±16.64 [a]	379.45±336.68 [a]	181.42±64.58 [a]	0.001
Hb Conc (g/l).	122.80±4.22 [a]	96.61±17.28 [a]	113.13±11.30 [a]	0.001
HCT (l/l)	0.362±0.02	0.336±0.07[b]	0.378±0.04	0.001

Key:
a = significant difference across all groups; b = significant difference between identified group and the other groups
For routinely analysed parameters, reference range for women are as follows; Serum ferritin 15-200 µg/l, Hb Conc. 120-150 g/l and HCT 0.36-0.46 l/l.

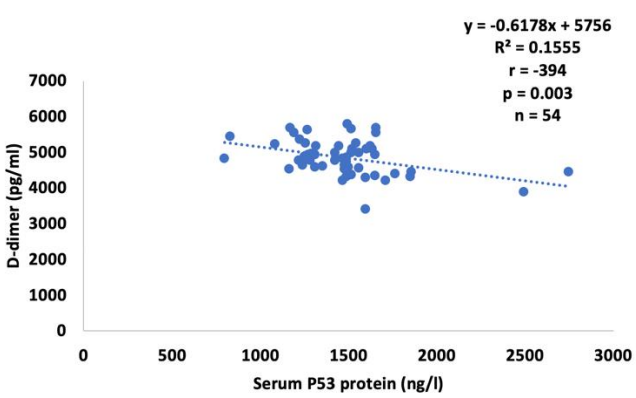

Figure 1
Correlation of Serum p53 protein and D-dimer among HAART-Naïve HIV–infected subjects

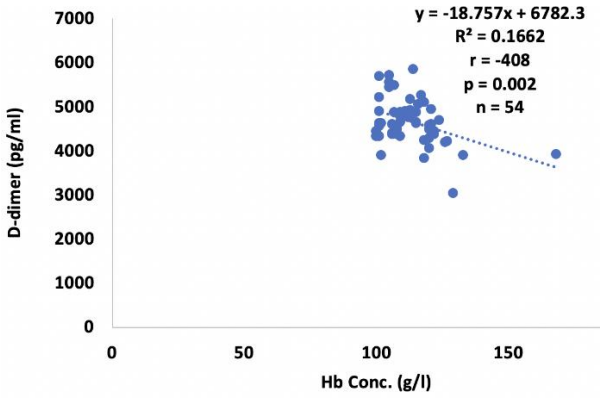

Figure 2
Correlation of Hb Conc. and D-dimer among HIV–infected subjects on HAART

Significant weak negative correlations between D-dimer and serum p53 protein level among the newly diagnosed as well as between D-dimer and Hb Conc. among subjects undergoing therapy (Figures 1and 2 respectively) were observed.

DISCUSSION

Haemoglobin concentration and haematocrit are both consideration tools used for screening of anaemia, which are influenced by age and gender among other factors. Such physiological variations may mask the degree to which such indicators are deranged when general populations are sampled. In view of the finding that women of reproductive age disproportionately carry the burden of HIV infection, it became necessary to segregate this group and investigate changes in some known and potential markers with regards to HIV infection and probable impact on reproductive health. Low Hb and elevated serum ferritin level were observed among persons living with HIV infection, particularly among the newly diagnosed followed by those undergoing antiretroviral therapy.

The latter group had more resolved values towards normalcy compared to the newly diagnosed. Interestingly, the values for Hb conc. would be expected to match the haematocrit across the groups, but the result revealed similar haematocrit values for both control and treated groups. This

observation in haematocrit values is at variance with the significantly lower value for Hb conc. of the group on treatment compared to controls. Increase in haematocrit for HIV-infected persons on treatment is attributable to both improvements in erythropoiesis as well as adverse effects from use of antiretroviral drugs that cause macrocytosis (Sullivan *et al.*, 1998; Okafor *et al.*, 2019). Imperatively, Hb conc. stands a better assessing parameter of anaemia for persons living with HIV, as haematocrit values alone may not be reliable.

Additionally, on effective erythropoiesis, serum ferritin serves as an indicator of stored iron but in chronic inflammatory conditions, it rather paints a picture of non-utilization of iron. Thus, the significant increase in serum ferritin observed in HIV infection as recorded in the present study supports the theory of iron sequestration commonly found in anaemia of chronic inflammation. The implication of this finding is that despite high iron stores, the release of iron for erythropoiesis remains insufficient. Although, being on antiretroviral therapy conferred some level of improvement, the infection itself remains an important aspect of HIV complication and a challenge in the management of the condition (Kyeyune *et al.*, 2014; Kallianpur *et al.*, 2016; Al-Kindi *et al.*,2017; Okafor *et al.*, 2019).

The CD4 count and p53 level were significantly reduced, while elevated D-dimer level existed in HIV infection. Significant weak negative correlations were also seen between D-dimer versus serum p53 protein level and haemoglobin concentration among newly diagnosed subjects and those undergoing therapy respectively. The degree of immuno-competence versus immunodeficiency, mainly assessed by CD4 T-helper cell count, is an important aspect in the management of HIV infection. Indicative of disease progression, CD4 T-helper cell count is important in routine monitoring of patients even after commencement of antiretroviral therapy. D-dimer level attests to the presence or otherwise of activated coagulation beyond the information obtainable from platelets as part of full blood count. Its downward resolution among those undergoing antiretroviral therapy was not statistically different from the newly diagnosed, altogether both differed significantly from control mean value. Owing to the risk of poor pregnancy outcome in the event of activated coagulation, D-dimer measurement serves as a screening assessment for the possibility of venous thromboembolism (Choi and Krishnamoorthy, 2018; Zaini *et al.*, 2019). Therefore, a finding of elevated D-dimer values in a group of women of reproductive age is of concern, particularly as planned pregnancy and preconception maternal care are not very common in developing countries such as Nigeria. More so, activated coagulation correlated with anaemia among subjects undergoing therapy. In tallying to HIV-associated anaemia, there may be need to look into activated coagulation and also its possible mediation in HIV-related neoplasms more directly in the management of HIV infection among women.

Currently in developing countries, there is paucity of information on p53 assessment in disease diagnosis. The p53 gene and its protein regulate cell division and aid apoptosis. Regulation of p53 expression is necessary for immune competence, otherwise abnormal proliferations emanating during cell growth would be left unchecked (Theoret *et al.*, 2008; Pinzone *et al.*, 2012). Unfortunately, cancer is among the AIDS-defining features in HIV infection. The observation of significant reduction in the p53 protein level among HIV-infected subjects suggests consumption of p53 protein possibly arising from increased tumour clones in HIV infection. The co-existence of activated coagulation and dwindling tumour suppression mechanisms among the newly diagnosed is a revealing observation capable of impacting reproductive health especially as conventional antenatal care is optional and influenced by socio-demographic dynamics. Anaemia on its own contributes immensely to maternal mortality, particularly, in developing countries. Added to the existing knowledge of HIV-associated anaemia, this study highlights heightened coagulation and diminishing tumour suppression indicators in HIV infection with the newly diagnosed being worse off. Unfortunately, it is not feasible to determine duration of infection prior to detection. It is also disturbing that maternal health in this region of the world is often considered in the context of pregnancy period with less attention on post-pregnancy period. Preconception aspect of maternal health for the general population is still evolving (Olowokere *et al.*, 2015; Ekem *et al.*, 2018; Akinajo *et al.*, 2019).

In addition to detecting anaemia and immunosuppression, this study has revealed ongoing coagulation disturbance as shown by higher D-dimer levels alongside lower tumour suppression protein levels among women of reproductive age living with HIV infection.

REFERENCES

Akinajo, O.R., Osanyin, G.E., Okojie, O.E. (2019). Preconception care: Assessing the level of awareness, knowledge and practice amongst pregnant women in a tertiary facility. J. Clin. Sci. 16(3): 87-92.

Akwiwu, E.C., Akpotuzor, J.O., Okon, J.E., Egharevba, O. (2017). Iron studies of HIV-infected subjects in Calabar- A Nigerian perspective. J. Med. Sci. Clin. Res. 5: 19572-19577.

Al-Kindi, S.G., Zidar, D.A., McComsey, G.A., Longenecker, C.T. (2017). Association of anisocytosis with markers of immune activation and exhaustion in treated HIV. Pathog. Immun.; 2: 138-150.

Biggar, R.J., Chaturvedi, A.K., Geodert, J.J., Engels, E.A. (2007). AIDS-related cancer and severity of immunosuppression in persons with AIDS. J. Natl. Cancer Inst. 99: 962-972.

Choi, H., Krishnamoorthy, D. (2018). The diagnostic utility of D-dimer and other clinical variables in pregnant and post-partum patients with suspected acute pulmonare embolism. Int J Emerg Med.; 11(1):10.

Coghill, A., Shiels, M.S., Suneja, G., Engels, E.A. (2015). Elevated Cancer-specific Mortality among HIV-infected Persons in the US. J Clin. Oncol. 33:2376-83.

Cummins NW, Badley AD (2010). Mechanisms of HIV-associated lymphocyte apoptosis. Cell Death Dis. 1:e99.

Ekem, N.N., Lawani, L.O., Onoh, R.C., Iyoke, C.A., Ajah, L.O., Onwe, E.O., Onyebuchi, A.K., Okafor, L.C. (2018). Utilization of preconception care services and determinants of poor uptake among a cohort of women in Abakiliki Southeast Nigeria. Journal of Obstetrics and Gynaecology 38(6): 739-744.

Federal Ministry of Health (2010). Nigeria National Guidelines on prevention of mother to child transmission of HIV in Nigeria. https://aidsfree.usaid.gov/sites/default/files/tx_nigeria_pmtct_2010.pdf

Gedefaw, L., Yemane, T., Sahlemariam, Z., Yilma, D. (2013). Anemia and Risk Factors in HAART Naïve and HAART

Experienced HIV Positive Persons in South West Ethiopia: A Comparative Study. PLoS ONE 8: e72202.

Kallianpur, A.R., Wang, Q., Jia, P., Hulgan, T., Zhao, Z., Letendre, S.L., *et al*. (2016). Anemia and Red Blood Cell Indices Predict HIV-Associated Neurocognitive Impairment in the Highly Active Antiretroviral Therapy Era. J Infect Dis. 213: 1065–1073.

Kyeyune, R., Saathoff, E., Ezeamama, A.E., Loscher, T., Fawzi, W., Guwatudde, D. (2014). Prevalence and correlates of cytopenias in HIV-infected adults initiating highly active antiretroviral therapy in Uganda. BMC Infect Dis. 14: 496.

Lopez-Calderon, C., Palacios, R., Cobo, A., Nuno, E., Ruiz, J., Marquez, M. *et al*. (2015). Serum ferritin in HIV-positive patients is related to immune deficiency and inflammatory activity. Inter J STD AIDS26: 393-397

Ogbe, P.J., Idoko, O.A., Ezimah, A.C., Digban, K.A., Oguntayo, B.O. (2012). Evaluation of iron status in anemia of chronic disease among patients with HIV infection. Clin. Lab. Sci. 25:7-12.

Okafor, A.O., Akwiwu, E.C.,Akpotuzor, J.O. (2019). Prevalence of Anaemia after Initiation of Antiretroviral Therapy among HIV-infected Patients attending University of Calabar Teaching Hospital Calabar, Nigeria. International Journal of Tropical Diseases and Health 35: 1-7.

Okafor, A.O., Usanga, E.A., Akwiwu, E.C. (2016). Iron related parameters of HIV-infected patients attending University of Calabar Teaching Hospital, Nigeria. Journal of Dentistry and Medical Sciences 15: 65-68.

Olowokere, A.E., Komolafe, A., Owofadeju, C. (2015). Awareness, knowledge and uptake of preconception care among women in Ife Central Local Government Area of Osun State, Nigeria. Journal of Community Meddicine and Primary Health Care 27(2): 83-92.

Panwar, A., Sharma, S.C., Kumar, S., Sharma, A. (2016). A study of anemia in human immunodeficiency virus patients: Estimating the prevalence, analyzing the causative effect of nutritional deficiencies, and correlating the degree of severity with CD4 cell counts. Med J DY Patil Univ 9: 312-318.

Pinzone, M.R., Fiorica, F., Di Rosa, M., Malaguanera, G., Malaguanera, L., Cacopardo. B., *et al*. (2012). Non-AIDS-defining cancers among HIV-infected people. Eur. Rev. Med. Pharmacol. Sci. 16: 1377-1388.

Riedel, D.T., Mwangi, E.I., Fantry, L.E., Alexander, C., Hossain, M.B., Pauza,, C.D. *et al*. (2013). High cancer-related morality in an urban, predominantly African American, HIV-infected population. AIDS 27: 1109-1171.

Sullivan, P.S., Hanson, D.L., Chu, S.Y., Jones, J.L.,Ward, J.W. (1998). Epidemiology of anemia in human immunodeficiency virus (HIV)-infected persons: results from the multistate adult and adolescent spectrum of HIV disease surveillance project. Blood 91: 301-308.

Theoret, M.R., Cohen, C.J., Nahvi, A.V., Ngo, L.T., Suri, K.B., Powell, D.J. Jr. *et al*., (2008). Relationship between p53 overexpression on cancers and recognition by anti-p53 T cell receptor-transduced T cells. Hum. Gene. Ther. 19: 1219-1232.

Ugochi V.E.,Akwiwu E.C. & Akpotuzor J.O. (2018). Factors associated with HIV Transmission and Infection among Persons Aged 0-17 years in Calabar Metropolis of Nigeria. Journal of Medical and Dental Science Research 5 (5): 27-30.

United Nations Programme on HIV/AIDS (2019). New survey results indicate that Nigeria has an HIV prevalence of 1.4%., https://www.unaids.org

Walsh, C.M., Hattingh, Z., Veldman, F.J., Bester, C.J. (2010). Iron status and anaemia of chronic disease in HIV infected African women in Mangung, Bloefontein. SA Family Practice 52: 55-59.

World Health Organization (2010). PMTCT strategic vision 2010-2015: Preventing mother-to-child transmission of HIV to reach the UNGASS and Millennium Development Goals. :https://www.who.int/hiv/pub/mtct/strategicvision.pdf

Yang, J., Su, S., Zhao, H., Wang, D., Wang, J., Zhang, F., *et al*. (2016). Prevalence and mortality of cancer among HIV-infected in patients in Beijing, China. BMC Infect. Dis.;16: 82

Yoon, C.H., Kim, S.Y., Byeon, S.E., Jeong, Y., Lee, J., Kim, K.P., *et al*. (2015). P53-derived host restriction of HIV-1 replication by protein kinase R-mediated Tat phosphorylation and inactivation.J Virol. 89: 4262-4280

Zaini, R., Al-Rehali, A., Kufia, R. (2019). Evaluation of plasma D-Dimer concentration among normal and complicated pregnancies, Saudi Arabia. International Journal of Women's Health and Reproduction Sciences 7(1):17-23.

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Full Length Research Article

Comparative Microscopic Assessments of the Effect of Aqueous and Ethanol Extracts of *Phoenix dactylifera L.* in a Rat Model of Mercury-Triggered Hippocampal Changes

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Summary: Mercury is an environmental neurotoxicant that triggers structural and physiological alterations in different brain parts. The hippocampus is associated with learning and memory, and injury to this brain part may lead to behavioural and cognitive changes. *Phoenix dactylifera* (date palm) has been demonstrated to possess a variety of medical benefits. This study comparatively assessed the neuroprotective property of aqueous and ethanol fruit pulp extracts of *P. dactylifera* in a rat model of mercury-triggered hippocampal changes using microscopic examinations. Twenty-eight Wistar rats were divided into seven groups (I–VII, n=4). Group I (control) was administered distilled water (2ml/kg); group II was administered mercuric chloride, $HgCl_2$ (5mg/kg); group III was administered vitamin C (100mg/kg) as reference drug +$HgCl_2$; groups IV and V were administered aqueous extract (250mg/kg and 500mg/kg, respectively) +$HgCl_2$, while groups VI and VII were administered ethanol extract (250mg/kg and 500mg/kg, respectively) +$HgCl_2$. Extracts' neuroprotective property were evaluated using histological and histometric assessments of CA1 and CA3 hippocampal sub-regions. Results revealed cytoarchitectural changes including karyopyknosis, basophilic necrosis and remarkably decreased histometric features of hippocampal pyramidal neurons in $HgCl_2$-treated group relative to control. Administration of the extracts remarkably ameliorated mercury-induced degenerative changes by preservation of cytoarchitectural features comparable to reference drug. Comparatively, neuroprotective efficacies of the extracts are relatively similar, especially at doses of 500mg/kg and could be attributed to antioxidant activities of constituent phytochemicals. Results suggest that aqueous and ethanol fruit pulp extracts of *P. dactylifera* may prove efficacious in ameliorating mercury-triggered microscopic alterations in the hippocampus of Wistar rats.

Keywords: *Neurodegeneration; Neuroprotection; Histology; Histometric; Oxidative stress*

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INTRODUCTION

Severe illness and sudden death in humans has been implicated to environmental pollution. Heavy metals including cadmium, lead and mercury have been classified among major causes of environmental pollution (Chen and Chen, 2001; Galadima et al., 2011; Branco et al., 2017). Mercury is a potent contaminant present in various environmental media and food across the globe at levels that adversely affect biological systems, exerting toxic effect on a variety of vital organs in the human body (UNEP, 2002; Jha et al., 2019). Mercury is an established neurotoxicant causing structural and physiological alterations in different brain parts by eliciting oxidative stress which results to neurological deficits (Xu et al., 2012; Phukan et al., 2019). Subcortical limbic structures like the hippocampus is associated with learning and memory, and injury to this brain part may lead to behavioural and cognitive changes (Snell, 2010; Owoeye and Farombi, 2015; Fogwe et al., 2020).

Several plants in the human diet, containing numerous natural compounds with vast medicinal benefits, have been used in folk medicine to treat different types of ailments like cognitive disorders, including neurodegenerative diseases (Kumar and Khanum, 2012). *Phoenix dactilyfera L.* (date palm) is a plant that belongs to the family Arecaceae (Ahmed et al., 2008); its fruits reported as a good source of energy and essential vitamins and elements (Usama et al.,

2009; Farooqui et al., 2019), and different parts of the plant claimed to have medicinal benefits used for the treatment of diversity of ailments including paralysis, memory disturbances, loss of consciousness and nervous disorders (Nadkarni, 1976; Saha et al., 2017). *P. dactilyfera* has been scientifically demonstrated to possess a variety of pharmacological activities including amelioration of the deleterious effects toxins, antioxidant and neuroprotective activities (Allaith and Abdul, 2005; Vyawahare et al., 2009; El-Far et al., 2016). There is need to empirically demonstrate the neuroprotective properties of certain solvent extract forms of *P. dactilyfera* against different environmental toxins in different brain regions.

This study comparatively assessed the neuroprotective property of aqueous and ethanol fruit pulp extracts of *P. dactylifera* in a rat model of mercury-triggered hippocampal changes using microscopic examinations.

MATERIALS AND METHODS

Plant Material: Dried *P. dactylifera* (date palm) fruits were obtained from Samaru Market in Zaria, Kaduna State, Nigeria and authenticated in the Herbarium Unit of the Department of Biological Sciences, Faculty of Life Sciences, Ahmadu Bello University (ABU), Zaria with the Voucher Specimen Number 7130.

Extraction and phytochemical screening of *P. dactylifera* fruit was carried out in the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, ABU, Zaria. The method of maceration as reported by Agbon *et al.* (2013) and Abdul-Wahab *et al.* (2010) for the preparation of aqueous fruit pulp extract of *P. dactylifera* (AFPD) and ethanol fruit pulp extract of *P. dactylifera* (EFPD), respectively, were adopted. The method described by Trease and Evans (2002) for qualitative phytochemical screening of secondary metabolites was adopted.

Experimental Animals: The experimental animals (Wistar rats) weighing 130 ± 20 g were obtained from Animal House of the Department of Human Anatomy, Faculty of Basic Medical Sciences, ABU, Zaria and housed in new cages in the same facility were rats acclimatized for a week before experimentation. The rats were housed under standard laboratory condition, light and dark cycles of 12 hours, and were provided with food (rat chow) and water *ad libitum*.

Drug: Mercury in the form of mercuric chloride ($HgCl_2$) was obtained and used as neurotoxicant for this study. The product is manufactured by British Drug Houses Chemicals, Poole, England.
Vitamin C (ascorbic acid) was obtained and used for this study as reference antioxidant drug. The product is manufactured by Emzor Limited, Lagos, Nigeria.

Experimental Protocol: Twenty-eight (28) Wistar rats were divided into seven (7) groups (I – VII) of four rats each. Hippocampal neurotoxicity was induced in rats by the administration of $HgCl_2$ as reported by Sheikh *et al.* (2013) Group I (control) was administered distilled water (2 ml/kg); group II was administered $HgCl_2$ (5 mg/kg); group III was administered vitamin C (100 mg/kg; Raghu-Jetti et al., 2014) as reference drug followed by $HgCl_2$ (5 mg/kg); groups IV and V were administered AFPD (250 mg/kg and 500 mg/kg, respectively), while groups VI and VII were administered EFPD (250 mg/kg and 500 mg/kg, respectively). Treatment period lasted fourteen (14) days. All administrations were via oral route (*See* Figure 1).

At the end of the experiment, rats were euthanized using chloroform anesthesia and brains harvested and fixed in a fixative, Bouin's fluid.

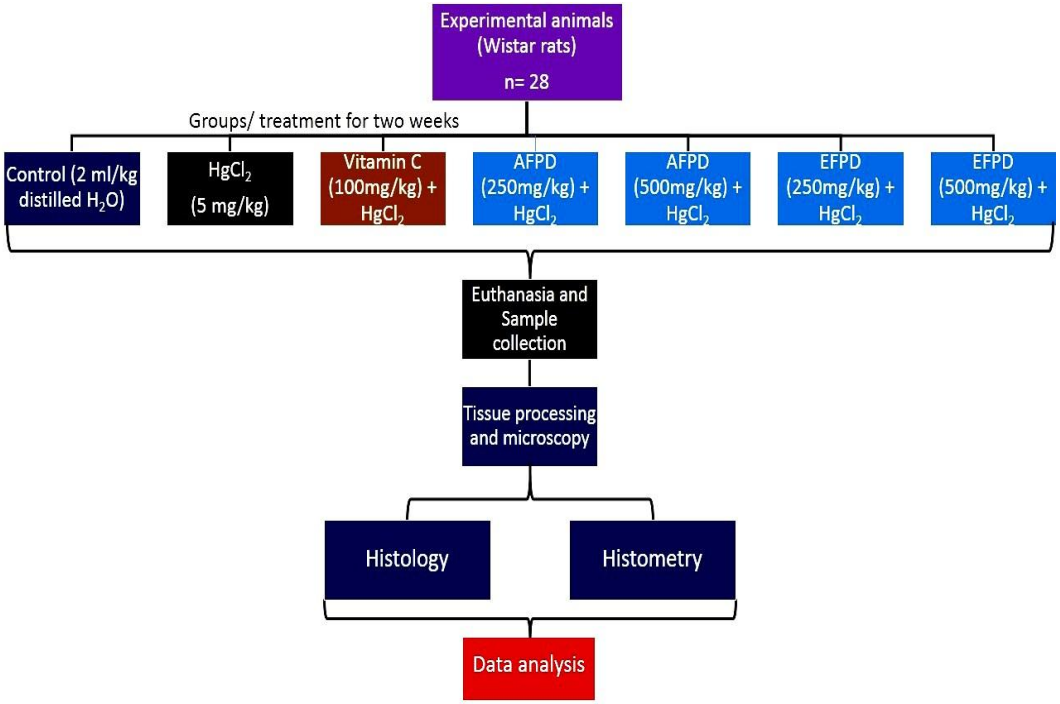

Figure 1:
Experimental protocol
n = 4; AFPD= Aqueous fruit pulp extract of Phoenix dactylifera; EFPD= Ethanol fruit pulp extract of Phoenix dactylifera; $HgCl_2$= Mercuric chloride

Physical Observation
During the period of experiment, the rats were observed for changes in physical activity and behavioral pattern, like playing and eating. Absolute body weight before (initial weight, IW) and after the experiment (final weight, FW) were weighed (using digital weighing scale, Kerro BL 20001, 0.1 g) and values were statistically compared.

