Research Article

Attenuation of N, N-Dimethylnitrosamine-Induced Liver Fibrosis in Rats by Ethanolic Leaf Extract of *Annona Muricata*

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Abstract: This study was carried out to investigate the effects of ethanolic leaf extract *Annona muricata* (soursop) on N,N-dimethylnitrosamine (DMN)-induced liver fibrosis in rats. Four groups of Wistar albino rats (12 rats/group) were used for the study. One group received intra-peritoneal injection of 10mg of DMN/kg body wt. thrice a week (on the first three days) for 14 days, in addition to the extract at a dose of 200mg/kg body wt. given by gavage. A second group received the same dose of DMN but without extract, while rats in the third group were administered the same dose of extract without DMN. Members of the fourth group were given physiological saline (vehicle), and served as controls. After 14 days, the rats were sacrificed by cardiac puncture. Blood samples were collected from the ocular vein, and the sera were analysed for aspartate transaminase, AST; alanine transaminase, ALT and alkaline phosphatase, ALP; while collagen, malondialdehyde, MDA; reduced glutathione, GSH, superoxide dismutase, SOD and catalase, CAT levels were assayed in liver tissue. All assays were done using standard methods. Liver sections were also fixed in formol-saline and subjected to histological analysis. DMN administration resulted in significant increases in serum AST, ALT and ALP; and in liver MDA and total collagen content (P 0.05). On the other hand, liver SOD and CAT activities were significantly reduced by the DMN treatment. Histological examination of liver sections from the DMN rats showed severe degenerative changes such as congestion, haemorrhagic necrosis and deposition of thick collagen fibers. These biochemical and histological changes were reversed significantly by the *Annona muricata* leaf extract.These results suggest that *Annona muricata* leaves may reverse hepatic fibrosis probably through maintenance/restoration of liver antioxidant status.
Keywords: *Annona muricata,* Liver Fibrosis, Collagen, N,N-Dimethylnitrosamine, DMN

INTRODUCTION

Nitrosamines are well-known carcinogenic agents [1, 2]. Humans are exposed to nitrosamines through various routes, the most important of which is the diet. Meat processing methods such as cooking, frying, smoking and grilling result in nitrosamine formation, especially if the meat was preserved with nitrite [3]. During these processes, the nitrite reacts with secondary amines in the meat to generate nitrosamines. Indeed all protein-containing foods can produce nitrosamines when exposed to a nitrogen source [3]. Thus humans are regularly exposed to nitrosamines in foods. For nitrosamines to exert their carcinogenic effects, they are activated through hydroxylation by liver cytochrome P_{450} 2E1 sub-family of phase I enzymes, to generate derivatives that form adducts with DNA [4-6]. Thus the liver is the principal site of action of many nitrosamines, especially DMN which primarily targets the hepatocytes [4,5]. Studies have shown that DMN exposure in rats leads to hepatic fibrosis, characterized by accumulation of extracellular matrix proteins or scar [7,8]. The pathogenesis of hepatic fibrosis is mediated through oxidative stress and hepatocyte injury, and is always accompanied by impaired hepatic metabolism and deposition of connective tissue components especially collagen and hyaluronan [7,8]. Fibrosis results in enhanced synthesis of matrix components specifically collagen, with a concomitant decrease in the expression of matrix degrading proteases and accumulation of several extracellular matrix, ECM proteins [9].

It has been well established that DMN-induced liver injury in rats is a reproducible and potentially valuable animal model for studying the pathogenesis of hepatic fibrosis and alcoholic cirrhosis, and for screening anti-fibrotic agents [10,11]. Although there are no perfect and effective anti-fibrogenic agents, the potential candidates include agents that reduce inflammation, suppress immune response and block the activation of ECM-producing cells [12]. Others include

antioxidants such as vitamin E [13] and agents that increase fibrillar ECM degradation [14].

