<u>Original Research Article</u>

Hippocrateaafricana root extract and fractions ameliorates carbon tetrachloride-induced oxidative stress and kidney injuries in rats.

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Abstract

Aim:*Hippocrateaafricana*root used locally in the treatment of poisoning was investigated to confirm its antidotal potential in rats.

Methodology: The root extract (200-600 mg/kg) and fractions; dichloromethane (DCM) and aqueous, 400 mg/kg, were evaluated for hepatoprotective activity against paracetamol-induced liver injury in rats. Assay of liver function parameters, antioxidative stress markers as well as histopathological study of the liver were used to assess hepatoprotective activity of root extract and fractions.

Results: Administration of the root extract (200-600 mg/kg) caused significant (*P*<.05-.001) reductions in the levels of liver biomarker enzymes (ALT, AST, and ALP), direct and total bilirubin and elevation of serum level of total protein elevated by paracetamol administration. The effects were dose-dependent in most cases. Histology of the liver sections of extract, fractions and silymarin-treated animals showed reductions in the pathological features compared to the organotoxic-treated animals. The chemical pathological changes were consistent with histopathological observations suggesting marked hepatoprotective potentials.

Conclusion: The results showed that root extract and fractions of *Hippocrateaafricana* have hepatoprotective antioxidative stress potentials against injurious agents which may be due to the activities of its phytochemical components.

Keywords: *Hippocrateaafricana*, anti-toxicant, oxidative stress, liverprotective, antioxidant

1.0 Introduction

Hippocrateaafricana (Willd.) Loes. ex Engl. (Celastraceae) syn. *Loeseneriellaafricana* (Willd.) N.Halléis a green forest perennial climber widely distributed in tropical Africa [1] It is commonly known as African paddle-pod and 'Eba enangenang' by the Ibibios of Nigeria. The plant root has been variously used traditionally by the Ibibios of the Niger Delta region of Nigeria to treat ailments such as fever, convulsion, malaria, body pains, diabetes and diarrhea [2]. The plant's root is also use for its antidote or antipoison potential to treat liver diseases such as jaundice and hepatitis [3-5].Previous reports showed that the root extract possess antimalarial [2,6], antioedema and antinociceptive [7], antidiabetic and hypolipidemic [8,9], antidiarrhoeal and antiulcer [10], hepatoprotective [11], antileishmanial, cytotoxicity and cellular antioxidant [12], antibacterial, anticonvulsant and depressant [13], *in vivo* alpha amylase and alpha glucosidase inhibitory [6], *in vitro* antioxidant[9,14] activities. Earlier studies had reported the presence of spirohexane-1-carboxylic acid, ethyl ester, 3-methoxy-2-methylphenol,2,3-benzofurandione,6-hydroxy-4-(p-hydroxybenzyl), $^{\delta}$-3-Carene and α-terpineol in ethyl acetate fraction[15]and the presence of monoterpenes (thujene, limonene, linalool, α-phellandrene, α-terpineol and sabinene) and sesquiterpenes (dehydromevalonic lactone), in the n-hexane fraction of the root extract[11]. Also, two xanthones; 1,3,6,7-tetrahydroxyxanthone and 1,3,6-trihydroxy-7-methoxyxanthone, have been isolated from the root of *H.*

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Africana[14]. We report hepatoprotective and antioxidative stress effects of the root extract and fractions of *H. africana*against paracetamol-induced liver injury in rats.

2.0 Materials and methods

2.1 Plants collection and preparation of extract and fractions

Fresh roots of *Hippocrateaafricana*were collected in bushes in 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. Fresh root of *H. africana* were washed, cut into smaller pieces and dried under shade for two weeks. They were powdered using 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

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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.

2.2 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.

