ORIGINAL ARTICLE

Protective Effect of *Combretum Hypopilinum* Diels: Root Bark Extract against CCl₄-Induced Hepatotoxicity in Wistar Rats

Kokou Idoh, Kossivi Dosseh, Tchazou Kpatcha, Amegnona Agbonon, Messanvi Gbeassor

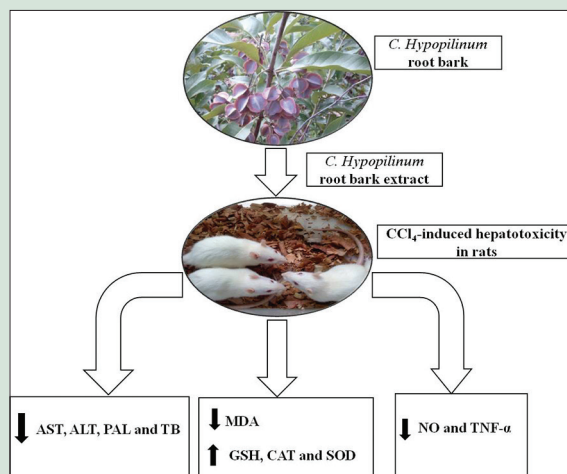Department of Physiology/Pharmacology, Faculty of Sciences, University of Lomé, Lomé, Togo

ABSTRACT

Background: Combretum hypopilinum is used in Togolese traditional medicine to alleviate various ailments including hepatic disorders. **Objective:** This study evaluates the hepatoprotective activities of crude ethanolic extract of C. hypopilinum root bark (CECH) and its possible mechanism(s) of action in Wistar rats. **Materials and methods:** In vitro antioxidant potential was evaluated using DPPH, Ferric-reducing power and hydroxyl radical scavenging assays. Hepatoprotective activity was assessed using CCl4-induced acute hepatotoxicity in rats. Animals were pretreated orally with different doses of CECH (200 and 400 mg/kg) once daily for 7 consecutive days and were subjected to intraperitoneal single injection of CCl4 (1 mL/kg). Serum markers of hepatic damage, hepatic endogenous antioxidants, NO, TNF-α and lipid peroxidation contents were measured out. **Results:** The pretreatment with CECH significantly decreased levels of hepatic enzymes ($p < 0.05$, $p < 0.01$, $p < 0.001$) and MDA in pretreated rats ($p < 0.05$, $p < 0.01$). CECH restored the decreased levels of hepatic antioxidants including SOD ($p < 0.01$), CAT ($p < 0.05$ at 400 mg/kg) and GSH ($p < 0.05$ at 400 mg/kg) and significantly inhibited hepatic production of NO ($p < 0.05$, $p < 0.01$). as well as the level of TNF-α ($p < 0.001$) in pretreated rats. Protective effects of CECH were confirmed by histopathological examinations. CECH exhibited antioxidant in vitro and contains also total phenol and flavonoid. **Conclusion:** This study suggests that CECH protected the liver from CCl4-induced hepatic damage possibly via antioxidant and anti-inflammatory mechanisms.

Key words: *Combretum hypopilinum*, hepatoprotective, oxidative stress, tumor necrosis factor-α

SUMMARY

- *C. hypopilinum* protected the liver against CCl4-induced hepatotoxicity in rats through antioxidant and anti-inflammatory mechanisms

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INTRODUCTION

The liver represents 2% of total body weight in humans and is the largest solid organ in the body.[1,2] The liver performs many vital functions including, drugs metabolism and other xenobiotics. Consequently, the liver is prone to xenobiotic-induced injury.[3] Liver injury can be caused by toxic chemicals, drugs, and virus infiltration from ingestion or infection.[4,5] However, conventional drugs used in modern pharmacotherapy have shown limited therapeutic benefits and are associated with serious risks sides effects. In the absence of a reliable liver protective drug in the modern system of medicine, natural extracts from medicinal plants considered to be effective and safe are recommended for the treatment of liver disorders.[6] These plants contain natural antioxidants in the form of tannins, flavonoids, alkaloids, etc. which synergistically provides a protective and beneficial effect to liver cells.[7,8]

Combretum hypopilinum Diels (Family: Combretaceae; Synonym: *Combretum collinum* Fresen. Subsp. *Hypopilinum* (Diels) Okafa) is a small to medium-sized shrub, with several stems, deciduous, up to

12–17 m tall. It grows in various soils, with semi-arid-to-moderate rainfall conditions. The plant has many uses in Africa, especially in traditional medicine. Infusion of fresh or dried leaves and decoction of roots bark are commonly taken as cholagogic, diuretic, and purgative and also to treat gastrointestinal disorders, including diarrhea, dysentery, and stomach aches.[9] Decoction of leaves and twigs is taken as a drink, and

