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Hepatoprotective potential of antioxidant potent fraction from *Urtica dioica* Linn. (whole plant) in CCl_4 challenged rats

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ABSTRACT

The aim of the present study was to isolate hepatoprotective component from *Urtica dioica* Linn. (whole plant) against CCl_4-induced hepatotoxicity *in-vitro* (HepG2 cells) and *in-vivo* (rats) model. Antioxidant activity of hydro alcoholic extract and its fractions petroleum ether fraction (PEF), ethyl acetate fraction (EAF), *n*-butanol fraction (NBF) and aqueous fraction (AF) were determined by DPPH and NO radicals scavenging assay. Fractions were subjected to *in-vitro* HepG2 cell line study. Further, the most potent fraction (EAF) was subjected to *in-vivo* hepatoprotective potential against CCl_4 challenged rats. The *in-vivo* hepatoprotective active fraction was chromatographed on silica column to isolate the bioactive constituent(s). Structure elucidation was done by using various spectrophotometric techniques like UV, IR, ^{1}H NMR, ^{13}C NMR and MS spectroscopy. Ethyl acetate fraction (EAF) of hydro-alcoholic extract of *U. dioica* possessed the potent antioxidant activity *viz.* DPPH (IC_{50} 78.99 ± 0.17 μg/ml) and NO (IC_{50} 101.39 ± 0.30 μg/ml). The *in-vitro* HepG2 cell line study showed that the EAF prevented the cell damage. The EAF significantly attenuated the increased liver enzymes activities in serum and oxidative parameters in tissue of CCl_4-induced rats, suggesting hepatoprotective and anti-oxidant action respectively. Column chromatography of most potent antioxidant fraction (EAF) lead to the isolation of 4-hydroxy-3-methoxy cinnamic acid (ferulic acid) which is responsible for its hepatoprotective potential. Hence, the present study suggests that EAF of hydro-alcoholic extract has significant antioxidant and hepatoprotective potential on CCl_4 induced hepatotoxicity *in-vitro* and *in-vivo*.

1. Introduction

Liver is one of the important organ of our body and plays a vital function in the maintenance, performance and regulating homeostasis of our body [45]. Liver disorders have become one of the serious health problems and a major cause of morbidity and mortality all over the world. Nearly 20,000 deaths and 250,000 new cases have been reported every year [42]. The percentage of liver toxicity due to various exposures is much higher in developing countries like India (8–30%) compared to advanced countries (2–3%) [51]. Oxidative stress plays a major role in the development of liver diseases. The liver injury is initiated by the various toxic agents produced by chemicals, alcohol, viruses or by their bio-activation to chemically reactive metabolites. These metabolites can be free radicals, which either elicits an immune response or directly affects the biochemistry of the cells by interacting with cellular macromolecules. Even after the advancement in modern system of medicine, there is absence of a reliable synthetic liver protective drug. Hence, natural extracts /products from medicinal plants are considered to be safe and effective for the treatment of liver disorders [62]. The plants are the rich source of bioactive compounds viz. natural polyphenols and a number of them are being used in medicine for liver ailments [65]. The phytoconstituents (polyphenols) are potent antioxidant and proved to be Hepatoprotective and are used in the treatment of chronic liver injuries [54].

Experimental models of hepatotoxicity can be produced by alcohol, paracetamol, CCl_4 *etc.* The CCl_4, is the most common hepatotoxic agent used for experimental induction of liver fibrosis [8].

Abbreviations: UD, *Urtica dioica*; PEF, petroleum ether fraction; EAF, ethyl acetate fraction; NBF, *n*-butanol fraction; AF, aqueous fraction; SGOT, serum glutamate oxaloacetate transaminase; SGPT, serum glutamate pyruvate transaminase; ALP, alkaline phosphatase; CCl_4, carbon tetrachloride; MDA, malondialdehyde; CAT, catalase; GSH, glutathione; OD, optical density; HepG2, human hepatocellular carcinoma cells.

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This model has been used in studies to examine the deposition of extracellular matrix in the fibrotic and cirrhotic liver [40]. CCl_4 is metabolized by cytochrome P4502E1 to the trichloromethyl radical ($^0CCl_3$) and peroxy trichloromethyl radical ($^0OOCCl_3$). It has been reported that one of the cause of CCl_4-induced liver injury is lipid peroxidation, which is induced and accelerated by free radical derivatives of CCl_4 [35].

Urtica dioica Linn. (UD) belonging to family Urticaceae is an annual and perennial plant which is commonly known as stinging nettle [32]. The vernacular names of this plant are Bichu Butti in Hindi and Punjabi, Vrishchhiyaa-shaaka in Sanskrit and Shisuun in (Kumaon) folk language [28,6]. Traditionally, the leaves and roots of this plant are used internally as a blood purifier, emmenagogue, diuretic, nasal and menstrual haemorrhage, rheumatic pain, colds and cough [48], liver insufficiency [63], stomachache [64], eczema, anemia, nephritis, haematuria, jaundice, menorrhagia and diarrhea [28,57,61]. The different types of medicinal important phytoconstituent present in UD are steroids [5], terpenoids [13], phenylpropanoids, coumarins [4], polysaccharides [59] and lectins [12], flavonol glycosides (kaempherol-3-*O*-glucoside, and -3-*O*-rutinoside; quercetin-3-*O*-glucoside, and -3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside, -3-*O*-rutinoside and -3-*O*-neohesperidoside) [5]. The plant has been reported to have immunostimulatory, anticarcinogenic, anti-inflammatory, antioxidant, antiallergenic [15,1], antiandrogenic [41], hepatoprotective [27], hypoglycemic [17], antiviral [2] activities. Supplementation of UD leaves beverage has been shown to have a significant protective effect against trichloroacetic acid induced liver injury [3,26]. However, there is not any report available on the bioactivity guided fractionation leading to isolation of hepatoprotective component. Hence, the present study was designed to investigate the hepatoprotective activity of potent antioxidant fraction (EAF) of *U. dioica* Linn. (whole plant) against CCl_4 induced hepatotoxicity *in-vitro* and *in-vivo*.

