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Selected Plant Extracts Attenuates Dimethylamine and Sodium Nitrite Induced Liver Injury in Experimental Mice

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Authors' contributions

Authors AA, OAO and IFA wrote the manuscript. Authors IFA, FAA and AA performed the experiments. Authors FAA, OSO, IFA performed literature review and the statistical analysis. Authors AA, OAO and OSO designed the research. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Dimethylamine (DMA) and sodium nitrite ($NaNO_2$) are present in numerous foods, food additive and environmental factors, which enhance chemical driven liver damage by inducing oxidative stress and cellular injury. Therefore, this study evaluated the possible therapeutic and protective effects of selected plant extracts on dimethylamine (DMA) and sodium nitrite ($NaNO_2$)-induced hepatotoxicity in mice.
The selected plants (*Morinda lucida, Securine gavirosa, Xylopia aethiopica, Piper guineense* and *Calotropis procera*) were extracted by maceration in distilled water and concentrated using freeze

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dryer. Swiss male albino mice were divided into Group I (control group) received distilled water; group II were administered orally with DMA (150 mg/kg body weight) and $NaNO_2$ (100 mg/kg body weight) twice every week for 4 weeks, group III were treated orally with extract every 48 hrs simultaneously with DMA and NaNO2 and continued until the end of the experiment, group IV were treated orally with extract and fractions (150 mg/kg body weight) every 48 hrs for four weeks after the administration of DMA and NaNO2 and continued until the end of the experiment (4 weeks) and group V were given 5-flourouracil every 48 hours after induction of liver toxicity in mice. Liver function (alanine amino transferase, aspartate amino transferase, gamma glutamyl transferase and alkaline phosphatase) tests were done in the serum of mice using standard method. Extract of *P. guineense* exhibited the best activity and was fractionated for further hepatoprotective studies. Liver sections of the mice treated with fractions of *P. guineense* were used for immuno-histochemical studies for p53, BCl-2, COX-2 and Ki-67 expression, and liver histological analysis. The antioxidant status of the mice treated with fractions of *P. guineense* was determined by measuring the catalase activity, sodium dismutase activity, reduced glutathione and malondialdehyde concentrations in the liver homogenates. Data were expressed as mean and considered significant at $p<0.05$ by one-way Analysis of Variance using Graph Pad Prism 5.
The results of this study showed that activities of liver enzymes were significantly ($p<0.05$) decreased in groups treated with aqueous extracts after liver toxicity induction in mice. Treatment with fractions of *P. guineense* enhanced the antioxidant status of the mice administered with DNA/$NaNO_2$. Oral administration of fractions of *P. guineense* to mice conferred hepatoprotection as evident from normal serum enzyme levels and reduced injuries on hepatocytes. Immuno-histochemical analysis of the liver samples revealed reduction in the expression of anti-apoptotic protein BCl_2 and COX-2 in the mice treated with fractions of *P. guineense* and non-expression of cell cycle regulator p53 and Ki-67 after toxicity induction in mice.
The ability of the selected extracts and fractions of *P. guineense* to impose certain ameliorative effects on DMA and $NaNO_2$ induced toxicities in mice provided some scientific basis for their use in traditional medicine. The extracts might be used for liver toxicity treatment and/or prevention.

Keywords: Liver; antioxidant; hepatoprotection; DMA and $NaNO_2$; tumour.

1. INTRODUCTION

Liver intoxication has increased globally because of exposure to high level of nitrite and amines used as food-flavouring agents, food additives and preservatives. Metabolism of nitrite and amines in many systems is linked to cancerous nitrosamine. Nitrosamines are one of the most important environmental carcinogen that has been suggested to cause oxidative stress and alter antioxidant defense system in tissues, thus may be one of the mechanism by which nitrosamines induced liver cancer [1 and 2]. There are available evidences that nitrosamines can be generated from nitrites or oxides of nitrogen and secondary amines in acidic condition *in-vivo* [3;4;5;6]. N-nitroso-dimethylamine could also be formed *in-vitro* during cooking of canned or smoked fish with nitrite [7].

Secondary amines, particularly DMA are common constituents of foodstuffs [8]. They are found in fish meals and products, cereals, tea, flavoring agents and tobacco [9 and 10]. Inorganic nitrites are also found in plant vegetables and water. Similar characteristic effects such as inhibition of mouse liver protein and mRNA synthesis, alkylation of guanine in liver RNA that are known with dimethylnitrosamine and methylbenzylnitrosamineshave been produced by combined administration of sodium nitrite and dimethylamine or methylbenzylamines [11]. Hepatoprotective agents especially plants with strong antioxidant ingredients are known to complement the defense mechanism of the liver [12]. This present study therefore investigated the protective and therapeutic effects of *M. lucida, S. virosa, X. aethiopica, C. procera* and *P. guineense* on the damage induced by combined administration of DMA and $NaNO_2$ in liver of mice.

2. MATERIALS AND METHODS

2.1 Plant Collection

The leaves of the selected plants were collected within Ogbomoso, Oyo State, Nigeria.

