Vol. 6/2 (2006) 147 - 152

JOURNAL OF NATURAL REMEDIES

Hepatoprotective effects of the triterpenes isolated from the stem bark of *Diospyros cordifolia* Roxb.

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Abstract

The hepatoprotective activity of the chemical constituents isolated from the stem bark of *Diospyros cordifolia* Roxb. was screened on male Wister strain rats against carbon tetrachloride induced toxic hepatitis. Significant hepatoprotective activity was observed in the active constituents, ursolic acid, lupeol and betulin isolated from the petroleum ether extract of stem bark. The hepatoprotective activity was assessed by the assay of liver function parameters *viz.* serum total bilirubin, total protein, alanine aminotransaminase, aspartate aminotransaminase and alkaline phosphatase activities. Histopathological studies also evidenced the hepatoprotective effect of the tested constituents. Ursolic acid showed more significant hepatoprotective activity than lupeol and betulin. The results have comparison with the standard hepatoprotective drug silymarin.

Keywords: *Diospyros cordifolia*, stem bark, ursolic acid, hepatoprotective effect.

1. Introduction

Diospyros cordifolia Roxb. (Ebenaceae) is a deciduous tree and popularly known as Indian ebony. In Karnataka State, India, the plant is sparsely distributed in the deciduous forests of Nagarahole National Park, Kollur reserve forest and Bhadra Wild Life Sanctuary [1, 2]. The plant is of great medicinal importance used for liver disorders, whooping cough, leprosy, skin eruptions, dysentery, eye infections, abdominal pains, wounds, ulcers, gonorrhea, fever, as emetic and anthelmintic [3, 4]. The alcoholic extract of the plant possesses anti-inflammatory, antipyretic, analgesic activity [5], CNS depressant, spasmolytic, produces bradycardia and hypotension [6]. The aqueous extract of the stem bark is traditionally used by the tribal

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people 'Soligas' of Biligiri Ranga Hill range to cure critical jaundicised condition [7].

Literature survey indicated that no systemic studies have been carried out on the clinical evaluation of hepatoprotective activity of stem bark of *Diospyros cordifolia* Roxb. The petroleum ether extract of the stem bark of *Diospyros cordifolia* Roxb. exhibited significant hepatoprotective activity against carbon tetrachloride induced toxic hepatitis on male Wister rats [8].

This paper is reporting the evaluation of the hepatoprotective effect of the constituents isolated from the petroleum ether extract of stem bark of *Diospyros cordifolia* against carbon tetrachloride induced toxic hepatitis. The results were also comparatively evaluated with the standard hepatoprotective drug silymarin.

2. Materials and methods

2.1 Plant

The stem bark and the flowering twigs of *Diospyros cordifolia* were collected from the Lakkavalli range forest of Bhadra Wild Life Sanctuary, Karnataka. The plant was authenticated by comparing with the herbarium specimen at Kuvempu University Herbaria (F. D. D. KUSF - 801) [9] and Sri Krishna-devaraya University Herbaria (S.K.H., CAB-1590) [10].

2.2 Preparation of plant extracts

The plant material was shade dried and powdered mechanically (sieve10/44). This was extracted with hot petroleum ether for 48 h. The extract so obtained was concentrated in vacuum using rotary flash evaporator (Buchi). The yield was 1.20%.

2.3 Isolation

The constituent ursolic acid was isolated from the petroleum ether extract according to the method of Suresh Chandra *et.al.* (1989) [11]. In addition, the other constituents lupeol and betulin were also isolated and characterized by the authors from the petroleum ether extract by the method of Kapil *et.al.* (1960) [12].

2.4 Drug formulation

Oral suspensions containing 10 mg/ml of each active constituents viz. ursolic acid, lupeol and betulin were prepared in 1% gum tragacanth.

2.5 Animals

Male Wister albino rats weighing 150-200 g were obtained from the Central animal house, National College of Pharmacy, Shimoga, Karnataka. They were maintained at standard housing conditions and fed with commercial diet (Hindustan Lever Ltd., Bangalore) and water *ad libitum* during experiment. The institutional animal ethical committee (Reg.No.144/1999/CPCSEA/SMG) permitted the study.

2.6 Evaluation of hepatoprotective activity

The animals were divided into six groups of six rats each. The animals of group-I served as control for 14 days and received 1 ml/kg /day of 1% gum tragacanth orally. The animals of groups II to VI received 0.1 ml/kg/day CCl_4 in 1:1 olive oil (0.1 ml/kg/day ip) [13] for 10 days. In addition to CCl_4 the animals of groups III were treated with silymarin (100 mg/kg/day orally) [14] for 14 days. While the animals of groups IV, V and VI received the test samples of ursolic acid (10 mg /kg /day orally) [15], lupeol [16] and betulin [17] (50 mg/kg/day orally) for 14 days along with CCl_4.

