

HEPATOPROTECTIVE EFFECT OF METHANOL STEM BARK EXTRACT OF *Diospyros mespiliformis* AGAINST CARBON TETRACHLORIDE INDUCED LIVER DAMAGE

ABSTRACT

Aim: The study was designed to evaluate the hepatoprotective effects of methanol stem bark extract of *Diospyros mespiliformis* against carbon tetrachloride-induced liver damage in rats.

Methodology: Acute toxicity study was determined using limit fixed dose test of 5000mg/kg for 14 days. Six (6) adult albino rats of both sexes were used and randomly divided into six groups of one rat each. Hepatoprotective study; a total of forty two (42) adult albino rats were divided in to seven groups, of six rats each. Methanol stem bark extract of *Diospyros mespiliformis* administered to different groups of rats at the doses of 50, 100, 150 and 200 mg/kg, P.O once a day for fourteen days followed by CCl₄-administration for every 72 hours. Silymarin a known hepatoprotective compound at a dose of 100mg/kg body weight per oral, once daily for fourteen (14) days followed by CCl₄ induction for every seventy two (72) hours was used as reference drug. Hepatoprotective effect was studied by assaying the activities of liver function indices (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and gamma glutamyl transferase, total protein, albumin, direct and total bilirubin) in serum.

Results: The LD₅₀ of methanol stem bark extract of *Diospyros mespiliformis* was found to be greater than 5000 mg/kg. The activities of all the marker enzymes revealed a significant ($P < 0.05$) elevated levels and decreased in the levels of total protein and albumin in CCl₄ treated rats. Group administered with the extract prior to CCl₄ induction show significantly low level of serum liver marker enzymes and increased levels of total protein and albumin compared to CCl₄-induced untreated group and this observations is supported by the histology of liver sections, the result indicate that methanol stem bark extract of *Diospyros mespiliformis* possess significant hepatoprotective property by inhibiting lipid peroxidation.

Conclusion: This property may be attributed to the phytochemical compounds present in the extract.

Key words: Hepatoprotective, *Diospyros mespiliformis*, Silymarin.

1. INTRODUCTION

The liver is the largest internal organ in the body, it essential in keeping the body functioning properly. It involved in almost all biochemical process: excretion, production, storage and detoxification [1]. Therefore it appears to be a sensitive target site for substances modulating biotransformation. While performing several of these metabolic functions, liver undergoes stress, involves oxidative stress leading to liver diseases (condition of liver inflammation or tissue damage that affects liver metabolic functions) ending in liver damage and serious health problems and death [2]. Therefore, maintenance of a healthy liver is essential for the overall well-being of an individual. Liver cell injury caused by various toxicants such as certain chemotherapeutic agents, carbon tetrachloride, thioacetamide, chronic alcohol consumption, viral and microbes are common [3]. In spite of tremendous strides in modern medicine, there are hardly any drugs that stimulate liver function, offer protection to the liver from damage or help regeneration of hepatic cell [2]. Many formulations containing herbal extracts are sold in the Nigerian market for liver disorders but management of liver disorders by a simple and precise herbal drug is still an intriguing problem [4].

Plants kingdom has proven to be the most useful in the treatment of diseases and provide important source of all the world pharmaceuticals [5]. All over the world, plants have served as the richest source of raw materials for traditional as well as modern medicine, particularly in Africa and Asia. Around 80% of the world population depends on medicine which is predominantly based on plant materials [6]. In Nigeria and other developing countries, people have relied on traditional herbal preparations for treatment of various diseases. There are a large number of medicinal plants whose scientific importance has not been explored [7]. *Moringa oleifera*, *Senna alata*, *Cochlospermum tinctorium*, *Uvaria afzelii*, *Vernoni ambigua*, *Acimum americanum* and *Ficus exasperate* are among the plant that have been extensively used in traditional medicine in Nigeria for treatment of various liver diseases [8]. Another plant that could be used for that purpose is *Diospyros mespiliformis*.

