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HEPATOPROTECTIVE ACTIVITY OF ETHYL ACETATE FRACTION OF *BOMBAX COSTATUM* (PELLEGR. ET VUILLET) STEM BARK AGAINST CARBON TETRACHLORIDE INDUCED ACUTE LIVER INJURY IN MICE

N. Mohammed[a,*], A. Saleh[b], J. Ya'u [c], B.N. Umar [d], R.M. Hamza[e], N.S. Ismail[f], K. Usman [c], A. M. Musa[g]

a= Department of Clinical Pharmacology and Therapeutics, Ahmadu Bello University Zaria
b= Department of Veterinary Pathology, Ahmadu Bello University Zaria, Nigeria
c= Department of Pharmacology and Therapeutics, Ahmadu Bello University Zaria, Nigeria
d= Department of Veterinary Microbiology, Ahmadu Bello University Zaria, Nigeria
e= Department of Human Anatomy, Federal University Dutse, Nigeria
f= Ahmadu Bello University Medical Centre, Ahmadu Bello University Zaria, Nigeria
g= Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University Zaria

***Corresponding author: Nuhu Mohammed, nuhumuhd8@gmail.com**, **+2348069675540**

ABSTRACT

Background: Hepatic disease is still a major public health problem due to susceptibility of the liver to a variety of metabolic, circulatory, neoplastic, xenobiotic and microbial insults. *Bombax costatum* stem bark was reported to possess hepatoprotective activity but high dose was found to be associated with hepatocellular necrosis. The search for new effective therapy with minimal side effect is still ongoing.

Objectives: To evaluate the hepatoprotective activity of ethyl acetate fraction of *B.costatum* stem bark against carbon tetrachloride (CCl_4) induced acute liver injury in mice.

Methodology: Phytochemical and acute toxicity studies of the ethyl acetate fraction of *B. costatum* stem bark were carried out. Single dose of 1 ml/kg of CCl_4 was administered intraperitoneally to treated groups of mice to induce acute hepatotoxicity while *in vitro* 1,1 - diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method was used for antioxidant study. Effects of the ethyl acetate fraction of *B.costatum* on DPPH radical, serum biochemical parameters and liver histopathology were analysed.

Results: Phytochemicals present include tannins, triterpenes and flavonoids. Oral median lethal dose was estimated to be >5000 mg/kg. Pre-treatment of mice with the ethyl acetate fraction of *B.costatum* stem bark at 62.5 and 125 mg/kg significantly (p<0.001) reduced alanine aminotransferase (55.6±6.27 and 54.2±2.73 respectively) and aspartate transaminase levels (76.8±5.16 and 53.2±3.44 respectively) compared to CCl_4 toxic group. These results were supported by the preservation of the liver against CCl_4 -induced hepatocellular necrosis in the ethyl acetate fraction treated groups.

Conclusion: The results obtained suggest that ethyl acetate fraction of *B.costatum* stem bark possesses hepatoprotective activity against CCl_4 induced acute liver injury. The hepatoprotective effect of the ethyl acetate fraction of *B.costatum* stem bark could be due to its antioxidant activity.

Keywords: *Bombax costatum*; Carbon tetrachloride; Ethyl acetate; Hepatoprotective; Necrosis

1.0 INTRODUCTION

Hepatic disease is still a major public health problem due to susceptibility of the liver to a variety of metabolic, circulatory, neoplastic, xenobiotic and infective insults. It accounts for 2 million deaths per year globally [1]. According to the European Association for study of Liver Disease

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[EASL] [2], liver disease is extremely costly in terms of human suffering, management and premature loss of productivity. It is also an important cause of morbidity and mortality globally. The pattern of liver disease varies in different geographical locations [3]. The search for effective, safe, accessible and affordable therapy for liver disease is still ongoing. The WHO strongly encourages strategic research and rational use of traditional medicines in the primary health care delivery system of developing countries [4] and this has led to the discovery of relevant plants with useful chemical constituents that can be used in treatment of various diseases. We previously reported the hepatoprotective effect of methanol stem bark extract of *Bombax costatum* (BC) against carbon tetrachloride (CCl_4) as well as paracetamol induced acute liver injury in rats. Lower doses of the extract were noted to exhibit more hepatoprotective activity while high doses were observed to be associated with hepatocellular necrosis [5]. This study therefore aimed to evaluate the hepatoprotective activity of ethyl acetate (EA) fraction of *B. costatum* against CCl_4 induced acute liver injury in mice.

