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Research Article

Piptandenia africana and *Nauclea latifolia* Protects Against Diethylnitrosamine-Induced Hepatic Tumors in Wistar Rats

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Abstract

We examined the effect of *Piptandenia africana* (PA) and *Nauclea latifolia* (NL) as phytoprotective candidates against N-nitrosodiethylamine (DEN)-induced hepatic toxicities and tumorigenesis. Wistar's rat were treated as follows: (1) Control, (2) DEN, (3) PA, (4)NL, (5)PA+NL, (6)DEN+ PA, (7)DEN+ NL, (8)DEN+PA+NL for 60 consecutive days. Hepatic transaminases were assessed in serum for hepatoxicity, in addition to bilirubin, blood glucose levels and hepatic antioxidants status. Furthermore, DEN-induced tumors were estimated, and sectioned for histopathological examination. Animals treated with DEN only, had mean tumor of five (T5), these were reduced to T2, T2 and T1.75 in groups VI, VII and VIII respectively. Blood glucose levels in DEN treated rats were lowest (87.5 ± 5.1) compared to other treated rats. Elevated markers of oxidative stress and decreases in antioxidant profile, suggests that group 2 animals were stressed oxidatively compared to other groups. Significantly higher markers of hepatic toxicity indicated DEN injuriousness was highest in DEN only treated animals. Limited histopathology indicated severe mononuclear cells infiltration of the portal triad, and multiple foci of hepatic necrosis in group 2 animals, compared to control. PA, SL and in combination treated animals were devoid of these pathological hallmarks emblematic in DEN treated animals. Administration of PA and NL mitigated DEN-induced hepatic toxicities and tumorigenesis. This phytoprotective capacity unveiled, maybe pertinent in averting hepatic damage from potential chemical carcinogens. Elucidating the phytochemistry and mechanisms of PA and NL mode of action is necessary, which could serve as potential anti-tumor drug candidate.

Key Words: *N-Nitrosodiethylamine, Piptandenia africana, Nauclea latifolia, hepatocarcinogenesis, hepatotoxicity*

INTRODUCTION

Hepatocellular carcinoma (HCC) is the commonest primary liver malignancy: the fifth (in men) and seventh (in women) most frequent cancer worldwide (Cardin *et al*., 2014) and the third cause of cancer deaths yearly (Colagrande *et al*., 2016, Diaz-Gonzalez *et al*., 2016). Management of cancer comes with a lot of pain (Nicholson *et al*., 2017, Pinato *et al*., 2017, Colagrande *et al*., 2016), The need for alternative methods with fewer adverse side effects, to augment already established methods for the treatment and management of cancer in general is ever increasing. Medicinal plants has been useful in the management of various ailments including hepatic malignancies (Huo *et al*., 2017) In folk's medicine, herbal extracts from plants (phytochemicals) have been used from time immemorial for the remediation of a plethora of ailments (Phillipson and Wright, 1991, Hajdu, 2016, Jaradat *et al*., 2016), which presently still remains a relevant avenue to explore (Phillipson, 1994, Wargovich *et al*., 2001, Wargovich, 2001) in the ever-urgent search for a cure for cancer. Two plants extracts from shrubs widely used in herbal medicines were the subjects of this study:

Nauclea latifolia (NL) is an evergreen multi-stemmed shrub widespread in the humid tropical rainforest and savannah woodlands of West-Central Africa. It has been used in West and South Africa by infusions and decoctions of the bark and leaves as treatment for stomach pains, fever, diarrhea, and against parasites like nematodes in humans and animals (Asase *et al*., 2005, Asase A., 2009, Kawanishi *et al*., 2002). It's believed to be efficacious against tropical diseases like malaria and also as a remedy against tuberculosis. *Piptandenia Africana (PA)*, bark, roots and leaves are commonly used in herbal medicine. Bark decoctions of *PA* finds medicinal application in the treatment of cough, bronchitis, headache, mental disorders, hemorrhoids, genito-urinary infections, stomachache, dysmenorrhea and male impotence (R., 1969). In addition, *PA*-bark extracts have been used as arrow poison, fish poison and when mixed with bits of food items can be help in controlling rodents.

Decoctions of *Nauclea latifolia (NL)* (Benoit-Vical *et al*., 1998) and *Piptandenia africana* (*PA)* have been used as co-constituents of poly-herbal remedy in managing protuberances and tumors -likened to cancers- of the abdomen without much documented evidence other than traditional oral transmission of such knowledge. Devoid of any scientific validation these

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remedies have served their purposes in the management of several ailment for ages. Earlier studies on *PA* and *NL* indicated anti-proliferative effects on a variety of cell lines *in vitro*.

In the absence of any scientific enquiry to examine the anti-tumorigenic activity, specifically in the liver, we, here examined further the effect of co-treatment of *PA* and *NL* on Diethylnitrosamine (DEN)-induced hepatic tumors in an *in vivo* model - albino Wistar rats. DEN is a known causative agent in the development of hepatocarcinogenesis. DEN has been used in the induction of hepatic tumors in experimental animals (Bhosale P, 2002, Nermin A.H. Sadik, 2008). Metabolites of DEN interacts with DNA and can result in mutations resulting in carcinogenesis (Nermin A.H. Sadik, 2008). DEN metabolism and induced oxidative stress may play a role in the induction of oncogenic mutations, while oxidative stress have been implicated in a plethora of diseases (Le Lay *et al*., 2014) including hepatocarcinogenesis (Takaki and Yamamoto, 2015). We further examined the effect of *PA* and *NL* treatment in mitigating oxidative stress *in vivo* by monitoring the antioxidant defense system of exposed rats in the presence and absence of DEN. Finally, we looked at the effect of PA and NL activity on protecting the genome from oncogenic mutations using the UMU bacteria chromo test.

