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Modulatory Effect of Ethanolic Leaf Extract of *Annona muricata* Pre-treatment on Liver Damage Induced by Dimethylnitrosamine (DMN) in Rats

Usunobun Usunomena[1*], Okolie P. Ngozi[2] and Eze G. Ikechi[3]

[1]Department of Basic Sciences (Biochemistry unit), Faculty of Basic and Applied Sciences, Benson Idahosa University, P.M.B 1100, Benin City, Edo State, Nigeria.
[2]Department of Biochemistry, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.
[3]Department of Anatomy, School of Basic Medical Sciences, College of Medicine, University of Benin, Benin City, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study aim to evaluate the modulatory effect of ethanolic leaf extract of *Annona muricata* against dimethylnitrosamine (DMN)-induced hepatic toxicity in rats.
Methodology: Twenty-four (24) male albino rats divided into four (4) groups of six (6) rats each were used for the study. Group 1 served as control and was untreated, group 2 and 3 were pre-treated with 400 mg/kg *Annona muricata* for one week while group 3 and 4 each received a single oral dose of 20 mg/kg DMN after one week. The rats were sacrificed 48 hrs after DMN administration.
Place and Duration of Study: University of Benin and Benson Idahosa University, Benin city, between January and April, 2015.
Results: In rats administered 20 mg/kg DMN, *Annona muricata* pre-treatment at 400 mg/kg body weight significantly reduced the levels of alanine aminotransferase (ALT), aspartate amino-

**Corresponding author: Email: usunsquare@yahoo.com;*

transferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), total cholesterol (TC), triglyceride (TG) and malondialdehyde (MDA) as well as significantly increased reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) compared to DMN-alone administered rats which showed the opposite. Histopathological study in corroborating biochemical analysis also showed significant reduction of liver damage in the rats.
Conclusion: *Annona muricata* significantly restored the antioxidant levels in the liver, counteracted oxidative hepatic dysfunction and exhibited significant protective effect against DMN-induced liver toxicity, which can be mainly attributed to the antioxidant property of *Annona muricata*.

Keywords: Annona muricata; dimethylnitrosamine; hepatic; ethanol; necrosis.

1. INTRODUCTION

Research on *Annona muricata* commonly called sour sop in Nigeria have shown that a novel set of phytochemicals (Annonaceous acetogenins) found in the leaves, seeds and stem are cytotoxic against various cancer cells [1-3]. Traditional practitioners often use *Annona muricata* leaves to treat headaches, liver problems, diabetes, insomnia and hypertension [4,5]. Phytochemical screening of the leaves of *Annona muricata* has shown it to consist of flavonoids, tannins, saponins, cardiac glycosides and alkaloids [6], annomuricin E, annomuricin C, muricatocin C, gigantetronenin and murica-pentocin with antioxidant and antitumor properties [7,8], as well as, essential oils such as β-caryophyllene, δ-cadinene, epi-α-cadinol and α-cadinol [9].

Dimethylnitrosamine (DMN), a member of the *N*-nitrosamines, is a potent hepatotoxin, carcinogen and mutagen which exerts its carcinogenic effects, causes fibrosis, cirrhosis and induces hepatic necrosis in experimental animals through metabolic activation by CYP2E1 [10,11]. DMN is generated from the *in situ* reaction of dimethylamine (DMA) with monochloroamine in the disinfection process or the nitrosation of DMA by nitrite [12]. The occurrence of DMN in our drinks and foods including fish, meat as well as fresh supermarket products is well established [13-16]. The aim of this study is to investigate the effects of *Annona muricata* ethanolic leaf extract pre-treatment on serum liver function, lipid profiles and biomarkers of oxidative stress in hepatocytes of DMN administered rats.

2. MATERIALS AND METHODS

2.1 Collection, Identification, Preparation and Extraction of Plant Leaves

Annona muricata leaves were collected from the tree in Upper Sakponba, Benin City, Edo state, Nigeria and thereafter identified by a Botanist in Benson Idahosa University, Benin City, Edo State. The leaves after separation from stalk were washed, air-dried at room temperature and then pulverized, crushed into fine powder and weighed.

