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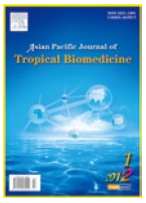

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Free radical scavenging activity of *Castanopsis indica* in mediating hepatoprotective activity of carbon tetrachloride intoxicated rats

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ABSTRACT

Objective: To investigate the free radical scavenging activity of methanol extract of *Castanopsis indica* (*C. indica*) in mediating hepatoprotective activity of carbon tetrachloride intoxicated rats. **Methods:** Free radical scavenging activity was evaluated by using DPPH, nitric oxide, peroxynitrite, superoxide radical, hydroxyl radical, hypochlorous acid scavenging and lipid peroxidation assay models. **Results:** The IC_{50} values of *C. indica* in DPPH, nitric oxide, peroxynitrite, superoxide radical, hydroxyl radical, hypochlorous acid scavenging and lipid peroxidation were found to be (43.86±1.85), (103.20±2.41), (327.00±7.23), (74.76±2.50), (35.39±1.51), (193.1m±6.74), (42.30±2.46) μg/mL, respectively. There was also a dose dependant increase in reductive ability of *C. indica* extract with increase in concentration. In case of hepatoprotective evaluation the levels of liver enzymatic, non-enzymatic systems (SGOT, SGPT, ALP), total bilirubin, total protein, catalase (CAT), reduce glutathione (GSH), superoxide dismutase and lipid peroxidation were restored towards the normal value in *C. indica* treated carbon tetrachloride intoxicated rats. The free radical scavenging and antioxidant activities may be attributed to the presence of phenolic compound, which was (318.70±1.39) μg in 1 000 μg of *C. indica* extract. **Conclusions:** Results obtained in the present study indicate that the *C. indica* is a potential hepatoprotective agent via its free radical scavenging.

1. Introduction

Oxidation is the transfer of electrons from one atom to another and represents an essential part of aerobic life and our metabolism, since oxygen is the ultimate electron acceptor in the electron flow system that produces energy in the form of ATP[1]. Oxygen is the most vital element in our environment for our survival. How can something so vital be so toxic? The answer lies in the fact that the problems may arise when the electron flow becomes uncoupled (transfer of unpaired single electrons), generating free radicals. Free radicals are two types: known as "reactive oxygen species" (ROS) and "reactive nitrogen species" (RNS)[2]. They contents superoxide (O^{2-}), peroxyl (ROO), alkoxyl (RO), hydroxyl (HO), and nitric oxide (NO). The hydroxyl (half-life of 10^{-9} sec) and the alkoxyl free radicals are very reactive and rapidly attack the molecules in nearby cells[3]. In addition to these ROS radicals in living organisms there are also other ROS nonradicals, such as the singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO). It is accepted that ROS play different roles *in vivo*. Some are positive and are related to their involvement in energy production, phagocytosis, regulation of cell growth, intercellular signaling and synthesis of biologically important compounds[2]. However, ROS may be very damaging, since they can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates and DNA, to induce oxidations, which may causes cancer, atherosclerosis, aging, immunosuppressant, inflammation, ischemic heart disease, diabetes, hair loss and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease[4,5].

Mammalian cells possess elaborate defense mechanisms for radical detoxification. Key metabolic steps are the superoxide dismutase (SOD) catalysis of the dismutation of superoxide to hydrogen peroxide and oxygen, the

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conversion of H_2O_2 into water and oxygen by catalase (CAT) and glutathione peroxidase (GPX) which destroys toxic peroxides[6]. In addition to antioxidant enzymes, several small-molecule play important roles that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reaction[3]. There are two basic categories of antioxidants, namely, synthetic and natural. In general, synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substitution, whereas natural antioxidants can be phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), or carotenoids as well as ascorbic acid[7]. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used as antioxidants since the beginning of this century. Restrictions on the use of these compounds, however, are being imposed because of their carcinogenicity. Thus, the interest in natural antioxidants has increased considerably[8]. Plant-derived antioxidants such as vitamin E, vitamin C, polyphenols including phenolic acids, phenolic diterpenes, flavonoids, catechins, procyanidins and anthocyanins are becoming increasingly suggested as important dietary factors. Therefore, recommendations have been made to increase the daily intake of fruit and vegetables, which can be lower the risk of chronic health problems associated with the diseases mentioned above[9].

Castanopsis indica (Roxb.) (*C. indica*) A.DC. (Family-Fagaceae) is commonly known as 'Dhalne katus' and 'Indian chestnut tree' found throughout the Himalayan region of North East India, Bangladesh, Laos, Myanmar, Nepal, Sikkim, Bhutan, Thailand, Vietnam. A decoction of the leaves applied to treat stomach disorder and skin diseases[10]. Powdered leaves are given to cure indigestion. A plant resin is given to treat diarrhea. A paste of leaves is applied for headache[10]. Bark paste is applied on chest to control chest pain[11]. Ethanol (50%) extract from stem bark have been reported to have anticancer activity in KB cancer cell and phostidylserine targeting antibody system[12]. However, in spite of traditional use, pharmacology of its leaves has not yet been explored scientifically. Previously isolated classes of constituents from the stem bark of *C. indica*, are identified as psoralen, β-sitosterol, angelicin, erythrodiol, ursolic acid, castanopsin, castanopson and castanopsol[12]. The objective of present study was to evaluate free radical scavenging activity of *C. indica* mediate hepatoprotective activity in carbon tetrachloride intoxicated rats.