Histological Studies
Fixed brains were processed using histological techniques by making a coronal section at the caudal region of cerebrum to target the hippocampus. Histological brain sections were stained for light microscopy with Haematoxylin and Eosin (H & E) and Cresly Violet (CV) stains to demonstrate cytoarchitectural features of *cornu ammonis* (CA; CA1 and CA3 regions) of the hippocampus. Tissues were processed in the Histology Unit, Department of Human Anatomy, ABU, Zaria and, light microscopy and micrography conducted in the Microscopy and Stereology Research Laboratory of the same facility.

Histometric studies: Histometric analysis was used as an objective basis for comparison of histological observation (Asuquo et al., 2007; Huda and Zaid et al., 2007). Briefly, histometry involved measuring the soma area and perimeter of pyramidal neurons of hippocampal CA1 and CA3 regions from CV (*CV is an excellent neuronal, cell body-specific stain (Suvarna et al., 2019))* stained micrographs (digital microscopic images). Histometric analysis was conducted using a light microscope (HM-LUX, Leitz Wetzlar, Germany) with a 40/ 0.65 x objective (x 400 magnification), micrometer slide (1 mm graduated in 0.01 mm units; that is divided 10x into 100 μm units) and computer running imaging software (AmScope MT *version* 3.0.0.5, USA) according to the manufacturer's instruction (Using the AmScope Microscope Cameras, 2012). Three different micrographic fields were randomly captured (Jelsing, 2006; Oliveira et al., 2015) in the CA1 and CA3 hippocampal regions and 5 - 10 neurons that met the criteria for selection (*that is, pyramidal neurons, with well-outlined nucleus in the cell profiles*) were randomly selected; using the AmScope imaging software polygon tool, soma area and perimeter were measured and analyzed.

Data Analysis
Data obtained were expressed as mean ± S.E.M; paired sample *t*–test was employed for the comparison of means and one way ANOVA with least significant difference (LSD) *post hoc test* for presence of significant difference among means of the groups. Values were considered significant when $p< 0.05$. Data were analyzed using the statistical software, Statistical Package for the Social Sciences (IBM SPSS *v 21.0* SPSS Inc., Chicago, USA) and Microsoft Office Excel 2013 for charts.

Physical observation: During the period of treatment, rats in the control group were observed to exhibit normal behavioural pattern of eating and physical activities, like movement and playfulness, while rats in other groups exhibited reduced activity, especially $HgCl_2$- treated group. The absolute body weights of rats in all groups were observed to have increased remarkably ($p<0.05$) when IW and FW were compared, except for $HgCl_2$-, Vitamin C+ $HgCl_2$ and EFPD (250 mg/kg)+ $HgCl_2$-treated groups (Table 2).

RESULTS

Phytochemical Analysis: Phytochemical screening of AFPD and EFPD produced positive reaction for secondary metabolites like, flavonoids, saponins and tannins (Table 1).

Physical observation: During the period of treatment, rats in the control group were observed to exhibit normal behavioural pattern of eating and physical activities, like movement and playfulness, while rats in other groups exhibited reduced activity, especially $HgCl_2$- treated group. The absolute body weights of rats in all groups were observed to have increased remarkably ($p<0.05$) when IW and FW were compared, except for $HgCl_2$-, Vitamin C+ $HgCl_2$ and EFPD (250 mg/kg)+ $HgCl_2$-treated groups (Table 2).

Table 1:
Phytochemical constituents of fruit pulp extracts of *P. dactylifera*

Constituents	Alkaloid	Anthraquinones	Cardiac glycoside	Carbohydrates	Flavonoids	Saponin	Tannin
Inference (AFPD)	+	–	–	+	+	+	+
Inference (EFPD)	+	–	–	+	+	+	+

+ = Positive (Present); **–** = Negative (Absent); **AFPD=** Aqueous fruit extract of *Phoenix dactylifera*; **EFPD=** Ethanol fruit extract of *Phoenix dactylifera*;

Table 2:
Absolute body weight comparison of Wistar rats

Group	Treatment	IW (g)	FW (g)	*t*	*p*-value
I	Control (H_2O 2 ml/kg)	116.63±13.28	138.50±12.01	8.919	0.000
II	$HgCl_2$ (5mg/kg)	109.50±20.67	131.00±38.92	2.261	0.073
III	Vit C (100mg/kg) + $HgCl_2$ (5mg/kg)	101.40±19.27	111.20±8.55	0.735	0.503
IV	AFPD (250mg/kg) + $HgCl_2$ (5mg/kg)	111.25±12.09	144.75±39.51	3.310	0.045
V	AFPD (500mg/kg) + $HgCl_2$ (5mg/kg)	104.57±16.55	129.86±13.23	2.881	0.028
VII	EFPD (250mg/kg) + $HgCl_2$ (5mg/kg)	144.25±36.37	159.25±59.36	1.730	0.182
VIII	EFPD (500mg/kg) + $HgCl_2$ (5mg/kg)	117.50±21.42	138.00±21.61	4.889	0.016

n= 4; mean ± SEM; Paired sample *t*- test; **AFPD=** Aqueous fruit extract of *Phoenix dactylifera*; **EFPD=** Ethanol fruit extract of *Phoenix dactylifera*; **Vit C**= Vitamin C; **$HgCl_2$**= Mercuric chloride; **FW**=Final weight; **IW**= Initial weight.

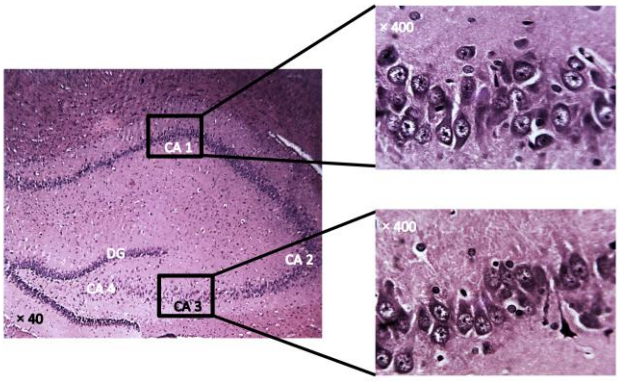

Plate 1:
Coronal section of the hippocampus of Wistar rat with subregions (CA1 - CA4). H and E stain. *Cornu ammonis* (CA); Dendate gyrus (DG)

Histological Examination: Histological examination of sections of the hippocampus (CA 1 and CA 3 regions) of rats, stained with H & E and CV stains revealed the following: The rat hippocampus (*hippocampus proprius*) is subdivided into four regions (CA1 - CA4) according to density, size and branching of axons and dentrites of the pyramidal cells. Each of these regions consists of three layers: *stratum molecular* (molecular layer), *stratum pyramidale* (pyramidal layer) which contains bodies of the pyramidal cells *and stratum multiforme* (multiform layer). The continuation of CA3 in the concavity of dendate gyrus (*fascia dentata*) is the CA4. CA1 is characterized by densely packed medium sized cells; CA3 is the region with large less densely packed cells, while CA2 is a transition field between CA3 and CA1 (Plate 1). The control group showed normal histoarchitecture of CA1 and CA3 regions: the characteristic pattern of an ordered sheet of neurons (pyramidal and granule cells) whose cell bodies are all packed together (Plates 2a and 3a). The large neurons are the giant pyramids of CA3 and other interneurons such as stellate, fusiform and basket cells of Cajal, which differ from the pyramidal and granule cells, observed most clearly in CA3 (Plates 2a and 3a).

Histological sections of $HgCl_2$-treated group revealed histoarchitectural distortion of CA1 and CA3 regions; neurodegenerative changes like, irregular arrangement of CA1 hippocampal neurons, satellitosis, perineuronal vacuolation, pyknotic and basophilic necrosis (Figures 2b, 3b, 4b and 5b). However, hippocampi of vitamin C + $HgCl_2$-, AFPD+ $HgCl_2$- and EFPD+ $HgCl_2$- treated groups, revealed mild histoarchitectural distortions of CA1 and CA3 regions relative to the control. The histological features of the vitamin C-treated group showed chromatolysis and karyorrhexis (Plates 2c, 3c, 4c and 5c); AFPD-treated groups showed histoarchitectural changes like karyopyknosis and satellitosis (Figures 2d - e, 3d - e, 4d - e and 5d – e) and EFPD-treated groups showed changes such as, karyorrhexis, eosinophilic necrosis, perineuronal vacuolations and pyknotic necrosis (Plates 2f - g, 3f - g, 4f - g and 5f - g). AFPD and EFPD administration conferred preservation of the histoarchitecture in a dose dependent manner compared to the control

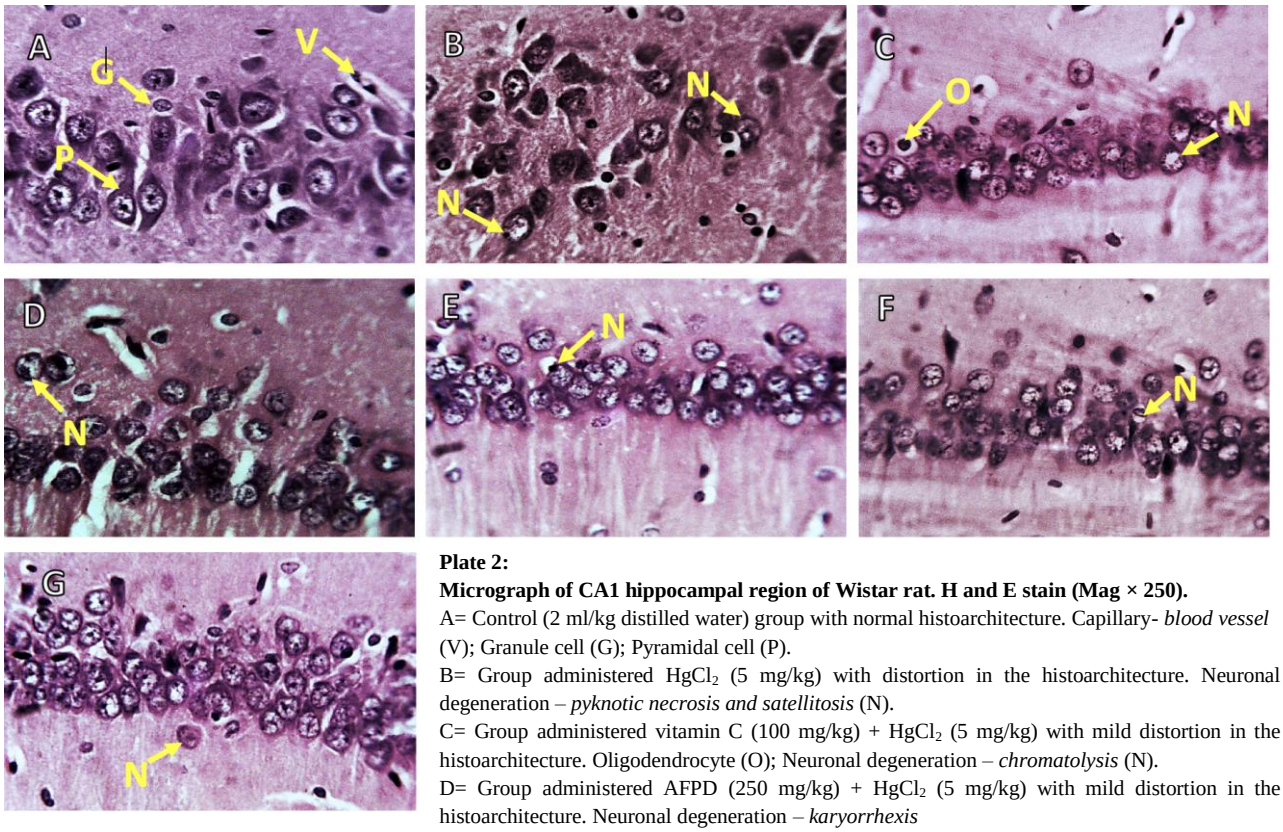

Plate 2:
Micrograph of CA1 hippocampal region of Wistar rat. H and E stain (Mag × 250).
A= Control (2 ml/kg distilled water) group with normal histoarchitecture. Capillary- *blood vessel* (V); Granule cell (G); Pyramidal cell (P).
B= Group administered $HgCl_2$ (5 mg/kg) with distortion in the histoarchitecture. Neuronal degeneration – *pyknotic necrosis and satellitosis* (N).
C= Group administered vitamin C (100 mg/kg) + $HgCl_2$ (5 mg/kg) with mild distortion in the histoarchitecture. Oligodendrocyte (O); Neuronal degeneration – *chromatolysis* (N).
D= Group administered AFPD (250 mg/kg) + $HgCl_2$ (5 mg/kg) with mild distortion in the histoarchitecture. Neuronal degeneration – *karyorrhexis*
E= Group administered AFPD (500 mg/kg) + $HgCl_2$ (5 mg/kg) with mild distortion in the histoarchitecture. Neuronal degeneration – *pyknotic necrosis and perineuronal vacuolation* (N); F= Group administered EFPD (250 mg/kg) + $HgCl_2$ (5 mg/kg) with mild distortion in the histoarchitecture. Pyramidal cell (P); Neuronal degeneration – *pyknotic necrosis and perineuronal vacuolation* (N); G= Group administered EFPD (500 mg/kg) + $HgCl_2$ (5 mg/kg) with mild distortion in the histoarchitecture. Pyramidal cell (P); Neuronal degeneration – *esinophilic necrosis* (N).

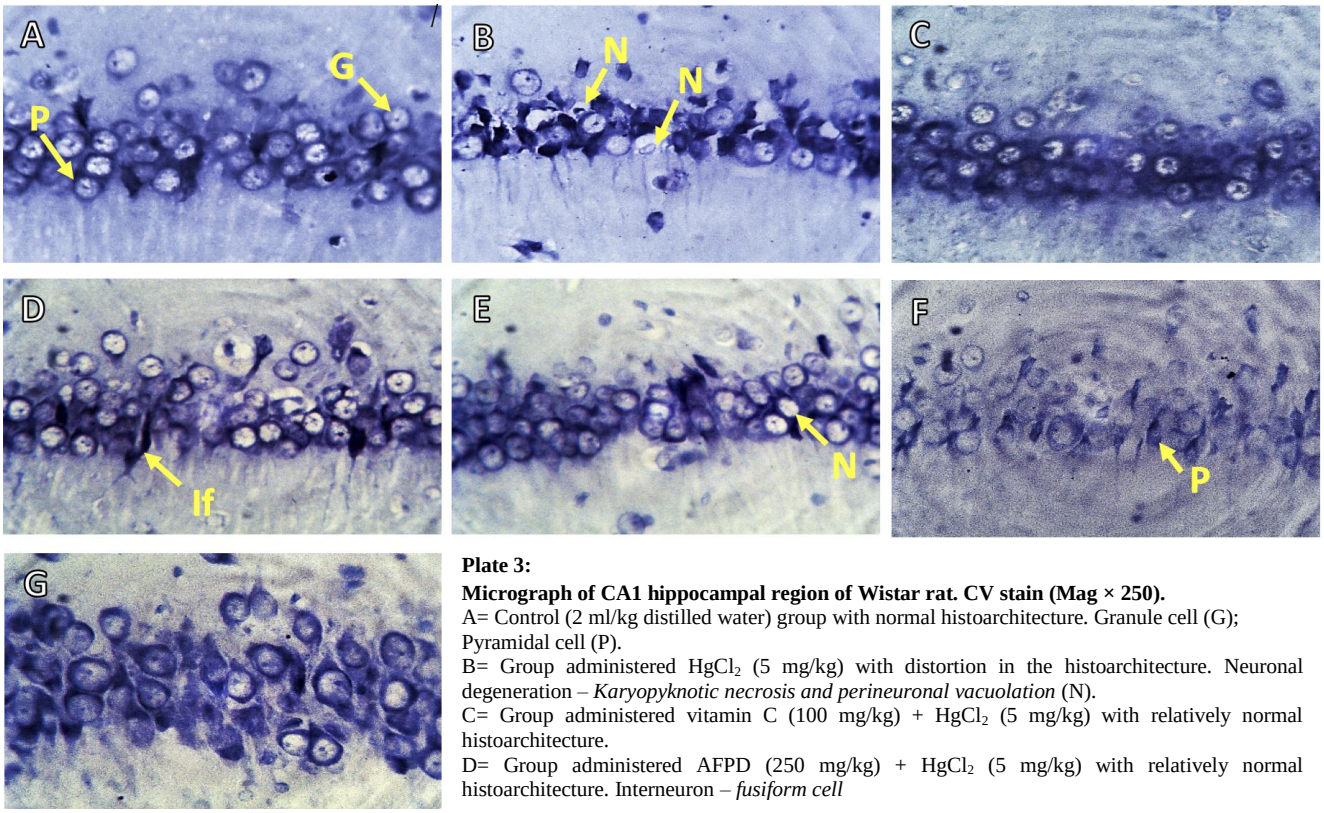

Plate 3:
Micrograph of CA1 hippocampal region of Wistar rat. CV stain (Mag × 250).
A= Control (2 ml/kg distilled water) group with normal histoarchitecture. Granule cell (G); Pyramidal cell (P).
B= Group administered $HgCl_2$ (5 mg/kg) with distortion in the histoarchitecture. Neuronal degeneration – *Karyopyknotic necrosis and perineuronal vacuolation* (N).
C= Group administered vitamin C (100 mg/kg) + $HgCl_2$ (5 mg/kg) with relatively normal histoarchitecture.
D= Group administered AFPD (250 mg/kg) + $HgCl_2$ (5 mg/kg) with relatively normal histoarchitecture. Interneuron – *fusiform cell*
E= Group administered AFPD (500 mg/kg) + $HgCl_2$ (5 mg/kg) with mild distortion in the histoarchitecture. Neuronal degeneration – *chromatolysis* (N).
F= Group administered EFPD (250 mg/kg) + $HgCl_2$ (5 mg/kg) with relatively normal histoarchitecture. Pyramidal cell (P).
G= Group administered EFPD (500 mg/kg) + $HgCl_2$ (5 mg/kg) with relatively normal histoarchitecture.

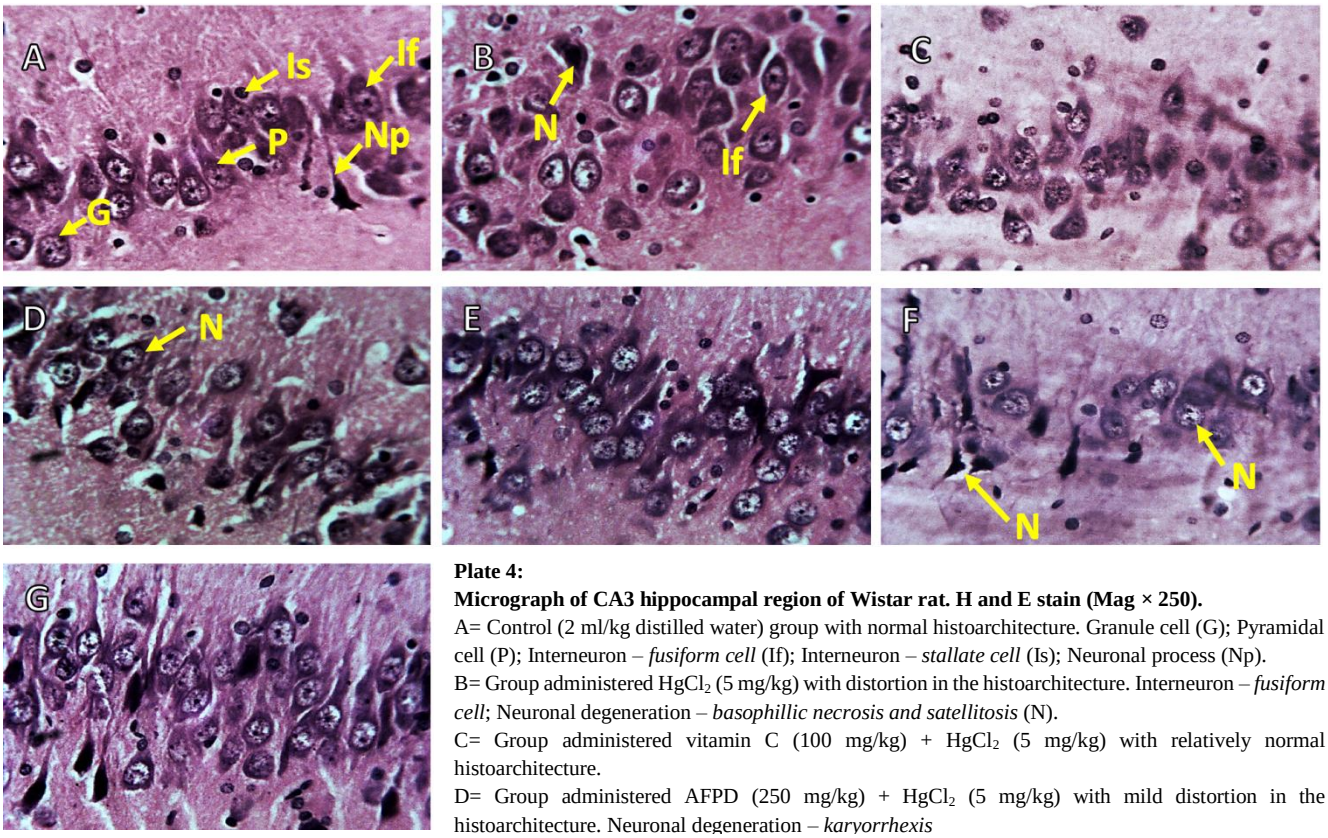

Plate 4:
Micrograph of CA3 hippocampal region of Wistar rat. H and E stain (Mag × 250).
A= Control (2 ml/kg distilled water) group with normal histoarchitecture. Granule cell (G); Pyramidal cell (P); Interneuron – *fusiform cell* (If); Interneuron – *stallate cell* (Is); Neuronal process (Np).
B= Group administered $HgCl_2$ (5 mg/kg) with distortion in the histoarchitecture. Interneuron – *fusiform cell*; Neuronal degeneration – *basophillic necrosis and satellitosis* (N).
C= Group administered vitamin C (100 mg/kg) + $HgCl_2$ (5 mg/kg) with relatively normal histoarchitecture.
D= Group administered AFPD (250 mg/kg) + $HgCl_2$ (5 mg/kg) with mild distortion in the histoarchitecture. Neuronal degeneration – *karyorrhexis*
E= Group administered AFPD (500 mg/kg) + $HgCl_2$ (5 mg/kg) with relatively normal histoarchitecture.
F= Group administered EFPD (250 mg/kg) + $HgCl_2$ (5 mg/kg) with mild distortion in the histoarchitecture. Pyramidal cell (P); Neuronal degeneration – *karyorrhexis and perineuronal vacuolation* (N).
G= Group administered EFPD (500 mg/kg) + $HgCl_2$ (5 mg/kg) with relatively normal histoarchitecture.

