Egyptian Journal of Basic and Applied Sciences 4 (2017) 264–269

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Egyptian Journal of Basic and Applied Sciences

journal homepage: www.elsevier.com/locate/ejbas

Full Length Article

Preventive and curative effects of *Cocculus hirsutus* (Linn.) Diels leaves extract on CCl_4 provoked hepatic injury in rats

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ARTICLE INFO

Article history:
Received 8 June 2017
Received in revised form 28 September 2017
Accepted 13 October 2017
Available online 20 October 2017

Keywords:
Antioxidants
Carbon tetrachloride
Cocculus hirsutus
Hepatoprotective effect
Lipid peroxidation

ABSTRACT

The present study was designed to estimate the protective or curative potency of an extract from *Cocculus hirsutus* leaves against CCl_4 intoxication *via* its antioxidant property in rats. Liver enzyme markers (SGOT, SGPT, ALP, LDH, and bilirubin) and oxidative stress markers {lipid peroxidation (LPO), enzymatic antioxidants [superoxide dismutase, catalase and glutathione peroxidase] and non-enzymatic antioxidants [reduced glutathione, vitamin C and E]} were analyzed by spectrophotometry. Histopathological studies on hepatic tissue were also performed by the method of Hematoxylin and Eosin staining. Rats administrated with 30% CCl_4 in olive oil intraperitoneally resulted in significant increase in the levels of SGOT, SGPT, ALP, LDH, and bilirubin compared to control rats. Significant elevation of hepatic LPO and depletion of enzymatic and non-enzymatic antioxidants levels were observed in CCl_4 induced rats. When CCl_4 induced rats were co-treated with *C. hirsutus* at doses of 250 and 500 mg/kg b·wt, all the altered levels of liver marker enzymes and oxidative stress markers were restored to near control values. Histopathological studies provided direct evidence of the hepatoprotective effect of *C. hirsutus*. In conclusion, extract from *C. hirsutus* could protect the liver from CCl_4-induced oxidative damage, by scavenging the free radicals generated during the metabolism of CCl_4.

1. Introduction

Hepatic-organ is the imperative organ, which regulates a wide range of physiological processes in the body and it is one of the major organs prone to the oxidative damage by the attack of reactive oxygen species [1,2]. Carbon tetrachloride (CCl_4) exerted hepatocellular damage has been largely evaluated and employed model for detecting the novel hepato-protective therapeutics. As the reactive oxygen species are the major cause for the deleterious effects in hepatic disorders, various plant extracts were reported for their hepato-protective activities through their antioxidant activities [3,4]. Natural antioxidants are especially considered as robust candidates to confer protection against chemical induced toxicity [5,6].

Since the emergence of civilization on the earth, herbal formulations have been used to maintain human health and to treat various diseases by the vast majority of the World's population [7–9]. Many traditional systems of medicine in India use a number of medicinal plants and their formulations to cure hepatic disorders [10]. In addition to these known plants, there are many other plants used by tribal and folk practitioners which are a potent source of effective hepatoprotective agents that remained unexplored.

Antioxidant property has been reported to play a crucial role in the hepatoprotective capacity of many plants such as *Spirulina maxima*, *Bauhinia hookeri*, and various medicinal herbs [8,11,12]. Treating liver disorders with plant drugs has been an age old tradition by Ayurveda, an indigenous system of medicine in India. Thus the search for potent natural source has become a prime focus for new drug development in pharmaceutical industry for hepatoprotection.

Cocculus hirsutus (Linn.) Diels (Menispermaceae), described in ayurvedic literature as patalagarudi is a straggling shrub, widely distributed all over India, especially in dry regions. The leaves and roots of this plant are largely employed in the Indian traditional medicine for a variety of diseases including, hepatic

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https://doi.org/10.1016/j.ejbas.2017.10.004
2314-808X/

obstruction, jaundice, bronchitis, diabetes mellitus, anorexia, gonorrhoea, and leprosy [13]. *C. hirsutus* is well documented for its anti-inflammatory, analgesic [14], antidiabetic and spermatogenic [15] activities. Considering its varied biological activities and traditional therapeutic use for hepatic disorders, the present study was aimed to evaluate the hepato-protective potential of this plant via its antioxidant property against the deleterious effects of CCl_4 induced oxidative damage.

