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Isolation and *in vivo* hepatoprotective activity of *Melothria heterophylla* (Lour.) Cogn. against chemically induced liver injuries in rats

Arijit Mondal[1*], Tapan Kumar Maity[1], Dilipkumar Pal[2], Santanu Sannigrahi[3], Jagadish Singh[1]

[1]*Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700 032, India*
[2]*School of Pharmaceutical Sciences, IFTM University, Moradabad- 244 001, India*
[3]*NSHM College of Pharmaceutical Technology, 124 B.L. Saha Road, Kolkata-700 053, West Bengal, India*

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ABSTRACT

Objective: To investigate hepatoprotective activity of ethanol extract of *Melothria heterophylla* Lour Cogn. (EEMH) against CCl_4-induced hepatic damage in rats. **Methods:** β -sitosterol was isolated by column chromatography and characterized spectroscopically. Two different doses (200 and 400 mg/kg bw) of EEMH were administered orally in alternate days. The hepatoprotective activity was studied in liver by measuring biochemical parameters such as serum aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), total protein and total bilirubin. Lipid peroxidation product and different antioxidant enzyme activities were assessed in liver homogenate. **Results:** EEMH reduced all biochemical parameters and lipid peroxidation, as well as it increased the antioxidant enzyme activities in comparison with silymarin. The protective effect of the extract on CCl_4 induced damage was confirmed by histopathological examination of the liver. **Conclusions:** This result strongly supports the protective effect of EEMH against acute liver injury, and may be attributed to its antioxidative activity.

1. Introduction

Melothria heterophylla Lour. Cogn.(*M. heterophylla*), family- Cucurbitaceae, popularly known as kudari, a scandent herb with tuberous roots found throughout India ascending up to 2 100 m in the hills. They are considered to be used by the tribal of Orissa for their stimulant, invigorating and purgative property[1]. The juices of the leaves are applied to the parts inflamed by the application of the marking nut juice (from *Semecarpus anacardium* Linn). It is established that diabetes is associated with low level of antioxidants and many plant show hypoglycemic activity because of their antioxidant potential[2,3]. As this plant is reported to have the antioxidant activity[4], it may also be useful in liver damage. Thus, the present study was to investigate the hepatoprotective activity of ethanol extract of aerial parts of *M. heterophylla* (EEMH) against CCl_4-induced hepatic damage in rats.

*Corresponding author: Arijit Mondal, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, West Bengal-700 032, India.
Tel: +91-9674124916.
E-mail: arijit_ncp@rediffmail.com

2. Materials and methods

2.1. Plant material

Aerial parts of *M. heterophylla* was collected from young matured plants during August to September, from the rural belt of Mayurbhanj district, Orissa and identified by taxonomist, Botanical Survey of India, Howrah, India. A voucher specimen (CNH/I-I (65) 2006/Tech.II/1661) was deposited in the Department of Pharmaceutical Technology, Jadavpur University. The collected plant materials were washed, shade-dried and then milled to coarse powder.

2.2. Preparation of extracts and isolation technique.

Dried powdered plant material (40-mesh size) was extracted with absolute alcohol by soxhlate apparatus for 48 h. Extracts were screened for the presence of various phyto-constituents employing standard screening test[5].

30 g of the methanol extract was adsorbed on silica gel (Silica gel 60 G, Merck, 600 g) and applied to a column of silica gel. A gradient of toluene: chloroform: methanol (4.5 : 3.5 : 1.5 v/v) was used to elute the column, collecting 100 fractions of 50 mL each. Fractions were combined into five groups (F_1-F_5) according to their similarity of TLC patterns. F_1 (2 g, fractions 1-25) which shows a single band, was rechromatographed in a second silica gel column eluted with hexane-$CHCl_3$ (8 : 2) mixtures to give one compound which is recrystallised with methanol to give pure β-sitosterol (25 mg).

2.3. Animals

Adult male Wistar albino rats weighing 150-180 g were used for the present investigation. All animal experiments were duly approved by Institutional Ethical Committee (CPCSEA/ORG/CH/2006/Reg.No.95), Jadavpur University, Kolkata, India.

2.4. Chemicals and drugs

Silymarin was purchased from Microlabs (Hosur, Tamilnadu, India), 1-chloro-2, 4-dinitrobenzene, bovine serum albumin was purchased from Sigma Chemical St.Louis, MO, USA. Thiobarbituric acid and nitrobluetetrazolium chloride were purchased from Loba Chemie, Mumbai, India. 5, 5′-dithio bis-2-nitrobenzoic acid and carbon tetrachloride purchased from SISCO Research Laboratory, Mumbai. All other chemicals and solvent were of analytical grade and commercially available.

2.5. Acute toxicity test

The EEMH suspension was administrated orally in increasing dose up to 2 000 mg/kg bw. These animals were observed for mortality and toxicity for 72 h[6].

2.6. Experimental design

Animals were divided into five groups (n=6). Group I served as a vehicle control, which received liquid paraffin, intraperitoneally. Group Ⅱ - Ⅴ were treated with CCl_4 in liquid paraffin (1 : 2) at the dose of 1 mL/kg bw intraperitoneally once in every 72 h for 16 days[7]. Aqueous suspension of EEMH at the doses of 200 mg/kg bw and 400 mg/kg bw, were administered orally to the animals in group Ⅲ to Ⅳ in alternate days for 16 days. Group Ⅴ received silymarin as a standard drug at the dose of 25 mg/kg bw, *po*. in alternate days for 16 days. On the17th day, all rats were sacrificed by cervical dislocation after collecting the blood from retro-orbital plexus under ether anesthesia for biochemical estimations. Blood samples were clotted and the serum was separated by centrifugation at 5 000 rpm for 5 min and used for the assay of biochemical marker enzymes.

