Original Article

Hepatoprotective effects of aqueous root bark extracts of *Anogeissus leiocarpus*, *Terminalia avicennioides*, and their mixture in diethylnitrosamine-treated rats

Abstract

Introduction: This study investigated the hepatoprotective activity of aqueous root bark extracts of Anogeissus leiocarpus, Terminalia avicennioides, and their 1:1 mixture in diethylnitrosamine (DEN)-treated rats. **Materials and Methods:** Rats were orally pretreated with extracts and 1:1 mixture (200, 400, and 800 mg/kg body weight) for 2 weeks before being challenged with DEN (30 mg/kg body weight) once a week on weeks 3 and 4. Curcumin (200 mg/kg body weight) was used as reference antioxidant agent, and distilled water was used as a control. The whole experiment lasted for 4 weeks. The activities of liver and serum alkaline phosphatase (ALP), aspartate and alanine aminotransferases (AST and ALT), and gamma-glutamyltransferase (GGT), as well as the levels of serum total protein, albumin, total, and conjugated bilirubin were evaluated. **Results:** The extracts and their mixture significantly ($P < 0.05$) prevented the DEN-induced alterations in the activities of ALP, AST, ALT, and GGT in the liver and serum of the animals; as well as the altered levels of serum total and conjugated bilirubin. **Conclusion:** Overall, the results of this study revealed that the extracts possess hepatoprotective activities against DEN-induced liver damage in rats.

Key words:
Anogeissus leiocarpus, curcumin, diethylnitrosamine, hepatoprotective activity, liver function, Terminalia avicennioides

Introduction

The liver, being the major site of metabolic activities, is continually assaulted when there is exposure to chemical agents. One example of such chemical compounds is diethylnitrosamine (DEN) or *N*-nitrosodiethylamine, found in the environment, foods and consumer products. It is a potent hepatotoxin,[1-3] causes lipid peroxidation and attacks the antioxidant defense mechanism of the cell leading to liver damage which can eventually lead to liver carcinogenesis.[4,5] Such processes can be prevented or reversed by the use of antioxidants and hepatoprotective agents such as curcumin and other natural products derived from botanicals. Examples of such plants are *Anogeissus leiocarpus* and *Terminalia avicennioides*, both of which are from the *Combretaceae* family and are widely distributed in many countries including Nigeria.

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A. leiocarpus (DC) Guill and Perr is known as chewing stick tree (English) and *Ayin* (Yoruba, South Western Nigeria). *T. avicennioides* Guill and Perr is known as *Baushe* (Hausa, Northern Nigeria), *Idi* (Yoruba), and *Edo* (Igbo, Southeast Nigeria). The aqueous root bark extracts of these plants have been reported to contain flavonoids, tannins, phenolics, saponins, alkaloids, and other phytochemicals.[6] They have also been reported to exhibit free-radical scavenging, antioxidant and anticancer activities.[6,7]

In view of the day-to-day exposure of man to industrial chemicals like DEN from various sources, it is pertinent to prevent the toxicological effects of such compounds on the cellular system. This study, therefore, aims at investigating the effects of aqueous root bark extracts of *A. leiocarpus*, *T. avicennioides*, and their mixture on parameters of liver damage; and the activities of cellular enzymes in liver of DEN-treated rats using curcumin as a reference antioxidant.

Materials and Methods

Plant materials

Fresh roots of *A. leiocarpus* and *T. avicennioides* were obtained from a farmland in Offa, Nigeria and authenticated at the IFE Herbarium, Ile-Ife, Osun State, Nigeria, with voucher numbers 13775 and 15428, respectively.

Experimental animals

Male albino rats (181.98 ± 9.36 g) of Wistar strain were obtained from the Animal House of Department of Biochemistry, University of Ilorin, Ilorin, Nigeria. The animals were housed in aluminum cages placed in well-ventilated house with optimum conditions (temperature: 22 ± 3°C; photoperiod: 12 h natural light and 12 h dark; humidity: 40–45%) and were allowed free access to rat pellets (Premier Feed Mill Co., Ltd., Ibadan, Nigeria) and tap water. The entire study was carried out in the Biochemistry and Nutrition Unit, Department of Chemical Sciences, Fountain University, Osogbo, Nigeria.

