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Hippocratea africana Ethanol Root Extract and Fractions Attenuate Doxorubicin-Induced Testicular Toxicity and Oxidative Stress

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Abstract

Hippocratea africana root, used locally in treating poisoning, was investigated to confirm its antidotal potential in rats. The root extract (200-600 mg/kg) and fractions; dichloromethane (DCM) and aqueous, 400 mg/kg) were evaluated for hepatoprotective activity against doxorubicin-induced testicular toxicity in male rats. Testosterone level, lipid profile indices, testicular oxidative stress markers, and testis histology were used to assess the testicular protective effect of the extract. The root extract (200-600 mg/kg) and fractions, dichloromethane (DCM) and aqueous, 400 mg/kg reduced the serum levels of total cholesterol, triglycerides, LDL, and VLDL that were elevated by doxorubicin. In contrast, the high-density lipoprotein (HDL) reduced by doxorubicin was increased by the extract and fractions co-administration. Testosterone level, which was significantly ($p<0.05$) reduced by doxorubicin, was significantly ($p<0.05$-0.01) elevated by the root extract and fractions co-administration. The levels of GSH, GST, SOD, GPx, and CAT that were decreased by doxorubicin were significantly ($p<0.01$) elevated, and raised MDA level was reduced by the root extract and fractions. Histology of the testes sections of extract/fractions -treated animals showed absent/or reductions in the pathological features compared to the organotoxic-treated animals. The chemical pathological changes were consistent with histopathological observations, suggesting marked testicular protective potential. The anti-toxic effect of this plant may in part be mediated through the chemical constituents of the plant. The plant, *H. africana* possesses anti-toxicant properties which can be exploited in the treatment of doxorubicin-related toxicities.

Keywords: *Hippocratea africana*; anti-oxidant; oxidative stress; testicular-protective; antioxidant.

INTRODUCTION

Doxorubicin, an anthracycline glycoside antibiotic with a broad spectrum of activity against various human solid tumors and hematological malignancies (Calabresi & Chamber, 1990) but limited clinical usefulness due to its diverse toxicities, including cardiac, hepatic, hematological, and testicular toxicity (Yilmaz *et al.*, 2006). The toxic, short-lived metabolite, semiquinone form of doxorubicin, triggers the generation of reactive oxygen species (ROS) through cascades of events. Doxorubicin-induced cardio, hepatic, testicular, and nephrotoxicities have been attributed to ROS generation, inflammatory processes, and lipid peroxidation (Injac *et al.,* 2009; Kalender *et al.,* 2005). Doxorubicin has also been proposed to promote free radical generation by enhancing the activities of extra-mitochondrial oxidative enzymes such as NADPH and xanthine oxidases and interfering with mitochondrial iron export (Bachur *et al.,* 1979). These free radicals cause distortion of the cell's membranes and cause organ dysfunction. Research on plants that can counteract the toxic effects of doxorubicin has been ongoing, especially on *H. africana* (Noah *et al*., 2023c).

Hippocratea africana (Willd.) Loes. Ex Engl. (Celastraceae) known in English as African paddle-pod and Eba enang enang' in the Ibibio language in Nigeria, is a climber perennial plant distributed widely in tropical Africa (Hutchison & Dalziel, 1973). Traditionally, the plant root has been utilized in various herbal preparations to treat diseases like malaria and diabetes (Okokon *et al.*, 2006), as well as liver diseases (Ajibesin *et al.,* 2008). Previous reports showed that the root extract possess antimalarial (Okokon *et al.,* 2006;2021), angioedema and antinociceptive (Okokon *et al.,* 2008), antidiabetic and hypolipidemic (Okokon *et al.,* 2010; 2022), antidiarrhoeal and antiulcer (Okokon *et al.,* 2011), nephroprotective (Noah *et al.,* 2023a; 2023b) hepatoprotective (Okokon *et al.,* 2013a;Noah *et al.,* 2023c, Noah *et al.,* 2023d), antileishmanial, cytotoxicity and cellular antioxidant (Okokon *et al.,* 2013b), antibacterial, anticonvulsant and depressant (Okokon *et al.,* 2014), genotoxic and cytotoxic (Jonhnny *et al.,* 2023). Also, earlier studies had reported the presence of $^{\delta}$-3-Carene and α-terpineol (Okokon *et al.*, 2017), isolation of 1,3,7-trihydroxy-6-methoxyxanthone [isoathyriol] and 1,3,6,7-tetrahydroxyxanthone [norathyriol] (Umoh *et al.,* 2021) from ethyl acetate

fraction. Monoterpenes and sesquiterpenes have been identified in the *n*-hexane fraction (Okokon *et al.*, 2013a). We report testicular protective and antioxidative stress potentials of the root extract and fractions of *H. africana* against doxorubicin-induced testicular toxicity in male rats.

