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Protective effect of *Croton zambesicus* leaf extract against carbon tetrachloride-induced cardiac toxicity in rats

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ABSTRACT

Background: The leaves of *Croton zambesicus* (CZ) Muell Arg. (Euphorbiaceae) are used as a spice for food and in traditional medicine for the management of several disease conditions, including hypertension and diabetes. The study was carried out to determine the cardioprotective effects of CZ leaf extract and n-butanol fraction on carbon tetrachloride (CCl_4) -induced toxicity. Male albino rats were treated for 5 days with CZ extract, n-butanol fraction, and ascorbic acid. **Materials and Method:** On day 6, CCl_4 was administered subcutaneously for 3 days to rats in Groups II-VII and treated concurrently for 3 days with aqueous leaf extract of CZ, n-butanol fraction, and ascorbic acid. Serum levels of cardiac function markers, creatine-kinase myocardial band (CK-MB) fraction, lactate dehydrogenase (LDH) were evaluated. Antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), oxidized GSH (GPx), and malondialdehyde (MDA) were also determined. **Results:** Aqueous leaf extract and n-butanol fraction significantly ($P < 0.05$) decreased serum concentration of CK-MB and LDH. In addition, there was a significant ($P < 0.001$) increase in activity of antioxidant enzymes (SOD, CAT, GSH, and GPx) with a significant ($P < 0.05$) decrease in the concentration of MDA. **Conclusion:** Results from this study revealed the aqueous leaf extract of CZ attenuated toxic effects of CCl_4 on the heart and thus found to have a protective potential.

Keywords: Antioxidant enzymes, aqueous leaf extract, carbon tetrachloride, cardioprotective, *Croton zambesicus*

INTRODUCTION

Medicinal plants and herbal species have been used in traditional medicine for the prevention and management of various diseases. The majority of the population in African countries resort to alternative therapies from herbal sources because they are easily accessible and affordable.[1,2] Plants are rich in secondary metabolites which have potent antioxidant activity. They decrease oxidative stress and are beneficial in the management of chronic non-communicable diseases.[3] Carbon tetrachloride (CCl_4) is often employed to induce oxidative stress by producing free radicals in the heart, kidney, liver, and brain of experimental animals.[4,5] Administration of CCl_4 induces cardiac toxicity by producing ROS that inhibits endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and oxidized GSH (GPx).[6]

The protective effect of medicinal plants is often produced through increased expression of antioxidants which act on free radicals to prevent their toxic effects on vital cells, tissues, and organs.[7]

Croton zambesicus (CZ) Muell Arg. (Euphorbiaceae) is a medicinal plant widely distributed in Nigeria and West African sub-region. The leaves are used by traditional medicine practitioners in the treatment of several ailments, including wounds, inflammation, epilepsy, hypertension, diabetes, and kidney disease.[8] The leaves and fruits are also used traditionally to make sauces, spices, and flavorings. The *in vitro* antioxidant,[9] hypotensive,[10] anti-diabetic,[11] and nephron-protective activities of CZ have been reported.[12] The aim of the present study was to evaluate the protective effect of the aqueous leaf extract and n-butanol fraction of CZ against CCl_4-induced cardiac toxicity in rats.

MATERIALS AND METHODS

Chemicals

CCl_4, n-butanol, acetylsalicylic acid purchased from (Sigma-Aldrich USA). Assay kit (ELISA) for the creatine-kinase myocardial band (CK-MB), lactate dehydrogenase (LDH), SOD, CAT, GSH, GPx, and malondialdehyde (MDA) Purchased from (Cusabio Technology, USA).

Collection and Identification of Plant Material

Fresh leaves of CZ were collected in Ilorin, Kwara State-Nigeria. Identification and authentication were done by Mr. Ibrahim of Botany Department, University of Ilorin, Nigeria. A voucher specimen was deposited in the herbarium of Pharmacognosy Department, University of Ilorin, Nigeria.

Preparation of Aqueous Leaf Extract

Leaves of CZ were dried in the shade for 2 weeks. A laboratory mill was used to reduce the leaves to smaller particle sizes. The milled-leaf was weighed and macerated with distilled water for 24 h. The aqueous extract obtained was filtered and concentrated over a water bath at 50–55°C.