Chemicals and reagents

Assay kits for alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyltransferase (GGT) were products of Randox Laboratories, County Antrim, UK. Curcumin and DEN were products of Sigma-Aldrich, St. Louis, USA. All other reagents were of analytical grade and prepared with distilled water.

Preparation of extracts

Aqueous extracts of the roots were prepared as previously described.[6] Briefly, fresh roots of both plants were separately oven-dried at 40°C for 3 weeks and pulverized with an AKIRA blender (model: BL-1531, Indonesia). A known amount (300 g) of each powder was extracted in 5 L of distilled water and placed on orbital shaker maintained at 300 rpm for 24 h. This was then filtered through Whatman No. 1 filter paper, and the filtrate concentrated on a water bath to yield 28.5 g *A. leiocarpus* (9.5%) and 30 g of *T. avicennioides* (10%). A 1:1 mixture of both extracts was also prepared for use in the present study.

Animal grouping and treatments

Eighty-four male albino rats were randomly assigned to twelve Groups (A-L) of seven rats each and treated orally with 0.5 ml of distilled water, extracts and their mixture (1:1) daily for 28 days as follows:

- Group A: Received distilled water only (daily) for 4 weeks
- Group B: Received distilled water (daily) for 4 weeks and DEN (30 mg/kg body weight in normal saline) on day 15 and day 22 (weeks 3 and 4)
- Group C: Pretreated with curcumin a reference antioxidant agent (200 mg/kg body weight in olive oil) daily for 4 weeks and DEN (as in Group B above)
- Group D: Pretreated with 200 mg/kg body weight *A. leiocarpus* extract daily for 4 weeks and DEN (as in Group B above)
- Group E: Pretreated with 400 mg/kg body weight *A. leiocarpus* extract daily for 4 weeks and DEN (as in Group B above)
- Group F: Pretreated with 800 mg/kg body weight *A. leiocarpus* extract daily for 4 weeks and DEN (as in Group B above)
- Group G: Pretreated with 200 mg/kg body weight *T. avicennioides* extract daily for 4 weeks and DEN (as in Group B above)
- Group H: Pretreated with 400 mg/kg body weight *T. avicennioides* extract daily for 4 weeks and DEN (as in Group B above)
- Group I: Pretreated with 800 mg/kg body weight *T. avicennioides* extract daily for 4 weeks and DEN (as in Group B above)
- Group J: Pretreated with 200 mg/kg body weight of the mixture daily for 4 weeks and DEN (as in group B above)
- Group K: Pretreated with 400 mg/kg body weight of the mixture daily for 4 weeks and DEN (as in Group B above) and
- Group L: Pretreated with 800 mg/kg body weight of the mixture daily for 4 weeks and DEN (as in Group B above).

The rats were sacrificed 24 h after the last doses. The study was approved by the Ethical Committee on the Care and Use of Experimental Animals of the College of Natural and Applied Sciences, Fountain University, Osogbo, Nigeria. The animals were also handled according to the Guidelines of the National Institute of Health (NIH; Bethesda, Maryland, USA), Guide for the Care and Use of Laboratory Animals.[8]

Preparation of serum and liver supernatants

The sera and liver supernatants were prepared as previously described.[9] The rats were anesthetized in a glass jar

containing cotton wool soaked in diethyl ether. The unconscious rat was quickly removed, and the neck area cleared of fur. The jugular veins were cut and the blood collected into clean and dry test tubes were allowed to clot at room temperature for 10 min and centrifuged at 894×*g* for 15 min. Sera were collected using Pasteur pipette and kept frozen. The rats were then dissected, the liver removed and cleaned, weighed and homogenized in ice-cold 0.25 M sucrose solution (1:4 w/v). The homogenates were then centrifuged at 1398×*g* for 15 min to obtain the supernatants, which were later transferred into specimen bottles and kept frozen.[9] The sera and supernatants were used within 24 h for their respective assays.