MATERIALS AND METHODS

Plants collection

Fresh root of *Hippocratea africana* were collected in bushes in the Uruan area, Akwa Ibom State, Nigeria in November 2021. The plant was identified and authenticated by a taxonomist in the Department of Botany and Ecological Studies, University of Uyo, Uyo, Nigeria. Hebarium specimen was deposited at Department of Pharmacognosy and Natural Medicine Herbarium, University of Uyo.

Preparation of extract and fractions

Fresh root of *H. africana* were washed, cut into smaller pieces and dried under shade for two weeks. They were powdered using an electric grinder. The pulverised root of *H. africana* (HAE) was soaked in ethanol (50%) for 72 h. The liquid filtrate obtained was concentrated in a rotary evaporator at 40˚C. The crude extract (20 g) was dissolved in 500 mL of distilled water and partitioned with equal volume of dichloromethane (DCM, 5 x 500 mL) till no colour change was observed, to obtain DCM and aqueous fractions. The extract and fractions were stored at 4˚C in a refrigerator until used for the experiment.

Animals

In this study, male albino Wistar rats were used. The animals were sourced from University of Uyo Animal house and sheltered in plastic cages. The rats were fed with pelleted standard Feed (Guinea feed) and given unlimited access to water. The study was approved by College of Health Sciences Animal Ethics Committee, University of Uyo.

Experimental design

This study used the repeated dose model earlier described by Raskovi *et al.* (2011) and Olorundare *et al.* (2020), which lasted for 14 days. Group I rats, which served as the untreated control, were orally pretreated with 10 mL/kg/day of distilled water. Group 2 rats were given normal saline (10 mL/kg/day) but equally treated on alternate days with 1.66 mg/kg of doxorubicin hydrochloride dissolved in 0.9% normal saline administered on alternate days for 14 days. Groups 3-5 rats were orally pretreated with 200 mg/kg/day, 400 mg/kg/day, and 600 mg/kg/day of *Hippocratea africana* dissolved in distilled water 2 hours before treatment with 1.66 mg/kg of doxorubicin in 0.9% normal saline administered intraperitoneally on alternate days for 14 days, respectively. Groups 6 and 7 were pretreated with 400 mg/kg of DCM and aqueous fractions, respectively. Group 8 rats, which served as the positive control group were equally pretreated with 100 mg/kg/day of silymarin two hours before treatment with 1.66 mg/kg of doxorubicin in 0.9% normal saline administered intraperitoneally on alternate days for 14 days.

Collection of blood samples and organs

After 14 days of treatment (24 hours after the last administration), the rats were weighed again and sacrificed under light diethyl ether vapour. Blood samples were collected by cardiac puncture and used immediately. Blood samples were collected into plain centrifuge tubes. The blood in the centrifuge tubes was centrifuged immediately at 1500 rpm for 15 mins to separate the serum at room temperature to avoid haemolysis and used for biochemical assays. The testes of the rats were surgically removed, weighed. One testis was fixed in 10 % formaldehyde for histological processes, while the other testis was briskly rinsed in ice cold 1.15% KCl solution and put in a clean sample bottle. These were stored in ice cold 0.9% NaCl.

Evaluation of progressive motility, viability, count, and the structural abnormality of sperm

The caudal piece of epididymis was isolated to retrieve the sperm samples. Initially, the epididymal part was finely minced in 5 mL of physiological-saline and was incubated for 30 min at 37◦C for spermatozoa releasing of the epididymal ducts. Sperm progressive motility percentage was noted through the phase-contrast microscope at 400X (Kenjale, 2008). Sperm viability was assessed, by eosin or nigrosin staining and accompanied by microscopic evaluation. Moreover, a hemocytometer was employed to count epididymal sperm in the suspension (Yokoi *et al.*, 2003). Furthermore, morphological anomalies of the head, tail, and mid piece of sperm were determined in percentage using the method of Filler (1993). The apparent abnormal characteristics included (i) the size and shape of spermatozoa heads (bigor small heads) with lighter and emphasized curvature;(ii) intermediary pieces' defects that result in untied heads; and (iii) defects of tails (short, multiple, folded, and broken tails).

Biochemical Assays.