2. Materials and methods

2.1. Plant material and preparation of extract

Leaves of the *C. indica* were collected from the middle hill region of Sikkim in the month of September 2010 and authenticated by Dr. Kanad Das Scientist at Botanical Survey of India, Gangtok, India. A voucher specimen (No. SHRC-5/5/2010/Tech.276) was deposited at Phytotherapy and Pharmacology Research Laboratory, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India. Leaves were shade dried at room temperature for 7 days and then powdered in a mechanical grinder. Finally powdered plant material (200 g) was successively extracted with petroleum ether (60-80℃) followed by methanol using Soxhlet extraction apparatus. Then solvent was completely removed under reduced pressure and stored in a vacuum desecrator. The yield of the petroleum ether and methanol extract was about 4% and 11%, respectively. Preliminary phytochemical study of the methanol extract of *C. indica* indicated the presence of steroid, triterpenoid, flavonoid, saponin and tannins. The *C. indica* extract were sealed in a glass beaker and stored at 20℃ for the assessment of the present experiment.

2.2. Chemicals

1, 1-diphenyl-2-picryl-hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. Nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), sodium nitroprusside (SNP), napthyl ethylene diamine dihydrochloride (NED), ascorbic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA), potassium nitrite (KNO_2), Sodium hypochlorite (NaClO), potassium ferricyanide [$K_3Fe(CN)_6$]. Diethylene triamine pentaacetic acid (DTPA) and 5, 5′-dithiobis (2 nitrobenzoic acid) (DTNB) was obtained from Spectrochem Pvt. Ltd, Mumbai, India. Folin-Ciocalteu's phenol reagent (FCR) was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Manganese dioxide (MnO_2) was obtained from SD Fine Chemicals, Mumbai, India. All other chemicals and solvents were used in high analytical grade.

2.3. In vitro free radical scavenging activity

2.3.1. DPPH radical assay

DPPH radical scavenging activity of *C. indica* was measured using the method proposed by Subhashini *et al* with some modifications[13]. 3 mL of reaction mixture containing 0.2 mL of DPPH (100 μM in methanol) and 2.8 mL of various concentrations of *C. indica* extract or standard ascorbic acid (10-200 μg/mL in methanol) was incubated at 37℃ for 30 min. The absorbance of the resulting solution was measured at 517 nm using Beckman model DU-40 spectrophotometer. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with extract) using the following formula.

Percentage inhibition= [(C-T)/C] × 100, Where, C = Absorbance of the control and T = Absorbance of the test sample.

2.3.2. Nitric oxide radical scavenging

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside solution, which interacts with oxygen to produce nitrite ions, which may be quantified by the Griess Illosvoy reaction[14]. The reaction mixture contained 1 mL of 10 mM sodium nitroprusside was mixed with 1

mL solution of various concentrations *C. indica* extract or standard ascorbic acid in phosphate buffer (pH 7.4) and the reaction mixture was incubated at 25℃ for 150 min. From the incubated mixture, 1 mL was taken out and 1 mL of Griess' reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) was added to it. Absorbance of the chromophore formed by the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was measured at 546 nm and percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

2.3.3. Measurement of peroxynitrite scavenging activity

Peroxynitrite ($ONOO^-$) is a cytotoxic intermediate produced by the reaction between the superoxide anion (O_2) and nitric oxide (NO). Synthesis of $ONOO^-$ was carried out according to the described method of Beckman *et al*[15]. The concentration of $ONOO^-$ was measured spectrophotometrically at 302 nm (€ = 1 670 M^{-1} cm^{-1}).

An Evans blue bleaching assay was used to measure peroxynitrite scavenging activity. The assay was performed by propose method of Karmakar *et al* with slight modification[16]. The percentage scavenging of $ONOO^-$ was calculated by using the above mention formula.

2.3.4. Superoxide anion scavenging activity

Superoxide radicals are generated in a PM β nicotinamide adenine dinucleotide (reduced form, NADH) system by oxidation of NADH and assayed by the reduction of NBT. Measurement of superoxide anion scavenging activity was done based on the method described by Koheil *et al* with some modifications[17]. The percentage inhibition of superoxide generation was calculated by using the above mention formula.

2.3.5. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured according to the method of Naskar *et al*[6]. EDTA (1 mM), $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM), and deoxyribose (10 mM), all solution were prepared in distilled deionized water. The assay was performed by adding 0.1 mL EDTA, 0.01 mL of $FeCl_3$, 0.1 mL H_2O_2, 0.36 mL of deoxyribose, 1.0 mL various concentrations of *C. indica* extract or standard ascorbic acid (10-200 μg/mL) was dissolved in distilled water, 0.33 mL of phosphate buffer(50 mM, pH 7.4) and 0.1 mL of ascorbic acid in sequence. The mixture was then incubated at 37℃ for 1 h. The pink chromogen was developed by adding 1.0 mL of 0.5% thiobarbituric acid followed by 1.0 mL of 10% trichloroacetic acid and heating in a boiling water bath for 20-30 min. The absorbance was measured at 532 nm and % inhibition of deoxyribose degradation is calculated by using above mention formula.