MATERIALS AND METHODS

Chemical and Assay kits: Diethylnitrosamine (DEN) was purchased from Sigma Chemical (St Louis, MO, USA). Randox™ diagnostic kit (Randox™ Laboratories, United Kingdom) used for the determination of hepatic transaminases - alanine transaminase (ALT), aspartate transaminase (AST), and bilirubin assessment. EBPI UMU-Chromotest™ kit (EBPI, Ontario, Canada) was purchased from Environmental Bio-detection Products Incorporation. All other reagents were of the highest quality available commercially.

Plant extracts: Extracts of *Piptandenia Africana* (PA) and *Nauclea latifolia* (NL) for this study was a generous contribution by a collaborator after extraction from the plants source.

Plant Materials: Stem bark of *Piptandenia Africana (PA)* and *Nauclea latifolia (NL)* were sourced Noun region of Cameroon and South Africa respectively, and the specimens were appropriately identified. *PA* and *NL* were washed thoroughly and air-dried in the dark at room temperature before being pulverized separately using Hammer mill. *PA* (2000g) and *NL* (1000g each) were macerated in the cold using methanol in a ratio of 1:10 respectively for 72hrs. The resulting decoction was filtered with a Whatman No.1 filter paper to remove any residual cellulose fiber under pressure Buchner flask. Extract of *PA* and *NL* were concentrated using a rotary evaporator (Buchi Model 240) at 35^0C to dryness. The extracts of *PA* and *NL* were scrapped out of the round bottom flask and weighed with approximate yield for *PA* (12%w/w) and *NL* (18.7% w/w). The extracts were preserved in a refrigerator at 4^0 C until needed for further experiment.

Phytochemical screening: A small portion of extract of *PA* and *NL* was subjected to phytochemical analysis using the method of Trease (Trease, 2009) to test for flavonoids, alkaloids, tannins, anthraquinones, saponins, and cardenolides.

Animals: Healthy Wistar rats (120-150 g) of both sexes purchased from the primate colony, University of Ibadan, Nigeria were used for this study. The rats were allowed to acclimatize for a week and were declared disease free with no obvious physical deformity, and were divided randomly into eight groups of 5 rats. Each group was housed in polycarbonated plastic cages placed in a well-ventilated room. The animals were provided with standard rat pellets purchased from Ladokun™ feeds, Mokola, Ibadan, Nigeria and were provided water *ad libitum*. All experimental were subjected to natural photoperiod of 12-hour light: dark cycle, during acclimatization (7 days) and experimental (60days) period until they were sacrificed.

Dose response studies and Treatment protocol:: Administration of *N*-nitrosodiethylamine (DEN: 25µg/g body weight) weekly by intraperitoneal (i.p.) injection was selected following established protocol for inducing hepatic tumor (Vesselinovitch and Mihailovich, 1983, Kyriazis *et al*., 1974, Heindryckx *et al*., 2009, Pitot and Dragan, 1994) The selection of *Piptandenia africana* (*PA*) and *Nauclea latifolia* (*NL*) doses suspended in dilute water and administered orally used experimentally were reached from a dose response study conducted for one week using doses of *PA* and *NL* 50, 100 and 150 mg/kg body weight. These treatment regimens did not result in any way a toxic response, limited food intake nor abnormal body weight change in treated animals (Data not shown). A dose of 100mg/kg body weight was therefore selected for further experimental purposes.

Group 1: Control
Group 2: *N*-nitrosodiethylamine (DEN: 25µg/g body weight) weekly by intraperitoneal (i.p.) injection.
Group 3: *Piptandenia africana* (*PA:* 100mg/kg body weight) orally, thrice weekly
Group 4: *Nauclea latifolia* (*NL:* 100mg/kg body weight) orally, thrice weekly
Group 5: *PA and NL* (100mg/kg body weight each) orally, thrice weekly
Group 6: DEN (25µg/g body weight: i.p.) + *PA* (100mg/Kg body weight) orally thrice weekly
Group 7: DEN (25µg/g body weight: i.p.) + *NL* (100mg/Kg body weight) orally thrice weekly
Group 8: DEN (25µg/g body weight: i.p.) +*PA* +*NL* (100mg/Kg body weight each) orally thrice weekly.
All animals were treated as described above and maintained for a period of 60 days.

Terminal point sacrifice of experimental animals: Twenty-four hours after the last treatment, the animals were sacrificed by cervical dislocation. Blood was collected in non-heparinized tubes by ocular puncture and allowed to clot for serum separation by centrifugation (4000g, for 15 minutes) with a table centrifuge. Liver were harvested quickly rinsed in ice-cold 1.15% potassium chloride solution, blotted dry, weighed, and processed for histopathological and biochemical assays. Liver sections were fixed in phosphate buffered formaldehyde for 24hrs. The fixed tissues were embedded in paraffin, sectioned and processed for limited histopathological examination stained with hematoxylin and eosin (H&E). In addition, pieces of liver section were homogenized in ice-cold phosphate buffer, pH 7.4 weight/volume as required for specific biochemical assays. Liver index was estimated

according to the formula: (rat liver weight / rat final body weight) x 100% and expressed as percentage.

Estimation of tumor burden, count and sizes: The incidences of DEN-induced tumors were counted macroscopically on each lobe of the liver and the sizes were calculated using a Delcast^{TM} Digital Caliper Model (DCAL-02).

Biochemical analysis

Determination of serum Alanine aminotransferase (ALT) and Aspartate amino transferase (AST)
The in *vitro* quantitation of ALT and AST was carried out using Randox^{TM} Assay kits. The serum ALT activity was determined as previously described (Reitman and Frankel, 1957).

Determination of Bilirubin: Total bilirubin was determined by colorimetric method as described (Heinemann and Vogt, 1988)

Determination of reduced Glutathione (GSH), Glutathione peroxidase (GPX) activities and Superoxide dismutase (SOD): Reduced glutathione (GSH) was estimated by the method of Beutler *et al*., (Beutler *et al*., 1977) and glutathione peroxidase (GPX) activity as described by Rotruck *et al*., (Rotruck *et al*., 1972) with modifications. Superoxide dismutase (SOD) activity was estimated by the method of Misra and Fridovich (Misra and Fridovich, 1972).

