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Antioxidant and Hepatoprotective Activity of Methanolic Extract of *Cassia sieberiana* Leaves in Carbon Tetrachloride-induced Hepatotoxicity in Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author GKM designed the study and wrote the protocol. Author SOO managed the animals, collected all data, performed the statistical analysis, and wrote the first draft of the manuscript. Author MIE did the literature search and also wrote part of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study was aimed at evaluating the hepatoprotective and antioxidant activity of the methanolic extract of *C. sieberiana* leaves.
Study Design: Hepatoprotective, *in vitro* and *in vivo* antioxidant activity.
Place and Duration of Study: Department of Veterinary Physiology, Pharmacology, Biochemistry and Animal Health and Production, College of Veterinary Medicine, Micheal Okpara University of Agriculture, Umudike, Umuahia, Abia State, June 2014.
Methodology: *Cassia sieberiana* leaves were extracted with 80% methanol for 48 h using cold maceration method. The hepatoprotective activity of *C. sieberiana* extract (100, 200 and 400 mg/kg) and silymarin (100 mg/kg) was evaluated using carbon tetrachloride (CCl_4) induced

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hepatotoxicity in albino rats. The antioxidant activity was determined using both *in vitro* (2, 2-diphenyl-1-picrylhydrazyl photometric assay) and *in vivo* (malondialdehyde and catalase level assay) models.

Results: The extraction of the *C. sieberiana* leaves for 48 h using cold maceration method yielded 9.5% w/w of dark green and pasty extract. The phytochemical spot test of the CSE showed the presence of saponins, alkaloids, flavonoids, terpene/sterol, glycosides and tannins. The extract (100, 200 and 400 mg/kg) and silymarin (100 mg/kg) produced a significant ($p < 0.05$) dose-dependent increase in alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphate levels in serum of treated rats, when compared with the negative control group. The extract (25 – 400 µg/ml concentration) produced a concentration-dependent increase in antioxidant activity in 2, 2-diphenyl-1-picrylhydrazyl (DPPH) photometric assay. The IC_{50} of the extract in DPPH photometric assay was 50 µg/ml concentrations. The extract and silymarin showed a significant ($p<0.05$) dose-dependent increase in catalase level in treated rats, when compared with the negative control group. Also, the extract (400 mg/kg) and silymarin (100 mg/kg) produced a significant ($p<0.05$) decrease in malondialdehyde level in treated rats, when compared with the negative control group.

Conclusion: This study demonstrates that *C. sieberiana* leaves have a potent hepatoprotective and antioxidant activities. It justified the use of *C. sieberiana* leaves in the treatment of liver injury related disease in folk medicine.

Keywords: Cassia sieberiana; carbon tetrachloride; catalase; malondialdehyde; hepatoprotective activity; silymarin.

1. INTRODUCTION

The liver plays important metabolic, detoxification, and secretory roles in the body. Liver disease is associated with distortion of these metabolic functions [1]. Thus, liver diseases remain one of the serious public health problems and its disorders are numerous with no effective treatment. Every year about 20,000 deaths are recorded due to liver disorders worldwide [2]. Despite considerable progress in the treatment of liver diseases by oral hepatoprotective agents, search for newer drugs continues because the existing synthetic drugs have several limitations [3-5]. So, the search for new medicines of plant origin is still ongoing due to the fact that liver performs many vital functions in the human body and damage of liver causes unbearable problems [6,7].

Cassia sieberiana DC belongs to the family Fabaceae. It is called “drumstick” in English and its indigenous names in Nigeria include “marga” in Hausa, “margaje” in Fulani, “kiskatigrai” in Kanuri and “ifo” or “aridantooro.” in Yoruba. It grows up to 10 – 20 m high with drooping branches and bright yellow flowers [8]. The fruit (pods) is cylindrical, smooth, 40 - 60 cm long and about 1.5 cm in diameter; indehiscent and dark brown. The plant is an open savannah tree found in drier areas of forest and thickets. The plant is widely distributed in the southern sahel and sudan savanna from Senegal to Cameroun, as far as Sudan and the Republic of Congo [9,10]. It is also found in most parts of Nigeria [8]. Various parts of the plants are used for the treatment of fever, jaundice, stomach ache, gonorrhea, piles and ulcers in traditional medicine [8]. The extract from various parts of the plant have been shown to possess some pharmacological activities like; antioxidant, anti-ulcer, antidiabetic, laxative, wound healing and anti malarial [11-13]. The present study was aimed at investigating the hepatoprotective effect of methanolic extract of *Cassia sieberiana* (CSE) leaves in carbon tetrachloride-induced hepatotoxicity in rats.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant Material

Fresh leaves of *Cassia sieberiana* were collected in March, 2013 from Nsukka Enugu state, Nigeria and identified by Mr A. O. Ozioko, a Taxonomist at Bioresource Development and Conservation Programme (BDCP), Enugu state, Nigeria. A voucher specimen catalogued MOUAU/VPP/2013/11 was deposited in the departmental herbarium for reference purposes.