2. MATERIALS AND METHODS

2.1 Equipment and Chemicals

Carbon tetrachloride (BDH Ltd Poole, England), Ethyl acetate, Methanol, Hexane (Sigma Aldrich, USA) Silymarin (Micro Labs Limited, India), Biochemical kits (Randox, UK), Haematoxyline and Eosin stain, ScopetekDCM500 Camera (Hangzhou S. Digital Technology Ltd. China), Leitz Microscope (Optical Institute, Germany), Spectrophotometer (Metsar Technologies Pvt. Ltd., Hyderabad), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) powder (Sigma Chemical Co. USA)

2.2 Experimental Animals

Male mice (25-30 g) were obtained from Animal house of Department of Pharmacology and Therapeutics, Ahmadu Bello University Zaria. They were kept inside clean cages in a well-ventilated room and fed with standard commercial mice feed (Vital feed®, Zaria). The mice were allowed free access to the feed and drinking water *ad libitum*. All experiment protocols were performed according to regulations governing the care and use of experimental animals as contained in "Principles of laboratory animal care" published by the National Institute of Health (NIH Publication No. 85-23, revised, 1996) and were approved by the Ahmadu Bello University Committee for Animal Use and Care (Approval number: ABUCAUC/2020/50).

2.3 Preparation of ethyl acetate fraction from stem bark of B. costatum

Stem bark of *B. costatum* was collected from Sabon Gari local Government, Kaduna, Nigeria in the month of July. It was authenticated by Namadi Sanusi, a taxonomist at the Herbarium Unit of the Department of Botany, Ahmadu Bello University, Zaria by comparing with an existing reference voucher specimen previously deposited in the herbarium (number 1211) for future reference. The stem bark of the plant was air dried under shade, mechanically powdered using mortar and pestle and stored in an air tight container. Methanol extract of *B. costatum* stem bark was prepared by subjecting 11 kg of the powdered stem bark to maceration using 40 L of 70% v/v methanol for 72 hours. The mixture was intermittently stirred during the 72 hours period and then filtered using Whatman filter paper (No. 1). The filtrate was concentrated to dryness over a water bath maintained at 50ºC. The extract was kept in an air tight container and then stored in a desiccator until required for further

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studies. To obtain the EA fraction of BC, 460g of methanol stem bark extract of *B. costatum* was subjected to liquid-liquid partitioning to separate the extract into different fractions. For each session, about 100 mg of methanol stem bark extract of *B. costatum* powder was dissolved in distilled water (500 ml) and exhaustively extracted by succesive liquid/liquid partition with hexane (500 ml), chloroform (500 ml) and ethyl acetate (500 ml) using a separating funnel (1000 ml). For each solvent, the mixture was allowed to stand for 30 minutes in the separating funnel until a clear separation line appeared before desorption. The solvent fractions were evaporated to dryness on a water bath maintained at 45 ^{0}C to obtain their corresponding fractions [6].

2.4. Preliminary phytochemical screening

Preliminary phytochemical screening of EA fraction of BC was conducted according to the method previously described [7]. The fraction was screened for presence or absence of saponins, anthraquinones, flavonoids, cardiac glycoside, alkaloids, tannins, triterpenes and steroids.

2.5. Acute toxicity study

Acute toxicity study of EA fraction of BC was carried out in mice according to Organisation for Economic Co-operation and Development (OECD) guideline 423 [8]. Before oral administration of a single dose of the fraction, the mice were deprived of food for 3 hours. Limit test at dose of 5000 mg/kg was conducted using 3 mice. The first mice was dosed at 5000 mg/kg and observed. The remaining 2 mice were also dosed at 5000 mg/kg. All the mice were observed for general behavioural changes, symptoms of toxicity and mortality after treatment for the first four hours, then over a period of 24 hours and thereafter daily for 14 days duration.