Determination of biochemical parameters

The biochemical parameters were determined using standard procedures as described for protein,[10] ALP,[11] aminotransferases,[12] GGT,[13] albumin,[14] and bilirubin.[15]

Statistical analysis

The group mean (n = 7) ± standard deviation for each analysis was calculated, and significant differences were determined by analysis of variance and Tukey's *post-hoc* test for multiple comparisons at 95% confidence level.

Results

The administration of DEN significantly (P < 0.05) reduced the liver ALP activity while pretreatment with curcumin and the extracts significantly (P < 0.05) attenuated the decrease in a dose-dependent manner. The 800 mg/kg body weight of the individual extracts produced the highest attenuation of ALP that compared favorably with the distilled water-treated control animals, whereas the activity of ALP by all the doses of the mixture compared well with the distilled water-treated control animals [Figure 1]. There was significant (P < 0.05) increase in ALP activities in the serum of animals following administration of DEN and this was dose-dependently reduced by the individual extracts and their mixture [Figure 2]. The reduction in the serum enzyme by the 800 mg/kg body weight of the individual extracts and their mixture compared well (P < 0.05) with the distilled water-treated control animals [Figure 2].

The activity of ALT in the liver of rats administered DEN was significantly reduced compared to distilled water-treated control. This trend of reduction was reversed by pretreatment with the extracts, as well as their mixture, in a manner similar to the animals treated with curcumin [Figure 3]. However, the 400 mg/kg body weight of *T. avicennioides* and the mixture were significantly (P < 0.05) higher than the enzyme activity produced by the curcumin-treated animals [Figure 3]. The administration of DEN significantly (P < 0.05) elevated the serum ALT activity and this trend was reduced by the pretreatment with the extracts and their mixture in a manner similar to that obtained with curcumin [Figure 4]. However, the activity of the serum enzyme obtained with 800 mg/kg body weight of *A. leiocarpus* and *T. avicennioides*, as well as that of 400 and 800 mg/kg body weight, of the mixture of the extracts compared favorably with those of the distilled water-treated control rats [Figure 4].

The reduction in the activity of AST in the liver of the animals by DEN was reversed by all the doses of the extracts and their mixture [Figure 5]. This pattern was similar to the DEN-treated animals administered curcumin. Furthermore, in all the treatment groups, despite the elevation in the liver AST, the increase in activity of the enzyme did not compare well with that of the distilled water-treated control animals [Figure 5]. There was significant (P < 0.05) increase in serum AST activities of rats administered DEN. This trend was reversed following the administration of the extracts and their mixture in a manner similar to that of curcumin-treated animals. The activity of the enzyme produced by the 800 mg/kg body weight of the extract-treated animals compared favorably (P > 0.05) with the curcumin and distilled water-treated animals [Figure 6].

The administration of DEN significantly reduced the liver GGT activity, and this was significantly (P < 0.05) attenuated by pretreatment with the extracts and their mixture, as well as curcumin, at all the experimental doses. The 800 mg/kg

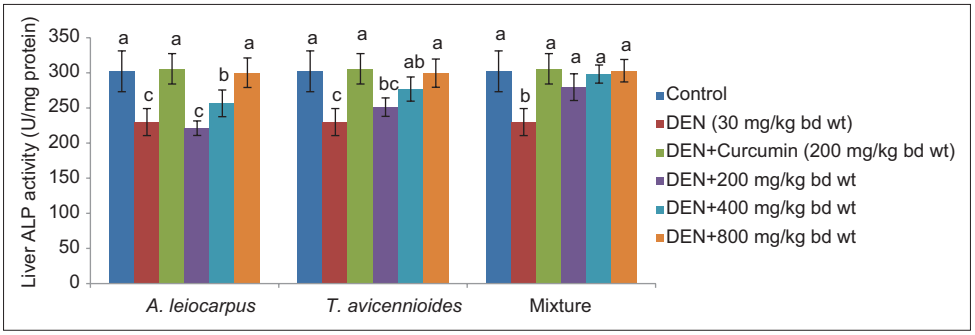

Figure 1: Liver alkaline phosphatase activities of diethylnitrosamine treated rats pretreated with aqueous root bark extracts of *Anogeissus leiocarpus*, *Terminalia avicennioides* and their mixture. Bars carrying different letters are significantly different (P < 0.05)