2.2 Preparation of the Plant Extract

Dried and pulverized leaves of *Cassia sieberiana* (200 g) were extracted by cold maceration

method for 48 hours at room temperature using 80% methanol in a Winchester bottle. Filteration was done using Whatmann No. 1 filter papers and the filtrate was concentrated in a hot air oven at 40ºC to give 19 g residue. The extract was stored in a refrigerator at 4℃ throughout the duration of this study.

2.3 Experimental Animals

Thirty five male Albino Wistar rats (120-140 g) obtained from the laboratory animal unit of the Department Of Veterinary Physiology, Pharmacology, Biochemistry, Animal health and production; Michael Okpara University Agriculture, Umudike, were used for this study. They were housed in aluminium cage at 5 rats per cage and were fed *ad libitum* with standard commercial pelleted feed (Vital feed®, Nigeria) with free access to clean drinking water. They were kept at normal environmental temperature and natural light/darkness daily cycle. They were maintained in accordance with the recommendations of the *Guide for the care and use of laboratory animals* [14]. They were allowed two weeks to acclimatize before the commencement of the experiment. The animal experiment protocol was approved by the Michael Okpara University of Agriculture animal ethics committee.

2.4 Experimental Procedures

2.4.1 Phytochemical spot test

The CSE was tested for the presence of alkaloids, flavonoids, tannins, glycosides, saponins, terpenes/sterols, carbohydrates, and starch using the standard procedures [15].

2.4.2 Oral acute toxicity study

The oral acute toxicity test of methanolic extract of *Cassia sieberiana* was determined according to the OECD guideline no. 425 (acute oral toxicity – Up-and-Down-procedure). Briefly, two groups of five rats each were dosed orally with 1000 and 2000 mg/kg of CSE, respectively and were observed for 48 h for signs of toxicity and death [16].

2.4.3 Effects of CSE on carbon tetrachloride (CCl_4)-induced hepatotoxicity rats

Twenty five male albino rats were randomly divided into five groups (I-V) of 5 rats each. Group I (negative control) received 10 ml/kg of distilled water, group II (positive control) received silymarin 100 mg/kg, while groups III – V received CSE at the doses of 100, 200 and 400 mg/kg, respectively. All the animals were dosed orally once daily for 5 consecutive days. Twenty four hours after the last treatment (day 6), 20% CCl_4 in liquid paraffin was administered intraperitoneally to each rat at the dose of 2 ml/kg. After 24 h of CCl_4 treatment, rats were anaesthetized with chloroform and blood was collected through direct cardiac puncture. Serum was separated by centrifugation at 2000 rpm for 10 min and aspirated into sterile plain sample bottles. The rats were later euthanized by cervical dislocation and the liver was dissected out immediately for preparation of liver homogenate.

2.4.4 Serum biochemical analysis

Assay for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were done by the method described by Reitman and Frankel [17] and serum level of alkaline phosphatase was measured by the method of Klein et al. [18] using Randox test kits. The serum total protein and albumin content was assayed using standard diagnostic kit (Randox kit, Randox Laboratories, U.K.).

2.4.5 Determination of the lipid peroxidation (LPO) using liver homogenate

The level of thiobarbituric acid reactive substance (TBARS), a commonly used marker of lipid peroxidation and malondialdehyde (MDA) production was measured in liver homogenate by modified method as described by Draper and Hadley [19]. The liver homogenate (50 μL) was deproteinized by adding 1 mL of 14% trichloroacetic acid and 1 mL of 0.6% thiobarbituric acid. The mixture was heated in a water bath at 100ºC for 30 min to complete the reaction and then cooled on ice for 5 min. After centrifugation at 2000 rpm for 10 min, the absorbance of the colored product (TBARS) was measured at 535 nm with a UV spectrophotometer. The concentration of TBARS was calculated using the molar extinction coefficient of malondialdehyde (1.56 × 105 mol/L/cm) using the formula, $A = \Sigma CL$, where A = absorbance, Σ = molar coefficient, C = concentration, and L = path length. The results were expressed in nmol/mg of protein.