Lipid Profile

Blood samples from the heart chamber were allowed to clot and then centrifuged at 5000 rpm to separate clear sera from the clotted blood samples. The clear samples were obtained for assays of the following biochemical parameters: serum testosterone, serum cholesterol, triglyceride and high density lipoprotein (HDL) levels of the treated rats were measured using standard colorimetric methods (Tietz, 1995). These lipid parameter determinations were done

spectrophotometrically using Fortress Diagnostic Kits® according to standard procedures of manufacturer's protocols. Serum testosterone levels were measured using the corresponding rat enzyme-linked immunosorbent assay kits (Testosterone, Cambridge, UK).

Effect of the root extract and fractions on testis oxidative stress markers

The oxidative marker assays were performed on the testes homogenates of rats that were used in this study. These oxidative stress markers were used to assess the antioxidant stress potentials of the extract.

Preparation of testis Homogenate

After the rats were sacrificed humanely under inhaled diethyl ether, the testes of the rats were surgically removed and weighed. They were briskly rinsed in ice cold 1.15% KCl solution and put in a clean sample bottle. These were stored in ice cold 0.9% NaCl. Homogenates were made in a ratio of 1 g of wet tissue to 9 ml of 1.25% KCl by using motor driven Teflon-pestle. The homogenates were centrifuged at 7000 rpm for 10 min at 4˚C and the supernatants were used for the assays of superoxide dismutase (SOD) (Marklund & Marklund, 1974), catalase (CAT) (Sinha,1972), glutathione peroxidase (GPx) (Lawrence & Burk,1976), reduced gluthathione (GSH) (Ellman,1959) & malondialdehyde (MDA) content (Esterbauer & Cheeseman, 1990). The assays were performed on testes homogenates of rats used in this study. These oxidative stress markers were used to assess the antioxidative stress potentials of the extract.

Histopathological studies

The excised testes fixed in 10 % buffered formalin were used for histological processes. They were processed and stained with haematotoxylin and eosin (H&E) (Drury & Wallington, 1980), according to standard procedures at Department of Chemical Pathology, University of Port Harcourt Teaching Hospital, Port Harcourt. Morphological changes observed and recorded in the excised organs of the sacrificed animals. Histologic pictures were taken as micrographs.

Statistical analysis

The Data collected were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-test (Graph pad prism software Inc. La Jolla, CA, USA). Values were expressed as mean ± SEM and significance relative to control were considered at $p<0.001$ and $p<0.05$.

RESULTS

Effect of root extract and fractions of *H. africana* on body and organs weights of rats with doxorubicin-induced toxicity

Administration of *H. africana* root extract and fractions to rats with doxorubicin-induced organ toxicities caused considerable improvement in the body weights compared to the organotoxic group. The crude extract caused a significant ($p<0.01$) dose-dependent effect compared to the organotoxic group, with the dichloromethane fraction treated group exerting the highest effect. The testis weight of the group treated with doxorubicin was only reduced when compared to that of the standart control group although it was not statistically significant ($p>0.05$). However, treatment of rats with doxorubicin-induced toxicities with the root extract and fractions of *H. africana* improved the testis weights insignificantly ($p>0.05$) except in the group treated with aqueous fraction (Table1).

Effect of leaf extract of *Hippocratea africana* on Seminal analysis

Table 2 shows the seminal analysis of semen from rats with doxorubicin-induced testicular toxicity. The semen appearances in all treatment groups were observed to be milky. However, the semen volume in the group administered with doxorubicin alone was found to be small (0.01 mL). There was a considerable dose-dependent increment in the semen volume of rats treated with the leaf extract (0.02 -0.04 ml). The PH of the semen samples from all the groups was 8.0. The extract/fractions-treated groups were found to have a higher percentage of viable sperm cells (75-90%) compared to the organotoxic group (55%). Silymarin-treated group had 90% viable cells while control had 80%. The viscosity of semen in all the groups was normal. At the same time, the percentage of active sperm cells in the extract/fractions-treated groups ranged from 70-90% dose-dependently compared to 55% recorded in the organotoxic group. The percentage of dead sperm cells in the organotoxic group was found to be about 40% compared to 30% in the low dose (200 mg/kg) group, 15% in the middle dose (400 mg/kg) and aqueous fraction- treated groups and 10% in the high dose (600 mg/kg) and 5% in DCM fraction and silymarin treated-groups. The sperm count for the organotoxic group was 200 cells, while 400, 450 and 600 cells were respectively recorded from 200, 400 and 600 mg/kg treated groups. The control and aqueous fraction-treated groups had sperm count of 500 cells,while silymarin, DCM and aqueous fractions-treated groups had sperm count of 600 cells (Table 2).

Effect of root extract and fraction on testes oxidative stress markers of doxorubicin-induced testes toxicity.