2.6 In vivo hepatoprotective study of ethyl acetate fraction of B. costatum against CCl_4 induced acute hepatotoxicity in mice

CCl_4 induced acute liver injury was carried out according to method previously described [9] with some modifications. The mice were divided into 6 groups with each group containing 5 mice. Group I (Normal control) received olive oil, 1 ml/kg, *p.o.* on the 8th day of the study. Mice in group II (Toxic group) received CCl_4 (1ml/kg, 1:1 in olive oil, *p.*o) on the 8th day. Group III (Standard group) was treated with silymarin (100 mg/kg *p.o.*) daily for 7 days and CCl_4 (1ml/kg, 1:1 in olive oil, *p.*o) on the 8th day. Mice in groups IV, V and VI received 31.25, 62.5 and 125 mg/kg of EA fraction of BC *p.o.* per day for 7 days and CCl_4 (1ml/kg, 1:1 in olive oil,.*p.*o) on the 8th day respectively. The animals in all the groups were euthanized 24 hours after injection of CCl_4. Liver tissues were rapidly dissected and the left lobes were fixed in a buffer solution of 10% formalin for histological analysis while blood samples were collected in plain sample bottles and then centrifuged at a speed of 4000 revolutions per minute to obtain serum. The sera were used for determination of AST and ALT using commercially available kit (Randox reagent UK) and a spectrophotometer for reading the absorbance.

2.7 DPPH radical scavenging activity of ethyl acetate fraction of B. costatum stem bark

The DPPH radical scavenging activity of the EA fraction of BC was determined by the DPPH radical scavenging method [10]. DPPH solution was prepared by dissolving 6 mg of DPPH in 100 ml of methanol. To 1ml of various concentrations of the EA fraction of *B. costatum* stem bark (20, 40, 60, 80, 100 µg/ml), 2 ml of DPPH solution (0.1mM) was added. An equal amount of

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methanol and DPPH was used as control. The mixture was shaken vigorously after being kept for 30 minutes. The absorbance of the resulting solution was taken using UV spectrophotometer at 520 nm. The experiments were performed in triplicate and the percentage scavenging activity of EA fraction of *B. costatum* stem bark on DPPH radical was calculated using the formulae below:

Radical Scavenging activity (%) = $[AB - AE/AB] \times 100$

Where AB= absorbance of control, AE= absorbance of EA fraction of BC

DPPH radical scavenging activity of the extract was expressed in terms of IC_{50} (concentration of the extract required to inhibit DPPH radical formation by 50%) which was calculated using linear regression equation from the graph of % inhibition against sample concentration on Microsoft excel software.

2.8 Histopathological Study

Sections of liver tissue were used for histopathological examination after dissecting the animals. Tissues were fixed in 10% buffered formalin (pH 7.2) and dehydrated through a series of ethanol solutions (70%, 90%, 96% and 100%). The tissues were cleared in xylene and embedded in paraffin. Sections of 5 µm thickness were cut using a rotary microtome and stained with haematoxylin and eosin for examination. The stained tissues were observed through a Leitz microscope at x250 magnification and photographed by a Scope tek DCM500 camera.

2.9 Statistical analysis of data

Data were presented using tables, chart and plates where applicable. Analyses of data were done using SPSS (version 20). Biochemical data were presented as Mean ± Standard Error of Mean (S.E.M.). Differences between mean were analysed using one way ANOVA followed by Tukey's post hoc test. The results were considered significant at *p*-values ≤ 0.05.

3.0 RESULTS

Percentage yield of EA fraction of BC

The percentage yield of the ethyl acetate fraction of *B.costatum* was calculated to be 5.10% $^{w}/_{w}$.

Phytochemical constituents of EA fraction of BC

Preliminary phytochemical screening of ethyl acetate fraction of *B. costatum* showed the presence of flavonoids, tannins, carbohydrate, triterpenes and steroids (Table 1).