2.3 Effect of ethanol root extract and fractions of *Hippocrateaafricana*on paracetamol-induced toxicity in rat

In this model, forty (40) rats were randomly divided into eight (8) groups of 5 rats each and treated as follows; Group 1- served as the control group (normal control) and animals in this group received 10 mL/kg of distilled water orally for eight consecutive days. Groups 2- served as the organotoxic group. The animals were administered with 10 mL/kg of normal saline orally for 8 days. Groups 3 - 5 served as the extract treated groups. Animals in groups 3- 5 were respectively and orally administered with 200, 400 and 600 mg/kg of root extract daily for 8 days. Animals in group 6 and 7 were pretreated with 400 mg/kg of DCM and aqueous fractions respectively for 8 days. Group 8 served as the positive control group. Animals were orally administered with 100 mg/kg of Silymarin for 8 days.

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On the eighth (8th) day, animals in groups 2 - 8 received paracetamol (2g/kg, i.p) dissolved in corn oil mixed at a ratio of 1:3. Twenty-four hours after paracetamol administration, all animals were weighed again and sacrificed under light diethyl ether vapour.

2.4 Collection of blood samples and organs

After 8 days of treatment (24 hours after the last treatment) the rats were weighed again and sacrificed under light diethyl ether vapour. Blood samples were collected by cardiac puncture and used immediately. Blood were collected into plain centrifuge tubes. The blood in the centrifuge tubes were centrifuged immediately at 2500 rpm for 15 minutes to separate the serum at room temperature to avoid haemolysis and used for biochemical assays. The livers were surgically removed, weighed and fixed in 10% formaldehyde for histological process.

2.5 Biochemical analysis

2.5.1 Liver function test

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The following parameters were determined; Aspartate transaminase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Total plasma protein and Total and direct bilirubin. The determinations were done spectrophotometrically using Randox analytical kits according to standard procedures of manufacturer's protocols[16] at the Chemical Pathology Department of University of Uyo Teaching Hospital.

2.5.2 Oxidative stress markers

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

2.5.3 Preparation of liver homogenate

After the rats were sacrificed humanely under inhaled diethyl ether, the liver of the rats was 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)[17], catalase (CAT)[18], glutathione peroxidase

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(GPx)[19], reduced gluthathione (GSH)[20]and malondialdehyde (MDA) content[21].The assays were performed on liver homogenates of rats that were used in this study. These oxidative stress markers were used to assess antioxidative stress potentials of the extract.

2.6 Histopathological studies

The excised livers fixed in 10 % buffered formalin were used for histological processes. They were processed and stained with haematoxylin and eosin (H&E)[22], according to standard procedures at Department of Chemical Pathology, University of Uyo Teaching Hospital, Uyo. Morphological changes observed and recorded in the excised organs of the sacrificed animals. Histologic pictures were taken as micrographs.

2.7 Statistical analysis and data evaluation

Data obtained from this work were analysed statistically using ANOVA (one –way) followed by a post test (Tukey-Kramer multiple comparison test). Differences between means were considered significant at 5% level of significance ie $P \leq .05$.

3.0 Results and Discussion

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The present study was done to investigate the hepatoprotective and antioxidative stress activities of *H. africana*root extract and fractions using adult male wistar rats against paracetamol-induced liver injury and oxidative stress. Effect of the *H. africana*root extract and fractions on liver function test, oxidative stress markers and histology were used as parameters to assess these properties.The administration of paracetamol to rats caused significant (P<.001) increase in liver weight when compared to normal control. However, the administration of root extract and fraction of *H. africana* to rats with PCM -induced toxicities caused a non-dose dependent significant (P<.001) reduction in liver weights of the rats especially in the extract treated groups when compared to paracetamol group. Similarly, paracetamol as well as the extract/fractions treatment did not cause any significant (P>.05) effect on the kidney weights of the rats (Table 1).

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Table 1: Effect of *H. africana*root extract on organs weights of rats with PCM-induced toxicity

Parameters/treatment	Dose mg/kg	Liver
Normal control	-	7.55±0.17
Paracetamol	2000	6.90± 0.18^{c}
Silymarin+PCM	100	6.00±0.13^{c,e}
Extract+PCM	200	5.41±0.14^{c,f}
	400	6.99±0.12
	600	5.40±0.16^{c,f}
Aqueous fraction	400	8.75±0.17^{c}
DCM fraction	400	7.97±0.23^{c}

Data were expressed as mean ±SD. significant at $^{c}P<.001$ when compared to normal control; $^{d}P<.05$, $^{e}P<.01$, $^{f}P<.001$ when compared to organotoxic control. $n = 5$.

