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Hepatoprotective Effects of Ethanol Extract of *Caesalpiniabonduc* against Carbon Tetrachloride Induced Hepatotoxicity in Albino Rats.

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ABSTRACT: The present study was carried out to evaluate the acute toxicity, hepatoprotective and in-*vivo* antioxidant activities of ethanolic extract of *Caesalpinia bonduc* leaf on carbon tetrachloride (CCl_4) induced liver damage using Swiss albino rats. The ethanolic extract of the plant of *C. bonduc* were suspended in 5 % tragacanth and then administered orally at doses of 250 and 500 mg/kg body weight for fourteen days before intraperitoneally injection of Carbon tetrachloride (CCl_4) at dose of 2 mL/kg body weight. The plant extracts at 250 and 500mg/kg b.wt showed a remarkable hepatoprotective and *invivo* antioxidant activities against carbon tetrachloride CCl_4 – induced hepatotoxity judged from the serum marker enzymes .The CCl_4 induced significant increase in aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phophatase (ALP), total bilirubin, and malondyaldehyde (MDA) with a reduction of total protein, catalase, and glutathione peroxidase. Treatment of rats with different doses of plant extract (250 and 500 mg/kg b.wt.) significantly ($P< 0.001$) altered serum maker enzymes and antioxidant levels to near normal levels. The study suggests that *C. Bonduc* specifically chloroform and ethyl acetate fraction may be good sources of natural antioxidant and hepatoprotective substance. © JASEM

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KEYWORDS:*Ceasalpiniabonduc*, hepatoprotective, intraperitoneally , antioxidant

Introduction

Liver is one the most complex organ in the body and it plays a key role in the metabolism, secretion, detoxification and excretion of xenobiotics(Alcamo 2004; Rowen et al., 2009).The main causes of liver injury are viral infection, xenobiotics, toxic chemicals, excessive drug therapy, environmental pollutants,chronic alcohol ingestion and chemotherapeutic drugs (King and Perry,2001; Sharma, et al., 1991; Subramonium and Pushpangadan 1991). Due to inadequacy of liver protective agents, researchers and traditional medicine practitioners have focused on herbal based remedies for various liver disorders. There are no safe hepatoprotective drug available for the treatment of liver disorders (Karan et al., 1999; Chaterrjee, 2000). Carbon tetrachloride (CCl4)-induced hepatotoxicity model is widely used for the study of hepatoprotective effects of drugs and plant extracts (Rubinstein, 1962; Suja et al., 2002).

C*aesalpinia bonduc* (Linn.) Roxb popularly called fever nut belonging to Caesalpiniaceae family is used to treat fevers and roasted seeds are used to treat diabetes (Chevallier, 1996). It is a prickly and extensive climber, very thorny shrubs, branches finely grew-downy, armed with hooked and straight hand yellow brickles. Vine stem, diameter up to 5cm recorded, usually grows as a vine but also flowers and fruits as a shrub. The leaves are with large, leafy branched, basal appendanges, 30-60cm long. The plant grows all over tropical part of the world and the whole plant of *Caesalpinia bonduc* contain all major chemical constituents such as alkaloid, steroidal saponin, fatty acid, hydrocarbon, phytosterols, isoflavones, amino acids and phenolics.

Patil *et al*. reported antidiabetic activity of bark and root of *Caesalpinia bonduc (*Patil *et al*., 2011). The seed extract were screened for adaptogenic activity using cold stree model and surium endurance model. It was also found that extracts proved efficient in controlling the hyperlipidaemic condition due to stress (Kannur *et al*., 2006). It was found that the extract showed significant anthelmintic activity (Wadkar *et al*., 2010).However, literature review reveals that *Caesalpiniabonduc* has not been scientifically investigated for its hepatoprotective properties. Therefore the plant is tested for its potential hepatoprotective in experimental animal models.

MATERIALS AND METHODS

*Collection and identification of plant material***:** Fresh

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leaves of *Caesalpiniabonduc* were collected in June 2011 from Igueben in Igueben Local Government Area of Edo State. It was identified and authenticated by Ugbogu O.A. and Shasanya O.S. of the Forest Research Institute of Nigeria (FRIN), Ibadan. A Specimen number 109493 was given and the plant specimen was deposited at the FRIN Herbarium.