2. Materials and methods

2.1. Chemicals

2,4-dinitro phenyl hydrazine (DNPH), Disodium phenylphosphate, Thiobarbituric acid (TBA), 1,1,2,2-tetraethoxy propane (TEP), epinephrine (Adrenaline), Glutathione reductase, reduced glutathione (GSH), Nicotinamide adenine dinucleotide phosphate reduced (NADPH) and cumene hydroperoxide were purchased from Sigma chemical company, USA. All the other chemicals utilized for this study were of analytical grade.

2.2. Plant material collection and extract preparation

Leaves from *Cocculus hirsutus* plant were collected in and around Tirumala Tirupathi hills, India and compared with the voucher specimen deposited at the Department of Botany, Sri Venkateswara University, Tirupati, India for its identification. The leaves were shade dried for a week and finely powdered using a blender. Ethanol extract of *C. hirsutus* (EECH) was prepared by soxhlation process. The plant powder (100 g) was extracted with 500 mL of 70% ethanol for more than 6 h and concentrated by rotary evaporation and vacuum drying. The yield of the plant extract was recorded (5.3%) and stored at −20 °C for further use.

2.3. Animals

Male Wistar rats (200 ± 50 g; Sri Raghavendra animal suppliers, Bangalore, India) were maintained under standard hygienic conditions at 25–28 °C with 12 h light/dark cycle and provided standard pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. The animal experiments in the present study were conducted by following the Institute Animal Ethics Committee regulations; approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

2.4. Acute oral toxicity study

This experiment was performed according to the Organization for Economic Cooperation Development (OECD) guidelines. Animals were divided into one control group and five plant extract treated groups (N = 6). After an overnight fast, rats were dosed orally with ethanol extract of *Cocculus hirsutus* (EECH) in distilled water at doses of 100, 250, 500, 1000, and 2000 mg/kg b. wt. Hourly observations of the animals for any signs of behavioral changes, toxicity and mortality was recorded over a period of 72 h [16].

2.5. Experimental design to access the hepato-protective activity of EECH against CCl_4 induced liver injury

Male albino rats were allocated into 4 groups each containing 6 rats. Group I received olive oil only (1 mL/kg body weight, i.p.) as a control, Group II (CCl_4 induced) received mL/kg bodyweight of 30% CCl_4 in olive oil, i.p. The EECH Extract (250 and 500 mg/kg body weight) was admintrated (orally) once in a day and CCl_4 was administered after every 72 h. Treatment period was 10 days [4]. Blood was collected from all the animals through retro-orbital plexus. After collecting the blood, the rats were sacrificed and their livers were excise, rinsed in ice cold normal saline followed by 0.15 M Tris-HCl (pH 7.4), blotted dry and then frozen at −80 °C for further biochemical analyses.

2.6. Estimation of enzyme levels in serum

Serum was separated from the blood samples by allowing the blood to clot at room temperature for 45 min followed by centrifugation (2500 rpm at 30 °C for15 min). Serum transaminases [glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT)], alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) was estimated according to standard protocol described previously [17–19].

2.7. Estimation of bilirubin in serum

Bilirubin content was estimated according to standard protocol by Jain et al. [20].

2.8. Preparation of liver homogenate

Liver homogenates (10%) were prepared by homogenizing the liver samples in 50 mM phosphate buffer (pH 7.0) and centrifuging the homogenates at 4 °C; 10,000×*g* for 15 min. The supernatant was collected and preserved at −20 °C for further Biochemical analytical purpose.

All the Biochemical parameters were expressed in the units of activity per mg protein. The protein content in each liver homogenate was estimated according to the standard protocol [21].

2.9. Estimation of lipid peroxides

LPO in the liver was determined by the method described earlier [4] by measuring the amount of malondialdehyde (MDA).