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fresh roots are chewed to treat lung problems such as cough, bronchitis, and tuberculosis and also to treat snake bites and jaundice.[10] Maceration or decoction of root bark is taken to treat gonorrhea and infertility in women and men. In addition, the infusion of leaves and roots is taken as a blood tonic.[11] Few pharmacological and phytochemical studies have been carried out on *C. hypopilinum*. Nevertheless, combretastatins A and B which are stilbenoids and several phenanthrens were isolated from the aerial parts of the plant.[12] Moreover, a series of amino acids have been isolated from gum exudates, the most important being aspartic acid, glycine, glutamic acid, and alanine.[13] It has been reported that an aqueous extract of stem bark and root showed antibacterial activity against *Proteus mirabilis* while different leaf extracts showed weak antifungal activities *in vitro*.[9]

In the Savanna region of Togo, *C. hypopilinum* is known as "Pupiong" (Moba), "Aléblé" (Lamba), or "magic tree" and it is widely used as traditional herbal medicine to alleviate a variety of diseases including headache and jaundice.[14] Thus, the current study was undertaken to assess the hepatoprotective activity and mechanism(s) of ethanolic root bark extract of *C. hypopilinum* on carbon tetrachloride (CCl_4)-induced acute hepatotoxicity in Wistar rat.

MATERIALS AND METHODS

Plant material and extraction

Roots of *C. hypopilinum* were collected in Dapaong (Far North of Togo, Savanna region) about 650 km from Lomé in March 2014. Botanical authentication was confirmed at the Department of Botany by Prof. Kokou Kwami, University of Lomé, where a voucher specimen of *C. hypopilinum* was deposited in the herbarium (N° TG230314). Roots were cleanly washed with tap water. Root barks were removed and then dried under air conditioning for 7 days. Dried root barks were coarsely reduced into powder. The powder (300 g) was macerated at room temperature with ethanol 95° (3 L) for 72 h under intermittent stirring. The solvent was removed using a rotary vacuum evaporator (Buchi, Switzerland) at 40°C and the crude extract of *C. hypopilinum* (CECH), representing a yield of approximately 10.4% (w/w), was stored at 2°C–8°C until administrated doses preparation, when it was dissolved in sterilized distilled water.

Animals and treatment

Male Wistar rats weighing 150–170 g used in the present study were provided by animals' facilities of the Laboratory of Physiology and Pharmacology of the Faculty of Sciences, University of Lomé. Animals were housed divided in cages (5 rats per cage) at ambient temperature and humidity with a 12 h day-light cycle, with free access to food and water *ad libitum*. Experimental protocols were based on the World Health Organization guidelines for care and use of laboratory animals, and the use of the animals was approved by the Ethics Committee of the University of Lomé, a branch of the National Ethics Committee for Control and Supervision of Experiments on Animals (N° SBM/UL/14/NS0004).

Chemicals

Silymarin, quercetin, trichloroacetic acid (TCA), sodium chloride, sodium hydrogenphosphate, potassium dihydrogenphosphate, hydrogen peroxide (H_2O_2), glacial acetic acid, potassium ferricyanide ($K_3Fe(CN)_6$), sodium nitrite ($NaNO_2$), 5′-dithio-bis-2-nitrobenzoic acid, sodium nitrate, sodium bicarbonate, sodium carbonate (Na_2CO_3), Folin–Ciocalteu's reagent, sulfanilamide, gallic acid (GA), and aluminum chloride ($AlCl_3$) were purchased from Sigma–Aldrich (St. Louis, MO, USA). CCl_4 was purchased from BDH Chemicals (Poole, England). Enzyme-linked immunosorbent assay (ELISA) kit for tumor necrosis

factor-α (TNF-α) assay (Rat TNF-α ELISA Kit) was purchased from RayBio® Inc., (Norcross, USA).

2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay

Free-radical scavenging activity of CECH was evaluated *in vitro* using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by McCune and Johns.[15] Briefly, 0.25 mL of a methanol solution of CECH at different concentrations (0–250 µg/mL) was mixed with 1.5 mL of DPPH (100 µM). After 15 min of incubation, the change in absorbance with respect to the control (containing DPPH only without sample, expressed as 100% free radicals) was measured at 517 nm. Quercetin was used to generate a standard curve for the 50% inhibition concentration (IC_{50}) determination. The assay was performed in triplicate.

Ferric-reducing antioxidant power assay

The ferric-reducing power of CECH was determined according to previously reported method.[16] Briefly, to 1 mL of a methanol solution of CECH at different concentrations (0–250 µg/mL), 2.5 mL of phosphate buffer (0.2 M, pH 6.6) was added followed by addition of 2.5 mL $K_3Fe(CN)_6$ solution (1%). The reaction mixture was incubated in water bath at 50°C for 15 min. After incubation, TCA (10% w/v, 2.5 mL) was added to the reaction mixture and was centrifuged at 7500 rpm for 15 min. To the aliquot of the upper layer (2.5 mL), distilled water (2.5 mL) and a solution of ferric chloride (0.1%, 0.5 mL) were added. Finally, the absorbance of the reaction mixture was measured at 700 nm. Quercetin was used as a standard. The assay was performed in triplicate.