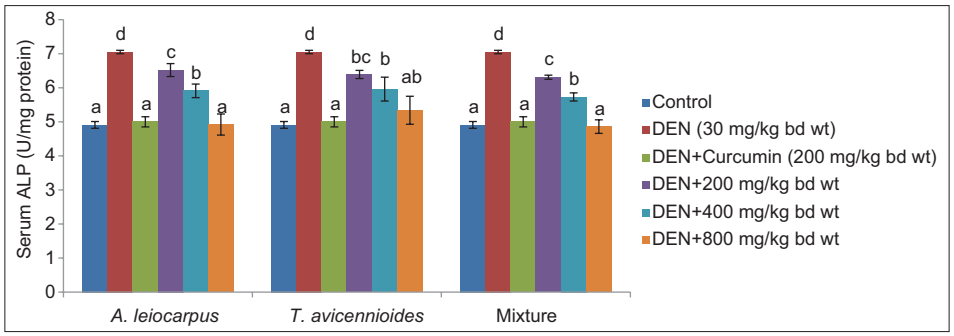

Figure 2: Serum alkaline phosphatase activities of diethylnitrosamine treated rats pretreated with aqueous root bark extracts of *Anogeissus leiocarpus*, *Terminalia avicennioides*, and their mixture. Bars carrying different letters are significantly different ($P < 0.05$)

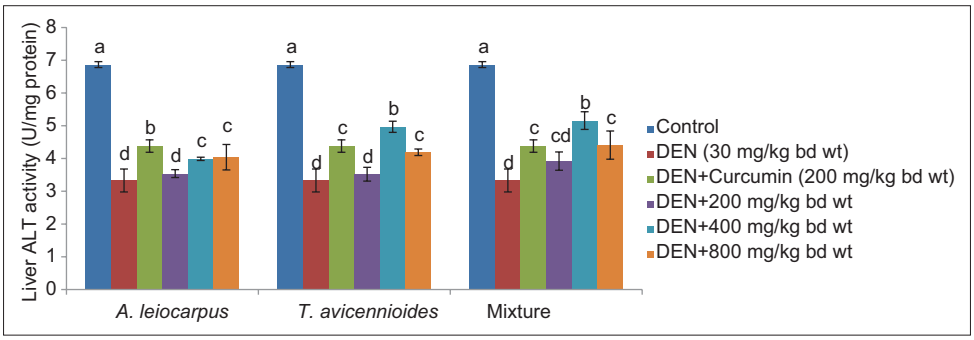

Figure 3: Liver alanine aminotransferase activities of diethylnitrosamine treated rats pretreated with aqueous root bark extracts of *Anogeissus leiocarpus*, *Terminalia avicennioides*, and their mixture. Bars carrying different letters are significantly different ($P < 0.05$)

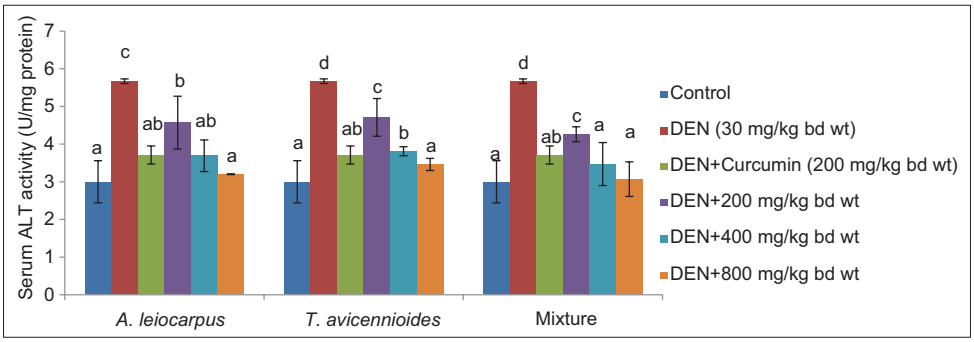

Figure 4: Serum alanine aminotransferase activities of diethylnitrosamine treated rats pretreated with aqueous root bark extracts of *Anogeissus leiocarpus, Terminalia avicennioides*, and their mixture. Bars carrying different letters are significantly different ($P < 0.05$)