*Processing & Extraction of crude powdered sample***:** The fresh leaves were air dried and the dried material was grounded into powder using an electric blender (pyeUnicam, Cambridge, England). The powdered plant material was subjected to cold extraction by soaking 800g of dry powder in 5L of distilled ethanol for three days (72hours) at room temperature with occasional shaking. The ethanol extract was filtered using a Buchner funnel and Whatman No.1 filter paper.Dried ethanol extracts were obtained after removing the solvent by evaporation under reduced pressure using Rotary evaporator.The extract was stored in an air-tight container and kept in the refrigerator at 4°C until use

*Animals***:** Swiss albino mice weighing 25-30 g and Wistar albino rats weighing 200-250 g were procured from the Animal House, Department of Pharmacology, Faculty of Pharmacy, University of Ibadan. Food and water were provided *ad libitum.* Animals were exposed to controlled environmental of temperature (28± 2°C), relative humidity (50± 5%) and 12 hour light or dark. The handling procedures were conducted in accordance with the Faculty of Pharmacy, University of Benin Ethical committee on experimental animals. The animals were also allowed two weeks under these conditions to acclimatize before the commencement of the experiments.

Preparation of the hepatotoxin: Carbon tetrachloride (CCl_4) was given with olive oil (1:1 v/v) at dose of 2 mL/kg body weight intraperitoneally.

*Hepatoprotective Activity***:** Rats were divided into five groups, each group consisting of six animals.

Group I: (Control) received 5 % tragacanth (1mL/kg b.w. orally) daily for 14 days and olive oil (1mL/kg b.wt. ip) on day 14.

Group II: (CCl_4 induced) received 5% tragacanth (1mL/kg b.wt. orally) daily for 14 days and CCl_4: olive oil (1mL/kg b.wt. ip) on day 14. **Group III:** (Standard) received silymarin (50 mg/kg b.w orally) for 14 days and CCl_4: olive oil (1ml/kg b.wt. ip) on day 14. **Group IV:**received ethanol leaf extract of *C. bonduc*(250 mg/kg orally) for 14 days CCl_4 : olive oil (1ml/kg b.wt. ip) on day 14.

Group V: received ethanol leaf extract of *C. bonduc*(500 mg/kg b.w orally) for 14 days and CCl_4 :olive oil (1ml/kg b.wt. ip) on day 14.

After 24 hours of the last treatment, all the animals were anaesthesized with chloroform and blood was collected via cardiac puncture. The blood was put into a plain sample tubes and sera was obtain from it by allowing it to stand for 2hrs at room temperature before centrifuging for at 2000rpm to separate the serum. The serum was used for estimation of various biochemical parameters.

*Assessment of liver function***:** *Serum Aspartate andAlanineTransferase(AST and ALT)* The activities of these enzymes were estimated by the method of Reitman and Frankel (1957). 0.2 ml of serum was added to 1 ml of phosphate buffer containing substrate, mixed and incubated for 30 min for ALT and 60 min for AST at 37°C, Then 1 ml of dinitrophenylhydrazine was added and incubated for 20 min at room temperature and 10 ml of 0.4% sodium hydroxide was added, mixed well and after five minutes read at 550 nm against sample blank .The value of AST and ALT are calculated from a series of standards curve.

*Alkaline Phosphatase***:** Serum alkaline phosphatase activity was measured following the method of King and Armstrong (1934),usingdisodium phenyl phosphate as substrate.Thecolour developed was read at 510 nm. Activities are expressed as KAU/L.

*Total Bilirubin***:** Serum total bilirubin was estimated following the method of King and Coxon (1950).In brief, 1 ml of serum was mixed with 0.5 ml of diazoreagent, followed by 0.5 ml $(NH_4)_2SO4$ .The volume was made up to 10 ml with 85% ethanol.The contents were mixed well and allowed to stand for 30 min for even distribution of the precipitate.The precipitate was filtered and measured using colorimeter

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Protein Content: Protein content of serum was determined by biuret method described Kingsley (1942). To 0.02 ml of sample, 1ml of solution 1 was added and mixed gently. The mixture was incubated for 30mins at 30°C, The absorbance of the sample and of the standard was read at 546 nm against the blank.

Determination of malondialdehyde (MDA) concentration.: The level of malondialdehyde was determined by modified procedure described by Guidet and Shah (Guidet and Shah, 1989).Briefly, 150µl of serum sample was mixed in a test tube with 1 ml of 17.5 %TCA.1ml of 0.6 % TBA was added, and the mixture was mixed well by vortexing, incubated in boiling water bath for 15 min, and then allowed to cool.

1ml of 70 % TCA was added, with mixing, the mixture was left to stand at room temperature for 20 minutes. The mixture was centrifuged at 2000 g for 15 minutes, and 1ml the supernatant was taken out for measuring the absorbance at 532 nm.

