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Hepatoprotective and Antioxidant Activity of Root Bark of *Calotropis procera* R.Br (Asclepediaceae)

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Abstract: In the present study, *Calotropis procera* (Asclepediaceae) was evaluated for its possible hepatoprotective and antioxidant potential. Hepatoprotective activity of the methanol extract (MCP) and phyto-constituents directed three sub fractions hexane (HCP), ethylacetate (ECP) and chloroform (CCP) of the root bark was determined using carbon tetrachloride (CCl_4) induced liver injury in mice. First the MCP extracts and then three sub fractions namely HCP and ECP and CCP from MCP extract evaluated, at an oral dose of 200 mg kg^{-1}. The animals were weighed each and divided in groups of six. Liver damage was achieved by injecting CCl_4 in olive oil (1:1) 0.8 mL kg^{-1}. The treatment groups pretreated with above extracts. Silymarin was used as reference standard drug. At the end of 7 days, blood was collected, liver extracted, weighed, processed for histopathological assessments and for antioxidant activity. The MCP and its sub fractions HCP and ECP exhibited a significant hepato-protective effect by lowering the elevated serum levels of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), Alkaline phosphatase (ALP), total and direct serum bilirubin, cholesterol and significantly increasing high density lipoprotein (HDL) and moderately increasing total protein and albumin. While, the CCP fraction does not show significant protective effect. These biochemical observations were supplemented by histopathological examination of liver sections. Further, the effects of the active fractions on antioxidant enzymes also have been investigated to elucidate the possible mechanism of its hepatoprotective activity. The fractions exhibited a significant effect by modifying the levels of reduced glutathione, super oxide dimutase, catalyse activity and malondialdehyde equivalent, an index of lipid peroxidation of the liver. These findings suggest the use of this plant for the treatment of liver toxicity in oriental traditional medicine.

Key words: *Calotropis procera*, hepatoprotective, Asclepediaceae, lipid peroxidation, antioxidant, silymarin, hepatotoxicity

INTRODUCTION

Liver is a vital organ of the body. It plays a pivotal role in the metabolism, secretion and storage. Any type of the injury or impairment of its functions may leads to many type of complication in one's health. Unfortunately, Hepatic dysfunction due to ingestion or inhalation of hepatotoxins is increasing worldwide. Management of liver disease is still a challenge to the modern medicine (Reddy *et al*., 1993). Due limited therapeutic options and disappointing therapeutic success of the modern medicine, uses of herbal drugs has increased worldwide (Stickel and Schuppan, 2007). Numerous medicinal plants and their formulations used for liver disorders in ethanomedical practices and in traditional system of medicine in India (Sethuraman *et al*., 2003). In this modern age it is very important to provide scientific proof to justify the medicinal uses of herbs. Efficacy of the drugs should be tested by standard experimental methods and there should be adequate data from studies to validate the therapeutic potential (Girish *et al*., 2009). In the present study, in order to search for a new natural remedy for hepatic disorder, the *Calotropis procera* root bark was evaluated for its possible hepatoprotective activity.

The genus *Calotropis* R. Br (Asclepediaceae) is distributed in tropical and subtropical region of Asia and Africa, while in India it is represented by two species viz., *Calotropis procera* and *Calotropis gigantea. C. procera* is large bushy shrub, more common in southwestern and central India and western himalayas (Phondke, 1992). In India the *C. procera* holds pride of place largely because of its other uses and economical values (Ahmed *et al*., 2005). The plant is also known for its use in folk medicines. Traditionally, the plant has been used as