2.10. Quantitative analysis of enzymatic antioxidants

The mean activities of superoxide dismutase SOD (units/min/mg protein) [22,23], catalase (CAT; μmol of H_2O_2 consumed/min/mg protein) [24] and glutathione peroxidase (GPx; μmol of glutathione oxidized/min/mg protein) was evaluated by the previously described standard methods [25].

2.11. Quantitative analysis of non-enzymatic antioxidants

Reduced glutathione (GSH) content was determined by its chromogenic reaction with dithio-bis-2-nitrobenzoic acid (DTNB) [26]. Vitamin C was estimated by following the standard procedure described by Santhrani et al. [27]. Vitamin E was determined according to the protocol of Ramanathan et al. [28].

2.12. Histopathological examination

Liver tissues were embedded in paraffin blocks and thin sectioning was performed according to paraffin slice techniques. The sections were further mounted on to the microscopic slides and stained with Hematoxylin and Eosin stains [29]. These microscopic slides were observed under the light microscope and photographed.

2.13. Statistical analysis

The data was calculated and analyzed by using Statistical Package for Social Sciences (SPSS) software; version 15.0. One-way Analysis of Variance for comparing the difference in the means across the groups was performed. Duncan's Multiple Range Test (DMRT) is used to identify significantly differing group means. The results presented in Tables are mean and standard error (me an ± SE).

3. Results

3.1. Acute oral toxicity

No mortality, abnormal signs and behavioral changes were observed in rats administered orally up to the dose of 2000 mg/k g b·wt. of EECH, which is the maximum recommended dose of any drug for testing acute toxicity by OECD guidelines [16].

3.2. Serum enzyme parameters

A significant increase in the levels of SGOT, SGPT, ALP and LDH in the CCl_4 administered rats (Group II) was observed, when compared to control (Group I) rats. However, a significant ($p < .05$) decrease in the levels of these enzymes was observed in the rats treated with two different doses of *C. hirsutus* extract (250 and 500 mg/kg body weight respectively) than in Group II rats (Table 1).

3.3. Serum bilirubin

A marked increase in serum bilirubin was noticed in CCl_4 exposed rats when compared to Control rats. Significant decrease in the levels of bilirubin was perceived in Group III and Group IV (250 and 500 mg *C. hirsutus* extract treated) rats on comparing to control (Group I) rats (Table 1).

3.4. Hepatic concentration of TBARS

A pronounced increase in the mean levels of TBARS was noticed in Group II (CCl_4 treated) rats on comparing to control (Group I) rats. Treatment with two doses of *C. hirsutus* ethanol extract to Group III and Group IV rats resulted in a significantly ($p < .05$) low concentrations of TBARS, apparently by hindering hepatic lipid peroxidation (Table 2).

3.5. Hepatic enzymatic and non-enzymatic antioxidants

Hepatic enzymatic and non-enzymatic antioxidants levels in all the four groups of rats are represented in Table 2. In comparison to control rats, a marked decline in the levels of both enzymatic and non-enzymatic antioxidants was noticed in the rats administered with CCl_4. Treatment with two doses of *C. hirsutus* extract in Group III and Group IV rats alleviated the CCl_4 damage by retrieving the declined levels of both enzymatic and non-enzymatic antioxidants to near control values.

3.6. Histopathological examination

Fig.1 represents the histopathological examination of hepatic tissue of all the four groups of rats. Extensive damage to the histoarchitecture of hepatic tissue (the disruption of the lattice nature of the hepatocyte, damaged cell membranes, disintegrated central vein and damaged hepatic sinusoids; Fig. 1B) of the rats exposed to CCl_4 was noticed compared to the histoarchitecture of hepatocytes in control rats (Fig. 1A). Minimum disturbance of the hepatic cellular structure was noticed in both the *C. hirsutus* extract treated groups (Fig. 1C and D), when compared to CCl_4 alone treated rats.

4. Discussion

Hepatic injuries caused by CCl_4 are the specific symptoms of xenobiotic-induced hepatotoxicity and regularly used models for the screening of hepatoprotective drugs [30]. The major causes of CCl_4 induced hepatic damage is generation of free radicals causing lipid peroxidation and contaminant decrease in antioxidant enzymes [30]. As free radicals play a vital role in CCl_4-induced hepatotoxicity, it seems reasonable that compounds that counteract such radicals may have hepatoprotective effect. Several natural products have been reported to protect against CCl_4-induced hepatotoxicity [31–34].