2. Materials and methods

2.1. Material

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), L-ascorbic acid, sodium nitroprusside, sulphanilamide (Sigma–Aldrich Co., Mumbai). Phosphoric acid (H_3PO_4), *N*-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) (Rankem Ltd., New Delhi). Fetal bovine serum (FBS), phosphate buffered saline (PBS) and dulbecco's modified eagle medium (DMEM) were obtained from Himedia Lab Pvt. Ltd., Mumbai. 3-(4,5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), carbon tetrachloride (CCl_4), silymarin, trichloro acetic acid (TCA), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), were purchased from sigma aldrich, Co., Mumbai. The diagnostic kits for serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), total protein (TP) and total bilirubin (TB) were purchased from calkine and coral private Ltd. All solvents used were of analytical grade and purchased from Rankem (Deejay Corporation, Jalandhar). Thin layer chromatography (TLC) was performed using silica gel $60F_{254}$ (E-Merck). Silica gel (60–120 mesh) used for column chromatography was purchased from CDH (Chemical Corporation, Ludhiana). ^1H NMR and ^{13}C NMR spectras were recorded on bruker 400 MHz spectrometer using TMS (Tetramethylsilane) as the internal standard and mass spectra were recorded on ESI-esquire 3000 bruker daltonics instrument. The HepG2 cell line was obtained from National Center for Cell Sciences NCCS, Pune (India).

2.2. Plant material

The whole plant of UD was collected from the local areas of Ranikhet, Uttarakhand, India (August–September 2013) and authenticated by Dr. Sunita Garg from NISCAIR, New Delhi. The voucher specimen (Ref. NISCAIR/RHMD/Consult/2008-9/1192/224) was deposited at the Department of Raw Material Herbarium and Museum (NISCAIR). Plant drug was shade dried (<40 °C), coarsely powdered and stored in air tight container.

2.3. Extraction and fractionation

The coarsely powdered drug (500 g) was extracted by continuous hot extraction process using soxhlet apparatus with 80% (v/v) alcohol. The hydro-alcoholic extract was filtered and concentrated under reduced pressure to obtain a green semi-solid residue. This hydro-alcoholic extract was suspended in water (500 ml) and sequentially partitioned with different solvents *viz.*, petroleum ether, ethyl acetate, *n*-butanol and aqueous in increasing order of polarity. The fractions obtained were concentrated under reduced pressure and yield was calculated.

2.4. Phytochemical screening

The UD extract and its fractions (PEF, EAF, NBF and AF) was qualitatively tested for the presence of phytochemicals as per described standard methods [11,21,56].

2.5. In-vitro *Free radical scavenging activity*

2.5.1. DPPH radical scavenging activity

The antioxidant activity of UD whole plant extract and its fraction were assessed by determining its ability to scavenge free radicals. 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical [49]. The 0.1 mM solution of DPPH in methanol was prepared. 1 ml of this solution was added to 2 ml of test drug solution at different concentration (50–250 μg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm. Ascorbic acid was used as standard. The percentage of scavenging activity was determined using the following formula:

$$\text{Percentageofinhibition}(\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

where, A_{control} − absorbance of DPPH, A_{sample} − absorbance of DPPH with test sample.

2.5.2. Nitric oxide scavenging activity

Nitric oxide radical scavenging activity was performed according to the method of [14]. Nitric oxide radical is generated from reaction mixture containing sodium nitroprusside (20 mM) in phosphate buffered saline (pH 7.4) when incubated at 25 °C for 30 min [37]. The nitric oxide radical thus generated interacts with oxygen to produce nitrite ion, which is assayed by mixing with an equal amount of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) [24] and its absorbance was measured at 570 nm. Decrease in absorbance in the presence of different concentrations of test sample (50–250 μg/ml) indicated the nitric oxide scavenging activity. The ascorbic acid was used as standard. The percentage of nitric oxide scavenging activity was determined using the Formula (1).

2.6. In-vitro *CCl_4 induced toxicity in HepG2 cell line*

The monolayer HepG2 cell culture was trypsinized and cell count was adjusted to 1.0×10^5 cells/ml using DMEM medium

containing 10% FBS. Cells were maintained in 5% CO_2 humidified incubator at 37 °C. Subculturing was done by trypsinization (0.25%) when they were reached 80% confluency. To investigate the possible toxic effect, the cells were treated with different fractions of UD at concentration ranging from (10–100 μg/ml) for 24 h. Similarly, to induce the toxicity, cells were treated with toxicant (medium containing 1% (v/v) CCl_4) at a concentration 100 μg/ml for 24 h prior to each experiment. The cells were pre-treated with different fraction of UD for 2 h before the addition of toxicant. After 24 h, cells viability was determined by MTT assay.

2.6.1. Cell viability study using MTT assay

MTT assay was performed as described previously [38,58]. HepG2 cells in the exponential phase were seeded onto 96 well plates (1×10^4 cells/well), allowed to stay (for 24 h), and treated with various concentrations of different fractions of UD, and standard (silymarin). The culture medium was removed and cells were washed with PBS. 100 ml of the MTT stock (5 mg/ml) was added to each well. After 4 h of incubation, solution was removed and 100 μl of DMSO was added. After 10 min, the absorbance (O.D) was read at 540 nm on an ELISA reader (Tecan, Austria). The data was recorded using the software. The percentage viability was calculated as follows:

$$\%\text{cell viability} = \frac{\text{Mean O.D. of treated wells} - \text{Mean O.D. of blank wells}}{\text{Mean O.D. of control wells} - \text{Mean O.D. of blank wells}} \times 100$$

Control well—cells without test drug, treated well—cells with test drug, blank well—media only.

2.7. In-vivo CCl_4 induced hepatotoxicity in rats

2.7.1. Experimental animals

Young Wistar rats (180–200 g) breed in the Central Animal House, I.S.F. College of Pharmacy, Moga, Punjab, (India) were used in the study. Animals were acclimatized to laboratory conditions at room temperature prior to experimentation and kept under standard conditions of a 12 h light/dark cycle with food and water ad libitum in polyacrylic cages. All the experiments were carried out between 09.00 and 16.00 h. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of college (ISFCP/IAEC/CPCSEA/2013/149) and carried out in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India on animal experimentation.

