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Protective Roles of *Annona senegalensis* Pers. Extract in N-Diethylnitrosamine-Induced Hepatocellular Carcinoma in Male Wistar Rats

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ABSTRACT

Hepatic cancer is a common and leading cancer around the world. Preventive measures have been emphasized in view of its prognosis and limited treatment. This study evaluates the protective roles of *Annona senegalensis* in N-diethylnitrosamine (DEN) – a model of hepatocarcinoma in male Wistar rats. Rats were intra-peritoneally induced weekly with 100 mg/kg of DEN for 3 weeks and treated with *Annona senegalensis* extract using a cannula for 21 days. Biochemical assays; aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lipid peroxidation were carried out. Reverse transcription-polymerase chain reactions for the expression of pro-inflammatory RNA, interleukin 6 (IL-6), were equally determined. *Annona senegalensis* extract (100 mg/kg and 200 mg/kg body weight) significantly ($p < 0.05$) reduced the activities of ALT and AST in DEN-induced hepatocarcinoma. Lipid peroxidation associated with the DEN model of the hepatocarcinoma was reversed, and the expression of the pro-inflammatory, IL-6 mRNA was also down-regulated with *Annona senegalensis* extract. Histopathological analysis revealed that the treatment (*Annona senegalensis* extract) restored the liver architecture in DEN model of hepatocarcinoma. Restoration of the liver enzymes, downregulation of the pro-inflammatory, IL-6 mRNA, and restoration of the liver architecture in the DEN model of hepatocarcinoma corroborated the protective potentials of *Annona senegalensis* extract against hepatocarcinoma.

***Keywords*:** N-diethylnitrosamine, *Annona senegalensis*, Hepatocellular carcinoma, Gene expression.

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer mortality across the globe.[1] HCC has been associated with various disease conditions, especially hepatitis C and B.[2] As a result of high liver tolerance, HCC is rarely detected at the preliminary stage, making it a notable health challenge as a result of poor prognosis.[3] The deterioration rate of HCC is high, and the patient's survival rate still very low. Surgical removal and transplant is still the most effective treatment for HCC. Reports have shown that over 70% of the world's population depends on plant-based medicine.[4] *Annona senegalensis*, commonly called wild soursop (English), Uburocha (Igbo), abo (Yoruba), and gwandar (Hausa) is widely distributed across Nigeria.[5] Virtually all parts of the plant have been reported to have medicinal effects. The leaves are used to treat smallpox, yellow fever, and tuberculosis.[5,6] The stem bark has been used in the treatment of hernia and snakebite,[7] while the root is used as an antidote for necrotizing toxins.[8,9] Despite the countless use of the plant, there is still insufficient scientific justification for such use of the plant. The present study thus evaluates the protective roles of *Annona senegalensis* extract in N-diethylnitrosamine induced hepatocellular carcinoma in male Wistar rats.

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Materials and Methods

Plant material

A. senegelensis leaves were collected from Ile-Ife in Osun State, Nigeria on 20th July, 2020, and they were identified by Dr. J.O. Popoola of the Biological Science Department, Covenant University, Ogun State, Nigeria. A voucher specimen was deposited at the Herbarium of Forestry Research Institute of Nigeria, Oyo State and allocated voucher number FHI 112597.

Preparation of extract

The leaves were air-dried at room temperature and pulverized using a manual electric blender. The powdered leaves (4.5 kg) were extracted using the maceration method in 2 L of hexane for 72 h, after which it was filtered using Whatman No.1 filter paper. The filtrate obtained was concentrated using a rotary evaporator at reduced temperature to give a yield of 161.8 g of the extract and a percentage yield of 3.6%.

Animals

Thirty-six healthy male Wistar rats with an average weight of 150 g were obtained from the Animal Holding unit of Lagos State University Teaching Hospital, Lagos, Nigeria. The animals were housed in clean plastic cages with appropriate ventilation conditions with free access to rat pellets and water *ad libitum*. The animals were handled following the guidelines of the National Institute of Health on the care and use of laboratory animals.[10] Ethical approval was obtained from the Research Ethical Committee of Covenant University, Ota, Nigeria, and was assigned an approval no NHREC/25/10/2018.

