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EFFECT OF *Spondias mombin* L. ON THE TUMOR MARKER, IMMUNOLOGICAL FUNCTION AND PROTEIN IN DIETHYLNITROSAMINE-INDUCED LIVER CANCER IN MALE WISTAR RATS

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ABSTRACT

Diethylnitrosamine (DEN) is a common rat model used in the study of the mechanism of chemical carcinogenesis and the response of Hepatocellular carcinoma (HCC) to anticancer drug therapy. The present study investigated the effect of the administration of *Spondias mombin* ethanolic stem-bark extract on tumor marker, cytokine mediators, protein, and albumin levels in DEN-induced liver cancer in male Wistar rats. Thirty male Wistar rats were randomly divided into six groups. Group 1 served as the normal control while Group 2 served as the negative control. Groups 3, 4, and 5 were orally treated with 200mg/kg body weight, 400 mg/kg body weight, and 800 mg/kg body weight of the extract daily respectively after induction with DEN. Group 6 served as the positive control. The level of Serum alpha-fetoprotein (AFP), activities of Interleukin-6 and Interleukin-10, protein, and albumin levels were determined using biochemical assays. From the results obtained, IL-10 activities were reduced for all treated rats when compared with negative control while rats treated with 800mg/kg showed a non-significant reduction in IL-6 activities compared with negative control. Total protein (rats treated with 800mg/kg body weight of extract) and albumin (rats treated with 400 and 800mg/kg body weight of extracts) levels were also increased non-significantly when treated with the extract after DEN administration compared to the negative control. Serum AFP which increased after DEN administration in animals was reduced except in rats treated with 800m/kg body weights of extract which showed a slight increase. The results show the therapeutic effect of *Spondias mombin* extract against induced hepato-carcinogenesis.

Keywords: Hepatocarcinoma, *Spondias mombin*, Diethylnitrosamine, serum alpha-fetoprotein, Interleukin-6, Interleukin-10, protein, albumin

INTRODUCTION

The most common form of liver cancer is Hepatocellular carcinoma (HCC). It commonly occurs in people with liver disease, particularly caused by infection with chronic hepatitis B and C (Féher and Lengyel, 2012; Akinyemiju *et al*., 2017). Hepatocarcinogenesis induced by Diethylnitrosamine (DEN), a hepato-carcinogen, is known to cause disturbance in the enzymes involved in deoxyribonucleic acid (DNA) repair-replication and is normally used as a

carcinogen to induce liver cancer in animal models (Nermin *et al*., 2008; Tolba *et al*., 2015). DEN is bio-transformed to its alkylating metabolites, and the reactive product interacts with DNA causing mutation in a pathway dependent on cytochrome P450 enzymes, which would lead to carcinogenesis (Abe *et al*., 2012; Tolba *et al*., 2015). The use of plants and plants parts in the treatment of various diseases have been an important part of drug history. Over the years, there has been an increased interest in the effectiveness of traditional medicinal plants in the treatment of diseases including cancer (Alabi *et al.,* 2020). *Spondias mombin* L., also known as Hog plum (Family: Anacardiaceae), is a tropical fruit tree that is cultivated in areas such as Brazil, Africa, America, and Asia (Cabral *et al.,* 2016). In Nigeria, *Spondias mombin* is known with various names (Iyeye in Yoruba, Ijikara in Igbo, and Tsardarmasar in Hausa) (Ibegbu *et al.,* 2018). It serves as a purgative, diuretic, emetic, and as a common remedy for cough and laxative given in fever and constipation, respectively. It is a common remedy for various digestive problems also, it is considered to have antiviral, antibacterial, antiseptic, and antimicrobial effects (Ayoka *et al.,* 2008; Ibegbu *et al.,* 2018) and its anti-inflammatory and antioxidant potential has also been confirmed (Ayoka *et al.,* 2008; Cabral *et al.,* 2016). Phytochemicals such as alkaloids, saponins, carotenoids, tannins, phenolic compounds, and eugenol (Igwe *et al.*, 2010; Ali *et al.,* 2014; Metibemu *et al.,* 2020). Compounds such as catechin, stigmasterol, geranin, ellagic acid, and chlorogenic acid have also been isolated from extracts of this plant (Cabral *et al.,* 2016; Osuntokun *et al.,* 2017; Sameh *et al.,* 2018). However, there is little information on the potential of the plant extract to treat hepatocellular carcinoma. This study aims to evaluate the effect of *S. mombin* stem bark extract on tumor and inflammatory markers in DEN-induced rat model of hepatocellular carcinoma.