Identification and authentication were done by a qualified Taxonomist in the Department of Pure and Applied Biology LAUTECH, Ogbomoso. The leaf samples were air dried in the laboratory for three weeks and then ground into powder using electric blender.

2.2 Extraction and Fractionation of Plant Extracts

One hundred (100) grams of each plant sample was weighed and soaked in 1000mL distilled water for 72hrs with constant shaking and was kept at 4°C. At the end of the 72hrs, the mixture was filtered separately using Whatman No.1 filter paper. The filtrate was freeze-dried, and the freeze-dried samples were kept until use at 4°C under refrigeration. Leaves of *Piper guineense* was initially extracted in methanol and thereafter subjected to fractionation using four different solvents (n-hexane<chloroform<ethylacetate<butanol) according to the method described by Bakht *et al*. [13]. The methanol sample was dissolved in distilled water and partitioned sequentially with n-hexane, chloroform, ethyl acetate and n-butanol respectively. N-hexane, which is the least polar, was added to the crude extract upon dissolution in water, it was stirred on magnetic stirrer and then poured into a separating funnel. Compounds soluble in the upper n-hexane phase (n-hexane being lighter than water) was collected and the lower aqueous phase was extracted with the next solvent in the increasing order of polarity i.e. chloroform<ethylacetate<butanol. Chloroform is heavier than water, ethyl acetate (lighter than water) and n-butanol (lighter than water). The fractions were concentrated at 39°C using rotary evaporator and stored until needed.

2.3 Experimental Animals and Design

One hundred and ten (110) male Swiss albino mice (20-25g) were obtained from Mercy Land Campus College of Health Sciences, Ladoke Akintola University of Technology, Osogbo, Nigeria. The animals were acclimatized for a period of one week in basket cages and kept under 12hrs light/dark cycle with assess to food and water *ad libitum*. They were divided into three experimental groups; the first for protective and the second for curative and fraction screening. Grouping and treatment of animals is as described in Tables 1 and 2.

Table 1. Grouping of animals and treatments for curative and protective studies

Groups	Treatment
A	*Morinda lucida*
B	*Securinega virosa*
C	*Xylopia ethiopica*
D	*Calotropis procera*
E	*Piper guineense*
F	Induction without treatment (Negative control)
G	1 % DMSO in distilled H_2O (Control)
H	Induction + 50 mg/kg body weight of 5-Fluorouracil (Positive control)

Mice in the protective study were administered DMA (150 mg/kg body weight) and $NaNO_2$ (100 mg/kg body weight) twice a week and simultaneously with the plant extracts (150 mg/kg body) every 48 hrs for four weeks. While the mice in curative study were administered with DMA (150 mg/kg body weight) and $NaNO_2$ (100 mg/kg body weight) twice a week for four weeks and thereafter treated with extracts every 48 hrs for four weeks.

Table 2. Grouping of animals and treatments for *P. guineense* fraction

Groups	Treatment
A	n-hexane fraction
B	chloroform fraction
C	ethyl acetate fraction
D	butanol fraction
E	aqueous fraction
F	Induction without treatment (Negative control)
G	1 % DMSO in distilled H_2O (Control)
H	Induction + 50 mg/kg body weight of 5-Fluorouracil (Positive control)

Induction: Mice were administered twice a week with DMA (150 mg/kg body weight) and $NaNO_2$ (100 mg/kg body weight) for four weeks and treated with fractions (150 mg/kg body weight).

2.4 Collection of Blood and Preparation of Homogenates

Forty-eight hrs after the last treatment, all animals were sacrificed by cervical dislocation and blood was collected by cardiac puncture into plane sterile bottles. The blood was allowed to clot and centrifuge at 4000rpm for 15mins. The supernatant sera were collected and the activity of ALP, ALT, AST and GGT was assessed. The liver samples were also harvested and excised into two. The liver sample for histopathological and immunohistochemical analysis was fixed in 10% formalin buffer and that for enzyme analysis was washed in phosphate buffer saline and stored at 4^oC until use. Liver homogenates was prepared by homogenization of frozen liver samples in ice-cold 150mM Tris-HCl buffer (pH 7.4) of 1:10 w/v ratio. Preparation of aliquots was done for biochemical analysis. The liver homogenate was centrifuged using cold centrifuge and the supernatant was used for the antioxidant study.

2.5 Bioassays

2.5.1 Estimation of liver marker enzymes

Determination of alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) activities was assessed Alkaline phosphatase assay was done in the liver, using a commercial kit, according to the manufacturer's instructions (Sigma UK).

2.5.2 Estimation of antioxidant status of mice in the liver homogenate

Antioxidants such as superoxide dismutase (SOD) [14], catalase (CAT) [15], and glutathione (GSH) content were assayed in tissue homogenate.Malondialdehyde (MDA) level, the main product of lipid peroxidation was determined as described by Ohkawa et al. [16]. MDA assay was based on the reaction of a chromogenic reagent, 2-thiolbarbituric acid with MDA at 25^oC and read at an absorbance 532 nm [17]. Determination of catalase activity was based on the measurement of the hydrogen peroxide substrate remaining after the action of catalase [18].Sodium dismutase activity was measured according to the method of Winterbourn et al., [19]. GSH activity was determined by the method described by Ellman, [20].