2.3.6. Hypochlorous acid scavenging activity

HClO was prepared immediately before use by adjusting a solution of NaClO to pH 6.2 with 0.6 M diluted sulfuric acid. Its concentration was determined spectrophotometrically at 235 nm using the molar absorption coefficient of 100 M^{-1} cm^{-1}. TNB was prepared according to a described method of Hazra *et al*[18]. Reaction mixture contained, 1.5 mM of HCLO and various concentrations (10-300 μg/mL) of *C. indica* extract or standard ascorbic acid was added and incubated at 37℃ for 1h. After that 30 mM taurine solution was added and again incubated for 30 min at 37℃. Thionitro benzoic acid added and measured the absorbance at 412 nm against blank and scavenging activity was calculated by using the standard formula[16].

2.3.7. Lipid peroxidation inhibitory activity

Lipid peroxide formation was measured by the method of Saha *et al* by measuring the color of thiobarbituric acid reactive substances (TBARS) formed at the end of reaction[19]. Reaction mixture (0.5 mL) containing rat liver homogenate 0.1 mL (25% W/V) in Tris HCl buffer (20 mM pH 7.0), 0.1 mL of KCl (30 mM), 0.1 mL of $FeS0_4$ (0.16 mM), 0.1 mL of ascorbic acid (0.06 mM) and 0.1 mL of various concentrations (10-100 μg/mL) of *C. indica* extract or standard ascorbic acid was incubated at 37℃ for 1 h. After that incubation reaction mixture was treated with 0.2 mL SDS (8.1%), 1.5 mL TBA (0.8%), 1.5 mL TCA (10%) and total volume was makeup to 4 mL by distilled water and then kept in a water bath at 95-100℃ for 30 min. After cooling, 1 mL of distilled water and 5 mL of n-butanol and pyridine mixture (15:1 V/V) was added to the reaction mixture, shaken vigorously and centrifuged at 4 000 rpm for 10 min. The organic layer was removed and absorbance was measured at 530 nm to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density of treatment with that of the control. The percentage inhibition was calculated by using the above mention formula.

2.3.8. Reductive ability

The Fe^{3+}-reducing power of the test samples was determined on the basis of the ability of their antioxidant principles to form colored complex with potassium ferricyanide, TCA and $FeCl_3$ and it was measured by the method reported by Warokar *et al*[20]. 1 mL of different concentrations of *C. indica* extract or standard ascorbic acid (10-200 μg/mL) was mixed with potassium ferricyanide (2.5 mL, 1%) and 2.5 mL of phosphate buffer (pH 6.6). The mixture was incubated at 50℃ for 20 min. After incubation 2.5 mL TCA (10%) was added to it and centrifuged at 3 000 rpm for 10 min. From the upper portion 2.5 mL of supernatant was taken out and to this 2.5 mL water and 0.5 mL $FeCl_3$ (0.1%) were added. The reaction mixture was left for 10 min at room temperature and absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power.

2.3.9. Determination of total phenolic compounds

Total soluble phenolic compounds in the *C. indica* extract were determined with Folin-Ciocalteu reagent according to the standard method[19], using pyrocatechol as a standard phenolic compound. The content of total phenolic compounds in *C. indica* extract was determined as microgram of pyrocatechol equivalent by using an equation that was obtained from standard pyrocatechol graph.

Absorbance at 760 nm = 0.001 × pyrocatechol (μg) + 0.003 3

2.4. Hepatoprotective activity

2.4.1. Animals

Healthy Wistar albino male rats (150-180 g) were divided into five group (n= 6) for the present study. These animals were maintained under standard laboratory conditions (temperature 25-30℃ and 55%-60% relative humidity with dark/light cycle 14/10 h).They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. All the procedures described were reviewed and approved by Jadavpur University Animal Ethics Committee (367001/C/ CPCSEA).

2.4.2. Experimental protocol

After seven days of acclimatization, these rats were divided into five groups (n=6). Treatment was done for 10 days as follows[21].

Group I: Normal control (0.9% Nacl; 1 mL/kg, *i.p.*)

Group II: CCl_4 control [CCl_4: liquid paraffin (1:2); 1 mL/kg, *i.p.*]

Group III: CCl_4 + *C. indica* extract (25 mg/kg, *i.p.*).

Group IV: CCl_4 + *C. indica* extract (50 mg/kg, *i.p.*)

Group V: CCl_4 + silymarin (25 mg/kg, *p.o.*).

Group II-V received CCl_4 solution once in every 72 h.

At the end of 10th day, all rats were fasted for 18 h and blood was collected from retro-orbital plexus under ether anesthesia. Blood samples were allowed to clot and the serum was separated by centrifugation at 2 500 g at 37℃ and was used for biochemical estimation. All animals were then sacrificed and liver tissues were dissected out to check the antioxidant status and histopathological observation.

2.4.3 Estimation of biochemical parameters

Serum was utilized for the estimation of various biochemical parameters namely serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) activities, alkaline phosphatase (SALP), serum bilirubin and total protein content were measured by using commercially available kits from Span Diagnostics Ltd. Mumbai, India.