Several studies have established the antioxidant properties of the leaves of Graviola (soursop), *Annona muricarta* [15,16]. In view of the diverse dietary sources of human exposure to nitrosamines, studies on mitigation of their toxic effects are of health significance. This study was therefore designed to investigate the protective effect of ethanolic extract of *A. muricarta* leaves against DMN-induced liver fibrosis in rats.

MATERIALS AND METHODS
Collection, identification and preparation of *Annona muricata* leaves
Fresh leaves of *Annona muricata* were purchased from a local market in Benin City, Edo state, Nigeria and identified by Dr. Chris Akoma, a Botanist in the Department of Basic Sciences, Faculty of Basic and Applied Sciences, Benson Idahosa University, Benin city, Edo State. The *Annona muricata* leaves were separated from the stalk, washed and air-dried at room temperature (24^oC) and then pulverized, crushed into fine powder and weighed.

Preparation of leaf extract
The ethanolic extract of the plant leaves was prepared by soaking 100g of the dry powdered plant leaves in 1000ml of absolute ethanol at room temperature for 48hrs (for thorough extraction). The extract was then filtered first through a Whatmann filter paper No. 42 (125mm) and then through cotton wool. The extract was thereafter concentrated in a rotary evaporator at 60∘C to one-tenth its original volume and then finally freeze-dried. The dried residue (crude extract) was then stored at 4°C. Portions of the crude extract were weighed and dissolved in distilled water for use on each day of our experiments.

Experimental animals, DMN and extract administration
Male Albino Wistar rats weighing 150-225 g were obtained from the Animal Unit of the University of Ibadan, Ibadan, Oyo state, Nigeria. The animals were housed in controlled environmental conditions (temperature—24 ± 2 ∘C; relative humidity—50–70%; 12 h light/dark cycle) in the Animal House of the Department of Biochemistry, University of Benin, Benin city, Edo state. The animals were provided standard pellet diet and water *ad libitum*. Institutional Animal Ethical Committee permission was obtained before performing the experiments.

The DMN used in this work was synthesized in a fume chamber at the Department of Biochemistry, University of Ibadan, Oyo state, Nigeria, according to the method of Vogel (1971).

Forty-eight (48) rats divided into four groups were used for the study. The *Annona muricata* leaf extract was reconstituted in distilled water and administered orally via gastric intubation to groups 1 and 2 rats at a dose of 200mg/kg for two weeks (14 consecutive days). One of these groups (group 2) received DMN in addition at a dose of 10mg/kg b.w (dissolved in 0.15M NaCl) via intraperitoneal injection in the first three days of each week for 14 days. Rats in group 3 received the same amount of DMN but without extract, while members of the fourth group received normal saline and served as controls. Hepatic fibrosis was confirmed two weeks after through histology and total collagen assay. Administration of extract and drug was between 9:00–10:00am each day. 24 hours after the end of the treatment period, the rats were sacrificed by cardiac puncture and blood collected via the ocular vein in plain tubes and allowed to stand for 45 min before centrifuging at 5,000 rpm for 20 min. The serum samples were stored at -20° C until analyzed.

Collection of tissue samples and preparation of liver homogenates
Following sacrifice, liver samples were quickly excised and rinsed with normal saline. A small portion of each liver sample was fixed in 10% phosphate-buffered formalin for histological examination while the remaining portions were stored at –20°C for biochemical analysis. 10% liver homogenate was prepared in physiological saline. The homogenate was centrifuged at 5000 x g for 15 minutes and the clear supernatant obtained used for biochemical analysis.