2.6 Histopathological and Immunohistochemical Studies

The histopathological analysis was performed by the haematoxylin and eosin staining [21]. Firstly, the samples were dehydrated through ascending grades of alcohol, cleared in xylene, and then impregnated in paraffin wax of melting point between 55^0C-56^0C for infiltration. Tissue sects were mounted on slides and then stained with haematoxylin and eosin (H & E).

2.7 Statistical Analysis

All data were expressed as mean and analysed by one-way analysis of variance at a significance level of $p<0.05$ using Graph Pad prism 5.0

3. RESULTS

3.1 Effects of Plant Extracts on Serum Enzyme Level of Mice Induced with DMA and $NaNO_2$

Table 3 showed the effects of selected plant extracts on the activities of marker enzymes (ALT, AST and GGT) in the serum of control and treated mice in the protective study. The activity of GGT was significantly ($p< 0.05$) decreased in all the extract treated groups when compared with group administered with DMA and $NaNO_2$. No significant ($p< 0.05$) difference was observed in the GGT activity of mice treated with *M. lucida, X. aethiopica* and *P. guineense* when compared with the group given distilled water (normal control). A significant ($p< 0.05$) decrease was observed in the ALT activity, of mice treated with *C. procera* and *P. guineense* when compared with DMA and $NaNO_2$ administered group. Only *M. lucida, S. virosa* and *P. guineense* treated groups exhibited significant ($p< 0.05$) decrease in the AST activity when compared with group treated with DMA and $NaNO_2$.

Table 4 showed the effects of extracts on the activity of serum GGT, AST and ALT in mice induced with DMA and $NaNO_2$ liver toxicity in the curative study. The result revealed a significant decrease ($p< 0.05$) in the GGT activity of mice across the extract treated groups when compared to the group treated with DMA and $NaNO_2$. There was an insignificant ($p< 0.05$) difference in AST activity of groups treated with *M. lucida, S. virosa* and *X. aethiopica* when

compared with the group treated with DMA and $NaNO_2$, but a significant ($p < 0.05$) decrease was observed in groups treated with *C. procera* and *P. guineense.* ALT activity in groups treated with *M. lucida*, *S. virosa* and *P. guineense* was observed to be significantly ($p < 0.05$) decreased when compared with the group treated with DMA and $NaNO_2$.

Table 3. Effect of aqueous plant extracts on serum enzyme activity of mice induced with $NaNO_2$ and DMA (protective study)

Groups	Liver enzymes		
	GGT (U/L)	AST (U/L)	ALT (U/L)
Morinda lucida	26.06±0.64*	90.50±3.01#	112.52±2.70*•
Securinega virosa	36.48±0.21*	65.50±4.30#	92.52±3.70*
Xylopia aethiopica	29.53±1.74*	62.64±0.00#	127.50±0.00#•
Calotropis procera	39.95±1.74*	56.93±3.58*	127.72±3.50•#
Piper guineense	25.17±0.90*	56.21±1.43*	98.92±1.50*•
Untreated control	57.33±1.06	65.14±4.50	129.62±0.80
Normal control	16.83±0.56	36.50±1.00	42.12±2.11

*Values were expressed as mean ± SEM. *significant when compared to the untreated group, # not significant when compared to the untreated group, •significant difference when compared to the control group. All group were induced with DMA (150 mg/kg body weight) and $NaNO_2$ (100 mg/kg body weight) except the normal group.*

Table 4. Effect of aqueous plant extracts on serum enzyme activity of mice induced with $NaNO_2$ and DMA (Curative study)

Groups	Liver enzymes		
	GGT (U/L)	AST (U/L)	ALT (U/L)
Morinda lucida	34.74±0.00*•	85.86±2.51•	88.62±2.00*•
Securinega virosa	38.21±0.00*•	46.93±4.29*	121.62±1.40•#
Xylopia aethiopica	39.96±0.69*•	105.50±4.86*	133.32±2.40•#
Calotropis procera	36.48±1.74*	78.00±2.50#	105.12±2.20*•
Piper guineense	30.40±1.45*	40.22±0.43*•	121.02±0.40#
Untreated control	57.33±1.06	65.14±4.50	129.62±0.80
Normal control	16.83±0.56	36.50±1.00	42.12±2.11

*Values were expressed as mean ± SEM. *significant when compared to the untreated group, # not significant when compared to the untreated group, •significant difference when compared to the control group. All group were induced with DMA (150 mg/kg body weight) and $NaNO_2$ (100 mg/kg body weight) except the normal group.*

Table 5. Effects of *. guineense* fractions on serum marker enzymes of mice induced with DMA and $NaNO_2$