MATERIALS AND METHODS

REAGENTS

Diethylnitrosamine (DEN) was procured from Sigma-Aldrich (USA). A commercial product of silymarin (rich in silibinin) called Silybon-140, manufactured by Micro Labs Limited (India) was used. The different solvents and chemicals used in the present study were of analytical grade and purchased from local vendors.

COLLECTION AND IDENTIFICATION OF PLANT

The stem-bark of *Spondias mombin* was purchased from a local vendor in Osogbo, Osun state, Nigeria. Their plant samples were identified and authenticated using its leaves by Dr. Nodza George, at the Herbarium of the Department of Botany, University of Lagos, Lagos, Nigeria. Identification Number: LUH 8790 was given.

PREPARATION OF PLANT EXTRACT

The stem-bark of *Spondias mombin* weighed was then washed with distilled water to remove debris and other particulate matter. The stem bark was kept in the shade for 12 days to dry at room temperature and then cut and ground into fine powder by using a blender. The powdered stem (2.35kg) was extracted by the cold extraction method (maceration) using ethanol as a solvent; the powder was soaked in 6.5 L of ethanol for 72-96 hours, during which the mixture was shaken twice daily to promote extraction. The solvent was filtered using a muslin bag and the filtrate was evaporated to dryness using a rotatory evaporator. The weight of the dried extract was 78.39g giving a yield of 3%. The obtained extract was placed in a refrigerator at 4^0C for further use.

EXPERIMENTAL ANIMALS

Thirty (30) healthy albino male Wistar rats, weighing 102-163g were used for this study. All animals were fed a standard diet and were given access to water *ad libitum*. Experimental techniques and protocols followed in this study were following the criteria given in the "Guide for the Care and Use of Laboratory Animals" edited by the Commission of Life Sciences, National Research Council (USA).

EXPERIMENTAL DESIGN

A total of 30 rats were weighed and distributed into six groups, five per group. The entire experiment was conducted in 2 weeks (14 days). Group 1 (Control) animals were orally administered normal saline daily. Group 2 (Negative) received 200mg/kg bodyweight of DEN single i.p. in normal saline) on day 0 and left for 14 days without further treatment. Group 3 animals (Positive control) received 200mg/kg bodyweight of DEN single i.p. in normal saline on day 0, followed by 50mg/kg bodyweight of Silymarin in distilled water (p.o.) daily for 14 days. Group 4 (Experimental group I) received 200mg/kg bodyweight of DEN single i.p. in normal saline on day 0 and 200 mg/kg bodyweight of extract in distilled water, (p.o.) daily, for 14 days. Group 5 (Experimental group II) received 200mg/kg bodyweight DEN single i.p. in normal saline) on day 0 and 400 mg/kg bodyweight extract in distilled water, p.o.) daily, for 14 days. Group 6 (Experimental group III;) received 200mg/kg bodyweight DEN single i.p. in normal saline) on day 0 and extract (800 mg/kg bodyweight in distilled water, p.o.) daily, for 14 days. Their weights were recorded at the beginning of the experiment and were taken every 3-4 days for the duration of the study.

SAMPLE COLLECTION

After completion of the experiment, the rats were sacrificed via cervical dislocation. The blood samples were collected from all the groups of rats via puncturing the retro-orbital plexus and were kept in labeled bottles. Serum samples were separated by centrifugation at 4000 rpm for 15 min.