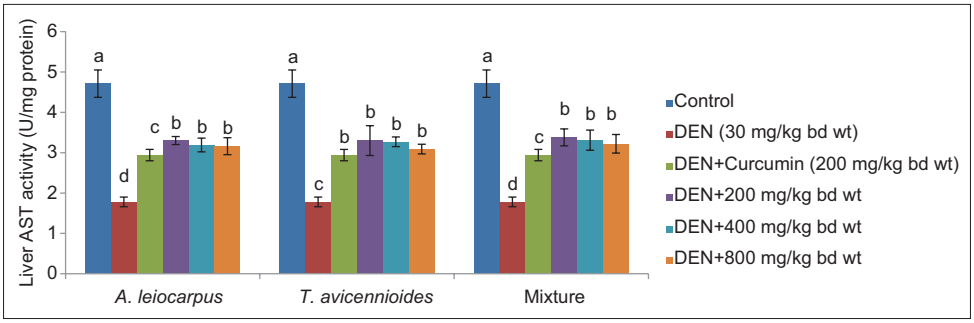

Figure 5: Liver aspartate aminotransferase activities of diethylnitrosamine treated rats pretreated with aqueous root bark extracts of *Anogeissus leiocarpus*, *Terminalia avicennioides*, and their mixture. Bars carrying different letters are significantly different ($P < 0.05$)

body weight of all the extracts produced the activity of GGT in the liver of the animals that compared favorably with the distilled water and curcumin–treated control animals [Figure 7]. Serum GGT activity was significantly elevated following DEN administration. This trend was significantly reversed by extract pretreatment at all the experimental doses. The reversal was, however, not total even in the curcumin-treated animals [Figure 8] as the enzyme activities were still significantly higher than the distilled water-treated control animals.

There were no significant ($P > 0.05$) differences in the serum total protein and albumin concentrations of all the treatment groups when compared with their respective control values [Table 1]. However, the total and conjugated bilirubin levels in DEN-treated rats were significantly ($P < 0.05$) elevated. These elevations were reversed following pretreatment with the extracts. The 800 mg/kg body weight of the individual and mixture of the extracts produced serum total bilirubin concentrations that compared well with the control [Table 2]. In contrast, all the doses of the extracts, as well as their mixture, produced serum conjugated bilirubin contents that were not significantly different from the distilled water and curcumin-treated animals [Table 2].

Discussion

Chemoprevention refers to the use of chemical agents including plant extracts to prevent a disease process. In the present study, aqueous extracts of *A. leiocarpus* and

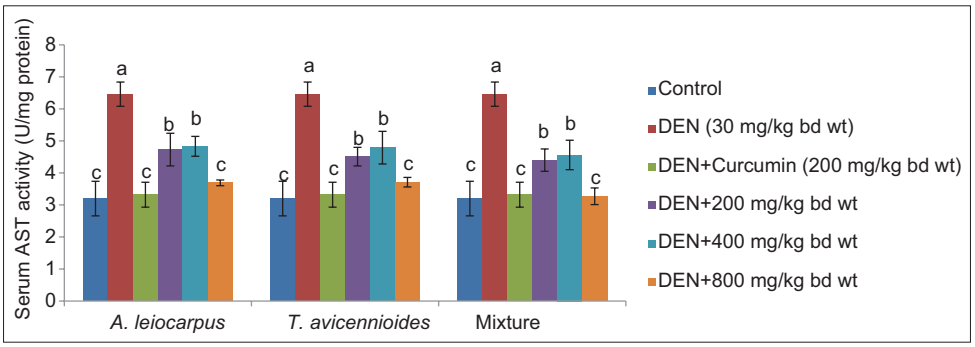

Figure 6: Serum aspartate aminotransferase activities of diethylnitrosamine treated rats pretreated with aqueous root bark extracts of *Anogeissus leiocarpus*, *Terminalia avicennioides*, and their mixture. Bars carrying different letters are significantly different ($P < 0.05$)