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Antifungal (Larhsini *et al*., 1997), antipyretic (Al-Yahya *et al*., 1985) and analgesic activity (Mohsin *et al*., 1989). Dried leaves used as an expectorant, as antiimflamatory (Kapur and Sarin, 1984), for treatment of paralysis and rheumatic pains (Sebastian and Bhandari, 1984). Dried latex and dried root used as an antidote for snake poisoning. It is also used as an abortificient (Basu *et al*., 1992), for treatment of piles (Gupta *et al*., 1996) and intestinal worms (Singh *et al*., 1980). The tender leaves of plant are also used to cure migraine (Ahmed *et al*., 2005). The capsulated root bark powder is effective in diarrhoea (Ahmed *et al*., 2005) and asthma (Singh *et al*., 1980). The previous pharmacological studies include reports of anticancer (Ahmed *et al*., 2005), antifungal (Hassan *et al*., 2006) and Insecticidal (Ahmed, *et al*., 2006) activity of *C. procera*. The flowers of the plant possessed hepatoprotective activity (Setty *et al*., 2007), anti-inflammatory, antipyretic, analgesic, antimicrobial properties and larvicidal activity (Mascolo *et al*., 1989; Markouk *et al*., 2000). The latex of the plant was reported to possess analgesic and wound healing activity (Dewan *et al*., 2000; Rasik *et al*., 1999), anti-inflammatory activity (Arya and Kumar, 2005) and antimicrobial (Sehgal *et al*., 2005). The roots are reported to have anti-fertility (Kamath and Rana, 2002) and anti-ulcer activities (Basu *et al*., 1997).

Earlier chemical examination of this plant has shown the presence of triterpenoids, calotropursenyl acetate and calopfriedelenyl; a norditerpenyl ester, calotropternyl ester oleanene triterpenes like calotropoleanyl ester, procerleanol A and B (Ansari and Ali, 2001) and cardiac glycosides calotropogenin, calotropin, uscharin, calotoxin and calactin (Ahmed *et al*., 2005). The plant also has been investigated for cardenolides (Seiber *et al*., 1982) and anthocyanins (Ahmed *et al*., 2005). The root bark also found to possess α-amyrin (Saber *et al*., 1969), β-amyrin (Saxena and Saxena, 1979), lupeol, β-sitosterol (Saber *et al*., 1969) and flavanols like quercetin-3-rutinoside (Lal *et al*., 1985). The rich source of phytoconstituents and there are no scientific bases or reports in modern literature regarding usefulness of root bark as hepatoprotective agent prompts us to evaluate root bark of plant for its possible hepatoprotective activity.

In the course of searching for hepatoprotective agents from medicinal plants, the MeOH extract of root bark of *C. procera* was evaluated against carbon tetrachloride induced hepatic damage. The results instigated us for further pharmacological investigation of phyto-constituents diretected fractions from MeOH extract to locate possible active phytoconstituents. The identified active fractions then also studied for *ex vivo* antioxidant activity to identify the possible mechanism of action.

MATERIALS AND METHODS

Plant material: Fresh, well-developed plants of *C. procera* were collected from Rajkot, Gujarat, in the month of September-2007. The authenticity of plants was confirmed by a taxonomist of Gujarat Ayurveda University, Jamnagar, Gujarat. Voucher specimen (HNS 11) was deposited in the department of Pharmacognosy, Shri H. N. Shukla Institute of Pharmaceutical Education and Research, Rajkot, Gujarat. Bark of the roots were separated and dried in the sun and reduced to powder (60 #).

Preparation of extract: Dried root bark powder (200 g) was extracted with methanol by soxhlet apparatus for 5 h. The methanolic extract of *C. procera* (MCP) was tested for qualitative phytoconstituents and indicated the presence of tri-terpenoids and their glycosides, flavanoids, alkaloids and steroids. Hepetoprotective activity of the methanolic extract was studied. Further, phytoconstituents directed fractionation was carried out using concentrated MeOH extract (30 g), suspended in H_2O, acidified with 2N H_2SO_4 and sequentially partitioned with n-hexane and Ethyl acetate. The Acidic layer was basified with dilute ammonium hydroxide ($_pH10$) and extracted with $CHCl_3$. Preliminary phytochemical testing and thin layer chromatography showed presence of terpenoids and steroids in the hexane fraction (HCP), flavanoids in the ethyl acetate (ECP) and alkaloids in chloroform fraction (CCP). All the three fractions were subjected for detailed hepatoprotective activity and *ex vivo* antioxidant activity.

Animals: BLAB/c albino mice (22-25 g) of either sex were used. The animals received a standard pellet diet (Lipton, Mumbai), water ad libitum and were maintained under standard temperature and humidity conditions. All the protocols followed for pharmacological assays were duly endorsed by the Institutional Ethical Committee of Smt. R. D. Gardi B. Pharmacy College, Rajkot, Gujarat.