BIOCHEMICAL ANALYSIS

The levels of protein and albumin in serum were determined using the biuret method (Gonall *et al.,* 1949) and Bovine Serum Albumin (BSA) as standard.

The activities of tumor marker- Alfa fetoprotein (AFP) in serum was assayed using an ELISA (Enzyme-linked immunoassay) kit (Calbiotech AFP ELISA Kit, California). Interleukin 6 and 10 (IL-6 and IL-10) were both determined using the Rat IL-6 and IL-10 ELISA Kit. The ELISA kit used the Sandwich-ELISA principle.

STATISTICAL ANALYSIS

The results were expressed as mean ± standard error mean (SEM) for three animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the GraphPad Prism software. Post hoc testing was performed for intergroup comparisons using Tukey's multiple comparisons. The comparison was made between the negative control group and test groups that received 200mg/kg, 400mg/kg, and 800mg/kg bodyweight of ethanol extract of *Spondias mombin* stem-bark. p values <0.05 was considered statistically significant and given respective symbols.

RESULTS

The bodyweight of the rats in the experimental and control groups were recorded and are as shown in Figure 1. There were no significant differences between body weight changes in the control and experimental groups.

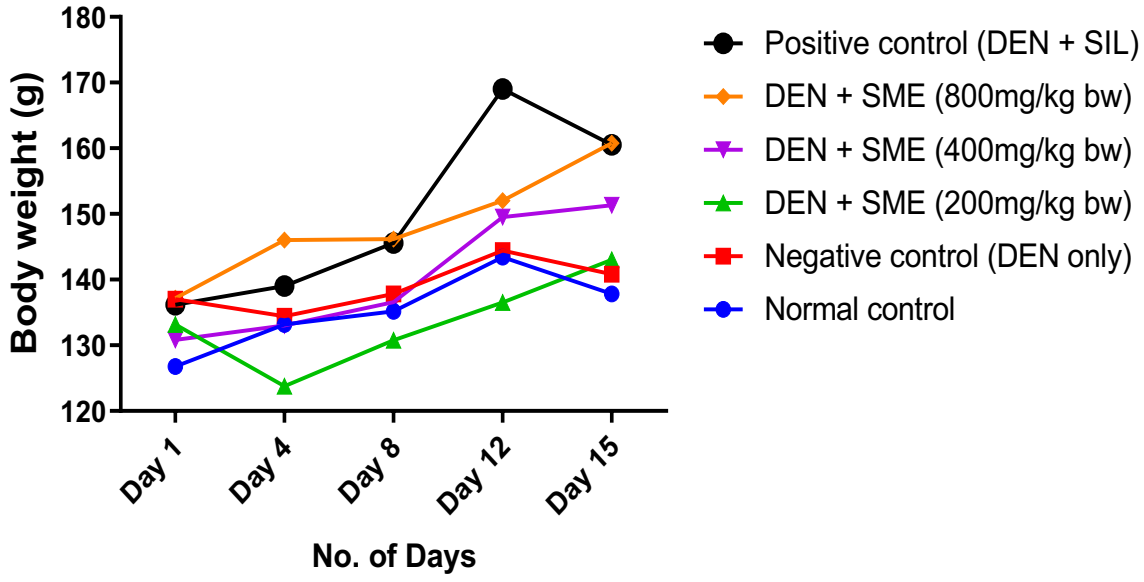

Figure 1: Effect of *Spondias mombin* ethanolic stem-bark extract and Silymarin on bodyweight of DEN-induced Wistar

*Results are given as mean ± S.D. Comparisons are made with negative control rats (DEN only). DEN - diethylnitrosamine; SME - Spondias mombin ethanolic stem-bark extract; SIL - Silymarin.