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The widely used painkiller and antipyretic drug, Paracetamol, has toxic effects at high doses that cause liver damage. The drug is bioactivated to the hazardous electrophile, N-acetyl p-benzoquinone imine (NAPQI), which binds to tissue macromolecules covalently and likely oxidizes lipids or the essential sulphydryl groups (protein thiols) as well as changes calcium homeostasis[23]. Massive reactive species formation may result in the loss of protective physiological moieties like glutathione and -tocopherol, which could harm the macromolecules in crucial biomembranes and cause liver damage[24].

Administration of paracetamol (2 g/kg) to rats caused significant (*P*<.01-.001) elevations in the levels of AST, ALT, ALP, total and combined bilirubin and decrease in total protein and albumin levels when compared to control. Pre-treatment with root extract and fractions of *H. africana* (200 – 600 mg/kg) caused observable significant (*P*<.05-.001) decreases of these enzymes levels and that of total and combined bilirubin in the extract/fractions-treated groups when compared with the organotoxic group. However, these decreases were non-dose dependent with the DCM fraction having the highest effect. Total protein and albumin levels were significantly (*P*<.01-.001) elevated non-dose dependently in the groups pre-treated with the root extract/fractions when compared to the paracetamol group with DCM fraction having the highest effect (Table 2).

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Liver function was assessed by estimating the activities of serum ALT, AST, ALP, bilirubin (total and direct) and total protein that are originally present in the cytoplasm[25]. These enzymes and chemicals leak into the bloodstream during hepatopathy, serving as a sign of the liver injury[26]. The abnormally high serum concentrations of ALT, AST, ALP, total and direct bilirubin, as well as the decrease in total protein level that were found in the paracetamol group in this study are signs of paracetamol-induced liver dysfunction and point to hepatic cell destruction.The ameliorative impact of the extract/fractions via inhibition of the leakage of intracellular enzymes by its membrane stabilizing activity may have contributed to the reversal of paracetamol-induced increased serum enzymes in the groups treated with the extract/fractions. Increase in serum level of ALP in the paracetamol group is due to increased synthesis, in the presence of increasing biliary pressure[27] and reflects the pathological alteration in biliary flow[28]. Therefore, an improvement in the level of ALP in the pre-treated rats suggests that the extract can be useful in management and prevention of conditions such as gallstone and cholescystitis.

In the present study, reduction in serum total protein and albumin levels were observed in the paracetamol-treated rats which may be associated with decreased number of hepatocytes resulting in decreased hepatic capacity to synthesize protein. The decreased level of total protein as recorded in paracetamol-treated rats revealed the severity of hepatopathy. This negative effect on total protein was significantly and

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dose-dependently improved in the extract/fractions pre-treated groups, indicating an improvement of the functional status of the liver cells by the root extract/fractions. Bilirubin, a metabolic product of hemoglobin, undergoes conjugation with glucuronic acid in hepatocytes to increase its water solubility. Any abnormal increase in the levels of serum bilirubin indicates hepatobiliary disease and severe disturbance of hepatocellular function. Hence, determination of serum bilirubin represents an index for the assessment of hepatic function[29]. Paracetamol caused elevated amount of bilirubin in the blood of the administered rats which were significantly lowered in the root extract and fractions pre-treated group. Decreased serum bilirubin level following extract treatment indicated the effectiveness of the extract to restore normal functional status of the liver.

Administration of paracetamol was found to significantly (P<.01-.001) reduced the levels of GSH, GPx, CAT, GST and SOD, while MDA level was highly increased. Treatment of rats with PCM-induced organs injuries with root extract and fractions *of H. africana* and silymarincaused significant (P<.05-.001) and non-dose dependent elevation in the levels of GSH, GPx, CAT, GST and SOD when compared to the organotoxic group. However, concomitant treatment of the rats with root extract and fractions of *H. africana* caused reductions in the levels of MDA of

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various treatment groups which were only significant in the groups treated with the highest dose (600 mg/kg) of the extract and silymarin (Table 3).