2.4.4. Evaluation of antioxidant properties and hispathological observation

900 mg of liver tissue was collected, washed in normal saline and soaked in filter paper. These tissues were then homogenized in 3.0 mL 0.15 M Tris HCl buffer (pH 7.4) and centrifuged at 3 000 rpm at 4℃ for 1 h. The supernatant was collected for the determination of lipid peroxidation (LPO) [19], enzymic assays like SOD[6], CAT[22], and non-enzymic assay reduce glutathione content (GSH)[23]. Hispathological observation was done by standard method[21].

2.5. Statistical analysis

All results were shown as Mean±SEM of three individual measurements. 50% inhibitory concentrations (IC_{50}) were calculated by plotting the data in the graph as concentration versus percentage inhibition using Graph Pad Prism software, version 5. For hepatoprotective activity, data were calculated by one way ANOVA followed by Dunnett's *post hoc* test using Graph Pad Prism software, version 5. P< 0.05 was considered as statistical significance.

3. Results

3.1. In vitro free radical scavenging activity

The free radical scavenging activity of *C. indica* extract was detected and compared with reference compound ascorbic acid on the above *in vitro* free radical scavenging assay models. The *C. indica* extract showed the dose dependent free radical scavenging activity in all *in vitro* assay models

Table 1.
Effect of *C. indica* extract on serum enzyme levels (SGOT, SGPT and ALP), total bilirubin and total protein in CCl_4 intoxicated rats.

Groups	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	Total Bilirubin (mg/100 mL)	Total protein (mg/dL)
Normal control	50.33 ± 3.59	48.33 ± 3.08	17.83 ± 3.75	0.57 ± 0.12	7.70 ± 0.30
Carbon tetrachloride (1 mL/kg)	131.80 ± 4.52$^{\#\#}$	107.30 ± 5.05$^{\#\#}$	63.67 ± 4.88$^{\#\#}$	2.37 ± 0.18$^{\#}$	4.03 ± 0.46$^{\#\#}$
C. indica (25 mg/kg)	111.00 ± 4.59*	84.83 ± 6.67**	41.61 ± 4.08**	1.53 ± 0.29	5.37 ± 0.29
C. indica (50 mg/kg)	74.67 ± 5.45**	61.33 ± 3.11**	32.50 ± 3.67**	1.10 ± 0.12*	6.08 ± 0.07*
Silymarin (25 mg/kg)	60.50 ± 3.83**	54.83 ± 4.78**	21.17 ± 4.24**	0.77 ± 0.07**	6.69 ± 0.31*

$^{\#}$ P< 0.05, $^{\#\#}$ P< 0.01 when carbon tetrachloride control compared with normal group. * P< 0.05, ** P < 0.01 when all treatment groups compared with carbon tetrachloride control groups.

Table 2.
Effect of *C. indica* extract on LPO, GSH, CAT and SOD levels of liver in CCl_4 intoxicated rats.

Groups	LPO [MDA content (nM/mg)]	GSH (μg/mg tissue)	CAT (μM of H_2O_2 decomposed/ min/mg tissue)	SOD (U/mg tissue)
Normal control	25.67 ± 4.07	6.33 ± 0.95	0.98 ± 0.09	11.50 ± 0.67
Carbon tetrachloride (1 mL/kg)	112.50 ± 5.23$^{\#\#}$	2.66 ± 0.55$^{\#\#}$	0.39 ± 0.01$^{\#}$	5.66 ± 0.71$^{\#\#}$
C. indica (25 mg/kg)	85.67 ± 7.14*	4.16 ± 0.47	0.63 ± 0.01	7.83 ± 1.16
C. indica (50 mg/kg)	46.67 ± 4.96**	5.16 ± 0.70*	0.86 ± 0.03*	8.66 ± 0.84*
Silymarin (25 mg/kg)	33.50 ± 4.09**	5.83 ± 0.70*	0.92 ± 0.06*	9.83 ± 1.53**

$^{\#}$ P < 0.05, $^{\#\#}$ P < 0.01 when carbon tetrachloride control compared with normal group. * P < 0.05, ** P < 0.01 when all treatment groups compared with carbon tetrachloride control groups.

(Figure 1-8). IC_{50} values of *C. indica* extract and standard ascorbic acid for DPPH, nitric oxide, peroxynitrite, superoxide radical scavenging, hydroxyl radical, hypochlorous acid scavenging activity and lipid peroxidation were found to be (43.86 ± 1.85) and (10.25 ± 1.02) μg/mL, (103.20 ± 2.41) and (34.62 ± 2.24) μg/mL, (327.00 ± 7.23) and (62.00 ± 3.25) μg/mL, (74.76 ± 2.50) and (11.42 ± 2.06) μg/mL, (35.39 ± 1.51) and (13.26 ± 1.45) μg/mL, (193.1 ± 6.74) and (144.0 ±3.78) μg/mL, (42.30 ± 2.46) and (28.56 ± 2.24) μg/mL, respectively (Figure 9). There was also a dose dependant increase in reductive ability with increase in concentration of *C. indica* extract and ascorbic acid. The leaves extract revealed content total phenolic compound (318.70 ± 1.39) μg in 1 000 μg of *C. indica* extract.