Biochemical assays
Serum AST and ALT activities were estimated colorimetrically according to the method of Reitman and Frankel [17], while ALP assay was done by monitoring change in absorbance at 405nm due to liberation of para-nitrophenol, using Randox kits (UK) according to manufacturer's instructions. Liver total collagen was assayed as hydroxyproline derivatives following hydrolysis using QuickZyme^R kits (Biosciences, Netherlands). MDA was estimated in a colorimetric reaction with thiobarbituric acid [18]. SOD was assayed based on the ability of the enzyme to inhibit the autooxidation of epinephrine according to the method of Misra and Fridovich [19]. The assay of catalase was carried out colorimetrically by measuring the first order rate constant for the decomposition of H_2O_2 in the presence of the enzyme [20]. Reduced glutathione was estimated colorimetrically by measuring the reduction of Ellman's reagent (5, 5'-di-thio-bis-2-nitrobenzoic acid) at 412nm as described by Ellman [21].

Histology
Liver sections fixed in formol-saline were processed for light microscopy at the Department of Anatomy, Faculty of Basis Medical Sciences, College

of Medicine, University of Benin. The resultant slides were read and interpreted by one of us, G.I.E., a qualified pathologist.

Statistical Analysis

Numerical data obtained from the study were expressed as mean ± SD. Differences between means were determined using Statistical Package for Social Sciences (SPSS). A probability level of less than 5% (p < 0.05) was considered significant.

RESULTS

Table 1 shows the effect of *Annona muricata* on liver total collagen and serum AST, ALT, and ALP in the DMN-treated rats and controls. Total liver collagen of the DMN group was significantly higher than in each of the other groups (p < 0.05). However, the extract administration led to significant decrease in liver total collagen ((p < 0.05). The DMN group also had significantly higher serum levels of ALT, AST and ALP when compared with corresponding values for each of the other two treatment groups and controls ((p < 0.05).

Table 2 shows liver levels of SOD, CAT and GSH in the 4 groups. The mean liver MDA levels of the DMN treated rats were significantly higher than those in the rats that received AME and intraperitoneal DMN injections as well as in the control and the group that received the extract alone (p < 0.05). The mean hepatic SOD and CAT levels of rats in the DMN group were significantly lower than corresponding values for DMN + extract group, extract-only group and control group (p < 0.05). The DMN group also had significantly lower liver GSH concentration than any of the other groups (p < 0.05).

Table-1: Effect of *Annona muricata* on liver total collagen and serum AST, ALT, and ALP in DMN-treated rats and controls

Treatment groups	Total Collagen (µg/ml of liver homogenate)	ALT (U/L)	AST(U/L)	ALP (U/L)
Control (normal saline)	$43.01 \pm .450^a$	$14.67 \pm .52^a$	$18.33 \pm .14^a$	28.01 ± 3.31^a
AME only (200mg/kg)	$43.78 \pm .992^a$	$17.75 \pm .59^a$	$21.00 \pm .94^a$	$30.00 \pm .13^a$
AME (200mg/kg) + DMN (10mg/kg)	$57.95 \pm .032^b$	$41.66 \pm .52^b$	$66.33 \pm .50^b$	$77.72 \pm .97^c$
DMN only (10mg/kg)	$106.08 \pm .946^c$	$81.09 \pm .20^c$	138.5 ± 8.49^z	128.53 ± 9.41^z

Values are mean± SD; n=5. Mean values in each column having different superscript (a, b, c, z) are significantly different (p < 0.05)

Table-2: Liver MDA, GSH, SOD and CAT in DMN-treated rats and controls

Treatment group	MDA(U/mg)	GSH(µM/mg)	SOD(U/mg)	CAT(U/mg)
Control(Normal Saline)	1.87 ± 0.28^a	48.14 ± 2.98^a	9.33 ± 0.58^a	52.05 ± 3.33^a
AME only (200mg/kg)	$1.40 \pm .22^a$	54.82 ± 5.89^a	11.83 ± 0.76^b	$61.21 \pm .25^b$
AME (200mg/kg) + DMN (10mg/kg)	3.10 ± 0.28^c	35.31 ± 2.15^c	5.86 ± 0.80^c	37.02 ± 3.98^c
DMN only (10mg/kg)	6.89 ± 0.11^z	13.79 ± 3.44^z	2.01 ± 0.10^z	12.75 ± 4.97^z

