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Synergistic, Attenuative and Modulatory Activity in Dimethylnitrosamine (DMN)-induced Fibrotic Rats Treated with *Vernonia Amygdalina* and *Annona Muricata* Leaves

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Abstract

Background: This study assessed the effect of *Vernonia amygdalina* and *Annona muricata* leaves in a combine dose on oxidative stress and extracellular matrix parameters in liver fibrosis induced by dimethylnitrosamine (DMN).

Methods: One group, the control received physiological saline; Second group was given 100mg/kg each of *Vernonia amygdalina* and *Annona muricata* ethanol leaf extract without DMN orally for 14 consecutive days. The third group received intra-peritoneal injection of 10mg of DMN/kg body weight three times a week (on the first three days) for two weeks, in addition to 100mg/kg each of Vernonia amygdalina and Annona muricata which were administered for 14 days consecutively while the last group was given only 10mg of DMN/kg. 14 days later, the rats were sacrificed, blood samples collected and sera analysed for hyaluronic acid (HA), alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) while collagen, catalase (CAT), reduced glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) levels were assayed in liver tissue homogenate. Sections of liver tissue were also fixed in formol-saline and subjected to histological analysis.

Results: DMN administration caused significant increases in kidney weight, serum ALP, AST, HA, ALT and in liver total collagen content and MDA ($P < 0.05$) and a significant drop in body and liver weight, liver CAT, GSH and SOD. Histological examination of liver showed that DMN caused congestion, centrilobular, haemorrhagic necrosis, thick collagen fibers deposition and fibrosis. However a combination of 100mg/kg each of *Vernonia amygdalina* and *Annona muricata* leaves remarkably attenuated and modulated above DMN-induced changes.

Conclusion: This study suggest that combination of *Vernonia amygdalina* and *Annona muricata* leaves possess fibrosuppressant, anti-oxidant and anti-hepatotoxic properties against DMN-induced hepatic fibrosis.

Keywords:*Annona muricata*, *Dimethylnitrosamine*, *Fibrosis, Liver*, *Vernonia amygdalina*

1. Introduction

Liver fibrosis is a pre-pathological cirrhotic state, signalized by collagen and extracellular matrix (ECM) proteins accumulation (produced in damaged liver by stellate Cells) in the space of Disse [1-3]. DMN, a liver mutagen and carcinogen has been suggested and proven to be a standard model for studying hepatic fibrosis and cirrhosis associated pathophysiological and biochemical changes [4-6].

Vernonia amygdalina (bitter leaf), a leafy vegetable is used for food and disease treatment including liver and kidney problems, diabetes, malaria, infertility, and gastrointestinal problems [7; 5]. Nutritional and phytochemical analysis *Vernonia amygdalina* leaves have revealed levels of crude protein, crude fiber, carbohydrate, phytochemicals (flavonoids, tannins, saponins etc), mineral components (Mg, Ca, K, Na, Mn, Zn, Fe etc) and antioxidant vitamins (A, C, E and riboflavin) [8-11].

Annona muricata, commonly called soursop have its various parts such as bark, roots and leaves for treatment of diseases and ailments including diabetes, liver disease, arthritis, bacterial and fungal problems [12-14; 6]. Phytochemical screening of Annona muricata leaves has shown it to consist of alkaloids, flavonoids etc as well as mineral elements such as Na, Ca, K, Fe, Zn, Mg etc. Previous studies on *Annona muricata* showed *Annonaceous acetogenins* found in the stem, seed and leaves to be cytotoxic against cancer cells [17-18]. This study is aimed at the possible fibro-suppressant and anti-oxidative attenuation of *Vernonia amygdalina* and Annona muricata leaves in combine dose on liver fibrosis induced by dimethylnitrosamine (DMN) in wistar rats.

2. Materials and Methods

2.1 Plant materials and Extraction

Annona muricata leaves were collected from the tree in Upper Sakponba while *Vernonia amygdalina* leaves were purchased from Oba market in Benin City, Nigeria and thereafter a Botanist identified the leaves. Washed and air-dried (at room temperature (24℃) Annona muricata and Vernonia amygdalina leaves were crushed into fine powder and then weighed. Ethanolic extracts of the powdered leaves were prepared by weighing and soaking 100g each of the powdered leaves in 1000ml of absolute ethanol for 48hrs (at room temperature). At the end of the 48hours, the extracts were filtered using Whatmann filter paper and cotton wool. The *Annona muricata* and *Vernonia amygdalina* ethanolic extracts were thereafter concentrated using a rotary evaporator (set at 60◦C and 40◦C respectively) to 1/10th its original volume followed by freeze drying. The dry powdered crude extract stored at 4° C were weighed, dissolved (in distilled water) and used for animal experimental study.

