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Oriental Pharmacy and Experimental Medicine 2006 **6(1)**, 45-52

Hepatoprotective activity of methanol extracts of *Berberis tinctoria*

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SUMMARY

The methanol extracts of the roots, root bark and stem of *Berberis tinctoria,* were investigated for their hepatoprotective activity against carbon tetrachloride (CCl_4) induced toxicity in freshly isolated rat hepatocytes, HEp-G2 cells and animal models. The methanol extracts were able to significantly normalise the levels of aspartate amino transferase, alanine aminotransferase, alkaline phosphatase, triglycerides, total proteins, albumin, total bilirubin and direct bilirubin, which were altered due to CCl_4 intoxication in freshly isolated rat hepatocytes and also in animal models. The anti-hepatotoxic effect of the methanol extracts *in vitro* were observed at 600 - 1,000 μg/ml concentrations. A dose dependent increase in the percentage viability was observed when CCl_4 exposed HEp-G2 cells were treated with different concentrations of the methanol extracts. The highest percentage viability of HEp-G2 was observed at a concentration of 1,000 μg/ml. The results from the present investigations also indicate good correlation between the *in vivo* and *in vitro* studies.

Key words: *Berberis tinctoria*; Hepatoprotective; HEp-G2; Rat hepatocytes

INTRODUCTION

Berberis tinctoria (Family: Berberidaceae) is a shrub or a small tree growing to about 1.8 to 2.4 m in height and armed with trifid spines. It is distributed in the temperate and sub-tropical parts of Asia, Europe and America. The roots are reputed in Ayurveda and are used in the treatment of jaundice, cholera and eye problems. It is widely used in the treatment of jaundice in the Nilgiris (personal communication). It is also a constituent of Liv-52^R, a well known hepatoprotective formulation in India. Four alkaloids, viz., berberine, berbamine, jatrorrhizime and palmatine have been isolated form the roots (Anonymous, 1998). The alkaloid, berberine receives the widest acclaim as the active component of *Berberis species.* Traditionally, species of *Berberis* were used to treat a large number of conditions in European and American herbalism, particularly for infections and stomach problems. There is also a history of its internal use to treat skin conditions (Duke *et al*., 1985). Many plants and their components, selected on the basis of their traditional use have been reported for their hepato protective properties (Vijayan *et al*., 2003; Oh *et al*., 2004; Gilani *et al*., 2005). In the present investigation the hepatoprotective effects of methanol extracts of root, root bark and stem of *Berberis tinctoria* were studied on carbon tetrachloride (CCl_4) induced hepatotoxicity on freshly isolated rat hepatocytes

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(Kiso *et al*., 1983), HEp-G2 cells (Ira *et al*., 1997) and in animal models (Rao *et al*., 1993). Biochemical parameters such as levels of aspartate amino transferase (ASAT), alanine aminotran sferase (ALAT), alkaline phosphatase (ALP), triglycerides, total proteins, albumin, total bilirubin and direct bilirubin, were estimated to assess the hepatic function (Harper, 1961).

MATERIALS AND METHODS

Reagents and cells

All chemicals were obtained from SD Fine Chemicals, Mumbai, India. (3-(4, 5-dimethyl thiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT), collagenase, insulin, dexamethasone were purchased from Sigma Chemical Co., St. Louis, MO, USA, Minimum Essential Medium of Eagle's (EMEM) and Ham's F 12 from Hi-Media Laboratories, Mumbai, India and Ecoline diagnostic kits from E. Merck, Mumbai, India. Liv-52® a marketed formulation (Himalaya Drugs Ltd., Bangalore, India) was used as a standard. Human liver derived HEp-G2 cell line was obtained from National Centre for Cell Science, Pune, India.

Plant material and extraction

Berberis tinctoria, entire plant was collected from Moyar Village, The Nilgiris, Tamilnadu, India in the month of May 2000. The plant was identified and authenticated by comparing with standard specimen preserved at Survey of Medicinal Plants and Collection Unit, Government Arts College, Ooty (Voucher No. SB 5). The root, root bark and stem were separated from the plant, shade dried and powdered. Each of the powder (250 g) was subjected to a single Soxhlet extraction using methanol (1 liter) for 24 h. The extracts were then concentrated to dryness under reduced pressure and controlled temperature to yield dark brown semisolids (yield, 5.12%, 3.52% and 3.68% respectively, for root, root bark and stem), which were preserved in refrigerated condition till further use.