The concentration of MDA $= \frac{\text{Absorbance}}{Lx\varepsilon}$ x D

Where; L: Light path (cm) ε: Extinction coefficient (1.56 x $10^5 M^{-1}cm^{-1}$)

D: Dilution factor $= \frac{\text{Volume used in Ref.(mL)}}{0.15} = \frac{1+1+1+0.15}{0.15} = 21$

*Determination of serum glutathione peroxidase (GPx) level***:** Glutathione peroxidase (GPx) was assayed according to the procedure of Rotruck*et al*. (1973). To 0.05ml sodium azide (10 mM), 0.2 mL of Sodium phosphate buffer (0.4 M, pH 7.0) was mixed with vortex mixer. Then, 0.1ml of reduced glutathione (4mM),0.05ml of hydrogen peroxide (2.5 mM H_2O_2) and 0.1ml distilled water were added. The mixture was mixed together with a vortex mixer.0.25 mL of serum were added to the mixture and the mixturewas incubated for 90 seconds.The reaction was terminated with 0.25 mL of 10 % TCA.The tubes were centrifuged, and then 1 mL of supernatant was taken. Then 0.15 mL of 0.4M-Phosphate buffer was added to each tube. 0.5ml of 0.04% DTNB reagent was added to each tube. The colour that was developed was read at 412 nm.

Enzyme activity was expressed in terms of µM/min in 1L of plasma.

Enzyme activity

$$= \frac{\text{Change in absorbance/ min x Vt}}{\varepsilon \text{ x Vs}} \text{ x } 1000$$

Where; V**t:** Total volume.

Vs: Volume of sample. ε: Extinction coefficient (0.04 x 10^3 $L.cm^{-1}.\mu mol^{-1}$

Catalase (CAT): The method of Luck (1974) was adopted to measure the activity of catalase. The enzyme extract (0.1ml) was added to the reaction mixture containing 3ml of H_2O and 0.01M phosphate buffer (pH 7.0) and the OD change was measured at 240nm, the time taken for decrease in the absorbance from 0.45 to 0.4 is noted as ΔT.The activity of the enzyme was expressed in the terms of µmole of H_2O_2 consumed /min/mg protein

*Statistical analysis***:** Statistical analysis of the results was done by one way analysis of variance (ANOVA) using SPSS software followed by Dunnet's comparison test for significance. Significance was set at ($p<0.05$). Results are presented as Mean± S.E

RESULTS AND DISCUSSION

The results of hepatoprotective effects of ethanol extract of Caesalpinia bonduc on rats induced with single dose of CCl_4 (1mL/kg b.w. ip) are as shown above (Table 1 and 2). The results indicate that rats administered with 1 ml /kg b.wt CCl4 recorded severe hepatic damage (group 2) when compared to control (group 1) and rats pretreated with Caesalpinia bonduc ethanol extract (group 4 and 5) respectively. This was evidenced by a marked increase in the levels of serum liver enzymes, (AST, ALT and ALP) in rats treated with CCl4 alone.

Antioxidant enzymes

The oxidative stress was assessed by measuring the levels of lipid peroxidation product, MDA, ,the antioxidant defense enzymes (GPx and CAT). Administration of CCl_4 significant ($p < 0.01$) exhibited elevation of MDA and reduction of antioxidant enzymes(GPx, and CAT) when compared to the control Group I rats . Whereas, Administration of *C. bonduc* extract at the doses of 250 and 500 mg/kg b.wt significantly ($P < 0.001$) increase the level of these serum enzymes, and thus countering the effect of CCl_4 induced hepatotoxicity in a dose independent manner. Dose of 500 mg/kg b.wt has been shown to be more protective than 250 mg/kg b.wt.

Table 1: The effect of *C. bonduc* on biochemical parameters in CCl_4 induced hepatotoxicity in rats.

Treament	Dose (mg/kg b.wt)	AST U/L	ALT U/L	ALP IU/L	Total Protein g/dL	Total Bilirubin mg/dL
Control		37.37 ± 5.35	22.18 ± 5.42	63.75±11.68	8.97 ± 0.53	0.207 ± 0.05
CCl_4 induced	2ml/kg	192.13±14.67[a]	163.78 ± 11.5[a]	115.26±7.44[a]	5.42 ±0.39[a]	0.428 ± 0.09[a]
Silymarin	50	137.60±29.20[a,b]	140.06 ± 1.47a	76.44±15.51[b]	9.21 ±0.51[b]	0.243 ± 0.10[b]
***C.bonduc* Leaf extract**	250	64.62±9.540[b]	120.41±15.67[a,b]	73.54±10.18[b]	9.35 ±1.06[b]	0.265 ± 0.05
	500	48.79±6.73[b]	101.52±17.59[a,b]	60.86±5.06[b]	9.16 ±0.39[b]	0.25 ± 0.09

Data represents mean ± standard error of mean (SEM).

n=6

[a]$P < 0.001$vs control

[b]P < 0.001vsCCl_4

Data were analyzed by using one way ANOVA followed by Tukey multiple comparison test.