Table 3 shows the effect of *H. africana* root extract/fractions on testes oxidative stress markers of the

rats. Administration of doxorubicin (1.66 mg/kg i.p) on alternate days for 14 days caused significant ($p<0.05$-0.001) decreases in liver antioxidant enzymes activities (SOD, GPx, GST, CAT) and GSH levels compared to control. The MDA level was also significantly ($p<0.05$) elevated by doxorubicin treatment when compared to standart control. However, concomitant administration of root extract/fractions of *H. africana* (200 - 600 mg/kg) with doxorubicin for 14 days caused significant ($p<0.05$-0.001) and non-dose-dependent elevations of the enzymatic and non enzymatic endogenous antioxidants in the treated rats groups when compared to the organotoxic groups, with the DCM fraction exerting the highest effect. Dose-dependent and significant ($p<0.05$) decreases in MDA levels of the extract/fractions treated groups were recorded with DCM having the highest effect. A Similar decrease was also observed in the silymarin-treated group compared to organotoxic control (Table 3).

Effect of the root extract and fractions of *H. africana* on serum testosterone level of rats with doxorubicin-induced testicular toxicity

Administration of doxorubicin (1.66 mg/kg i.p) on alternate days for 14 days was found to significantly ($p<0.001$) caused decreases of serum testosterone levels of rats when compared to control. However, concomitant administration of root extract and fractions of *H africana* (200-400 mg/kg) and silymarin with doxorubicin for 14 days caused significant (p<005-0.01) dose-dependent elevation of the testosterone levels of the treated rats when compared to the organotoxic groups with the DCM fraction having the highest effect (Figure 1).

Effect of root extract and fraction of *H. africana* on lipid profile of rats with doxorubicin –induced organs toxicities

Administration of doxorubicin (1.66 mg/kg) was observed to caused significant ($p<0.05$-0.001) elevation in levels of total cholesterol, triglyceride, low density lipoprotein, and very low density lipoprotein, while the high density lipoprotein level was not significantly ($p>0.05$) elevated. The total cholesterol and low density lipoprotein levels were significantly ($p<0.05$-0.001) reduced compared to organotoxic group following concommitant treatment with root extract and fractions of *H. africana* and silymarin. Similarly, triglyceride and very low density lipoprotein levels were also reduced but the reductions were only significant ($p<0.05$) at the highest dose (600 mg/kg) of the extract. However, the high density lipoprotein level was not affected by the concommitant treatment of the doxorubicin intoxicated rats with the root extract/fractions as well as silymarin (Table 4).

Effect of root extract and fractions of *H. africana* on histology of rat testis in doxorubicin-induced testicular toxicity

Histological sections of testes of rats receiving various treatments at magnification (x400) stained with the H&E method revealed that Group 1 (standard control, A) rat treated with distilled water (10 mL/kg) showed a well preserved cellular and connective tissue architecture, multiple stages of seminiferous tubules with lumen and developing germinal cell layers, normal tunica albuginea and no evidence of pathological changes were seen. The organotoxic group (Group 2, B) treated with doxorubicin (1.66 mg/kg) showed multiple seminiferous tubules with ceased germinal cell maturation, focal dilated congested blood vessels were seen. The basal seminiferous tubule showed germinal epithelium degeneration (Figure 2). Rats in group 3 (C) treated with 200 mg/kg of *H. africana* root extract and doxorubicin (1.66 mg/kg), group 4 (D) treated with 400 mg/kg of *H. africana* root extract and doxorubicin (1.66 mg/kg), group 5 (E) treated with 600 mg/kg of *H. africana* root extract and doxorubicin (1.66 mg/kg), group 6 (F) treated with 400 mg/kg of aqueous fraction of *H. africana* root and doxorubicin (1.66 mg/kg), group 7 (G) treated with 400 mg/kg of dichloromethane fraction of *H. africana* root and doxorubicin (1.66 mg/kg) and group 8 (H) treated with 100 mg/kg of silymarin of *H. africana* root and doxorubicin (1.66 mg/kg) had testis sections that showed well preserved cellular and connective tissue architectures, multiple stages of seminiferous tubules with lumen, developing germinal cell layers, normal tunica albuginea and no evidence of pathologic changes were found except focal germinal cell vacuolation that was seen in group 6 (Figure 2 A-H).

Table 1. Effect of *H. africana* root extract on body and testis weights of rats with doxorubicin-induced toxicity.