10 mg of the methanolic extracts of the root, roots bark and stem of *Berberis tinctoria* were dissolved in 1 ml of dimethyl sulphoxide (DMSO) and the volume was made up to 10 ml with Ham's F_{12}/ MEM to obtain a stock solution of 1 mg/ml concentration and stored at -20°C till use. Further dilutions were made to obtain different concentrations with respective media and used for *in vitro* investigations. A suspension of Liv-52 powder was also prepared (250 μg/ml) in a similar manner. The methanol extracts and the Liv-52 powder were suspended in sodium CMC (0.3%) in distilled water separately and used for *in vivo* investigations.

Hepatoprotective effect of plant extracts in freshly isolated rat hepatocytes

Liver cells were isolated by the modified procedure of Seglen (1994). The calcium free HEPES buffer and collagenase solution were warmed to 37°C in a water bath. The abdomen of the rat was opened under phenobarbital sodium (35 mg/kg body weight) anesthesia. A midline incision was made and a loosely tied ligature was placed around the portal vein approximately 5 mm from the liver and the cannula was inserted up to the liver and then the ligature was tightened and heparin was injected into the femoral vein (1,000 IU). The inferior vena cava was cut below the renal vein. Perfusion was performed for 20 min with calcium free HEPES buffer, which contained 1% bovine serum albumin fraction V, at a flow rate of 30 ml/min. The liver swells during this time slowly changing colour from dark red to pinkish white. The swollen liver was then perfused with TPVG (trypsion, Phosphate buffered saline, versine, glucose) solution (50 ml) followed by perfusion with calcium free HEPES buffer, which contained additional collagenase (0.075%) and calcium chloride (4 mM) at a flow rate of 15 ml/min for 20 min.

After the perfusion, the lobes were removed and transferred into a sterile petridish containing calcium free HEPES buffer and dispersed gently. It was transferred into a sterile conical flask and the cell suspension was stirred with the help of a magnetic stirrer for 5 min to release hepatocytes into the

solution. The cell suspension was filtered through a nylon mesh (250 μ) to and the preparation was centrifuged at 1,000 rpm for 15 min. The supernatant was aspirated off and the loosely packed pellet of cells were gently re-suspended in calcium free HEPES buffer. This washing procedure was repeated three times. Cell viability was determined by the trypan blue dye exclusion method (Freshney, 2000). These isolated hepatocytes were cultured in Ham's F_{12} medium, supplemented with 10% new born calf serum, antibiotics, 10^{-6}M dexamethasone and 10^{-8} bovine insulin. The cell suspension was incubated at 37°C for 30 min in a humidified incubator under 5% CO_2.

CCl_4 induced *in vitro* hepatocytes injury

CCl_4 induced hepatocytes injury assay was carried out. After an incubation of 24 h, the hepatocytes were exposed to the fresh medium containing CCl_4 (1%) along with/without various concentrations of the methanol extracts or the medium containing the same amount of DMSO used for extracts alone (as control). After 60 min of CCl_4 challenge, concentrations of ASAT, ALAT, ALP, TGL, total proteins, albumin, total bilirubin and direct bilirubin in the medium were measured as an indication of hepatocytes necrosis using Ecoline diagnostic kits (Yoshinobu *et al*., 1983).