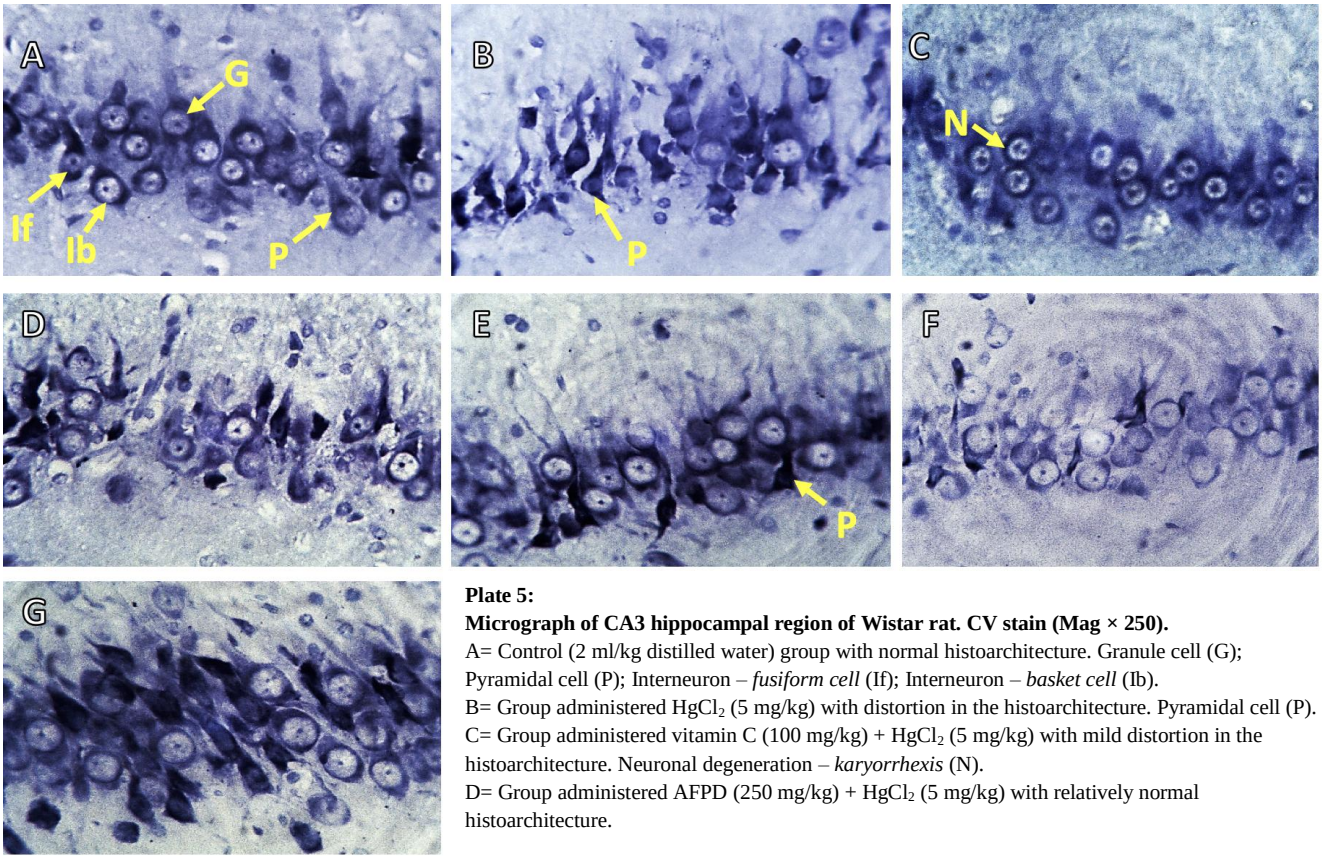

Plate 5:
Micrograph of CA3 hippocampal region of Wistar rat. CV stain (Mag × 250).
A= Control (2 ml/kg distilled water) group with normal histoarchitecture. Granule cell (G); Pyramidal cell (P); Interneuron – *fusiform cell* (If); Interneuron – *basket cell* (Ib).
B= Group administered $HgCl_2$ (5 mg/kg) with distortion in the histoarchitecture. Pyramidal cell (P).
C= Group administered vitamin C (100 mg/kg) + $HgCl_2$ (5 mg/kg) with mild distortion in the histoarchitecture. Neuronal degeneration – *karyorrhexis* (N).
D= Group administered AFPD (250 mg/kg) + $HgCl_2$ (5 mg/kg) with relatively normal histoarchitecture.
E= Group administered AFPD (500 mg/kg) + $HgCl_2$ (5 mg/kg) with relatively normal histoarchitecture. Pyramidal cell (P).
F= Group administered EFPD (250 mg/kg) + $HgCl_2$ (5 mg/kg) with relatively normal histoarchitecture.
G= Group administered EFPD (500 mg/kg) + $HgCl_2$ (5 mg/kg) with relatively normal histoarchitecture.

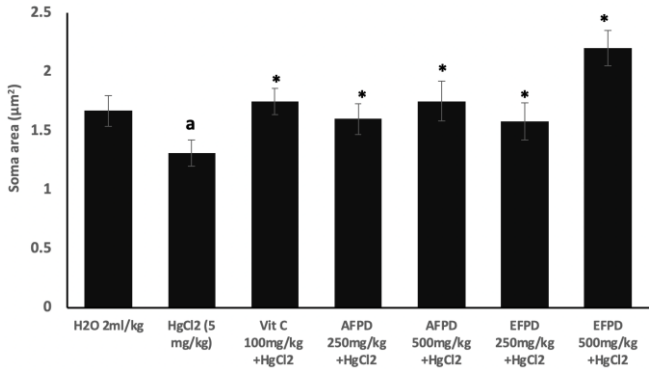

Figure 2a
Effect of *P. dactylifera* on histometric characteristic (soma area) of pyramidal neuron in the CA1 hippocampal region of Wistar rats.
n = 20 ± 5 (*5-10 cells/ 3 fields*); mean ± SEM; One way ANOVA *LSD post hoc test*, **a** =p<0.05 when compared with the control (2 ml/kg distilled H_2O), ***** =p<0.05 when compared with $HgCl_2$. **AFPD=** Aqueous fruit extract of *Phoenix dactylifera*; **EFPD=** Ethanol fruit extract of *Phoenix dactylifera*; **Vit C**= Vitamin C; **CA**= *Cornu Amonis.*

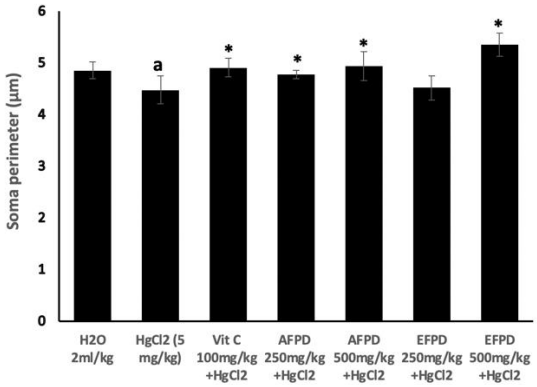

Figure 2b
Effect of *P. dactylifera* on histometric characteristic (soma perimeter) of pyramidal neuron in the CA1 hippocampal region of Wistar rats.
n = 20 ± 5 (*5-10 cells/ 3 fields*); mean ± SEM; One way ANOVA *LSD post hoc test*, **a** =p<0.05 when compared with the control (2 ml/kg distilled H_2O), ***** =p<0.05 when compared with $HgCl_2$. **AFPD=** Aqueous fruit extract of *Phoenix dactylifera*; **EFPD=** Ethanol fruit extract of *Phoenix dactylifera*; **Vit C**= Vitamin C; **CA**= *Cornu Amonis*

Histometric Analysis: Histometric features of CA1 pyramidal neuron revealed remarkable ($p<0.05$) decrease in soma area and perimeter of $HgCl_2$- treated group compared to the control. Relative to the $HgCl_2$-treated group, striking ($p<0.05$) difference in histometric features were observed with the vitamin C + $HgCl_2$-, AFPD+ $HgCl_2$- and EFPD+ $HgCl_2$ -treated groups (Figures 2a and b).

Histometric features of CA3 pyramidal neuron revealed remarkable ($p<0.05$) decrease in soma area of $HgCl_2$-treated group relative to the control. Moreover, relative to the $HgCl_2$-treated group, striking ($p<0.05$) difference in neuronal soma area was observed with vitamin C + $HgCl_2$-, AFPD+ $HgCl_2$- and EFPD+ $HgCl_2$ -treated groups (Figure 3a). Neuronal soma perimeter revealed significant decrease with $HgCl_2$- and AFPD (250 mg/kg) + $HgCl_2$-treated groups when compared to the control. Comparing neuronal soma perimeter of $HgCl_2$-treated group with AFPD+ $HgCl_2$- and EFPD+ $HgCl_2$ -treated groups revealed remarkable difference (Figure 3b).

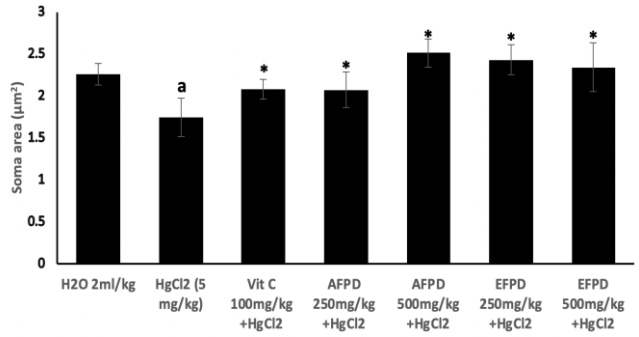

Figure 3a
Effect of *P. dactylifera* on histometric characteristic (soma area) of pyramidal neuron in the CA3 hippocampal region of Wistar rats.
n = 20 ± 5 (*5-10 cells/ 3 fields*); mean ± SEM; One way ANOVA *LSD post hoc test*, **a** =p<0.05 when compared with the control (2 ml/kg distilled H_2O), ***** =p<0.05 when compared with $HgCl_2$. **AFPD=** Aqueous fruit extract of *Phoenix dactylifera*; **EFPD=** Ethanol fruit extract of *Phoenix dactylifera*; **Vit C**= Vitamin C; **CA**= *Cornu Amonis*

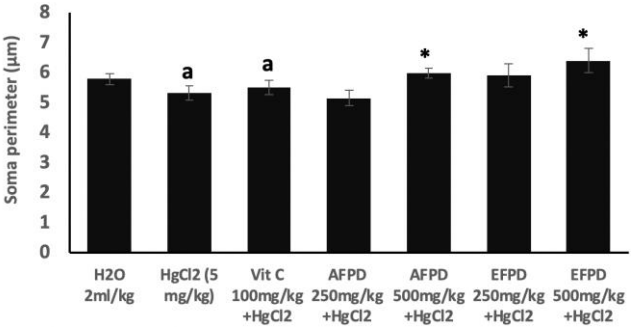

Figure 3b
Effect of *P. dactylifera* on histometric characteristic (soma perimeter) of pyramidal neuron in the CA3 hippocampal region of Wistar rats.
n = 20 ± 5 (*5-10 cells/ 3 fields*); mean ± SEM; One way ANOVA *LSD post hoc test*, **a** =p<0.05 when compared with the control (2 ml/kg distilled H_2O), ***** =p<0.05 when compared with $HgCl_2$. **AFPD=** Aqueous fruit extract of *Phoenix dactylifera*; **EFPD=** Ethanol fruit extract of *Phoenix dactylifera*; **Vit C**= Vitamin C; **CA**= *Cornu Amonis*

DISCUSSION

In this study, phytochemical analysis of AFPD and EFPD were conducted and, the neuroprotective effect of AFPD and EFPD against mercury-triggered hippocampal changes was comparatively evaluated by the assessment of Wistar rats' physical activity, absolute body weight changes and microscopic features of CA1 and CA3 hippocampal regions.

Phytochemical analysis of AFPD and EFPD revealed the presence of similar metabolites like flavonoids, saponins, and tannins which have been reported to possess antioxidant and neuroprotective properties in models of neurological disorders (Chen et al., 2015; Sarian et al., 2017). Findings agree with reported phytochemicals present in fruit extract of *P. dactylifera* (Raghu-Jetti et al., 2014; Bouhlali et al., 2017).

Altered physical activity exhibited by $HgCl_2$-treated group is suggestive of treatment-related toxicity. Loss of appetite and sluggishness has been associated with drug-related toxicity (Salawu et al., 2009; Agbon et al., 2014). Body weight change is a sensitive pointer to the general health status of an animals and serves as indicator of the deleterious effects of drugs and chemicals (Mukinda and Syce, 2007; Salawu et al., 2009). Observed striking increase in the trend of absolute body weight with AFPD and EFPD-treated groups, when IW and FWs were compared, could be attributed to the high caloric content in *P. dactylifera* which has been reported as a good source of energy and rich in nutrients (Ghnimi et al., 2017; Al-Mssallem et al., 2019; Hussain et al., 2020).

The hippocampus has several functions, playing critical role in short- and long-term memory and spatial navigation (Eichenbaum and Cohen, 2014; Ekstrom and Ranganath, 2018). The relevance of hippocampal CA1 and CA3 subregions in hippocampal functions have been reported by several researchers (Kesner et al., 2005; Kesner, 2013; Dimsdale-Zucker et al., 2018).

Observed histoarchitectural distortion of the hippocampal regions, like irregular arrangement of CA1 hippocampal neurons, pyknosis and basophilic necrosis are indicative of $HgCl_2$ treatment-triggered neurodegenerative changes. Findings are in accordance with the reports of Falluel-Morel et al. (2007) and Ranjan et al. (2015) on the vulnerability of hippocampus to mercury showing detrimental changes; cytoarchitectural distortion of hippocampal neurons in response to mercury exposure.

The integrity of CA1 architecture is important because of the particular vulnerability of the neurons in the pyramidal layer to toxic events (Seidman, 2011). Typically, the neurons are packed together, arranged in one or two very dense rows (Slomianka, 2011). Depolarization or irregular arrangement of CA1 neurons observed in this study is suggestive of treatment-related degenerative changes. Finding is in line with the reports on preferential degeneration of CA1 pyramidal cells following insult (Ruan et al., 2007; Seidman, 2011). Wu *et al.* (2016) observed disorganization and decrease in the number of hippocampal neurons in Sprague Dawley rats exposed to mercury. Observed basophilia of cytoplasm and pyknosis of neurons, which histologically characterizes neuronal atrophy associated with a wide variety of irreversible neuronal injuries resulting to progressive cell death in several degenerative disorders (Seilhean et al., 2004) is indicative of treatment related toxicity. Several lines of evidence have associated the main neurotoxic mechanism of mercury to induction of oxidative stress (Shanker et al., 2004; Farina et al., 2013; Abdel-Zaher et al., 2017).

In this study, administration of vitamin C ameliorated mercury-induced hippocampal changes. Findings are in agreement with reports on neuroprotective activity of ascorbic acid following heavy metal intoxication (El-Sokkary and Awadalla, 2011; Kumar et al., 2018). Raghu-Jetti *et al.* (2014) reported less severe neurodegenerative changes in hippocampal subregions of fluoride exposed animals treated with ascorbic acid. Treatment with AFPD and EFPD ameliorated mercury-induced hippocampal changes by conferring histoarchitectural preservation. Findings are in consistence with reports related to neuroprotective properties of plant products which had been shown to exert neuroprotective effects (Kim et al., 2008; Khazdair et al., 2019; Phukan et al., 2019) against experimentally-induced neuronal injury.

Mild neurodegenerative changes observed in hippocampal CA1 and CA3 subregions of vitamin C-, AFPD- and EFPD-treated groups is suggestive of histoarchitectural preservation and neuroprotection. Neuroprotection refers to the relative preservation of neuronal structure and/ or function (Casson et al., 2012; Sairazi and Sirajudeen, 2020). Relative preservation of neuronal integrity implies a reduction in the rate of neuronal

damage in the presence of a neurodegenerative insult such as mercury. Ascorbic acid participates in several beneficial cellular functions including antioxidant protection which plays critical roles in the reversion of mercury-induced injury by forming inert complexes and inhibiting toxic effects on neurons (Kumar et al., 2018; Teleanu et al., 2019). Neuroprotective properties of natural agents including plants have been attribute to antioxidant activities (Hwang et al., 2012; Kim et al., 2015; Sairazi and Sirajudeen, 2020). Plant phytochemical constituents including flavonoids and tannin have been reported to exhibit great antioxidant activities which provide protection against oxidative stress (Chang et al., 2012; Hwang et al., 2015). Thus, AFPD and EFPD neuroprotective property is comparable to that of the reference drug, vitamin C.

Histometric quantification provides for accurate statistical grading for comparison of histological observation, increasing precision and improving assessment of certain histological change compared with direct visual appraisal (Huda and Zaid et al., 2007; Agbon et al., 2016). In this study, remarkably decreased histometric features (soma area and perimeter) of CA1 and CA3 pyramidal neurons in $HgCl_2$-treated group are an indication of treatment-related cytoarchitectural changes. Decreased perikaryal size, an attribute of neuronal cytoplasmic shrinkage, has been associated to stress-induced cytoarchitectural changes (Insausti et al., 1997; Mohammad et al., 2012). Histometric parameters are directly applied and related to tissue function (Pearse and Mark, 1974; Huda and Zaid et al., 2007). Heavy metals, including mercury, have been reported to undergo redox cycling reactions and possess the ability to produce reactive radicals such as superoxide anion radical and nitric oxide in biological systems (Jomova and Valko, 2011; Farina et al., 2013; Cariccio et al., 2018). Neuronal shrinkage has been reported as direct toxic effect or role of mercuric chloride in neurodegenerative progression (Ramzi and Stanely, 1994; Ghusoon et al., 2012; Jha et al., 2019).

Relative to the $HgCl_2$-treated group, striking difference in histometric features observed in vitamin C-, AFPD- and EFPD-treated groups is suggestive of cytoarchitectural preservation and neuroprotection. Neuroprotective effects of various phytochemicals are associated with reduced levels of oxidative stress (Kumar and Khanum, 2012; Gombeau et al., 2019). Wan Ismail and Mohd Radzi (2013) reported increased levels of endogenous antioxidants in the brain and remarkable reduction in neuronal damage in form of neuronal shrinkage, atrophy and necrosis in rats treated with *P. dactylifera* fruit. Histometric findings corroborate with histologic observations in this study. Thus, AFPD and EFPD have potentials to preserve neuronal cytoarchitectural features exposed to $HgCl_2$-triggered degenerative changes. Comparatively, the neuroprotective efficacy of AFPD and EFPD are relatively similar, especially at doses of 500 mg/kg.

Antioxidant activities have been implicated for neuroprotective property of *P. dactylifera* (Kalantaripour et al., 2012; Agbon et al., 2017; Essa et al., 2019). Phytochemicals including flavonoids, saponins and tannins have been reported to be potent scavengers of reactive oxygen species, metal ions chelators (El Sohaimy et al., 2015; Komaki et al., 2015), protectors of neurons from lethal damage induced by neurotoxins (Pujari et al., 2011) and, exert multiplicity of neuroprotective actions in *in vivo* and *in vitro* models of neurological disorders (Lobo et al., 2010; Chang et al., 2012; Hussain et al., 2020). Relevance of the structural and physiological integrity of CA1 and CA3 hippocampal subregions in hippocampal functionality has been established by several researchers (Kesner et al., 2005; Drew and Huckleberry, 2017; Liang et al., 2020). Ross *et al.* (2009) and Farovik *et al.* (2010) have reported severe memory impairment after selective CA3 and CA1 damage. The potential ability of *P. dactylifera* phytochemicals to suppress neuroinflammation and neuronal injury indicates its potential to promote memory, learning and cognitive function (Komaki et al., 2015).

In conclusion, results suggest that aqueous and ethanol fruit pulp extracts of *P. dactylifera* may prove efficacious in ameliorating mercury-triggered microscopic alterations in the hippocampus of Wistar rats. Neuroprotective property was dose dependent and relatively similar efficacy for both extract forms, AFPD and EFPD. Neuroprotective property could be attributed to antioxidant activities of constituent phytochemicals. Thus, aqueous and ethanol fruit pulp extracts of *P. dactylifera* are potential candidates for application in the management and treatment of mercury-induced neurodegenerative changes and related disease conditions.

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REFERENCES

Abdel-Zaher, M., Taha, M.M.M., Ahmed, H.G. (2017). Neurodegenerative disorders associated with mercuric chloride toxicity in mice and the role of some antioxidant. Int. J. Sci. Res. 6(4):1253-1260.

Abdul-Wahab, A., Mabrouk, M.A., Joro, J.M., Oluwatobi, S.E., Bauchi, Z.M., John, A.A. (2010). Ethanol extract of *phoenix dactylifera l.* prevents lead induced hematotoxicity in rats. Cont. J. Biomed. Sci. 4:10-15.

Agbon, A.N., Abubakar, M.G., Enemali, F.U., Mahdi, O., Bobbo, K.A., Sule, H., Yahaya, M.H., Okoh C.C. (2017). Assessment of Ethanol Fruit Extract of *Phoenix dactylifera L*. (Date Palm) on Mercuric Chloride-induced Cerebral and Cerebellar Alterations in Wistar Rats. J. Anat. Sci. 8(1):188-201.

Agbon, A.N., Ingbian, S.D., Dahiru, A.U. (2014). Preliminary histological and histochemical studies on the neuroprotective effect of aqueous fruit extract of *Phoenix dactylifera L*. (Date Palm) on atesunate – induced cerebellar damage in Wistar rats. Sub-Saharan Afr. J. Med. 1:204-209.

Agbon, A.N., Kwanashie, H.O., Hamman, W.O., Ibegbu, A.O., Akpulu, P.S., Ogirima, N.A. (2016). Histological and histochemical studies on the neuroprotective effect of aqueous fruit extract of *Phoenix dactylifera L.* (date palm) on mercury-induced cerebellar damage in Wistar rats. J. Anat. Sci. 7(2):44-54.

Agbon, A.N., Kwaneshie, H.O., Hamman, W.O. (2013). Antidiarrheal activity of aqueous fruit extract of *Phoenix dactylifera* (date palm) in Wistar rats. Br. J. Pharmacol. Toxicol. 4(3):121-127.

Ahmed, M.B., Hasona, N.A., Selemain, H.A. (2008). Protective effects of extract from dates (*Phoenix dactylifera* l.) and ascorbic acid on thioacetamide-induced hepatotoxicity in rats. Iran J. Pharm. Res. 7(3):193-201.

Allaith, F., Abdul, A.A. (2005). *In vitro* evaluation of antioxidant activity of different extracts of *Phoenix dactylifera* fruits as

functional foods. Deutsche Lebensmittel Rundschau. 101:305-8.

Al-Mssallem, M.Q., Alqurashi, R.M., Al-Khayri, J.M. (2019). Bioactive compounds of date palm (*Phoenix dactylifera L.*). Springer Nature Switzerland AG. p. 2

Asuquo, O.R., Igiri, A.O., Olawoyin, O.O., Eyong, E.U. (2007). Correlation of histological and histometric changes in rats testes treated with chloroquine phosphate. Nig. J. Physiol. Sci. 22(1-2):135-139.

Biglari, F., Abbas, F.M.A., Azhar, M.E. (2008). Antioxidant activity and phenolic content of various date palm (*Phoenix dactylifera*) fruits from Iran. Food Chem. 107:1636-1641.

Bouhlali, E.T., Ramchoun, M., Alem, C., Ghafoor, K., Ennassir, J., Zegzouti, Y.F. (2017). Functional composition and antioxidant activities of eight Moroccan date fruit varieties (*Phoenix dactylifera L.*). J. Saudi Soci. Agri. Sci. 16:257–264.

Branco, V., Caito, S., Farina, M., Teixeira da Rocha, J., Aschner, M., Carvalho, C. (2017): Biomarkers of mercury toxicity: Past, present, and future trends, J. Toxicol. Environ. Health, Part B. 1-36.

Cariccio, V.L., Samà, A., Bramanti, P., Mazzon, E. (2018). Mercury Involvement in Neuronal Damage and in Neurodegenerative Diseases. Biol. Tr. Elem. Res. 187(2):341-356.