PARAMETERS/ TREATMENT	Dose mg/kg	Testis(g)	Body weight(g)		
			Before	After	% increase in body weight
Normal control	-	2.42±0.29	135.6±18.34	151.0± 12.33	11.35
Doxorubicin	1.66	1.78±0.5	130.0 ± 9.45	126.3± 10.43	-2.84
Silymarin+DOX	100	2.39±0.35	132.6± 14.55	143.2 ± 3.15	7.99
Extract+DOX	200	2.20±0.35	142.8± 10.56	153.0± 5.29	7.14
	400	2.42±0.03	140.3± 7.36	151.6 ± 6.22	8.05
	600	2.41±0.19	138.4 ± 8.54	149.6 ± 8.48	8.09
Aqueous fraction	400	1.87±0.24	138.4 ± 6.26	144.6± 10.22	4.47
DCM fraction	400	2.50±0.14	134.3± 8.50	146.8± 13.20	9.30

Data are expressed as mean ±SEM. Not significant at $p>0.05$-compared to normal control and organotoxic control. n = 6.

Table 2. Effect of root extract of *H. africana* on seminal analysis of rats with doxorubicin-induced testicular toxicity.

Parameters	Control	Doxorubicin	Silymarin	Extract 200 mg/kg	Extract 400 mg/kg	Extract 600 mg/kg	DCM fraction	Aqueous fraction
App	Milky	Milky	milky	milky	milky	milky	milky	milky
Volume	0.03mL	0.01mL	0.02mL	0.02mL	0.03mL	0.03mL	0.043mL	0.02mL
PH	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Viability	80%	55%	90%	78%	80%	90%	90%	80%
Viscosity	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Normal	80%	55%	90%	70%	80%	80%	90%	80%
Abnormal	20%	45%	10%	32%	20%	10%	10%	20%
Active	80%	50%	90%	70%	75%	80%	90%	75%
Sluggish	10%	10%	5%	10%	10%	10%	5%	10%
Dead	10%	40%	5%	30%	15%	10%	5%	15%
Sperm	500	200	600	400	450	600	600	500

Table 3. Effect of *H.africana* root extract and fractions on testes oxidative stress markers of rats with doxorubicin-induced toxicity.

Treatment	Dose mg/kg	SOD (U/mL)	CAT (U/g of protein)	GPx (µg/mL)	GSH (µg/mL)	GST	MDA (µMol/mL)
Control	10	0.55± 0.02	1.32±0.01	0.071±0.002	1.41±0.11	0.46±0.02	0.24±0.01
Doxorubicin	1.66	0.20 ± 0.01^{c}	0.50 ± 0.01^{a}	0.030 ± 0.001^{a}	0.46 ± 0.01^{c}	0.28 ± 0.02^{b}	0.66 ± 0.02^{c}
Crude extract	200	$0.35\pm0.04^{a,d}$	1.34 ± 0.15^{c}	0.061±0.001	$1.02\pm0.15^{a,e}$	0.59 ± 0.01^{f}	0.51 ± 0.01^{b}
	400	$0.33\pm0.04^{a,d}$	1.18 ± 0.10^{c}	0.069 ± 0.001^{f}	1.19 ± 0.06^{f}	0.52 ± 0.01^{e}	$0.45\pm0.02^{b,e}$
	600	0.42 ± 0.02^{e}	$1.26\pm0.22^{c,e}$	0.058±0.003	1.28 ± 0.12^{f}	0.43 ± 0.02^{e}	$0.42\pm0.01^{a,d}$
Aqueous fraction	400	0.29 ± 0.03^{c}	1.05 ± 0.36^{f}	0.045 ± 0.005^{c}	$0.74\pm0.15^{b,d}$	0.48 ± 0.03^{e}	$0.50\pm0.01^{b,d}$
DCM fraction	400	0.48 ± 0.02^{e}	$1.12\pm0.42^{c,d}$	0.070 ± 0.002^{f}	1.25 ± 0.11^{f}	0.59 ± 0.05^{f}	$0.40\pm0.01^{a,e}$
Silymarin	100	0.50 ± 0.04^{f}	1.14 ± 0.31^{c}	0.057 ± 0.002^{c}	1.37 ± 0.11^{f}	0.45 ± 0.02^{f}	0.35 ± 0.02^{f}

Data are expressed as MEAN ± SEM, Significant at $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$, when compared to control; Significant at $^{d}p<0.05$, $^{e}p<0.01$, $^{f}p<0.001$ compared to organotoxic group.. (n=6)

Table 4. Effect of *H.africana* root extract and fractions on lipid profile parameters of rats with doxorubicin-induced toxicity.