Chemicals

The test kits for alanine aminotransferase and aspartate aminotransferase were products of Randox Laboratories. Every other reagent used was sigma products.

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Induction of hepatocellular carcinoma

Rats were induced intraperitoneally with 100 mg/kg bodyweight of DEN followed by injection of 0.5 mL/kg bodyweight of carbon tetrachloride (CCl_4) once a week for 3 weeks to ensure the development of liver cancer following the method described by Sundaresan and Subramanian.[11]

Experimental design

Thirty-six male Wistar rats were grouped into six groups; three groups were induced with 100 mg/kg of DEN once a week for 3 weeks while all groups aside the normal control were treated with different doses of the extract (based on toxicity test report) for three weeks as follows:
Group 1: normal control rats fed with standard diet and water
Group 2: negative control group induced with 100 mg/kg DEN and 0.5 mL/kg of CCl_4 (toxicant)
Group 3: rats induced with toxicant and treated with 100 mg/kg of *A. senegalensis*
Group 4: rats induced with toxicant and treated with 200 mg/kg of *A. senegalensis*
Group 5: rats administered 100 mg/kg of *A. senegalensis* only
Group 6: rats administered 200 mg/kg of *A. senegalensis* only

Table 1: Gene-Specific Primer sequence

TARGET GENE	FORWARD 5'-3'	REVERSE 5'-3'
IL 6	GACTTCCAGCCAGTT GCCTT	GCAGTGGCTGTCAAC AACAT

Biochemical assays

Liver enzyme activity (alanine aminotransferase and aspartate aminotransferase) were determined in plasma following the kit's manufacturer's instruction. The level of microsomal lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS).[13]

Histopathology

Liver tissues fixed in 10% formalin were cut into thin slices of 2.1 mm thick and further dehydrated using ethanol, treated with paraffin wax, and cast into blocks; sections of the tissue were split into 5 µm and left on a slide to dry. The hematoxylin-eosin stain was used to stain the blocks and then analyzed using a light microscope.[14,15]

Statistical analysis

Statistical analyses were carried out using GraphPad Prism version 6.0. One-way ANOVA was used for comparisons between groups. Tukey test was used as the Post Hoc test. All values were expressed as mean± SEM (standard error of the mean) using a 95% confidence interval.

At the end of the experimental period; the animals were fasted overnight and sacrificed the next morning using cervical dislocation.

Sample collection

Blood was collected into heparin bottles and centrifuged at 4000 rpm for 10 min, and the plasma obtained was kept in Eppendorf tubes at -20^0C until needed for analysis. The liver was also removed, rinsed in ice-cold potassium chloride buffer, and a portion was fixed in 10% formalin while the remaining was stored at -80^oC. A part of the liver was homogenized for the lipid peroxidation assay.

RNA isolation and reverse transcription-polymerase chain reaction

The total RNA of the liver tissues was isolated using TRIzol reagent (Gibco, Location). The RNA was dissolved with the RNA-free DNase (Roche, Switzerland) for 15 min at a temperature of 37°C. The RNeasy kit (Qiagen,Germany) was used to purify the RNA. The primers were designed using Snap gene software and ordered from Sigma-Aldrich, USA. Beta-actin (β-actin) is the internal control gene. The genes were amplified for 50 cycles, two hours, and 20 min using the thermocycler. The amplified PCR products were run on 1.0% agarose gels and visualized with the aid of ethidium bromide (EtBr) staining.[12]