Hydroxyl radical scavenging assay

Antioxidant effects of *C. hypopilinum* on hydroxyl radical (OH•) was determined according to a previous procedure with slight modification.[17] Briefly, 1 mL of CECH solution at different concentrations (0–250 µg/mL) was added to a mixture solution containing 1 mL $FeSO_4$ (100 mM), 1 mL salicylic acid-ethanol (100 mM), and 1 mL H_2O_2 (200 mM). The resultant mixture was incubated 60 min at 37°C and its absorbance was measured at 510 nm against the blank. The assay was performed in triplicate.

Determination of total flavonoid content

Total flavonoid content in CECH was measured according to colorimetric method using $AlCl_3$.[18] CECH or standard (100 µl, 1 mg/mL) in ethanol was mixed with 0.4 mL of distilled water and 0.03 mL of 5% $NaNO_2$ solution. After 5 min, 0.02 mL of a 10% $AlCl_3$ solution was added. To the mixture, 0.02 mL of Na_2CO_3 (1 mol/L) was added, and 5 min later, 0.25 mL of distilled water was added. The solution was well stirred and the absorbance was read at 510 nm. Quercetin was used as a standard to obtain the standard curve (0–500 µg/mL). Results are expressed as milligram quercetin equivalent per gram of extract (mg Q Eq/g extract).

Determination of total phenol content

Total phenol content was determined by the Folin–Ciocalteau's method according to Al-Farsi *et al.*[19] The extract (100 µL) was mixed with 750 µL of Folin–Ciocalteu's reagent (previously diluted 10-fold with distilled water) for 5 min at room temperature. About 750 µL of aqueous Na_2CO_3 (60 g/L) was added, and the mixture was homogenized and allowed to stand at room temperature for 90 min. After incubation, the absorbance was measured at 725 nm. GA (0–450 µg/mL) was used as the standard for the calibration curve. Total phenol content was expressed as milligram of GA equivalent per gram of extract (mg GA Eq/g extract).

Hepatoprotective activity of *Combretum hypopilinum* root bark extract

Twenty-five male Wistar rats are used in this study. Animals were randomly divided into 5 groups of 5 each: Group I (control) served as normal control, Group II (CCl_4) served as CCl_4-induced hepatotoxicity model; both Groups I and II were orally given distilled water (10 mL/kg) once daily for 7 consecutive days, Group III (S 100 mg/kg + CCl_4) served as positive control group and was orally given Silymarin (100 mg/kg) once daily for 7 consecutive days, and Groups IV and V (CECH 200 mg/kg + CCl_4 and CECH 400 mg/kg + CCl_4) were orally given CECH, respectively, at 200 or 400 mg/kg once daily for 7 consecutive days. Two hours after last administration of extract or Silymarin, Groups II–V were injected intraperitoneally with a single dose of CCl_4 (1 mL/kg, i. p.) mixed in equal volume of corn oil (v/v), while the normal control group received equal volume of corn oil (1 mL/kg, i. p.). On the 8th day, blood samples were collected from overnight fasted rats through retroorbital sinus puncture for biochemical analysis.[20] Later, rats were sacrificed by cervical dislocation under mild ether anesthesia and liver was excised and washed in fresh phosphate buffer saline (pH 7.4). Blood was allowed to coagulate and serum was separated by centrifugation at 3000 rpm for 15 min using electric centrifuge (Shimadzu Scientific Corporation Tokyo, Japan). Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), total cholesterol (TC), triglycerides (TG), total bilirubin (TB), and total protein (TP) were estimated using commercial kits purchased from Human GmbH (Wiesbaden, Germany).

Measurement for hepatic antioxidant markers

Livers were immediately excised, weighed, and homogenized (20%, w/v) in PBS (pH 7.4). The homogenates were then centrifuged as previously. Supernatant was collected and stored at −80°C for further estimations of lipid peroxidation using malondialdehyde (MDA) assay,[21] superoxide dismutase (SOD) assay,[22] catalase (CAT) assay,[23] glutathione (GSH) assay,[24] and hepatic TP content.[25]

Measurement for hepatic nitric oxide

Hepatic nitric oxide (NO) content was estimated using Griess reagent according to Fermor *et al.*[26] Briefly, 500 μL of Griess reagent (Sulfanilamide [1%], *N*-(1-Naphthyl) ethylenediamine [0.1%], and phosphoric acid [2.5%]) were added to 500 μL of liver homogenate. The mixture was homogenized and incubated for 10 min in the darkness. After incubation, the absorbance of the mixture was measured at 570 nm against the blank. Calculations were done after generating a standard curve from $NaNO_2$ in the same buffer as used for preparation of homogenates. Results are expressed in nmol/mg proteins.