Effect of EA fraction of BC on ALT and AST

Significant ($p \leq 0.001$) increase in serum AST and ALT were observed in mice treated with CCl_4 in CCl_4 toxic group (117.2±2.06 and 86.2±3.98 respectively) when compared with normal control group (49.2±4.22 and 24.0±2.28 respectively) (Table 2).

However, pre-treatment with sylimarin (100 mg/kg) and ethyl acetate fraction of *B. costatum* at all tested doses caused significant ($p \leq 0.001$) reduction in serum level of AST when compared with that of CCl_4 toxic group (Table 2). Pre-treatment of mice with silymarin (100 mg/kg) and ethyl acetate fraction of *B. costatum* (62.5 and 125 mg/kg) also caused significant ($p \leq 0.001$) reduction in serum level of ALT (60.4±5.39, 55.6±6.27 and 54.2±2.73 respectively) when compared with CCl_4 toxic group.

Effect of EA fraction of BC on histopathological changes on liver

Photomicrograph of liver section from the CCl_4 toxic group showed moderate hepatocellular necrosis (Plate Ib). Pre-treatment of mice with 31.25 mg/kg of the EA fraction of BC did not protect the liver from toxic effect of CCl_4 (Plate Id) but pre-

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treatment with silymarin (100 mg/kg) and EA fraction of *B. costatum* (62.5 and 125 mg/kg) protected the liver from CCl_4 induced hepatotoxicity (Plate Ic, Ie and If respectively).

DPPH free radical scavenging activity of EA fraction of BC

The IC_{50} of the EA fraction of *B. costatum* stem bark was found to be 53 µg/mL (Table 3, Figure 1).

4.0 DISCUSSIONS

This study evaluated the effect of EA fraction of BC against CCl_4 induced acute liver injury in mice. Flavonoids were reported to possess anti-inflammatory and free radical scavenging activities [11],[12]. This may be responsible for the observed hepatoprotective activity of the EA fraction of BC against CCl_4 induced acute liver injury in mice.

The aminotransferases enzymes (ALT and AST) are sensitive indicators of liver cell injury and are most helpful in recognizing acute hepatocellular diseases. Liver cell damage can cause loss of hepatocytes cell membrane integrity leading to leakage of liver enzymes out of the hepatocytes and their elevation in the serum [13]. CCl_4 is a fast acting hepatotoxic agent that can cause morphological changes in the liver appearing as early as 15 minutes after parenteral administration [14].

This study demonstrated that single intraperitoneal injection of 1ml/kg of CCl_4 to mice caused significant ($p \leq 0.001$) elevation in the serum level of ALT and AST as well as distortion of liver architecture. These findings were in agreement with results of previous study [9], in which CCl_4 was used to induce acute liver damage that led to significant elevation in the serum levels of ALT and AST.

Free radical scavenging activity of substances can be evaluated using the DPPH assay. DPPH is a stable free radical compound and has an absorbance in its oxidized form within 515-520 nm [15, 16]. Change in colour, from purple to yellow indicates presence of antioxidant substance [17]. This demonstrates that the antioxidant found in a mixture solution interact with the free radicals in DPPH [18].

IC_{50} is the concentration of the extract required to inhibit DPPH radical formation by 50%. The IC_{50} of the EA fraction of *B. costatum* stem bark was found to be 53 µg/mL. This implies that EA fraction of *B. costatum* stem bark posses higher *in vitro* antioxidant activity than the crude methanol stem bark extract of *B. costatum* previously reported [5]. The lower the IC_{50} value, the higher the antioxidant activity of the sample [19]. This further validated the findings of previous study where EA fraction of *B. costatum* was reported to have *in vivo* antioxidant potential [20]. Oxidative stress is a key driver of hepatic inflammation and fibrosis. Under normal physiological conditions, oxygen-containing reactive molecules regulate key physiological activities in the body. These physiological activities include cell growth, proliferation, migration, differentiation, and apoptosis [21]. However, elevated intracellular reactive oxygen species (ROS) concentrations can induce damage to cell's structure, organelles and macromolecules due to oxidative stress. In the liver, ROS can induce apoptosis and necrosis of hepatocytes. It can also stimulate the production of profibrogenic mediators by Kupffer cells and recruitment of circulating inflammatory cells. Antioxidants play essential role in the prevention of oxidative stress-related diseases and in the reduction of total mortality [22]. Flavonoids commonly accumulate in the epidermal cells of plant organs such as flowers, leaves, stems, roots, seeds and fruits. Studies have shown that flavonoids and tannins exert their antioxidant properties via free radical scavenging effect [23]. In this study, EA fraction of BC was found to possess both antioxidant and hepatoprotective activities against CCl_4-induced acute liver damage. This could be due to presence of flavonoids and tannins. Previous studies have shown that plants rich in natural antioxidant possess good hepatoprotective activity [24].