2.2 Experimental animals

Wistar rats (48 males, 150-225g) obtained from Animal Unit facility of the University of Ibadan, Ibadan, Nigeria were housed in wooden cages and used for the study. The rats acclimatized for one week, had free access to drinking water and commercial pelleted rat chow (Bendel Feed & Flour Mill Ltd., Ewu, Nigeria) ad libitum. Ethical clearance/permission were obtained from Institution Animal Ethical Committee before performing the experiments. The DMN was synthesized at the department of Biochemistry, University of Ibadan according to the method of Vogel [19].

The *Annona muricata* and *Vernonia amygdalina* leaf extracts weighed and dissolved in distilled water were administered orally using gavage to rats in second and third groups at a dosage of 100mg/kg for 14 consecutive days (two weeks). Rats in third group were in addition given DMN (via intraperitoneal injection) at a dose of 10mg/kg b.w (dissolved in 0.15M NaCl) in the first three days of each week (for two weeks). Rats in forth group were given same amount of DMN as in third group but without leaf extract, while rats in first group (control group) were given normal saline. By day 15, all the rats were sacrificed (cardiac puncture), blood collected and allowed to stand for 1 hr before centrifuging at for 20 min. The serum samples were stored at -20 ° C until analyzed.

Serum was used for determination of hyaluronic acid (HA) level, total bilirubin (TB), total protein (TP), aspartate aminotransaminase (AST), Lactate dehydrohenase (LDH), alanine aminotransaminase (ALT), and alkaline phosphatise (ALP). The livers were immediately excised, washed in cold normal saline and blotted individually. A portion of the weighed liver tissue was fixed in formalin for histopathological examinations while the other portion was stored at -20°C for analysis. 10% liver tissue homogenate were prepared using normal saline and resulting clear supernatant used for determination of total collagen (ECM component), GSH, CAT, SOD and MDA.

2.3 Biochemical assays

Serum LDH, AST, ALT, ALP, TP and TB were determined spectrophotometrically using RANDOX Kit. Serum HA level was determined using ELISA assay kit as described by Chichibu et al [20]. Liver total collagen was quantified using QuickZymeR kits. MDA was determined in a colorimetric reaction with thiobarbituric acid [21]. SOD was determined according to the method of Misra and Fridovich [22]. The catalase assay was by measuring the first order rate constant colorimetrically for H2O2 decomposition [23]. GSH was determined according to Ellman [24].

2.4 Statistical analysis

Data obtained at the end of this study were expressed as mean ± SD using Statistical Package for Social Sciences (SPSS). A probability level of less than 5% ($p < 0.05$) was considered significant.

3. Results

The result of liver function enzymes as shown in table 1 showed that dimethylnitrosamine caused massive elevation in ALT, AST, ALP and LDH after two weeks of administration indicating liver toxicity while simultaneous treatment with a combine dose of 100mg/kg each of *Vernonia amygdalina* and *Annona muricata* significantly reduced the spillage of the enzymes into the blood stream. No significant difference was observed in control rats and rats given VAE and AME alone.

Table 1: Effect of *Vernonia amygdalina* and *Annona muricata* on serum liver function enzymes in DMN-induced hepatic fibrotic rats

Treatment groups	ALT (U/l)	AST (U/l)	ALP (U/l)	LDH (U/l)
Control (normal saline)	11.89 ± 3.02^{a}	20.01 ± 1.99^{a}	25.22 ± 2.78^{a}	200.00 ± 4.07^{a}
VAE alone (100mg/kg) + AME alone (100mg/kg)	13.99 ± 4.01^{a}	21.32 ± 3.12^{a}	28.94 ± 4.25^{a}	198.89 ± 5.53^{a}
VAE(100mg/kg)+AME (100mg/kg) + DMN(10mg/kg)	47.96 ± 7.37^{b}	70.33 ± 3.03^{b}	74.53 ± 3.21^{b}	325 ± 10.09^{b}
DMN alone(10mg/kg)	89.74 ± 7.08^{c}	144.50 ± 6.87^{d}	132.01 ± 7.09^{d}	543.54 ± 15.01^{c}

Values are means ± SD; n=5, VAE = *Vernonia amygdalina* ethanolic extract, AME = *Annona muricata* ethanolic extract, DMN = Dimethylnitrosamine, ALT= Alanine aminotransaminase, AST=Aspartate aminotransaminase, ALP=Alkaline phosphatase, LDH=Lactate dehydrogenase

Mean values in each column (between groups) having different superscript (a, b, c, d) are significantly different ($p < 0.05$).