2.7.2. Experimental protocol and procedure

Rats were divided into six groups consisting of six animals in each group.

Group I received distilled water containing 0.5% sodium carboxymethylcellulose (CMC–Na) (1 ml/kg body weight, p.o.) for 7 days, and olive oil (1 ml/kg body weight, s.c.) on days 2 and 3.

Group II (CCl_4) received 0.5% CMC–Na (1 ml/kg body weight, p.o.) for 7 days, and a 1:1 mixture of CCl_4 and olive oil (2 ml/kg body weight, s.c.) on days 2 and 3.

Group III was treated with the standard drug silymarin (50 mg/kg body weight, p.o.) [53] daily for 7 days and also received the CCl_4–olive oil mixture (1:1, 2 ml/kg body weight, s.c.) on days 2 and 3, 30 min after administration of silymarin.

Groups IV–VI (test group animals) was administered a dose of 20, 40, and 80 mg/kg body weight of EAF (p.o.) for 7 days. Additionally, 30 min after administration of EAF, they received a dose of the CCl_4–olive oil mixture (1:1, 2 ml/kg, s.c.) on days 2 and 3.

On day 7, animals were anaesthetized by ketamine, blood was collected by retro-orbital puncture, allowed to clot, and serum was separated for assessment of enzyme activity. The rats were sacrificed by cervical dislocation; the livers were carefully dissected, rinsed with ice-cold isotonic saline (0.9% sodium chloride) and weighed. A 10% (w/v) tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at $10{,}000 \times g$ for 15 min and aliquots of the supernatants were separated and used for tissue biochemical estimation. Some parts of the liver tissue were immediately transferred into 10% formalin for histopathological investigation.

2.7.3. Estimation of serum biochemical parameters

Biochemical parameters were assayed according to standard methods. Activity of the following serum enzymes was measured: serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP), using the method of [29]. Total bilirubin (TB) was measured by the method of [36]. Serum biochemical parameters were estimated using commercial enzymatic biochemical diagnostic kits.

2.7.4. Estimation of Tissue biochemical parameters

2.7.4.1. Measurement of lipid per oxidation. The extent of lipid per oxidation in the liver was determined quantitatively by performing the method as described by [43]. The amount of malondialdehyde (MDA) was measured by reaction with thiobarbituric acid at 532 nm using Schimadzu spectrophotometer (Japan). The values were calculated using the molar extinction coefficient of chromophore ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$) and expressed as percentage of control.

2.7.4.2. Estimation of nitrite. The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide was determined by a colorimetric assay with Greiss reagent (0.1% *N*-(1-Napththyl) ethylenediamine dihydrochloride, 1% sulphanilamide and 5% phosphoric acid). Equal volumes of the supernatant and the Greiss reagent were mixed and the mixture was incubated for 10 min at room temperature in the dark. The absorbance was measured at 540 nm using Schimadzu spectrophotometer (Japan). The concentration of nitrite in the supernatant was determined from sodium nitrite standard curve and expressed as percentage of control [19].

2.7.4.3. Estimation of reduced glutathione levels. Reduced glutathione was estimated according to the method described by [10]. 1 ml supernatant was precipitated with 1 ml of 4% sulphosalicylic acid and cold digested for 1 h at 4 °C. The samples were then centrifuged at $1200 \times g$ for 15 min at 4 °C. To 1 ml of the supernatant obtained, 2.7 ml of phosphate buffer (0.1 mmol/l, pH 8) and 0.2 ml of 5,5′dithio-bis (2-nitrobenzoic acid) (DTNB) was added. The yellow color developed was measured at 412 nm using Schimadzu spectrophotometer (Japan). Results were calculated using molar extinction co-efficient of the chromophore ($1.36 \times 10^4\ (mol/l)^{-1}\ cm^{-1}$) and expressed as percentage of control.

2.7.4.4. Catalase estimation. Briefly, the assay mixture consisted of 12.5 mM H_2O_2 in phosphate buffer (50 mM of pH 7.0) and 0.05 ml of supernatant from the tissue homogenate (10%) and the change in absorbance was recorded at 240 nm. The results were expressed as mM of H_2O_2 decomposed per milligram of protein/min [33].

2.7.4.5. Protein estimation. The protein content was estimated by Biuret method [18] using bovine serum albumin as a standard.

Table 1
Physical properties of hydroalcoholic extract of UD and its various fractions.

Extract /fraction	Color	Consistency	% Yield (w/w)
Hydro-alcoholic extract	Greenish brown	Semi-solid	11.95
PEF	Greenish yellow	Solid mass	1.30
EAF	Dark green	Semi-solid	4.50
NBF	Dark brown	Semi-solid	2.90
AF	Light brown	Semi-solid	3.25

2.7.5. Histopathological studies

Liver tissues were fixed in 10% formalin for at least 24 h, embedded in paraffin, and cut into 5 μm-thick sections using a rotary microtome. The sections were stained with Haematoxylin–eosin dye and observed under a microscope (Olympus, Japan) to observe histopathological changes in the liver.

2.7.6. Statistical analysis

All experiments were done in triplicate and results were reported as mean ± S.E.M. ($n = 6$). The data were analyzed by one-way ANOVA, and statistically significant effects were further analyzed by means comparison using Tukey's multiple comparison analysis. The $p < 0.05$ was considered to be statistically significant.

2.8. Isolation of compound

On the basis of *in vitro* (antioxidant, cell line studies) and *in vivo* (hepatoprotective studies), potent fraction EAF (5.00 g) was charged into silica gel (60–120 mesh size) column. The column was eluted in gradient manner by using Hexane; Hexane: DCM, (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9), DCM; DCM: ethyl acetate (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9), ethyl acetate; ethyl acetate: methanol (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9) and methanol. Total 580 fractions were collected. Eluents were monitored using TLC on different solvent system. The similar fractions were pooled in to 7 major sub fraction (Fr-A, B, C, D, E, F) all these sub fraction were subjected to antioxidant study. The potent fraction was kept for crystallization for isolation of pure compounds. Structure elucidation of the isolated compound(s) was carried out by melting point and spectral techniques; IR, ^{1}H NMR, ^{13}C NMR and MS.