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5.0 CONCLUSION

The results obtained suggest that ethyl acetate fraction of *B.costatum* stem bark possesses hepatoprotective activity against CCl_4 induced acute liver injury. The antioxidant and hepatoprotective effect of the EA of *B. costatum* could be due to presence of flavonoids and tannins which are known to possess anti-inflammatory and free radical scavenging activities.

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Conflict of Interest

The authors declared that they have no conflict of interest.

Table 1: Phytochemical Constituents of Ethyl Acetate fraction of *B. costatum*

Phytoconstituents	Test	Inference
Flavanoids	Shinoda	+
Saponins	Frothing	-
Tanins	Ferric chloride	+
Anthraquinons	Bontregers	-
Carbohydrate	Molisch	+
Triterpenes and steroid	Liebermann-Bucchard	+
Alkaloid	Dragendorff	-

Table 2: Effect of Ethyl Acetate Fraction of *B. costatum* on AST and ALT in CCl_4 induced liver injury

Treatment	AST (U/L)	ALT (U/L)
OO	49.2±4.22	24.0±2.28
CCl4	117.2±2.06 *	86.2±3.98*
SLY	59.2±4.5♯	60.4±5.39♯
EA31.25mg//kg	85.2±2.89♯	77.0±8.02
EA62.5mg/kg	76.8±5.16♯	55.6±6.27♯
EA125mg/kg	53.2±3.44♯	54.2±2.73♯

+ = Present, - = Absence

Data were presented as Mean ± SEM. (n = 5), One way ANOVA followed by Tukey's post hoc test.*= $p \leq 0.05$ compared with normal control group, ♯ = $p \leq 0.05$ compared to CCl_4 toxic group. ALT: Alanine transaminase, AST: Aspartate transaminase, OO: Olive oil, SLY: Silymarin, EA: Ethyl acetate fraction of *Bombax costatum*, CCl_4: Carbon tetrachloride

Table 3: Quantitative DPPH Assay of the Stem Bark Extract of Ethyl Acetate Fraction of *B. costatum*

S/N	Concentration (µg/ml)	AB	AE	% Inhibition
1	20	0.548	0.328	40.15
2	40	0.452	0.206	54.42
3	60	0.532	0.215	59.59
4	80	0.812	0.212	73.89
5	100	0.698	0.122	82.52

Keys: Where AB= absorbance of control, AE= absorbance of the Extract, SN= Serial number