Table 2: The effect of *C. bonduc* on antioxidant level in CCl_4 induced hepatotoxicity in rats.

Treatment	Dose (mg/kg b.wt)	MDA	Catalase	GPx
Control		14.63 ± 4.32	1.40 ± 3.37	68.1 ± 3.98
CCl4	2ml/kg	46.64 ± 2.86[a]	0.18± 0.029[a]	33.8 ± 3.92[a]
Silymarin	50	15.84 ±1.62[b]	1.63 ± 0.14 [b]	56.15±2.84[a,b]
***C. bonduc*Leaf extract**	250	17.98 ± 0.87[b]	1.67 ± 0.21[b]	66.3 ± 2.52[b]
***C. bonduc*Leaf extract**	500	16.89 ± 1.74[b]	1.17 ± 0.43[b]	62.45± 2.97[b]

n = 6, values are expressed as mean + SEM
[a]P< 001 Vs control
[b]P< 0.001 Vs CCl_4
Data were analyzed by one way ANOVA followed by Tukey multiple camparison test.
MDA:Malondialdehyde
CAT:Catalase
GPX: Glutathione peroxidase

It is well established that CCl_4 is metabolized in the liver by cytochrome p450 to the highly reactive trichloromethyl radical (Johnson and Kroenmy, 1998).

These activated radicals bind covalently to the macromolecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. These result in changes of structures of the endoplasmic reticulum and other membrane, loss of metabolic enzyme activation, reduction of protein synthesis leading to liver injury (Recknagel and Glende, 1973; Azri*et al*.,1992). This is evidenced by an elevation in the serum marker enzymes namely AST, ALT, ALP, total bilirubin and decrease in biochemical parameter such as total protein. Membrane damage releases the enzyme into circulation. ALT catalyzes the convertion of alanine and glutamate to alpha -ketoglutarate and pyruvate and is released during membrane damage. AST is more specific to the liver and is thus a better parameter for detecting liver injury (Palanival*et al.*, 2008). It catalyzes the conversion of aspartate and glutamate to alpha-ketoglutarate and oxalo-acetate. Serum ALP, total bilirubin and total protein levels on the other hand are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis in presence of increasing biliary pressure (Muriel and Garcipiana, 1992). ALP catalyzes the conversion p-nitrophenylphosphate (p-NPP) to p-nitrophenol.

Pretreatment with CCl_4 caused a significant (P <0.001) elevation of enzymes level such as AST, ALT, ALP, catalase ,MDA and biochemical parameter such as total bilirubin with decrease in total protein, , catalase and glutathione peroxidase when compared to control. There was a significant (P < 0.001) restoration of these enzyme levels on pretreatment with extracts in a dose independent manner and also by Silymarin at dose of 50 mg/kg b.wt.

The restoration by the extract may be due to the prevention of the leakage of intracelluller enzymes by its membrane stabilizing activity. This is in consonance with the commonly accepted view that serum level of transaminase return to normal with the healing.

The increase in MDA level in the group induced by CCl_4 suggests enhanced lipid peroxidation leading to tissue change and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. pretreament with *C. bonduc* significantly reverse these changes.

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues and the highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide radicals. *C. bonduc* extract enhanced significantly (P < 0.001) the protective enzyme, catalase and glutathione peroxidase when compared with CCl_4 induced group. CCl_4 further depleted the levels of these enzymes whereas,*C. bonduc* extract and Silymarin pretreatment reversed this effect (P < 0.001). Flavonoids, tannins, and microelements have been reported in *caesalpiniabonduc* and act as antioxidants and exert their antioxidant activity by scavenging the lipid peroxidase (Ubhenin et al 2013and Yutung*et al.*, 1990).

Therefore, the present work provides conclusive evidence for the hepatoprotective effect of *C. bonduc* aganist carbon tretrachloride-induced hepatotoxicity. The plausible mechanism of the hepatoprotective action of *C. bonduc* might be at least partly due to its antioxidant effects. Research is in progress to isolate, characterize and elucidate the structure of bioactive compound responsible for hepatoprotective effect of the plant.

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