The result of the effect of *the Vernonia amygdalina* and *Annona muricata* on oxidative stress parameters (table 2) showed that while DMN caused a significant increase in malondialdehyde (MDA) after two weeks of administration, *Vernonia amygdalina* and *Annona muricata* when combined in usage significantly reduced the MDA level indicating protection. Also, table 2 results showed significant decline in CAT, GSH and SOD in DMN administered rats whereas *Vernonia amygdalina* and *Annona muricata* when administered simultaneously significantly increased GSH, SOD and catalase.

Table 2: Effect of *Vernonia amygdalina* and *Annona muricata* on oxidative stress parameters in DMN-induced hepatic fibrotic rats

Treatment groups	MDA (U/mg wet tissue)	Reduced Glutathione (µM/mg protein)	SOD (U/mg tissue)	Catalase (U/mg tissue)
Control (normal saline)	1.87 ± 0.28^{a}	50.70 ± 3.02^{a}	10.75 ± 1.18^{a}	50.43 ± 4.12^{a}
VAE alone (100mg/kg) + AME alone (100mg/kg)	1.49 ± 0.65^{a}	57.89 ± 5.03^{a}	13.08 ± 0.95^{b}	62.93 ± 3.04^{a}
VAE(100mg/kg) + AME (100mg/kg)+ DMN(10mg/kg)	4.16 ± 0.08^{c}	31.64 ± 2.06^{c}	6.88 ± 0.87^{c}	33.06 ± 2.13^{c}
DMN alone(10mg/kg)	8.15 ± 0.23^{d}	15.04 ± 5.00^{d}	2.12 ± 0.091^{d}	13.94 ± 5.18^{d}

Values are means ± SD; n=5, VAE = *Vernonia amygdalina* ethanolic extract, AME = *Annona muricata* ethanolic extract, DMN = Dimethylnitrosamine, MDA=Malondialdehyde, SOD=Superoxide dismutase
Mean values in each column (between groups) having different superscript (a, b, c, d) are significantly different ($p < 0.05$).

The result of Vernonia amygdalina and Annona muricata on ECM proteins in fibrotic rats induced by DMN as shown in table 3 showed a several folds increase in Hyaluronic acids (HA) and total collagen in rats administered DMN alone whereas in rats simultaneously treated with Vernonia amygdalina and Annona muricata, HA and total collagen where greatly and significantly reduced. Also table 3 showed that Vernonia amygdalina and Annona muricata significantly reduced serum total bilirubin (TB) and significantly increased serum total protein (TP) when compared to rats given DMN alone. There was however no significant difference in control rats and rats given Vernonia amygdalina and Annona muricata alone.

Table 3: Effect of *Vernonia amygdalina* (VAE) and *Annona muricata* (AME) on hyaluronic acid (HA), total collagen, total bilirubin (TB) and total protein (TP) in DMN-induced hepatic fibrotic rats

Treatment groups	HA (ng/ml)	Total Collagen (µg/ml)	TB (mg/dl)	TP (g/l)
Control (normal saline)	60.59 ± 12.78^{a}	41.01 ± 2.15^{a}	0.51 ± 0.04^{a}	62.18 ± 4.06^{a}
VAE alone (100mg/kg) + AME alone (100mg/kg)	61.22 ± 3.16^{a}	42.17 ± 3.13^{a}	0.61 ± 0.02^{a}	65.23 ± 2.13^{a}
VAE (100mg/kg) + AME (100mg/kg) + DMN (10mg/kg)	195.80 ± 10.14^{b}	59.76 ± 7.58^{b}	1.10 ± 0.05^{c}	53.01 ± 5.42^{b}
DMN alone (10mg/kg)	460.11 ± 10.21^{c}	126.01 ± 6.94^{c}	2.91 ± 0.21^{d}	32.92 ± 2.22^{d}

Values are means ± SD; n=5, VAE = *Vernonia amygdalina* ethanolic extract, AME = *Annona muricata* ethanolic extract, DMN = Dimethylnitrosamine, HA=Hyaluronic acid, TB=Total Bilirubin, TP=Total protein
Mean values in each column (between groups) having different superscript (a, b, c, d) are significantly different ($p < 0.05$).