Casson, R.J., Chidlow, G., Ebneter, A., Wood, J.P., Crowston, J., Goldberg, I. (2012). Translational neuroprotection research in glaucoma: a review of definitions and principles. Clin. Exp. Ophthalmol. 40(4):350–357.

Chang, C.L., Lin, C.S., Lai, G.H. (2012). Phytochemical characteristics, free radical scavenging activities, and neuroprotection of five medicinal plant extracts. Evidence-Based Comp. Alt. Med. 1-8.

Chen, Y., Chen, M. (2001). Heavy metal concentrations in nine species of fishescaught in coastal waters off Ann-ping S.W. Taiwan. J. Food Drug Anal. 9(2):219.

Chen, Y., Sun, H., Huang, L., Li, J., Zhou, W., Chang, J. (2015). Neuroprotective Effect of Radix Trichosanthis Saponins on Subarachnoid Hemorrhage. Evidence-Based Compl. Alt. Med. 2015;1-10.

Dimsdale-Zucker, H.R., Ritchey, M., Ekstrom, A.D., Yonelinas, A.P., Ranganath, C. (2018). CA1 and CA3 differentially support spontaneous retrieval of episodic contexts within human hippocampal subfields. Nature Communications, 9(294): 1-8.

Eichenbaum, H., Cohen, N.J. (2014) Can we reconcile the declarative memory and spatial navigation views on hippocampal function? Neuron. 83:764–770.

Ekstrom, A.D., Ranganath, C. (2018) Space, time, and episodic memory: the hippocampus is all over the cognitive map. Hippocampus. 28:680–687.

El Sohaimy, S.A., Abdelwahab, A.E., Brennan, C.S., Aboul-enein, A.M. (2015). Phenolic content, antioxidant and antimicrobial activities of Egyptian date palm (*Phoenix dactylifera L.*) fruits. Australian J. Basic Appl. Sci. 9:141–147.

El-Far, A.H., Hazem, M.S., Mohamed, M.A., Soad, K.A., Shaker, A.M. (2016). Date palm (*Phoenix dactylifera*): Protection and remedy food. Curr. Tr. Nutr. 2016;1(2):1-10.

El-Sokkary, G.H., Awadalla, E.A. (2011). The protective role of vitamin c against cerebral and pulmonary damage induced by cadmium chloride in male adult albino rat. *The Open* Neuroendocrinology J. 4:1-8.

Essa, M.M., Singh, V., Guizani, N., Manivasagam, T., Thenmozhi, A.J., Bhat, A., Ray, B., Chidambaram, S.B. (2019). *Phoenix dactylifera L.* fruits date fruit ameliorate oxidative stress in 3-NP intoxicated PC12 cells. Int. J. Nutr. Pharm. Neurol. Dis. 9:41-7.

Falluel-Morel, A., Sokolowski, K., Sisti, H.M., Zhou, X., Shors, T.J., Dicicco-Bloom, E. (2007). Developmental mercury exposure elicits acute hippocampal cell death, reductions in neurogenesis, and severe learning deficits during puberty. J. Neurochem. 103:1968-81.

Farina, M., Avila, D.S., da Rocha, J.B., Aschner, M. (2013). Metals, oxidative stress and neurodegeneration: a focus on iron, manganese and mercury. Neurochem. Intern. 62(5):575-594.

Farooqui, S., Khan, W., Pandey, S., Hussain, Z. (2019). Study of sustenance properties of dates. J. Emer. Tech. Inn. Res. 6(1):1-5.

Farovik, A., Dupont, L.M., Eichenbaum, H. (2010). Distinct roles for dorsal CA3 and CA1 in memory for sequential nonspatial events. Learn Mem.17:12-17.

Fogwe, L.A., Reddy, V., Mesfin, F.B.(2020) Neuroanatomy, Hippocampus In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing.

Galadima, A., Garba, Z.N., Leke, L., Almustapha, M.N., Adam, I.K. (2011). Domestic water pollution among local communities in Nigeria - causes and consequences. Euro. J. Sci. Res. 52(4):592-603.

Ghnimi, S., Umer, S., Karim, A., & Kamal-Eldin, A. (2017). Date fruit (*Phoenix dactylifera L.*): An underutilized food seeking industrial valorization. NFS J., 6:1-10.

Ghusoon, A.K.A., Rajiha, A.A.N., Eman, H.Y.A. (2012). Assessment the therapeutic effects of aqueous extracts of *Cilantro* and *Garlic* in mercuric chloride poisoning in rats. Iraqi J. Vet. Med. 36(2):231-243.

Gombeau, K., Bezerra de Oliveira, R., Sarrazin, S.L.F., Veras Mourão, R.H., Bourdineaud, J.P. (2019). Protective effects of *Plathymenia reticulata* and *Connarus favosus* aqueous extracts against cadmium- and mercury-induced toxicities. Toxicol. Res. 35(1):25-35.

Huda, M.A., Zaid, A.A. (2007). Photo-histometry a modified computer assisted morphometric measurement program. Fac. Med. Baghdad. 49(1):1.

Hussain, M.I., Farooq, M., Syed, Q.A. (2020). Nutritional and biological characteristics of the date palm fruit (*Phoenix dactylifera L.*) – A review. Food Biosci. 34(100509):1-12.

Hwang, S., Shih, P., Yen,G. (2015). Citrus flavonoids and effects in dementia and age-related cognitive decline. Diet Nutr. Dem. Cogn. Decl. 80:869–878.

Hwang, S., Shih, P., Yen, G. (2012). Neuroprotective effects of citrus flavonoids. J. Agric. Food Chem. 60(4):877–885.

Insausti, A.M., Gaztelu, J.M., Gonzalo, L.M., Romero-Vives, M., Barrenechea, C., Felipo, V. (1997). Diet induced hyperammonemia decreases neuronal nuclear size in rat entorhinal cortex. Neurosci. Lett. 231:179–181.

Jelsing, J., Gundersen, H.J.G., Nielsen, R., Hemmingsen, R., Pakkenberg, B. (2006). The postnatal development of cerebellar Purkinje cells in the Göttingen minipig estimated with a new stereological sampling technique – the vertical bar fractionators. J. Anat. 209:321-331.

Jha, A., Saidullah, B., Bubber, P. (2019). A study on prooxidative and neurotoxic effects of mercury chloride in rats. EC Pharmacol. Toxicol. 7(2):112-124.

Jomova, K., Valko, M. (2011). Advances in metal-induced oxidative stress and human disease. Toxicol. 10,283(2-3):65-87.

Kalantaripour, T.P., Asadi-Shekaari, M., Basiri, M., Gholaamhosseinian, A.N. (2012). Cerebroprotective effect of date seed extract (*Phoenix dactylifera*) *on* focal ischemia in male rats. J. Biol. Sci. 12(3):180-185.

Kesner, R.P. (2013). A process analysis of the CA3 subregion of the hippocampus. Frontiers in Cell. Neurosci. 7.78:1-17.

Kesner, R.P., Hunsaker, M.R., Gilbert, P.E. (2005). The role of CA1 in the acquisition of an object-trace-odor paired associate task. Behav. Neurosci. 119:781-786.

Khazdair, M.R., Anaeigoudari, A., Hashemzehi, M., Mohebbati, R. (2019). Neuroprotective potency of some spice herbs, a literature review. J. Trad. Comp. Med. 9:98-105.

Kim, S., Nah, S.Y., Rhim, H. (2008). Neuroprotective effects of ginseng saponins against L-type Ca^{2+} channel-mediated cell death in rat cortical neurons. Biochem. Biophys. Res. Comm. 365(3):399–405.

Kim, W., Kim, D.W., Yoo, D.Y., Jung, H.Y., Kim, J.Y., Kim, D. *et al.* (2015). Antioxidant effects of Dendropanax morbifera Léveille extract in the hippocampus of mercury-exposed rats. BMC Comp. Alt. Med. 15:247.

Komaki, A., Hoseini, F., Shahidi, S., Baharlouei, N. (2015). Study of the effect of extract of Thymus vulgaris on anxiety in male rats. J. Trad. Com. Med.1-5.

Kumar, A., Saini, R.V., Saini, A.K. (2018). Neuroprotective role of ascorbic acid: antioxidant and non-antioxidant functions. Asian J. Pharm. Clin. Res. 11(10):30-33.

Kumar, G.P., Khanum, F. (2012). Neuroprotective potential of phytochemicals. Pharmacogn. Rev. 6(12)81-90.

Liang, X., Hsu, L.M., Lu, H., Ash, J. A., Rapp, P. R., Yang, Y. (2020). Functional connectivity of hippocampal CA3 predicts neurocognitive aging via CA1–frontal circuit. Cerebral Cortex. 30:4297-4305.

Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacog Rev* 2010;4:118-126.

Mohammad, H., Ebrahim, E., Ali, H. (2012). The effect of prenatal exposure to restraint stress on hippocampal granule neurons of adult rat offspring. Iran J. Basic Med. Sci. 15(5):1060-1067.

Mukinda, J.T., Syce, J.A. (2007). Acute and chronic toxicity of the aqueous extract of Artemisia afra in rodents. J. Ethnopharmacol. 112:138-144.

Nadkarni, K.M. (1976). Indian Materia Medica, Vol. 1, Popular Prakashan. Bombay. p. 1175–81.

Oliveira, A.C., Pereira, M.C., Santana, L.N. da S., Fernandes, R.M., Teixeira, F.B., Oliveira, G. B., et al. (2015). Chronic ethanol exposure during adolescence through early adulthood in female rats induces emotional and memory deficits associated with morphological and molecular alterations in hippocampus. J. Psychopharmacol. 29(6):712-724.

Owoeye, O., Farombi, E.O. (2015). Tomato pomace protects against mercuric chloride-induced neurodegeneration and motor abnormality in adult rat. Int. J. Biol. Chem. Sci. 9(3):1142-1153.

Pearse, A., Marks, R. (1974). Measurement of section thickness in quantitative microscopy with special reference to histometry. J. Clin. Path. 27:615.

Phukan, P., Namasudra, S., Bawari, M., Sengupta, M. (2019). Neuroprotective effects of aqueous extract of *Hydrocotyle javanica* in ameliorating neurobehavioral alteration induced by mercury . Asian J. Pharm. Clin. Res.12(1):374-379.

Pujari, R.R., Vyawahare, N.S., Kagathara, V.G. (2011). Evaluation of antioxidant and neuroprotective effect of date palm (*Phoenix dactylifera L*.) against bilateral common carotid artery occlusion in rats. Indian J. Exp. Biol. 49(8):627-633.

Raghu-Jetti, R., Raghuveer, C.V., Mallikarjuna-Rao, C., Somayaji, S.N., Prakash-Babu, B. (2014). Neuroprotective effect of Ascorbic acid and *Ginkgo biloba* against Fluoride caused Neurotoxicity. IOSR JESTFT. 8(1):30-36.

Ramzi, V.K., Stanely, I. (1994). Robbins pathological basis of diseases. 5th ed. Philadelphia, London, Toronto, Monterial. p.183-5.

Ranjan, B., Husain, S.M.D., Kumar, K., Maheshwari, T.P. (2015). Comparative study of histo-pathological effects of mercury on cerebrum, cerebellum and hippocampus of adult albino rats. Ann. Int. Med. & Den. Res. 1(1):21-4.

Ross, R.S., Brown, T.I., Stern, C.E. (2009). The retrieval of learned sequences engages the hippocampus: Evidence from fMRI. Hippocampus.19:790-799.

Ruan, Y.W., Zou, B., Fan, Y., Li, Y., Lin, N., Zhang, Y. *et al.* (2007). Morphological heterogeneity of CA1 pyramidal neurons in response to ischemia. J. Neurosci. Res. 85:193-204.

Saha, S., Barua, B., Sikdar, D. (2017). Phytochemical screening, phenolic content and antioxidant activity of wild date palm (*Phoenix sylvestris* Roxb.) fruit extracted with different solvents. Int. Food Res. J. 24(6):2534-2542.

Sairazi, N.S.M, Sirajudeen, K.N.S. (2020). Natural products and their bioactive compounds: neuroprotective potentials against neurodegenerative diseases. Evidence-Based Comp. Alt. Med. 2020:1-30

Salawu, O.A., Chindo, B.A., Tijani, A.Y., Obidike, I.K., Salawu, T.A., Akingbasote, A.J. (2009). Acute and sub-acute toxicological evaluation of the methanolic stem bark extract of *Crossopteryx febrifuga* in rats. Afr. J. Pharm. Pharmacol. 3(12):621-626.

Sarian, M.N., Ahmed, Q.U., So'ad, S.Z., Alhassan, A.M., Murugesu, S., Perumal, V., Syed- Mohamad, S.N., Khatib, A., Latip, J. (2017). Antioxidant and antidiabetic effects of flavonoids: A structure-activity relationship based study. Biomed. Res. Int. 2017:1-14.

Seidman, R.J.(2011). Normal gross brain and microscopy. Neuropathology, Department of Pathology Stony Brook University.

Seilhean, D., De Girolami, U., Gray, F. (2004). Basic pathology of the central nervous system. In textbook of Escourolle and Poirier Manual of Basic Neuropathology 4th ed. Butterworth Heinemann an imprint of Elsevier, Philadelphia. p.2-4.

Shanker, G., Aschner, J.L., Syversen, T., Aschner, M. (2004). Free radical formation in cerebral cortical astrocytes in culture induced by methylmercury. Brain Res. Mol. Brain Res. 128:48-57.

Sheikh, T.J., Patel, B.J., Joshi, D.V., Patel, R.B., Jegoda, M.D. (2013). Repeated dose oral toxicity of inorganic mercury in Wistar rats: biochemical and morphological alterations. *Vet. World.* 6(8):563-567.

Slomianka, L. (2011). Hippocampal pyramidal cells: the reemergence of cortical lamination. Brain Str. Func. 216(4):301-17.

Snell, R.S. (2010). Introduction and organization of the nervous system. Clinical Neuroanatomy, 7th ed. Philadelphia, Lippincott Williams & Wilkins. 1: p 2.

Suvarna, S.K., Layton, C., Bancroft, J.D. (2019). Stains for Nissl substance; Techniques for staining neurons in Bancroft's Theory and Practice of Histological Techniques. 8th Ed., Elsevier, China. p. 308.

Teleanu, R.L., Chircov, C., Grumezescu, A.M., Volceanov, A., Teleanu, D.M. (2019). Antioxidant therapies for neuroprotection-A Review. J. Clin. Med. 8(1659):1-23.

Trease, G.E., Evans, W.C. (2002). Phytochemicals in: Pharmacognosy. 15th ed. W.B. Sanders Publishers, London.

United Nations Environment Programme, UNEP: Global Mercury Assessment, UNEP Chemicals, Geneva, Switzerland, 2002.

Usama, B., El-Gazzar, A.H., El-Far Hussein, A.A. (2009). The ameliorative effect of *Phoenix dactylifera* extract on CCl_4 hepatotoxicity in New Zealand rabbits. J. Appl. Sci. Res. 5(9):1082-1087.

Using the AmScope Microscope Cameras (2012). Part 1, AmScope Microscope, USA. p. 1–12.

Vyawahare, N., Pujari, R., Khsirsagar, A., Ingawale, D., Patil, M., Kagathara, V. (2009). *Phoenix dactylifera*: An update of its Indegenous uses, phytochemistry and pharmacology. Internet J. Pharmacol. 7:1:1531-2976.

Wan Ismail, W.I., Mohd Radzi, M.N.F. (2013). Evaluation on the benefits of date palm (*Phoenix dactylifera*) to the Brain. Alt. Integ. Med. 2(4):1-3.

Wu, J., Cheng, G., Lu, Z., Wang, M., Tian, J., Bi, Y. (2016). Effects of methyl mercury chloride on rat hippocampus structure. Biol. Trace Elem. Res. 171:124-30.

Xu, F., Farkas, S., Kortbeek, S., Zhang, F., Chen, L., Zamponi, G.W. *et al.* (2012). Mercury-induced toxicity of rat cortical neurons is mediated through N-methyl-D-Aspartate receptors. Mol. Brain. 5:(30)1-14.

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Full Length Research Article

Methanolic extract of *Citrullus lanatus* Seeds Abates Testicular Degeneration and Dose-Dependently Modulates Testicular Function in Hyperlipidemic Male Wistar Rats

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Summary: Hyperlipidemia is emerging as an important cause of adverse health outcomes including cardiovascular complications, obesity, metabolic disorders, and infertility. A total of twenty-five (25) male albino Wistar rats were divided into five groups (n=5): Normal control, Hyperlipidemic control group which was administered (intra-peritoneal) with 0.2ml/10g body weight of egg yolk and then terminated after twenty-four (24) hours, Hyperlipidemic non-treated group which were administered with 0.2ml/10g body weight of egg yolk and were left throughout the treatment period. Hyperlipidemic low-dose treated group (administered 0.2ml/10g body weight of egg yolk, 800mg/kg body weight of methanolic extract of *Citrullus lanatus* seed-MECLS), and Hyperlipidemic high-dose treated group (administered 0.2ml/10g body weight of egg yolk, and 1600mg/kg body weight of MECLS). No significant change was observed in testosterone levels and sperm count across all groups. However, a statistically significant increase (P<0.05) in luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels was observed in Hyperlipidemic non-treated and Hyperlipidemic low-dose treated group when compared to Normal Control Group. They also showed marked testicular damage and significantly decreased (P<0.05) sperm cell motility and significantly increased sperm cell abnormalities. Hyperlipidemic low and high dose groups exhibited moderate and complete regeneration of testicular histo-architecture respectively. Furthermore, high dose treated group showed a significant decrease in sperm count, motility, LH and FSH levels. This study suggests that MECLS dose dependently ameliorates testicular damage induced by hyperlipidemia but may affect sperm cell characteristics.

Keywords: *Hyperlipidemia, Sperm count, testosterone, LH, FSH, Testicular histology*

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INTRODUCTION

Male infertility accounts for 40–50% of the reasons why couples are unable to conceive (Brugh and Lipshultz, 2004). Various abnormal metabolic and disease conditions increase the risk of male infertility (Ventimiglia *et al.,* 2016). Hyperlipidemia, or high cholesterol, means that one or more fat proteins in the blood is elevated beyond normal levels. It is believed that with rapid socioeconomic development, increasing prevalence of sedentary life styles and dietary changes, hyperlipidaemia is emerging as an important cause of adverse health outcomes globally (Zappalla and Gidding, 2009). An increment in circulatory lipids may induce oxidative stress causing significant production of oxygen free radicals, which may lead to oxidative modification in lipoproteins (Mishra et al., 2011) which predisposes individuals to development of type 2 diabetes (Zhou *et al.,* 2014), atherosclerosis (Snehalatha *et al,* 2014), stroke (Djelilovic-Vranic *et al.,* 2013; Tziomalos *et al.,* 2009) and cardiovascular diseases (Vergani and Lucchi, 2012).

Tanaka *et al.,* (2001) have suggested that hypercholesterolemia is an independent risk factor for testicular dysfunction in animal models. Studies have shown its detrimental effect on testicular histology and functions including spermatogenesis and steroidogenesis, epididymal sperm maturation process, sperm quality parameters, sperm fertilizing capacity and fertility index (Ashrafi *et al.,* 2013; Zhang *et al.,* 2012). The exact forms of Cholesterol and mechanisms by which they potentiate this negative effect remain subject to contemplation. Zmuda *et al.,* 1997 reported a negative correlation between testosterone levels and triglycerides levels, Garcia-Cruz *et al*.,(2012) suggests that hyperlipidemic men have significantly lower testosterone levels compared to non-hyperlipidemic controls.

The medicinal value of any plant depends on its chemical composition that elicits a specific physiological action on the human body. Over the years, man has explored the potential of plants for a number of other purposes, hence their dependency on plants increased both directly and indirectly (Ali and Qaiser, 2009). *Citrullus lanatus* (commonly known as water melon) is among the variety of fruits and vegetables consumed globally (Erhardt *et al.,* 2003). Water melon seeds have both nutritional and health importance, the seed contain vitamins B2, minerals (such as magnesium, potassium, phosphorous, sodium, iron, zinc,

manganese and copper), riboflavin, fat, carbohydrates, proteins (Lazos, 1986) and phytochemicals (Braide *et al.*, 2012). Seeds of watermelons are also known to be used in the preparation of snacks, flour sauces, cooking Oil and production of cosmetics (Jensen *et al.*, 2011).

This study was aimed at evaluating the potential effect of methanolic extract of *C. lanatus* (watermelon) seeds (MECLS) on testicular function (sperm profile and tissue histology) and some key reproductive hormones (FSH, LH and Testosterone) in hyperlipidemic animal models.

MATERIALS AND METHODS

Experimental animal: A total of twenty-five male albino Wistar rats weighing between 140 -180g was obtained from the animal house of the Faculty of Basic Medical Sciences, Alex Ekwueme Federal University Ndufu Alike Ikwo, (AE-FUNAI) for the experiment. The rats were acclimatized for two weeks before the experiment commenced. The rats were fed normal rat's pellets and water ad libitum and the animals were kept under standard conditions. The animal room was properly ventilated with a temperature range of 27-29^{0}C under 12hours day/light photoperiod regimen.

Plant Material: The seeds were obtained from 30 fruits *of citrullus lanatus* (watermelon) from a local market in Ikwo, Ebonyi State. The plant was authenticated by plant taxonomist from Department of Biology, Faculty of Science, AE-FUNAI. The flesh was removed and the seed collected was washed, sun-dried and milled into fine powder.

Preparation of Extracts: The fruits were thoroughly washed and cut open in order to get the seeds, then seeds were air-dried for three weeks after which it was weighed and the weight was 1kg;. Then pulverized using mortar and pestle under aseptic conditions and ground to powder using a blender (waring commercial blender, model no. HGB2WTS3). The method of extraction employed was percolation. After grinding, the powdered form of the seeds weighed 0.9kg which was soaked into 1500ml of methanol in air tight container kept at room temperature for a period of three days. After three days, it was filtered using cheese cloth in order to remove the chaff after which the filtrate was evaporated using evaporator water bath machine. The extract was constituted using normal saline as a vehicle where by 20g of the extract was dissolved 100ml of the vehicle and 30g of the extract was dissolved in 100ml of the vehicle for low and high dosage respectively.

Experimental Design: Twenty-five (25) male albino Wistar rats was randomly selected and divided into five (5) groups, consisting of five (5) rats per group viz: Control group**.,** Hyperlipidemic control group (Sacrificed 24 hours after inducing hyperlipidemia), hyperlipidemic untreated group (left for the duration of treatment after inducing hyperlipidemia)**,** Hyperlipidemic low-dose treated group: (received 800mg/kg body weight of methanolic extract of *Citrullus lanatus* seed (MECLS) for twenty-one days) and Hyperlipidemic high-dose treated group: received 600mg/kg body weight of (MECLS) for twenty-one days. All rats were allowed free access to drinking water.

Inducing hyperlipidemia: Hyperlipidemia was induced by intra-peritoneal administration of egg yolk (0.2ml/10g body weight) for a day (Song *et al,.* 2013 and Anuwat *et al.*, 2017). After which the HCG were sacrificed after 12 hours fasting period to ascertain the attainment of hyperlipidemia.

Experimental Procedure: The treatment regimen was done through oral gavage. After which blood sample was collected from the animals from all groups via retro-orbital puncture using heparinized capillary tubes.

Laboratory Analysis:

Serum lipid profile: Total cholesterol and triglyceride level was estimated using Enzymatic Method (Allain *et al.*, 1974). Lipoprotein analysis was estimated using the Direct method (Sugiuchi *et al.*, 1995; Rifai *et al.*, 1992).