Hepatoprotective activity: Animals were divided into four groups each of six animals. Group I and II served as normal and intoxicated control, respectively and received only the vehicle (0.5% Tween-80; 1 mL kg^{-1} p.o). Group III animals were treated with standard silymarin at an oral dose of 100 mg kg^{-1} and group IV received the MCP at an

oral dose of 200 mg kg^{-1}. The treatment was continued for 7 days, once daily. On the day of 2nd, 4th and 6th for groups II, III, IV 30 min post-dose of extract administration animals received CCl_4 at the dose of 0.8 mL kg^{-1} (1:1 of CCl_4 in olive oil) orally. Twenty four hours after CCl_4 administration, blood was obtained from all groups of mice by puncturing retro-orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30°C for 15 min and analyzed for various biochemical parameters, Aspartate aminotransferase (SGOT), Alanine aminotransferase (SGPT), Alkaline phosphatase (ALP) and Billirubin (Total and Direct) using Span diagnostic kits.

Detail hepatoprotective study of sub fractions from methanolic extract: Group IV, V and VI received the HCP, ECP and CCP fractions respectively (200 mg kg^{-1}, p.o.) as a fine suspension of 0.5% aqueous Tween-80 and Group VII animals received silymarin (100 mg kg^{-1}, p.o). The treatment was continued for 7 days, once daily. On the day of 2nd, 4th and 6th for groups III-VI received CCl_4 (1:1 of CCl_4 in olive oil) 0.8 mL kg^{-1} I.p. 30 min after the dose of extracts administration. The animals were sacrificed after 36 h of administration of acute dose of CCl_4. The blood was collected and serum was separated out and used for estimation of aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT), alkaline phosphatase (ALP), albumin, billirubin (Total and direct), total protein (TP), cholesterol and HDL using Span diagnostic kits. The liver was immediately dissected out and the liver-tissue was used for estimation of malondialdehyde equivalent, an index of lipid peroxides (LPO), reduced glutathione (GSH), Super Oxide Dismutase (SOD) and Catalase Activity (CAT). A section of liver was processed for histological studies.

Assessments of oxidative stress

Preparation of tissue antioxidant: The livers were rinsed with ice cold distilled water followed by sucrose solution (0.25 M). And again rinsed with distilled water and immediately stored at -20°C till further biochemical analysis. One gram of liver tissue homogenized in 10 mL of ice cold Tris-hydrochloride buffer. The prepared homogenates were centrifuged and used for the assay of determination lipid peroxidation (LPO) by measuring the release of malondialdehyde (MDA) by the method of Slater and Sawyer (1971) and the estimation of reduced glutathione enzyme (GSH) (Moron *et al*., 1979).

Post Mitochondrial Supernant preparation (PMS): The homogenates were centrifuged at 800 rpm for 5 min at 4°C to separate debris. The supernatant so obtained was centrifuged at 10,500 rpm for 20 min at 4°C to get the post mitochondrial supernant (PMS) which was used to assay catalase (CAT) (Aebi, 1984) and superoxide dismutase enzyme(SOD) activity (Misra and Fridovich, 1972).

Histopathological study: The tissues of liver were fixed in 10% formalin and embedded in paraffin wax. Sections of 4-5 microns thickness were made using rotary microtome and stained with haematoxylin-eosin. Histological observations were made under light microscope (Galighor and Kozloff, 1976; Luna, 1968).

Statistical analysis: The results are expressed as Means±SD. The differences between experimental groups were compared by one-way ANOVA (toxic control versus treatment, tukeys method; using Graph pad prism statistical software, version 5.0) and were considered statistically significant at $p<0.05$.