Hepatoprotective effect in HEp-G2 cell line

The screening of hepatoprotective activity was based on the protection of human liver derived HEp-G2 cells against CCl_4 induced damage (Ira *et al*., 1997) determined by estimating mitochondrial synthesis using microculture Tetrazolium (MTT) assay (Ke *et al*., 1999). HEp-G2 cells were routinely grown and subcultured as monolayers in DMEM supplemented with 10% newborn calf serum. The experiments in this investigation were conducted with cells that had been initially batch cultured for 10 days. At this stage, the cells were harvested and plated at approximately 30,000 cells/well in 96 well microtitre plates (Nunclon) and left to rest for 24 h at 37°C in a humidified atmosphere of 5% CO_2. The cells were then exposed to toxicant (medium containing 1% CCl_4) along with/without various concentrations of the methanolic extracts of root, root bark and stem of *Berberis tinctoria* or the medium containing the same amount of DMSO used for extracts alone (as control). At the end of the period, cytotoxicity was assessed by estimating the viability of HEp-G2 cells by MTT reduction assay. After 1h incubation, the test solution from each well was removed by aspiration and replaced with 50 μl of MTT prepared in MEM without phenol red (MEM-PR^-). The plates were gently shaken and incubated for 3 h at 37°C in a humidified 5% CO_2 atmosphere. The supernatant was removed and 50 μl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at 540 nm.

***In vivo* hepatoprotective effect**

Colony bred Wistar strain adult albino rats (180 - 200 g) of either sex were used for the investigations. All the animals were maintained under standard husbandry conditions with food and water *ad libitum*. The experimental procedures were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai (Proposal No: 19/29, JSSCP, Ooty). The animals were divided into 6 groups consisting of six animals in each group. Liver damage was induced by administration of CCl_4 (1 ml/kg body weight) intra peritoneally a day prior to the treatment. Group I received the vehicle (Sodium CMC, 0.3%) and served as control and was not treated with the toxicant. The second group served as CCl_4 treated control and received the vehicle (Sodium CMC, 0.3%). Groups III, IV and V received a suspension of the methanolic extracts of roots, root bark and stem of *Berberis tinctoria* at 250 mg/kg body weight respectively. Group VI received the standard drug Liv-52 (Himalaya Drug Co., Bangalore, India) at 250 mg/kg body weight. After 24 h of

intoxication, the animals received these treatments by oral route for a period of 6 days. On the 8th day, blood was collected in sterile centrifuge tubes and allowed to clot. Serum was separated and used for the estimation of ASAT, ALAT, ALP, TGL, total proteins, albumin, total bilirubin and direct bilirubin using Ecoline diagnostic kits.

Statistical analysis

Statistical analysis was carried out using Student's *t*-test. The results were judged significant if $P < 0.05$.

RESULTS

The effects of the methanol extracts of root, root bark and stem of *Berberis tinctoria* on freshly isolated rat hepatocytes intoxicated with CCl_4 are recorded in Table 1. A significant increase in the levels of ASAT, ALAT, ALP, total bilirubin, direct bilirubin ($P < 0.001$) and a significant reduction in the levels of TGL, total proteins and albumin ($P < 0.001$) was observed in hepatocytes exposed to CCl_4 when compared to normal hepatocytes. These cells when treated along with the methanolic extracts of *Berberis tinctoria* showed a significant restoration of the altered biochemical parameters towards the normal ($P < 0.001$, when compared to CCl_4 treated group) and were dose dependent. A similar result was obtained when CCl_4 intoxicated hepatocytes were treated with the Liv-52. However, the hepatoprotective effect of extracts of the root, roots bark and stem of *Berberis tinctoria* was observed at 600 - 1,000 μg/ml concentrations.

The CCl_4 exposed HEp-G2 cells showed a percentage viability of 24%. These exposed cells when treated with different concentrations of the methanolic extracts of *Berberis tinctoria* showed a dose dependent increase in percentage viability. The percentage viability ranged between 29 - 35% for the extracts of the roots and the root bark, and 32 - 37% for the stem extracts at 200 - 1,000 μg/ml concentrations and was found to be less effective when compared to the Liv-52 (Table 2).

The effects of methanol extracts of root, root bark and stem of *Berberis tinctoria* on CCl_4 intoxicated rats are recorded in Table 3. Intoxication of rats treated with CCl_4 significantly ($P < 0.01$ and $P < 0.001$) altered the biochemical parameters when compared with normal control rats. Treatment with methanol extracts of roots, root bark and stem of *Berberis tinctoria* at 250 mg/kg body weight showed a significant ($P < 0.01$ and $P < 0.001$) decrease in ASAT, ALAT, ALP, total bilirubin, direct bilirubin and a significant ($P < 0.01$ and $P < 0.001$) elevation in the TGL, total proteins and albumin levels in serum when compared with CCl_4 treated rats. However, the Liv-52 at 250 mg/kg body weight exhibited better results with no mortality.