Genotoxicity study
SOS-Chromotest:
Activation of lyophilized *E. coli* PQ37: Under aseptic techniques, one vial (one unit) of the bacteria was mixed with 12mL of SOS Chromotest growth media to activated lyophilized *E. coli* PQ-37. The resulting mixture was shaken to ensure a thorough mix and incubated overnight for 16 h at 37^0C. Upon activation bacterial solution was visually examined for turbidity (indicating bacterial growth and viability). The turbid bacterial solution was further diluted with SOS growth media to obtain a suspension with a final absorbance of 0.05 at OD600 and set aside until needed for use. 20 µL each of *PA*, *NL* and 4-NQO (a standard genotoxin - positive control) were dispensed into designated well of a pre-labeled 96-well microplate preloaded with 10 µL of saline solution. The samples were serially diluted (2 fold dilutions) to obtain five different concentrations in designated columns. Activated E. *coli* PQ-37 (100 µL) was then added to each well containing *PA*, *NL* and 4-NQO and incubated for 2hours at 37^0C. After incubating, 100µL of reconstituted alkaline phosphatase-blue chromogenic substrate were added to each test well and incubated for another 90 min until a green colour was produced. The overall reaction was then terminated by the addition of 50µL of the stop solution. The viability of *E. coli* PQ37 and potential genotoxicity of *PA*, *NL* were obtained spectrophotometrically by reading the absorbance at (405nm) and (615nm) respectively. A test compound is classified as genotoxic only when they fulfill the following criteria: (a) test sample exhibits a dose-response relationship; and (b) the number of times a test sample O.D was higher than that of the negative control (SOS Induction Factor (SOSIF)). A SOSIF response is considered significantly genotoxic for a test sample when (SOSIF>1.5). SOSIF<1.5 is classified as probably genotoxic and SOSIF<1 is regarded as non-genotoxic. The SOS induction potency (SOSIP) from the test was also used to indicate the degree of genotoxicity of a sample. The SOSIP was obtained from the linear portion of the dose response curve of the SOS Chromotest $[O.D._{.1}\text{-}O.D._{.3\,(615nm)}] / [C_1 – C_3]$ in this case reflecting the SOS inducing ability of *PA*, *NL* under investigation.

Histopathology
Limited histopathological analysis was conducted on sections of liver tissue. Tissues were fixed in 10% phosphate buffered formalin for 24 hours, and subsequently embedded in paraffin following dehydration serially in an ethanol gradient followed by xylene. Sections of 5-µm thickness were cut, fixed on glass slides, de-paraffinized and rehydrated. Liver sections were stained with hematoxylin and eosin (H&E) and examined by Carl Zeiss light microscope.

Statistical analysis
All values were expressed as the mean ± S.D of five animals. Data were analyzed using one-way analysis of variance (ANOVA) of biochemical data using SPSS (10.0) statistical software and graph pad prism for the plotting of graphs. P values < *0.05* were considered statistically significant.

RESULTS

Effect of DEN *PA* and *NL* treatment on serum hepatic transaminases alanine amino transferase (ALT) and aspartate amino transferase (AST):
Figure 1 and 2 show the effects of DEN, *PA* and *NL* on serum levels of hepatic transaminases (ALT and AST) in treated rats. DEN treatment significantly ($p<0.05$) elevated these values. Conversely *PA* and *NL* mitigated the effect of DEN, more significantly in the presence of *PA+NL*

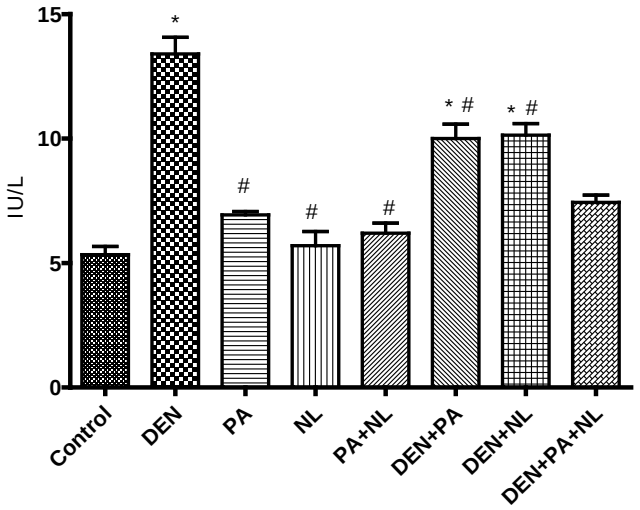

Figure 1:
Effect of diethylnitrosamine (DEN), *Piptandenia africana* (*PA)* and *Nauclea latifolia (NL)* treatment on serum alanine amino transferase (ALT) in rats. Each bars represent mean ±S.D with 5 rats per group treated for 60 consecutive days. DEN (25µg/g); *PA* (100mg/kg); and *NL* (100mg/kg body); *Significantly ($p< 0.05$) different compared with normal control; #different ($p< 0.05$) compared to DEN.

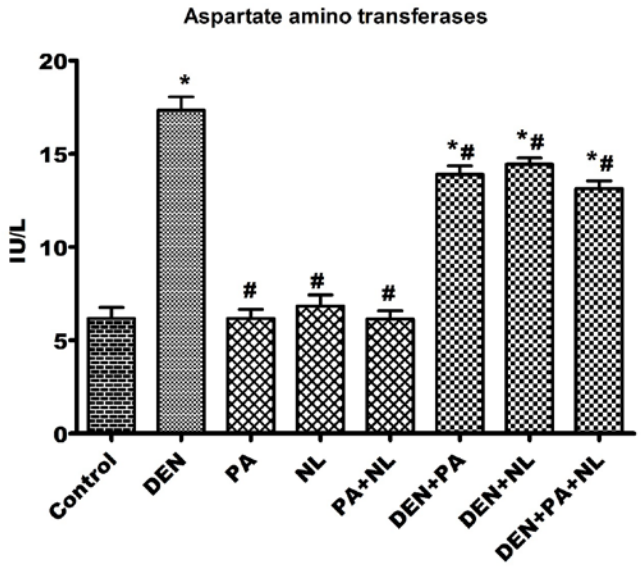

Figure 2:
Effect of diethylnitrosamine (DEN), *Piptandenia africana* (*PA)* and *Nauclea latifolia (NL)* treatment on serum aspartate amino transferase (AST) in rats. Each bars represent mean ±S.D with 5 rats per group treated for 60 consecutive days. DEN (25µg/g); *PA* (100mg/kg); and *NL* (100mg/kg body); *Significantly ($p< 0.05$) different compared with normal control; #different ($p< 0.05$) compared to DEN.

