

Effect of *Annona muricata* pre-treatment on liver synthetic ability, kidney function and hematological parameters in dimethylnitrosamine (DMN)-administered rats

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Abstract

Background: Dimethylnitrosamine (DMN) is a potent hepatotoxin, carcinogen and mutagen which exerts carcinogenic effects and induces hepatic necrosis in experimental animals through metabolic activation by CYP2E1. This study thus evaluated efficacies of *Annona muricata* pre-treatment on acute dimethylnitrosamine (DMN)-induced anemia and biochemical alterations in male albino rats.

Methods: 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 400mg/kg *Annona muricata* ethanol leaf extract for one week while group 3 and 4 each received single dose of 20mg/kg DMN (orally) after one week. The rats were sacrificed 48hrs after DMN administration.

Results: In rats administered 20mg/kg DMN, toxicity was clearly shown by decreased activities of serum liver synthetic molecules namely total protein, albumin, increased total bilirubin, increased kidney parameters such as creatinine and urea as well as decreased body weight gain. The toxic effect of DMN was also indicated by significantly decreased levels of red blood cells (RBC), hemoglobin (Hb), packed cell volume (PCV), white blood cells (WBC) and platelet count (Plt count). However, in rats pre-treated with 400mg/kg *Annona muricata* prior to DMN administration, there were significant reversals in the activities of serum liver synthetic molecules, kidney profiles, and hematological parameters when compared to DMN-alone administered rats.

Conclusion: *Annona muricata* pre-treatment exhibited *in vivo* protective and anti-anemic effects against DMN-induced injury.

Keywords: *Annona Muricata*; Anaemia; Dimethylnitrosamine; Liver Synthetic Ability; Rats.

1. Introduction

Dimethylnitrosamine (DMN), a liver toxicant, is a member of a family of extremely potent carcinogens, the N-nitrosamines (U.S. EPA 2002). It is generated from the *in situ* reaction of dimethylamine (DMA) with monochloroamine in the disinfection process or the nitrosation of DMA by nitrite (Gerecke & Sedlak 2003).

Annona muricata is known as soursop in English-speaking countries and is referred to by numerous common names (Blench & Dendo 2007). Soursop trees are widespread in the tropics and frost-free sub-tropics of the world (Morton 1973). Many bioactive compounds and phytochemicals including flavonoids, saponins, alkaloids and tannins have been found in *Annona muricata* (Usunobun *et al.* 2015, Usunobun & Okolie 2015). Research on *Annona muricata* have shown that a novel set of phytochemicals (Annonaceous acetogenins) found in the leaves, seeds and stem are cytotoxic against various cancer cells (Chang 2001, Liaw *et al.* 2002). This study hope to ascertain the protective effect of *Annona muricata* on kidney function, hematology parameters and liver synthetic molecules in 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 300g 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 (125mm) followed by cotton wool. The filtrate was thereafter reduced to one-tenth its volume using a rotary evaporator with the water bath set at 60°C and then finally freeze dried. The dried residue (crude extract) was then stored at 4°C. Before use, portions of *Annona muricata* ethanolic leaf extract were weighed and reconstituted in distilled water and administered orally.

2.2. Experimental animals, DMN and extract administration

Male wistar albino rats divided into four (4) groups of six (6) rats each, weighing between 160-195g 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— $24\pm 2^{\circ}\text{C}$; 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 (1971).

A total of 24 rats divided into 4 groups were used. Group 1 served as control, Group 2 received 400mg/kg *Annona muricata* only for seven (7) consecutive days, Group 3 received 400mg/kg *Annona muricata* for 7 consecutive days followed by oral administration of a single dose of 20mg/kg DMN (dissolved in 0.15M NaCl), on day 8 while Group 4 received oral administration of single dose of 20mg/kg DMN (dissolved in 0.15M 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 either EDTA bottles for hematology or in plain tubes. Blood collected in plain bottles were allowed to stand for 45 min before it was centrifuged at 4,000 rpm for 30 min. Serum was stored at -20°C until analyzed.