Treatment	Dose mg/kg	TOTAL CHOLESTEROL (mMol/L)	TRIGLYCERIDE (mMol/L)	HDL-C (mMol/L)	LDL-C (mMol/L)	VLDL (mMol/L)
Control	10	2.25± 0.09	0.75±0.07	1.25±0.03	1.34± 0.11	0.34± 0.03
Doxorubicin	1.66	3.63 ± 0.20^{c}	1.45 ± 0.04^{c}	1.51±0.11	2.77 ± 0.18^{c}	0.66 ± 0.01^{c}
Crude extract	200	2.86 ± 0.20^{d}	1.29 ± 0.11^{b}	1.38±0.10	2.03± 0.25	0.58 ± 0.05^{b}
	400	3.03 ± 0.13^{a}	1.31 ± 0.03^{b}	1.33±0.06	2.43± 0.18	0.59 ± 0.01^{b}
	600	2.46 ± 0.14^{f}	0.97 ± 0.10^{e}	1.38±0.06	1.52 ± 0.16^{e}	0.43 ± 0.04^{a}
Aqueous Fraction	400	2.85 ± 0.06^{f}	1.39 ± 0.04^{c}	1.31±0.09	1.37 ± 0.12^{f}	0.63 ± 0.02^{c}
DCM fraction	400	2.33 ± 0.12^{f}	1.32 ± 0.15^{b}	1.36±0.08	1.57 ± 0.12^{e}	0.60 ± 0.07^{b}
Silymarin	100	2.30 ± 0.15^{f}	1.37 ± 0.04^{c}	1.41±0.03	1.30 ± 0.13^{f}	0.62 ± 0.01^{b}

Data are expressed as MEAN ± SEM, Significant at $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$, when compared to control;Significant at $^{d}p<0.05$, $^{e}p<0.01$, $^{f}p<0.001$ compared to organotoxic group. (n=6)

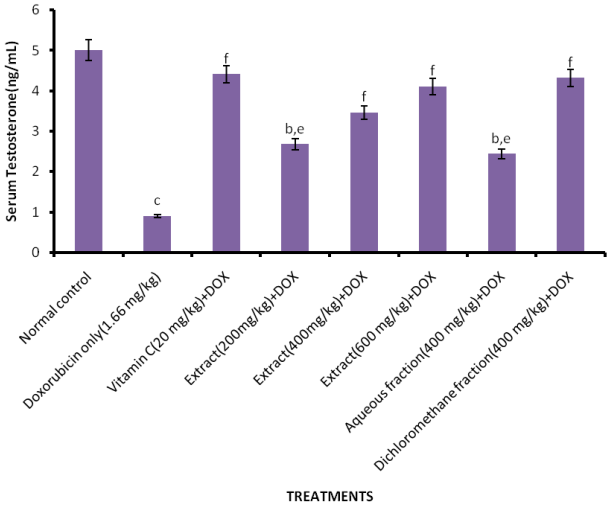

Figure 1. Effect of root extract and fractions of *H. africana* on serum testosterone levels of rats with doxorubicin-induced testicular toxicity.

Data are expressed as MEAN ± SEM, Significant at $^{b}p<0.01$; $^{c}p<0.001$ compared to standart control; $^{e}p< 0.01$, $^{f}p< 0.001$, compared to organotoxic control. (n=6).