Measurement for hepatic tumor necrosis factor-α

Hepatic TNF-α contents in supernatants were quantified using commercially available rat TNF-α ELISA Kit from RayBio® Inc., (Norcross, USA). Measures were performed strictly according to the manufacturer's instructions. The ELISA kits used was specific for the measurement of rats specific TNF-α without cross-reactivity with other cytokines. TNF-α concentration was expressed in ng/g proteins.

Histopathological study

Liver samples were fixed in 10% formalin, dehydrated in graded (50%–100%) alcohol and embedded in paraffin. Thin sections were cut with microtome and stained with hematoxylin and eosin (H and E) stain for microscopic assessment. Microphotographs of each of the slides were taken with light microscope at ×40 magnification.

Statistical analysis

Results are expressed as mean ± standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests to evaluate significant differences between groups. Results were considered to be significant at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 5 from GraphPad Software, Inc. (California, USA).

RESULTS

In vitro antioxidant activity

The antioxidant potential of CECH was investigated using *in vitro* assays of DPPH radical, ferric-reducing power, and OH· scavenging. All antioxidant assays provide support to antioxidant capacity of CECH. In DPPH radical scavenging assay, CECH showed an IC_{50} of CECH extract: 10,17 ± 2,05 μg/mL while quercetin showed an IC_{50} of 7.79 ± 1.66 μg/mL. Regarding OH· scavenging capacity of CECH, it was noticed to be low to intermediate and increased in a dose-dependent manner [Figure 1a]. Observably, at the same concentration (250 μg/mL), OH· scavenging capacity of CECH was 57.68% whereas that of quercetin was 88.56%. Ferric-reducing power of CECH showed a certain degree of ferric-reducing antioxidant potential in a dose-dependent manner. At the same concentration (250 μg/mL), ferric-reducing power of CECH was 69.91%, whereas that of quercetin was 95.15% [Figure 1b]. This study showed that CECH was active scavenger of OH·s and active reducer of Fe^{3+}. However, its OH· scavenging and ferric-reducing potentials are inferior to those of quercetin.

Total phenol and total flavonoid content

Total phenol and flavonoid contents were estimated as 583.56 ± 5.95 mg GA Eq/g extract and 76.11 ± 5.97 mg Q Eq/g extract, respectively, showing that CECH contains a nonnegligible amount of polyphenols and flavonoids.

Hepatoprotective activity

Effect of CECH at the dosages of 200 and 400 mg/kg was studied on serum marker enzymes of liver damage, TP s, and TB in CCl_4-intoxicated rats. Compared to control group (Group I), injection of CCl_4 (Group II) induced significant increase in serum markers of liver injury including
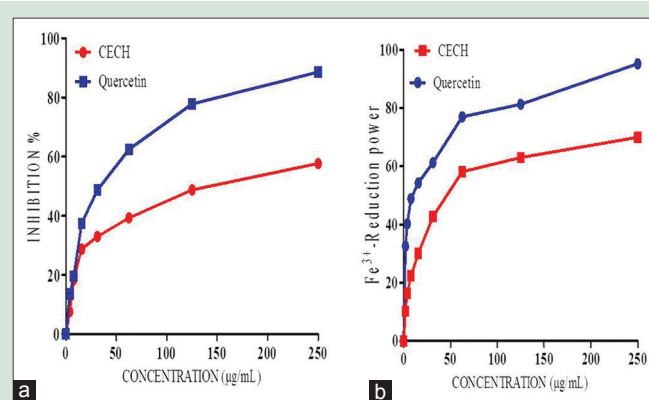

Figure 1: Ferric-reducing power and hydroxyl radical scavenging assays of various concentrations of CECH (a): OH·-scavenging capacity and (b): ferric-reducing antioxidant power. CECH: Crude extract of *Combretum hypopilinum*; OH·: Hydroxyl radical

transaminases ($P < 0.001$), ALP ($P < 0.001$), and TB ($P < 0.001$). However, pretreatment with *C. hypopilinum* root bark extract has considerably reduced serum concentrations of livers injury markers [Table 1]. Effect of CECH on CCl$_4$-induced acute hepatitis is significant at 400 mg/kg ($P < 0.001$) for AST. ALT concentrations also dropped down significantly at 400 mg/kg ($P < 0.05$), while those of ALP and TB began to fall from 200 mg/kg ($P < 0.001$, $P < 0.01$) to 400 mg/kg ($P < 0.001$, $P < 0.01$). LDH levels also decreased but not significantly ($P > 0.05$) as compare to Group II. The same trend is observed in TPs contents where a nonsignificant ($P > 0.05$) increase induced by the extract is observed. Silymarin-pretreated animals also showed significant ($P < 0.001$, $P < 0.01$, $P < 0.05$) protection against CCl$_4$-induced liver damage through the above-mentioned markers [Table 1]. The assayed results of serum TG and TC levels are shown in Table 2. It can be seen that CCl$_4$ induced a systemic accumulation of TG and TC in CCl$_4$-injected rats as compare to control rats (Group I). Observably, there is a significant difference in serum levels of TG and TC between control and CCl$_4$-injected groups ($P < 0.05$). However, extract of *C. hypopilinum* reduced serum levels of TG and TC in pretreated animals. This diminution was significant at 400 mg/kg ($P < 0.01$) as for TC and at 200 and 400 mg/kg ($P < 0.01$) as for TG.