Annona muricata ethanolic extract was prepared by soaking 300 g of the dry powdered plant leaves in 1 litre of absolute ethanol at room temperature for two (2) days. The extract was then filtered using Whatmann filter paper No. 42 (125 mm) followed by cotton wool. The filtrate was thereafter reduced to one-tenth its volume using a rotary evaporator with the water bath set at 60ºC and then finally freeze dried. The dried residue (crude extract) was then stored at 4ºC. Before use, portions of *Annona muricata* ethanolic leaf extract were weighed and reconstituted in distilled water and administered orally via gastric intubation

2.2 Experimental Animals, DMN and Extract Administration

Male wistar albino rats divided into four (4) groups of six (6) rats each, weighing between 160-195 g were obtained from the Animal Unit facility of the University of Ibadan, Oyo state, Nigeria and housed in wooden cages in the animal house of the Department of Biochemistry, University of Benin. The rats were maintained under controlled environmental conditions (temperature- 24±2ºC; relative humidity-50-70%; 12 h light/dark cycle), housed for one week after their arrival to the animal house for acclimatization. The rats had free access to drinking water and normal pellet diet (NPD) *ad libitum* until they were assigned to individual groups.

Also the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments were examined and approved by the appropriate ethics committee"

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 [17].

A total of 24 rats divided into 4 groups were used. Group 1 served as control, Group 2 received 400 mg/kg *Annona muricata* only for seven (7) consecutive days, Group 3 received 400 mg/kg *Annona muricata* for 7 consecutive days followed by oral administration of a single dose of 20 mg/kg DMN (dissolved in 0.15 M NaCl), on day 8 while Group 4 received oral administration of single dose of 20 mg/kg DMN (dissolved in 0.15 M NaCl) on day 8. Without the use of anticoagulant, all rats were sacrificed on the tenth day of the study by cardiac puncture and blood collected via the ocular vein in plain tubes and allowed to stand for 45 min before it was centrifuged at 4,000 rpm for 30 min. Serum was stored at -20ºC until analyzed.

2.3 Collection of Tissue Samples and Preparation of Liver Homogenates

Following sacrifice, liver samples were quickly excised and rinsed with normal saline and a small portion of each fixed in 10% phosphate-buffered formalin for histological examination while the remaining portions were stored at -20ºC 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 determination of MDA, SOD, CAT and GSH.

2.4 Biochemical Assays

Serum AST and ALT activities were estimated colorimetrically according to the method of Reitman and Frankel [18]. Serum ALP, GGT, triglyceride and total cholesterol were estimated using Randox kits (UK) according to manufacturer's instructions. GSH was estimated colorimetrically by measuring the reduction of Ellman's reagent (5, 5'di-thio-bis-2-nitrobenzoic acid) at 412 nm as described by Ellman [19]. SOD was assayed based on the ability of the enzyme to inhibit the autooxidation of epinephrine according to the method of Misra and Fridovich [20] while CAT was carried out colorimetrically based on the measurement of the rate of decomposition of H_2O_2 after the addition of the sample containing the enzyme by reacting it with excess $KMnO_4$ and then measuring the residual $KMnO_4$ spectrophotometrically at 480 nm [21]. MDA was estimated in a colorimetric reaction with thiobarbituric acid [22].

2.5 Histology

Liver sections fixed in formol-saline were processed for light microscopy at the Department of Anatomy, School 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 consultant pathologist.

2.6 Statistical Analysis

Numerical data obtained from the study were expressed as mean value ± standard deviation. Differences between means of control and tested groups were determined using Statistical Package for social scientist (SPSS). A probability level of less than 5% (P<0.05) was considered significant.

3. RESULTS

Table 1 shows the serum hepatic marker enzyme levels of control and experimental rats. The levels of serum hepato-specific enzymes such as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) were significantly increased in DMN-administered rats, when compared with control rats. However pre-treatment with *Annona muricata* (400 mg/kg) prior to DMN administration significantly decreased the level of serum hepato-specific enzymes when compared to DMN- alone treated rats.

Serum total cholesterol and triglycerides were significantly elevated (P<0.05) in DMN-treated rats compared to control rats while *Annona muricata* pre-treatment improved total cholesterol and triglycerides towards normal values as shown in table 2.

The changes in the levels of hepatic lipid peroxidation content and antioxidants in control and experimental rats are shown in Table 3. The levels of malondialdehyde (MDA) content was significantly increased (P<0.05) in DMN-treated rats when compared with normal control rats while in rats pre-treated with *Annona muricata* prior to oral administration of a single dose of DMN, there was a significantly low levels of MDA when compared to DMN- alone treated rats. A significant depletion (P<0.05) in the levels of

GSH, CAT and SOD were noticed in rats treated with DMN when compared to normal control rats. Pre-treatments with *Annona muricata* prior to DMN administration significantly (P<0.05) restored the antioxidant levels towards near normalcy when compared with DMN-alone treated rats.