2.9. HPTLC fingerprinting analysis of potent antioxidant fraction (EAF) of UD

EAF was analyzed for the presence of compound by comparing with R_f value and spectral comparison with co-chromatographic standard compound ferulic acid. Chromatography was performed on precoated aluminium silica gel $60F_{254}$ (E-Merck) (4 cm × 10 cm) plates. EAF and standard compound of known concentrations were applied to the layers as 6 mm-wide bands positioned 15 mm from the bottom and 15 mm from side of the plate, using Camag Linomat 5 automated TLC applicator with the nitrogen flow providing a delivery speed of 90 nL/s from the application syringe. These conditions were kept constant throughout the analysis of the samples. Following sample application, layers were developed in a Camag twin through glass chamber that had been presaturated with the mobile phase of toluene: ethyl acetate: formic acid (8:2:0.4), the developed plate were dried with a hair dryer and scanned at the 366 nm with Camag U.V. scanner.

3. Results

The physical properties and percentage (%) yield of hydro alcoholic extract and its various fractions are mentioned in Table 1.

Table 2
Preliminary phytochemical screening of hydroalcoholic extract of UD and its fractions.

Class of compound	Hydro alcoholic extract	PEF	EAF	NBF	AF
Carbohydrates	+	–	–	–	+
Glycosides	+	–	–	+	+
Proteins	+	–	–	–	+
Steroids and triterpenoids	+	+	+	+	–
Phenolic compounds	+	–	+	+	+
Flavonoids	+	–	+	–	+
Amino acids	+	–	–	–	+
Alkaloids	–	–	–	–	–
Saponins	+	–	–	–	+

(+) Present, (–) Absent.

3.1. Phytochemical studies

Preliminary phytochemical screening of hydroalcohalic extract and its various fractions are shown in Table 2.

3.2. In-vitro *free radical scavenging activity*

3.2.1. DPPH radical scavenging activity

The antioxidant activity of hydro alcoholic extract and its fractions was determined by its capacity to scavenge DPPH radical. The hydro alcoholic extract and its fractions PEF, EAF, NBF and AF showed DPPH radical scavenging activity with an IC_{50} of 140 ± 0.76 μg/ml, 215.96 ± 0.06 μg/ml, 78.99 ± 0.17 μg/ml, 168.24 ± 0.34 μg/ml, 302.90 ± 0.14 μg/ml respectively. Ascorbic acid (IC_{50} 26.24 ± 0.19 μg/ml) showed an excellent activity. The EAF has shown significant free radical quenching capacity when compared to hydroalcoholic extract and other fractions.

3.2.2. Nitric oxide scavenging activity

The nitric oxide scavenging activity of hydroalcoholic extract and its fractions was determined based on the inhibition of nitric oxide radical generation from sodium nitroprusside in buffer saline and measured by Griess reagent. The hydro alcoholic extract and its fractions PEF, EAF, NBF and AF showed NO radical scavenging activity with an IC_{50} of 161.29 ± 0.41 μg/ml, 172.38 ± 0.63 μg/ml, 101.39 ± 0.30 μg/ml, 141.23 ± 0.80 μg/ml and 202.26 ± 0.67 μg/ml respectively. The standard ascorbic acid IC_{50} was 45.76 ± 0.62 μg/ml. The EAF has shown the significant NO free radical scavenging ability in comparison to hydroalcoholic extract and other fractions.

3.3. In-vitro *CCl_4 induced toxicity in HepG2 cell line*

3.3.1. Cytoprotective effect of UD fractions in HepG2 cells

The exposure of HepG2 cells to various concentrations (10–100 μg/ml) of UD fractions (PEF, EAF, NBF, and AF) alone for 24 h did not alter the viability. However, exposure of cells to 1% (v/v) CCl_4-induced significant cell death. The cell viability was almost half of control after 24 h exposure (40.66 ± 1.85). Following pretreatment of cells with various concentrations (10–100 μg/ml) of UD fractions, exposure to 1% (v/v) CCl_4 did not drastically affect the cell viability. The pretreatment with EAF have prevented, the cell death and percentage cell viability was concentration dependent. The result of cell viability are depicts in Table 3.

3.4. In-vivo *CCl_4 induced hepatotoxicity in rats*

3.4.1. Effect of potent antioxidant fraction (EAF) on hepatic markers

The hepatoprotective effect of EAF was assessed by measuring liver-related biochemical parameters following CCl_4 induced

Table 3
Protective effect of various fraction of UD on CCl_4 induced toxicity in HepG2 cell line.

Group no.	Experimental groups	Cell viability (%)
Control	Normal control	100
Toxicant control	CCl_4 control (1%, v/v)	40.66 ± 1.85
Silymarin treatment		
	Silymarin (10 μg/ml) + CCl_4 (1% v/v)	54.36 ± 2.58
	Silymarin (25 μg/ml) + CCl_4 (1% v/v)	68.15 ± 1.80
	Silymarin (50 μg/ml) + CCl_4 (1% v/v)	77.08 ± 1.59
	Silymarin (100 μg/ml) + CCl_4 (1% v/v)	87.94 ± 3.30
UD fractions treatment		
	PEF (10 μg/ml) + CCl_4 (1% v/v)	35.36 ± 2.01
	PEF (25 μg/ml) + CCl_4 (1% v/v)	38.45 ± 1.99
	PEF (50 μg/ml) + CCl_4 (1% v/v)	46.39 ± 1.69
	PEF (100 μg/ml) + CCl_4 (1% v/v)	50.20 ± 1.88
	EAF (10 μg/ml) + CCl_4 (1% v/v)	52.17 ± 1.08
	EAF (25 μg/ml) + CCl_4 (1% v/v)	65.71 ± 1.23
	EAF (50 μg/ml) + CCl_4 (1% v/v)	74.38 ± 2.13
	EAF (100 μg/ml) + CCl_4 (1% v/v)	83.23 ± 1.60
	NBF (10 μg/ml) + CCl_4 (1% v/v)	34.24 ± 2.22
	NBF (25 μg/ml) + CCl_4 (1% v/v)	41.70 ± 2.41
	NBF (50 μg/ml) + CCl_4 (1% v/v)	59.32 ± 3.36
	NBF (100 μg/ml) + CCl_4 (1% v/v)	63.35 ± 2.12
	AF (10 μg/ml) + CCl_4 (1% v/v)	37.27 ± 2.27
	AF (25 μg/ml) + CCl_4 (1% v/v)	46.20 ± 2.94
	AF (50 μg/ml) + CCl_4 (1% v/v)	50.47 ± 3.05
	AF (100 μg/ml) + CCl_4 (1% v/v)	54.75 ± 3.16