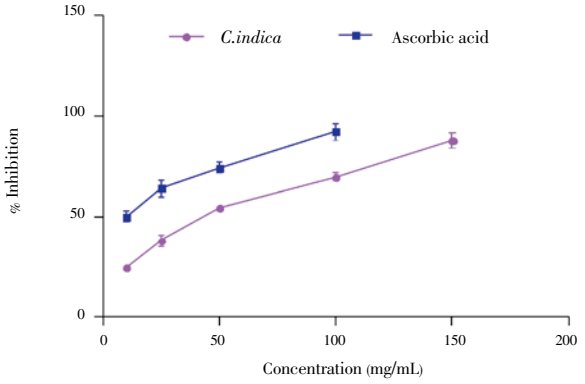

Figure 1. DPPH scavenging activity of *C. indica* extract and the standard ascorbic acid.

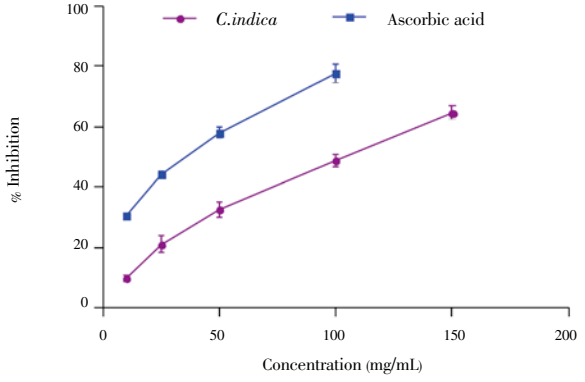

Figure 2. Nitric oxide radical scavenging activity of *C. indica* extract and standard ascorbic acid.

3.2. Hepatoprotective activity

3.2.1. Estimation of biochemical parameters

The effect of *C. indica* extract on SGOT, SGPT, ALP, total bilirubin and total protein levels in CCl_4 intoxicated rats are summarized in Table 1. There was a significant increased in SGOT, SGPT, ALP and bilirubin level and the total protein level was significantly decreased in the CCl_4 intoxicated rats, when compared with those of normal group. Treatment with *C. indica* extract showed a significant decrease in all elevated serum marker levels and reversed the altered total protein level to almost normal. Comparative results were also found with the standard drug silymarin.

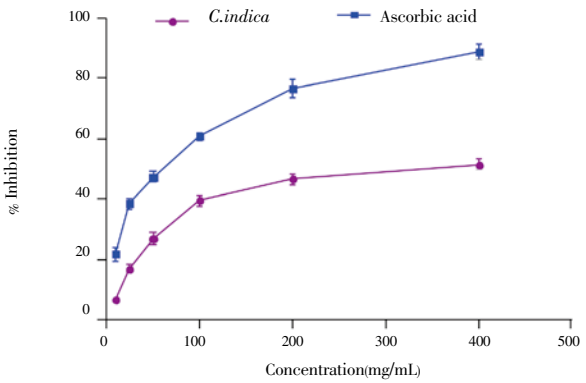

Figure 3. Peroxynitrite anion scavenging activity of *C. indica* extract and standard ascorbic acid.

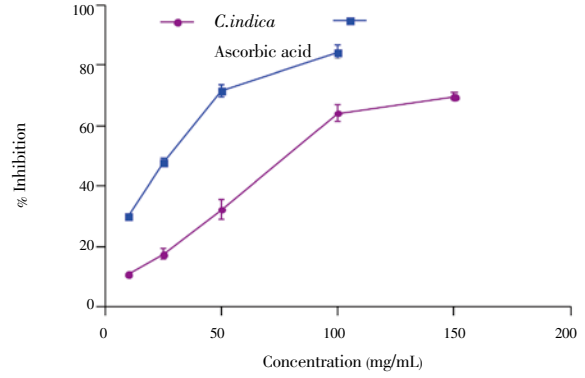

Figure 4. Superoxide radical scavenging assay of *C. indica* extract and the standard ascorbic acid.

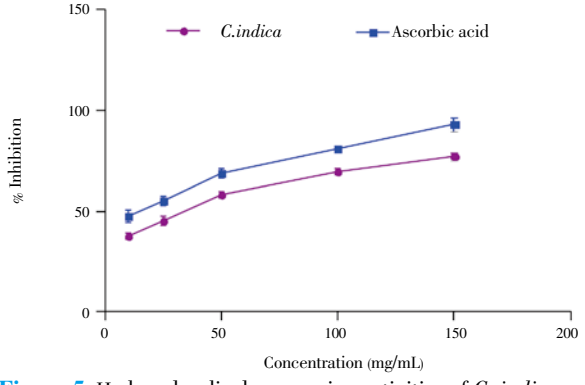

Figure 5. Hydroxyl radical scavenging activities of *C. indica* extract and standard ascorbic acid.