Histopathological study

H and E-stained sections of liver are shown in Figure 2. Normal liver lobular architecture and cell structure were shown in control group (A). While hepat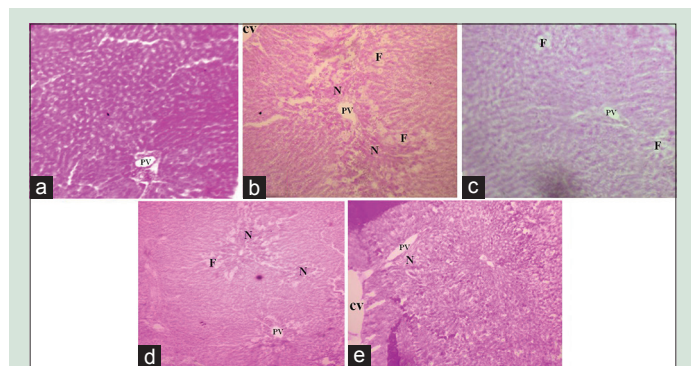ic tissue of CCl$_4$-iduced hepatotoxicity model rats showed apparent morphological changes including large areas of extensive cell necrosis with loss of hepatocyte architecture (B). However, pretreatment with CECH decreased the injured area and necrotic cells (D and E).

Effects of crude extract of *Combretum hypopilinum* on hepatic antioxidants and malondialdehyde contents

Effects of different treatments on hepatic contents of GSH, SOD, and CAT are shown in Table 3. Results showed clearly that activities of antioxidant enzymes (CAT and SOD) and GSH content significantly ($P < 0.001$, $P < 0.01$) decreased in CCl$_4$-induced hepatotoxicity model group (Group II) as compare to control group (Group I). However, pretreatment with CECH (Groups IV and V), for 7 consecutive days, induced significant increase in hepatic CAT and SOD as well as hepatic GSH content. Thus, SOD activity significantly increased at 200 and 400 mg/kg ($P < 0.01$) as compare to Group II. CAT content also increased from 65.0 ± 4.6 U/min/mg protein at 200 mg/kg ($P > 0.05$) to 72.6 ± 6.1 U/min/mg protein at 400 mg/kg ($P < 0.05$). GSH content ranges from 4.47 ± 0.12 nmol/mg protein at 200 mg/kg ($P > 0.05$) to 5.84 ± 0.52 nmol/mg protein at 400 mg/kg ($P < 0.05$).

The hepatic tissue of CCl$_4$-intoxicated rats (Group II) showed significantly higher level of MDA as compare to nonintoxicated rats ($P < 0.05$). However, pretreatments with CECH reduced
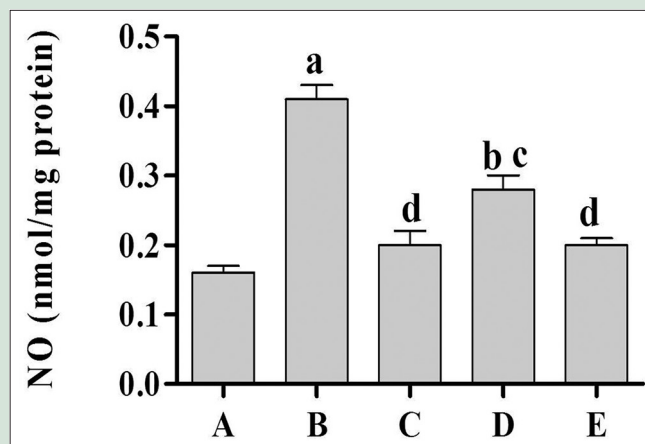

Figure 2: Effects of CECH on histopathological changes. Histological examination was performed under a light microscope (×40) with hematoxylin and eosin staining. (a) Healthy liver showing normal structure of hepatic cells. (b) Showing massive fatty changes, necrosis, and ballooning degeneration. (c) Liver section after administration of 100 mg/kg of silymarin. (d) Liver section after administration of 200 mg/kg of CECH. (e) Liver section after administration of 400 mg/kg of CECH. N: Necrosis; F: Fatty acid accumulation; PV: Portal vein; CV: Central vein; CECH: Crude extract of *Combretum hypopilinum*