Diospyros mespiliformis (Hochst) (Ebenaceae) is a medicinal plant commonly called Jackal-berry or African ebony, locally called Kaiwa or kanya among Hausa's [9]. *Diospyros mespiliformis* is reportedly one of the most important genera of Ebenaceae which have been used for the treatment of various ailments which included liver disease. Research on medicinal plants with acclaimed effects in reducing hepatic disorders therefore becomes imperative in order to improve the quality of life of the people. In view of the fact that there are claimed by the traditional healers regarding the use of this traditional medicinal plant stem bark (*Diospyros mespiliformis*) in the management of liver diseases. Moreover, there are little to no available scientific report regarding the use of the plant stem bark as hepatoprotective agent. The study was designed to evaluate the hepatoprotective effect of the methanol stem bark extract of *Diospyros mespiliformis* in CCl₄-induced rat liver damaged. Thus, the hepatoprotective activity of the plant reported in this study could provide scientific evidence of its claimed medicinal properties.

2.1 METHODOLOGY

2.1.1 Study area.

2.1.1 Plant sample collection and identification

The plant sample of *Diospyros mespiliformis* stem bark was collected from Ngaski Local Government area of Kebbi State, Northern Nigeria. The plant was taken to the Herbarium section of the Department of Plant Science and Biotechnology, Kebbi State University of Science and Technology Aleiro; where it was identified and authenticated by a plant taxonomist Prof. Dramendra Singh. A voucher specimen number was obtained: V. N: 182 and a voucher specimen with number was deposited at the Herbarium for future reference.

2.1.2 Drug used

Silymarin: a known hepatoprotective drug purchase sigma chemical company, USA.

2.1.3 Chemicals and Reagents

All chemicals and reagents used were of analytical grades. Carbon tetrachloride was purchased from Benzer Multitech, India. Methanol, ferric chloride, hydrochloric acid (HCl), sulphuric acid (H₂SO₄), benzene, sodium hydroxide (NaOH) and acetic anhydride were purchase from British Drug House (BDH), England. Chloroform (GPR) was purchased from Sigma-Aldrich chemical limited, UK, Normal saline was purchased from Kernel and distilled water (AR) was purchased from SD chemical limited. Similarly some reagents used for experiments were commercial kits, which are products of Randox, (UK), Chemelex Lab kit S.A and Cayman Chemicals, USA.

2.1.4 Experimental animals

Sixty (60) albino rats of both sexes weighing 150-200g were obtained from animal's house, Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto. The animals were kept in a well-ventilated room and allowed to acclimatize to laboratory condition for two (2) weeks with free access to food and water ad libitum. Dark and light cycles were also maintained at 12 hours each.

2.2 METHODS

2.2.1 Preparation of plant sample and extraction

The identified stem bark of *Diospyros mespiliformis* was washed with distilled water (To remove sand particles) and air dried (away from sun, dust and intense heat) under the shade in the laboratory for two (2) weeks. The stem bark was pounded into powder, using a wooden mortar and pestle. The stem bark was powder was weighed and stored in a specimen bottle until required for used.

The plant material (200g) was extracted with two liters of 98% methanol and was left in an air tight aspirator bottle for 72 hours with occasional shaken. The mixture was then filtered with sterile Muslim cloth; the filtrate was evaporate using hot air ovum at 45oC and subsequently dried in a drying cabinet at 45oC. Extract was subsequently weighed and recorded for calculation of percentage yield, labeled and stored in a closed container until required for reconstitution in distilled water (for oral administration).

2.2.2 Experimental design for hepatoprotective study

Induction of hepatotoxicity was done with slight modification according to the method of Rao et al. [11].

Forty two (42) adult albino rats weighting 150-200g were divided in to seven groups, of six rats each.

Group I: Served as normal control, received distilled water (1 ml/kg body weight P.O) for fourteen days, in addition to normal diet.

Group II: Induction control, received 30% carbon tetrachloride (1ml/kg body weight, I.P) in liquid paraffin for every 72 hrs for fourteen (14) days, in addition to normal diet.

Group III, IV, V and VI: Received the methanol stem bark extract of *Diospyros mespiliformis* at dose of 50, 100, 150 and 200mg/kg respectively; once a day for fourteen days following by CCl₄ induction for every seventy two (72) hours.

Group VII: received silymarin a known hepatoprotective compound (sigma chemical company, USA), at a dose of 100mg/kg body weight per oral, once daily for fourteen (14) days followed by CCl₄ induction for every seventy two (72) hours.

2.2.3 Collection and preparation of blood sample

At the end of the experiment, the animals in various groups were sacrificed after 48 hours of 30% CCl₄ induction under chloroform anesthesia, blood and liver samples were collected. A blood sample collected into clean non-heparinised bottles was allowed to clot and the serum was separated by centrifuging at 3000rpm for 5 minutes. The serum was collected according to groups using pasteur pipette into the sample bottle. The serum was used for biochemical estimations (GGT, AST, ALT, ALP, Total protein, albumin, bilirubin).