Histopathological studies showed that treatment with DMN caused severe liver damage including sinusoidal dilation, severe centrilobular hemorrhagic necrosis, vacuolization, inflammatory cell infiltration (Fig. D) when compared with control liver (Fig. A). The above changes were reduced in the liver of rats treated with *Annona muricata* and DMN (Fig. C). The histological pattern were normal in rats treated with *Annona muricata* alone (Fig. B).

4. DISCUSSION

Liver function tests are commonly used in clinical practice to screen for liver disease, monitor the progression of a known disease and determine the effects of potentially hepatotoxic drugs [23]. In this study, rats treated with DMN alone showed a significant increase in serum levels of AST, ALT, ALP and GGT compared to controls. The abnormally high level of serum AST, ALT, ALP and GGT is a consequence of DMN-induced liver damage. Also, alterations in GGT and ALP are likely to affect membrane permeability and produce derangement in the transport of metabolites. However, DMN-administered rats orally pre-treated with extracts of 400mg/kg *Annona muricata* showed significantly decreased levels of serum AST, ALT, ALP and GGT compared to DMN alone treated rats. The decrease in AST, ALT, ALP and GGT levels by the extract in tested groups indicate the protection of structural integrity of hepatocytic cell membrane or regeneration of damaged liver cells. The findings in this study agree with those of Kim et al. [24] and Kusunose et al [25] who reported significant increases in serum AST and ALT levels, as well as with the findings of Lukivskaya et al. [26], which showed a significant rise of ALP level after DMN treatment. Our result is also in agreement with those of Farombi et al [27,28] who reported that pre-treatment with curcumin as well as kolaviron significantly lowered serum activities of AST, ALT and GGT that were released as a consequence of DMN-induced hepatic damage. Similarly, Patel and Sail [29] reported that a single dose of 12mg/kg DMN caused significant elevations of plasma AST, ALT and ALP compared to controls while dietary Vitamin E significantly reversed the hepatotoxic effect of the DMN intoxication.

Alterations in the concentration of major lipids of animals such as cholesterol and triglycerides can give useful information on lipid metabolism as well as predisposition of the animals to cardiovascular risk [30]. The results of this study show that DMN caused a significant (P <0.05) increase in the levels of total cholesterol and triglycerides. The increase in cholesterol in DMN intoxicated rats might have resulted from damage of hepatic parenchymal cells that lead to disturbance of lipid metabolism in liver. It's also possible that damage to the liver by DMN affected reverse cholesterol transport due to decreased synthesis of HDL-cholesterol leading to elevated serum cholesterol. The significant increase in serum total cholesterol and triglycerides in DMN intoxicated group is in agreement with those of Augusti et al. [31] who reported that injection with CCl_4 increased serum and tissue lipid profile. However, pre-treatment with extract of 400 mg/kg Annona muricata on DMN toxicity showed a significant (P < 0.05) decrease in serum total cholesterol and triglyceride levels.

Table 1. Effect of ethanolic leaf extract of *Annona muricata* pre-treatment on serum liver function enzymes in DMN toxicity

Treatment	AST (U/l)	ALT (U/l)	ALP (U/l)	GGT (U/l)
Control (normal saline)	19.50±2.18^a	13.50±1.66^a	25.67±1.25^a	14.01±1.01^a
AME alone (400 mg/kg)	20.00±4.24^a	12.00±3.16^a	25.00±3.56^a	12.53±1.97^a
AME (400 mg/kg)+DMN (20 mg/kg)	127.50±5.06^b	80.25±2.68^b	49.02±2.82^b	30.01±1.52^b
DMN alone (20 mg/kg)	198.00±5.41^c	135.25±5.26^c	89.67±2.06^c	49.00±2.02^c

Values are expressed as Mean ± SD, (n=5), AME = Annona muricata, DMN = Dimethylnitrosamine, AST = Aspartate aminotransferase, ALT = Alanine aminotransferase, ALP = Alkaline phosphatase, GGT = Gamma-glutamyl transferase. Mean values in each column having different superscript (a, b, c) are significantly different (P < 0.05) while mean values with same superscript is not significantly different (P < 0.05).