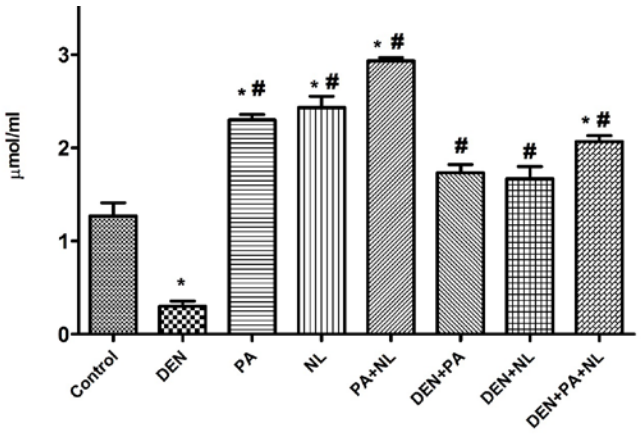

Figure 3:
Effect of diethylnitrosamine (DEN), *Piptandenia africana* (*PA)* and *Nauclea latifolia (NL)* treatment on glutathione peroxidase in rats**.** Each bars represent mean ±S.D with 5 rats per group treated for 60 consecutive days. DEN (25µg/g); *PA* (100mg/kg); and *NL* (100mg/kg body); *Significantly ($p< 0.05$) different compared with normal control; #different ($p< 0.05$) compared to DEN.

Effect of DEN *PA* and *NL* treatment on markers of oxidative stress:

Glutathione peroxidase levels increased significantly ($p<0.05$) in *PA* and *NL* treated group compared to DEN only (figure 3) and baseline level. In the presence of DEN, the observed increases were dependent on the presence of *PA* and *NL;* indicative of *PA* and *NL* ability to alter antioxidant levels in DEN exposed rats.

Reduced glutathione increased significantly ($p<0.05$) in the presence of DEN+*PA*, DEN+*NL* and DEN+*PA*+*NL* (figure 4) when compared to the DEN only treated groups. *PA*, *NL* and *PA*+*NL* treatment elevated reduced glutathione levels above baseline.

There were no significant ($p<0.05$) increases in glutathione -*S*- transferase (GST) in *PA, NL* and *PA*+*NL* treated rats (figure 5), whereas exposure to DEN only significantly ($p<0.05$) stimulated the expression of GST. In the presence of the extracts (DEN+*PA*, DEN+*NL* and DEN+*PA*+*NL)* GST expression was significantly reduced when compared to the DEN only treated groups.

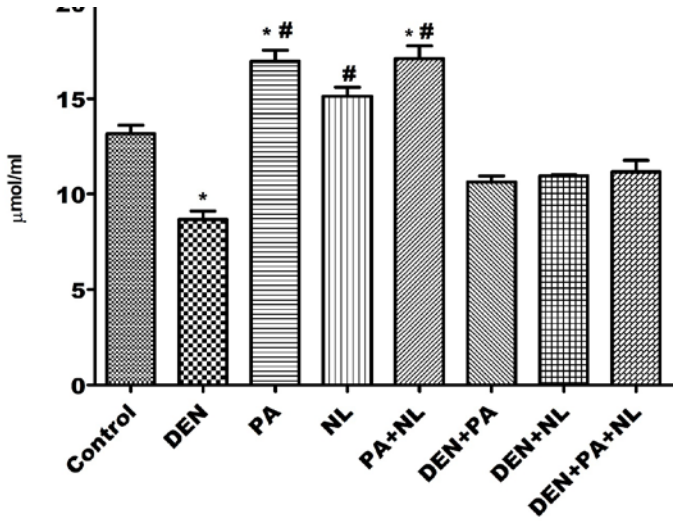

Figure 4:
Effect of diethylnitrosamine (DEN), *Piptandenia africana* (*PA)* and *Nauclea latifolia (NL)* treatment on reduced glutathione in rats**.** Each bars represent mean ±S.D with 5 rats per group treated for 60 consecutive days. DEN (25µg/g); *PA* (100mg/kg); and *NL* (100mg/kg body); *Significantly ($p< 0.05$) different compared with normal control; #different ($p< 0.05$) compared to DEN.

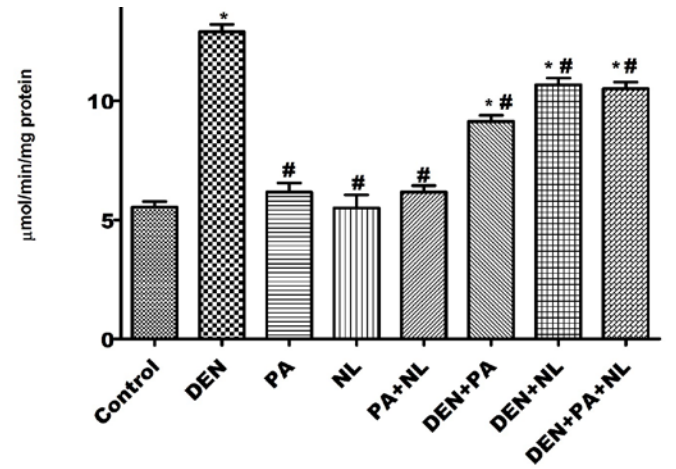

Figure 5:
Effect of diethylnitrosamine (DEN), *Piptandenia africana* (*PA)* and *Nauclea latifolia (NL)* treatment on glutathione-s-transferase in rats. Each bars represent mean ±S.D with 5 rats per group treated for 60 consecutive days. DEN (25µg/g); *PA* (100mg/kg); and *NL* (100mg/kg body); *Significantly ($p< 0.05$) different compared with normal control; #different ($p< 0.05$) compared to DEN.