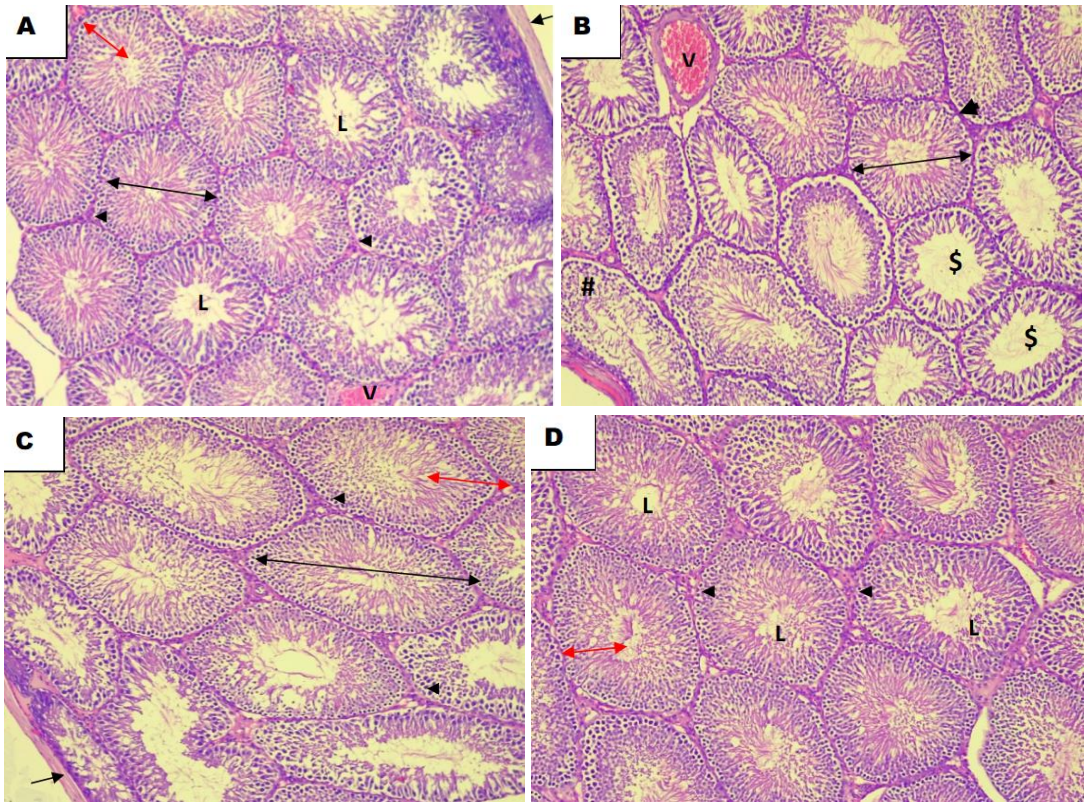

Figure 2. Photomicrograph of testis section of rat treated with (A) distilled water (10mL/kg),(B) doxorubicin (1.66 mg/kg), (C) 200 mg/kg of *H. africana* root extract and doxorubicin (1.66 mg/kg), (D) 400 mg/kg of *H. africana* root extract and doxorubicin (1.66 mg/kg) (D) showing a well preserved cellular and connective tissue (**arrowhead**) architecture, multiple stages of seminiferous tubules (**double end arrow**) with lumen (**L**), developing germinal cell layers (**Red double end arrows**), normal tunica albuginea (**thin arrow**), multiple seminiferous tubule with with ceased germinal cell maturation (**$**) , focal dilated congested blood vessel (**V**), basal seminiferous tubule showing germinal epithelium degeneration (**#**).H&E stain x100 magnification.

DISCUSSION

Doxorubicin, an anthracycline used in the treatment of tumours (Konopa *et al.*, 1988; Thorn *et al.*, 2012), exhibits undesirable severe effects on the male reproductive system (Sridevi *et al.*, 2012), such as disruption of normal reproductive functions via the induction of oxidative stress. Investigation of agents with antagonistic potentials to counteract the toxic effects of doxorubicin is ongoing. This study was carried out to

assess the effects of root extract of *H. africana* on doxorubicin-induced male reproductive system toxicity.

The exact mechanisms through which doxorubicin induces toxicity in the male reproductive system, though reported widely, have not been adequately elucidated. The drug reportedly retards testicular growth, suppresses spermatogenesis, leading to male infertility through imposing oxidative stress and cellular apoptosis (Yeh *et al.,* 2007). It also causes double-strand DNA breaks and cell death by intercalating into DNA strands (Konopa *et al.,* 1988; Gewirtz *et al.,* 1999) in meiotically dividing spermatocytes and spermatogonia (Cabral *et al.,* 2014). Spermatogenesis suppression, sperm motility impairment, increased percentage of abnormal spermatozoa, decreased body and testicular weights, reduced testosterone levels, and testicular failure through oxidative stress and cell apoptosis in testicular tissue have also been reported (Howell & Shalet, 2005). In this study, doxorubicin was found to cause a significant increase in the percentages of dead and abnormal cells. Also, decreased seminal volume and sperm count were observed in the doxorubicin alone treated group. Similarly, testes from rats treated with doxorubicin alone were found to have marked degenerated germ cells and spermatozoa, supporting previous reports (Howell & Shalet, 2005). However, all these toxic effects of doxorubicin were alleviated by concomitant administration of root extract and fractions of *Hippocratea africana*, as animals treated with the root extract/fractions had high sperm cell count, greater seminal volume, low level of dead cells as well as standart testicular architecture, portraying the extract's potentials in preventing doxorubicin-induced testicular toxicity probably through the antioxidative stress activity of its phytochemical components. The antioxidative burst and antioxidant activities of the root extract and fractions of *H. africana* had previously been reported (Okokon *et al.,* 2013a; 2022; Umoh *et al.,* 2021). Moreover, the antioxidative stress activities of the root extract and fractions observed in this study further support the antioxidant potential of the plant. These activities may have contributed to the observed protective effects in this study.