Spotlight of the present study was on investigating the role of *C. hirsutus* against the hepatotoxicity of CCl_4 and to understand the possible mode of action in hepatoprotection. Direct evidence of CCl_4 causing liver damage was observed by the manifestation of alterations in various hepatic parameters {elevated MDA concentration, depleted levels of non-enzymatic antioxidants (GSH, Vitamin C and E), reduced levels of antioxidant enzymes (SOD, CAT, GPx)} in CCl_4 alone administered (Group II) rats in comparison to control rats. The increase in levels of SGOT, SGPT, ALP and LDH in Group II rats also suggests the hepatotoxicity in these rats. To elucidate the hepatoprotective activity of ethanol extract of *C. hirsutus*, this plant extract was administered in the concentrations of 250 and 500 mg/kg body weight to the respective group of rats. This concentration of the extract was chosen on the basis of earlier related studies suggestion that this dose would be effective [14,35].

Serum transaminases, ALP, and LDH are well chosen specific markers of hepatic damage [36]. CCl_4 induced damage of hepatocytes can leads to effect on their transport function and membrane permeability, leading to these enzymes leakage from the cells and releasing them into the blood stream [37]. In the present study, rats induced with CCl_4 showed significant elevation of all these enzymes demonstrating serious damage of hepatocytes. Hyper bilirubinaemia which is the indicator of severe hepatic damage

Table 1
Effects of ethanol extract from *Cocculus hirsutus* on serum GOT, GPT, ALP, LDH, and bilirubin levels in control and experimental rats against CCl_4 induced toxicity.

Parameter	Group I	Group II	Group III	Group IV	ANOVA F-value
SGOT (IU/L)	90.3 ± 10.3^{a}	298 ± 8.5^{b}	185 ± 6.8^{c}	110 ± 7.3^{d}	617.01
SGPT (IU/L)	51.6 ± 12.3^{a}	321 ± 9.81^{b}	138 ± 5^{c}	98.3 ± 2^{d}	927.22
ALP (IU/L)	173.6 ± 10.1^{c}	318 ± 6.7^{a}	254 ± 5^{d}	218 ± 6^{b}	895.25
LDH (IU/L)	941 ± 14.4^{d}	2481 ± 20.4^{a}	1468 ± 11^{b}	1043 ± 15^{c}	511.19
Bilirubin (mg/dL)	1.21 ± 0.03^{c}	2.1 ± 0.06^{a}	1.8 ± 0.05^{d}	1.4 ± 0.05^{b}	38.77

Group I: (control); Group II: CCl_4 treated; Group III: CCl_4 + 250 mg/kg EECH; Group IV: CCl_4 + 500 mg/kg EECH.
Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.05 levels by Duncan's Multiple Range Test (DMRT).
All the enzyme activity units = IU/L: International Units/Liter; mg/dL: milligrams/deciliter.

Table 2
Effect of ethanol extract of *Cocculus hirsutus* on hepatic levels of TBARS, enzymatic and non-enzymatic antioxidants in control and experimental rats against CCl_4 induced toxicity.

Parameter	Group I	Group II	Group III	Group IV	ANOVA F-value
LPO	1.28 ± 0.02^a	2.48 ± 0.06^b	1.93 ± 0.04^c	1.53 ± 0.03^d	125.89
SOD	0.402 ± 0.03^d	0.21 ± 0.01^b	0.31 ± 0.01^a	0.39 ± 0.02^c	101.59
CAT	64.32 ± 5.63^c	43.12 ± 3.61^a	51.21 ± 2.61^b	59.31 ± 1.32^d	339.73
GPx	53.46 ± 3.43^d	36.46 ± 1.21^c	39.86 ± 1.05^b	44.32 ± 1.25^a	495.52
GSH	5.48 ± 0.69^a	2.43 ± 0.11^b	3.61 ± 0.06^c	4.18 ± 0.01^d	398.41
Vitamin C	3.19 ± 0.06^c	1.54 ± 0.16^d	2.01 ± 0.13^b	2.94 ± 0.03^a	580.66
Vitamin E	1.53 ± 0.02^a	0.71 ± 0.01^b	0.89 ± 0.03^c	1.19 ± 0.04^d	179.72