Hormonal Assay: An enzyme-based immunoassay system was used to measure testosterone, FSH and LH levels in serum samples collected. Blood serum was introduced into micro–plate well for each sample to be measured. Thereafter, an enzyme antigen linked conjugate for each hormone was added respectively and then incubated for 1 h at room temperature. The plate was then washed with micro plate washer to remove all unbound material. After washing, excess fluids were taped off. Then color was developed by adding color reagent to determine the bound hormone. Quantitative test result was obtained by measuring the absorbance. The color intensity was checked by tasking the ELISA plate to an ELISA reader which is attached to spectrophotometer that read the absorbance.

Testicular histology: The testes were fixed in 10% Formalin, after complete fixation the blocks was embedded in paraffin and sections cut at 5mm (micron) using a microtome and then stained with hematoxylin and eosin and mounted. Microscopic examination of the sections was then carried out under a light microscope and later the microscopic slides of the testes were photographed at magnification ×400.

Sperm profile: Sperm morphology was investigated using a microscope, Sperm motility was evaluated by an earlier method by saalu *et al.*, 2010. Sperm count was done using methods by Neubauer's counting chamber method.

RESULTS

Lipid profile: High cholesterol intake is known to induce hyperlipidemia. As shown in Table 1, we recorded a significant increase of total cholesterol, triglyceride, low density lipoprotein, and a significant decrease (P<0.05) in HDL in hyperlipidemic control group when compared to other groups. This finding demonstrates that intra-peritoneal administration of egg yolk induced hyperlipidemia. This is consistent with other studies by Pashaie *et al.*, 2017 and Sumbul and Ahmed, 2012. Results from Table 1 although shows that over time, there was a significant recovery. With cholesterol levels returning back to normal in hyperlipidemic non-treated group. Subsequent findings however convey the fact that despite this recovery, significant alterations in reproductive parameters were still persistent

Table 1
Lipid profile of the various groups.

Lipid profile	Normal control group (*n*=5)	Hyperlipidemic control group (*n*=5)	Hyperlipidemic non-treated group (*n*=5)	Hyperlipidemic low-dose treated group (*n*=5)	Hyperlipidemic high-dose treated group (*n*=5)	*P*- value
Total cholesterol (mmol/l)	1.20±0.01	4.44 ± 0.21*	1.44 ± 0.05	1.08± 0.03	1.45± 0.02	<0.001*
Triglyceride (mmol/l)	0.45± 0.01	4.03± 0.11*	0.53±0.05	0.36± 0.01	0.53± 0.08	<0.001*
HDL (mmol/l)	1.13± 0.02	0.43± 0.12*	0.86± 0.02	1.00± 0.11	1.05± 0.01	<0.001*
LDL (mmol/l)	0.31± 0.01	1.58± 0.58*	0.32± 0.04	0.19± 0.01	0.29± 0.02	<0.006*

mmol/l = milimole per litre. HDL= High density lipoproteins, LDL = low density lipoproteins. Values are mean ± SEM, (n=5). One way ANOVA followed by Newman-Keuls Multiple Comparison Test was used for statistical significance. "" denotes statistically significant compared to other groups (P < 0.05).*

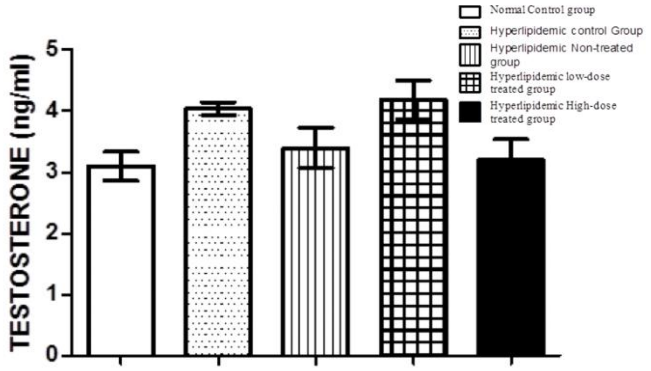
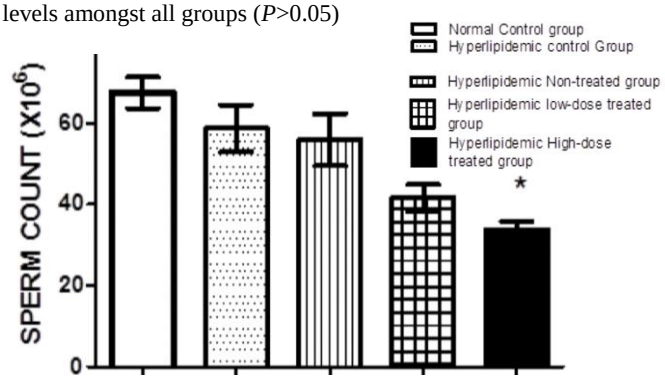


Figure 1:
A comparison of Testosterone levels among the various groups. There was no statistical significance difference in testosterone levels amongst all groups (*P*>0.05)



Testosterone Levels: Figure 1 shows that there was no significant change in testosterone levels among the various group. It remained steady both in hyperlipidemic rats and hyperlipidemic treated rats.

Sperm Count: We observed a significant decrease (*P*>0.05) in sperm cell count in hyperlipidemic high-dose treated group when compared to hyperlipidemic control and hyperlipidemic non-treated groups (Figure 2).

Mid-piece Abnormality: We observed statistically significant increase (*P*<0.05) in sperm cell with mid-piece abnormality in hyperlipidemic non-treated group and hyperlipidemic low-dose treated group when compared to normal control group and hyperlipidemic control group. (Figure 3).

Active Motility: Figure 4 shows a statistically significant decrease (*P*<0.05) in sperm cell with active motility in hyperlipidemic control group when compared to normal control and hyperlipidemic control group. There was also a significant decrease (*P*<0.05) in hyperlipidemic high-dose treated group when compared to other groups.

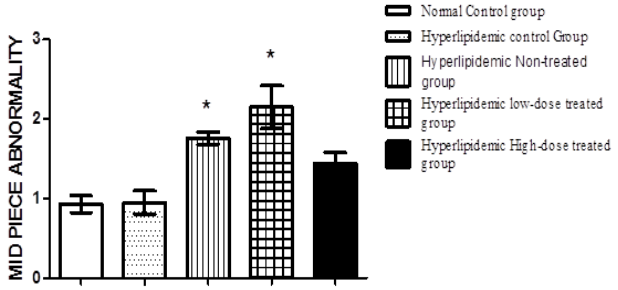


Figure 3:
Comparison of sperm cell with mid-piece abnormalities. *= statistically significant difference amongst the various groups (*P*<0.05).

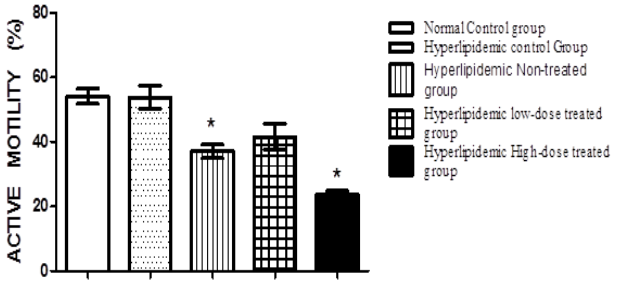


Figure 4:
Comparison of sperm cells with active motility. * = Significant decrease (*P*<0.05).

Luteinizing Hormone: Results from this study (Figure 5) shows that there was a significant increase in LH levels in Hyperlipidemic non-treated group and Hyperlipidemic low-dose treatment group when compared to another group.

Follicle Stimulating Hormone: A statistically significant increase (*P*<0.05) in FSH levels in Hyperlipidemic control group, Hyperlipidemic non-treated group and Hyperlipidemic low-dose treated group when compared to normal control group. A statistically significant decrease (*P*<0.05) in FSH levels in hyperlipidemic high-dose treated group when compared to Hyperlipidemic control group, Hyperlipidemic non-treated group and Hyperlipidemic low-dose treated group.

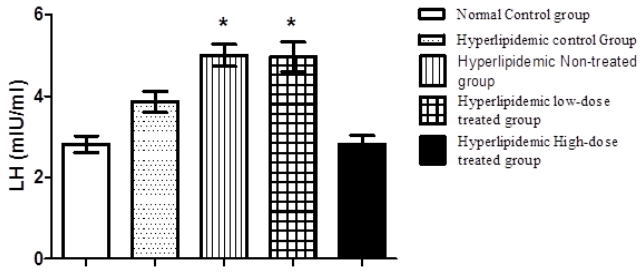


Figure 5:
Effect of *Citrullus lanatus* seed on the luteinizing hormone (LH) level of the groups. * = significant difference, which means that there is a significant increase (P<0.05).

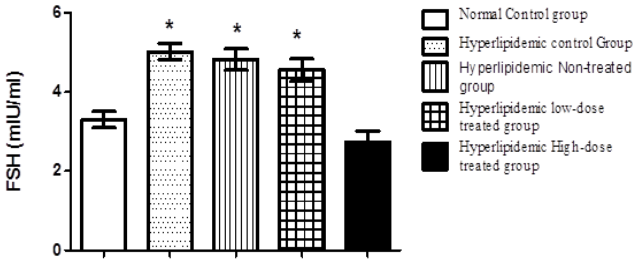


Figure 6:
Comparison of the FSH levels amongst the various groups *= statistically significant difference in that group (P<0.05).

Histological Findings:

Normal control group: Light microscopic examination of the testis showed normal testicular architecture with seminiferous tubule (ST) that are lined with interstitial cells of leydig (ICL), sertoli cell (SC) and well enhanced spermatogenesis (WES). The overall feature appears normal.

Hyperlipidemic control group: Light microscopic examination of the testis showed moderate to severe degeneration with moderate arrest of the spermatogenesis (MAS), focal area of hemorrhage (FAH)within the lumen and moderate apoptosis of the interstitial cell of leydig (MAICL).

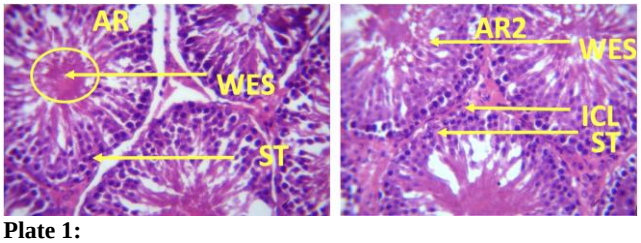


Plate 1:
Photomicrograph of section of testis (x400)(H/E) for normal control group.

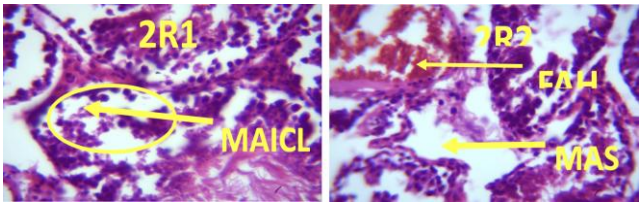


Plate 2:
Photomicrograph of section of testis of hyperlipidemic control groups (x400) (H/E).

Hyperlipidemic non-treated group: Light microscopic examination of the testis showed severe degeneration with severe arrest of the spermatogenesis (SAS), focal area of necrosis (FAN), moderate aggregate of inflammatory cell in the background of the necrotic area coupled with moderate apoptosis of the interstitial cell of leydig (MAICL).

Hyperlipidemic non-treated group: Light microscopic examination of the testis showed severe degeneration with severe arrest of the spermatogenesis (SAS), focal area of necrosis (FAN), moderate aggregate of inflammatory cell in the background of the necrotic area coupled with moderate apoptosis of the interstitial cell of leydig (MAICL).

Hyperlipidemic low-dose treated group: Shows moderate regeneration with moderate enhanced spermatogenesis (MES) in R2. Mild distortion of the testicular architecture (MDTA) and moderate apoptosis of the interstitial cell of leydig (MAICL) in R1.

Hyperlipidemic high-dose treated group: Shows a well regenerated testicular tissue coupled with well-established spermatogenesis (WES) and mild focal area of inflammation (MFAI) otherwise normal.

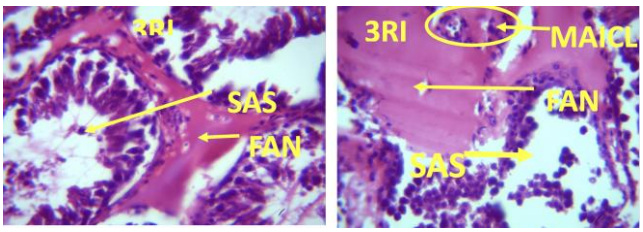


Plate 3
Photomicrograph of section of testis hyperlipidemic non-treated group. (x400) (H/E).

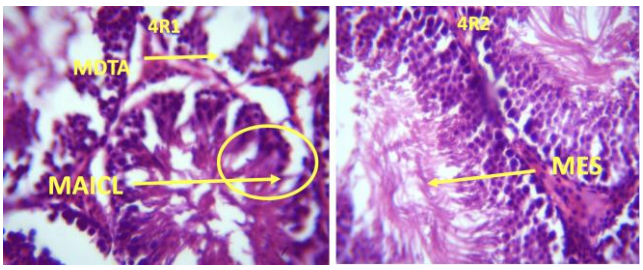


Plate 4
Photomicrograph of section of testis of hyperlipidemic low-dose treated group (x40o)(H/E).

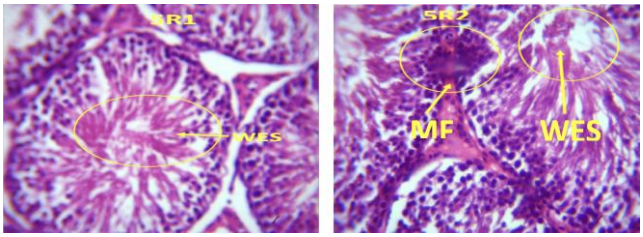


Plate 5
Photomicrograph of section of testis of hyperlipidemic group treated with a high dose of MECLS).(x400)(H/E).

DISCUSSION

It is well established that hypercholesterolemia induces reproductive and testicular damage by increasing lipid peroxidation and excessive generation of free radicals and increased oxidative stress, which are cytotoxic to spermatozoa (Bashhandy, 2007). Testicular histo-pathological reports from this study reveals that there was a severe degeneration with marked cell inflammation, necrosis, moderate apoptosis of interstitial cell of leydig and severe arrest of the spermatogenesis in Hyperlipidemic control group and Hyperlipidemic non-treated group when compared to Normal control group These findings are similar to those of Zhang *et al.,* 2012 who reported degenerative changes in testicular tissues in hyperlipidemic rats.

Sperm parameters such as count, motility and morphology are reportedly key indices of male fertility; being fair markers of spermatogenesis and epididymal maturation of spermatocytes (Morakinyo, 2010). There are valid evidences that hyperlipidemia negatively affects sperm cell structure and motility (Yamamoto *et al.,* 1999; Bashandy, 2007; Ghanayem *et al.,* 2010). Comparably, in this study we observed that over time, hyperlipidemia seem to induce morphological abnormalities in spermatocyte as a significant increase in sperm cells with mid-piece abnormalities in Hyperlipidemic non-treated group when compared to Normal control group and Hyperlipidemic control group. The mid-piece regions of sperm cells are known to contain numerous amounts of mitochondria which provide energy for motility (Mukai and Travis, 2012). Furthermore, a proportionate significant decrease in sperm cell motility was seen in Hyperlipidemic non-treated group. This decrease could thus be a complementary effect of the structural abnormality in mid piece section.

Lipid peroxidation is an important factor that may induce morphological changes in the spermatozoa (Sanchez *et al.,* 2006), however, Various testicular mechanisms involved in lipid homeostasis in male reproductive tract (Maqdas *et al.,* 2013; Lobaccaro *et al.,* 2012). From this study, hyperlipidemia had no significant effect on testosterone levels and sperm count. These findings with those of Zarei *et al.,* 2014 and Saez *et al.,* 2010.

The production of male gametes depends on the concerted action of the two gonadotropins FSH and LH on the testis (Simon *et al*., 1999). LH plays an important role in regulating testosterone secretion by the interstitial cells of the testes and FSH helps in increases germ cell proliferation (Vihko *et al.,* 1991; McLachlan *et al.,* 1995). There was a significant increase in LH and FSH levels in hyperlipidemic non-treated group and low dose treatment group when compared to normal control group. This increase may perhaps be due to the endocrine systems response to testicular damage induced by hyperlipidemia, which successively may have bolstered testosterone levels and sperm count from declining. A surge in LH elicits a proportionate increase in testosterone (O'Donnell *et al.,* 2006). However, the surge in LH observed in the hyperlipidemic non-treated group didn't potentiate a corresponding increase in testosterone levels. There is a possibility that hyperlipidemia may have activated some regulatory mechanisms such as the unique rennin-angiotensin system localized in the testis which stabilizes steroidogenesis and consequently limiting the increase in testosterone production (Martinez-mortos *et al.,* 2011).

Methanolic extract of *C. Lanatus* seed (MECLS) is known to improve testicular function and morphology (Kolawole *et al.,* 2014). Histo-pathological studies as shown in Figure 10, are affirmative that mild dose (800mg/kg bw) of MECLS to an extent, ameliorated structural damages on testicular tissues induced by hyperlipidemia. This was reflective in hyperlipidemic low-dose treated group as moderate regeneration with moderate enhanced spermatogenesis, mild distortion of the testicular architecture and moderate apoptosis of the interstitial cell of the testis. However, these ameliorating effects wasn't reflected in testicular function because there was no significant change in sperm count. Also, the increase in sperm cells with mid-piece abnormality in hyperlipidemic non-treated group was persistent in hyperlipidemic low-dose treated group when compared to Normal Control Group (NCG) and Hyperlipidemic Control Group (HCG),

Methanolic extract of *C.Lanatus* seed had earlier been reported to increase LH and FSH levels (Kolawole *et al*., 2014), results from this study is in tandem with prior reports as a sustained significant increase in LH and FSH levels was observed in hyperlipidemic low-dose treatment group when compared to NCG and HCG. This sustenance could be as a result of the physiological compensatory feedback mechanism ensuring steady levels of testosterone and sperm count.

Furthermore, Amedu and Idoko (2016) had earlier reported that Methanolic extract of C. Lanatus improves sperm count and increases FSH and LH secretion. From this study, in the hyperlipidemic, high-dose treated group, there was regeneration of testicular tissue coupled with well-established spermatogenesis. However, there was an observable significant decrease for FSH levels, LH levels and sperm count in hyperlipidemic high-dose treated group when compared to Hyperlipidemic low-dose, Hyperlipidemic non-treated and hyperlipidemic control groups. This decrease suggests a possibility of a negative (inhibitory) effect of a high dose of methanolic extract of *C. Lanauis* seed on the anterior pituitary which inhibits the release of both FSH and LH.

In conclusion, hyperlipidemia induced an increase in FSH and LH levels without affecting sperm count and testosterone levels respectively, but decreased sperm quality via its cytotoxic effect on testicular tissues. Mild dose MECLS seems beneficial in repairing damages induced by hyperlipidemia on testicular tissues. However, a high dose of MECLS (despite provoking testicular rejuvenation), has an inhibitory effect on gonadotropic hormones and testicular function.

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REFERENCES

Ali, H., Qaiser, M., (2009). The Ethnobotany of Chitral Valley, Pakistan with Ali SI Significance of Flora with Special to Reference *Pakistan. Pak.J.Bot*.**41 (4)**: 2009-2041.

Allain, C.C., Pon, L.S., Chan, C.S.G., Richmond, W., (1974). Enzymatic determination of total cholesterol. *Clinical Chemistry*, **20**: 470-475.

Amedu, N.O., Idoko, U.P., (2016). Dietary Consumption of Citrullus lanatus can Ameliorate Infertility Potential of Carica papaya Seeds Extract in Male Rats. *British Biotechnology Journal*, 11(2): 1-9.

Anawat, T., Kanokporn, S., Supap, S., (2017). Hypoglycemic and hypolipidemic properties of herbal tea on Wistar rat. *Chiang Mai Veterinary Journal*.**15(1):** 25-35.

Ashrafi, H., Ghabili, K., Alihemmati, A., Jouyban, A., Shoja, M.M., (2013). The effect of quince leaf (Cydonia oblonga Miller) decoction on testes in hypercholesterolemic rabbits: a pilot study. *Afr J Tradit Complement Altern Med*. **10**: 277-282.

Bashandy, A.E.S., (2007). Effect of fixed oil of nigella sativa on male fertility in normal and hyperlipidemic rats. *Int J Pharmacol*. **3**: 27-33.

Bashandy, A.E.S., (2007). Effect of fixed oil of nigella sativa on male fertility in normal and hyperlipidemic rats. *Int J Pharmacol*. 3: 27-33.

Braide, W., Odiong, I. J., Oranusi, S., (2012). Phytochemical and Antibacterial properties of the seed of watermelon (Citrullus lanatus). *Prime Journal of Microbiology Research.* **2**(3): 99-104.

Brugh, V. M., Lipshultz, L. I., (2004). Male factor infertility. *Medicinal clinics of North America*. **88** (2): 367-85.

Djelilovic-Vranic, J., Alajbegovic, A., Zelija-Asimi, V., Niksic, M., Tiric-Campara, M., Salcic, S., Celo, A., (2013). Predilection role diabetes mellitus and dyslipidemia in the onset of ischemic stroke. *Med.Arch.*. **67**: 120–123.

Erhadt, J.G., Meisner, C., Bode, C., (2003). Lycopene, Beta carotene and colorectal Adenomas. *American Journal Of Clinical Nutrition*. **78**: 1219 – 1224.

Garcia-Cruz, E., Piqueras, M., Huguet, J., Gosalbez, D., Peri, L., Izquierdo, L., (2012). Hypertension, dyslipidemia and overweight are related to lower testosterone levels in a cohort of men undergoing prostate biopsy. *Int J Impot Res.* **24**(3):110-113.

Ghanayem, B.I., Bai, R., Kissling, G.E., Travlos, G., Hoffler, U., (2010). Diet-induced obesity in male mice is associated with reduced fertility and potentiation of acrylamide-induced reproductive toxicity. *Biol Reprod.* 82: 96-104.

Jensen, B.D., Toure, F.M., Hamattal, M.A., Toure, F.A., Nantoumé, D.A., 2011. Watermelons in the Sand of Sahara: Cultivation and use of indigenous landraces in the Tombouctou Region of Mali. *Ethnobotany Research and Applications.* **9**: 151-162.

Kolawole, T. A., Dapper, D. V., Ojeka, S. O., (2014). Ameliorative Effects of the Methanolic Extract of the Rind of Citrullus lanatus on Lead Acetate Induced Toxicity on Semen Parameters and reproductive Hormones of Male Albino Wistar Rats. *European Journal of Medicinal Plants.* 4(9): 1125-1137.

Lazos, E. S., (1986). Nutritional, fatty acid and oil characteristics of pumpkin and melon, Lipid concentrations and semen quality. *The LIFE study Andrology* .**2**: 408-415.

Lobaccaro, J.M.A., Brugnon, F., Volle, D.H. Baron, S., (2012). Lipid metabolism and infertility: is there a link? *Clin Lipidol*. **7**: 485-488.

Maqdas, S., Baptissart, M., Vega, A., Baron, S. Lobaccaro, J.M., (2013). Cholesterol and male fertility: what about orphans and adopted? *Mol Cell Endocrinol* .**368**: 30-46.

Martínez-Martos, J.M., Arrazola, M., Mayas, M.D., Carrera-González, M.P., García, M.J., (2011). Diet-induced hypercholesterolemia impaired testicular steroidogenesis in mice through the renin-angiotensin system. *Gen Comp Endocrinol*. **173**: 15-19.

McLachlan, R.I., Wreford, N.G., de Kretser, D.M., Robertson, D.M., (1995). The effects of recombinant follicle-stimulating hormone on the restoration of spermatogenesis in the gonadotropin-releasing hormone-immunized adult rat. *Endocrinology*. **136(9):** 4035-43.