Treatments	Parameters			
	ALP(U/L)	AST(U/L)	ALT(U/L)	GGT(U/L)
n-hexane	13.20±0.59#	24.21±0.87*	15.29±2.35#	6.29±3.82#
Chloroform	16.34±7.16#	16.17±6.02*	7.17±2.15#	6.74±4.80#
Ethyl acetate	3.05±0.22*	7.05±3.19#	2.44±0.8*	6.37±0.43#
n-Butanol	9.30±2.72*	8.81±3.94#	8.02±2.85#	8.81±3.29#
Aqueous	5.47±1.90*	9.41±1.70#	7.12±1.33#	12.62±2.95*
Untreated	14.73±4.34	7.27±1.93	6.18±1.02	5.01±2.96
Control	9.97±2.39*	14.25±3.07#	3.28±0.76*	4.21±1.67#
5-florouracil	2.88±0.34*	1.91±0.29*	8.85±0.82#	7.84±2.95#

*Values were expressed as mean ± SEM. *significant when compared to the untreated group, # not significant when compared to the untreated group, •significant difference when compared to the control group. All group were induced with DMA (150 mg/kg body weight) and $NaNO_2$ (100 mg/kg body weight) except the normal group.*
Key; Untreated = induced but not treated
Control = 1 % DMSO in distilled water only
5-florouracil = standard drug

3.2 Effects of Fractions of *P. guineense* on Serum Enzyme Activity of Mice Induced with DMA and $NaNO_2$

Effects of treatment with the fractions of *P. guineense* on serum markers enzymes were shown in Table 5. ALP activity was significantly ($p< 0.05$) decreased in the ethyl acetate, n-butanol, aqueous fractions and 5-florouracil treated groups when compared with DMA and $NaNO_2$ treated group, while an insignificant ($p< 0.05$) increase was observed in the n-hexane and chloroform, fractions group. No significant ($p< 0.05$)difference was observed in the AST activity across the treated groups except in the n-hexane and chloroform fraction treated groups where a significant ($p< 0.05$) increase were observed when compared with DMA and $NaNO_2$ treated group. There was an insignificant ($p< 0.05$) difference in ALT and GGT activity in all the extract treated groups except in the ethyl acetate fraction group where a significant ($p< 0.05$) decrease was observed in the ALT activity and the aqueous fraction group where a significant ($p< 0.05$) increase was observed in the GGT activity. No significant ($p< 0.05$) difference was observed in the ALT and GGT activity of mice when compared with the control group.

3.3. Liver Antioxidant Status of Mice Induced with DMA and $NaNO_2$ and Treated with the Fractions of *P. guineense*

Effects of fractions on liver antioxidant status of mice induced with DMA and NaNO2 are shown in Table 6. Catalase activity was significantly ($p< 0.05$) increased in the entire fraction treated groups when compared with the group induced with DMA and $NaNO_2$ and the standard drug. There were no significant ($p< 0.05$) difference in the SOD activity of the fraction treated groups when compared with the group given1% DMSO. GSH concentration was significantly ($p< 0.05$) increased in the ethyl acetate and butanol fraction treated groups when compared with the group induced with DMA and $NaNO_{2.,}$ but an insignificant ($p< 0.05$) difference in the n-hexane, chloroform, aqueous fractions and control group. MDA concentration was significantly ($p< 0.05$) decreased in the 5-florouracil, n-hexane, chloroform, ethyl acetate and aqueous fraction treated groups when compared with the group induced with DMA and $NaNO_2$,while an insignificant ($p< 0.05$) difference was observed in the butanol and control groups.

3.4 Immunohistochemical (IHC) Analysis of Liver of Mice Induced with DMA and NaNO2 and Treated with Fractions of *P. guineense*

P53 and Ki-67 expression by IHC were graded as negative (< 5 % tumour cells) or positive (> 5 % tumour cells) and there was negative expression in all the groups. The expression of the COX2 was estimated as percentage of positive cells in the chloroform (35 %), butanol (70 %), aqueous (60 %) and control groups (20 %) and negative expression in the n-hexane, untreated and 5-fluorouracil treated group.

Table 6. Effect of *P. guineense* fractions on liver antioxidant status of mice induced with DMA and $NaNO_2$

Treatments	Parameters			
	CATALASE (µmol/minutes/mg Protein)	SOD(U/mg protein)	GSH (mM)	MDA(µM)
n-hexane	0.36±0.11*	0.02±0.004*	12.45±8.18#	15.68±8.48*
Chloroform	0.39±0.08*	0.02±0.005*	6.48±3.49#	16.44±4.05*
Ethyl acetate	0.33±0.09*	0.01±0.004#	20.49±3.07*	19.66±3.01#
n-Butanol	0.22±0.04*	0.01±0.002#	20.93±6.39*	31.92±4.82#
Aqueous	0.25±0.02*	0.02±0.001*	11.42±2.27#	18.21±2.95*
Untreated	0.15±0.07	0.01±0.004	14.45±2.82	21.21±11.71
Control	0.29±0.04*	0.02±0.003*	13.97±5.37#	20.99±9.26#
5-florouracil	0.13±0.06*	0.01±0.003#	17.71±5.47*	15.20±8.38*