3.2.2. Evaluation of antioxidant properties and hispathological observation

Toxic effects of CCl_4 significantly reduced the activities of enzymic (CAT, SOD), non-enzymic (GSH) antioxidant system and increased LPO level of liver homogenate in the CCl_4 intoxicated rats, when compared with those of normal group (Table 2). There was significantly increase in both of the enzymic and non-enzymic antioxidant systems and the elevated LPO level were found to be decreased towards the normal value in *C. indica* extract as well as silymarin treated rats. Histopathological observation of liver sections of normal control group showed normal cellular architecture with

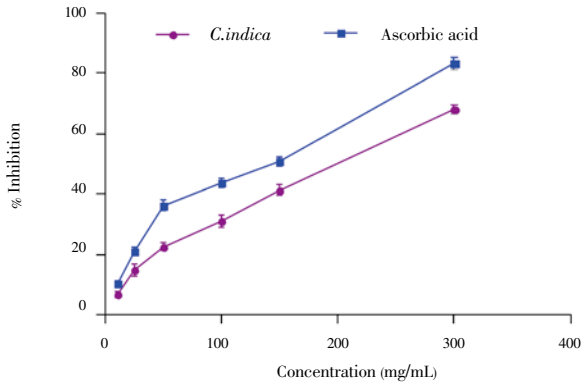

Figure 6. HClO scavenging activity of *C. indica* extract and standard ascorbic acid.

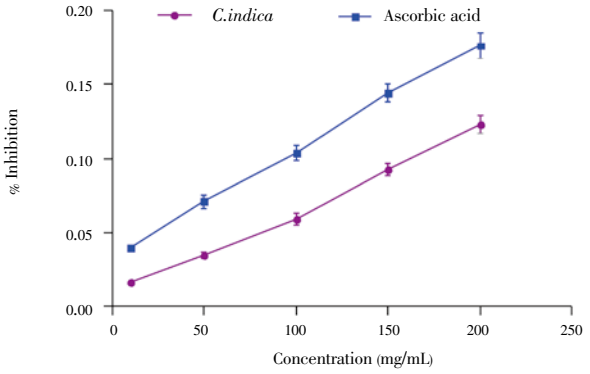

Figure 8. Reductive effect of *C. indica* extract and standard ascorbic acid.

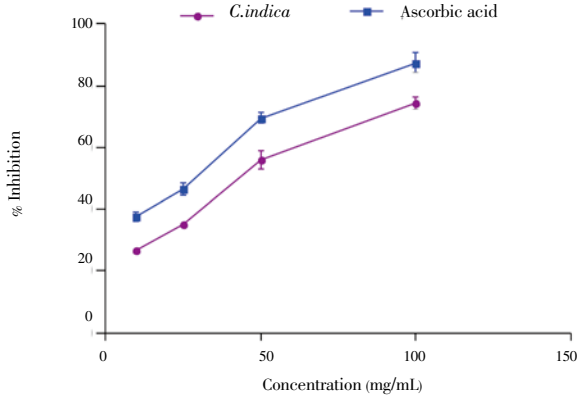

Figure 7. Lipid peroxidation activity of *C. indica* extract and standard ascorbic acid.

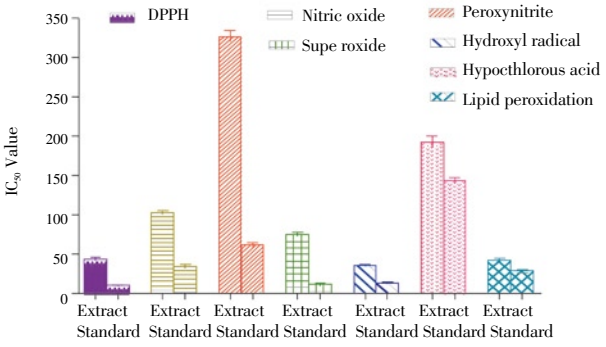

Figure 9. IC_{50} values.