Mishra, P. R ., Panda, P.K., Apanna, K.C. Panigrahi, S., (2011). Evaluation of acute hypolipidemic activity of different plant extracts in Triton WR-1339 induced hyperlipidemia in albino rats. *Pharmacologyonline*. **3**: 925-934.

Morakinyo, A.O, Achema, P.U., Adegoke O.A., (2010). Effect of Zingiber Officinale (Ginger) on Sodium Arsenite Induced Reproductive Toxicity in Male Rats. *Afr. J. Biomed. Res.* **13 (1):** 39 – 45.

Mukai, C., Travis, A.J., (2012). What Sperm Can Teach us About Energy Production. *Reprod Domest Anim.* **47(4):** 164–169.

O'Donnell, L., Meachem, S.J., Stanton, P.G., McLachlan, R.I., (2006). Endocrine regulation of spermatogenesis. In: Neill JD editor, editor; ; Plant TM, editor; , Pfaff DW, editor; , Challis JRG, editor; , de Kretser DM, editor; , Richards JS, editor; , Wassarman PM, editor. , section editors. Knobil and Neill's Physiology of Reproduction. Sydney, Australia: Elsevier. p. 1017-70

Pashaie, B, Hobbenaghi, R., Malekinejad, H., (2017). Anti-atherosclerotic effect of Cynodon dactylon extract on experimentally induced hypercholesterolemia in rats. *Vet Res Forum*. **8(3):**185–193.

Rifai, N, Iannotti, E, DeAngelis, K., Law, T., (1998). Analytical and clinical performance of a homogeneous enzymatic LDL-cholesterol assay compared with the ultracentrifugation-dextran sulfate-Mg2+ method. *Clin Chem*: 44: 1242-1250.

Saalu, L.C., Kpela, T., Benebo, A.S., Oyewopo, A.O., Anifowope, E.O., Oguntola, J.A., (2010). The dose-dependent testiculoprotective and testiculotoxic potentials of Telfairia occidentalis Hook f. leaves extract in rat. *Int. J. Applied Res. Nat. Prod*., **3**: 27-38.

Saez-Lancellotti, T.E., Boarelli, P.V., Monclus, M.A., Cabrillana, M.E., Clementi, M.A., (2010). Hypercholesterolemia impaired sperm functionality in rabbits. PLoS One 5: e13457.

Simoni, M., Weinbauer, G.F., Gromoll, J., Nieschlag, E., (1999). Role of FSH in male gonadal function. *Ann Endocrinol (Paris).* 60(2):102-6.

Snehalatha, C., Nanditha, A., Shetty, A.S., Ramachandran, A., (2011). Hypertriglyceridaemia either in isolation or in combination with abdominal obesity is strongly associated with atherogenic dyslipidaemia in Asian Indians. *Diabetes Res. Clin. Pract*.. **94**: 140–145.

Song L., Dong L., Bo H., Yuxin C., Xiaocong L., Youwei, W., (2013). Inhibition of pancreatic lipase, α-glucosidase, α-amylase, and hypolipidemic effects of the total flavonoids from Nelumbonucifera leaves. *J. Ethnopharmacol*. **149(1):** 263–269.

Sugiuchi, H., Irie, T., Uji, Y., Ueno, T., Chaen, T., Uekama K,, (1998). Homogenous assay for measuring low-density lipoprotein cholesterol in serum with triblock copolymer and α-cyclodextrin sulfate. *Clin Chem.* 44:522-531.

Sumbul, S., Ahmed, S.I., (2012). Anti-hyperlipidemic Activity of Carissa carandas (Auct.) Leaves Extract in Egg Yolk Induced Hyperlipidemic Rats. *J Basic Appl Sci*. **8:** 124-134.

Tanaka, M., Nakaya, S., Kumai, T., Watanabe, M., Matsumoto, N., (2001). Impaired testicular function in rats with diet-induced hypercholesterolemia and/ or streptozotocin-induced diabetes mellitus. *Endocr Res*. **27**: 109-117.

Tziomalos, K., Athyros, V.G., Karagiannis, A. Mikhailidis, D.P., (2009). Dyslipidemia as a risk factor for ischemic stroke. *Curr. Top. Med. Chem*.**9**: 1291–1297.

Ventimiglia, E., Capogrosso, P., Colicchia, M., Boeri, L., Serino, A. and Castagna, G., (2016). Metabolic syndrome in white European men presenting for primary couple's infertility: investigation of the clinical and reproductive burden. *Andrology.* **4**(5):944-951.

Vergani, C., Lucchi, T., (2012). Plasma HDL cholesterol and risk of myocardial infarction. *Lancet.* **380**: 1989–1990.

Vihko, K.K., LaPolt, P.S., Nishimori, K., Hsueh, A.J., (1991). Stimulatory effects of recombinant follicle-stimulating hormone on Leydig cell function and spermatogenesis in immature hypophysectomized rats. *Endocrinology*. **129:**1926-32.

Yamamoto, Y., Shimamoto, K., Sofikitis, N., Miyagawa, I., (1999). Effects of hypercholesterolaemia on Leydig and Sertoli cell secretory function and the overall sperm fertilizing capacity in the rabbit. *Hum Reprod.* **14:** 1516-1521.

Zappalla, F.R., Gidding, S.S., (2009). Lipid management in children. *Endocrinol Metab Clin North Am*. **38**: 171-183.

Zarei, A., Ashtiyani, S.C., Vaezi, G.H., (2014). A study on the effects of the hydroalcholic extract of the aerial parts of Alhagi camelorum on prolactin and pituitary-gonadal activity in rats with hypercholesterolemia. *Arch Ital Urol Androl.* **86:** 188-192.

Zhang, K., Lv, Z., Jia, X., Huang, D., (2012). Melatonin prevents testicular damage in hyperlipidaemic mice. *Andrologia*. **44**: 230-236.

Zhou, X., Zhang, W., Liu, X., Li, Y., (2014). Interrelationship between diabetes and periodontitis: Role of hyperlipidemia. *Arch. Oral. Biol*.**60**:667–674.

Zmuda, J.M., Cauley, J.A., Kriska, A., (1997). Longitudinal relation between endogenous testosterone and cardiovascular disease risk factors in middle aged men. A 13-year follow-up of former Multiple Risk Factor Intervention Trial participants. *Am J Epidemiol.* **146**: 609-617.

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Full Length Research Article

Anti-Hyperlipidemic and Antioxidant Potentials of Aqueous Leaf Extract of *Telfairia occidentalis* (Hook. F.) in Male Sprague-Dawley Rats

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Summary: In Africa traditional medicine, certain plant leaves are employed in the treatment of metabolic disorders such as dyslipidaemia. *Telfairia occidentalis* is named among Nigerian plants that are under investigation for anti-hyperlipidemic activity. The antihperlipidemic and antioxidant potentials of *Telfairia occidentalis (TO)* aqueous leaf extract were studied in male Sprague- Dawley rats. Twenty-four healthy male Sprague-Dawley rats were grouped into four of six rats thus: Group A (control) received normal saline (10mg/Kg); treated groups B, C and D, received, 50mg/kg; 100mg/kg; and 150mg/kg of *Telfairia occidentalis* aqueous leaf extract for 14 days respectively. At the end of the experiment, serum cholesterol (CHOL), triglyceride (TG), high density lipoprotein (HDL) and low-density lipoprotein (LDL), aspartate amino transferase (AST), alanine amino transferase (ALT), Alkaline phosphatase (ALP) wer assessed. Serum level of creatinine was determined and antioxidants such as reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were investigated. 'Malonaldehyde (MDA) level was also assessed. Results from the current study showed a significant decrease in CHOL and LDL levels at all the doses tested compared with control. *Telfairia occidentalis produced a significant increase in HDL level in all the tested doses compared with control* However, TG was significantly decreased in groups C and D compared with control. *Telfairia occidentalis* aqueous leaf extract produced a significant decrease in AST level in all the tested doses compared with control. ALT level significantly decreased in groups C and D 100mg/kg and 150mg/kg doses compared with control while ALP level significantly increased in all the doses tested compared with control. Creatinine level was significantly decrease in groups B and C compared with control. Results from the antioxidant analysis revealed a significant increase in SOD, GSH and CAT with concomitant reduction in MDA level in all the doses tested MDA's lipid peroxidation when compared with control. The current findings revealed that *Telfairia occidentalis* aqueous leaf extract possesses anti-hyperlipidemic, hepatoprotective effects and improved oxidative balance. However, care has to be taken during its use as it has ability to elevate LDL and activities of liver enzymes at higher dose which may be deleterious to the body system.

Keywords: *Antioxidant, cholesterol, Liver, protein, Sprague-Dawley, fluted pumpkin*

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INTRODUCTION

The use of plants in traditional medicine referred to as herbalism or botanical medicine (Evans, 2002) falls outside the mainstream of the Western or Orthodox medicine. It has been estimated that about 75% of the world's population (mainly in the developing countries) rely on traditional medicine as their primary form of health care (Sumner, 2000). The use of traditional medicine in the treatment and management of diseases in the Africa cannot fade away and this could be attributed to the socio-cultural, socio-economic, lack of basic health care and qualified personnel (Eujoba *et al*., 2000).

Plants contain active components such as flavonoids, glycosides, saponins, tannins, etc., which possess medicinal properties that are harnessed for the treatment of different diseases (Chevalier, 2000). The active ingredients for a vast number of pharmaceutically -derived medications contain components originating from phytochemicals. These active substances that contain the healing property are known as the active principles and differ from plant to plant.

Telfairia occidentalis is an edible vegetable plant that belongs to the family Cucurbitaceae. It is a tropical vine grown mainly in West Africa for its vegetable (Akoroda, 1990). In Nigeria, it is known locally as 'Ubong' by Ibibios, 'Ugu' by Igbos and 'Iroko' by Yorubas. The Ghanians refer to it as 'okrobonka' while to the Sierra- Leoneans, it is known as 'Oroko' (Abiose, 1990). The concentration of photosynthesis in leaves makes them rich in protein, minerals and sugar because of their nutritional value leaves is prominent in the diet of many animals, including humans as leaf vegetables (Leaf, 2011). The leaf has high nutritional, medicinal and industrial values being rich in protein 29%, fat 18%, minerals and vitamins 20% (Ndor *et al*., 20130. The aqueous extract of *T. occidentalis* has been shown to be

hepatoprotective against garlic-induced oxidative stress (Olorunfemi *et al*., 2005, Oboh *et al*., 2007) while both aqueous and ethanolic extracts have demonstrated hypoglycaemic properties both in normoglycaemic and alloxan-induced diabetic rats (Zhang and Yao, 2002). The haematinic capacity of this plant fresh leaf concoction has a high-value health tonic for impotent men with a cheap and fast remedy for acute anemia (Kayode and Kayode, 2011 Nwozo *et al*., 2004). It was revealed according to Veral *et al*., (2014) that the fluted pumpkin seed shell served majorly as a source of dietary fibre. Also, most cultures consider fruit shells as waste in the strictest sense and therefore avoid their use even in earthnomedicine (Verla *et al.*, 2012). The seeds are cooked and eaten like beans as the shoots and leaves while eaten like vegetables. The leaves contain vitamins and minerals the body needs to stay healthy. The leaves are also a good source of iron (*Telfairia occidentalis* , 2009).

Telfairia occidentalis is a very good vegetable plant, popularly known in Nigeria for its ornamental purposes. It is hard to believe that anyone would ever think of consuming its leaves as a meal. Although, there are claims concerning the medicinal/health benefits of the leaves hidden from the consumers. Moreover, *Telfairia occidentalis* is going to extinction in most countries especially in Nigeria, where limited research work has been done to verify the claims concerning the nutritional-health values. However, in the current study the anti-hperlipidaemic, hepatic and antioxidant effects of *Telfairia occidentalis* in male Sprague-Dawley rats were investigated.

MATERIALS AND METHODS

Plant collection and extraction: *Telfairia occidentalis leaves* were harvested from Itori Ewekoro Local Government Area of Ogun State, Nigeria and the collected samples were identified at FRIN Ibadan Oyo state, Nigeria. The fresh leaves of *Telfairia occidentalis* were air dried until constant weight of 250g was. The dried leaves were powdered using mortar and pestle. 250g of powdered leaves were macerated using distilled water for 48 hours. The extract was filtered and evaporated at 40ºc under reduced pressure. The yield of the dark browned colored dried extract obtained was 38.5g and the weighed extract was stored at 4ºc until use in which case distilled water was used for reconstitution immediately and was given orally to the experimental animals.

Animals: Twenty-four male Sprague-Dawley rats weighing between 150-200g were obtained from the animal house of the College of Medicine of the University of Lagos. They were kept in well-ventilated, hygienic compartments maintained under standard environmental conditions, acclimatized for three weeks before the experiment. They were fed with standard rodent diet and water ad libitum. The experimental procedures used were in accordance with the provisions of the Experimentation Ethics Committee on Animals Use of the College of Medicine of the University of Lagos, Lagos State and the United States National Academy of Sciences Guide for the Care and Use of Laboratory Animals (2011).

Phytochemical screening: The phytochemical screening of the plant was carried out on dried sample as described by Harbone, (1973) to identify the active components present in *Telfairia occidentalis*

Acute Toxicity test (LD_{50}): The acute toxicity test was carried out as described by Lorke (1983).

Animal treatment and experimental groups: After successful acute toxicity test, the extract was given orally to the rats for two weeks as follows: Group A (Control, 10mg/kg of normal saline), group B (50mg/kg of the extract), group C (100mg/kg of the extract) and group D (150mg/kg of the extract).

Collection of blood sample: Five (5ml) of blood sample was taken by retro-orbital puncture. Blood was allowed to clot for 1 hour at 4^0C then centrifuged at 3,000 rpm for 10 minutes and the serum samples were kept at -20^0C until assayed (Morakinyo *et al*., 2018)

Blood lipids: Serum and liver homogenate lipid levels of triglycerides (TG), Cholesterol (CHOL), low density lipoprotein (LDL), and high density lipoprotein (HDL) after treatment were determined by automatic biochemistry analyzer (Mindray BS-120, Chema Diagnostica, Italy) using diagnostic kits for each, purchased from BioSystems® (S.A Costa Brava of Barcelona, Spain).

Liver and kidney functions: Albumin, alkaline phosphatase (ALP), alkaline amino transferase (ALT) and aspartate amino transferase (AST) were determined using serum samples by an automated analyzer (Mindray BS-120, Chema Diagnostica, Italy). The machine was equally used for the determination of creatinine.

Antioxidant studies

Determination of superoxide dismutase (SOD) activity: Briefly; SOD activity was measured by the inhibition autoxidative capacity of pyrogallol. The SOD activity was evaluated using a spectrophotometer at 420 nm. A calibration curve was constructed using SOD as standard. A 50% inhibition of autoxidation of pyrogallol was defined as one SOD unit (DinizVilela *et al*., 2016)

Determination of reduced glutathione (GSH) activity: The protein content of the samples was initially precipitated by metaphosphoric acid (MPA) at the ratio of 1:1 (homogenate/MPA). The samples were centrifuged at 3000rpm for 10 minutes. The supernatant was collected and mixed with sodium phosphate buffer (0.1M, pH 7.4), containing EDTA (5mM) and ortho-phthaldialdehyde (1 mg/mL in methanol). The mixture was incubated in the dark at room temperature for 15 min and fluorescence was measured at 350 nm (excitation) and 420 nm (emission). A standard curve of GSH (0.001–0.1 mM) was used for linear regression (DinizVilela *et al*., 2016)

Determination of catalase (CAT) activity: Briefly, sample (1ml) was mixed with 49 ml of distilled water to give a 1 in 50 dilution of the sample. The assay mixture contained 4ml of H_2O_2 solution (800 μmoles) and 5ml of Phosphate buffer in a 10ml flat bottom flask. 1ml of properly diluted enzymes

preparation was rapidly mixed with the reaction mixture by a gentle swirling motion. The reaction was run at room temperature. A 1ml portion of the reaction mixture was blown into 2ml of dichromate acetic acid reagent at 60s intervals**.** Catalase (CAT) activity was determined by measuring the exponential disappearance of H_2O_2 at 240nm and expressed in units/mg of protein (Aebi, 1984).

Determination of malonaldehyde (MDA) activity: Briefly, the most abundant individual aldehyde resulting from lipid peroxidation breakdown in biological systems, MDA was estimated with the method of Uchiyama and Mihara (1978) which is based on its interaction with thiobarbituric acid (TBA) to form pink complex with absorption at 535nm. Absorbance was read using Microlab 300 recording spectrophotometer (UV 160) in all measurements.

Statistical analysis: All results are presented as the mean and standard error of mean (SEM). Statistical analyses were conducted using GraphPad Prism Software (GraphPad, Inc., La Jolla, CA, USA). Data analyses were performed by one-way analysis of variance (ANOVA) with post hoc Tukey's multiple comparison test. Statistical significance was set at $p < 0.05$.

RESULTS

Table 1 shows the presence of molisch, tannin, glycosides, terpenoid magnesium chip, flavonoid and fehlings following phytochemical screening. The acute toxicity tests at stages A and B revealed no toxicity at different doses tested except at 5000mg/kg in which there was swollen legs in the group tested. (Table 2 and 3).

Table 1:
Phytochemical screening of the aqueous leaf extract of *Telfairia occidentalis*

Phytochemicals	Present, (+) Strongly present (++)
Molisch	++
Tannin	++
Glycosides	+
Terpenoid	+
Magnesium Chip	++
Flavonoid	++
Fehlings	++

k**Table 2:**
Stage A of the acute toxicity (LD_{50}) of the *Telfairia occidentalis* aqueous leaf extract

GROUPS	DOSAGE	MORTALITY
Group B	10	Nil
Group C	100	Nil
Group D	1000	Nil

Table 3:
Stage B of the acute toxicity (LD50) of the *Telfairia occidentalis* aqueous leaf extract

GROUP S	DOSAGE	MORTALITY
Group B	1600	Nil
Group C	2900	Nil
Group D	5000	ST

ST= sign of toxicity

Lipid profile: There was no significant difference ($p>0.05$) in CHOL level in groups B (27±0.13), C (2.53±0.21) and D (2.55±0.16), compared with control ()2.72±0.192). *Telfairia occidentalis* produced a significant decrease ($p<0.05$) in TG level in groups C (0.95±0.16) and D (0.70±0.11) compared with control (1.18±0.07). TG also showed a significant decrease in group D (0.70±0.11) compared with group B (1.14±0.13) ($p<0.05$). HDL showed a significant increase ($p<0.05$) in groups B (1.56±0.13) compared with control (1.25±0.10). LDL showed a significant increase in groups B (0.62±0.11) and D (0.48±0.10) compared with control (0.83±0.06) ($p<0.05$) and D (0.48±0.10) significantly decreased compared with group B (0.62±0.11) (Table 4).

Liver functions: The results showed a significant decrease ($p<0.05$) in AST level in groups C (35.50±3.12) and D (39.25±6.61) compared with control (58.50±4.38) and a significant decrease in group D (39.25±6.61) compared with group B (57.40±7.51) ($p<0.05$) (Table 5). Table 5 also showed a significant increase ($p<0.05$) in ALT levels in groups B (25.20±0.65), C (20.00±0.29) and D (17.00±0.24) compared with control (17.67±0.67). Also, *Telfairia occidentalis* produced a significant decrease ($p<0.05$) in ALT in groups C (20.00±0.29) and D (17.00±0.24) compared with group B (25.20±0.65), and a significant decrease ($p<0.05$) in ALT in group D (17.00±0.24) compared with group C (20.00±0.29).

Table 4:
Effect of *Telfairia occidentalis* aqueous leaf extract on Cholesterol (CHOL), Triglycerides (TG),high density lipoprotein (HDL) and low density lipoprotein (LDL) levels

Parameters (mmol/l)	Group A	Group B	Group C	Group D
CHOL	2.72±0.192	27±0.13	2.53±0.21	2.55±0.16
TG	1.18±0.07	1.14±0.13	0.95±0.16*	0.70±0.11*#
HDL	1.25±0.10	1.56±0.13*	1.40±0.20	1.40±0.16
LDL	0.83±0.06	0.62±0.11*	0.70±0.07	0.48±0.10*#

Values represent Mean ± SEM. n=6. Significant (*$p<0.05$ vs. group A, #$p<0.05$ vs group B)

Table 5:
Effect of *Telfairia occidentalis* aqueous leaf extract on Aspartate amino transferase (AST), Alanine amino transferase (ALT) and alkaline phosphatase (ALP) levels

Parameters	Group A	Group B	Group C	Group D
AST u/l	58.50±4.38	57.40±7.51	35.50±3.12*#	39.25±6.61*#
ALT u/l	17.67±0.67	25.20±0.65*	20.00±0.29*#	17.00±0.24*#α
ALP u/l	68.00±3.98	121.60±4.86*	157.25±16.15*#	111.00±12.43*α

Values represent Mean ± SEM. n=6. Significant (*$p<0.05$ vs. group A, #$p<0.05$ vs group B, α$p<0.05$ vs. group C

Table 5 also showed a significant increase ($p<0.05$) in ALP levels in groups B (121.60±4.86), C (157.25±16.15) and D (111.00±12.43) compared with control (68.00±3.98). Also, *Telfairia occidentalis* produced a significant increase ($p<0.05$) in ALP level in group C (157.25±16.15) and a significant decrease in group D (111.00±12.43) compared with group B (121.60±4.86), with a significant decrease ($p<0.05$) in ALP levels in group D (111.00±12.43) compared with group C (157.25±16.15).

Creatinine: Aqueous leaf extract of *Telfairia occidentalis* displayed a significant decrease ($p<0.05$) in creatinine level in groups B (27.92±0.36). and C (29.95±0.64). compared with control (34.30±0.54). The result also showed a significant decrease ($p<0.05$) in creatinine level in group D (32.38±0.58) compared with groups B (27.92±0.36) and C (29.95±0.64).

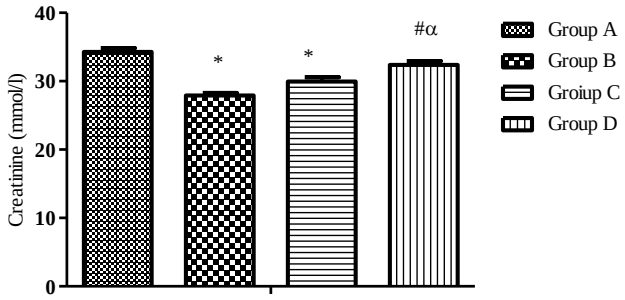

Figure 1:
Effect of *Telfairia occidentalis* aqueous leaf extract on creatinine level. Values represent Mean ± SEM. n=6. Significant ($^{*}p<0.05$ vs. group A, #$p<0.05$ vs group B, $^{\alpha}p<0.05$ vs. group C)

Table 6:
Effect of *Telfairia occidentalis* aqueous leaf extract *on Superoxide dismutase (SOD), reduced glutathione (GSH) and Catalase (CAT) activities*

Parameters	Group A	Group B	Group C	Group D
SOD (mg/ml)	1.11 ±0.03	1.48 ±0.02*	1.47 ±0.02*	1.43 ±0.02*#
CAT (mg/ml)	7.25 ±0.05	10.10 ±0.03*	9.49 ±0.09*	8.32 ±0.07*#α
GSH (µmol/ml)	599.62 ±24.38	835.76 ±19.26*	784.9 0±7.51*#	647.36 ±8.26*#α

Values represent Mean ± SEM. n=6. Significant ($^{*}p<0.05$ vs. group A, #$p<0.05$ vs group B, $^{\alpha}p<0.05$ vs. group C)

Antioxidant enzymes: Assay of antioxidant enzymes showed a significant decrease in SOD in groups B (1.48±0.02), C (1.47±0.02) and D (1.43±0.02) compared with control (1.11±0.03) while group D (1.43±0.02) showed a significant decrease ($p<0.05$) compared with groups B (1.48±0.02), and C (1.47±0.02) (Table 6). There was a significant decrease in CAT in groups B (10.10±0.03), C (9.49±0.09) and D (8.32±0.07) compared with control (7.25±0.05) while group D (8.32±0.07) showed a significant decrease ($p<0.05$) compared with groups B (10.10±0.03) and C (9.49±0.09) (Table 6). *Telfairia occidentalis* aqueous leaf extract a significant decrease in GSH in groups B (835.76±19.26), C (784.90±7.51) and D (647.36±8.26) compared with control (599.62±24.38) while group D (647.36±8.26) showed a significant decrease ($p<0.05$) compared with groups B (835.76±19.26), and C (784.90±7.51) (Table 6). *Telfairia occidentalis* produced a significant decrease in MDA level in group D (8.01±0.05) ($p<0.05$) compared with control (8.74±0.07), groups B (8.53±0.08) and C (8.71±0.05) (Figure 2).