2.4.6 Estimation of the effects of CSE on catalase activity in serum

The catalase activity in serum was determined using the modified method as described by Aebi [20] and Atawodi [21]. Briefly, the method is as follows: serum (10 μL) was added to test tube containing 2.80 mL of 50 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1 mL of fresh 30 mM hydrogen peroxide and the decomposition rate of hydrogen peroxide was measured at 240 nm for 5 min on a spectrophotometer. A molar extinction coefficient of 0.041 $mM^{-1}cm^{-1}$ was used to calculate catalase activity. The results were expressed in µmol/mg of protein.

2.4.7 Determination of *in vitro* antioxidant activities of CSE using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) photometric assay

The free radical scavenging activity of the extract was analyzed by the DPPH Assay [22] using spectrophotometer. The test extract (2 ml) at different concentrations (25, 50, 100, 200 and 400 µg/ml) each were mixed with 0.5 mM DPPH (in 1 ml of methanol) in a cuvette. The absorbance at 517 nm was taken after 30 minutes of incubation in the dark at room temperature. The concentrations were prepared in triplicates and the percentage antioxidant activity calculated as follows.

% antioxidant activity (AA) = 100-[{(absorbance of sample — absorbance of blank) ×100}/ absorbance of control].

One mililiter of methanol plus 2.0 ml of the extract was used as the blank while 1.0 ml of the 0.5 mM DPPH solution plus 2.0 ml of methanol was used as the negative control. Ascorbic acid (vitamin C) was used as reference standard [23].

2.5 Statistical Analysis

Data obtained were presented as mean ± SEM and analyzed using one-way analysis of variance (ANOVA). The variant mean were separated by least significant difference (LSD) of the different groups. Significance was accepted at the level of $p<0.05$.

3. RESULTS

3.1 Preparation of the Extract

The weight of CSE recovered was 19 g, and the percentage yield was 9.5% w/w dry matter. The extract was dark green in color and pasty in consistency.

3.2 Phytochemical Spot Test

The phytochemical spot test showed that CSE contained saponins, tannins, terpenes/sterols, glycosides, alkaloids and flavonoids.

3.3 Oral Acute Toxicity Test

The oral administration of the CSE was well tolerated in all doses (1000–2000 mg/kg) as no death or clinical signs of toxicity were observed throughout the period of observation.

3.4 Effects of *CSE* on the Liver Function Markers of Carbon Tetrachloride Treated Rats

The effects of CSE on the serum ALT, AST and ALP activities of carbon tetrachloride treated rats is presented in Table 1. The extract (100, 200 and 400 mg/kg) and silymarin (100 mg/kg) significantly ($p<0.05$) reduced the activities of ALT, AST and ALP in the serum of treated rats in a dose-dependent manner, when compared with the negative control rats. Also, the extract (100, 200 and 400 mg/kg) and silymarin (100 mg/kg) produced no significant ($p>0.05$) difference on the serum albumin and total protein levels in the treated rats when compared with the negative control rats.

3.5 *In vivo* Antioxidant Effect of CSE on Carbon Tetrachloride Treated Rats

The *In vivo* antioxidant effect of CSE on carbon tetrachloride treated rats is presented on Table 2. All doses of the extract (100, 200 and 400 mg/kg) and silymarin (100 mg/kg) produced a dose-dependent reduction in the levels of malondialdehyde in liver homogenate of the treated rats. The malondialdehyde levels of the groups treated with CSE (400 mg/kg) and Silymarin (100 mg/kg) was significantly ($p<0.05$) lower when compared with the negative control group. Also, the extract (100, 200 and 400 mg/kg) and silymarin (100 mg/kg) caused a significant ($p<0.05$) dose-dependent increase in

the serum catalase level in treated rats, when compared with the negative control rats.

3.6 *In vitro* Antioxidant Activity of CSE Using DPPH Photometric Assay

The in vitro antioxidant activity of CSE using DPPH photometric assay is presented in Fig. 1. The extract produced concentration-dependent increase in the DPPH radical scavenging activity. The extract at 200 and 400 µg/ml concentration produced an effect that is comparable to the effect of ascorbic acid. The optimum effect of the extract was produced at 400 µg/ml concentration.