Values were as expressed mean ± S.E.M. of three independent experiments carried out in triplicates.

hepatotoxicity. The activity of the enzymes SGOT, SGPT, ALP and TB levels were significantly increased in the CCl_4-control group compared to the normal control group ($p < 0.05$). However, rats treated with EAF (20, 40 and 80 mg/kg) significantly attenuated the increase activities of liver enzymes (SGOT, SGPT and ALP) and TB levels in dose dependently in the CCl_4-treated rats ($p < 0.05$) suggesting hepatoprotective potential. Moreover, the administration of standard silymarin (50 mg/kg) showed a significant ($p < 0.05$) hepatoprotective potential against CCl_4 induced liver injury (Table 4).

3.4.2. *Effect of EAF on oxidative stress parameters (lipid peroxidation, nitrite, Catalase and reduced glutathione) in CCl_4 induced hepatotoxicity in rats*

Chronic administration of CCl_4 significantly caused oxidative stress (increased MDA level, nitrite concentration, depleted catalase and reduced glutathione enzyme activity) as compared to vehicle treated group. The antioxidant fraction (EAF) (20, 40 and 80 mg/kg) treated group of rats significantly attenuated oxidative stress (MDA levels, nitrite concentration and restored the level of endogenous antioxidant enzyme viz. catalase and reduced GSH) dose dependently as compared to CCl_4 treated rats indicating antioxidant effect. Moreover, the administration of standard silymarin (50 mg/kg) significant ($p < 0.05$) attenuated the oxidative damage in CCl_4 induced liver injury (Fig. 1a–d).

3.4.3. *Histopathological studies*

The presence of cell injury in livers by CCl_4 was revealed by histopathological examinations. In the photomicrographs of hematoxylin eosin stained liver tissues, normal control hepatocytes had normal architecture (Fig. 2A). Severe hepatocyte necrosis, fatty degeneration, vacuolation were found in rats 24 h after CCl_4 treatment (Fig. 2B). The effects of silymarin (50 mg/kg body weight) on liver histopathology of CCl_4 treated rat are presented in (Fig. 2C). Pretreatment of EAF of UD at 20, 40 and 80 mg/kg body weight reduced the severity of hepatocells of CCl_4 induced liver injury (Fig. 2D–F). These results clearly indicate the protection provided by potent antioxidant EAF of UD.

3.5. *Structure elucidation of isolated compound*

The sub fraction (Fr-E) isolated from column has shown the significant antioxidant potential with (IC_{50} value 40.21 ± 0.20 μg/ml) as compared to other fraction in DPPH free radical scavenging assay. The potent sub fraction (Fr-E) subjected to crystallization. A pure compound obtained as colorless crystal, 18 mg; R_f 0.39 (toluene: ethyl acetate: formic acid, 6:3.5:0.5); having a melting point 168 °C. Compound gave positive $FeCl_3$ test for phenolics [39]. UV λmax (methanol): 318 nm, The IR (KBr) cm^{-1} spectrum showed the absorption band 3436 (—OH str.), 2923 (—CH_3 aliphatic str.), 1690 (>C=O str.), 1664 (>C=C< str.), 1466 (>C=C<), 1035 (C—O str.). The molecular formula, $C_{10}H_{10}O_4$ was determined by Mass spectrum with [M+H] at m/z 194.0. Further, when compound was subjected to ^{1}H NMR (DMSO-d6, 400 MHz) chemical shift, δ in ppm, coupling constant, 3.86 (3H,s), 6.27(1H,d, J = 16.0 Hz), 6.81(1H,d, J = 8.0 Hz), 7.0 (IH, dd, J = 2.0 Hz, J = 2.0 Hz.), 7.49 (1H,d, J = 16.0 Hz.), 7.15 (1H,s), 9.32(1H,s), 11.96(1H,s). ^{13}C NMR (DMSO-d6, 100 MHz) δ: 55.48(—OCH_3), 110.49(C5 (Ar.), 115.36(C6, C1′ (Ar.), 122.41(C2 (Ar.), 125.68(C1 (Ar.), 144.26(C2′(>C=C<), 147.67–148.89(C3, C4 (Ar.), 168.00(—COOH). On the basis of spectral analysis the compound was characterized as ferulic acid (Fig. 3).

3.6. *HPTLC fingerprinting analysis of potent antioxidant fraction (EAF) of UD*

The optimized high resolution HPTLC profile was achieved in the mobile phase of toluene: ethyl acetate: formic acid (8:2:0.4) at wavelength of 366 nm. The HPTLC analysis confirmed the presence of ferulic acid (R_f 0.39) (Figs. 4 and 5).

Table 4
Effect of potent antioxidant fraction (EAF) of UD on biochemical parameters of CCl_4 damaged livers in rats.

Groups	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	TB (mg/dl)
Normal-control	25.98 ± 3.76	14.23 ± 4.42	128.1 ± 7.04	0.25 ± 0.02
CCl_4-control	183.60 ± 5.67[a]	159.1 ± 7.07[a]	275.9 ± 6.79[a]	1.19 ± 0.04[a]
Silymarin (50 mg/kg)	48.42 ± 6.04[b]	36.66 ± 3.89[b]	150.5 ± 6.95[b]	0.31 ± 0.02[b]
EAF (20 mg/kg)	138.40 ± 5.79[b]	116.1 ± 6.42[b]	237.9 ± 7.26[b]	0.80 ± 0.03[b]
EAF (40 mg/kg)	75.99 ± 4.02[b,c]	72.60 ± 4.42[b,c]	191.4 ± 11.67[b,c]	0.55 ± 0.02[b,c]
EAF (80 mg/kg)	53.25 ± 5.51[b,c,d]	44.52 ± 3.93[b,c,d]	157.0 ± 5.99[b,c,d]	0.39 ± 0.02[b,c,d]

Values were expressed as mean ± S.E.M.
[a] $p < 0.05$ *vs.* normal control.
[b] $p < 0.05$ *vs.* CCl_4 control group.
[c] $p < 0.05$ *vs.* EAF fraction (20 mg/kg).
[d] $p < 0.05$ *vs.* EAF fraction (40 mg/kg).