The membranes of the male germ cells have a high amount of polyunsaturated fatty acids, one of the targets of reactive oxygen species (Robinson *et al.,* 1992). Thus, spermatozoa are vulnerable to oxidative damage because of the high amount of lipids in their membranes, making them lose their integrity and become less motile (Robinson *et al.,* 1992). This effect could have contributed to the low sperm count, high percentages of dead and abnormal cells observed in the group treated with doxorubicin only in this study. Doxorubicin has been shown to impair male fertility by causing germ cell oxidative stress and apoptosis (El-Maddawy and El-Naby, 2019., Aksu *et al.,* 2019). It has also been demonstrated to impair spermatogenesis (Kato *et al.,* 2001) and steroidogenesis (Rizk *et al.,* 2014; Olusoji *et al.,* 2017). Exposure to doxorubicin appears to affect testicular integrity at both prepubertal and post-pubertal stages of development. *In vitro* studies with prepubertal mouse testis have demonstrated significant loss in germ cell number following exposure to doxorubicin at concentrations that were equivalent to human therapeutic doses (Smart *et al.,* 2018). Further, studies have demonstrated early testicular developmental arrest and long-term germ cell DNA damage following prepubertal doxorubicin exposure (Vendramini *et al.,* 2010). The study observed these effects as marked degenerated germ cells and spermatozoa were seen in the histological sections of the testes. However, concomitant administration of the root extract/fractions protected the testes as the toxic effects observed in the doxorubicin alone treated group were absent or mild compared to that group treated doxorubicin alone. The high susceptibility of testes and sperm to doxorubicin -induced testicular oxidative stress may be due to a weak anti-oxidant defence system in testicular tissue and semen (Nowrouzi *et al.,* 2019). Besides, earlier studies demonstrated that antioxidant supplementation improved the quality of the semen profile in infertile men (Talevi *et al.,* 2013).

Lipids are an essential part of the reproductive system. Cholesterol is considered to be the precursor of steroid hormones. Steroidogenesis plays an essential role in the synthesis of spermatogenesis hormones. The biosynthesis of testosterone from pregnenolone is carried out by steroidogenesis enzymes including 17β hydroxysteroid dehydrogenase (17β-HSD) 3β and hydroxysteroid dehydrogenase (3β- HSD). It has been reported that doxorubicin results in the downregulation of these enzymes (Prahalathan *et al.,* 2006; Takashima, *et al.,* 2009). Adipocytes are the primary sites for triacylglycerol storage. It has been found that DOX downregulates adipogenesis *in vitro* by decreasing the expression of PPARγ (Arunachalam *et al.,* 2012). Doxorubicin inhibits spermatogenesis by causing defects in epididymal adipose tissue (Tirupathi Pichiah *et al.,* 2012), which is crusial for normal spermatogenesis (Chu *et al.,* 2010). Also, studies using adult rat models of DOX exposure have reported decreased testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels, decreased sperm count, motility and viability, and increased abnormally formed spermatozoa (Das *et al.,* 2012). The results of this study showed that testosterone level was significantly reduced in the group treated with doxorubicin alone compared to standart control. However, the root extract and fractions-treated groups had improved levels of testosterone which further point to the protective effect of the extract against doxorubicin-induced toxicity. This result agrees with an earlier report by Ndem and Johnson (2017) in which improved testosterone concentration was reported following *H. africana* root administration.

Oxidative stress plays an essential role in doxorubicin-induced toxicity by forming reactive oxygen species (ROS) (Arunachalam *et al.,* 2021). The results of

the doxorubicin alone treated group in this study revealed a significant elevation of testicular MDA levels and a significant decreases in testicular SOD, CAT, GST, GPx and GSH when compared with the DOX group. These results are from previous reported studies (Khodir *et al.*, 2021). However, co-treatment of root extract/fractions and DOX elevated the levels of these endogenous antioxidants revealing the free radicals scavenging potentials of the root extract/fractions and its antioxidative stress activity which is due to the activities of its phytochemical constituents such as monoterpenes, sesquiterpenes and xanthones earlier reported by Okokon *et al*.(2013a) and Umoh *et al.*, (2021) to be present in this root extract.

CONCLUSION

The findings of this study showed that the root extract and fractions of *Hippocratea africana* possess testicular protective potentials against doxorubicin-induced testicular toxicity. These properties can be attributed to its phytochemical constituents' antioxidant and antioxidative stress activities. Thus, the root can be used to alleviate and/or prevent doxorubicin-induced male reproductive system toxicity.

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