*Values were expressed as mean ± SEM. *significant when compared to the untreated group, # not significant when compared to the untreated group, •significant difference when compared to the control group. All group were induced with DMA (150 mg/kg body weight) and $NaNO_2$ (100 mg/kg body weight) except the normal group. Key; Untreated = induced but not treated; Control = 1 % DMSO in distilled water; 5-florouracil = standard drug*

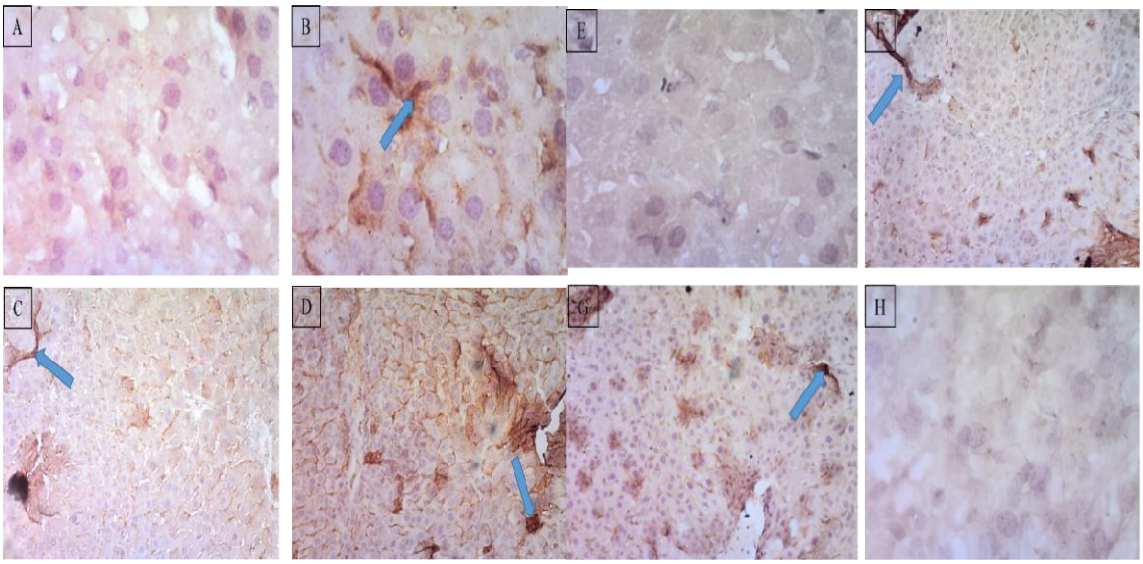

Fig.1. Representative immunohistochemical stained liver section of mice induced with DMA and $NaNO_2$ and treated with fractions of *P. guineense*. The paraffin embedded liver tissue section was subjected to immunohistochemical staining for the expression of Bcl-2. (MG X 100)

A= n-hexane treated group; B= chloroform treated group; C= ethyl acetate treated group; D= butanol treated group; E= aqueous treated group; F= group induced with DMA and $NaNO_2$ and not treated; G= 1 % DMSO in distilled water only (control); H= 5-Fluorouracil group

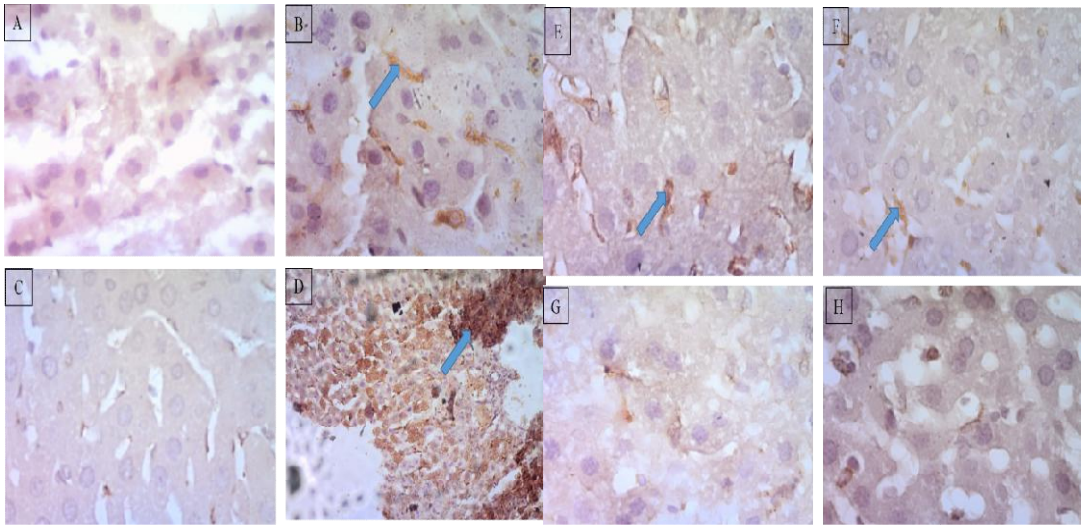

Fig. 2. Representative immunohistochemical stained liver section of mice induced with DMA and $NaNO_2$ and treated with fractions of *P. guineense*. The paraffin embedded liver tissue section was subjected to immunohistochemical staining for the expression of COX-2. (MG X 100)