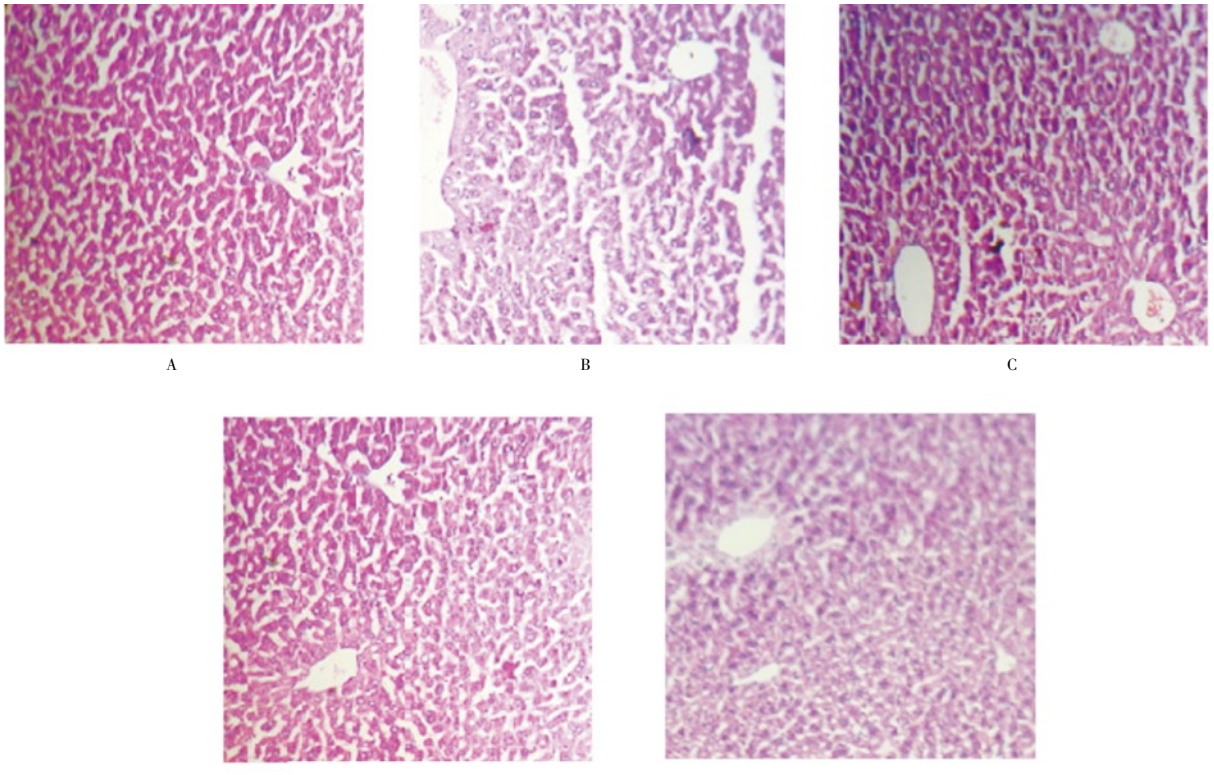

Figure 10. Histopathological effect of methanol extract of *C. indica* on carbon tetrachloride intoxicated rats liver.
Normal control (A), Carbon tetrachloride control (B), *C. indica* extract 25 mg/kg (C), *C. indica* extract 50 mg/kg (D) and Standard drug silymarin (E).

distinct hepatic cells, sinusoidal spaces and central vein (Figure 10A). Disarrangement of normal hepatocytes with centrilobuler necrosis, vacuolization of cytoplasm and fatty changes were observed in CCl_4 intoxicated rat livers (Figure 10B). These liver sections of rats treated with *C. indica* extract low dose (Figure 10C), high dose (Figure 10D) and silymarin (Figure 10E) showed a sign of protection against CCl_4 intoxication as evident by presence of normal hepatic cords and absence of necrosis with minimal inflammatory conditions around the central vein.

4. Discussion

Cells in humans and other organisms are constantly exposed to a variety of oxidizing agents, some of which are necessary for life. In the status of normal metabolism, levels of oxidants and antioxidants in humans are maintained in balance, which is important for sustaining optimal physiological conditions[24]. Overproduction of oxidants in certain conditions can cause an imbalance, leading to oxidative damage to large biomolecules such as lipids, DNA, and proteins. Many synthetic drugs protect against oxidative damage but they have adverse side effects[25]. Recently, there has been a considerable interest in finding natural antioxidants from plant materials to replace synthetic ones. Natural antioxidant substances are presumed to be safe since they occur in plant foods, and are seen as more desirable than their synthetic counterparts. Data from both scientific reports and laboratory studies show that plants contain a large variety of substances called ''plant chemicals'' or ''phytochemicals'' that possess antioxidant activity[8].

DPPH assay is the most widely reported method for screening antioxidant activity of many plant drugs, based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 516 nm, which is proportional to concentration of free radical scavenger added to DPPH reagent solution. DPPH is a stable, nitrogen-centered free radical which produces violet color in ethanol solution. It was reduced to a yellow colored product, diphenylpicryl hydrazine, with the addition of *C. indica* extract in a concentration-dependent manner[26].

Nitric oxide plays a vital role in various inflammatory processes. Higher levels of these radical are toxic to tissue and contribute to the vascular collapse, various carcinoma and ulcerative colitis. The toxicity of nitric oxide increases when it reacts with superoxide radical forming highly reactive peroxy nitrate anion ($ONOO^-$)[27]. *C. indica* extract decreases the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. This may be due to the antioxidant principle in *C. indica* extract, which competes with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite.

Peroxynitrite (($ONOO^-$) is now recognized by researchers as the culprit in many toxic reactions that were previously ascribed to its chemical precursors, superoxide and nitric oxide. Generation of excess $ONOO^-$ leads to oxidative damage and tissue injury[18]. Peroxynitrite bleaches Evans Blue by oxidizing it. According to present results, the *C. indica* extract in a concentration-dependent manner inhibits Evans Blue bleaching by scavenging peroxynitrite.

Superoxide dismutase catalyzes the dismutation of highly reactive superoxide anion to oxygen and hydrogen peroxide. Superoxide anion is also very harmful to cellular components and produced from molecular oxygen due to oxidative enzyme of body as well as via non-enzymatic reaction such as autoxidation by catecholamines[5]. The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm, *C. indica* extract indicated ability to quench superoxide radicals in the reaction mixture.

Hydroxyl radicals are major active oxygen species causing lipid peroxidation and enormous biological damage. Hydroxyl radical scavenging capacity of *C. indica* extract is directly related to its antioxidant activity. This method involves *in vitro* generation of hydroxyl radicals using Fe^{3+} /ascorbate/EDTA/H_2O_2 system using Fenton reaction. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid[28]. When *C. indica* extract was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction.