SOD and CAT are significant antioxidant enzymes in the enzymatic antioxidant defence system since they participate in the scavenging of free radicals to produce hydrogen peroxide and safer molecules, thereby lowering the harmful effect caused by these radicals[30]. The root extract and fractions have been observed in this study to increase hepatic enzymatic and non-enzymatic antioxidants such as SOD, GPx, CAT and GSH and also decrease MDA level, indicating the involvement of reactive free radicals scavenging activity, thereby reducing oxidative damage to the tissues besides improving activity of hepatic antioxidant enzymes. This finding corroborates earlier report by[11].

This effect could be due to the free radicals scavenging potentials of the root extract/fractions and its antioxidative stress activity [6, 11], which can be attributed to the activities of its phytochemical constituents such as monoterpenes, sesquiterpenes and xanthones earlier reported to be present in this root extract[6,11,14].

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Table 2: Effect of *H. africana*root extract and fractions on liver function parameters of rats with Paracetamol-induced toxicity

Treatment	Dose mg/kg	Total protein(g/dL)	Albumin (g/dL)	Total Bilirubin (µmol/L)	ALT (U/L)	ALP (U/L)	AST (U/L)	Combined Bilirubin (µmol/L)
Control	10	75.0± 2.55	47.50±0.64	3.95±0.18	9.15± 0.18	30.75± 2.42	17.0± 1.08	2.57± 0.22
Paracetamol	2000	56.0± 2.01^{c}	34.0±1.68^{c}	8.37±0.30^{b}	32.25± 1.49^{c}	61.75± 2.75^{c}	41.50± 2.02^{c}	5.85± 0.42^{c}
Crude extract	200	76.50±1.55^{f}	45.50± 1.32^{f}	7.32±0.19^{b,f}	29.75± 1.88^{b}	44.0±1.08^{c,d}	36.50± 0.64^{b}	4.32± 0.23^{a,d}
	400	69.0±1.68^{f}	41.25±1.49^{d}	7.42±0.51^{b,f}	28.0± 1.95^{b}	44.25± 3.56^{c,d}	36.5± 2.59^{b}	3.15± 1.04^{b}
	600	69.75±2.01^{f}	41.50± 1.55^{d}	6.80±0.51^{a,f}	21.5± 2.90^{e}	37.25± 1.75^{f}	33.50±2.50^{b,d}	4.95± 0.49^{a,e}
Aqueous	400	60.50±1.32^{a}	40.5±1.04	4.90±0.28^{f}	14.25± 0.90^{f}	47.25± 0.85^{c,e}	24.25± 0.85^{f}	2.57± 0.22^{f}
Fraction DCM fraction	400	65.0±1.22^{d}	41.25±1.49^{d}	4.92±0.26^{f}	13.95± 0.75^{f}	51.75± 3.68^{c}	24.25± 1.37^{f}	2.35± 0.15^{f}
Silymarin	100	66.50±2.32^{d}	41.0±2.18	3.52±0.17^{f}	11.12± 2.17^{e}	40.75± 1.25^{f}	13.75± 0.85^{f}	1.90± 0.16^{f}

Data is expressed as MEAN ± SD, 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. $n = 5$.

Table 3: Effect of *H. africana*root extract and fractions on liver oxidative markers of rats with paracetamol-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	0.35± 0.03	5.85±0.24	0.091±0.002	2.01± 0.08	0.53±0.07	0.41± 0.03
Paracetamol	2000	0.18± 0.01[c]	1.41±0.18[c]	0.036±0.02[b]	0.93± 0.07[c]	0.31±0.03[b]	0.61± 0.02[b]
Crude extract	200	0.20±0.01[b]	2.14± 0.25[f]	0.053±0.04[d]	1.56± 0.16	0.36±0.02[a]	0.59± 0.01[a]
	400	0.29±0.02	1.91±0.30[b]	0.046±0.09[a]	1.02± 0.21[b]	0.33±0.02[a]	0.51±0.03
	600	0.32±0.03	2.63± 0.15[f]	0.068±0.01[d]	1.48± 0.24	0.55±0.03[e]	0.48±0.04[d]
Aqueous Fraction	400	0.18±0.01	2.40±0.16[f]	0.048±0.01[a]	1.12± 0.16[e]	0.24±0.03[c]	0.59± 0.02[a]
DCM fraction	400	0.28±0.02	2.16±0.15[f]	0.040±0.001[b]	1.82± 0.03[f]	0.35±0.01[a]	0.50± 0.02
Silymarin	100	0.43±0.01[f]	3.56±0.32[f]	0.046±0.001[a]	1.04± 0.01[d]	0.40±0.01[d]	0.34± 0.02[e]