2.2.4 Biochemical Analysis

Serum Alanine Aminotransferase and Serum Aspartate Aminotransferase (AST) activities were ascertained using the method of Reitman and Frankel [11] (Assay kit: Randox laboratories, UK).

Total protein in the blood was determined by Biuret method of Young [12]. Total and conjugated bilirubin was determined using the method of Jendrassik and Grof [13] (Assay kit: Randox laboratories, UK), Alkaline phosphatase was estimated using Colorimetric method of Young (2001) (Assay kit: Labkit Chemelex S.A), Albumin was determined by the dye binding technique utilizing Bromocresol green (BCG) as modified by Dumas et al., [14] was employed. Gamma-Glutamyl Transferase Activity Assay (GGT) by the method of Szasz and Bergmeyer [15] (Assay kit: Randox laboratories, UK).

2.3.0 Statistical Analysis

The results were expressed as the mean \pm standard error of mean (SEM). For data comparison between the groups, one-way analysis of variance (ANOVA) was applied using statistical package for the Social Sciences (SPSS) version 20.0. Duncan post Hoc comparison test was used to check differences between the individual group and values were considered statistically significant at $P < 0.05$.

3.0 RESULTS AND DISCUSSION

3.1 Methanol Extract of *Diospyros mespiliformis* stem bark

The extract obtained was soluble in water, dark-brown in colour, with a characteristic of un-pleasant smell. The percentage yield of extract obtained was = 13.50%

3.3 Acute Toxicity Studies (LD₅₀)

3.3.1 Effect of acute administration of 5000 mg/kg body weight of methanol stem bark extract of *Diospyros mespiliformis* (MSEDM)

Acute administration of 5000mg/kg body weight of methanol stem bark extract of *Diospyros mespiliformis* (Table 1) produced no mortality after 48 hrs of observation. The median lethal dosage (LD₅₀) of the MSEDM was therefore estimated to be greater than 5000 mg/kg body weight. The extract did not produced any grossly negative behavioral changes such as etching, depression, tremor, weakness, food and water refusal, salivation, discharged from eyes and ears, skin changes and hair removal.

Table 1: The effect of acute oral administration of MSEDM

Dose	Groups	No. of Animal	No. of Death
DTW (1ml/kgbw)	A	1	0
MSEDM 5000 mg/kgbw	B	1	0
MSEDM 5000 mg/kgbw	C	1	0
MSEDM 5000 mg/kgbw	D	1	0
MSEDM 5000 mg/kgbw	E	1	0
MSEDM 5000 mg/kgbw	F	1	0

Key: DTW-distilled water MSEDM- methanol stem bark extract of *Diospyros mespiliformis*. The A- received distilled water and served as control. The remaining B to F were administered with single oral dose of 5000mg/kg body weight of methanol stem bark extract of *Diospyros mespiliformis*.

3.4.0 Assessment of Serum Liver Function Indices

3.4.1 Effect of administration of MSEDM on serum liver function indices

Administration of MSEDM at different doses on treated groups showed a remarkable protective effect across the various serum liver function indices. The CCl₄-induced control group showed significant ($p < 0.05$) increased in alanine aminotransferase (ALT), aspartate aminotransferase

(AST), total bilirubin (TB), direct bilirubin (DB), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) levels, while albumin and total protein levels decreased significantly ($p < 0.05$) compared to normal control (Table 2).

Table 2: Effects of administration of methanol stem bark extract of *Diospyros mespiliformis* on serum liver biochemical indices in rats with CCl₄-induced hepatotoxicity.