Results and Discussion

DEN is a carcinogen in the environment that can lead to oxidative stress via reactive oxygen species (ROS) generation, thus altering the antioxidant defense mechanism in the body system.[16] It is known to give rise to hepatocyte death and trigger proliferation in the liver.[17,18] DEN generates a radical based hepatic metabolism, mostly the mechanism underlying the hepatic damage observed following its administration.[19] This study documents the protective role of *Annona senegalensis* leaf extract in N-Diethylnitrosamine-induced hepatocellular carcinoma in male Wistar rats. The histological features of the liver of DEN-induced untreated rats showed cytoplasmic fat infiltration and necrotic hepatocyte, which could be associated with oxidative stress causing inflammatory cell infiltration, membrane damage, eventually leading to hepatic carcinoma.[20] However, the administration of *A. senegalensis* helped in restoring the liver's histological features (Figure 4). Enzyme leakage into the blood has been linked to cellular damage.[21] Serum ALT, AST are sensitive markers used in the hepatic damage diagnosis.[22] The rise in the activities of these enzymes in the blood might be as a result of leakage from damaged hepatocytes following DEN administration. Treatment with *A. senegalensis* significantly reversed the levels of the enzymes (Figure 1) to normal, perhaps by maintaining the integrity of the hepatocellular membrane. Lipid peroxidation has been widely used as an oxidative stress marker due to the susceptibility of membrane lipids to ROS.[23] When the concentration of generated ROS exceeds the antioxidant status, it leads to oxidative damage of cells.[24]

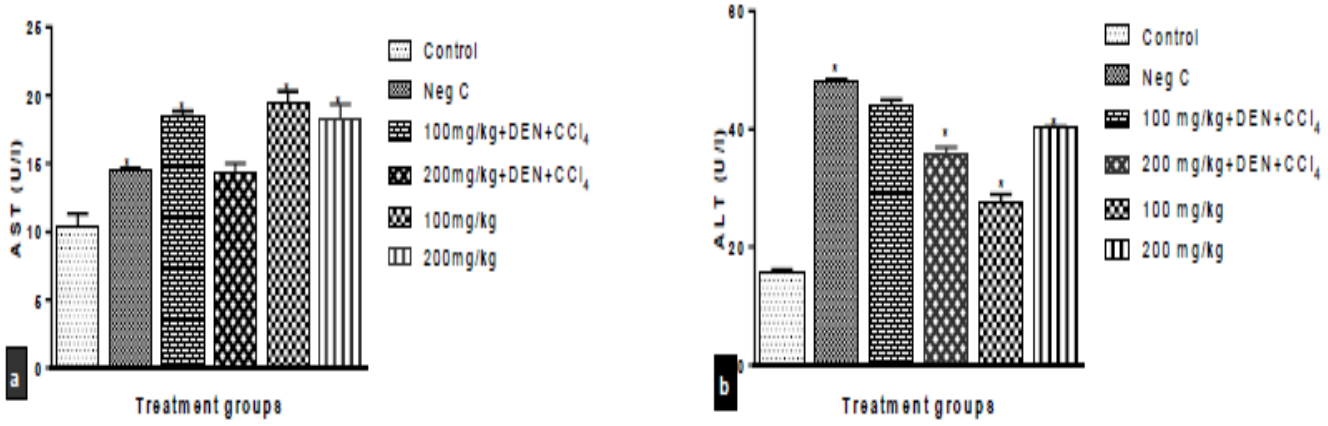

Figure 1: Effect of *Annona senegalensis e*xtract on liver function parameters in albino Wistar rats. **(a)** AST: Aspartate aminotransferase; **(b)** ALT: Alanine aminotransferase

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In this study, the significant increase in the level of LPO following the administration of DEN, as observed in the untreated group (Figure 2), suggests oxidative stress arising from the reactive metabolites of DEN. Administration of *A. senegalensis* significantly reduced LPO levels. This could be as a result of the phytochemicals present in the extract, which are capable of scavenging the ROS and preventing subsequent peroxidative damage to the hepatocytes. Terpenoids, the prominent phytochemical in *A. senegalensis*, has been reported to inhibit tumor cell proliferation and growth by apoptosis induction.[25] Interleukin-6 (IL-6) is a pro-inflammatory cytokine found to intervene in diseases, including cancer.[26] It has been reported to be a distinct marker in cancer research. IL-6 was found to be up-regulated in the induced untreated group (B), which is in line with the work of Li *et al.*[27] where they stated that IL-6 expression sustains hepatocellular carcinoma progression. Cancer- inducing potential of DEN/CCl_4 has been linked to pro-inflammation hence the high expression of IL-6 in the negative control group. The extract at 200 mg/kg body weight was seen to reduce the expression of IL-6 significantly.