At sites of inflammation, the oxidation of Cl^- ions by the neutrophil enzyme myeloperoxidase results in the production of harmful ROS, hypochlorous acid[16]. HClO has the ability to inactivate the antioxidant enzyme catalase through breakdown of the heme prosthetic group. Catalase inactivation is inhibited in the presence of the *C. indica* extract, signifying its HClO scavenging activity.

Peroxidation of membrane lipids initiated by oxygen radicals may lead to cell injury. Initiation of a peroxidation sequence in a membrane or polyunsaturated fatty acid is due to abstraction of a hydrogen atom from the double bond in the fatty acid. The free radical tends to be stabilized by a molecular rearrangement to produce a conjugated diene, which easily reacts with an oxygen molecule to give a peroxy radical that can abstract a hydrogen atom from another molecule give a lipid hydroperoxide, ROOH[29]. Thiobarbituric acid (TBA) test is one of the most frequently used tests for measuring the peroxidation of lipids. Method involves isolation of microsomes from rat liver and induction of lipid peroxides with ferric ions leading to the production of small amount of malonaldehyde (MDA). TBA reacts with MDA to form a pink chromagen, which can be detected spectrophotometricaly at 530 nm[19]. The lipid peroxidation inhibitory activity of *C. indica* extract was detected and

compared with reference compound ascorbic acid.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. For the measurement of the reductive ability, the Fe^{3+}-Fe^{2+} transformation was investigated in presence of the *C. indica* extract. Presence of reductants causes the reduction of the Fe^{3+}/ ferricyanide complex to the Fe^{2+} form. This Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm[16]. Increase in absorbance of the reaction mixture indicates the reductive capabilities of *C. indica* extract in concentration dependent manner when compared to the standard ascorbic acid.

The high content of phenolic compounds is known to have direct antioxidant property due to presence of hydroxyl groups which can function as hydrogen donor. Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses. The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups. *C. indica* extract showed significantly higher inhibition percentage (stronger hydrogen donating ability) positively correlated with total phenolic content[24].

Carbon tetrachloride (CCl_4) is one of the most commonly used hepatotoxins in the experimental study of liver disease. The hepatotoxicity induced by CCl_4 is due to its metabolite $CCl_3^{\bullet}$, a free radical that binds to lipoprotein and leads to peroxidation of lipids of endoplasmic reticulum[30]. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects[31].

Estimation of serum marker enzymes like SGOT, SGPT and ALP can make assessment of liver function. When liver cell was in damage, a variety of enzymes normally located in the cytosol are released into the blood stream[21]. The enhanced levels of these serum marker enzymes were observed in CCl_4-treated rats in our experiment. Restoration of levels of these enzymes to/towards near normal values in the *C. indica* extract and silymarin treated animals is a clear manifestation of anti-hepatotoxic effect of *C. indica* extract and silymarin. Increase in serum bilirubin was reflected the depth of jaundice, which was attenuated in *C. indica* extract and silymarin treated groups, indicating its hepatoprotective effect further[32]. The lowered level of total protein in CCl_4 challenged animals is attributed to the damage produced localized in the endoplasmic reticulum which results in the loss of P450 leading to its functional failure with a decrease in protein synthesis[33]. The rise in protein levels in the treated groups suggests the stabilization of endoplasmic reticulum leading to protein synthesis.

Lipid peroxide has been considered to be one of the destructive processes of liver damage due to CCl_4 intoxication. The level of MDA, end product of lipid peroxidation was found to be high in CCl_4 control group, leading to tissue damage failure of antioxidant defense mechanisms against free radicals[34]. Treatment with *C. indica* extract and silymarin significantly reversed these changes. Our body has an effective defense system against free radical induced damage. It consists of a set of endogenous antioxidant enzymes including CAT, SOD as well as non-enzymic antioxidants, such as GSH[5]. In CCl_4-intoxicated hepatotoxicity, the balance between ROS production and these antioxidant defenses may be lost. Treatment with *C. indica* extract and silymarin showed significant improvement in the level of these antioxidant systems over those in CCl_4 control animals[29].

Histopathological examination of these liver sections reveals that the normal liver architecture was disturbed by hepatotoxin intoxication[21]. In the sections obtained from the rats treated with *C. indica* extract or silymarin and intoxicated with hepatotoxin, the normal cellular architecture was retained as compared to those of the normal control rats, thereby confirming the protective effect of the extract or drug and thus the observation substantiates other results of the experiment.

On the basis of these results obtained in the present study, it is concluded that *C. indica* extract showed good hepatoprotective activity. The *in vitro* free radical scavenging assays indicate that this plant extract is a significant source of natural antioxidant which is mainly responsible for its hepatoprotective activity. Preliminary phytochemical analysis of the crude *C. indica* extract reveals the presence of flavonoids, saponins, tannins and steroids but the components which are responsible for the antioxidant and hepatoprotective activity are currently unclear. Therefore, further investigation is needed to isolate and identify the compounds present in the plant this extract prior to clinical use.

Conflict of interest statement

We declare that we have no conflict of interest.

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