Table 1. The effects of *CSE* on the liver function markers of carbon tetrachloride treated rats

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	Total protein g/dl	Albumin g/dl
Distilled water 10 ml/kg	415.00±5.00	250.50±3.50	129.51±1.76	6.90±0.02	3.41±0.05
Silymarin 100 mg/kg	224.50±10.50*	135.00±8.00*	64.01±9.99*	4.47±1.59	2.17±0.79
CSE 100 mg/kg	347.67±4.97*	203.33±6.01*	64.53±6.02*	4.85±0.08	2.38±0.84
CSE 200 mg/kg	316.67±2.73*	175.00±2.89*	62.60±7.06*	4.51±0.22	3.05±0.09
CSE 400 mg/kg	278.00±3.79*	159.67±1.76*	60.17±5.46*	4.76±0.18	2.34±0.28

**P<0.05 when compared to the negative control*

Table 2. *In vivo* antioxidant effect of CSE on carbon tetrachloride treated rats

Group	MDA (nmol/mg protein)	CATALASE (µmol/mg protein)
Distilled water 10 ml/kg	0.60±0.14	1.07±0.29
Silymarin 100 mg/kg	0.25±0.02*	3.49±0.19*
CSE 100 mg/kg	0.47±0.08	1.23±0.18
CSE 200 mg/kg	0.39±0.02	2.20±0.54*
CSE 400 mg/kg	0.27±0.10*	3.38±0.10*

**P < 0.05 when compared to the negative control*

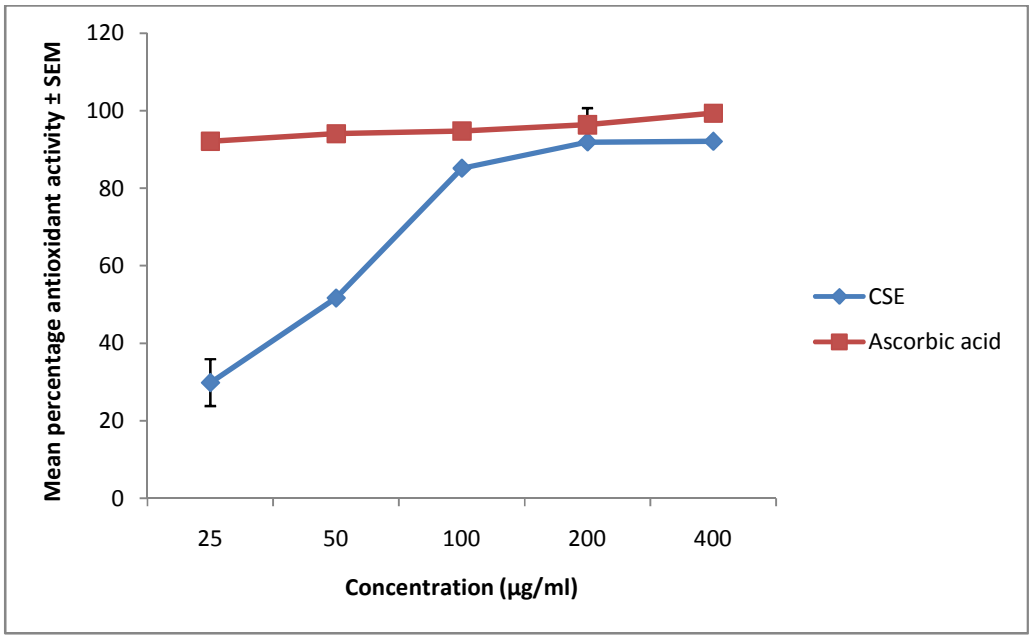

Fig. 1. The *in vitro* antioxidant activity of CSE using DPPH photometric assay

4. DISCUSSION

The hepatoprotective effect of the methanolic extract of *C. sieberiana* was studied using carbon tetrachloride-induced hepatoxicity. The antioxidant activity was studied using both *in vivo* (MDA and catalase) and *in vitro* (DPPH radical scavenging) models.

The extraction of the *C. sieberiana* leaves for 48 h using cold maceration method yielded 9.5% w/w of dark green and pasty extract. The phytochemical spot test of the CSE showed the presence of saponins, alkaloids, flavonoids, terpene/sterol, glycosides and tannins. This is in consonant with the result of Toma et al. [8]. The hepatoprotective action of the extract may be due to the presence of one or more of the phytochemical constituents [24].