Figure 3: Effect of CECH on hepatic nitric oxide production rats were received oral administration of different doses of CECH once daily for 7 consecutive days and were subsequently subjected to intraperitoneal single injection of CCl$_4$ (1 mL/kg) and hepatic NO content was measured. Values are expressed as mean ± standard error of the mean (n = 5). [a]$P < 0.001$, [b]$P < 0.05$ as compare to control group (a); [c]$P < 0.05$, [d]$P < 0.01$ as compare to CCl$_4$-induced hepatotoxicity model group (b). CECH: Crude extract of *Combretum hypopilinum*; NO: Nitric oxide

Table 1: Effect of *Combretum hypopilinum* on biochemical markers of hepatic injury

Groups	Treatments	AST[A]	ALT[A]	ALP[A]	LDH[A]	TB[B]	TP[C]
I	DW (10 mL/kg)	165.3±11.80	96.6±6.8	489.6±20.8	272.3±24.7	0.81±0.1	59.57±1.0
II	DW (10 mL/kg) + CCl$_4$	1750±267.8[b]	3052±761.6[b]	790.3±64.5[b]	446.3±120.6	2.83±0.6[b]	48.46±1.7
III	Silymarin (100 mg/kg) + CCl$_4$	198.2±28.24[e]	125.2±12.5[e]	396.6±20.1[e]	248.9±28.5	2.21±0.3[a]	63.36±1.6[d]
IV	CECH (200 mg/kg) + CCl$_4$	908.2±191.9[a,e]	1616±246.0[a]	424.4±24.3[e]	226.5±25.6	1.09±0.1[d]	58.78±5.8
V	CECH (400 mg/kg) + CCl$_4$	661.1±173.8[e]	1565±260.1[a,c]	401.6±19.2[e]	303.9±27.8	1.05±0.1[d]	55.68±4.1

[a]$P<0.05$, [b]$P<0.001$ as compare to Group I; [c]$P<0.05$, [d]$P<0.01$, [e]$P<0.001$ as compare to Group II. [A]U/L, [B]mg/L, [C]g/L. Rats were received oral administration of different doses of CECH once daily for 7 consecutive days and were subsequently subjected to intraperitoneal single injection of CCl$_4$ (1 mL/kg) and serum biochemical markers of liver injury were measured. Values are expressed as mean±SEM (n=5). CECH: Crude extract of *Combretum hypopilinum* root bark; DW: Distilled water; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; LDH: Lactate dehydrogenase; TB: Total bilirubin; TP: Total proteins; CCl$_4$: Carbon tetrachloride; SEM: Standard error of the mean

significantly ($P < 0.05$, $P < 0.01$; at 200 and 400 mg/kg, respectively) MDA contents indicating the effectiveness of CECH against CCl_4-induced hepatotoxicity [Table 3].

Effects of crude extract of *Combretum hypopilinum* on hepatic nitric oxide and tumor necrosis factor-α production

Results in Figure 3 showed high levels of NO in CCl_4-induced hepatotoxicity model rats (B) compared to control group (A) ($P < 0.001$). Pretreatment with *C. hypopilinum* root bark extract remarkably inhibited the production of NO in the liver tissue. NO levels in rats pretreated with CECH decreased dose-dependently at the doses of 200 and 400 mg/kg, respectively ($P < 0.05$, $P < 0.01$).

CCl_4 injection in rats increased significantly hepatic TNF-α content as compare to control animals [Figure 4]. Interestingly, pretreatment with *C. hypopilinum* root extract, at the highest dosage (400 mg/kg), significantly inhibited hepatic TNF-α production ($P < 0.001$) as compare to CCl_4-induced hepatotoxicity model animals [Figure 4].

DISCUSSION

The present study was designed to investigate the protective effect of CECH root bark on CCl_4-induced hepatotoxicity and its possible mechanism(s) of action. Results showed that CECH exhibited protective activity on CCl_4-induced liver damage. CECH showed *in vitro* antioxidant potentials and contains total phenolic and flavonoids compounds.

CCl_4 is one of the most widely used toxicants for inducing experimental liver injury in animals, and CCl_4-induced acute hepatotoxicity is commonly used to investigate the hepatoprotective activity of natural compounds.[27-30] It has been suggested that CCl_4 causes cellular leakage and a loss of functional integrity of cell membranes in the liver tissue. Damage to the liver tissues alters their transport function and membrane permeability, leading to leakage of enzymes from the cells.[31] Therefore, the marked release of AST, ALT, ALP, and LDH into the circulation indicates severe damage to hepatic cell membranes due to CCl_4 intoxication. Hence, administration of CCl_4 in rats could cause a variety of hepatic histological changes, such as hepatocellular necrosis, leukocyte infiltration, portal inflammation, and structural damage.[32] In the present study, general indicators of CCl_4-induced hepatotoxicity including AST, ALT, ALP, and LDH activities and serum TB, TP, TG, TC levels and histopathological were evident. However, pretreatment with CECH inhibited liver enzymes leakage into the systemic circulation and attenuated histological changes in rats. These results suggest that CECH significantly suppressed CCl_4-induced liver injury through the recovery of function and structure of hepatic cells.