DISCUSSION

Plant drugs are known to play a vital role in the management of liver diseases. There are numerous plants and polyherbal formulations claimed to have hepatoprotective activities. Nearly 150 phytoconstituents from 101 plants have been claimed to possess liver protecting activity (Doreswamy and Sharma, 1995). The preventive action in liver damage induced by CCl_4 has been widely used as an indicator of the liver protective activity of drugs or medicinal plant extracts, by *in vivo* and *in vitro* techniques (Kiso *et al*., 1983; Allis *et al*., 1990). CCl_4 produces an experimental liver damage, which histologically resembles that of viral hepatitis (James and Pickering, 1976). Most of the studies on hepatoprotective plants were carried out using chemical induced liver damage in rodents as models (Handa *et al*., 1989; Rao *et al*., 1993; Evans, 1996). *In-vitro* systems based on cultured immortalized hepatoma cell lines from man are widely used for studies on toxicity, xenobiotic metabolism and carcinogenesis (Hikimo and Kiso, 1988). The use of cells from man rather than animals not only avoids the killing of animals, but also has further advantage that possible species differences in responses, both to hepatotoxins and to plant extracts, are avoided.

Table 1. Effects of treatment of methanolic extract of root, root bark and stem of *Berberis tinctoria* on the biochemical parameters of CCl_4 intoxicated freshly isolated rat hepatocytes (Number of independent experiments = 3, 5 replicates, mean ± SEM

Sl. No	Treatment	Concentration	ASAT U/L	ALAT U/L	ALP U/L	TGL mg/dl	Total Protein gm/dl	Albumin G/l	Total Bilirubin mg/dl	Direct Bilirubin mg/dl
1	Control	-	11 ± 0.39	15 ± 0.01	27 ± 0.46	180 ± 4.08	0.762 ± 0.04	1.213 ± 0.04	0.204 ± 0.005	0.034 ± 0.002
2	CCl_4 treated	1%	69 ± 0.48[a]	56 ± 0.53[a]	79 ± 2.86[a]	97 ± 3.06[a]	0.107 ± 0.06[a]	0.3 ± 0.01[a]	0.518 ± 0.01[a]	0.174 ± 0.03[a]
3	CCl_4 and standard	250 µg/ml	20 ± 0.83[b]	27.3 ± 1.24[b]	27 ± 0.97[b]	190 ± 2.47[b]	0.65 ± 0.024[b]	1.1 ± 0.03[b]	0.275 ± 0.002[b]	0.059 ± 0.002[b]
4	CCl_4 and methanolic root extract	1,000 µg/ml	3.4 ± 0.167[b]	11.4 ± 0.509[b]	16.2 ± 0.81[b]	251.4 ± 10.6[b]	0.706 ± 0.04[b]	1.215 ± 0.07[b]	0.242 ± 0.009[b]	0.035 ± 0.001[b]
		800 µg/ml	9.2 ± 0.374[b]	16.4 ± 0.51[b]	27.4 ± 1.81[b]	239.8 ± 10.66[b]	0.558 ± 0.02[b]	0.878 ± 0.04[b]	0.324 ± 0.009[c]	0.072 ± 0.004[b]
		600 µg/ml	17.6 ± 0.707[b]	30.1 ± 1.27[b]	37.8 ± 1.36[b]	225.2 ± 11.55[b]	0.464 ± 0.02[b]	0.792 ± 0.03[b]	0.401 ± 0.02[d]	0.081 ± 0.04[b]
5	CCl_4 and methanolic roots bark extract	1,000 µg/ml	12.8 ± 0.66[b]	9.4 ± 0.42[b]	19.4 ± 0.9[b]	257.2 ± 11.6[b]	0.682 ± 0.02[b]	1.201 ± 0.06[b]	0.264 ± 0.01[b]	0.038 ± 0.002[b]
		800 µg/ml	21.6 ± 1.07[b]	11.8 ± 0.62[b]	22.6 ± 1.1[b]	205.6 ± 9.84[b]	0.593 ± 0.03[b]	0.986 ± 0.05[b]	0.312 ± 0.02[c]	0.068 ± 0.003[b]
		600 µg/ml	26.6 ± 1.56[b]	21.4 ± 1.05[b]	33.4 ± 1.32[b]	202.6 ± 9.53[b]	0.506 ± 0.04[b]	0.888 ± 0.00[b]	0.418 ± 0.02[d]	0.075 ± 0.004[b]
6	CCl_4 and methanolic stem extract	1,000 µg/ml	6.4 ± 0.509[b]	12.8 ± 0.66[b]	24.4 ± 1.1[b]	281.4 ± 11.6[b]	0.876 ± 0.04[b]	1.174 ± 0.05[b]	0.221 ± 0.009[b]	0.046 ± 0.002[b]
		800 µg/ml	11.2 ± 0.583[b]	21.4 ± 1.02[b]	30.4 ± 1.2[b]	260.5 ± 10.81[b]	0.636 ± 0.02[b]	0.988 ± 0.05[b]	0.337 ± 0.01[c]	0.071 ± 0.004[b]
		600 µg/ml	21.4 ± 1.1[b]	30.6 ± 1.2[b]	38.2 ± 1.36[b]	221.2 ± 11.37[b]	0.503 ± 0.03[b]	0.862 ± 0.04[b]	0.418 ± 0.02[d]	0.083 ± 0.004[b]