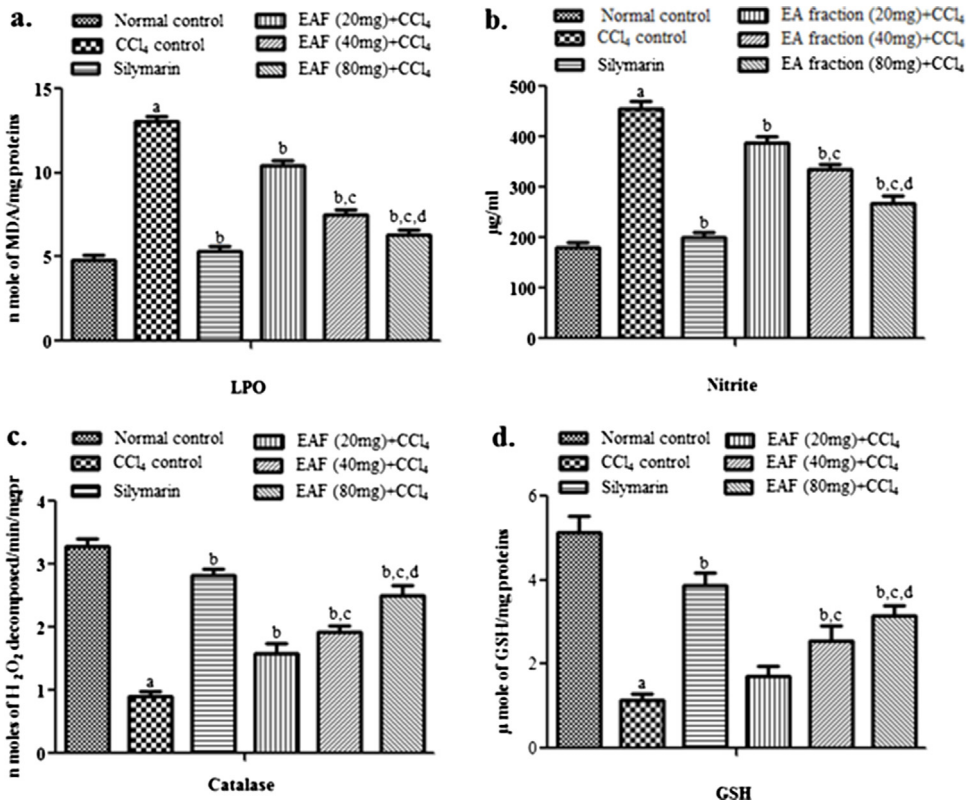

Fig. 1. Effect of antioxidant fraction (EAF) of UD on biochemical alteration in CCl_4 treated rats. a. MDA level b. Nitrite concentration c. Catalase d. Reduced glutathione (GSH). Results are expressed as mean ± S.D; $^{a}p < 0.05$ *vs.* normal control; $^{b}p < 0.05$ *vs.* CCl_4 control group; $^{c}p < 0.05$ *vs.* EAF (20 mg/kg), $^{d}p < 0.05$ *vs.* EAF (40 mg/kg).

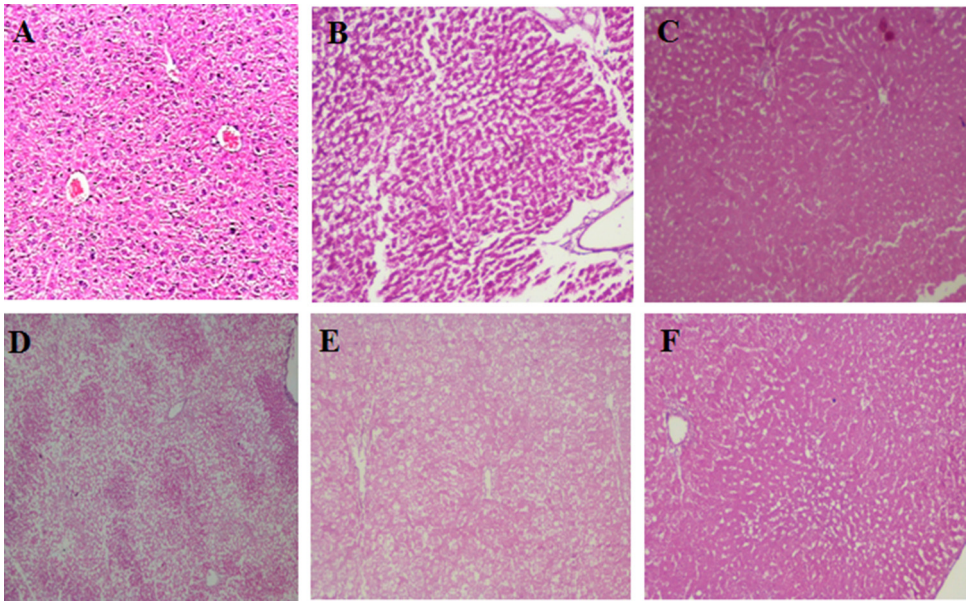

Fig. 2. Effect of EAF on hepatic cells in liver tissue of CCl_4 induced liver injury in rats. Sections are 6 μm thick and photomicrographs are taken at 100×. (A) Normal control group; (B) CCl_4 control group; (C) Silymarin standard groups; (D) EAF (20 mg/kg) treatment group; (E) EAF (40 mg/kg) treatment group; (F) EAF (80 mg/kg) treatment group.