A= n-hexane treated group; B= chloroform treated group; C= ethyl acetate treated group
D= butanol treated group; E= aqueous treated group; F= group induced with DMA and $NaNO_2$ and not treated; G= 1 % DMSO in distilled water only (control); H= 5-Fluorouracil group

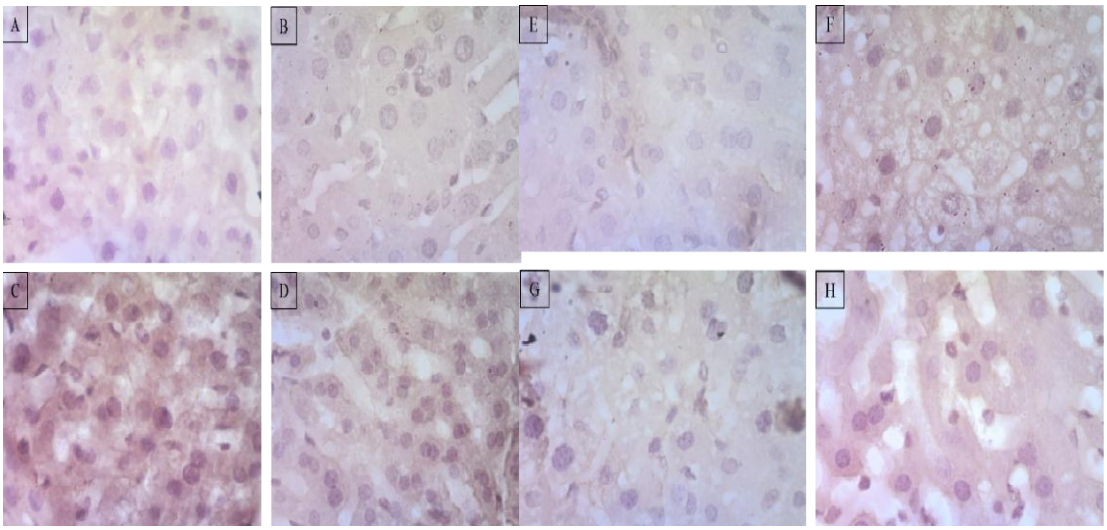

Fig. 3. Representative immunohistochemical stained liver section of mice induced with DMA and $NaNO_2$ and treated with fractions of *P. guineense*. The paraffin embedded liver tissue section was subjected to immunohistochemical staining for the expression of Ki-67 (MG X 100)

A= n-hexane treated group; B= chloroform treated group; C= ethyl acetate treated group; D= butanol treated group; E= aqueous treated group; F= group induced with DMA and $NaNO_2$ and not treated; G= 1 % DMSO in distilled water only (control); H= 5-Fluorouracil group

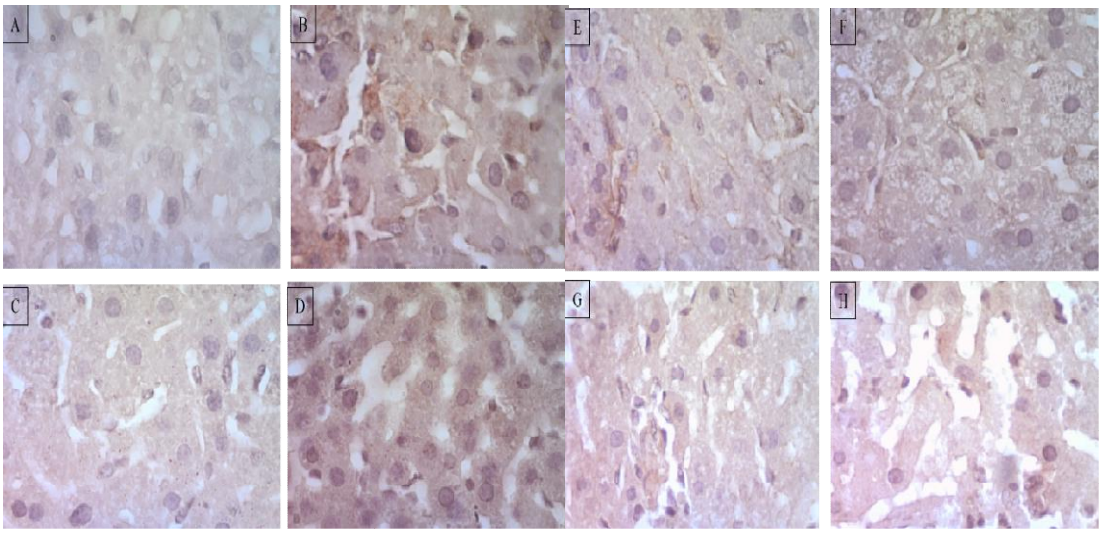

Fig. 4. Representative immunohistochemical stained liver section of mice induced with DMA and $NaNO_2$ and treated with fractions of *P. guineense*. The paraffin embedded liver tissue section was subjected to immunohistochemical staining for the expression of p53 (MG X 100)