4. Discussion

Connective tissue proteins accumulation such as Collagen and Hyaluronic acid (HA) has been reported and its measurement can serve in quantifying fibrosis [25]. In this study by day 15, total collagen was increased linearly following administration of DMN (Table 3), an indication of hepatic fibrosis similar to previous reports [4; 26-28]. The hyaluronic acid increase in this study could be explained by its increased synthesis by activated hepatic stellate cells (HSCs) resulting in its simultaneous leakage into the blood stream along with the liver function enzymes. The high rise in hyaluronic acid may also be as a result of its degradation by hyaluronidases enzymes [29]. However, the combination of *Vernonia amygdalina* and *Annona muricata* leaves showed anti-fibrogenic activity expressed in decrease of total collagen and hyaluronic acid content most probably due to the antioxidants and phytochemicals present in the leaves. Liver CYP2E1 is the main CYP involved in DMN activation. It is possible that the decrease in hepatic CYP2E1 activity by *Vernonia amygdalina*

and *Annona muricata* leaves affected the activation of DMN resulting in a reduction of Hepatic stellate cells activation and consequent decrease in total collagen and HA. The decrease of HA activity by *Vernonia amygdalina* and *Annona muricata* leaves may also be due to HA degrading enzymes activation. The protective effect of *Vernonia amygdalina* and *Annona muricata* leaves is in tandem with similar studies by Shin and Moon [30]; Yan et al [31]; George et al [32].

In this study, DMN administration caused a marked rise in serum AST, LDH, ALT, ALP and bilirubin, indicating damage of the liver and consequent cytolysis of hepatic cells similar to previous reports [4; 26; 33-35]. The release of the liver function enzymes from cytoplasm into blood can be said to be due to rupture of plasma membrane and cell damage. The high rise in AST compare to ALT may be due to mitochondrial AST release into the blood stream, a consequence of severe hepatocyte damage. The rise in serum bilirubin could be due to hemolysis. The results of George [36-37] were similar to our findings which reported significant increase in liver function enzymes in mice/rats treated with DMN intraperitoneally. Consequently, a combined dose of *Vernonia amygdalina* and *Annona muricata* leaves against DMN-induced fibrosis significantly reduced the levels of liver function enzymes compared to rats given only DMN, an indication that the leaves offered protection against liver cell damage. The reverse in liver marker enzyme activities may also be attributed to the ability of *Vernonia amygdalina* and *Annona muricata* leaves to inhibit hepatic P4502E1 activity. The protection offered by *Vernonia amygdalina* and *Annona muricata* in this study is similar to previous studies of George et al [32] and Shin and Moon [30]. In a related study by Sharma and Singh [38], Operculina turpethum ethanolic root extract showed therapeutic effects by restoring AST, ALT and ALP levels.

Lipid peroxidation (with Malondialdehyde, MDA considered its most significant indicator) is the most recognized mechanism in studying liver injury pathogenesis by several toxic agents [39-41]. In this study, the massive rise in MDA is an indication that DMN-induced damage of liver cell membrane results in free radical production thereby enhancing oxidative stress. In this study, however, simultaneous supplementation with combined dose of *Vernonia amygdalina* and *Annona muricata* leaves significantly reduced MDA levels, an indication that the plant leaves can scavenge and detoxify free radicals similar to previous findings of Shin and Moon [30] and George et al [32].

Also in this study, antioxidant component of self-defense system (GSH, CAT, and SOD) were reduced in rats given DMN, a finding similar to previous investigations [42-45]. The continuous mitochondria production of superoxide radical might be responsible for the reduction in liver SOD and CAT in rats given DMN. The drop in CAT, SOD and GSH biosynthesis during liver cell damage might be responsible for the depletion in the non-enzymatic and enzymatic self-defense component. However, treatment with combined dose of *Vernonia amygdalina* and *Annona muricata* leaves simultaneously with DMN significantly raised the antioxidants (SOD, CAT and GST) compared to rats given only DMN. *Vernonia amygdalina* and *Annona muricata* leaves contain phytochemical compounds such as flavonoids with strong free radical scavenging potentials. These protection offered by combined dose of both leaves is similar to previous work of Sharma and Singh [38].

5. Conclusion

In conclusion, *Vernonia amygdalina* and *Annona muricata* leaves possess synergistic, anti-fibrotic, free radical scavenging and hepatoprotective potentials against DMN-induced liver disease and the bioactive agents including flavonoids in both leaves may be responsible.

Conflict of Interest

We the authors hereby declare no conflict of interest.

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