Data is expressed as MEAN ± SD, Significant at [a]$P<.05$, [b]$P<.01$, [c]$P<.001$, when compared to control; Significant at [d]$P<.05$, [e]$P<.01$, [f]$P<.001$ compared to organotoxic group. $n= 5$.

Histological sections of livers of rats receiving various treatments revealed that Group 1 (normal control, A) treated distilled water (10 mL/kg) had normal cellular profile of portal triad, bile duct, hepatic artery, hepatic vein, hepatocytes and nucleus, all within normal cellular architecture (Figure 1). The organotoxic group (Group 2, B) treated with paracetamol (2 g/kg) revealed cellular area of cellular abnormalities including diffused necrotic area evidence with karyolysis and haemorrhagic parenchyma. Very few lobules showed normal parenchymal with normal arrays of hepatocyte when compared to control group (Figure 1). Group 3 (C) rat treated with 200 mg/kg of *H. africana* root extract and PCM showed partly normal area with normal arrays of hepatocytes and mildly heamorrhagic sinusoidal spaces. The parenchymal showed activated kupfer cells and focal fatty changes. Also seen were multifocal area of hepatocytes drop out and confluence necrosis (Figure 1). Rats in group 4 (D) treated with 400 mg/kg of *H. africana* root extract and PCM showed partly normal areas with arrays of hepatocytes and hydropic hepatocytes (ballooning degeneration). The parenchymal showed multiple areas of confluence necrosis. Group 5 (E) rats treated with 600 mg/kg of *H. africana* root extract and PCM had liver section showing multifocal area of hepatocytes drop out and confluence necrosis, area of inflammatory infiltrate and hyaline degeneration. Normal multiple lobule with normal hepatocytes were present. Liver section of rats in group 6 (F) treated with aqueous fraction of *H. africana*root and PCM (2000 mg/kg) showed normal arrays of liver cells and moderately congested vessels. Liver section of rats in group 7 (G) treated with dichloromethane fraction (400 mg/kg) of *H. africana* root and PCM (2000 mg/kg) showed normal arrays of hepatocytes and portal triad without any lesion observed. The silymarin treated rats with PCM-induced toxicity (Group 8, H) had liver section that revealed normal arrays of hepatocytes and blood vessels with no visible lesion seen (Figure 1).

Histologically, paracetamol was observed to have caused severe cellular degeneration and other abnormalities which were much reduced in the root extract/fractions pre-treated groups.

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Histology result agrees with values obtained from liver function parameters that the extract may exert a dose – dependent hepatoprotective effect on paracetamol induced liver toxicity.