2.3. Biochemical assays

Serum total bilirubin was determined using the RANDOX Kit according to the manufacturer's instructions by the method of Jendrasik and Grof (1938). Serum total protein was determined using the RANDOX Kit according to the manufacturer's instructions as described by Tietz (1995) while serum albumin was determined by using the RANDOX Kit according to the manufacturer's instructions based on the Bromocresol green (BCG) method as described by Doumas et al (1971). Serum urea was determined using the RANDOX Kit according to the manufacturer's instructions following the method of Fawcett and Scott (1960) while serum creatinine was determined by using the Jaffe' method. Hematology was carried using full automated blood cell counter PCE -210N (ERMA INC., USA).

2.4. Statistical analysis

Numerical data obtained from the study were expressed as mean value $\pm$ 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 results of the body and relative tissue weights with a marked reduction in body weight gain of DMN administered rats when compared to control rats. However, DMN administered rats pretreated with ethanolic leaf extract of 400mg/kg *Annona muricata* caused an increase in the body weight gain compared to DMN alone administered rats. There was no significant difference in relative weight of kidney between the different treatment groups. On the other hand, there was a significant increase ($P < 0.05$) in relative liver weight in DMN administered rats as compared to control rats. However, DMN administered rats pretreated with 400mg/kg *Annona muricata* had a decline in relative liver weight as compared to the DMN alone administered rats which were insignificant.

Table 1: Effect of *Annona muricata* on Body and Relative Organ Weight in Acute DMN-Induced Toxicity in Rats.

Treat-ment	Initial body weight (g)	Final body weight (g)	Weight gain (g)	Relative liver weight (%)	Relative kidney Weight (%)
Control (normal saline)	168.10 $\pm$ 1.67	190.00 $\pm$ 4.15	21.80 $\pm$ 3.08	3.07 $\pm$ 0.05 ^a	0.71 $\pm$ 0.03 ^a
AME alone (400mg/kg)	163.43 $\pm$ 2.87	190.50 $\pm$ 6.02	27.07 $\pm$ 3.15	3.06 $\pm$ 0.04 ^a	0.68 $\pm$ 0.03 ^a
AME (400mg/kg) + DMN (20mg/kg)	174.40 $\pm$ 3.20	189.43 $\pm$ 9.39	15.03 $\pm$ 6.19	3.42 $\pm$ 0.07 ^b	0.70 $\pm$ 0.01 ^a
DMN alone (20mg/kg)	190.19 $\pm$ 2.06	197.07 $\pm$ 5.16	6.87 $\pm$ 3.10	3.48 $\pm$ 0.10 ^b	0.72 $\pm$ 0.12 ^a

Values are expressed as Mean $\pm$ SD, (n=6), AME = *Annona muricata*, DMN = Dimethylnitrosamine

The effect of ethanolic leaf extract of *Annona muricata* on acute DMN-induced liver and kidney injury was evaluated by determining the levels of creatinine, urea, total protein, albumin and total bilirubin as shown in Figure 1 - 5. *Annona muricata* to a significant level lowered the levels of creatinine, urea and total bilirubin that were released into serum as a consequence of acute DMN-induced hepatic and renal damage. Pretreatment of the rats with 400mg/kg *Annona muricata* significantly decreased ($p < 0.05$) acute DMN-induced ($p < 0.05$) creatinine, urea and total bilirubin levels when compared to DMN alone group. Also, pretreatment of the rats with *Annona muricata* prior to acute DMN induction significantly increased ($p < 0.05$) total protein and albumin when compared to acute DMN alone group.

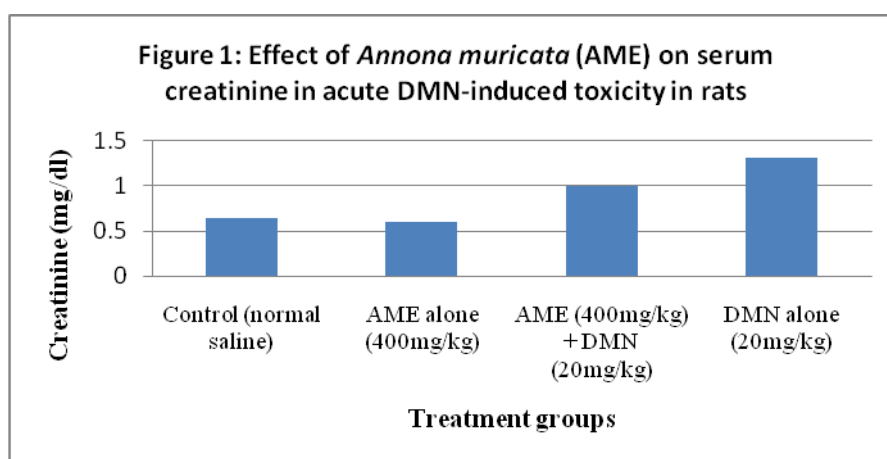


Fig. 1: Effect of *Annona muricata* (AME) on Serum Creatinine in Acute DMN-induced Toxicity in Rats.