RESULTS

In the present study, it was seen that administration of CCl_4 elevates the levels of serum marker enzymes SGPT, SGOT and ALP (Table 1). It can also be seen from the Table 1 that the animals pretreated with methanolic extract of *C. procera* (200 mg kg^{-1}; p.o.) showed significant ($p<0.001$) decrease in the serum enzyme values compared to those of toxic control values. Motivated by these results, phytoconstituent directed sub fractionation of the methanolic extract was done to identify the active fractions. Three fractions from methanolic extract, the HCP, ECP and CCP were studied for detailed hepatoprotective activity assay. The results are shown in Table 2. The animals treated with toxic doses of CCl_4 showed markedly elevated values of the serum SGPT, SGOT, ALP, total and direct bilirubin, cholesterol and decreased total protein, albumin and HDL compared to normal mice, indicating acute hepato-cellular damage. Pretreatment with HCP and ECP (200 mg kg^{-1}; p.o.) fraction significantly ($p<0.001$) decreased the value of SGOT, SGPT, ALP, billirubin (total and direct) and cholesterol and prevented diminution of HDL value. It suggested clear indication of the improvement of the functional status of the liver cells. Both the fraction showed marginal improvement in the values of total protein and albumin. The CCP did not exhibit significant improvement in serum enzyme values.

Table 1: Effect of MCP on CCl_4 –induced hepatotoxicity in mice

Groups	Treatment	Biochemical parameters SGOT	SGPT	ALP	Billirubin Total	Direct
I	Normal control	107.2±7.2	65.40±5.0	13.6±3.1	0.30±0.03	0.083±0.01
II	CCl_4 treated	309.2±33.1*	684.2±29.2*	48.2±7.6*	1.03±0.2*	0.33±0.06*
III	MCP+ CCl_4	224.9±10.8†	170.8±12.1†	16.3±1.8†	0.30±0.06†	0.20±0.01†
IV	Silymarin + CCl_4	165.8±14.1†	112.5±11.3†	14.3±2.5†	0.3±0.03†	0.11±0.03†

Values are expressed as Mean±SD of six animals in each group. ANOVA Statistical comparisons are as follows: *p<0.001 as compared with group I. †p<0.001as compared with group II

Table 2: Effect of various extracts on CCl_4 -induced hepatotoxicity in mice

Groups	Treatment	Biochemical parameters SGOT	SGPT	ALP	Billirubin Total	Direct	Total protein	Albumin	Cholesterol	HDL
I	Normal	112.5±7.0	73.7±7.4	12.1±2.3	0.23±0.02	0.06±0.005	7.9±0.5	4.7±0.3	133.1±12.4	13.0±0.7
II	CCl_4 Treated	203.3±29.7 A***	733.4±61.4 a***	45.0±6.3 a***	0.84±0.088 a***	0.3±0.045 a***	5.7±2.04 a*	3.6±0.3 a***	188±21.1 a***	2.7±0.4 a***
III	HCP+ CCl_4	136.5±11.0 B***	153.1±20.1 b***	12.2±0.6 b***	0.20±0.014 b***	0.07±0.014 b***	7.7±0.4 b*	4.3±0.3 b*	149.4±4.5 b***	12.1±0.6 b***
IV	ECP + CCl_4	146.2±13.0 B***	207.2±37.3 b***	13.1±2.9 b***	0.195±0.010 b***	0.05±0.013 b***	7.9±0.6 b**	4.2±0.4	158.1±8.2 b***	13.3±0.5 b***
V	CCP+ CCl_4	174.2±21.1	682.8±32.6	38.2±3.3 b*	0.48±0.126 b***	0.22±0.07 b***	6.2±1.5	3.9±0.2	187.7±5.3	3.9±0.2 b*
VI	Silymarin+ CCl_4	128.2±18.0 B***	78.8±18.7 b***	14.9±2.5 b***	0.21±0.021 b***	0.06±0.008 b***	7.2±0.8	4.5±0.1 b**	150.8±5.7 b***	10.1±0.4 b***

Values are expressed as Mean±SD (n = 6); *p<0.05, **p<0.01, ***p<0.001, ANOVA Statistical comparisons are as follows: a vs. Group I; b vs. Group II

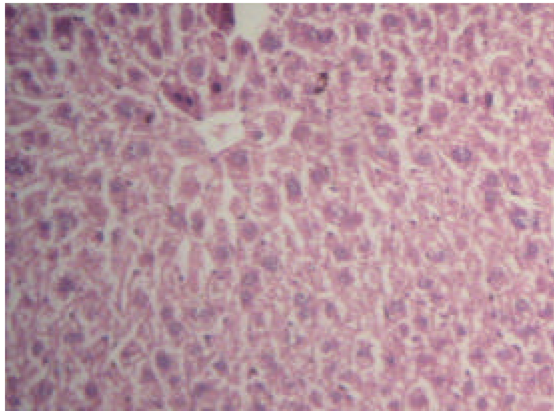

Fig. 1: Microphotograph of normal control mice liver section (x 200)