Figure 6 shows the effect of DEN, *PA* and *NL* treatment on superoxide dismutase (SOD) level in the rats. There was a significant ($p<0.05$) increase in the DEN only treated group, which was suppressed in the presence of *PA, NL* and *PA+NL* co-exposure with DEN. On the other hand *PA+NL* only elevated SOD above baseline levels.

Effect of DEN *PA* and *NL* treatment on direct bilirubin

DEN only treatment significantly ($p<0.05$) elevated direct bilirubin levels; in the presence of *PA* and *NL* bilirubin levels were still high and so much so in the presence of *PA* and *PA+NL*. On the other hand, *PA* and *NL* treatment only did not impact the level of direct bilirubin in experimental animals (figure 7) considerably.

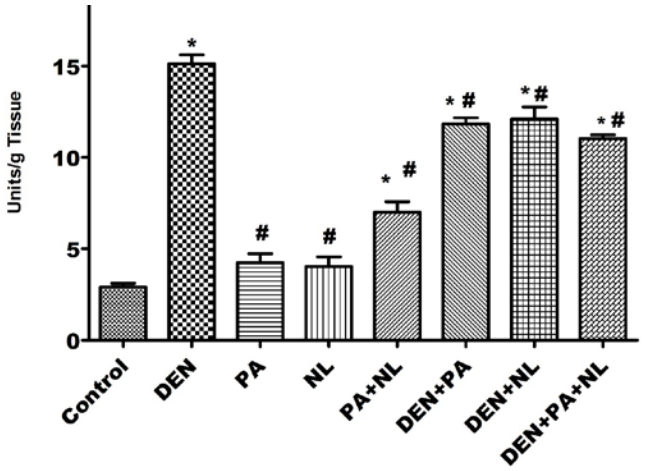

Figure 6:
Effect of diethylnitrosamine (DEN), *Piptandenia africana* (*PA)* and *Nauclea latifolia (NL)* treatment on superoxide dismutase in rats. Each bars represent mean ±S.D with 5 rats per group treated for 60 consecutive days. DEN (25µg/g); *PA* (100mg/kg); and *NL* (100mg/kg body); *Significantly ($p< 0.05$) different compared with normal control; #different ($p< 0.05$) compared to DEN.

Effect of DEN *PA* and *NL* treatment on body and organ weight and food intake

There was significant ($p< 0.05$) increase in the final body weight in experimental animals (table 1), but a decrease ($p< 0.05$) in food consumption and body weight in the DEN only treated animals. Liver weight in (DEN+*NL*, DEN+*PA+NL*, *PA* and *NL*) significant ($p< 0.05$) increased when compared to the control while there was a significant ($p< 0.05$) decrease in relative liver weight when compared to control animals.

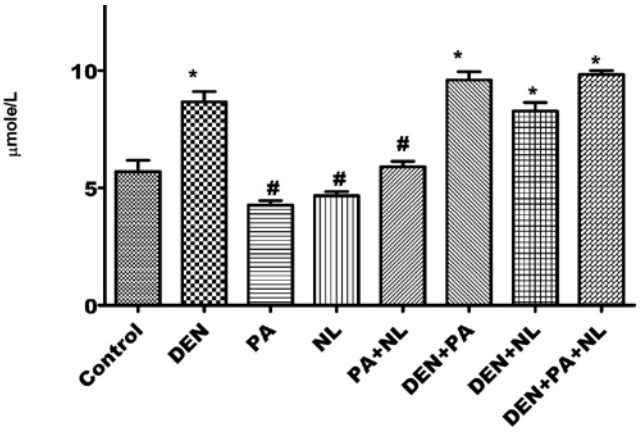

Figure 7:
Effect of diethylnitrosamine (DEN), *Piptandenia africana* (*PA)* and *Nauclea latifolia (NL)* treatment on direct bilirubin in rats. Each bars represent mean ±S.D with 5 rats per group treated for 60 consecutive days. DEN (25µg/g); *PA* (100mg/kg); and *NL* (100mg/kg body); *Significantly ($p< 0.05$) different compared with normal control; #different ($p< 0.05$) compared to DEN.

Effect of DEN *PA* and *NL* treatment on blood glucose levels

The highest mean blood glucose level was observed in control animal (119.4±4.1) compared to all other groups. Whereas blood glucose level was lowest in DEN only treated animals (87.5±5.1). In the presence of *PA* and *NL*, singly and in combination blood glucose levels were increased in DEN treated animals. Blood glucose in *PA, NL* and *PA + NL* groups significantly ($p< 0.05$) decrease compared to levels in control animals (table 2).

Table 1:
Effect of Diethylnitrosamine (DEN), *Piptandenia Africana* (*PA*) and *Nauclea latifolia* (*NL*) on organs and body weight of male Wistar's rat.

	IBW (g)	FBW (g)	WC (g)	LW (g)	RLW (g)
1. Control	184.2±24.9	218.2±28.9	34±4.00	6.50±0.5	2.9±1.0
2. DEN	124.2±24.9	118.2±28.9	6.00±4.00	5.0±0.5	4.3±1.0
3. PA	165.4±24.5	202.2±17.8*	37.2±7.3*	4.9±0.5	2.4±0.1
4. NL	165±27.9	210±16.9*	45.0±1.9*	7.1±0.4*	3.3±0.1
5. PA+NL	164.4±23.4	215.4±31.9*	51.0±8.5*	6.7±0.5*	3.1±0.6
6. DEN+PA	166.4±22.6	234.75±7.8*	68.71±14.8*	6.1±0.8*	2.5±0.2
7. DEN+NL	161.4±22.6	204.4±19.0*	43.0±3.6*	6.4±0.4*	3.1±0.1
8.DEN+PA+NL	129.6±9.8	222.4±18.2*	93.2±8.4*	5.6±0.3	2.5±0.1

The values are expressed as mean of five animals per group ± S.D. *Figures are significant when compared with control *$p <$ 0.05. IBW: Initial body weight;
FBW: final body weight; WC: weight change; LW: liver weight; RLW: relative liver weight.