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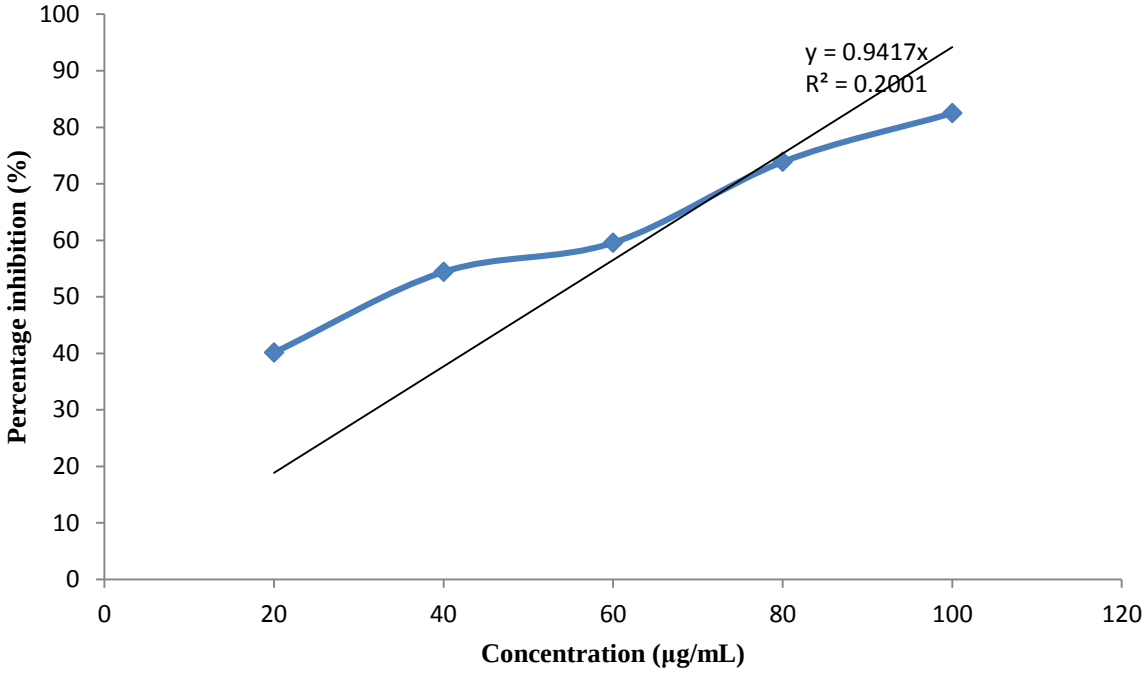
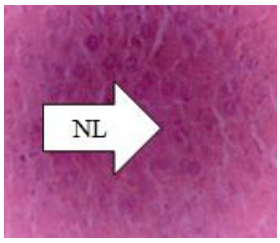
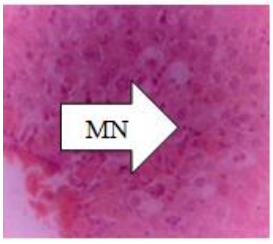


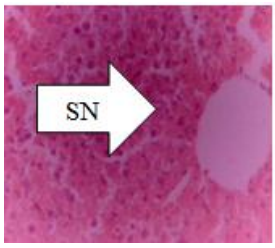
Figure 1: DPPH radical scavenging activity of methanol extract of *B. costatum* stem bark. Results represents mean of triplicates of different concentrations analysed. Wavelength =520nm.



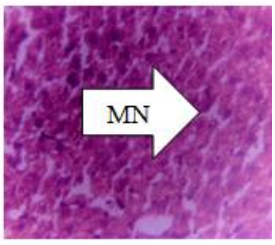
(Ia) OO: Normal hepatocyte



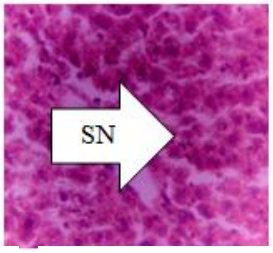
(Ib) CCl_4: Moderate necrosis



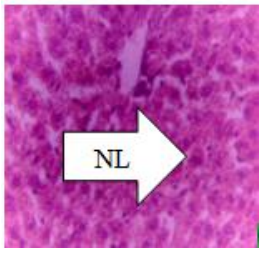
(Ic) SLY 100: Slight necrosis



(Id) EA31.25: Moderate necrosis



(Ie) EA62.5: Slight necrosis



(If) EA125: Normal liver

Plate I: Photomicrograph of Liver Showing Effect of Ethylacetate fraction of *B. costatum* in CCl_4-Induced Acute Liver Injury in mice (H and E ×250).

OO= Olive oil, CCl_4= Carbon tetrachloride, SLY= Silymarin, MN= Moderate necrosis, SN= Slight necrosis, NL= Normal liver, EA= Ethyl acetate

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