[a]=$P < 0.001$ when compared to normal group, [b]=$P < 0.001$, [c]=$P < 0.01$, [d]=$P < 0.05$ when compared to CCl_4 group. (U/L = Units/litre, mg/dl = milligram/decilitre, gm/dl = gram/decilitre, G/l = gram/litre)

Table 2. Hepatoprotective activity of the methanolic extracts of roots, roots bark and stem of *Berberis tinctoria* on CCl_4 intoxicated HEp-G2 cells

Sl. No.	Treatment	Concentration (μg/ml)	% Viability*
1	Control	-	100
2	CCl_4	1%	24.3 ± 2.16^{a}
3	CCl_4 (1%) + Methanolic extract of root	1,000	$35.4 \pm 1.56^{a,c}$
		500	$31.2 \pm 1.59^{a,c}$
		400	$30.8 \pm 1.27^{a,b}$
		300	$29.7 \pm 1.03^{a,b}$
		200	$29.1 \pm 1.22^{a,b}$
4	CCl_4 (1%) + Methanolic extract of root bark	1,000	$35.1 \pm 1.61^{a,c}$
		500	$33.8 \pm 1.03^{a,c}$
		400	$31.8 \pm 1.12^{a,c}$
		300	$31.4 \pm 1.52^{a,c}$
		200	$29.7 \pm 2.01^{a,b}$
5	CCl_4 (1%) + Methanolic extract of stem	1,000	$37.2 \pm 2.46^{a,c}$
		500	$34.3 \pm 2.19^{a,c}$
		400	$33.2 \pm 1.99^{a,c}$
		300	$32.6 \pm 1.46^{a,c}$
		200	$32.1 \pm 1.09^{a,c}$

(*Average of 5 replicates) [a] = $P < 0.001$ when compared to control, [b]= $P < 0.01$, [c]= $P < 0.001$, when compared to CCl_4 group.