Table 2:
Effect of Diethylnitrosamine (DEN), *Piptandenia Africana* (*PA*) and *Nauclea latifolia* (*NL*) weekly blood glucose level in male Wistar's rat.

	Mean Weekly Blood Glucose Level in Treated Rats							
	Wk1	**Wk2**	**Wk3**	**Wk4**	**Wk5**	**Wk6**	**Wk7**	**Wk8**
1. Control	100.4 ±5.2	112.8±4.4	108±4.2	109.4±4.1	106±6.1	103±9.3	101.9±3.8	110±5.6
2. DEN	83.4±5.2*	86.8±4.4*	88.6±4.2	90.4±4.1	74.23±5.3	70.4±2.8	73.5±7.5	76.3±10.4
3. *PA*	98.6±8.6	100±11.0	97±4.6	98.4±9.2	100±6.4	97.6±4.4	98.3±6.1	101±7.5
4. *NL*	96.8±6.2	91.0±5.1	98.3±4.0	87.0±7.0	94.0±2.8	98.3±5.2	99.1±2.8	102.55±1.8
5. *PA+NL*	94.4±7.4	95.4±7.9	97.4±4.9	96.0±12.3	100.2±2.4	98.3±3.6	99.7±4.2	95.6±8.4
6. DEN+*PA*	109± 21.5*, #	106.2± 6.2*, #	96.4 ±6.1*, #	87.0 ±7.5*, #	97 ± 4.3*, #	97± 3.7*, #	95.8± 4.3*, #	90± 5.2*, #
7. DEN+*NL*	111.0± 8.2*, #	97.8± 11.4*, #	95.7± 7.3*, #	83.2± 8.0*, #	90 ± 3.6*, #	84.8± 6.8*, #	89± 1.8*, #	86.2 ±2.3*, #
8. DEN+*PA+NL*	102.4± 7.1*, #	97.4± 7.7*, #	97.4± 7.7*, #	76.2± 2.8*, #	87± 8.4*, #	86.0± 5.3*, #	87± 3.4*, #	83.4 ±7.5*, #

The values are expressed as mean of five animals per group ± S.D. Figures are significant compared with control* and compared with DEN only treated animals#, $P < 0.05$. Wk.: week, DEN: Diethylnitrosamine, *PA*: *Piptandenia Africana* and *NL*: *Nauclea latifolia*

Effect of DEN, *PA* and *NL* treatment on tumor burden, numbers and sizes

No tumor burden was observed in any lobe of the liver; in the control, *PA*, *NL* and *PA+NL* treated animals (table 3a). Animals treated with DEN+*PA* exhibited the following tumor profile: two rats had no visible tumor (T0) but three other rats had (T1) each on the largest lobe (LL) of their livers. In DEN+*NL*: one rats had (T1) on the LL, while two other rats had (T3) and (T1) on the liver's smallest lobe (SL). Two rats had (T0), one rat had (T1) on the LL of rats treated with DEN+*PA+NL*. One rat had (T3) on LL and (T2) and (T1) on the intermediate lobe (IL) while the last rat (T0) on LL and (T1) on SL.

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Table 3 a:
Effect of Diethylnitrosamine (DEN), *Piptandenia Africana* (*PA*) and *Nauclea latifolia* (*NL*)
on DEN induced tumor, sizes and numbers of tumors.

Treatment groups	**Tumor number count**	**Mean tumor sizes (mm)**
I. Control	**Nil**	**Nil**
II. DEN	**5.00**	**3.84**
III. PA	**Nil**	**Nil**
IV. NL	**Nil**	**Nil**
V.PA+NL	**Nil**	**Nil**
VI. DEN+PA	**2**	**2.55**
VII. DEN+NL	**2**	**3.15**
VIII. DEN+PA+NL	**1.75**	**2.62**

Genotoxic effect of *PA* and *NL* treatment on *E. coli* PQ37 and SOS induction (SOSIP)

Extracts of *Piptandenia Africana* and *Nauclea latifolia* is not genotoxic at (100mg/kg body weight) using the SOS-chromotest response (Table 3B).

Table 3b: Genotoxic effect of *Piptandenia Africana* (*PA)* and *Nauclea latifolia* (*NL)* treatment on *E. coli* PQ37 and SOS Induction Potency (SOSIP). Extracts of *PA* and *NL* is not genotoxic at (100mg/kg body weight) estimated by SOS-chromotest response.

	SOSIP	**Genotoxicity**	***E. coli* viability**
Control	Nil	Nil	>100
3NQO	>1.5	Positive	<100
PA	<1	Nil	>100
NL	<1	Nil	>100
PA+NL	<1	Nil	>100

A SOSIP is considered significantly genotoxic for a test sample when (SOSIF>1.5). SOSIF<1.5 is classified as probably genotoxic and SOSIF<1 is regarded as non-genotoxic. *PA: Piptandenia Africana*, *NL: Nauclea latifolia*, 3NQO: 3-nitroquinoline oxide; SOSIP: SOS induction potency. The values are expressed as mean of triplicate wells in a 96-well plate

Histological changes in rat's liver cells following administration of DEN

Histopathology of liver sections showed sever portal cellular infiltration by mononuclear cells, congestion and multiple foci of severe hepatic necrosis in DEN only treated group compared with normal control (Figure 9A-D) 1), in addition to diffuse hydropic degeneration of hepatocytes. In contrast DEN+*PA* treated animals exhibited less dramatic histological changes characterized by milder portal cellular infiltration with moderate Kupffer cell hyperplasia. DEN+*NL* treated animals presented with sever but diffuse vacuolar degeneration of hepatocytes. Mild portal congestion, occasioned by cellular infiltration by mononuclear cells was observed in DEN+*PA+NL* treated animals. Conversely, Control, treatment with *PA* and *NL* alone and in combination exhibited no visible lesions or cellular congestion or infiltration of inflammatory mediatory cells