Values are means ± SD; n=5. Mean values in each column having different superscript (a, b, c, z) are significantly different (p < 0.05)

Histological examination of the liver sections revealed that while the control and extract-alone groups had normal features DMN caused severe degenerative changes such as hemorrhagic necrosis, bridging fibrosis and early cirrhosis (Plate 1). These changes were significantly mitigated in the rats given extract and DMN (Plate 2). The features of liver sections from the control were normal (Plates 1), while liver sections from the group given DMN alone had severe degenerative changes marked by hemorrhagic necrosis, bridging fibrosis and extensive collagen deposition (Plates 2A &2B).
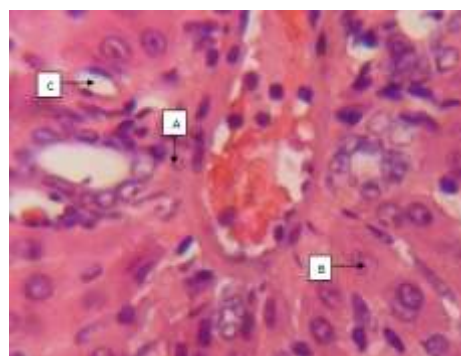

Plate-1: Photomicrograph of liver section from control rats showing liver parenchyma with central vein and radiating column of hepatocytes, with portal triad (A), hepatocytes (B) and sinusoid (C). Portal tracts appear normal (H&E staining; x 40)
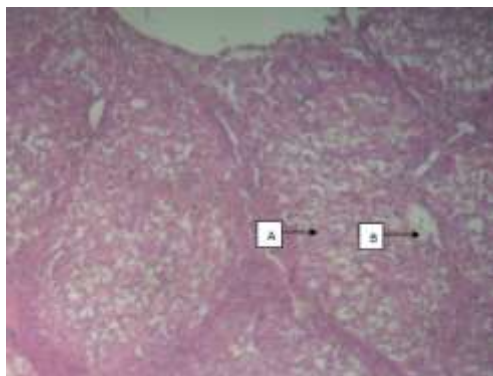
Plate-2A: Liver section from rats treated with DMN alone (10mg/kg) and sacrificed after 14 days. Well developed bridging fibrosis, cirrhosis and extensive deposition of collagen (A); and hemorrhagic necrosis (B) are evident (H&E x 5)
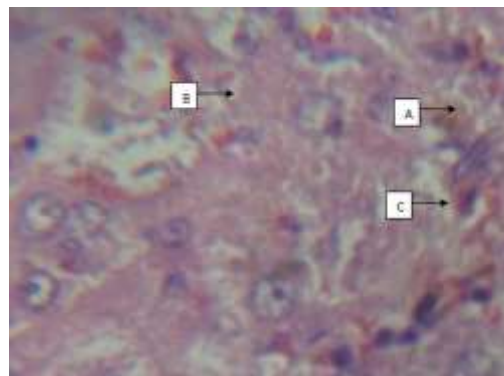

Plate-2B: 2A at a higher magnification (H&E staining; x 10)

Plate-3: Rat Liver treated with *Annona muricata* alone (200mg/kg) and sacrificed after 14 days showing mild vascular congestion A and mild kupffer cells activation B (H&E x 40)