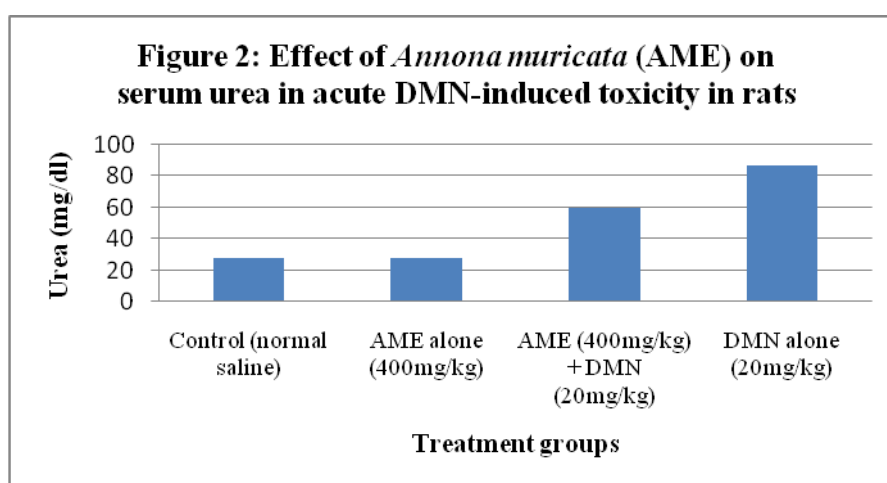


Fig. 2: Effect of *Annona muricata* (AME) on Serum Urea in Acute DMN-induced Toxicity in Rats.

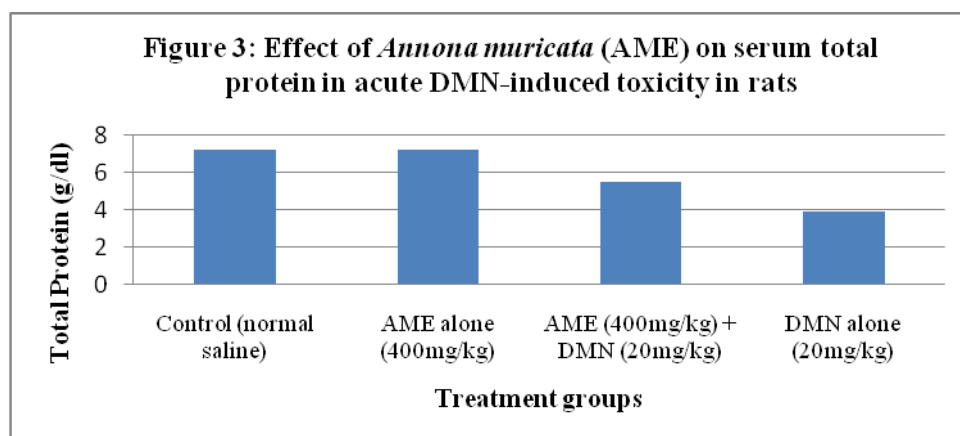


Fig. 3: Effect of *Annona muricata* (AME) on Serum Total Protein in Acute DMN-induced Toxicity in Rats.