The effect of oral administration of *Spondias mombin* ethanolic Stem-bark extract on Serum AFP, IL-6, and IL-10 level is as shown in Table 1. When compared to the negative control, all rats treated with SME extracts after DEN administration showed a non-significant reduction in AFP activities except for animals treated with 800mg/kg body weight which showed a slight increase. IL-10 activities were reduced for all treated rats when compared with negative control however no significance was recorded. Rats treated with 800mg/kg body weight showed a non-significant reduction in IL-6 activities compared with negative control (Table 1).

Table 1: Effect of orally administered *Spondias mombin* ethanolic stem-bark extracts on the AFP, IL-10, and IL-6 levels in the plasma of DEN-induced Wistar rats.

Groups	Treatment	AFP (ng/ml)	1L-10 (pg/ml)	IL-6 (pg/ml)
1	Normal Control	3.983±0.27	47.97±3.25	216.3±43.19
2	Negative Control	5.143±0.47	61.57±30.99	552.3±45.76
3	DEN + SME (200mg/kg bw)	4.917±0.39	43.27±9.77	1428±518.5
4	DEN + SME (400mg/kg bw)	4.945±0.67	40.04±13.09	609.7±424.0
5	DEN + SME (800mg/kg bw)	5.166±0.1	31.88±2.72	162.6±37.26
6	Positive Control (DEN + SIL)	6.842±1.0	59.98±23.24	2286±1744

Results are given as mean ± SEM for 3 male albino Wistar rats. Comparisons are made with negative control rats (DEN only). DEN, diethylnitrosamine; SME - *Spondias mombin* ethanolic stem-bark extract; SIL - Silymarin; AFP - Alpha-fetoprotein; IL-6 - Interleukin 6; IL-10 - Interleukin 10.

The effect of oral administration of *Spondias Mombin* Ethanolic Stem-bark extract on Total Protein Levels is as shown in Figure 2. There was no significant change ($p<0.05$) in protein concentration between groups. The comparison was made with negative control. Administration of a single dose of DEN (Negative Control) in rats produced a reduction in levels of protein in the plasma. Treatment with SIL (Positive control) after DEN administration saw a non-significant increase in protein levels. Treatment with 200 mg/kg and 400mg/kg body weight SME extracts after DEN administration in rats both produced a non-significant reduction in protein levels. 800mg/kg extract treatment after DEN administration saw a slight increase in protein levels.

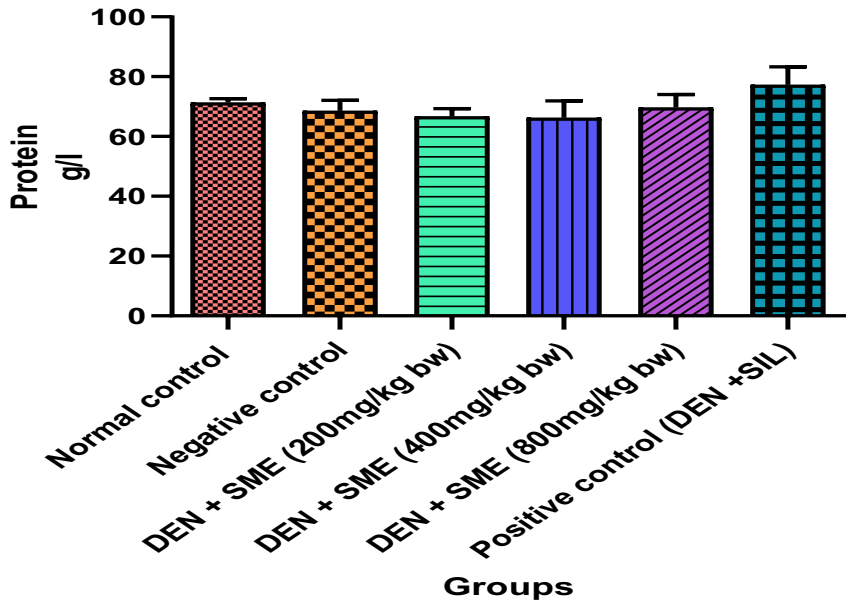

Figure 2: Effect of orally administered *Spondias mombin* ethanolic stem-bark extracts on the total protein levels in the plasma of DEN-induced Wistar rats.