2.7 Assessment of hepatoprotective activity

All the animals were sacrificed on 14^{th} day under light ether anesthesia. The blood samples of each animal were collected separately by carotid bleeding into sterilized dry centrifuge tube and allowed to coagulate for 30 min at 37°C. The

clear serum was separated at 2500 rpm for 10 min and was subjected for liver function biochemical investigations *viz.* estimation of total bilirubin [18] total protein [19], serum alanine aminotransaminase (ALT) and serum aspartate aminotransaminase (AST) [20] and serum alkaline phosphatase (ALP) [21].

The results of the experiment were evaluated as mean ± SE of six animals in each group. The data was subjected to one-way ANOVA followed by Tukey's multiple comparison test. P values <0.001 were considered as statistically significant.

2.8 Histopathology

After blood draining, liver samples were excised from all the experimental animals and washed with normal saline separately and processed for dehydration, infiltration and embedding [22]. Initially the materials were fixed in 10% buffered neutral formalin for 48 h and then with bovine solution for 6 h. They were infiltered and embedded with paraffin. The microtome technique sections were taken at 5-micron thickness, processed in alcohol-xylene series and were stained with alum haematoxylene and eosin. The sections were examined microscopically, for the evaluation of histopathological changes.

3. Results

From stem bark of *Diospyros cordifolia* Roxb. the triterpenoids *viz.* ursolic acid, lupeol and betulin were isolated from the petroleum ether extract. The identity of the compounds was confirmed by the IR, HNMR and Mass spectral studies.

The administration of CCl_4 to the animals resulted in a marked increase in the levels of total bilirubin (2.389 ± 0.133), serum alanine amino transaminase (ALT) (2194.33 ± 42.547), serum aspartate amino transaminase (AST) (1390.25 ± 28.281) and serum alkaline phosphatase (SALP) (423.30 ± 18.109). However, the serum level of total protein was decreased (5.966 ± 0.315) as shown in the

Table 1
Hepatoprotective effect of the constituents isolated from the stem bark of *Diospyros Cordifolia* on CCl_4 induced hepatotoxicity in rats.

Group (N)	Total Bilirubin mg/dl	Total Protein gm %	AST IU/L	ALT IU/L	ALP IU/L
Control	0.492 ± 0.03	9.246 ± 0.122	51.07 ± 0.611	148.9 ± 0.362	173.60 ± 2.565
CCl_4 Treated	2.389 ± 0.133	5.966 ± 0.315	1390.25 ± 3.281	2194.33 ± 4.54	423.3 ± 18.109
CCl_4+ Silymarin	0.503 ± 0.002*	8.717 ± 0.013*	89.04 ± 0.420*	205.05 ± 0.924*	181.67 ± 0.523*
CCl_4 + Ursolic acid	0.531 ± 0.002*	8.432 ± 0.033*	115.54 ± 0.603*	235.18 ± 1.214*	193.37 ± 0.424*
CCl_4 + Lupeol	0.589 ± 0.003	7.395 ± 0.029	244.08 ± 1.243	340.66 ± 0.996	266.51 ± 0.798
CCl_4 + Betulin	0.630 ± 0.007	6.986 ± 0.027	385.33 ± 0.985	640.45 ± 2.101	290.98 ± 0.804

N = 6 animals in each group; P<0.001 indicates significant when compared to control
Values are expressed as mean ± S.E.

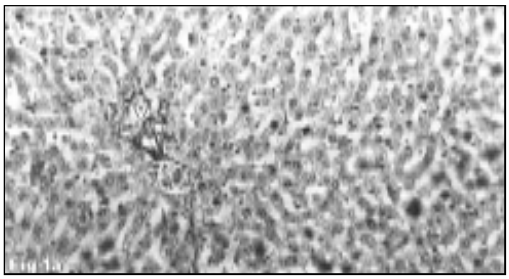

Fig. 1 (a)

Liver section of control animal showing ormal histology, portal triad (Vein V, Artery Arrow, Hepatic duct Arrow head) (H & E stain, 100x).

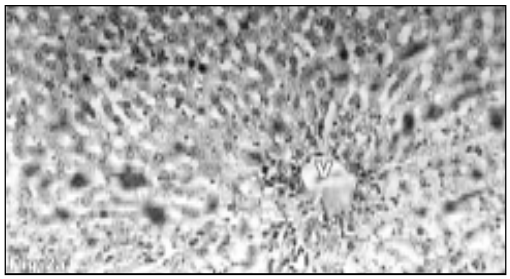

Fig. 2 (a)

Section of the liver tissue of Ursolic acid treated animal showing normal hepatocytes around central vein (V), (H & E stain, 100x).

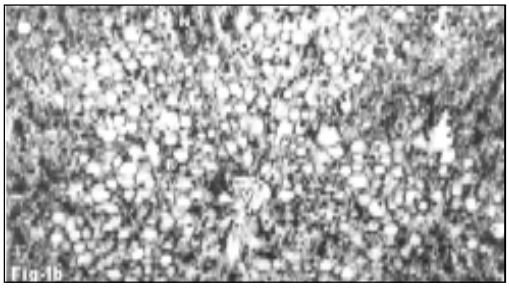

Fig. 1 (b)

Liver section of CCl4 treated animal showing necrosis (N), central vein (V), and fatty vacuole (F), (H & E stain, 100x).