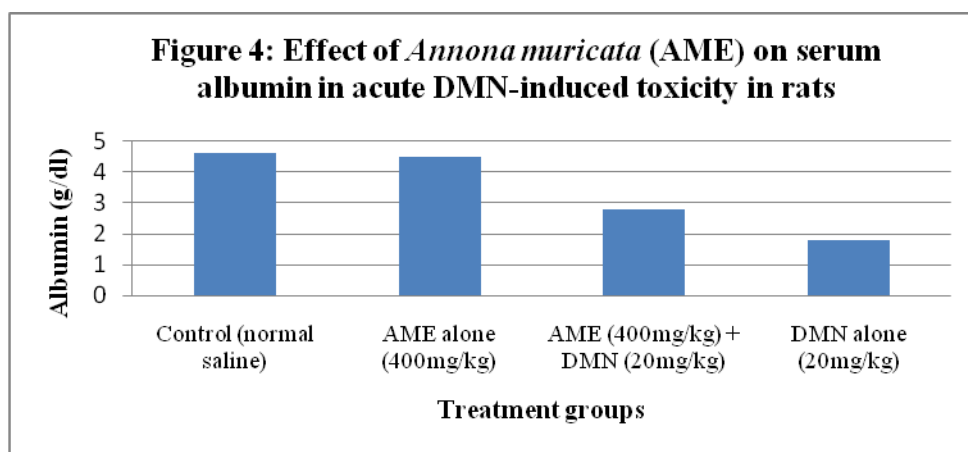


Fig. 4: Effect of *Annona muricata* (AME) on Serum Albumin in Acute DMN-induced Toxicity in Rats.

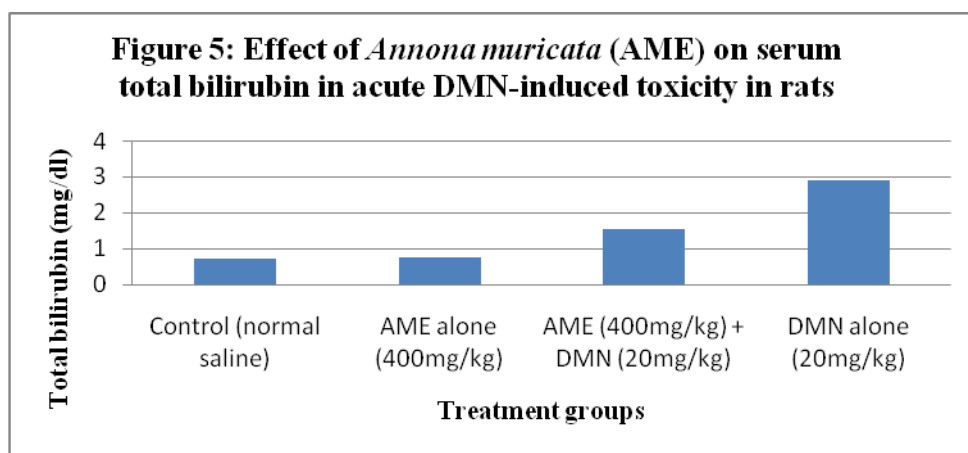


Fig. 5: Effect of *Annona muricata* (AME) on Serum Total Bilirubin in Acute DMN-induced Toxicity in Rats.

Hematological assessment shows that rats administered DMN had significantly decreased RBC, Hb, PCV, WBC and Plt Count while rats pretreatment with *Annona muricata* significantly increased the hematological parameters compared to rats that received DMN alone.

Table 2: Effect of *Annona muricata* on Haematological Parameters in Acute DMN-Induced Toxicity in Rats.

Treatment groups	WBC (x 10 ³ /μl)	RBC (x 10 ⁶ /μl)	Hb (g/dl)	PCV (%)	Plt Count (x 10 ³ /μl)
Control(normal saline)	5.70±0.33 ^a	6.02±0.52 ^a	14.6±0.66 ^a	50.91±0.07 ^a	507±42.48 ^a
AME alone (400mg/kg)	7.97±0.13 ^z	6.23±0.22 ^a	13.83±0.83 ^a	48.00±0.89 ^z	564.33±74.89 ^a
AME (400mg/kg) + DMN (20mg/kg)	3.99±0.29 ^b	4.91±0.04 ^b	9.30±0.10 ^b	28.95±0.36 ^c	95.00±7.71 ^b
DMN alone (20mg/kg)	2.50±0.08 ^d	3.23±0.12 ^d	8.00±0.36 ^d	20.61±1.43 ^d	41.00±4.32 ^d

Values are means ± SD; n=3, AME = *Annona muricata* ethanolic extract, DMN = Dimethylnitrosamine, WBC = White blood cells, RBC = Red blood cells, Hb = Hemoglobin, PCV = Packed cell volume, Plt count = Platelet count.

Mean values in each column having different superscript (a, b, c, d, and z) are significantly different (P < 0.05).