Plate-4: Rat Liver treated with *Annona muricata* (200mg/kg) and DMN(10mg/kg) and sacrificed after 14 days showing moderate hepatocytes preservation A, mild hemorrhagic necrosis B and moderate kupffer cells activation C (H&E x 40). Bridging fibrosis is absent.
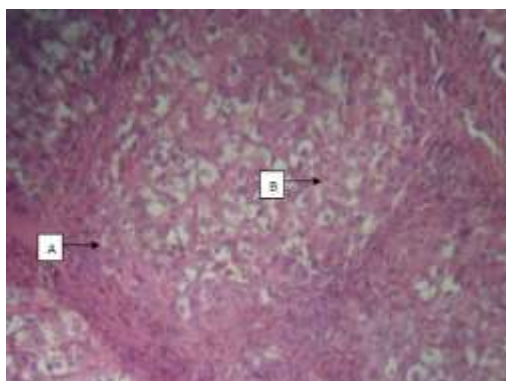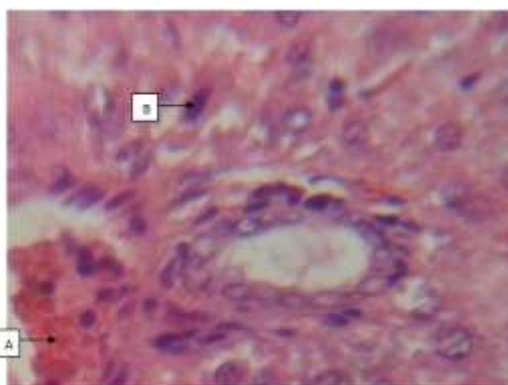
DISCUSSION

It has been reported that administration of DMN induces inflammation of hepatocytes, necrosis and increases in serum marker enzymes of hepatotoxicity [22-24]. Our results on DMN-induced elevation of serum marker enzymes and histological evidence of hepatocellular necrosis are in agreement with these reports. However the administration of *Annona muricarta* extract reversed these tissue lesions and serum enzyme profiles. Transaminases and ALP are sensitive indicators of hepatocellular injury [25]. Our results are in agreement with data from several studies which shave shown that DMN-induced increases in serum levels of enzyme markers of hepatoxicity are reversed by administration of drugs such as curcumin and silymarin [9]; ingestion of grape skin and seeds [26], ingestion of ethanolic root extract of *Operculina turpethum* (Sharma & Singh, 2014) and treatment with 1-*O*-hexyl-2,3,5-trimethylhydroquinone(HTHQ) [27].

DMN is one of the many agents that exert their hepatotoxic effects through oxidative stress Sathish *et al,* [28]. Oxidative stress causes increased membrane lipid peroxidation and cell membrane damage. Lipid peroxidation, the oxidative alteration of polyunsaturated fatty acids is generally accepted as a critical factor in the pathogenesis of hepatic injury by a number of toxic agents [29,30]. Malondialdehyde (MDA) is considered the most significant indicator of membrane lipid peroxidation resulting from the interaction between ROS and cell membrane lipids [31, 32]. In the present investigation, oxidative stress, marked by elevation in MDA was noticed in the liver of DMN-treated rats. This observation is similar to previous reports [30, 22, 33]. Numerous harmful effects of MDA have been reported [34, 35]. MDA also causes mutations by reacting with guanine nucleotide in DNA [36]. Liver MDA levels in the DMN rats were significantly higher than in the other three groups. However simultaneous administration of the *A. muricarta* leaf extract reduced