4. Discussion

Oxidative stress is a process where the physiological balance between pro-oxidants and antioxidants is disrupted, resulting in potential damage for the organism [34]. Alteration in oxidative defence balance is responsible for liver related disorders which remains one of the serious health problems worldwide [25]. The natural antioxidants counteract the oxidative stress induced by

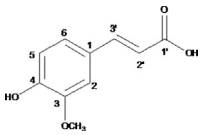

Fig. 3. Structure of 4-hydroxy-3-methoxy cinnamic acid (ferulic acid).

hepatotoxins [52]. Therefore, the present study was designed to investigate the hepatoprotective potential of potent antioxidant fraction of *U. dioica* Linn. (whole plant) against CCl_4 induced hepatotoxicity *in-vitro* and *in-vivo*. Preliminary phytochemical screening of EAF showed the presence of triterpenoids, flavonoids and phenolic compounds. These compounds have been previously reported to have antioxidant as well as hepatoprotective potential [30,47]. EAF of UD showed promising antioxidant activity in DPPH and NO radical scavenging assay.

Antioxidant activity of UD fraction on DPPH and NO radicals may be attributed to a direct role in trapping free radicals by donating hydrogen atom or electron. The antioxidant activity of (EAF) may be due to the high flavonoids and phenolic contents as phenolic compounds received attention for their high antioxidant activity [46].

HepG2 cells are considered as a reasonable model for studying *in-vitro* xenobiotics metabolism and toxicity to liver, since they maintain majority of specialized functions like normal human hepatocytes [31]. The percent cell viability has been determined using MTT assay. It is helpful to predict the cell damage [26]. The percentage cell viability in MTT assay showed that EAF significantly ($p < 0.05$) prevented the damage that was induced by CCl_4 in the HepG2 cells.

As per the *in-vitro* antioxidant and cell line study, the EAF fraction has shown the promising antioxidant potential and cytotoxic potential, so it was selected for *in-vivo* studies. The results demonstrated that potent antioxidant fraction (EAF) of UD attenuates the CCl_4-induced elevation of serum SGOT, SGPT, ALP and TB levels and oxidative damage (attenuated lipid peroxidation, nitrite levels; restored catalase and GSH levels).

CCl_4 is conventionally used to induce liver injury in rats, followed by testing of plant extract for their liver protecting property. CCl_4 is actively metabolized in the liver tissues to its highly reactive trichloromethyl free radical $CCl_3{}^0$. Trichloromethyl free radical reacts with cellular macromolecular protein and polyunsaturated fatty acids in presence of molecular oxygen to form more toxic trichloromethyl peroxyl radicals along with H_2O_2, O_2, OH that leads to liver damage [34].

The liver injury induced by CCl_4 elevates the liver marker enzymes and release them in to the blood [9]. Treatment with potent antioxidant fraction (EAF) of UD decreased the serum levels of SGOT and SGPT toward their respective normal value that is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by toxicant. Instead of ALP is a marker of pathological alteration in biliary flow [44]. CCl_4 induced elevation of ALP is in line with high levels of serum bilirubin. The depletion of increased ALP activity with simultaneous suppression of raised bilirubin level indicates the stabilization of biliary dysfunction in rat liver during the hepatic injury. The effective control of ALP and bilirubin levels in treated groups points toward an early improvement in the secretary mechanism of hepatocytes.

Increase in MDA levels, as evident in CCl_4 treated experimental rats, suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. The antioxidant system of liver is also affected through the lipid peroxidative degradation of biomembrane, which is the major cause of hepatotoxicity [16].

The EAF has significantly reduced the elevated nitrite concentration. The exposure to reactive and nitrogen species RNOS, may cause the lipid peroxidation in cell membranes, which generates reactive species that damage the cell proteins and promote their degradation [7]. Nitrite is a stable metabolite of NO. It can be used as marker of the overall formation of NO. The increased nitrite level as a result of increased NOS activity have been observed in liver homogenate of rats when exposed to CCl_4, indicating that animal suffered from the oxidative and nitrosative stress.

Catalase plays a vital role in protection against the deleterious effects of hydrogen peroxide and lipid peroxidation in diseases related to oxidative stress [66,60]. The GSH act as non-enzymatic antioxidant bio-molecules present in tissue. It is to remove the free oxygen species, such as H_2O_2, superoxide anions & alkoxy radicals, maintenance of membrane protein thiols, and it acts as a substrate for GP*x* and glutathione *S*-transferase (GST) [55]. GSH maintaining the body's antioxidant defence mechanism conjugates with free radicals directly to protect the integrity of cell membranes [22].

Further, EAF significantly restored the reduced GSH and CAT level and thus prevented the lipid peroxidation. The EAF has

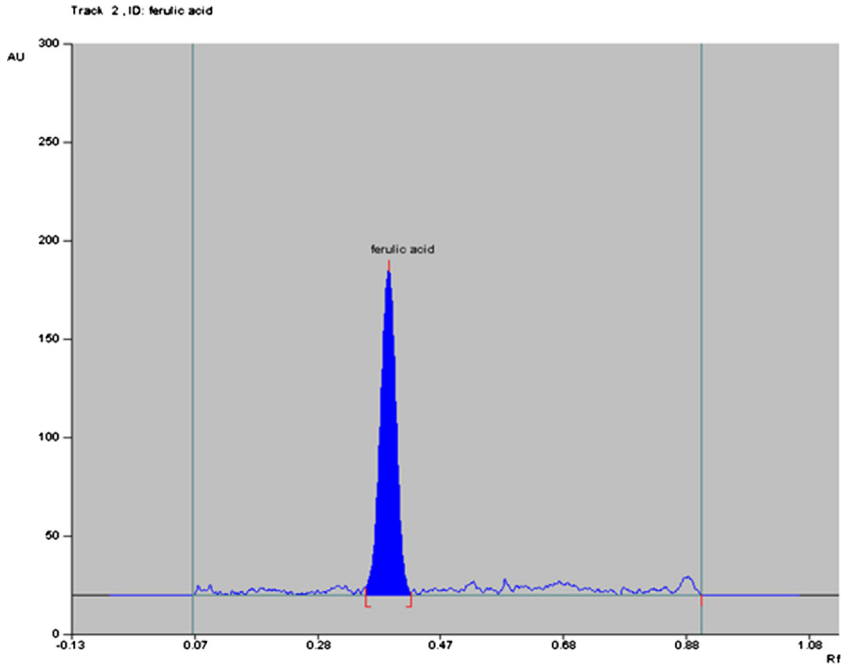

Fig. 4. HPTLC densitometric scan (at 366 nm) of ferulic acid.