Partitioning of Aqueous Leaf Extract of CZ

The concentrated extract (20 g) was weighed and dissolved in 200 ml water. The aqueous solution was transferred into a separating funnel containing 200 ml of n-butanol. The funnel was shaken gently, left to stand for a few minutes before collecting the aqueous and n-butanol fractions in separate beakers. This procedure was repeated twice, the aqueous fraction was discarded, and n-butanol fraction was concentrated over a water bath at 50–55°C.

Ethical Approval

Ethics clearance with approval number (UERC/ASN/2018/1110) was obtained from the University of Ilorin's Ethics Review Committee. Experiments were carried out in accordance with the International Animal Care and Use Committee guideline in Nigeria.

Study Design

The dose of aqueous and n-butanol extracts is different and was determined from a pilot study. The n-butanol dose used was the potent dose with activity. Albino rats were divided into groups and treated through the oral route for 5 days as follows: Groups I and II (normal saline, 0.2 ml of 0.9 %); Groups III and IV (CZ aqueous extract 200 and 400 mg/kg); Groups V and VI (n-butanol fraction 20 mg and 40 mg/kg); and Group VII (ascorbic acid 10 mg/kg).[13] In addition, CCl_4 in olive oil (2 ml/kg of 40% subcutaneously) was administered starting from day 6 of the experiment to rats in Groups II-VII and were treated concurrently for 3 days with aqueous leaf extract and n-butanol fraction of CZ. On day 9, rats were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (7.5 mg/kg). Animals were sacrificed, blood collected, and heart removed for biochemical analysis.

Preparation of Serum and Tissue Homogenate

Blood sample was collected through the cardiac puncture, put in plain bottles, and allowed to clot. The serum obtained was stored at −20°C for the determination of serum creatinine MB and lactate dehydrogenase activity. The heart was removed and placed immediately on ice-cold 0.25 M sucrose. A piece of heart tissue was homogenized and centrifuged at 5000 rpm for 10 min at 4–6°C. The supernatant obtained was transferred into Eppendorf tubes and stored on ice.

Determination of Markers of Cardiac Function in Serum

Serum (CK-MB) and LDH activity were determined using the methods described by Young 1990[14] and Lorentz *et al*. 1993,[15] respectively.

CK-MB activity was determined by spectrophotometric assay and rate of nicotinamide adenine dinucleotide phosphate (NADPH) formation was measured at 340 nM.

LDH was determined by pipetting Tris-HCl, nicotinamide adenine dinucleotide (NADH), and sodium pyruvate into a cuvette. This was incubated in the spectrophotometer for 4–5 min. To determine LDH activity, the diluted enzyme was added and reduced form of NADH was measured at 340 nm.

Determination of Cardiac Tissue Antioxidant Enzyme

SOD, CAT, GSH reductase, GSH peroxidase, and MDA were all determined by methods of Boveris,[16] Aebi,[17] Iqbal,[18] and Draper and Hadley,[19] respectively.

SOD activity was determined by measuring the rate of inhibition of autocatalytic adrenochrome formation in a reaction buffer containing adrenaline and glycine-sodium hydroxide at 560 nm. CAT activity was determined by sonicating homogenates in phosphate buffer and supernatant assayed by measuring the rate of decrease in hydrogen peroxide absorbance at 240 nm. GSH reductase activity was determined by assays measuring the oxidation of NADPH in the presence of exogenous GSH, cumene hydroperoxide, and GSH reductase. Absorbance was measured at 340 nm. GSH peroxidase activity was determined by adding homogenate to a reaction mixture consisting of phosphate buffer, ethylenediaminetetraacetic acid, sodium azide, GSH reductase NADPH, and hydrogen peroxide. Absorbance was read in microplate wells at 340 nm.

MDA activity (lipid peroxidation) was evaluated by adding trichloroacetic acetic to homogenates and centrifuged. The supernatant was mixed with thiobarbituric acid and incubated. Absorbance was measured at 532 nM.