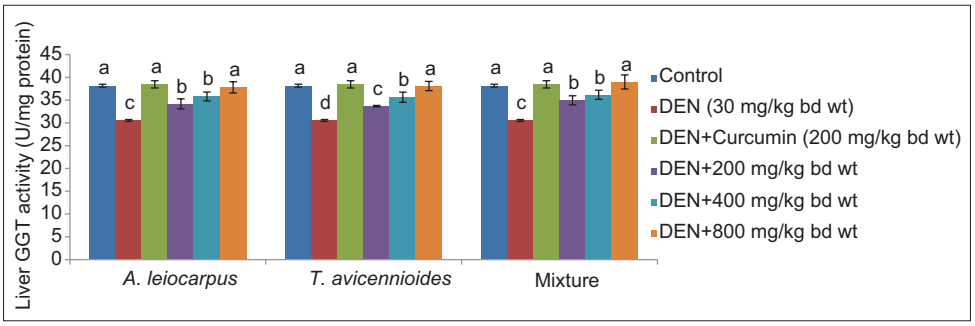

Figure 7: Liver gamma-glutamyltransferase activities of diethylnitrosamine treated rats pretreated with aqueous root bark extracts of *Anogeissus leiocarpus*, *Terminalia avicennioides*, and their mixture. Bars carrying different letters are significantly different ($P < 0.05$)

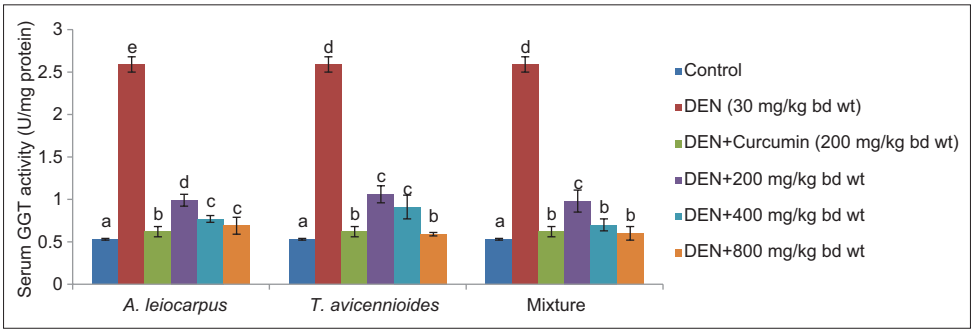

Figure 8: Serum gamma-glutamyltransferase activities of diethylnitrosamine treated rats pretreated with aqueous root bark extracts of *Anogeissus leiocarpus, Terminalia avicennioides*, and their mixture. Bars carrying different letters are significantly different ($P < 0.05$)

Table 1: Serum protein and albumin contents of DEN-treated rats pretreated with aqueous root bark extracts of *A. leiocarpus, T. avicennioides* and their mixture

Treatment	Total protein (mg/mL)			Serum albumin (mg/mL)		
	A. leiocarpus	*T. avicennioides*	Mixture	*A. leiocarpus*	*T. avicennioides*	Mixture
Control	45.40±2.83[a]	45.40±2.83[a]	45.40±2.83[a]	26.06±0.08[a]	26.06±0.08[a]	26.06±0.08[a]
DEN (30 mg/kg bd wt)	43.20±2.28[a]	43.20±2.28[a]	43.20±2.28[a]	25.05±0.32[a]	25.05±0.32[a]	25.05±0.32[a]
DEN + curcumin (200 mg/kg bd wt)	43.87±2.83[a]	43.87±2.83[a]	43.87±2.83[a]	26.07±0.28[a]	26.07±0.28[a]	26.07±0.28[a]
DEN + extract (200 mg/kg bd wt)	45.13±0.42[a]	44.90±0.51[a]	45.20±1.92[a]	24.06±0.76[a]	23.81±0.19[a]	24.09±0.35[a]
DEN + extract (400 mg/kg bd wt)	43.97±0.10[a]	44.67±1.54[a]	46.20±5.12[a]	24.31±0.19[a]	25.73±0.50[a]	25.97±0.43[a]
DEN + extract (800 mg/kg bd wt)	46.00±0.90[a]	45.14±1.77[a]	44.00±3.08[a]	25.99±0.11[a]	25.89±0.97[a]	26.33±0.13[a]