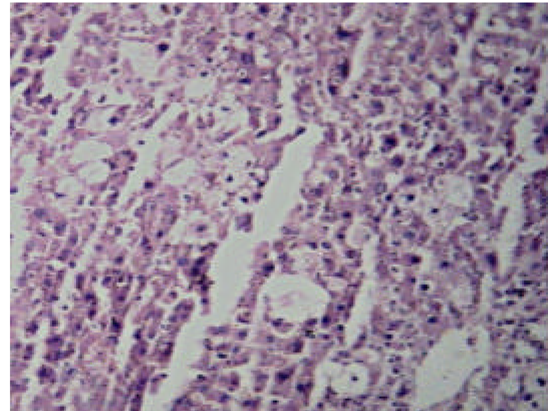

Fig. 2: Microphotograph of mice liver section treated with CCl_4 (x 200)

Further, the results also supported by histopathological examination of liver sections of normal control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein (Fig. 1). Disarrangement of normal hepatic cells with centrilobular necrosis, vacuolization of cytoplasm and fatty degeneration were observed in CCl_4 intoxicated animals (Fig. 2). The liver sections of the mice treated with HCP (Fig. 3), ECP (Fig. 4) and Silymarin (Fig. 5) followed by CCl_4 intoxication showed a sign of protection as it was evident by the absence of necrosis and vacuoles.

In order to elucidate the possible mechanism of the hepatoprotective activity, the effect of HCP and ECP on antioxidant enzymes have also been investigated as the level of these enzymes has been found to be of great importance in the assessment of liver damage. The CCl_4 treated animals had increased tissue lipid peroxide values and decreased SOD, CAT and GSH (Table 3).

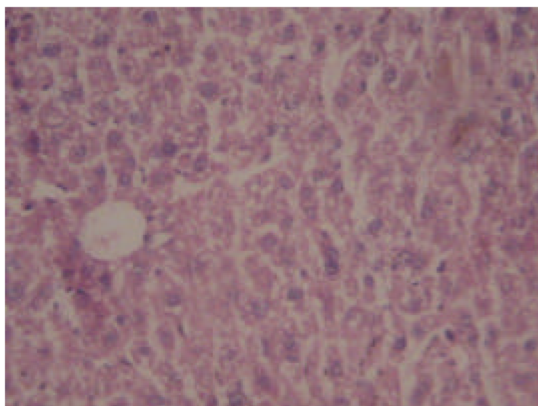

Fig. 3: Microphotograph of liver section of HCP and CCl_4 treated mice (x 200)

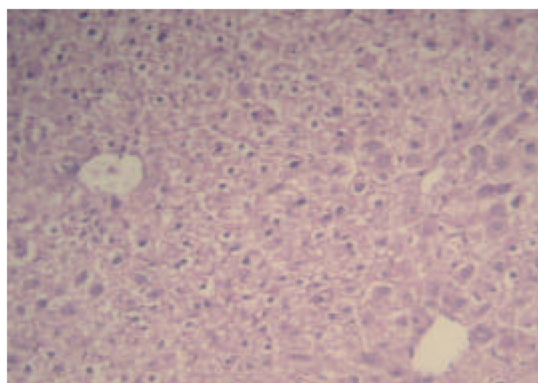

Fig. 4: Microphotograph of liver section of ECP and CCl_4 treated mice (x 200)

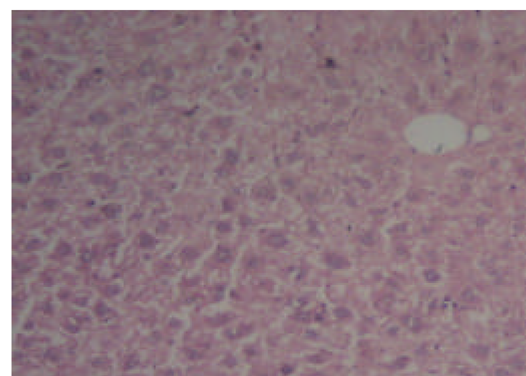

Fig. 5: Microphotograph of liver section of silymarin and CCl_4 treated mice (x 200)

Administration of both the fractions significantly reduced tissue lipid peroxide level and significantly increased the level of SOD and GSH. While, moderate improvement was seen in catalase activity. Hence, protection against liver necrosis could be obtained through antioxidant effect of HCP and ECP.