Statistical Analysis

GraphPad Prism Software Version 6.0 was used for analysis and data expressed as mean ± standard deviation. One-way ANOVA was used to compare the control and treated groups. Statistical significance was taken at $P < 0.001$.

RESULTS

Extract of CZ on Serum CK-MB and LDH in CCl_4-induced Cardiac Toxicity

Administration of CCl_4 through subcutaneous route to rats in Group II produced a significant ($P < 0.05$) increase in serum CK-MB concentration (6.39 ± 0.55 ng/ml) compared to animals in control group (3.54 ± 0.41 ng/ml). Treatment of rats with aqueous extract (400 mg/kg) produced significant decrease ($P < 0.001$) in concentration of CK-MB (1.97 ± 0.01 ng/ml). N-butanol fraction (40 mg/kg) caused a significant increase ($P < 0.001$) in serum CK-MB (8.32 ± 0.50 ng/ml). There was a significant ($P < 0.05$) decrease in serum LDH (8.01 ± 0.01 U/l) with n-butanol fraction (40 mg/kg) compared to CCl_4 treated group (10.50 ± 2.84 U/l). Ascorbic acid (10 mg/kg) also produced a decreased LDH (8.10 ± 5.72 U/l) concentration [Table 1].

Aqueous Leaf Extract of CZ and n-butanol Fraction on Antioxidant Enzymes in Cardiac Tissue

CCl_4 administration to rats in Group II produced significant ($P < 0.05$) decrease in SOD, CAT, GR, and GPx concentration compared with control Group I. The SOD concentration for CCl_4 treated group was (6.10 ± 0.21 units/mg protein) with aqueous extract (400 mg/kg) and n-butanol fraction (40 mg/kg) reversing this effect and produced a significant ($P < 0.001$) increase in SOD (32.51 ± 4.44 and 23.39 ± 1.22 units/mg protein), respectively. In addition, there was a significant ($P < 0.001$) increase in CAT concentration (46.46 ± 1.45 and 49.27 ± 2.12 units/mg protein), respectively, compared with CCl_4 control (31.12 ± 0.36) and increased concentration of GR (29.14 ± 0.46 and 30.07 ± 0.28 units/mg protein), respectively, compared with CCl_4 control (19.75 ± 0.75) in Groups III and IV animals treated with (200 and 400 mg/kg) aqueous extract of CZ, respectively [Table 2].

The aqueous extract (200 and 400 mg/kg) produced an increase in activity of GPx (35.21 ± 5.01 and 43.96 ± 8.55 units/mg protein), respectively, compared with the CCl_4 treated group (1.67 ± 0.24 unit/mg protein) and (12.71 ± 1.46 unit/mg protein) for ascorbic acid treated group [Table 2].

CCl_4 also produced a significant ($P < 0.05$) increase in MDA concentration (12.99 ± 3.38 unit/mg protein) and this effect was reversed by 200 and 20 mg/kg of leaf extract and n-butanol fraction (8.59 ± 0.20 and 9.53 ± 0.85 unit/mg protein), respectively [Table 2].

Table 1: Effect of aqueous leaf extract of *Croton zambesicus* and n-butanol fraction on serum CK-MB and LDH concentration

Group	Treatment	CK-MB ng/ml	LDH U/L
I	Control	8.76±0.04	8.09±0.00
II	CCl_4	14.75±0.28	10.5±2.84
III	CZ 200 mg/kg + CCl_4	11.70±0.00	12.14±0.00
IV	CZ 400 mg/kg + CCl_4	10.33±2.38**	10.12±2.02
V	NBF 20 mg/kg + CCl_4	20.23±1.00	9.16±1.06
VI	NBF 40 mg/kg + CCl_4	19.26±1.23	8.01±0.00*
VII	Ascorbic acid 10 mg/kg	11.00±084**	8.10±4.05*

*$P<0.05$, **$P<0.001$ versus Group II, $n=5$. CCl_4: Carbon tetrachloride, NBF: n-butanol fraction, CK-MB: Creatine-kinase myocardial band, LDH: Lactate dehydrogenase

DISCUSSION

CZ leaf is used as a food spice and by traditional medicine practitioners in the treatment of chronic disease conditions. Reactive oxygen species often cause oxidative stress and have been linked to the pathogenesis of chronic diseases.[20] In experimental animals, the administration of CCl_4 often causes the production of reactive oxygen species, resulting in oxidative stress.[21] Subcutaneous administration of CCl_4 in rats produces acute toxic effects in cardiac tissue, resulting in an increase in serum concentration of CK-MB and LDH.