Values are mean of seven determinations±SD. Values with superscripts similar to the control, a, down the column are not significantly different when compared with control ($P>0.05$). DEN – Diethylnitrosamine; SD – Standard deviation; *A. leiocarpus – Anogeissus leiocarpus; T. avicennioides – Terminalia avicennioides*

Table 2: Serum total and conjugated bilirubin contents of DEN-treated rats pretreated with aqueous root bark extracts of *A. leiocarpus, T. avicennioides* and their mixture

Treatment	Total bilirubin (mg/dL)			Conjugated bilirubin (mg/dL)		
	A. leiocarpus	*T. avicennioides*	Mixture	*A. leiocarpus*	*T. avicennioides*	Mixture
Control	15.80±2.53[a]	15.80±2.53[a]	15.80±2.53[a]	13.11±1.11[b]	13.11±1.11[b]	13.11±1.11[b]
DEN (30 mg/kg bd wt)	30.07±3.00[c]	30.07±3.00[c]	30.07±3.00[c]	15.86±0.54[a]	15.86±0.54[a]	15.86±0.54[a]
DEN + curcumin (200 mg/kg bd wt)	19.60±2.14[ab]	19.60±2.14[a]	19.60±2.14[ab]	13.53±1.16[b]	13.53±1.16[b]	13.53±1.16[b]
DEN + extract (200 mg/kg bd wt)	21.78±1.11[b]	22.01±1.09[ab]	22.53±2.84[b]	11.96±1.49[b]	12.00±1.73[b]	12.21±1.06[b]
DEN + extract (400 mg/kg bd wt)	21.00±0.55[ab]	21.36±0.42[ab]	21.87±2.38[b]	13.01±0.09[b]	12.75±0.10[b]	12.18±1.43[b]
DEN + extract (800 mg/kg bd wt)	20.09±0.07[a]	19.13±1.71[a]	17.47±5.34[a]	13.66±1.02[b]	13.09±1.22[b]	13.44±1.44[b]

Values are mean of seven determinations±SD. Total bilirubin values with superscripts b or c different from the control, a, are significantly different when compared with control ($P<0.05$). Total bilirubin values with superscripts ab are not significantly different when compared with control, a, and when compared with b ($P>0.05$). Conjugated bilirubin values with superscripts a different from the control, b, are significantly different when compared with the control ($P<0.05$). DEN – Diethylnitrosamine; SD – Standard deviation; *A. leiocarpus – Anogeissus leiocarpus; T. avicennioides – Terminalia avicennioides*

T. avicennioides root barks have been used to prevent DEN-induced liver damage. DEN has been reported to cause oxidative stress and peroxidation of lipids in membranes, arising from excessive reactive oxygen species (ROS) production by hydroxylation and subsequent activation of DEN by cytochromes P_{450}. This eventually releases a carbocation that attacks cellular macromolecules such as proteins, lipids, and nucleic acids.[3,4,16] Such attack on cellular membranes leads to peroxidation and distortion of the ordered phospholipid bilayer thereby compromising membrane integrity and the loss of membrane and intracellular components such as enzymes.

The measurement of the activities of enzymes in tissues and body fluids plays a significant role in disease investigation, diagnosis, assault on the organs/tissues and to a reasonable extent, the toxicity of drugs, and other chemical compounds. This is because the activities of enzymes sum up the catalytic influences of various factors like inhibitors and activators during such pathological conditions.[17]