Table 3: Effect of extracts on LPO, antioxidant enzymes and GSH in livers of CCL_4–induced hepatotoxic mice *exvivo*

Groups	Treatment	LPO[K]	SOD[L]	CAT[M]
I	Normal	4.5±0.7	16.1±1.89	2271.9±155.8
II	CCl_4 treated	33.7±2.6*	3.7±0.87*	536.7±138.9*
III	HCP+CCl_4	7.0±0.6†	10.4±0.79†	860.3±175.9††
IV	ECP+CCl_4	11.9±0.9†	10.73±0.99†	1536.1±178.0†
V	Silymarin+CCl_4	8.4±0.7†	12.5±0.55†	2294.0±147.6†

Values are expressed as Mean±SD of six animals in each group. ANOVA Statistical comparisons are as follows: *$p<0.001$ as compared with group I. †$p<0.001$ as compared with group II (except †† where, $p<0.01$). K= n mole of MDA/mg of protein. L: Units mg^{-1} of protein, M: µmole of H_2O_2 consumed/min/mg of protein. N: µg mg^{-1} of protein

DISCUSSION

Although *Calotropis procera* is reported to possess varied medicinal uses as discussed earlier, there is no previous report about the hepatoprotective activity of the root bark of the plant. The present investigation reports the hepatoprotective activity of the MCP and its sub-fractions HCP and ECP.

In the present study, hepatotoxicity model in mice was successfully produced by administering CCl_4 (1:1 in olive oil, 0.8 mL kg^{-1}) intraperitonially. It is well established that hepatotoxicity by CCl_4 is due to enzymatic activation to release CCl_3 radical in free state, which in turn disrupts the structure and function of lipid and protein macromolecule in the membrane of the cell organelles (Mujumdar *et al*., 1998). CCl_4 also plays a significant role in depletion of Intracellular antioxidant reduced glutathione (GSH), increased lipid peroxidation, membrane damage, depression of protein synthesis and loss of enzymes activity (Recknagel *et al*., 1989). As the damage marker enzymes SGOT and SGPT are cytoplasmic in location (Sallie *et al*., 1962) they get released in serum (Chenoweth and Hake, 1962). So increase in the level of SGPT, SGOT, ALP, total and direct bilirubin, cholesterol and HDL is conventional indicator of liver injury. CCl_4 challenges significantly decrease the levels of SOD, GSH and catalase in liver. The level of MDA which is produced as a result of lipid peroxidation is significantly increased.

As discussed in results, MCP, HCP and ECP showed significant hepatoprotective activity on CCl_4 induced hepatotoxic animals. In preliminary study, MCP significantly reduced the elevated levels of the different enzyme values. Further, in order to find out active fraction detailed study was done with the treatment of phytoconstituent directed three fractions, of which HCP and ECP showed significant hepatic protective activity. In addition, HCP and ECP also showed appreciable increase

in the levels of GSH, SOD and catalase whereas decreased the lipid peroxidation. Plant demonstrated superoxide scavenging activity there for it may be inferred that the antioxidant property of the plant may prevent formation of free radical and so inhibit the lipid peroxidation and offers the hepatoprotection against CCl_4 toxicity. Further, the improvement of GSH level by HCP and ECP treatment also indicate the natural tissue protection mechanism is kept intact and oxidative degeneration of the liver tissues prevented.

The results for antioxidant study suggest that the reason for hepatoprotective effect of the extracts may be that *C. procera* contains terpenoids (Sunitha *et al*., 2001) and flavanoids (Janbaz *et al*., 2002) which might have scavenged the free radical offering hepatoprotection.

In conclusion, the present study scientifically demonstrates that the root bark of *Calotropis procera* possess hepatoprotective property. In addition the hepatoprotective property may be attributed to the antioxidant principles of the plant.

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