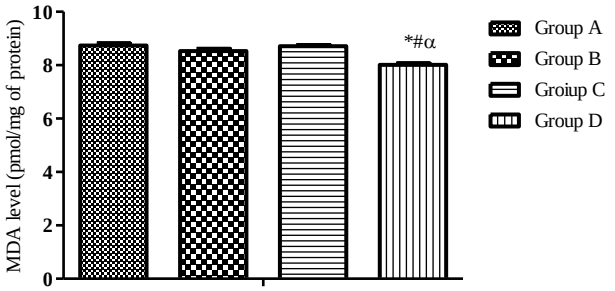

Figure 2:
Effect of Telfairia occidentalis aqueous leaf extract on *Malonaldehyde* (MDA) level. Values represent Mean ± SEM. n=6. Significant ($^{*}p<0.05$ vs. group A, #$p<0.05$ vs group B, $^{\alpha}p<0.05$ vs. group C)

DISCUSSION

Hyperlipidemia is a heterogenous group of disorders characterized by high level of lipids in the bloodstream. It may be caused by disorders of some endocrine glands, kidneys, effects of certain drugs, dietary intake containing high amount of fat, risky life style and ageing (Durrington *et al*., 1988). It is one of the risk factors in development of atherosclerosis (Nwodo *et al*., 2014). In the current study, decreased cholesterol and DL levels were observed which is suggestive suggestive of the hypolipidemic effect of the leaves of *Telfairia occidentalis* . It was also observed that the leaves extract of *Telfairia occidentalis* possesses hypoytriglyceridemic effect with the significant reduction of TG in all the doses tested. The leaf of *Telfairia occidentalis* supplemented in diet has however been reported to lower plasma cholesterol and low density lipoprotein levels (Eseyin *et al*., 2007).

The reduction in the low-density lipoprotein (LDL) level observed in the current study is in agreement with the reports of Ugwu *et al*., (2011) and Onuegbu *et al*., (2015) in which the leaf of *Telfairia occidentalis* produced a decrease in low-density lipoprotein level. This could be as a result of the activity of the fibre content of *Telfairia occidentalis* (Barakat and Mahmond, 2011). Fibre has been reported to decrease LDL by interrupting cholesterol and bile acid absorption and increasing LDL receptor activity (Venkateson *et al*., 2003). Low density lipoprotein is a bad cholesterol which facilitates transport of cholesterol into the cell (Marcel *et al*., 1980) thus, the significant reduction in LDL is suggestive and justifies the cholesterol lowering effect of *Telfairia occidentalis* observed in the current study, thereby, further reducing the risk of metabolic disorder such as obesity and atherosclerosis.

There have been reports on the lipid profile of various plants including *Telfairia occidentalis* and some of which is in accordance with the present study (Harword *et al*., 2005; Venkatesan *et al*., 2003; Vinson *et al*., 1998; Ikeda and Sugano, 1998). Thus, there could be alterations in the concentration of the various lipid metabolism and

predisposition of the heart to atherosclerosis and its associated coronary heart diseases. The current study is in agreement with the previous study which reported that it is used as anti-hypercholesterolemic in ethnobotany (Nwozo *et al*., 2004).

In addition, increased alkaline phosphatase observed at 150mg/kg may be suggestive of possible liver exhaustion while at doses 50mg/kg and 100mg/kg, the enzymes activities were not significantly elevated. Literature has shown that the destruction of liver architechture is the principal culprit for the elevation of the liver enzymes. This destruction often occurs in the presence of high amount of toxins and xenobiotics which becomes challenges to the liver (Ejike *et al*., 2008)

Oxidative stress results when the antioxidant system is overwhelmed by the generation of excess reactive oxygen species (ROS) (Halliwell and Gutteridge, 1999). These reactive species like superoxide radical anion (O^{-2}), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^-) cause severe damage to macromolecules, tissues and organs through the process of lipid peroxidation (LPO), protein modification and DNA strand breaks (Sun and Chen, 1998; Zaidi and Banu, 2004). Oxidative stress resulting from the generation of these free radicals is known to contribute immensely to several pathological conditions like aging, cancer, cardiovascular disorder, neurodegenerative diseases among others (Halliwell and Gutteridge, 1999; Abuja and Albertini, 2001).The SOD and GSH properties in this study were investigated because of their synergistic ability to work hand in hand, SOD catalyses the breakdown of superoxide, the most common free radical in the body into oxygen and hydrogen peroxide while GSH catalyses the breakdown of hydrogen peroxide to water.

Furthermore, in all the doses tested leave extract *Telfairia occidentalis* produced a significant reduction of SOD, CAT and GSH suggestive of reduced production of oxidative radicals from the current study. This is in agreement with previous study which reported that the presence of antioxidant and antimicrobial properties, its minerals (especially Iron), vitamins (especially vitamin A and C) and high protein contents were found to prevent oxidative radicals' production (Kayode and Kayode, 2011). This could also be due to the presence of secondary metabolites like tannins, glycosides, saponins, fehlings and terpenoids. A significant reduction was observed in MDA's lipid peroxidation level in all the doses tested suggestive of effective oxidative balance. MDA has been shown to be a biomarker of oxidative stress, excessive production of which has been linked to dyslipideamia and atherosclerosis.

The current study disagrees with Saalu *et al*., (2010) who reported that administration of *Telfairia occidentalis* leaf extract to rat at high dosage caused increased lipid peroxidation. The MDA level observed in this study is however agrees to the report of Ajani and Akinyemi (2016). There have been reports on antioxidant activities of various plants as well, some of which correlates with the present study. For instance, *Pelargonium reniforme* which is used locally for liver disorders, has strong antioxidant activities as a result of its tannin and flanovoid content (Fernandes *et al*., 2004). *Mallotus oppsitifolium*, a Nigerian plant rich in flavonoids has been said to possess antioxidant as well as anti-inflamatory activities (Farombi *et al*., 2001). These strongly agree with the present findings antioxidant activity observed

In conclusion, the results of the present study revealed that leaf extract of *Telfairia occidentalis* possesses anti-hyperlipidemic and antioxidant activities. It is hepato-protective and this is in agreement with its use in folk medicine in combating many diseases in the body. However, since it is dose dependent care has to be taken during therapeutic use as it has potential to increase alkaline phosphatase level.

REFERENCES

Abiose (1999). Assessment of the extent of use of indigenous African foods, introduced foods and imported foods in hotels and other commercial eating places in Southwestern Nigeria. Pp. 50-55. In: *Africa's Natural Resources Conservation and Surveys (Baidu- Forson, J.J. ed.) Unn/Inra, Accra.*

Aebi, H., (1984). Catalase in vitro. Methods Enzymology, 105:121-126.

Ajani, R.S, and Akinyemi, A.R (2016). *Telfairia occidentalis* leaf and seed extract as possible preventive and therapeutic agents for induced benign prostatic hyperplasia. Eur J Med Plants. 2016;12(1):1-11.

Akoroda (1990). Ethnobotany of *Telfairia occidentalis (cucubitaceace) among Igbos* of Nigeria. *Economic botany. 44 (1): 29-39.*

Barakat, LA. and Mahmond, R.H (2011). The antiatherogenic, renal protective and immunomodulatory effects of purslane, pumpkin and flax seeds on hypercholesterolemic rats. North Am J Med Sci; 3:351-357.

Chevalier (2000). Natural Health Encyclopedia of Herbal Medicine; 2nd ed. Darling

DinizVilela, D., Gomes Peixoto, L., Teixeira, R.R., BeleleBaptista, N., CarvalhoCaixeta, D., Vieira de Souza A., (2016). The Role of Metformin in Controlling Oxidative Stress in Muscle of Diabetic Rats. Oxid Med Cell Longev; 2016:6978625. https://doi.org/10.1155/2016/6978625PMID: 27579154

Durrington, P.M., Hunt, I., Ishola, M., Arrol, S., Bhatnagar, D. (1988). Apolipoproteins (a), AI, and B and parental history in men with early onset ischaemic heart disease. Lancet; 1:1070-1073

Ejike, C.C., Alumanah, L.U.S., Ezeanyika, A.N., Ojefua, E.E. (2008). Antibiotics administration and its possible liver damage. Bio Res.; 6:351-354

Eseyin, O.A., Udoh, I, Ekpo, A., Edoho, E.J., Igboasoiyi, A.C. (2007). Biochemical effects of the seed extract of *Telfairia occidentalis* in rats. Int J Pharmacol;3(2):198-200.

Eujoba, A.A., Odeleye, O.M., Ogunyemi, C.M. (2005). Traditional medicine development for medical and dental primary health care delivery system in Africa. *African Journal of Traditional, Complementary and Alternative Medicines*; **2**(1): 46-61.

Evans (2002). Pharmacognosy 15th ed. W.B Saunders, Edinburgh; p. 585. *Food and Agriculture*; 40(2): 151-155.

Fernandes, A.C., Cromarty A.D., Albrecht, C., Jansen Van Renburg, C.E. (2004). The antioxidant potential of Sutherlandia frutescens. *J. Ethnopharmacol*; 95; 1:1-5.

Halliwell, B., Gutteridge, J. M. C., (1999). Oxidative stress: adaptation, damage, repair and death. In: Halliwell B, Gutteridge JMC, editors. Free radicals in biology and medicine. Oxford, UK: Oxford University Press; 284–330.

Halliwell, B., Gutterridge, J. M. C., (1994). Lipid peroxidation, oxygen radicals, cell damage and antioxidant therapy. Lancet, 1: 1396–1397.

Harborne (1973). *Phytochemical Methods*. A Guide to Modern Technology of Plant Analysis, 2nd ed. Chapman and Hall, New York, Pp. 88-125.

Harword, H.J. Jr., Chandler, C.E., Pellarin, L.D., Bangerter, F.W., Wilkins, R.W., Long C.A. (2005). Pharmacologic consequences of cholersterol absorption inhibition: alteration in cholesterol concentration induced by the synthetic saponin β-tigogenin in cellobioside (CP-88818; tiqueside). *J. Lipid. Res*. 34: 377-95.

Ikeda, I., Sugano, M., Imoh, E.U., Julia, O.M. (2005). Nutrient requirement for the growth of water leaf (*Talinum triangulare*) in Uyo Metropolis, Nigeria. *The Environ*. 21; 153-159.

Kayode, A.A., and Kayode, O.T. (2011). Some medicinal values of *Telfairia occidentalis :* **2**: 36-42.

Leaf 2011, Wikipedia, the free encyclopedia, retrieved on 19th July. http://en.wikipedia.org/wiki/leaves

Lorke (1983). Determination of acute toxicity. *Arch. Toxicol*. **53; 275**

Marcel, Y.L., Vezina, C., Edmond, D., Suzue, G. (1980). Heterogenecity of human high density lipoprotein: Presence of lipoproteins with and without apo E and their roles as substrates for lecithin cholesterol acyl transferase reaction. Proc Natl Acad Sci; 77:2969-2973.

Morakinyo, A.O., Iranloye B.O., Ogunsola, O.A. (2018). Glucometabolic effects of single and repeated exposure to forced-swimming stressor in Sprague-Dawley rats. Endocr Regul., 52(2):85-92. doi: 10.2478/enr-2018-0010.

National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. (2011). Guide for the Care and Use of Laboratory Animals, 8th ed, The National Academies Collection: Reports funded by National Institutes of Health. National Academies Press (US), Washington (DC).

Ndor, E., Dauda, S. N., and Garba, M. N. (2013): Growth and Yield Performances of Fluted Pumpkin (*Telfairia occidentalis Hook F*.) under Organic and Inorganic Fertilizer on Ultisols of North Central Nigeria. *International Journal of Plant and Soil Science, 2 (2): 212 – 221, 2013. Article no: IJPSS.2013.004*

Nwodo, N.J., Nnadi, C.O., Ibezim, A., Mbah, C.J. (2014). Plants with hypolipidaemic effects from Nigeria flora. In Tech. Chapt;10: 241-255.

Nwozo, S.O., Adaramoye, O.A., and Ajaiyeoba, E.O. (2004). Antidiabetic and

Oboh, G., Nwanna, E.E. and Elusiyan, C.A. (2006). Antioxidant and antimicrobial properties of *Telfairia occidentalis (*Fluted pumpkins) leaf extract. *Journal of Pharmacology and Toxicolog*; 1:167-175

Olorunfemi, A.E., Arnold, C. I., Emmanuel, O., Nkaima, Akeem, A. (2005). Hypoglycaemic activity of *Telfairia occidentalis* in rats. *Journal of Pharmacology and Bioresearch*; 2: 36-42

Onuegbu, A.J., Olisekodiaka, J.M., Ude, T., Amah, K.U., Okwara, E.J, Ifeadike, C. (2015). Effects of *Telferia occidentalis* seeds on the serum lipid profile and atherogenic indices of male albino wistar rats. Pak J Nutri;14(9):557-562

Saalu, L.C., Kpela, T., Benebo, A.S., Oyewopo, A.O., Anifowope, E.O., and Oguntola, JA. (2010). The dose-dependent testiculoprotective and testiculotoxic potentials of *Telfairia occidentalis* (Hook F) leaves extract in rat. Int J of Applied Research in Natural Products;3(3):23-38.

Sumner, J. (2000). The Natural History of Medicinal Plants. 1st ed. Timber Press. Portland; P 235. *Toxicology*; **1**: 167-175.

Sun, A. Y., and Chen, Y. M. (1998). Oxidative stress and neurodegenerative disorders. J. Biomed. Sci, 5: 401-414.

Gootz, J. F., Barret, H. E., Zaidi, S.M., and Banu, N. (2004). Antioxidant potential of vitamins A, E and C in modulating oxidative stress in rat brain. Clin. Chim. Acta, 340: 229–33.

Telfairia occidentalis . (2009) Wikipedia, the free encyclope-dia, retrieved on 4th July, http://en.Wikipedia. Org /Wiki/ *Telfairia _ Occidentalis*

Uchiyama, M., and Mihara, M. (1978). Determination of malonaldehyde precursor in tissues by thiobarbituris acid test. Anal of Biochemistry*;* 86: 271-278.

Ugwu, C.E., Olajide, J.E., Alumana, E.O., and Eeanyika, C. (2011). Comparative effects of the leaves of *Vernonia amygdalina* and *Telferia occidentalis* incorporated diets on the lipid profile of rats. Afri J Biochemistry Research;5(1):28-32.

Venkateson, N., Devaraj, S., Devaraj, H. (2003). Increased binding of LDL and VDL to apo B, E receptors of hepatic plasma membrane of rats treated with fibernat. Eur J Nutri; 42:262-271.

Verla, A.W., Verla, P., Adowei, A., Briggs, E., Awa, M., Horsfall, JNR and Spiff, A.I. (2014). Merit Res. J. EnvironSci. Toxicol. Vol. 2(4) pp. 064-070,

Vinson, J.A., Hu, S.JJung, S, Stanski, A.M. (1998). A citrus extract plus ascorbic acid decreases lipids, lipid peroxidation, lipoprotein oxidation susceptibility, and atherosclerosis in hypercholesterolemic hamsters. *J. Agric Food Chem*; 46.

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Full Length Research Article

Knowledge and Perception of Veterinary Students in Ghana on Telemedicine

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Summary: This study aimed at assessing the knowledge base and perception of telemedicine among veterinary students in Ghana. It involved a cross sectional survey and online structured questionnaires were administered to 114 veterinary students to access their knowledge of telemedicine and the perceived utilisation, benefits, complexity and disadvantages of telemedicine. Descriptive statistics analysis was performed on the data collected using SPSS version 20 and Pearson Chi-square test was used to determine the significant association between categorical variables which were grouped. Statistical significance was tested at 5% significance level. The students exhibited good knowledge and perception of telemedicine with a mean response of 4.947 ± 1.374 and 3.473±1.115; represented by 86.8% and 78.9% of the students respectively. 92.1% had heard of the term 'telemedicine' from the internet and social media platforms. The level of study of veterinary students had a significant effect ($p<0.05$) on their knowledge of telemedicine; knowledge increases with an increase in the year of study. Veterinary students showed good knowledge level and perception of telemedicine. However, exposure to the use of telemedicine system is low. Issues of patients' information privacy being threatened by the usage of telemedicine were indicated. Development, incorporation of telemedicine in the curriculum in formative years of veterinary students training is critical to ensure effective and efficient training of students in telemedicine in the COVID era.

Keywords*: Telemedicine, Knowledge, Perception, Students, Ghana*

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INTRODUCTION

The advent of technology, its use and implementation has found its way into the field of medical practice in different forms. One of such technological development in the healthcare system is telemedicine. Telemedicine is the application of information and communication technology to provide healthcare access remotely including diagnosing, treating and evaluating patients through remote information exchange (Brown 1995). Telemedicine technologies have been in use for some time in the developing world, but on a relatively smaller scale compared to developed countries. Telemedicine in the veterinary perspective is a subcategory of telehealth that involves the digital exchange of information from a distance regarding a patient's clinical health status within an existing Veterinarian-Client-Patient-Relationship (VCPR) (American Veterinary Medical Association, 2019). Advantageously, telemedicine provides intra and inter hospital communication, cuts down the cost associated with providing quality healthcare, eliminates the need to construct new hospital facilities, which developing countries like Ghana generally lack (Wootton *et al*., 2011). In spite of the benefits derived from the use of telemedicine, the receptivity of the advancement in medicine, is generally a necessary precondition for sustained telemedicine use (Kirsten *et al*., 2019). More to the point, the knowledge, perception and attitudes toward telemedicine are likely to influence the nature telemedicine will take and it will play a central role in determining its ultimate success (Kirsten *et al*., 2019). In Ghana, telemedicine seems to be a new wave of technological advancement in health care system but the understanding and perception of the populace has not contributed much to its successful implementation. According to literature, an appreciable collection of research has focused on the knowledge, perception and attitudes towards telemedicine (Ayatollahi *et al*., 2015, Frimpong *et al*., 2016, Biruk *et al*., 2018, Mohamed *et al*., 2020). Most of these studies have been geared more towards the clinicians, health professionals and postgraduate medical students as well as that of veterinarians' receptivity and adoption of telemedicine (Watson *et al*., 2019) with little focus on the knowledge and perception of telemedicine amongst veterinary medical students.

It is expedient to note that the perceptions of veterinary students, is desirable in the bid to the extensive adoption, use and the improvement of telemedicine in Ghana especially during this COVID pandemic. With this background knowledge and the existing research gap, this research work was conducted to evaluate the knowledge and perception of telemedicine among veterinary students in Ghana. The outcome of this study will be useful in the design and implementation of intervention programs to improve telemedicine usage and practice among veterinary students in Ghana.

MATERIALS AND METHODS

Research Design and Study Area: This study was designed as a cross-sectional survey to assess the knowledge

and perception of telemedicine among veterinary students in Ghana. The study area was focused on the two main Schools of Veterinary Medicine (SVM) in Ghana. These were the SVM in Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi – Ghana and SVM in University of Ghana (UG), Legon, Accra-Ghana. These two Schools of Veterinary Medicine operate a 6-years academic programme with students in first, second and third years of study classified as pre-clinical students whilst students in clinical years are those in fourth, fifth and sixth years of study respectively.

Study Population, Sampling technique and sample size: The study population in this current research comprised all the students of the two Schools of Veterinary Medicine in Ghana. The overall population of the students in the two Schools of Veterinary Medicine as at the time of data collection for this current study stood at a total of two hundred and eighty-three (283) students in different years of study: 185 students in SVM-KNUST and 98 students in SVM-UG. Characteristically, the student population comprised of different individuals with different socio-economic backgrounds and from different ethnicities including some international students from some African countries including Sierra Leone, Tanzania, Nigeria and Uganda.

The sampling technique employed for this study was the simple random sampling technique. This method of sampling technique was used so as to avoid biases on the part of the researcher as well as to ensure that each student was provided with the chance of being selected to be a part of the respondents for this study. A total of one hundred and fourteen (114) students were able to give responses to the administered questionnaires within the time frame of the study. This number of students served as the sample size used in this study.

Data collection instrument and procedure: In this study, a well-structured questionnaire (Additional file 1: Telemedicine Questionnaire) which was designed by researchers with the help of Google Forms served as the data collection instrument. The questionnaire comprised questions on the demographic characteristics of the respondents as well as the questions on the knowledge of telemedicine and the perceived utilisation, benefits, complexity and disadvantages of telemedicine among veterinary students. The questionnaires were successfully administered to the respondents after obtaining their consent. Students were contacted via Student Association WhatsApp platforms, informing them of the research study, its purpose, and a link to an online questionnaire. A constant daily reminder of the questionnaire was submitted to the student WhatsApp platforms as well as to the students. The responses to the questionnaires were retrieved from Google Forms in a Microsoft Excel format by the researcher after a one-month time frame of data collection (from August, 2020 to September, 2020) has elapsed.

In data collection on knowledge of the students, a 'Yes' or 'No' format was used in assessment of the knowledge part. A score of '1' was given for 'Yes' and '0' for 'No'. A student can score a minimum of 0 and a maximum of 8 in this section. An average score of 4 (50%) from the eight questions was used as a cutoff point to determine the level of knowledge of telemedicine. The mean knowledge score of less than 4 (50%) was labeled as poor whilst a mean knowledge score of greater than 4 (50%) was labelled as good. With perception, questions were rated on a 5- point Likert scale that ranged from '1=strongly disagree' to '5=strongly agree. Mean scores were calculated and a mean of less than 2.5 (50%) was labeled as poor, 2.6 (51%)–3.0 (60%) as moderate, and greater than 3.0 (60%) is labeled as good.

Data Analysis: Statistical data analysis was performed on all the data collected using Statistical Package for Social Sciences (SPSS Version 20). Descriptive statistics were used to analyse the demographic characteristics as well as the students' knowledge and perception of telemedicine. Data was summarized using mean, standard deviation in quantitative data and using frequency and percentages for categorical data. Pearson Chi-square test was used to determine the significant association between categorical variables which were grouped. Statistical significance was tested at 5% significance level or 95% confidence interval which meant that p-values less than .05 were considered as statistically significant.

RESULTS

Demographic Characteristics of students

Results obtained for the demographic characteristics of the veterinary students are displayed in Table 1 below. The findings indicated that out of the one-hundred and fourteen (114) respondents, 78 respondents representing 68.4% were below 22 years of age whilst the remaining 36 respondents representing 31.6% were above 22 years of age. Findings on gender of the respondents showed that 73.7% of the respondents were males whilst the remaining 26.3% were females. The religious affiliation of the respondents indicated that 94.7% were Christians whilst 7.9% were Muslims. In terms of the marital status of the respondents, 94.7% of the respondents were unmarried whilst 5.3% were married. The findings showed that 57.9% of the respondents were clinical year students in year 4 to year 6 whilst the remaining 42.1% of the respondents were preclinical year students in year 1 to year 3 (Table 1).