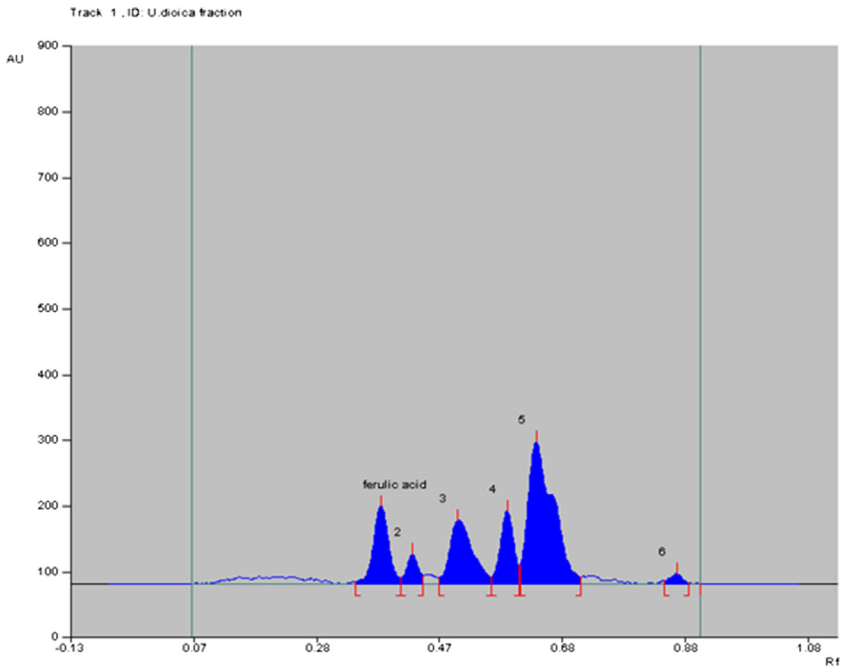

Fig. 5. HPTLC densitometric scan (at 366 nm) of potent antioxidant fraction (EAF).

also scavenged reactive free radicals that lessen oxidative damage to the liver tissue and improve the activities of the hepatic antioxidant enzymes. The hepatoprotective potential of the EAF is dose-dependent as the result have shown (80 mg/kg) maximum reduction in MDA level, nitrite concentration and resorted the catalase, reduced GSH level (Fig. 1).

Additionally, histological examination of liver sample showed chronic necrosis in CCl_4 treated rat. When severe liver injury induced by CCl_4 was markedly reduced by the administration of EAF (20, 40, 80 mg/kg) and silymarin (50 mg/kg), as evident by presence of normal cellular boundaries, lesser fatty changes, absence of necrosis, and ballooning degeneration, broad infiltration of lymphocytes. The *in-vitro* and *in-vivo* antioxidant activities of EAF may be associated with the flavonoids, phenolic, and terpenoidal compounds present in the fraction which has been known for their antioxidant and hepatoprotective activities [23].

EAF was subjected to silica gel column, 7 sub fractions (Fr-A, B, C, D, E, F) were obtained. Further Fr-E showed significant antioxidant potential (IC_{50} value 40.21 ± 0.20 μg/ml) as compared to other fractions. The potent sub fraction Fr-E was subjected to crystallization and it gave one pure compound. The melting point of that compound was found to be 168 °C and it gave positive $FeCl_3$ test for phenolics. Structure of isolated compound was elucidated by spectroscopical studies. The IR spectrum data revealed the absorption bands characteristics of hydroxyl group (3436 cm^{-1}), methyl group (2923 cm^{-1}), alkane group (1664 cm^{-1}), carbonyl group (1690 cm^{-1}) and phenolic group (1035 cm^{-1}). The molecular formula, $C_{10}H_{10}O_4$ of this compound was determined by ESI–MS spectrum with [M + H] at *m/z* 194.0. The compound, when subjected to ^1H NMR exhibited the carboxylic proton at 11.96 whereas the phenolic proton showed broad singlet at 9.32. There is sharp peak of three proton of methoxy group attached to the aromatic ring. The vinylic proton showed at 6.27 and 7.49 which are *Trans* to each other having $J = 16.0$ Hz. The aromatic protons appeared at 6.81 (C-6) and 7.15 (C-2). In ^{13}C NMR spectrum of compound, the aromatic carbon C1, C2, C3, C4, C5 and C6 appeared at 125.68, 122.41, 147.67, 148.89, 110.49, 110.49 and 115.36 respectively. The vinylic carbon appeared at 144.26 whereas the carboxylic carbon showed signal at 115.36. The carbon of methoxy group attaches at C-3 appeared at 55.48. From the above spectral data of compound was identified as 4-hydroxy-3-methoxy cinnamic acid which was reported as ferulic acid. The ferulic acid reported to have significant antioxidant potential as well as hepatoprotective activity, hence significant hepatoprotective effect of the potent antioxidant fraction (EAF) is due to ferulic acid.

EAF was standardized by HPTLC analysis using ferulic acid as a marker. The quantitative HPTLC analysis has shown the presence of 0.13% w/w ferulic acid. Moreover the ferulic acid already reported to have hepatoprotective potential [50]. This further supports our finding that the ferulic acid is responsible for hepatoprotective potential of UD.

5. Conclusion

The present study scientifically confirms that potent antioxidant fraction EAF of UD supports the highest percentage of hepatoprotective potential due to its ability to act as free radical scavenger, as evident by *in-vitro* and *in-vivo* antioxidant potential. The results suggested that the plant exhibited hepatoprotective effect due to the presence of phenolic compounds such as ferulic acid which act as antioxidants. Thus the study provides experimental evidences and clearly justifies the traditional claims and use in the treatment of liver diseases.

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