CCl_4 requires biotransformation by hepatic microsomal CYP450 to produce degraded metabolite trichloromethyl (CCl_3) free radical which induces oxidative stress and subsequently liver injury.[27] This product is unstable free radical and exhibits strong affinity for binding to proteins and lipids of the cell membrane or abstracting a hydrogen atom from unsaturated lipid, thereby triggering lipid peroxidation and causing liver damage. Hepatic MDA formation is commonly used as an indicator of

Table 2: Effect of *Combretum hypopilinum* on total cholesterol and triglycerides levels

Groups	Treatments	Total cholesterol (mM)	Triglycerides (mM)
I	DW (10 mL/kg)	0.87±0.02	0.99±0.04
II	DW (10 mL/kg) + CCl_4	1.75±0.45[a]	1.28±0.09[a]
III	Silymarin (100 mg/kg) + CCl_4	1.22±0.06	1.12±0.03
IV	CECH (200 mg/kg) + CCl_4	1.06±0.15	0.85±0.05[b]
V	CECH (400 mg/kg) + CCl_4	0.88±0.14[b]	0.84±0.05[b]

[a]$P<0.05$ as compare to Group I, [b]$P<0.01$ as compare to Group II. Rats were received oral administration of different doses of CECH once daily for 7 consecutive days and were subsequently subjected to intraperitoneal single injection of CCl_4 (1 mL/kg) and serum total cholesterol and triglycerides were measured. Values are expressed as mean±SEM (n=5). CECH: Crude extract of *Combretum hypopilinum* root bark; DW: Distilled water; CCl_4: Carbon tetrachloride; SEM: Standard error of the mean
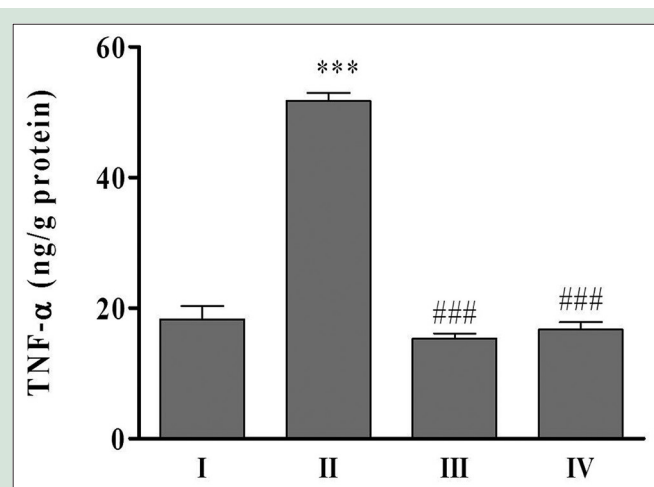

Figure 4: Effect of CECH on hepatic TNF-α content rats were received oral administration of different doses of CECH once daily for 7 consecutive days and were subsequently subjected to intraperitoneal single injection of CCl_4 in equal volume of corn oil (1 mL/kg, v/v) and TNF-α was measured in liver homogenate. (I) Control group, (I): CCl_4-induced hepatotoxicity model group, (III) S (100 mg/kg) + CCl_4 group, (IV) CECH (400 mg/kg) + CCl_4 group. Values are expressed as mean ± standard error of the mean (n = 5). ***$P < 0.001$, versus Group I, ###$P < 0.001$, versus Group II. CECH: Crude extract of *Combretum hypopilinum*; TNF-α: Tumor necrosis factor-α

Table 3: Effect of crude extract of *Combretum hypopilinum* on hepatic antioxidant markers

Groups	Treatments	MDA (nmol/mg proteins)	GSH (nmol/mg proteins)	SOD (U/min/mg proteins)	CAT (U/min/mg proteins)
I	DW (10 mL/kg)	0.96±0.14	5.94±0.56	4.30±0.43	123.3±12.3
II	DW (10 mL/kg) + CCl_4	1.85±0.34[a]	2.50±0.32[b]	1.58±0.21[c]	37.1±8.7[c]
III	Silymarin (100 mg/kg) + CCl_4	1.25±0.21	3.79±0.28	3.10±0.21[f]	105.8±8.2[f]
IV	CECH (200 mg/kg) + CCl_4	0.99±0.19[d]	4.47±0.12	3.37±0.44e	65.0±4.6[c]
V	CECH (400 mg/kg) + CCl_4	0.58±0.09[e]	5.84±0.52[d]	3.29±0.09e	72.6±6.1[b,d]