Group I: (control); Group II: CCl_4 treated; Group III: CCl_4 + 250 mg/kg EECH; Group IV: CCl_4 + 500 mg/kg EECH.
Values expressed are Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.05 level by Duncan's Multiple Range Test (DMRT). Lipid peroxidation (LPO) – nano moles of MDA produced/mg protein; CAT–μmoles of H_2O_2 utilized/min/mg protein; SOD–units/min/mg protein; GPX – μmoles of GSH oxidized/min/mg protein; GSH – μg of reduced glutathione/mg protein; Vitamin C – μg/mg protein; Vitamin E – μg/mg protein.

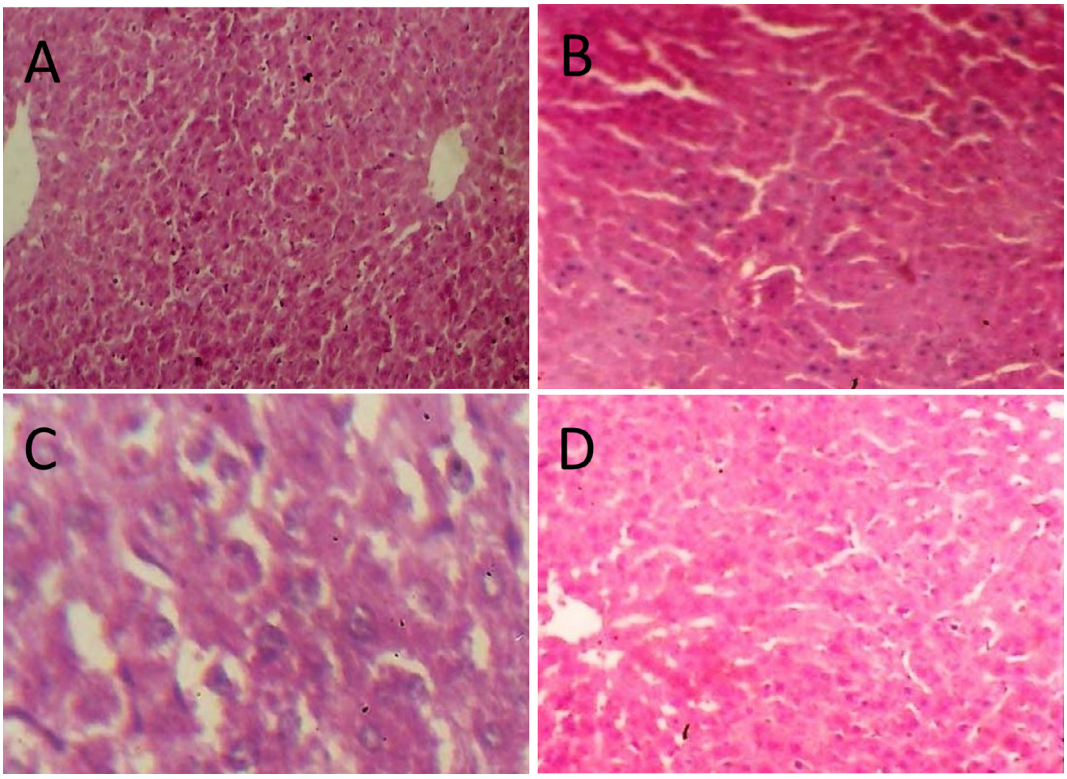

Fig. 1. Light microscopic photographs of liver histology in control and experimental rats. (A) Control mice showing normal histoarchitecture of hepatocytes, (B) A liver section of rat received CCl_4 showing hepatocellular damage, (C) A liver section of rat received CCl_4 + 250 mg/kg b·wt. EECH showing minimal damage to liver cells, and (D) A liver section of rat received CCl_4 + 500 mg/kg b. wt. EECH showing almost normal architecture of liver. Hematoxilin and Eosin stain; Original magnification ×10.