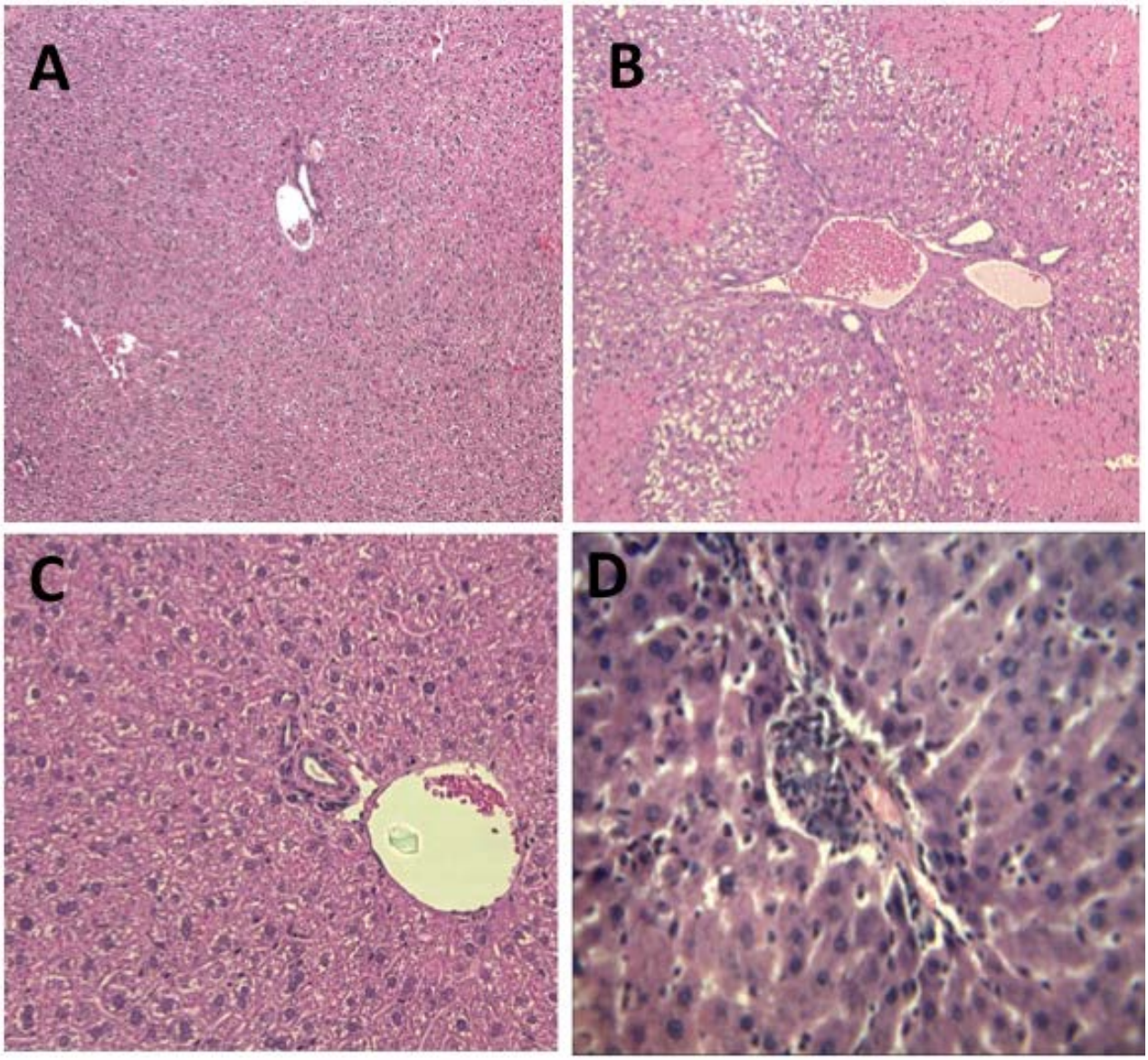

Plate 1:
Photomicrograph of liver sections from rats treated with Diethylnitrosamine (DEN), Piptandenia *Africana* (*PA*) and *Nauclea latifolia* (*NL*). (A) Control with no visible lesion seen; (B) DEN treatment characterized by severe portal cellular infiltration, congestion and necrotic stricture of hepatocytes with a characteristic periportal fibroplasias. (C) *PA* and *NL* only treated group, hepatocytes architecture appears similar to control but with clear vacuolar spaces and (D) DEN+*PA*+*NL* treatment with mild portal cellular infiltration and necrotic changes less severe compared with DEN only treated group (B).Limited histology of *PA* and *NL* treated groups (not pictured above) showed no visible lesions with limited portal congestion and cellular infiltration by mononuclear cells.

DISCUSSION

Death from cancers of any type remains an epidemiological challenge despite groundbreaking advances in medical sciences. Hepatocellular carcinoma from various etiology and known carcinogens such as Diethyl nitrosamine (DEN) is still elusive to curative strategies. Exposures to DEN is potentially harmful (David *et al*., 2005) and exist in many forms including foodstuffs, especially those cooked, smoked, or cured meat or fish, in addition to being present in beer and tobacco smoke (Najm, 2001). Protection from the deleterious effect of DEN is necessary since inadvertent exposure of human population over a period of time is implicated in the carcinogenesis process, especially of the liver.