The aqueous leaf extract of CZ prevented the toxic effect of CCl_4 with a resultant decrease in serum concentration of CK-MB and LDH, respectively. This effect was similar to that produced by ascorbic acid (an antioxidant drug). The aqueous extract increased serum markers of cardiac function and was nontoxic compared to an n-butanol fraction. A significant increase in CK-MB concentration was observed in animal treated with 20 and 40 mg/kg n-butanol fraction when compared with untreated control Group II that received CCl_4 only. CK-MB is an enzyme in the myocardium that indicates the extent of injury to cardiac cells.[22]

The increased serum CK-MB concentration in this group may be due to the toxic effect of n-butanol fraction on cardiac tissue. An increased concentration of CK-MB in serum has been attributed to a disturbance in the membrane of

Table 2: Effect of aqueous leaf extract of *Croton zambesicus* and n-butanol fraction on serum SOD, CAT, GR, and GPx concentration

Group	Treatment	SOD Units/mg protein	CAT Units/mg protein	GR Units/mg protein	GPx Units/mg protein	MDA Units/ mg protein
I	Control	10.22±0.31	35.17±7.48	24.46±0.64	3.2±095	7.95±0.46
II	CCl_4	6.10±0.21	31.12±0.36	19.75±0.75	1.67±024	12.99±3.38
III	CZ 200 mg/kg + CCl_4	11.16±1.05	46.46±1.45**	29.14±0.46**	35.21±5.01**	8.59±0.20
IV	CZ 400 mg/kg + CCl_4	32.51±4.44**	49.27±2.12**	30.07±0.28**	43.96±8.55**	10.34±0.05
V	NBF 20 mg/kg + CCl_4	10.50±0.70	29.35±3.75	22.14±0.19	4.58±1.18	9.53±0.85
VI	NBF 40 mg/kg + CCl_4	23.39±1.22**	36.98±3.20	25.33±1.34	4.37±0.29	10.81±0.94
VII	Ascorbic acid 10 mg/kg	13.18±0.25	42.90±1.57**	27.17±0.62**	12.71±1.46**	7.12±0.15

*$P<0.05$, **$P<0.001$ versus Group II, $n=5$. CCl_4: Carbon tetrachloride, NBF: n-butanol fraction, SOD: Superoxide dismutase, CAT: Catalase, GPx: Oxidized glutathione, GSH: Glutathione, MDA: Malondialdehyde

cardiac cells from peroxidation caused by free radicals. This results in leakage of enzymes into plasma with an increased concentration in serum.[23]

There was a decrease in the concentration of SOD, CAT, GSH, and GPx with subcutaneous administration of CCl_4. The aqueous extract prevented the toxic effect of CCl_4 with increased activity of antioxidant enzymes (SOD, CAT, GSH, and GPx) in cardiac tissue. In addition, an increased concentration of MDA produced by CCl_4 was inhibited by aqueous extract and n-butanol fraction. These findings are similar to those obtained from studies on the protective activity of leaf extracts of *Carissa opaca*[24] and *Cornus mas*.[25]

The aqueous leaf extract of CZ significantly attenuated the toxic effects of CCl_4 by restoring the alterations in cardiac function and activity of antioxidant enzymes. Antioxidant enzymes have been reported to inhibit the formation or scavenge free radicals and thus play a significant role in protecting various tissues and organs.[8]

Preliminary phytochemical analysis of the aqueous leaf extract of CZ revealed presence of flavonoids, tannins, saponins, triterpenes, alkaloids, and cardiac glycosides.[26] The protective effect may be attributed to the antioxidant activity of phytochemicals present in the aqueous leaf extract.[8]

CONCLUSION

The aqueous leaf extract of CZ was found to have protective potential and ameliorated the toxic effects induced by CCl_4 on cardiac tissue.

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