*Results are given as mean ± SEM for 3 male albino Wistar rats. Comparisons are made with negative control rats (DEN only). DEN, diethylnitrosamine; SME - *Spondias mombin* ethanolic stem-bark extract; SIL - Silymarin;

Effect of *Spondias mombin* ethanolic Stem-bark extract on albumin levels is as shown in Figure 3. There was no significant change ($p<0.05$) in albumin concentration between groups Administration of a single dose of DEN (Negative Control) produced a reduction in levels of albumin in the plasma. Treatment with SIL (Positive control) after DEN administration saw a slight non-significant decrease for albumin levels compared with negative control. Treatment with SME (200 mg/kg body weight) after DEN administration produced a non-significant reduction in albumin levels compared to the negative control. Treatment with both 400mg/kg and 800mg/kg extracts after DEN administration saw a non-significant increase in albumin levels compared to the negative control.

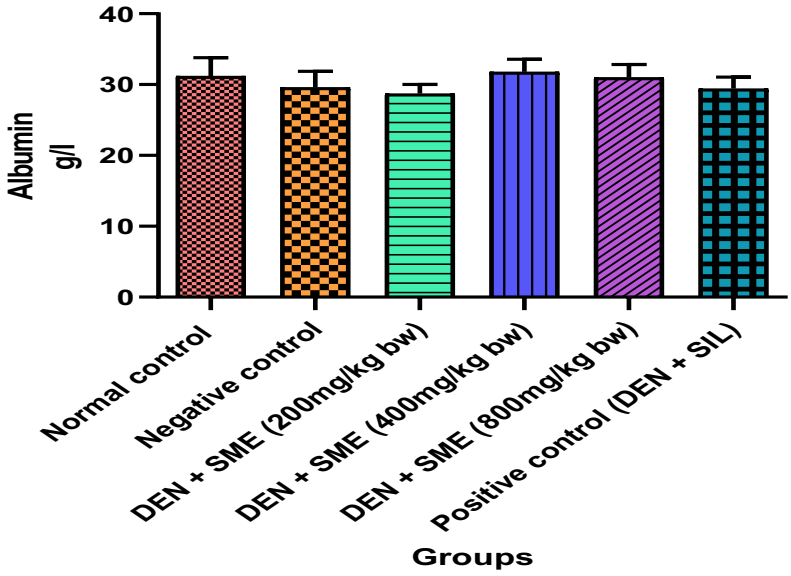

Figure 3: Effect of orally administered *Spondias mombin* ethanolic stem-bark extracts on the albumin levels in the plasma of DEN-induced Wistar rats.

*Results are given as mean ± SEM for 3 male albino Wistar rats. Comparisons were made with negative control rats (DEN only). DEN, diethylnitrosamine; SME - *Spondias mombin* ethanolic stem-bark extract; SIL - Silymarin;

DISCUSSION

Hepatocarcinoma, one of the most recognized forms of liver cancer affects many people worldwide each year (Globocan, 2020) and its exact mechanism has not been fully elucidated leading to hindrances in the development of effective therapy. This study was conducted to provide an alternative means of treatment using traditional medicine. The effect of *Spondias mombin* on inflammation formed the basis for this study to assess its possible role in Diethylnitrosamine (DEN) induced hepatocarcinogenesis. Previous studies carried out have shown the anti-inflammatory effect of *Spondias mombin* extracts on hepatocarcinogenesis (Ayoka *et al.,* 2008; Chukwuemeka *et al.,* 2011; Cabral *et al.,* 2016). The present biochemical study depicts the anti-inflammatory effect of *Spondias mombin* ethanolic stem bark (SME) extract using liver biochemical parameters and pro-inflammatory and anti-inflammatory cytokine mediators. One of the observations associated with HCC is body weight reduction (Tanaka *et al.,* 1993; Abe *et al.,* 2012; Nwidu *et al.,* 2018). In this study, there was a marked reduction in body weights of the DEN only administered group while the body weights of rats in the extract-treated groups increased with time. α-fetoprotein (AFP), is a protein of the serum