A= n-hexane treated group; B= chloroform treated group; C= ethyl acetate treated group; D= butanol treated group; E= aqueous treated group; F= group induced with DMA and $NaNO_2$ and not treated; G= 1 % DMSO in distilled water only (control); H= 5-Fluorouracil group

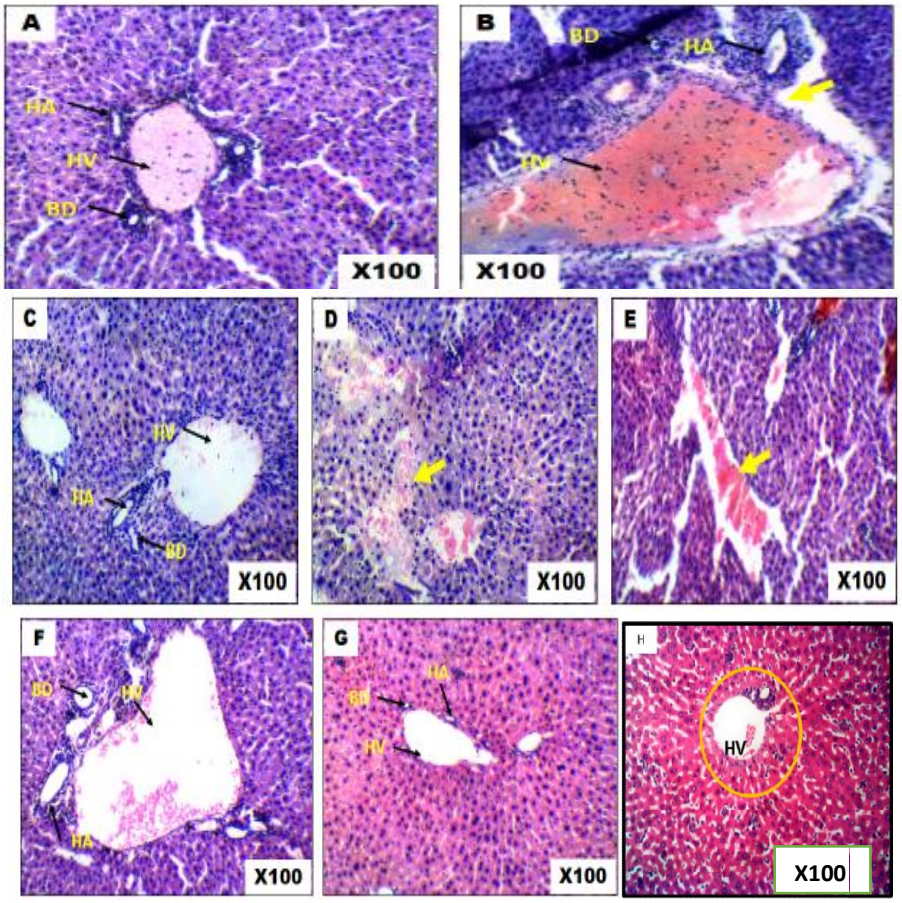

Fig. 5. Photomicrograph of representative groups in the DMA and NaNO2 induced liver toxicity showing liver general morphological presentations in mice across the various fractions

Haematoxylin and Eosin stain (MG X100). The portal triad (PT), hepatocytes (H), central vein (CV), hepatic vein (HV), hepatic artery (HA) and bile duct (BD), liver sinusoids (S), proliferating cells, infiltration and darken cells (black arrow) are well demonstrated across study groups. A= n-hexane treated group, B= chloroform treated group, C= ethyl acetate treated group, D= butanol treated group, E= aqueous treated group, F= group induced with DMA and $NaNO_2$ and not treated, G= 1 % DMSO in distilled water only (control), H= 5-Fluorouracil group

There was positive expression of Bcl-2 in chloroform (30 %), ethylacetate (20%), butanol (25 %), untreated (65 %) and control group (20 %) and a negative expression was observed in n-hexane, aqueous fractions and 5- fluorouracil treated group (Figs. 1-4).

3.5 Histological Analysis of Liver of Mice Treated with *P. guineense* Fractions after DMA and $NaNO_2$ Induced Toxicity

Fig. 5 showed the photomicrograph of liver section of mice induced with DMA and NaNO2 and treated with fractions of *P. guineense*. Observations include; altered panoramic morphological presentation of the hepatic cytology in chloroform, butanol and aqueous fraction treated group which is characterized with mild to severe pathological changes (yellow arrows). Poor outline of the hepatic vessels, fibrosis, hemorrhage and some necrotic changes were all observed in the treated groups.

4. DISCUSSION

Medicinal plants have been examined for use in a wide variety of liver disorders [22]. The present

study investigated the ameliorative effects of five selected plants in DMA and $NaNO_2$-induced liver damage. Certain enzyme markers are often used to evaluate the extent of damages to the liver. Among the marker enzymes are gamma glutamyl transferase (GGT), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALT) [23]. The serum activities of these enzymes are increased during liver injury or damage because of their release into the blood [24]. These enzymes are localized in the cell cytoplasm [25], increase in their activity in blood is indicative of cellular membrane damage [26]. In this study, the elevated level of serum GGT, AST and ALT in the DMA and $NaNO_2$ treated mice indicated the damaging effects of DMA co-administered with $NaNO_2$ on the mice liver. Conversely, treatments with the plant extracts in both the protective and curative studies showed reduction in the level of some of these marker enzymes. Therefore, the present study showed that the selected plants have some hepatoprotective potentials.