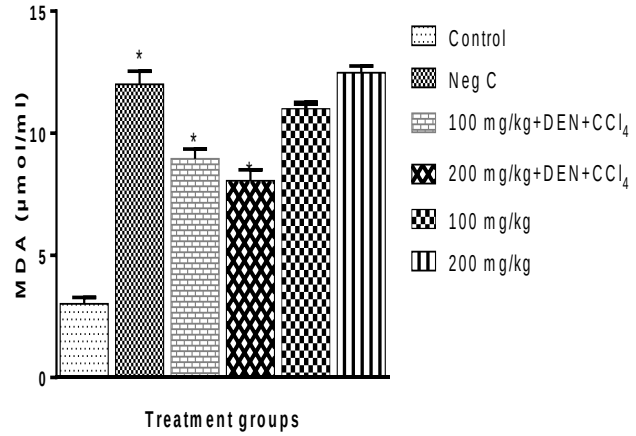

Figure 2: Malondialdehyde (MDA) concentrations in various treatment groups

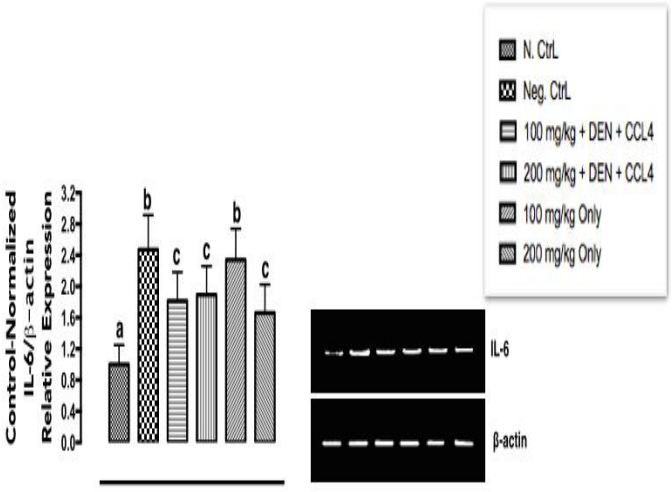

Figure 3: Effect of *Annona senegalensis* on IL-6 expression in hepatic tissue of rat

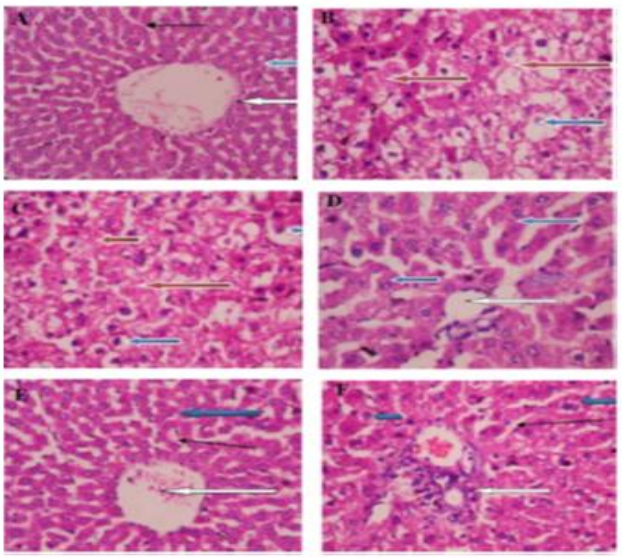

Figure 4: Photomicrograph of liver tissues (H and E, x400) of rats **A)** with normal liver morphology in control group, **B)** induced with the toxicant, **C)** treated with 100 mg/kg, **D)** treated with 200 mg/kg, **E)** administered 100 mg/kg, **F)** administered 200 mg/kg body weight of *A. senegalensis* extract.

Conclusion

We demonstrated the protective potential of *A. senegalensis* in DEN-induced liver cancer, which is achieved by reducing lipid peroxidation and downregulation of the pro-inflammatory gene (IL-6).

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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