The present study reveals the hepatoprotective effect of *Berberis tinctoria* against CCl_4 induced toxicity in isolated rat hepatocytes, HEp-G2 cells and in animal models. Freshly isolated hepatocytes have a distinct advantage that the cells express differentiated functions of liver and more importantly the activities of hepatic cytochrome P450 involved in the activation of drugs and chemicals, including CCl_4, which is used as a toxicant in this study. HEp-G2 cells retain many of the morphological and biochemical characteristics of normal cells (Jover *et al*., 1994). A reproducible microplate screening assay based on the protection of cells of the human-liver derived HEp-G2 cell line against toxic damage have been used (Ira *et al*., 1997) for the rapid identification of the protective activity of the plant extracts. Since most of the plant extracts are toxic above 1,000 μg/ml, the highest concentration was limited to 1,000 μg/ml *in vitro* studies. CCl_4 has been found to induce extensive liver damage within a period of 24 h following intra-peritoneal administration. Intoxication with CCl_4 in rat hepatocytes and in animals caused elevated serum levels of hepatospecific enzymes such as ASAT, ALAT, ALP as well as alterations in different liver parameters (Table 1 and 3). Treatment with the methanolic extracts of *Berberis tinctoria* exhibited significant restoration of the altered biochemical parameters towards normal in CCl_4 intoxicated rat hepatocytes and in rats. These results and the absence of mortality observed with extracts treated group are indicative of the potent hepatoprotective action of the plant. The maximum degree of protection was observed with the highest dose of extracts. Among the three extracts studied the stem extract was found to be more effective. The hepatoprotective effect *in vivo* study at 250 mg/kg body weight is comparable with that of the Liv-52 at the same dose. The investigation when carried out in human liver derived HEp-G2 cells against CCl_4 induced damage also confirmed the hepatoprotective nature of the methanolic extracts. The results from the present study also indicate good correlation between the *in vivo* and *in vitro* studies.

Table 3. Effects of treatment with methanolic extracts on the biochemical parameters of CCl_4 intoxicated rats

Sl. No	Treatment	Dose	ASAT U/L	ALAT U/L	ALP U/L	TGL mg/dl	Total Protein gm/dl	Albumin G/l	Total Bilirubin mg/dl	Direct Bilirubin mg/dl
1	Normal	-	63.25 ± 3.65	25.6 ± 1.32	257.6 ± 13.5	69.0 ± 5.09	7.04 ± 0.39	3.72 ± 0.17	0.408 ± 0.02	0.164 ± 0.013
2	CCl_4 treated	1 ml/kg bw	110.6 ± 5.09[a]	57.2 ± 1.52[a]	469.6 ± 16.91[a]	25.6 ± 1.5[a]	4.1 ± 0.19[a]	1.96 ± 0.02[a]	1.014 ± 0.04[a]	0.482 ± 0.016[a]
3	CCl_4 and methanolic root extract	1 ml/kg bw + 250 mg/kg bw	45.2 ± 2.03[b]	25.4 ± 1.32[b]	344.6 ± 10.03[b]	63.6 ± 2.36[b]	5.92 ± 0.156[d]	3.44 ± 0.14[d]	0.484 ± 0.028[c]	0.204 ± 0.009[b]
4	CCl_4 and methanolic roots bark extract	250 mg/kg bw	51.2 ± 2.55[b]	28.6 ± 1.36[b]	389.4 ± 12.03[b]	64.2 ± 2.37[b]	6.09 ± 0.256[d]	3.36 ± 0.13[d]	0.408 ± 0.021[b]	0.118 ± 0.009[b]
5	CCl_4 and methanolic stem extract	250 mg/kg bw	45.8 ± 3.39[b]	28.2 ± 1.31[b]	340.2 ± 11.33[b]	61.6 ± 2.95[b]	6.08 ± 0.18[d]	3.31 ± 0.08[d]	0.40 ± 0.025[b]	0.21 ± 0.011[b]
6	CCl_4 and standard	250 mg/kg bw	54.8 ± 3.03[b]	24.4 ± 1.02[b]	316.8 ± 10.9[b]	51.2 ± 3.48[b]	6.94 ± 0.46[d]	3.88 ± 0.14[d]	0.39 ± 0.03[b]	0.17 ± 0.01[b]

[a] = $P < 0.001$ when compared to normal group, [b] = $P < 0.001$, [c] = $P < 0.01$, [d] = $P < 0.05$ when compared to CCl_4 treated group. bw = body weight. (U/L = Units/litre, mg/dl = milligram/decilitre, gm/dl = gram/decilitre, G/l = gram/litre)

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