Nitrosamine metabolism has been demonstrated to cause oxidative stress that might have roles in the induction of hepatocellular necrosis, carcinogenesis, and tumour formation [27 and 28]. In addition, enhancement of oxidative stress has been implicated with dimethylnitrosamine-induced fibrosis [29]. Treatment with the plant extracts ameliorated the damage done to liver tissues by DMA and $NaNO_2$ both in the curative and protective study. The plant extracts might have restored the hepatic integrity via a mechanism that involves antioxidant activity. *P. guineense* showed the most hepatoprotective effect, it was therefore selected for further study and analysis.

Treatment of mice with *P. guineense* fractions significantly attenuated the elevations of liver marker enzymes. This improvement in enzyme activities of group treated with fractions of *P. guineense* might be due to therapeutic effects of fractions of *P. guineense.* In the process of nitrite toxicity, nitrite taken into the body is converted to nitrosoniumions, which in turn react with amines and amides to form nitrosamines and nitrosamides, respectively. Nitrosamine had been shown to induce free radicals production [30] and induce rapid oxidative stress in mouse liver tissue [31]. Cellular health is known to be strictly regulated by the balance between the oxidative and anti-oxidative systems and increased oxidative stress and/or decreased anti-oxidative mechanisms result with cellular degeneration and death [32]. The strong antioxidant potential (as reflected in GSH, catalase, SOD assays) exhibited by the fractions of *P. guineense* and healthy liver antioxidant status of mice treated with fractions of *P. guineense* might be the explanations for the nullification of oxidative stress caused by the DMA and $NaNO_2$ in mice.

$NaNO_2$ and DMA induce liver fibrosis in a highly reproducible manner, first inducing a central hemorrhagic necrosis followed by the formation of septa and establishing micronodular cirrhosis [33]. This report was confirmed by the histopathological study, which revealed that the liver showed foci of necrosis in the untreated group. However, administration of fractions of *P. guineense* to mice induced with $NaNO_2$ and DMA decreased the expression of necrosis in the liver. Therefore, this study showed that *P. guineense* fractions could interrupt tumour formation in the liver.

The immunohistochemical analysis revealed that the liver of mice treated with fractions of *P. guineense* did not show expression of p53 gene. Under normal homeostatic conditions, p53 (a tumour suppressor gene) is a short-lived protein that is regulated mainly through changes in its protein stability [34]. There are always increase cellular level of p53 where there is DNA damage and activated oncogene [35]. The mutational inactivation or down-regulation of p53 is a common scene in many cancers [36], sustained elevated levels of p53 are good indicators of its growth inhibition properties. Therefore, non-expression of p53 in the group of mice treated with fractions of *P. guineense* indicated the strong hepatoprotective potential of *P. guineense* fractions against tumour formation in mice. In addition, Ki-67 is a non-histone protein [37] and has been assigned a solid tumour proliferation marker and high expression level of Ki-67 in the tumour tissue is reported to be a prognostic marker [38]. Expression of Ki-67 gene was not observed in the liver of mice treated with the fractions. This is an indication that mice treated with the various fractions of *P. guineense* prevented liver tumour cell cycle proliferation in mice induced with liver toxicity.

Furthermore, the inflammatory marker (COX-2) was expressed in mice treated with chloroform, butanol and the aqueous fractions. Hence, the ability of n- hexane and ethyl acetate treated mice to suppress inflammation may seem to be

beneficial for preventing liver toxicity. Likewise, a negative expression of Bcl-2 was observed in n-hexane, aqueous and 5- fluorouracil treated group. It is well known that the Bcl-2, anti-apoptotic proteins are critical modulators of the intrinsic death pathway [39]. It is suggested that fractions of *P. guineense* (n-hexane and aqueous) might have prevented or suppressed liver tumor through the modulation of apoptotic induction.The hepatoprotective effects demonstrated by the fractions of *P. guineense* fractions might be by the synergistic, anti-proliferative, antioxidant and anti-tumour activities of these compounds.

5. CONCLUSION AND RECOMMENDATIONS

In this study, liver toxicities have been demonstrated following combined administration of rats with sodium nitrite and DMA. Results of this study are of practical relevance in view of the widespread dietary occurrence of dimethylamine and sodium nitrite. The consequence of these findings to *in vivo* biosynthesis of nitrosamines in man, particularly at normal dietary levels of nitrites and secondary amines, requires further consideration. Necessary precaution should be directed to the use of secondary amines as food-flavoring agents as well as their use as drug. In addition, drinking of water with elevated nitrate levels and the use of nitrates and nitrites as food preservatives in meat and fish should be discouraged. Hence, the permissible concentrations of nitrite in food additives, drugs and water should be strictly monitored by necessary government agencies.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s)

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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