albumin family that is usually found in elevated concentrations in conditions such as hepatocarcinoma and pregnancy as it is required in fetal development but found in minute amounts in normal adults (Jahan *et al.,* 2011; Zacharakis *et al.,* 2018). Such elevated concentrations are induced in humans and animal models through exposure to hepatotoxic agents or hepatocarcinogens (Jahan *et al.,* 2011). In this study, elevated levels of serum AFP were observed in the negative control group (DEN only) which decreased following treatment with SME indicating a positive response to the use of SME extract to treat liver cancer. The decrease in the levels of AFP on treatment with SME extract after DEN administration indicates tumor growth inhibition and a reduction in hepatic tumors, suggesting that the extract possesses antitumor properties. Diethylnitrosamine (DEN) is a widely known carcinogen and hepatotoxin which on the accumulation in target organ elicits the release of free radicals. These radicals attack important cellular macromolecules such as DNA causing inflammation (Abe *et al.,* 2012; Tolba *et al.,* 2015; Iweala *et al.,* 2019). This inflammatory response causes the release of soluble immune proteins such as cytokines, chemokines, and so on by macrophages, mast cells, lymphocytes, and neutrophils. Cytokines are generally released by the immune system in cases of stress such as inflammation and carcinogen-induced injury (Budhu and Wang, 2006; Hammerich and Tacke, 2014).

An increase in proinflammatory mediators such as IL-6 in the blood is usually characteristic of the activation of the immune system as expected in the incidence of tissue damage and cytotoxicity. The released cytokine then binds to its receptor on the cells such as hepatocytes. Levels of IL-6 in the serum of animals increase rapidly after organ inflammation and thus is used as a diagnostic marker to monitor inflammatory conditions (Hammerich and Tacke, 2014). Increased IL-6 production is associated with increased HCC development (Yeh and Chen, 2010). Their main function is to stimulate immune responses that result in the elimination of invading pathogens or damaged and dying cells. Anti-inflammatory mediators such as IL-10 are also produced to protect the host's body from exaggerated immune responses, limit organ damage, and deactivate monocyte/macrophage proinflammatory cytokine synthesis thereby inhibiting the production of proinflammatory mediators such as IL-6 and maintaining normal tissue hemostasis (Iyer and Cheng, 2012). The role of IL-10 is to reduce and if possible, terminate inflammation.Hence, when the harmful stimuli are removed or reduced considerably, the production of interleukins is no longer needed, and inflammation subsides. However, if the stimulus persists, inflammation can become chronic and induce a variety of inflammatory diseases, which often have fatal consequences for the host organism. Moreover, excess production of anti-inflammatory cytokines can suppress effective immune responses against malignant cells, thus promoting tumor growth. A reduction in IL-10 and IL-6 levels after DEN administration indicates the therapeutic effect of the SME extract and may suggest inhibition of carcinogenesis (Budhu and Wang, 2006; Hammerich and Tacke, 2014; Rico Montanari *et al.,* 2021; Hassan *et al.,* 2019). A recorded decrease was seen in the levels of liver biochemical parameters-total protein and albumin of the negative control group compared to those of the normal control group. The SME extract-treated groups saw some of the values of these enzymes increase to normal levels. Albumin is considered a negative acute-phase protein, meaning as inflammation and other acute physiologic processes occur, its levels decrease. SME extracts showed it could contribute to or repair liver damage as it affected the release of these enzymes, giving hepaprotective activity and hindering carcinogenesis.

CONCLUSION

In this study, *Spondias mombin* stem bark ethanolic extract was found to exhibit hepatoprotective effects at higher concentration by promoting repair of hepatic tissues and augmenting endogenous immune systems, thereby limiting inflammatory responses. These results provide the premise that requires further investigation of the promising therapeutic potential of *S. mombin* in hepatocarcinogenesis.

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