[a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ as compared to Group I; [d]$P<0.05$, [e]$P<0.01$, [f]$P<0.001$ as compared to Group II. Rats were received oral administration of different doses of CECH once daily for 7 consecutive days and were subsequently subjected to intraperitoneal single injection of CCl_4 (1 mL/kg) and hepatic MDA, SOD, CAT, and GSH levels were measured. Values are expressed as mean±SEM (n=5). CECH: Crude extract of *Combretum hypopilinum*; DW: Distilled water; MDA: Malondialdehyde; SOD: Superoxide dismutase; CAT: Catalase; GSH: Reduced glutathione; CCl_4: Carbon tetrachloride; SEM: Standard error of the mean

liver tissue damage and increase in hepatic MDA content suggests the enhanced lipid peroxidation, resulting in tissue damage and failure of the antioxidant defense mechanisms to prevent the formation of excessive free radicals.[33,34] Previous studies reported that natural compounds from plants reduce CCl_4-induced lipid peroxidation in animal models.[35,36] In the current study, CCl_4 caused an increase in hepatic MDA content in CCl_4-induced hepatotoxicity model group, as compared to the normal group. However, pretreatment with CECH significantly ($P < 0.05$, $P < 0.01$) decreased hepatic level of MDA, suggesting that CECH inhibited lipid peroxidation reaction triggered by CCl_4.

In human organism, cells have a number of mechanisms to defend themselves from the toxic effect of reactive oxygen species (ROS) including free-radical scavengers and chain reaction terminators such as SOD, CAT, and GSH.[37] SOD removes superoxide radicals by converting them into H_2O_2 which can be rapidly converted into water by CAT and GSH.[37] Our results showed deep decrease in hepatic SOD, CAT, and GSH contents in CCl_4-induced hepatotoxicity model group, revealing that acute CCl_4-induced oxidative liver injury had already occurred, and these results were in agreement with a previous study.[38] Interestingly, the CECH pretreatment at the doses of 200 and 400 mg/kg significantly reversed the trend. Pretreatment with CECH markedly elevated hepatic levels of SOD, CAT, and GSH. The present study suggests that CCl_4 increased ROS production in the liver and it was inhibited by the CECH pretreatment. This protective action may arise due to the potent free-radical scavenging activities of CECH. It was clear that CECH protected the liver by means of improving enzymatic and nonenzymatic antioxidant defense system to effectively alleviate the generation of free radicals activated by CCl_4. Furthermore, it has been suggested that natural compounds can act as direct antioxidants through scavenging ROS or inhibiting their formation and also as indirect antioxidants through upregulation of endogenous antioxidant defenses.[39] Hence, scavenging free radicals with various natural products provides quite important means to protect against CCl_4-induced liver oxidative stress. In the current study, CECH exerted a good scavenging activity against DPPH and OH· free radicals. The extract exhibited a dose-dependent activity as a free-radical scavenger, with a dose-dependent manner regarding ferric-reducing antioxidant power assay. CECH contains also total phenol and flavonoid in a nonnegligible proportion. These results suggest that CECH not only upregulated hepatic endogenous antioxidants but also it might possibly act as CCl_4-derived free-radical scavenger.

CCl_4-induced hepatotoxicity is characterized by two subsequent phases: a direct oxidative stress leading to hepatocyte death in the first phase and secondary damage from Kupffer cells. In fact, CCl_4 can cause liver inflammation and activate Kupffer cells. Kupffer cells, once activated, release pro-inflammatory cytokines including TNF-α and other pro-inflammatory cytokines.[40-43] An early increase in the TNF-α level induces expression of inflammatory mediators, including inducible NO synthase (iNOS) which produces NO. NO could subsequently cause liver damage by producing ROS through a reaction with a superoxide anion.[44] Furthermore, recent reports demonstrated that iNOS and COX-1 and COX-2 overexpression occurs in the liver of rats with CCl_4-induced acute liver injury.[45,46] Therefore, anti-inflammatory therapy may ameliorate CCl_4-induced liver damage.

Huang *et al*.[47] suggested that antrosterol from *Antrodia camphorata* submerged whole broth protects against CCl_4-induced acute liver injury in mice through the decrease of TNF-α levels and NO production. Results from the current study revealed that pretreatment with CECH significantly decreased hepatic contents of NO and TNF-α, suggesting CECH might suppress the inflammatory response through the inhibition of inflammatory cytokines and NO production. Altogether, the above

results suggest that CECH might ameliorate CCl_4-induced liver injury in rats through suppressing the CCl_4-induced inflammatory response as well as elevating rats' antioxidant capacities.

CONCLUSION

The current study demonstrated that CECH protected the liver against CCl_4-induced hepatotoxicity, possibly by scavenging free radicals, improving the endogenous antioxidant system, and inhibiting inflammation response by decreasing NO and pro-inflammatory cytokine TNF-α production.

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Conflicts of interest

There are no conflicts of interest.

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