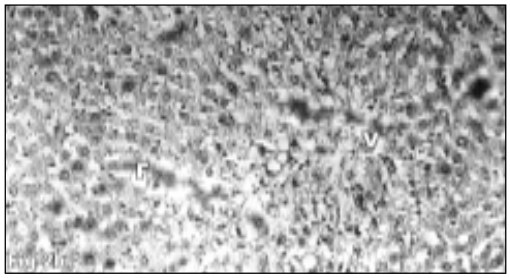

Fig. 2 (b)

Liver section of Lupeol treated animal showing moderate fatty lobule (F) and central vein (V), (H & E stain, 100x).

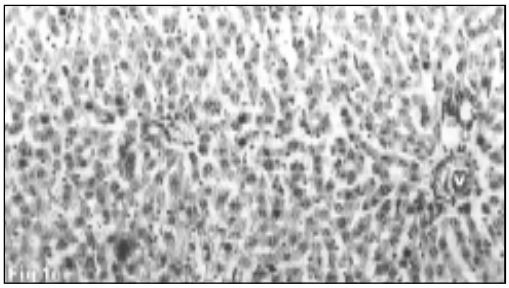

Fig. 1 (c)

Liver section of Silymarin treated animal showing normal hepatocytes (Vein V, Artery Arrow, hepatic duct Arrow head), (H & E stain, 100x).

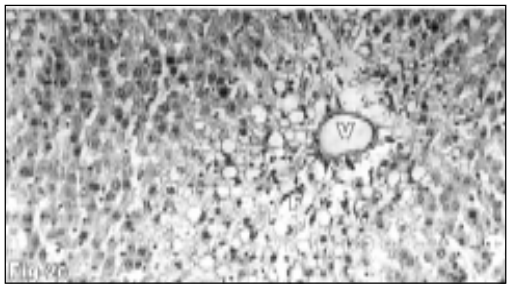

Fig. 2 (c)

Section of the liver tissue of the animal treated with Betulin showing increased fatty lobules (F) and central vein (V), (H & E stain, 100x).

Table 1. On the contrary, the blood samples of ursolic acid, lupeol and betulin treated animals exhibited hepatoprotective activity by controlling the toxic effect of CCl_4. Among these treated groups the extent of liver damage was of lesser in magnitude in the animals treated with ursolic acid and the standard drug silymarin.

The histopathological profile of the normal rat liver is shown in Fig. 1a. Histological observations of group II animals exhibited severe intense centrilobular necrosis and vacuolization, ballooning degeneration of hepatocytes due to fatty infiltration (Fig.1b).

The sections of the liver taken from the animals treated with standard drug silymarin (Fig.1c) and ursolic acid (Fig.2a) showed significant signs of hepatoprotection as evident from the presence of normal hepatic cords, absence of necrosis and fatty infiltration. On the contrary, sections of liver taken from the animals treated with lupeol and betulin showed moderate accumulation of fatty lobules (Fig. 2b and 2c respectively).

4. Discussion

The injury and dysfunction of liver caused by the toxic effect of carbon tetrachloride in experimental animals simulated the human viral hepatitis model [23]. In most of the studies this toxic chemical was used to induce hepatotoxicity [24]. In CCl_4 induced toxic hepatitis, a toxic reactive metabolite namely trichloromethyl (CCl_3) was produced by the microsomal oxidase system. This activated radical binds covalently to the macromolecules of the lipid membranes of the adipose tissue and cause peroxidative degradation.

As a result, fats from the adipose tissue get translocated and accumulated in the liver [25]. The increase in the levels of liver function parameters was a clear indication of cellular leakage and loss of functional integrity of the cell membrane [26].

Among the isolated constituents of *Diospyros cordifolia* stembark, ursolic acid was highly effective in preventing the toxic effect of CCl_4. While the therapeutic efficacy was less in animals treated with lupeol and betulin.

However, significant protection against the liver damage was also observed in animals treated with the standard drug silymarin. Chun-Ching Lin (1995) [27], Bahar Ahmad *et.al.* (2000) [28] have also used silymarin as the standard for the evaluation of the hepatoprotective activity of the plant extracts. Our results suggest that the constituents prevent fatty liver and exhibit membrane stabilizing acivity [29].

The hepatoprotective activity of constituents may either be due to either their ability to induce microsomal enzymes which accelerates the excretion of CCl_4 or inhibition of lipid peroxidation. [30] Reports also revealed that the triterpenoids have the anti-oxidant property [31]. It is also suggested that the hepatoprotective activity may be due to the inhibition of liver cytochrome-P-450 enzymes so as to prevent the oxidation of CCl_4 into the toxic radical [32].

The screening of the drugs of ethnomedical importance for the protection of the liver against damage is meaningful.

5. Acknowledgements

The authors are grateful to Prof. B. Abdul Rahiman, Chairman, Dept. Biotechnology, Kuvempu University and National Education Society. Shimoga for providing necessary facilities to carry out this work.

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