Table 2. Effect of ethanolic leaf extract of *Annona muricata* pre-treatment on serum Total cholesterol and triglyceride levels in DMN toxicity

Treatment	Triglyceride (mg/dl)	Total cholesterol (mg/dl)
Control (normal saline)	91.50±2.54[a]	90.50±10.45[a]
AME alone (400 mg/kg)	84.00±11.01[a]	85.00±7.58[a]
AME (400 mg/kg) + DMN (20 mg/kg)	123.00±4.00[b]	137.75±7.50[b]
DMN alone (20 mg/kg)	138.50±8.03[c]	165.50±4.03[c]

Values are expressed as Mean ± SD, (n=5), AME = Annona muricata, DMN = Dimethylnitrosamine. Mean values in each column having different superscript (a, b, c) are significantly different (P < 0.05) while mean values with same superscript is not significantly different (P < 0.05)

The *Annona muricata* extract might have prevented cholesterol increase by inactivating HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. The significant reduction in the concentration of total cholesterol and triglyceride by *Annona muricata* extract compared to DMN-alone rats may also be due to the presence of antioxidants and hypolipidaemic agents and this could be due to a decrease in absorption of cholesterol or an increase in HDL cholesterol [32]. Our result is similar to that of Kothavade et al. [33], which reported that administration of livomyn to ketokonazole treated rats led to decreased cholesterol level compared to ketokonazole alone treated rats. In a study by Adewole and Ojewole [34], serum total cholesterol and triglycerides were significantly elevated ($p<0.05$) in Streptozotocin-treated rats while treatment with aqueous extract of *Annona muricata* significantly reduced the total cholesterol and triglyceride towards normal values.

Free radical scavenging enzymes such as SOD and CAT provide the first defense against oxygen toxicity by catalyzing the dismutation of superoxide anion to H_2O_2 and decomposition of H_2O_2 to H_2O and O_2, respectively [35]. This study shows that DMN caused a significant decrease in GSH, SOD and CAT level with concomitant increase in MDA levels in DMN-treated rats compared to controls. There was clear evidence that DMN-induced hepatic injury was associated with free radical injury and oxidative stress. Oxidative stress was characterized by increased lipid peroxidation and/or altered non-enzymatic and enzymatic antioxidant systems. The increase in MDA indicates increased oxidative damage to cell membranes, inhibition of several important enzymes, reduced cellular function, and cell death [36]. The observed decrease in liver GSH in DMN-treated rats may be due to non-enzymatic interaction of GSH with excessive free radicals generated by the toxic insult in rat liver [37]. However, pre-treatment with extract of *Annona muricata* prior to DMN administration showed significant increase in GSH, CAT and SOD as well as significant reduction in MDA levels compared to DMN-alone treated rats. The reduced MDA may be related to the antioxidant properties of the phytochemical compounds found in the extracts [6]. Chemical compounds such as flavonoids and tannins have been reported to exert antioxidant activity by scavenging free radicals that cause lipid peroxidation [6]. In earlier studies, Farombi et al. [27,28] reported that DMN administered rats pre-treated with curcumin as well as kolaviron had significantly reduced MDA and significantly increased GSH levels compared to DMN alone treated rats.

Table 3. Effect of ethanolic leaf extract of *Annona muricata* on oxidative stress parameters in acute DMN toxicity

Treatment	MDA (U/mg wet tissue)	GSH(µM/mg tissue)	SOD (U/mg wet tissue)	CAT (U/mg wet tissue)
Control (normal saline)	2.75±0.19[a]	50.34±4.42[a]	10.03±0.99[a]	51.83±1.21[a]
AME alone (400 mg/kg)	2.23±0.14[z]	61.59±2.26[z]	13.76±0.85[z]	58.70±0.98[z]
AME(400 mg/kg) + DMN (20 mg/kg)	4.04±0.09[b]	36.76±1.73[b]	7.58±0.51[b]	35.46±0.82[b]
DMN alone (20 mg/kg)	8.19±0.14[c]	22.02±2.68[c]	4.29±0.11[c]	23.78±1.22[c]

Values are expressed as Mean ± SD, (n=5), AME = Annona muricata, DMN = Dimethylnitrosamine, MDA=Malondialdehyde, GSH=Reduced glutathione, SOD = Superoxide dismutase, CAT = Catalase Mean values in each column having different superscript (a, b, c, z) are significantly different (P < 0.05) while mean values with same superscript is not significantly different (P < 0.05)

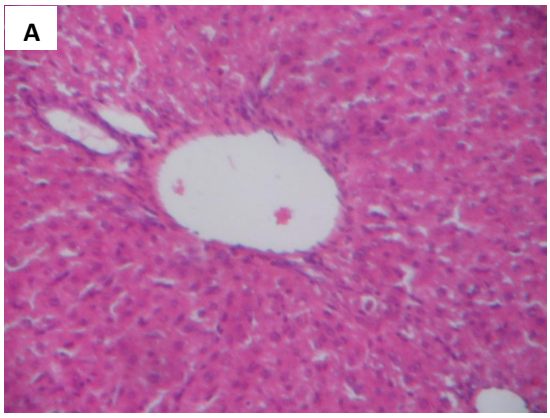

Fig. A. Photomicrograph A. Section of liver of control rats composed of portal vein, hepatocytes separated by sinusoids (H & E x 100)