Oral acute toxicity test showed that the extract was well tolerated as no mortality or any clinical signs were observed even at the highest administered dose of 2000 mg/kg used in this study. Similar conclusion has been drawn by other workers [16,25,26].

Hepatotoxicity was induced by single intraperitoneal injection of 20% CCl_4 in liquid paraffin at the dose of 2 ml/kg. Carbon tetrachloride-induced liver toxicity is a commonly used model for the screening of hepatoprotective drugs [27]. The hepatotoxic effect of CCl_4 is mediated by its active metabolite, trichloromethyl radical [28,29]. The trichloromethyl radical covalently bind to macromolecules and induce peroxidative degradation of the membrane lipids of endoplasmic reticulum rich in poly unsaturated fatty acids [30]. The lipid peroxidation gives rise to the generation of malondialdehyde (MDA), which causes damage to biomembranes. The membrane damage leads to the elevation in levels of the cytosolic enzymes in serum [31]. In this work, the induction of hepatotoxicity was evidenced by the increased serum levels of AST, ALT, ALP and increased level of MDA in the liver homogenate of the negative control group of rats.

The pretreatment of the rats with CSE (100, 200 and 400 mg/kg) and silymarin (100 mg/kg) before the induction of hepatoxicity caused a significant ($p<0.05$) reduction in the serum level of AST, ALT and ALP in the treated rats when compared with the negative control group. This is an indication that CSE may caused plasma membrane stabilization as well as repair of hepatic tissue damage caused by CCl_4. This is in agreement with the report of other previous workers; that the serum level of transferase and phosphatase return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes [30,32,33].

There was no significant difference ($P>0.05$) in the serum level of total protein and albumin of both the treated and control group and this may be attributed to the duration of the study [34]. Serum albumin levels are important in determining the chronicity and severity of the liver disease. In acute liver disease the serum albumin levels are often normal or within the reference range [35].

The *in vitro* free radical scavenging activity of CSE was determined using DPPH photometric assay. The use of DPPH photometric assay model for screening of free radical scavenging activities of chemicals is well documented [33,36]. The CSE produced a concentration-dependent increase in the free radical scavenging activity. The scavenging of DPPH radical is related to the inhibition of lipid peroxidation [37].

The lipid peroxidation was assayed by the measurement of the level of MDA in the liver homogenate. The treatment of the rats with CCl_4 caused an increased level of MDA in the liver homogenate as seen in this study. The pretreatment with silymarin and CSE produced a reduction in the MDA level which suggests that the extract may have inhibited the reductive dehalogenation of CCl_4 to trichoromethyl radical, catalysed by cytochrome P_{450} in the liver cell endoplasmic reticulum. The trichloromethyl radical react rapidly, attacking microsomal lipids leading to its peroxidation and also covalently binds to microsomal lipids and proteins ultimately initiating a site of secondary biochemical processes which are the main cause of CCl_4 hepatotoxicity [30,38].

The reduced catalase level observed in the negative control is due to increased reactive oxygen species (ROS) occasioned by the CCl_4 treatment [31]. The pretreatment of the rats with silymarin (100 mg/kg) and CSE (100, 200 and 400 mg/kg) increased the level of catalase in both the silymarin and CSE treated groups when compared with the negative control group. This may be due to the inhibition of the metabolic processes that would have lead to the generation of trichloromethyl radicals and other ROS from CCl_4 and subsequent exhaustion of the catalase

[31]. Catalase catalyzes the conversion of hydrogen peroxide to water [39].

The results obtained from the antioxidant evaluation suggest that the possible mechanism of the hepatoprotective activity of *C. sieberiana* may be through the antioxidant activity. This is supported by the presence of flavonoids, saponins, tannin and terpenoids, which have been shown to possess antioxidant and hepatoprotective activities [30,40].

5. CONCLUSION

In conclusion the results of this work demonstrate that *C. sieberiana* extract has a potent hepatoprotective action in carbon tetrachloride-induced liver damage in rats. This may be due to its antioxidant and free radical scavenging properties or may be as result of the presence of some phytochemicals. Further investigation is recommended to isolate the active hepatoprotective principle and determine its mechanism of action.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and therefore have been performed in accordance with the ethical standards laid down in the 1964 Declarations of Helsinki and Michael Okpara University of Agriculture, Umudike, Nigeria.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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