BIOCHEMICAL PARAMETERS	AST(U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)	ALB (g/dl)	TP (g/dl)	TB (g/dl)	DB (g/dl)
GROUP I	61.17±2.34 ^a	48.50±1.87 ^a	245.49±3.17 ^a	76.61±3.32 ^a	6.90±0.56 ^{cd}	9.53±0.65 ^d	1.57±0.31 ^a	0.97±0.18 ^{ab}
GROUP II	83.17±3.87 ^c	67.50±2.65 ^b	381.19±4.61 ^e	126.99±1.82 ^d	2.89±0.23 ^a	4.90±0.82 ^a	3.93±0.18 ^c	2.35±0.35 ^c
GROUP III	68.33±5.30 ^{ab}	58.50±3.26 ^{ab}	340.48±3.10 ^d	113.23±3.20 ^c	3.32 ±0.30 ^a	5.94±0.26 ^{ab}	4.00±0.30 ^c	2.06±0.22 ^c
GROUP IV	68.33±5.30 ^{ab}	56.83±3.81 ^{ab}	304.46±1.23 ^c	103.37±1.53 ^b	4.70 ±0.19 ^b	7.59±0.33 ^b	3.09±0.38 ^b	1.77±0.24 ^{bc}
GROUP V	65.83±2.59 ^a	51.33±3.81 ^a	265.11±2.33 ^b	93.38±1.42 ^a	4.27±0.15 ^b	8.08±0.62 ^{bc}	2.13±0.33 ^a	1.17±0.28 ^{ab}
GROUP VI	63.67±1.61 ^a	62.17±1.54 ^a	259.29±2.47 ^b	82.21±9.96 ^a	6.10 ±0.42 ^c	8.74±0.33 ^{ab}	1.83±0.38 ^a	1.37±0.41 ^{ab}
GROUP VII	61.00±4.57 ^a	49.16±0.98 ^a	247.68±5.2 ^a	79.27±1.90 ^a	6.86±0.28 ^d	9.47±0.40 ^{cd}	1.64±0.22 ^a	1.02±0.18 ^a
GROUP VII	61.00±4.57 ^a	49.16±0.98 ^a	247.68±5.2 ^a	79.27±1.90 ^a	6.86±0.28 ^d	9.47±0.40 ^{cd}	1.64±0.22 ^a	1.02±0.18 ^a

Values were expressed as mean ± standard error of mean (SEM), n = 6 in each group. Figures in parenthesis are percentage of protection of the activity to normal values as compared to CCl₄ control. Values are expressed as mean ± Standard error of mean. Mean values having different superscript letters in a column are significantly different at (p<0.05).

Key: ALB-Albumin, TP-Total protein, ALT-Alanine aminotransferase, AST-Aspartate aminotransferase, TB-Total bilirubin, DB-Direct bilirubin, ALP-Alkaline phosphatase, GGT -Gamma Glutamyl Transferase

Group I: received liquid paraffin (1ml/kg body weight P.O)

Group II: received 1ml/kg body weight I.P of 30% CCl₄ in liquid paraffin for every 72 hrs for 14 days

Group III: received 50 mg/kg body weight of the extract once daily and 1ml/kg, body weight of 30 % CCl₄ in liquid paraffin for every 72 hrs for 14 days

Group IV: received 100 mg/kg body weight of the extract once daily and 1ml/kg body weight of 30 % CCl₄ in liquid paraffin for every 72 hrs for 14 days

Group V: received 150 mg/kg body weight of the extract once daily and 1ml/kg body weight of 30% CCl₄ in liquid paraffin for every 72 hrs for 14 days

Group VI: received 200 mg/kg body weight of the extract once daily and 1ml/kg body weight of 30 % CCl₄ in liquid paraffin for every 72 hrs for 14 days

Group VII: received 100 mg/kg peros of silymarin once daily and 1ml/kg body weight of 30 % CCl₄ in liquid paraffin for every 72 hrs for 14 days.

5.0 DISCUSSION

The acute oral toxicity test of MSEDMD did not produce any negative behavioral changes such as etching, depression, weakness, food and water refusal, salivation, restlessness, excitement, respiratory distress and coma were not observed and also there was no lethal effect observed after a single oral administration of 5000mg/kg of MSEDMD for the 14 days of the experiment. According to Loomis and Hayes classification [16], a test substance administered orally and having an LD₅₀ within the range of 5000-15000 mg/kg is considered as practically non-toxic. The main objective of toxicity testing is basically to classify substances according to their toxicity profile or potency, this is aim to protect public health by regulating exposure to potentially dangerous substances [17]. Therefore, estimated LD₅₀ of methanol stem bark extract of *Diospyros mespiliformis* is above 5000mg/kg.