4. Discussion

In this study, DMN administration caused marked decrease in body weight gain compared to controls. However, pre-treatment with 400mg/kg *Annona muricata* prior to DMN administration caused an increase in the body weight gain compared to DMN-alone administered rats. In a similar study, Ismail *et al* (2009)

reported decreased feed intake and decreased body weight gain for CCl₄ group compared to control rats. The present study also showed a significant increase in relative liver weight in DMN-administered rats compared to control rats which are in agreement with the results of Patel and Sail (2006) who reported that liver and kidney weight showed significant increase in DMN administered rats compared to the control rats after 48hrs of intoxication. However, pre-treatment with 400mg/kg *Annona muricata* prior to DMN administration led to a decrease of relative liver weight compared to DMN-alone administered rats. The decrease in relative liver weights in DMN-administered rats pre-treated with *Annona muricata* obtained in this study is similar to that of Sadeek (2011) who reported that daily administration of 8ml/kg beet root or radish juice lead to a significant decline of liver weight/body weight % as compared to CCl₄ alone rats.

In rats administered DMN-alone, the significant increase in total bilirubin level compared to control and extract pre-treated rats gives credence to our previous study where we reported increases in AST, ALT, ALP and GGT (Usunobun, 2014, Usunobun *et al.* 2015). The abnormal high level of serum total bilirubin indicates liver dysfunction and thus damaged structural integrity of the liver.

However, DMN-administered rats orally pre-treated with *Annona muricata* showed significant decrease in total bilirubin level compared to DMN-alone treated rats. Thus, the extracts protected the hepatocytes from DMN-induced injuries. The total bilirubin reduction in the extract pre-treated groups may be due to *Annona muricata* inhibiting effects on cytochrome P₄₅₀ or/and promotion of its glucuronidation (Al-Qarawi *et al.* 2003). Adesanoye & Farombi (2009) in an earlier study, reported hepatoprotective effects of *Vernonia amygdalina* in rats treated with carbon tetrachloride via reduction of bilirubin. The hepato-protective effect of *Annona muricata* leaves may be due to presence of flavonoids, saponins,

alkaloids, tannins and ascorbic acid (Usunobun *et al.* 2015, Usunobun & Okolie 2015).

In this study, DMN induced liver damage in rats which was indicated by the decrease in levels of serum albumin and total protein. This study agrees with Ekam *et al.* (2012), who reported a significant decrease ($P < 0.05$) in total protein in paracetamol group compared to that of the normal control group. However, pre-treatment of ethanolic leaf extract of 400mg/kg *Annona muricata* significantly protected the rats against DMN-induced hepatotoxicity as evidenced by the significant increase in serum total protein and albumin compared to DMN-alone treated rats. In an earlier study, oral administration of chloroform and ethyl acetate fractions of *Astercantha longifolia* and also silymarin to CCl₄-treated rats showed significantly increased serum protein level (Muthulingam 2002).

Studies demonstrate that nephrotoxicity induced by chemical agents are one of the consequences of the accumulation of certain metabolites in kidneys (Sener *et al.* 2003). Creatinine, urea and uric acid are major catabolic products of muscle, protein and purine metabolism respectively. Marked increase in serum urea and creatinine noticed in this study in DMN administered rats may be an indication of functional renal damage as the kidney is unable to excrete them. However, *Annona muricata* pre-treatment prior to DMN administration significantly lowered the serum urea and creatinine levels, thus enhancing renal function.

In this study, DMN decreased white blood cells WBCs, Platelets, RBCs, PCV, and Hb in DMN treated group compared to control groups similar to our previous study (Usunobun, 2014). However, pre-treatment with 400mg/kg *Annona muricata* prior to DMN administration significantly ($P < 0.05$) enhanced the hematological parameters, thus indicating that *Annona muricata* improve immunity function and decrease inflammation. Reduction in platelets count in experimental animals has been reported to indicate adverse effect on the oxygen-carrying capacity of the blood as well as thrombopoietin (Li *et al.* 1999, McLellan *et al.* 2003).

The ability of *Annona muricata* leaves to modulate acute DMN toxicity may be probably due to the plant richness in chemical compounds including acetogenins, tannins, phenols and flavonoids as well as its ability to enhance endogenous enzymic and non-enzymic antioxidants.

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