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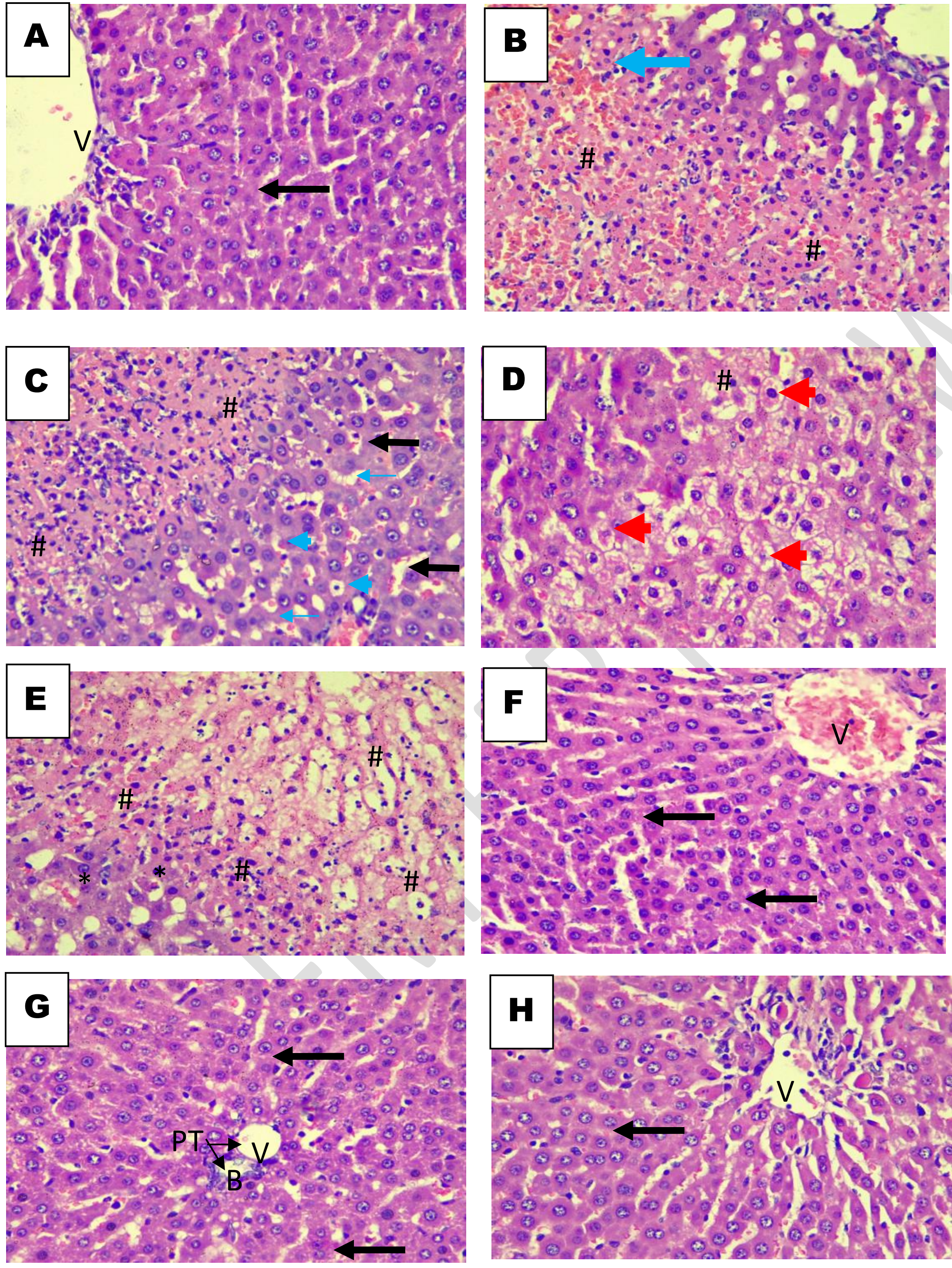

Figure 1: Liver histological section of rat treated with distilled water 10 mL/kg(A), Paracetamol 2g/kg (B), *H. africana* extract 200 mg/kg (C), 400 mg/kg, (D), 600 mg/kg (E), Aqueous fraction (F), DCM fraction (G), Silymarin 100 mg/kg (H) showing normal arrays of liver cells (black arrows) and moderately dilated vessels (V),confluence necrosis (#), area of inflammatory infiltrate and hyaline degeneration (asterisk),activated kupfer cells and focal fatty changes (white arrow) H&E stain, x400 magnification.

4.0 Conclusion

The results of this study suggest that the root extract and fractions of *H. africana* possess liver protective and antioxidative stress potentials against injurious substance which is through the activities of its phytochemical constituents.

Ethical approval

All authors hereby declare that 'Principles of laboratory animal care' (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. The study was approved by College of Health Sciences Animal Ethics Committee, University of Uyo.

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