DEN-induce carcinogenesis by alkylating deoxyribonucleic acids (DNA) upon metabolic activation, to α-hydroxyl nitrosamine (Verna *et al*., 1996), mediated by cytochrome P_{450}, enzymes with high activity in the centrilobular region of hepatocytes. In addition, oxidative damage may result from DEN metabolism and can contribute to hepatocarcinogenesis (Kolaja and Klaunig, 1997, Heindryckx *et al*., 2009, Qi *et al*., 2008). Reactive oxygen species (ROS) generated from CYP_{450} enzymatic system may exacerbate baseline but tolerable levels of cellular oxidative stress (OS). Furthermore, any cellular imbalance that promotes the increased production of ROS, known to cause DNA, protein and lipid damage can play a role in promoting carcinogenesis (Kawanishi *et al*., 2002), especially in a target organ like the liver. Plant extract containing phytochemicals

rich in polyphenol (Shahidi, 1992) and flavonoids are useful in scavenging ROS (Blois), generated from cellular metabolism, thus conferring protection to cellular macromolecules from damaging ROS effects (Owumi, 2012). The protective effect of *Nauclea latifolia (NL)* and *Piptandenia Africana (PA)*, were examined on DEN-induced OS and tumours induction in rats. Increasing evidence abounds establishing a link between chemical/drug exposure, generation of ROS and OS, believed to be a causative factors for various diseases. OS is characterized by an imbalance between prooxidant and antioxidants. Mitigating DEN-induced OS that can contribute to hepatocarcinogenesis (Kolaja and Klaunig, 1997, Qi *et al*., 2008), may help in ameliorating if not prevent the carcinogenesis process.

As expected DEN only treated animals showed increased levels of serum hepatic transaminases (ALT and AST) standard markers for early acute hepatocyte damage (Navarro, 2006)due to their localization in periportal hepatocytes. However, in the presence of *PA*, *NL* and in combination (*PA+NL*), co-treatment with DEN mitigated DEN-induced hepatocytes damage significantly ($p<0.05$) (Figures 1 and 2), indicative of a protective role of these plant extracts against DEN toxicity. *PA*, *NL* therefore maybe showing promises that can be explore that can be of medicinal values and in managing toxic effect that may arise from a wide range of chemical or drug compounds implicated in cellular toxicity or damages through an ROS generating and oxidative damaging mechanisms.

Bilirubin is product of normal haem catabolism, clearing aged red blood cells; elevated levels of bilirubin may indicate diseases state, marked by the background straw yellow colour of urine via its breakdown product, urobilin (Pirone *et al*., 2009). In line with previous findings (Rezaie *et al*., 2013), DEN treatment elevated direct bilirubin estimation in treated animals, whereas, *PA, NL* significantly ($p<0.05$) decreased baseline levels of direct bilirubin (Rui *et al*., 2014) compared to DEN only treated animals, and in combination *PA+NL* had less significant ($p<0.05$) implications on bilirubin levels, indicating that their presence does not alter haem metabolism appreciably.

ROS production that exceeds critical levels can overwhelm cellular enzymatic antioxidant defense system resulting in OS (Hanasaki *et al*., 1994). When localized to the hepatocyte can case extensive hepatic damage depending on the severity of cellular ROS concentration. Superoxide dismutase (SOD) -an enzymatic antioxidants- can abrogate the damaging effect of the superoxide anion (Tremellen, 2008), hence an important line of cellular defense against OS. In the presence of DEN only, expression of SOD was highest (figure 6) compared to control and *PA, NL* treated animals. However, SOD expression was lower in the presence of DEN indicative of a protective role of *PA and NL* and reduced need for SOD required for detoxification.

Furthermore, the present study revealed alterations in the activity of glutathione peroxidase (GPx) in the presence of DEN, causing a decline (figure 3), elevation of glutathione-s-transferase (GST) (figure 5) and reduction in cellular glutathione (GSH) levels (figure 4). These observations, in part, may be due to cellular enzymatic antioxidant defense system response to DEN-induced OS resulting from its metabolites. However, alterations in GPx, GST and GSH level in the presence of *PA, NL* and co-administered with DEN, appears to have mitigated DEN-induced toxic responses by plants polyphenols and flavonoids present in *PA* and *NL*, corroborating other findings on the efficacies of phytochemicals (Tharappel *et al*., 2008, Thangapazham *et al*., 2006, Surh *et al*., 2005) with such potentials.

Relative liver weight changed significantly in DEN only treated animals (table 1) compared to control and other groups, indicative of DEN-induced hyperplasia (Thirunavukkarasu *et al*., 2004, Chuang *et al*., 2000). In addition blood glucose sugar monitored weekly appeared to decrease from week 5 through 8 in the DEN only treated group (table 2) this may be as a result of a switch to aerobic respiration pathway of glucose uptake and utilization preferred by transformed and cancerous cells (Gatenby, 2004, Devic, 2016). The presence of *PA* and *NL* did not reverse this increased glucose uptake, indicating that *PA* and *NL* could not completely alter the processes from a biologically efficient anaerobic glucose utilization pathway to the less efficient aerobic one. Hepatic tumor induction by DEN was characterized by reduced tumor burden in the presence of *PA* and *NL* (table 3a). In addition, *PA* and *NL* were not genotoxic on examination by the *E. coli* PQ37 based UMU chromotest (table 3b). Its important to therefore suggest that the phytochemical composition of *PA* and *NL* may have played a role in reducing tumor burden in the rats, while on their own, *PA* and *NL* are not genotoxic. Limited histopathological findings suggest that rat treated with *PA, NL* and in combination did not present with pathological hallmarks characteristic of DEN treated rats. Conversely the presence of *PA* and *NL* mitigated DEN-induced histopathological changes plate 1. Taken together, numerous studies have established the toxic nature of DEN, when exposed to experimental animals, and it's used widely as experimental carcinogen in animal models often resulting in the induction of hepatic tumors. Our findings presented here, reinforces further DEN's capacity for inducing hepatic damage probably involving an OS component. Conversely the presence of plant-derived phytochemicals (*Piptandenia Africana and Nauclea latifolia)* confers hepatic protection and exhibits promising effect in mitigating the toxic and morphologically altering action of exposure to DEN inadvertently or otherwise and in the onset of hepatocarcinogenesis.

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