Table 1:
Veterinary students' demographic characteristics

Variable	Category	Frequency (n)	Percentage (%)
Age	<22	36	31.6
	>22	78	68.4
Gender	Male	84	73.7
	Female	30	26.3
Religion	Christianity	105	92.1
	Islam	9	7.9
Marital Status	Single	108	94.7
	Married	6	5.3
Year of Study	(Year 1- Year 3)	48	42.1
	Clinical (Year 4-Year 6)	66	57.9

Values are expressed in frequencies and percentages except otherwise stated

Table 2:
Veterinary students' response to survey questions on knowledge of telemedicine

Questions on knowledge of Telemedicine	Response		Overall knowledge (Mean and SD)
	Yes	No	
Have you heard of the term 'telemedicine'	105 (92.1%)	9 (7.89%)	4.947 ± 1.374
Have you seen a telemedicine system before	15 (13.2%)	99 (76.8%)	
Are you familiar with tools like teleconference or teleconsultation	51 (44.7%)	63 (54.3%)	
Telemedicine is useful in continuing medical diagnosis	96 (84.2%)	18 (15.8%)	
It is useful in chronic disease management and surgery	87 (76.3%)	27 (23.7%)	
It disseminates patient health information from one department to another	96 (84.2%)	18 (15.8%)	
Telemedicine provides sufficient understanding of a patients problem	66 (57.9%)	48 (42.1%)	
It does not includes administrative meetings and continuing medical education	84 (73.7%)	30 (27.3%)	

Sources of knowledge on Telemedicine: In addition, the study sought to examine the sources from which the veterinary students obtained the knowledge of telemedicine. The findings are displayed in Figure 1 below. The results indicate that 42.1% of the obtained information on telemedicine from the internet, 26.3% obtained their information from social media platforms, 15.8% obtained their knowledge of telemedicine from multimedia (radio, television) whilst 10.5% of the respondents had knowledge of telemedicine from their lecturers. Colleagues were cited by 2.6% of the students as their source of knowledge of telemedicine whilst 2.6% also cited scientific journals as their source of knowledge on telemedicine (Figure 1 below).

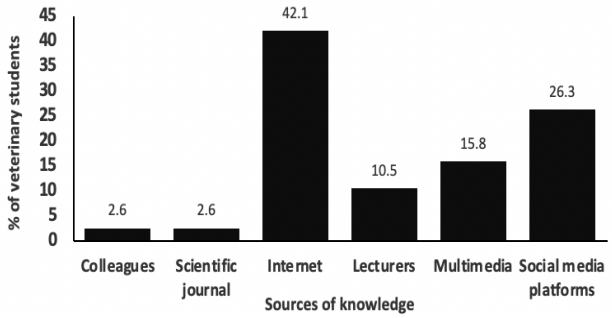

Figure 1:
Response on the source of veterinary students' knowledge of telemedicine.

Perception towards Telemedicine: This study examined the perception of veterinary students with respect to the utilization, benefits, complexity and disadvantages of telemedicine. The findings as shown in Table 3 below indicated that the veterinary students have an overall good perception toward telemedicine with a mean of 3.473±1.115. The recorded mean response for utilization and benefits of telemedicine was 3.65±1.105 whilst that of complexity and disadvantages of telemedicine was 3.05±1.139. The mean responses indicate clearly that the students had a very good perception of how beneficial telemedicine can be to the veterinary practice as well as the possible disadvantages that will be encountered in the adoption and usage of telemedicine in veterinary practice.

With respect to the utilization and benefits of telemedicine, 76.3% of the respondents responded by agreeing that telemedicine will help capture and store medical information for future use. 44.7% of the students were confident that telemedicine will reduce cost of patients assessing healthcare services whiles 36.8% were neutral. Majority of the students (57.9%) agreed that telemedicine will increase doctor and patient communication. 31.6% of the students agreed that medical errors can be reduced when telemedicine is used while 42.1% were neutral.

A greater percentage (67.2%) of the students indicated that telemedicine will reduce visits to veterinary clinics. It was agreed by 71% of the respondents that telemedicine use can held quick healthcare delivery while 50% agreed that telemedicine has the ability to enhance emergency medical delivery as well as 63.1% agreed to the likelihood of an increase in the number of patients to be attended to when telemedicine is adopted in veterinary practice (Table 3). With telemedicine improving clinical decisions in veterinary practice, facilitating effective and efficient diagnosis, enhancing quality of healthcare delivery, and reducing the number of hours to be used in surgery, 50%, 42.1%, 63.1% and 23.7% of the students respectively agreed to the above mentioned benefits of telemedicine whilst 50% of the students disagreed with telemedicine having the ability to reduce the time to be used in veterinary surgeries (Table 3).

With respect to the perceived complexity and disadvantages of telemedicine, majority of respondents (55.3%) agreed that telemedicine will add extra responsibility to the healthcare provider and 34.3% agreed telemedicine is not cost-effective to use in veterinary practice. Concerning telemedicine threatening patient information privacy, 39.5% agreed to it whilst 34.2% remained neutral. Telemedicine adoption and use in veterinary medicine being a tedious and stressful process was agreed upon by 23.6% of the respondents whilst most of the students (57.9%) remained neutral. With the issue telemedicine being perceived as not an effective use of time in veterinary practice, 60.5% of the students disagreed whiles 7.9% agreed to telemedicine being an ineffective use of veterinary practice (Table 3).

Overall Knowledge and Perception of Veterinary Students towards Telemedicine: The results on the overall knowledge and perception of veterinary students with respect to telemedicine are displayed in Table 4 below. The findings showed that, 99 (86.8%) of the students had a good knowledge of telemedicine whilst 15 (13.2%) had poor knowledge of telemedicine. In terms of perception, 90 (78.9%) of the students had a good perception on telemedicine, 21 (18.5%) of the respondents recorded a moderate perception on telemedicine whilst only 3 (2.6%) had a poor perception toward telemedicine in terms of its benefits, utilization, complexity and disadvantages (Table 4).

Table 3:
Perception of Telemedicine among veterinary students

	SD	D	N	A	SA	Mean & SD
Utilisation and Benefits of Telemedicine						
Capture and store medical information for future use	0 (0.0%)	6 (5.3%)	21 (18.4%)	30 (26.3%)	57 (50%)	4.21±0.935
Reduce cost of patients assessing health care services	6 (5.3%)	15 (13.2%)	42 (36.8%)	18 (15.8%)	33 (28.9%)	3.50±1.202
Increase doctor and patient communication	3 (2.6%)	12 (10.5%)	33 (28.9%	18 (15.8%)	48 (42.1%)	3.84±1.175
Medical error in veterinary practice can be reduced	9 (7.9%)	21 (18.4%)	48 (42.1%	15 (13.2%)	21 (18.4%)	3.16±1.175
Telemedicine reduces visits to veterinary clinics	3 (2.6%)	15 (13.2%)	21 (18.4%	30 (26.3%)	45 (39.5%)	3.87±1.095
Telemedicine can help quick healthcare delivery	0 (0%)	9 (7.9%)	24 (21.1%	39 (34.2%)	42 (36.8%)	4.00±0.959
Enhance emergency medical delivery	9 (7.9%)	6 (5.3%)	42 (36.8%	24 (21.1%)	33 (28.9%)	3.58±1.030
Increases number of patients attended to in veterinary practice	3 (2.6%)	9 (7.9%)	30 (26.3%	30 (26.3%)	42 (36.8%)	3.87±1.095
Improve clinical decisions in veterinary practice	0 (0.0%)	12 (10.5%)	45 (39.5%	18 (15.8%)	39 (34.2%)	3.74±1.057
Facilitates effective and efficient medical diagnosis	3 (2.6%)	6 (5.3%)	57 (50%)	18 (15.8%)	30 (26.3%)	3.58±1.200
Enhance quality of health care delivery	3 (2.6%)	9 (7.9%)	30 (26.3%	33 (28.9%)	39 (34.2%)	3.84±1.079
Reduce the number of hours used in veterinary surgery procedures	24 (21.1%)	33 (28.9%)	30 (26.3%	15 (13.2%)	12 (10.5%)	2.63±1.261
Utilisation and benefits total mean response						**3.65±1.105**
Complexity and Disadvantages of Telemedicine						
Adds extra responsibility to the healthcare provider	3 (2.6%)	12 (10.5%)	36 (31.6%	27 (23.7%)	36 (31.6%	3.71±1.113
Not cost-effective to use in veterinary practice	15 (13.2%)	24 (21.1%)	36 (31.6%	24 (21.1%	15 (13.2%	3.00±1.230
Threatens patient information privacy in healthcare delivery	18 (15.8%)	13 (11.4%)	39 (34.2%	30 (26.3%	15 (13.2%	3.11±1.247
Telemedicine adoption and use in veterinary medicine is a tedious and stressful process	9 (7.9%)	12 (10.5%)	66 (57.9%	9 (7.9%)	18 (15.7%	3.13±1.070
Telemedicine use is not an effective use of time in veterinary practice	27 (23.7%)	42 (36.8%)	36 (31.6%	3 (2.6%)	6 (5.3%)	2.29±1.037
Complexity and Disadvantages total mean response						**3.05±1.139**
Overall Perception Mean Response						**3.473±1.115**

Table 4:
Level of knowledge and Perception of Telemedicine

Parameter	Level	Frequency (n)	Percentage (%)
Knowledge of telemedicine	Good	99	86.8
	Poor	15	13.2
Perception towards telemedicine	Good	90	78.9
	Moderate	21	18.5
	Poor attitude	3	2.6

Demographic characteristics and knowledge of telemedicine: Findings on the effects of demographic characteristics of the veterinary students on the knowledge of telemedicine were displayed on Table 5 below. Generally, the knowledge level was good; however, the knowledge level of telemedicine among the students was not significantly (P>0.05) related to their age (P=0.084), religion (P=0.214), and marital status (P=0.236), but was statistically significant (P<0.05) with the gender (P=0.043) and year of study (P=0.025) of the students.

With respect to gender, more male students (63.1%) had a good knowledge of telemedicine as compared to the female students (23.7%). According to the year of study, more clinical students (52.6%) had good knowledge of telemedicine as compared to the preclinical students (34.2%). These differences in knowledge level were statistically significant (P<0.05).

Demographic characteristics and perception Towards telemedicine: Findings on the relatedness of demographic characteristics of the veterinary students to their perception of telemedicine were displayed in Table 6 below. From the findings, the general perception of telemedicine was good among the various categories of the demographic variables. Nevertheless, the differences in the students' perception was not significantly (P>0.05) related to their age (P=0.189), religion (P=0.887), marital status (P=0.993), gender (P=0.464) and year of study (P=0.280) of the students (Table 6).

Table 5:
Relationship between student demographics and knowledge of telemedicine.

		Level of Knowledge			
Variable	**Category**	**Good**	**Poor**	X^2	**P-value**
Age	<22	27 (23.7%)	9 (7.9%)	9.158	0.084
	>22	72 (63.1%)	6 (5.3%)		
Gender	Male	72 (63.1%)	12 (10.5%)	12.066	0.043*
	Female	27 (23.7%)	3 (2.6%)		
Religion	Christianity	90 (78.9%)	15 (13.2%)	8.662	0.214
	Islam	9 (7.9%)	0 (0%)		
Marital Status	Single	93 (%)	15(%)	11.259	0.236
	Married	6 (%)	0 (0.0%)		
Year of Study	Preclinical	39 (34.2%)	9 (7.9%)	3.218	0.025*
	Clinical	60 (52.6%)	6 (5.3%)		

** = statistical significance at 5% significance level (p<0.05).*

DISCUSSION

This current study examined the knowledge and perception of telemedicine among Ghanaian veterinary medical students. The findings revealed that students enrolled in veterinary school in Ghana have a relatively good knowledge of telemedicine with a mean response of 4.947 ± 1.374 which represented 86.8% of the students.

Table 6:
Relationship between student demographics and perception of telemedicine

		Level of Perception				
Variable	**Category**	**Good**	**Moderate**	**Poor**	X^2	**P-value**
Age	<22	36 (31.6%)	0 (0.0%)	0 (0.0%)	22.187	0.189
	>22	54 (47.4%)	21 (18.4%)	3 (2.6%)		
Gender	Male	69 (60.5%)	12 (10.5%)	3 (2.6%)	18.231	0.464
	Female	21 (18.4%)	9 (7.9%)	0 (0.0%)		
Religion	Christianity	84 (73.7%)	18 (16.1%)	3 (2.6%)	21.956	0.887
	Islam	6 (5.3%)	3 (2.6%)	0 (0.0%)		
Marital Status	Single	84 (73.7%)	21 (18.4%)	3 (2.6%)	14.602	0.993
	Married	6 (5.3%)	0 (0.0%)	0 (0.0%)		
Year of Study	Preclinical	45 (39.5%)	3 (2.6%)	0 (0.0%)	19.198	0.280
	Clinical	48 (42.1%)	18 (16.1%)	3 (2.6%)		

From the findings of this study, the major source of information was the internet and social media platforms while the information through the lecturers was low. This outcome however showed the need for the incorporation of the concept of telemedicine in the curriculum of veterinary medicine thereby making lecturers the major source of information on telemedicine. In this study, majority of the students (92.1%) had heard of the term 'telemedicine' which is similar to reports of Ameh (2008) among medical school students in Nigeria but higher than the report of Gour and Srivastava (2010) who indicated 58% of the students have heard of telemedicine in India. The differences in the outcomes could be due to geographical location as well as technological advancement of the countries involved.

In spite of hearing of telemedicine, 45.7% of the students were familiar with teleconferencing and teleconsultation while 76.8% of the students have not seen a telemedicine system. This could be attributed to the challenges with telecommunication infrastructure development in resource limited country with lack of telemedicine systems in hospitals and veterinary medical schools for teaching and learning purposes. The use of telemedicine to help in continual medical education as well as to disseminate patient health information from one department to another was known by 84.2% respondents. This finding is in accordance with report of Frimpong *et al*., (2016) where 90% of the respondents affirmed that telemedicine is a very useful tool in medical information sharing among health workers in Ghana.

Although it was observed that there is an increase in the knowledge base of veterinary students on telemedicine, this was statistically significant ($p<0.05$) with the gender ($p=0.043$) and year of study ($p = 0.025$) of the students. The significance in the knowledge of telemedicine as related to the gender could be attributed to the fact that more male students are enrolled into the veterinary programme in Ghana. The good knowledge of telemedicine exhibited by the clinical students (52.6%) than preclinical students (34.2%) could be as a result of the exposure of the clinical students to veterinary clinical practices and the need for advancements in a technologically advancing world while the preclinical students are adjusting to the basics and theoretical aspects of the veterinary training.

The perception of students on the utilisation, benefits, complexity and disadvantages of telemedicine among the veterinary students was good with a mean response of 3.473±1.115 represented by 78.9% of the students used in this study. This finding is in agreement with the findings of Hawk (2018) who indicated that veterinary students among other younger veterinarians held a more positive attitude and perception towards the use of telemedicine especially with the changing world.

This current study furthermore revealed that the students agreed that telemedicine will advantageously reduce visits to veterinary clinics (65.8%) ensure quick healthcare delivery (71.0%), enhance emergency medical delivery (50%), increase the number of patients to be attended to (63.1%) as well as facilitate effective and efficient diagnosis (42.1%) in veterinary practice. This perception from the students in this study buttresses the current position of governments on healthcare delivery in this era of COVID-19, which has created a path of reduced physical contact with humans. The responses of the students however are in accordance with use of telemedicine in veterinary practice being the way forward since there is the possible elimination of that fear of the clinician or the client contracting infectious diseases which are either nosocomial and or contagious in nature.

The use of communication devices such as telephones, video chats among others in the practice of telemedicine will enhance the work of the veterinarian as a lot of patients can be reached across the country especially in rural areas and also ensure the timely intervention of a veterinarian to save a patient in cases where time is a crucial factor and distance is a barrier.

The benefits of telemedicine improving clinical decisions in veterinary practice, reducing cost of patients assessing healthcare services, and facilitating effective and efficient diagnosis was agreed by 50%, 44.7% and 42.1% of the students in this study. These findings come to shed more light on previously reported benefits of telemedicine in veterinary practice (Bishop *et al*., 2015). This shows that in the use of telemedicine in veterinary practice, client comfort in accessing veterinary services will be ensured as well as the financial burdens comprising of transportation costs among other unforeseen costs will be reduced. Based on these perceptions, the adoption and usage of telemedicine in veterinary practice can help address the current short falls of delivering veterinary care in Ghana which includes reduced number of veterinary clinics in an area, distance barriers, among others. Individuals and farmers living in remote areas can still enjoy the services of veterinarians without stressing their animals through transportation of such ill

animals over longer distance travels to receive veterinary care (Bishop *et al*., 2015, Bragg *et al*., 2015).

Again this current study has shown that 76.3%, 57.9% and 63.1% of the students were in agreement to telemedicine being helpful in the capture and the storage of medical information for future use, increase doctor and patient communication, and enhance the quality of healthcare delivery respectively. These perceived benefits indicate that telemedicine will ensure effectiveness in the field of work on the part of the veterinarians. In addition, clinically unusual yet useful cases and their outcomes can be properly stored and retrieved for future reference to clients as well as useful in teaching and learning purposes.

Nevertheless, with the issue of telemedicine reducing the number of hours to be used in surgery, only 23.7% of the students agreed to it. This shows that most of the students perceive that it will be quite challenging when using telemedicine during surgery. This perception could be attributed to the non-exposure of veterinary students to the feasibility of telemedicine use in surgical sessions in Ghana. The adoption of telemedicine was agreed by the students that it will add extra responsibility to the veterinarians (55.3%). These extra responsibilities could be in the form of either ensuring technical effectiveness and efficiency of the telemedicine system or performing administrative and legal responsibilities which have not been the routine of veterinarians in recent times. These concern as raised in this study corroborates the findings of other studies conducted by Joseph *et al*., (2007) and Sheikhtaheri *et al*., (2016) where clinicians and hospital workers were much more concerned about the extra responsibilities associated with telemedicine adoption in the form of both administrative and legal responsibilities.

Telemedicine was also deemed by 34.3% of the students as not cost-effective in veterinary practice. The non-cost-effectiveness as indicated by some of the students could be in the form of the cost involved in purchasing a telemedicine system, installation and its maintenance which could be perceived to be relatively high as compared to the traditional way used by veterinarians in carrying out their practice. In addition, some students (39.5%) in this study raised the concern of telemedicine having the capability of threatening patient information privacy and hence can defeat client s' information confidentiality; which is a major concern for most clients. These concerns corroborate the perception of healthcare policy makers in a study conducted by Alaboudi *et al*., (2016). Isabalija *et al*., (2011) also in their study reported the issue of client information confidentiality as one of the factors affecting the implementation and adoption of telemedicine in Uganda. Therefore, for a successful implementation of telemedicine in veterinary practice in Ghana, securing patient information confidentiality should be of outmost importance and consideration.

In conclusion, based on the outcome of this current study, it was concluded that the veterinary students in Ghana exhibited very good knowledge of telemedicine of which their major source of knowledge was from the internet. They also possessed a good perception of the benefits, utilisation, complexity and disadvantages of telemedicine. However, their exposure to a telemedicine system is low. The gender and level of study had a significant effect on level of knowledge of telemedicine among veterinary medicine students. Furthermore, the issue of patients' information privacy being threatened by the usage of telemedicine was indicated by the students.

It is recommended that administrators and educational policymakers should acknowledge, develop and incorporate the teaching of telemedicine in the curriculum in the formative years of veterinary students training; so as to enhance the adoption and usage of telemedicine in veterinary medicine training and practice in the COVID era. Furthermore, it is important to establish a working telemedicine centre in the school of veterinary medicine to ensure effective and efficient training of students in telemedicine

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REFERENCES

Alaboudi A, Atkins A, Sharp B, *et al*. Barriers and challenges in adopting Saudi telemedicine network: The perceptions of decision makers of healthcare facilities in Saudi Arabia. J Infect Public Health. 2016;9(6):725–733.

Ameh N, Kene T. and Ameh E. Computer knowledge amongst clinical year medical students in a resource poor setting. Afr Health Sci, 2008; 8:40–43.

American Veterinary Medical Association. Veterinary Telemedicine. https://www.avma.org/KB/Policies/Pages/Telemedicine.aspx. Accessed 15 May 2019.

Ayatollahi H, Sarabi FZ and Langarizadeh M. Clinicians' knowledge and perception of telemedicine technology. Perspect Health Inf. Manag. 2015;12(Fall):1c.

Biruk K and Abetu E. Knowledge and attitude of health professionals toward telemedicine in resource-limited settings: a cross-sectional study in North West Ethiopia. J Healthc. Eng. 2018; 2018:1–7.

Bishop G, Evans B, Kyle K, Kogan L. Owner satisfaction with use of videoconferencing for recheck examinations following routine surgical sterilization in dogs. J Am Vet Med Assoc. 2015; 253(9):1151–7.

Bragg R, Bennett J, Cummings A, Quimby J. Evaluation of the effects of hospital visit stress on physiologic variables in dogs. J Am Vet Med Assoc. 2015; 246(2):212–5.

Brown N. A brief history of telemedicine. Telemedicine Information Exchange (Book). 1995; 105:833–5.

Frimpong, K.O., Asare, S., Kwakye Nomah, D., Antwi-Asante, D.A. Knowledge and perception of Telemedicine among Health Professionals at the Koforidua Regional Hospital, Ghana. International Journal of Healthcare Sciences, 2016; Vol. 4, Issue 2, pp: (96-103).

Gour N, and Srivastava D. Knowledge of computer among healthcare professionals of India: A key toward e-Health. Telemed J E Health, 2010; 16:957–962.

Hawk C. Veterinary telemedicine perception and utilization intention. University of Tennessee Honors Thesis Projects. (2018).https://trace.tennessee.edu/utk_chanhonoproj/2234.

Isabalija M, Kituyi A, Rwashana VW. Mbarika Factors affecting adoption, implementation and sustainability of telemedicine information systems in Uganda. J Health Inform Dev Ctries. 2011; 5:300–316.

Joseph L A, Shittu L, Olamide A, Mathew C, Izegbu C, Oluwole A, Oyewopo A, Arigbabuwo A. O, Ashiru O. Knowledge and perception of health workers towards tele-medicine application in a new teaching hospital in Lagos. Sci Res Essays. 2007; 2(1):016–019.

Kirsten E. Cowan, Alastair J. McKean, Melanie T. Gentry, Donald M. Hilty. Barriers to Use of Telepsychiatry: Clinicians as Gatekeepers. Mayo Clinic Proceedings; 2019; 94:12, 2510-2523.

Mohamed L. Elsaie, Hany A. Shehata, Noha S. Hanafi, Shady M. Ibrahim, Hany S. Ibrahim & Ayman Abdelmaksoud. Egyptian dermatologists attitude toward telemedicine amidst the COVID19 pandemic: a cross-sectional study, Journal of Dermatological Treatment, DOI:10.1080/09546634.2020.1800576.

Sheikhtaheri A, Sarbaz M, Kimiafar K, *et al*. Awareness, attitude and readiness of clinical staff towards telemedicine: a study in Mashhad, Iran. Stud Health Technol Inform. 2016; 228:142–146.

Watson, K., Wells, J., Sharma, M., Robertson, S., Dascanio, J., Johnson, W.J., Davis E.R., and Nahar K.V. A survey of knowledge and use of telehealth among veterinarians. BMC Veterinary Research, 2019; 15:474.

Wootton R, Bahaadinbeigy K, and Hailey D. Estimating travel reduction associated with the use of telemedicine by patients and healthcare professionals: proposal for quantitative synthesis in a systematic review. BMC Health Serv Res. 2011;11(1):1–10.