ALP is a "marker" enzyme of the integrity of cell membrane and endoplasmic reticulum.[18,19] It has been used to indicate liver damage.[3,20-22] The reduction in activities of liver ALP following the administration of 30 mg/kg body weight of DEN, which was accompanied by a corresponding increase in activities of ALP in the serum of the animals, could be attributed to loss of membrane components, including ALP, from the liver. The increase in the serum enzyme is an indication that the enzyme might have escaped from the liver cells into the extracellular fluid.[23,24] DEN has been reported to cause lipid peroxidation and damage to many enzymes through ROS production.[3,4,16] Peroxidation of the lipid membrane of cells by DEN could have been responsible for the loss of membrane components including ALP from the liver to the serum. The reversal of these effects by various doses of the extracts and their mixture is an indication of the ability of the phytochemicals such as tannins, phenolics, and flavonoids to act as antioxidants and prevent damage to liver cells. Aqueous extracts of *A. leiocarpus* and *T. avicennioides* root barks have been reported to contain flavonoids, tannins, saponins, and phenolics and also to possess antioxidant activity.[6,7] These findings agree with an earlier report that intraperitoneal injection of 200 mg/kg body weight of DEN in corn oil caused an increase in serum ALP of rats, which was reversed by administration of paeonol, a phenolic component of Moutan Cortex (*Paeonia suffruticosa*).[3]

The aminotransferases (AST and ALT) are "markers" of liver damage caused by exposure to chemicals with ALT

being more liver specific.[25,26] The reduction in activities of liver ALT and AST following administration of DEN, which was accompanied by corresponding increase in activities of serum ALT and AST could be attributed to escape of the enzymes into the extracellular space due to the compromised membrane. The increase in activities of these cytosolic enzymes in the serum further indicates that the cellular membrane was disrupted by the DEN. The reversal of these effects by the extracts and their mixture is an indication that they possess membrane-protecting activities. This is also an indication that the extracts possess chemopreventive activities. These findings also agree with a previous study, where DEN-induced activities of ALT and AST were reversed by a chemopreventive agent paeonol.[3]

GGT is involved in the amino acid transfer across the cellular membrane and in glutathione metabolism. It is a membrane-bound enzyme most sensitive to hepatobiliary disease.[27] The significant decrease in liver GGT activity accompanied by a corresponding significant increase in the serum enzyme further suggests membrane damage induced by DEN. These findings agree with a previous study, which reported an increase in serum ALP, ALT, AST, and GGT activities after a single intraperitoneal injection of 200 mg/kg body weight of DEN in rats.[3] Another study also reported an increase in the activities of ALP, AST, and ALT by 0.01% DEN in drinking water for 15 weeks, which were reversed by administration of extracts of *Morinda citrifolia* roots.[5] The cytoprotective effects of these extracts in the present study, justify the folkloric use of these plants, as well as previous reports especially the mixture, in the treatment of various diseases including cancer.[6,7]

Liver function indices such as serum total protein, albumin, total, and conjugated bilirubin are used to assess the functional activity of the liver.[9] Serum proteins (albumin and globulin) are synthesized by the liver and thus could be a useful marker to indicate the synthetic activity of this vital organ. Serum bilirubin is an index of the secretory activity of the liver.[9] DEN has been reported to cause severe hepatic injury by excessive ROS production and lipid peroxidation.[3,4]

The absence of an alteration in the levels of serum total protein and albumin in the present study could indicate that the synthetic ability of the liver was not adversely affected. This observation contrasts previous studies that reported significant reductions in the levels of these biomolecules after a single administration of 200 mg/kg body weight of DEN.[3] The disparity could be due to differences in the dose, duration, and route of administration of DEN. The significant increase in total and conjugated bilirubin concentrations following DEN treatment could be attributed to impaired bilirubin metabolism. This could indicate impaired uptake of bilirubin by hepatocytes or impaired bilirubin excretion caused by biliary tract obstruction, which could lead to the compromised excretory ability of the liver or liver dysfunction. This might have resulted from the decrease in liver enzyme activities earlier reported in the present study. The ability of the extracts to attenuate this loss of hepatic excretory function may suggest their hepatoprotective ability, or better still, their chemopreventive ability since DEN is a potent inducer of hepatocellular carcinoma.[3,4] A previous study also reported an increase in bilirubin concentration following DEN administration, which agrees with the findings from the present study.[5]

The results of this study have shown that aqueous extracts of *A. leiocarpus* and *T. avicennioides* root barks, as well as their mixture, possess hepatoprotective abilities that compared well with curcumin. The prevention of alterations in the parameters of liver function and activities of liver enzymes was most pronounced at the highest dose (800 mg/kg body weight) of the individual extracts and all the doses of the mixture.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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