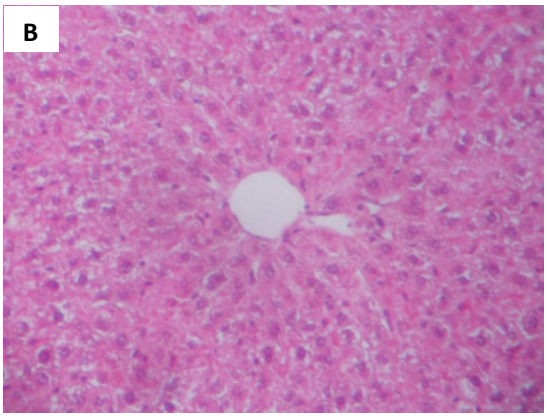

Fig. B. Photomicrograph B.Section of liver of rats pre-treated with Annona muricata (400 mg/kg) only for 7 days showing Slightly unremarkable hepatocytes and moderate vascular congestion (H & E x 100)

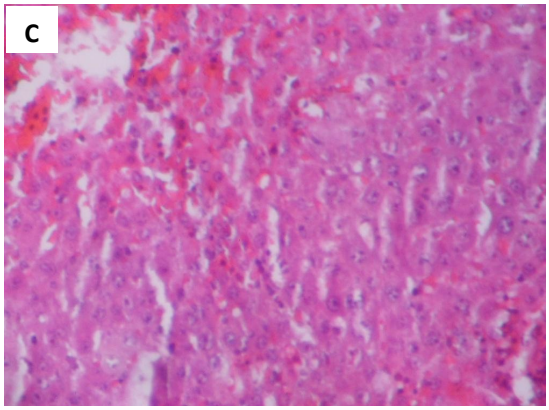

Fig. C. Photomicrograph C. Section of liver of rats pre-treated with 400 mg/kg Annona muricata for 7 days followed by 20 mg/kg DMN on day 8 showing focal haemorrhagic necrosis and viable hepatocytes(H & E x 100)

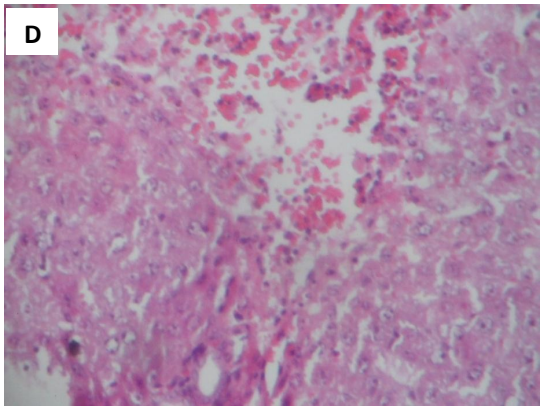

Fig. D. Photomicrograph D. Section of liver of rats treated with 20 mg/kg DMN, 48hours before sacrifice showing severe parenchymal haemorrhage and necrosis

Histopathological study of structure and chemical composition of tissues of animals are related to their function. In this study, when the rats were treated with DMN alone, the liver exhibited massive and severe haemorrhagic necrosis at the centrilobular zone as well as severe vacuolation of hepatocytes. This is in agreement with the previous report by Farombi et al. [27] who reported massive hepatocyte necrosis at centrilobular zone in DMN administered rats. This study also agrees with Jin et al. [38] that a high dose of DMN administered by a single injection in experimental animals cause central necrosis and hemorrhage and necrosis at its acute phase similar to human fulminant hepatitis. Coen et al. [39] reported marked changes in liver such as vacuolated hepatocytes, necrosis and congested sinusoids in paracetamol treated rats. However, pre-treatment with extract of *Annona muricata* prior to DMN administration mitigated the above histopathological changes as the integrity of the hepatocytes were relatively well preserved by inhibiting further tissue necrosis and inflammatory cell infiltration. In a related study, Farombi et al. [27,28] reported that pre-administration of kolaviron and curcumin to DMN treated rats enhanced the hepatocytes integrity as they were relatively preserved.

5. CONCLUSION

The anti-inflammatory effect observed in ethanolic leaf extract of *Annona muricata* against DMN toxicity may be attributable to their flavonoids, tannins, alkaloids and other phytochemical content as we previously published [6]. In addition, the anti inflammatory effects exhibited by these extracts to topical model of acute inflammation justify the traditional use of the plants leaves in the management of painful inflammatory conditions.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors declare that no competing interests exist.

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