Liver cell injury caused by various toxicants such as carbon tetrachloride and paracetamol is well study. In the present work, CCl₄-treated rats showed significant elevated AST, ALT, ALP, GGT, total and direct bilirubin and a remarkable decrease in total protein and albumin levels after CCl₄ administration as compared with that of normal as well as the extract and silymarin treated groups. Elevated levels of serum AST and ALT are due to alteration or increase in the permeability of the hepatocyte membrane and increased synthesis or decreased catabolism of amino transferases [18]. The remarkable decrease in total protein and albumin level implies alteration of the liver synthetic function. One of the major functions of liver is protein synthesis; albumin is a major part of the protein made specifically by the liver [19]. Liver damage causes disruption and disassociation of polyribosomes on endoplasmic reticulum and thereby reducing the synthesis of protein [20]. Decreased total protein level including albumin levels are due to defective protein biosynthesis arising from hepatocellular injury due to the CCl₄ intoxication [21]. These results are in agreement with the findings of Hassan *et al.* [22], Alhassan *et al.* [23] and Abbas *et al.* [18]; who reported that the serum levels of ALT, AST, ALP, GGT, total and direct bilirubin were elevated and decreased in total protein and albumin levels in CCl₄-treated group in comparison with the normal control group. Therefore, the elevated serum level of AST, ALT and ALP in CCl₄ treated animals indicated cellular breakage and loss of functional integrity of cell membranes and increased biliary pressure in the liver [24]. GGT, a membrane bound enzyme is a well-known indicator of cell and tissue damage by toxic substances. Bilirubin is a useful index of the excretory function of the liver. Elevated level of serum conjugated bilirubin implies regurgitation of bilirubin glucuronides from hepatocytes back into plasma, usually because of intrahepatic or extrahepatic obstruction to bile outflow and cholestasis. It may also be an indication of erythrocytes degradation caused due to liver injury (Holt, 2008). The significant elevation in serum total and direct bilirubin levels in CCl₄ treated group could be due to the regurgitation of bilirubin glucuronides from hepatocytes back into plasma, usually because of intrahepatic or extrahepatic obstruction to bile outflow [18].

Being a potent hepatotoxin, CCl₄ is the most extensively used chemical agent for investigation of hepatoprotective activity on various experimental animal model [24]. The hepatotoxicity induced by CCl₄ is due to its metabolite generated during its metabolism. The metabolism of CCl₄ begins with the formation of trichloromethyl and proxyl chloromethyl free radicals via the activity of oxygenase system of cytochrome P₄₅₀ in endoplasmic reticulum, which in turn, cause lipid peroxidation of the cellular membrane leading to the necrosis of hepatocyte. AST, ALT and ALP are the serum hepatobiliary enzymes present normally in the liver in high concentrations [19]. Upon hepatic dysfunction or damage these enzymes will be leaked into the circulation; raising serum concentration of these enzymes. Assessment of these liver functions indices revealed the liver damage was occurred in CCl₄ treated group, which indicate cellular leakage, loss of functional integrity of the hepatocytes and increased in biliary pressure in the liver [25]. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects [20].

Reduction in the levels of serum/liver homogenate AST, ALT and GGT after treatment with the MSEDMD is an indication of regeneration process of hepatocytes. Reduction in ALP level with decreases in the raised bilirubin levels suggests the stability of biliary function [26]. The serum total protein and albumin levels were declined in CCl₄ treated group. Treatment with the MSEDMD restored serum total protein and albumin levels towards their control values. The increase in protein and albumin levels is an indication of stabilization of endoplasmic reticulum leading to protein synthesis [25].

Administration of MSEDMD at different doses support the hepatoprotective role of plant extracts in present study. The obtained results indicated a high degree of protection at 200 mg/kg, after treatment with the MSEDMD. The ability of the MSEDMD to significantly protect the level of serum total and direct bilirubin may

also suggest the potential of the extract in clearing the level of bilirubin in the serum when it is elevated. The extract also protects decrease in protein and albumin levels are an indication of stabilization of endoplasmic reticulum leading to protein synthesis [26]. The present study reveals the effect of deferent doses of treatment of MSEDm had been effective in offering protection, which is comparable to Silymarin a known hepatoprotective agent. Silymarin has both hepatoprotective and regenerative actions. Silymarin metabolically stimulates hepatic cells and activates the RNA synthesis of ribosome to stimulate protein formation [26]. The hepatoprotective effect of the extract overall at 200mg/kg was comparable with that of silymarin. These results suggest that the stem bark extract of *Diospyros mespiliformis* possibly protect the structural integrity of the cell membrane of hepatocytes via a mechanism similar to that of silymarin.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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