the MDA concentration close to control levels. Since MDA exerts its toxic effect through oxidative stress, the significant reduction in the levels of MDA in the liver tissues of rats simultaneously treated with *Annona muricata* and DMN is most likely a consequence of extract-induced reduction in lipid peroxidation and elevation/stabilization of tissue antioxidant enzyme activities. This is so because *Annona muricata* leaves have been shown to possess antioxidant properties, due to the presence of acetogenins, which probably play the role of effective free radical scavengers [37]. In agreement with these findings, a study by Shin and Moon [26] on the effect of dietary supplementation of grape skin and seeds on DMN-induced liver fibrosis in rats found that MDA toxicity was significantly reduced by feeding the rats with grape skin or seeds. Earlier-on, George *et al.* [9], reported that silymarin and curcumin significantly reduced MDA levels in rats treated with DMN. Vendemiale *et al.* [29] reported that N-acetylcysteine, a glutathione precursor, was found to reduce the level of free radicals in DMN-treated rats. Moreover, the patterns of MDA levels in the various groups tally with their contents of enzymatic and non-enzymatic antioxidants. Thus reduced glutathione (GSH) and enzymatic components; CAT, and SOD were significantly decreased in the rats treated with DMN relative to control group. These results are consistent with previous reports which indicated that DMN brought about significant decreases in liver SOD, CAT and GSH [22-24,38]. The reduction in GSH levels might be a consequence of reduced hepatic activity of glucose-6-phosphate dehydrogenase. It has been reported that reduced glucose-6-phosphate dehydrogenase activity leads to increased cellular ROS levels, depletion of glutathione stores and heightened oxidative stress [39]. The significant reductions in CAT, SOD and GSH in DMN treated rats might be due to lowered rate of production as a consequence of DMN-induced hepatocellular damage, or probably due to their increased rate of utilization in free radical scavenging arising from DMN toxicity. These decreases in SOD and CAT activities in DMN-treated rats are similar to reports from other animal studies where hepatocellular damage was induced by administration of acetaminophen, carbon-tetrachloride, ethanol, or galactosamine/endotoxins [40-43]. On the other hand, simultaneous treatment of the DMN rats with ethanolic leaf extract of *Annona muricata* significantly increased liver SOD, CAT and GSH when compared to rats given DMN alone. Thus the extract significantly prevented liver damage by maintaining the integrity of the plasma membrane through reduced rate of lipid peroxidation, thereby suppressing the leakage of liver marker enzymes. Again the reduced rate of lipid peroxidation arising from increases in levels of SOD, CAT and GSH due to *Annona muricata* extract treatment can be attributed to the antioxidant properties of the extract. The antioxidant effect of *Annona muricata* leaf extract has been demonstrated by its ability to significantly reduce serum ROS, GSSG and MDA, while

significantly raising hepatic levels of CAT, GTPx, SOD and GSH in streptozotocin diabetic rats [44]. In a more recent study, DMN-induced lipid peroxidation in hepatocytes and decreases in SOD, CAT and GSH were significantly annulled by administration of *Operculina turpethum* [45].

Accumulation of connective tissue proteins, especially collagen has been reported in DMN-treated fibrotic rats, and measurement of these parameters is suggested as a valuable tool in the quantification of fibrosis as well as assessment of potency of anti-fibrotic drugs during therapeutic trials [46]. In the present study, there was a near 3-fold increase in liver collagen in the DMN-treated rats, indicating the establishment of hepatic fibrosis which is in agreement with results on DMN-induced increases in liver collagen, and their reduction by a variety of agents [9-11, 47, 48-51,26]. In injured liver, hepatic stellate cells (HSCs) also called Ito are primarily responsible for the increased collagen synthesis [52-55,10]. These cells are activated during necrosis, and in a manner similar to myofibroblasts, they initiate synthesis of large amounts of collagen [10]. The near 3-fold increase in liver collagen in DMN-treated rats is supported by histological evidence of bridging fibrosis seen in these rats. Again, simultaneous administration of the *Annona muricata* extract with DMN produced a protective anti-fibrogenic effect manifested by significantly decreased liver total collagen and very minimal fibrotic changes. Thus liver sections from this group had less damage than liver sections from the DMN group, indicating that the *Annona muricarta* extract produced a protective effect.

The results obtained in this study provide strong evidence for the anti-fibrotic effect of ethanolic leaf extract of *Annona muricarta* in DMN-treated rats. This is the first report on the anti-fibrotic potential of this plant, and it is considered crucial especially in Nigeria with increasing incidents of alcohol and drug-induced liver cirrhosis and fibrosis. *Annona muricarta* may well come to the rescue due to its ready availability and cheapness, being a plant that grows practically in all household gardens in Southern Nigeria. Moreover since there is as yet no effective clinical treatment for liver fibrosis, these findings may offer a basis for development of novel anti-fibrotic agents from leaves of *Annona muricarta.*

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