[38], was noticed in rats treated with CCl_4. Treatment with ethanol extract of *C. hirsutus*, restored all the altered levels of liver marker enzymes and bilirubin in a dose dependent manner demonstrating its curative potential to maintain the normal functional status of the hepatic tissue.

The level of TBARS is related to lipid peroxidation and the lipid peroxide levels in liver were found to be considerably elevated in CCl_4-challenged rats [39]. These free radicals trigger cell damage through two mechanisms namely covalent binding to cellular macromolecules and lipid peroxidation which affect the ionic permeability of the membrane preventing the disintegration and solubilization of membrane structure. The reduced TBARS formation after treatment with the EECH may be attributed to the antioxidant activity of the plant by scavenging the CCl_3-radical generated due to the metabolic transformation of CCl_4 in the liver. The antioxidant enzymes SOD, catalase and GPx constitute primary defense mechanisms against reactive oxygen species (ROS).

The antioxidant enzymes SOD, CAT and GPx constitute a mutually supportive group of defense against ROS. The decrease in the levels of these antioxidant enzymes in liver of CCl_4-treated rats may be due to the elevated lipid peroxidation or inactivation of the enzymes by cross-linking with malondialdehyde; this will cause massive accumulation of free radicals, which could further stimulate lipid peroxidation [40]. Administration of EECH significantly increased the activities of SOD, CAT, and GPx in the rats induced with CCl_4; this suggests administration of *C. hirsutus* ethanol extract appears to have brought about a remarkable improvement in the activity of these antioxidant enzymes in CCl_4 intoxicated rats. Similar improvement of antioxidant enzymes by supplementation of phytochemicals and extracts such as polyphenols from *Bauhinia hookeri* [41] and *Juniperus phoenicea* [42] extract against CCl_4 induced oxidative stress was recently reported.

Glutathione, Vitamin C and E protect cells against CCl_4 induced toxicity by preventing the formation of electrophiles, oxidation of

proteins sulfhydryl groups and by scavenging free radicals [43]. The key protective mechanism of glutathione against oxidative stress is acting as a co-factor for several detoxifying enzymes and also regenerate Vitamins C and E back to their active forms [38]. In the present study, rats administered with two doses of EECH leaves had observed significantly higher levels of GSH, vitamin C and E when compared with CCl_4 induced group and also near to the values obtained in normal rats. These results suggests that *C. hirsutus* extract appears to be a potent hepatoprotective agent against CCl_4 induced oxidative damage by maintaining the GSH, vitamin C and E levels. In the present study, replenishment of GSH, Vitamins C and E by the supplementation of plant extract to confer protection against CCl_4-induced liver damage, goes in accordance with Lavanya et al. (2012) [4] and Manubolu et al. (2014) [22] reports.

To provide direct evidence on protective effect of EECH against CCl_4 induced toxicity, histopathological changes in liver tissues were evaluated. Marked disruption of the cellular structure of liver was noted in rats challenged with CCl_4. Minimal disruption of the hepatocellular structure was noted in two doses of EECH treated groups of rats; these results provided supportive evidence for biochemical analysis (SGOT, SGPT, SALP, and LDH activities and MDA levels approximated to the levels in normal rats).

In conclusion, the results of this study indicated that EECH has a potent hepatoprotective activity on carbon tetrachloride induced hepatocellular destruction in rats. From the present experiments it was elucidated that the hepatoprotective nature of EECH may be due to its antioxidative and free radical scavenging properties. It also reveals that *C. hirsutus* is a robust medicinal herb for developing as a phytomedicine against hepatic disorders and further studies are essential in the direction of isolating the active principle of the plant which acts as an effective antihepatotoxic agent.

Authors' contribution

GL: Designed the research, conducted the research, analyzed the data, drafted and revised the manuscript. MM: helped with portions of conducting research and revised the manuscript.

PK and PRP: revised the manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interests

The authors declare that there are no conflicts of interest. None of the authors had any financial or personal interests in any company or organization sponsoring the research currently or at the time of research was done.

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