2.7. Biochemical estimations

Different biochemical parameters like aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatatse (ALP), total bilirubin and total protein were determined by using commercially available kits (Span Diagnostic Limited, Surat, India).

2.8. In vivo antioxidant status

2.8.1. Lipid peroxidation

900 mg of liver tissue was collected from each experimental rat, washed in normal saline and soaked in filter paper. 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[8]. The supernatant was collected and estimated for lipid peroxidation. The level of lipid peroxides was expressed as μ mole malondialdehyde (MDA)/g liver tissue.

2.8.2. Glutathione content

A 30% w/v liver homogenate was prepared in 0.15 M Tris-HCl buffer (pH 7.4) and trichloroacetic acid was added to precipitate proteins. Samples were centrifuged at 15 000 rpm at 4 ℃ for 1 h. The supernatant was analyzed for content of reduced glutathione and expressed as μg/g of liver tissue[9].

2.8.3. Catalase activity

900 mg liver tissue was homogenized in 3.0 mL M/150 phosphate buffer in ice and centrifuged at 30 000 rpm for 1 h at 4 ℃. The supernatant was taken to determine catalase activity[9].

2.9. Histological observation

Washed liver tissues were fixed by using fixative (picric acid, formaldehyde and 40% glacial acetic acid) for 24 h and dehydrated with alcohol. Liver tissues were cleaned and embedded in paraffin (melting point 58-60 ℃), cut in 3-5 μm sections, stained with the haematoxylin-eosin dye and finally, observed under a photomicroscope and morphological changes such as cell necrosis, ballooning degeneration, fatty changes or inflammation of lymphocytes were observed[10].

2.10. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Results are expressed as mean ± SD for six rats in each group. Differences among groups were considered significant at P<0.05 level.

3. Results

3.1. Phytochemical screening and isolation of β-sitosterol

Preliminary phytochemical screening of the ethanol extract of *M. heterophylla* revealed the presence of steroids, glycosides, saponin and flavonoids. Different compositions of the mobile phase were tested and the desired resolution of β-sitosterol with symmetrical and reproducible peak was achieved by using the mobile phase of toluene: chloroform: methanol (4.5 : 3.5 : 1.5 v/v).

β-sitosterol, has been quantified by gas chromatography (GC) and tandem mass spectrometry using gas chromatography (GC-MS). The gas chromatogram showed a single peak of β-sitosterol (Figure 1). This is the first report on isolation and identification of β-sitosterol from *M. heterophylla*. The structure of the compound was characterized by UV, IR, GC, GC-MS and 13C-NMR methods as β-sitosterol (Figure 2). Structures and the IR, 13C-NMR and GC-MS data obtained independently in these studies are in close conformity with reported literature.

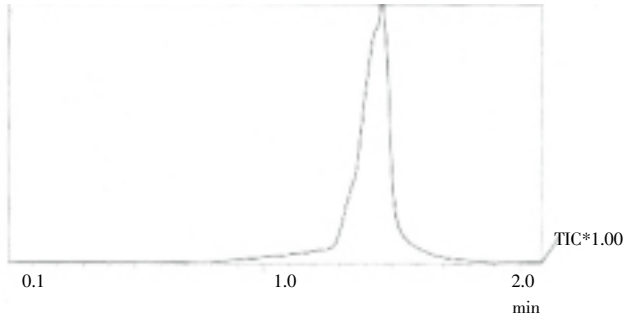

Figure 1. Gas chromatogram showing a single peak of β-sitosterol.

White solid; UV λ_{max} (C_2H_5OH): 206 nm; m.p. 136-138 ℃. MS m/z 414.4 (calculated value $C_{29}H_{50}O$, 414). 13C NMR (75 MHz, $CDCl_3$) δ 140.74, 121.73, 71.78, 56.74, 56.02, 50.10, 45.80, 42.30, 39.75, 37.23, 36.49, 36.13, 33.92, 31.88, 31.63, 29.11, 28.24, 26.02, 24.29, 23.04, 21.06, 19.82,19.39, 19.01, 18.76, 11.97, 11.85. IR (KBr) v cm^{-1}, 3 426, 2 924, 2 855, 1 738, 1 460, 1 375, 1 259, 1 057, 756.

3.2. Acute toxicity study

No mortality was observed up to doses 2 000 mg/kg, orally and it was considered as safe. No lethality or any toxic reactions were found up to the end of the study period.

3.3. Hepatoprotective activity

Rats treated with CCl_4 developed a significant hepatic damage and oxidative stress. There were significant increase in serum GPT, GOT, ALP and bilirubin levels in CCl_4 treated rats compared to normal rats (P<0.01). Whereas, total protein levels were significantly decreased in CCl_4 intoxicated rats (P<0.01). EEMH at the doses of 200 and 400 mg/kg *po*. significantly decreased the elevated serum marker enzymes (P<0.01). Total bilirubin and total proteins were found to be restored to almost normal level. Effects of EEMH on serum GOT, GPT, ALP, bilirubin and total protein levels in CCl_4 intoxicated rats are summarized in Table 1.

Lipid peroxidation and glutathione levels were significantly changed in CCl_4 treated rats when compared with normal rats (P<0.01). The increase in MDA suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent the formation of excessive free radicals. Effects of EEMH on lipid peroxidation expressed in terms of MDA, glutathione level and catalase level in rat liver tissue (Table 2). Treatment with EEMH at the doses of 200 and 400 mg/kg *po*. significantly decreased the elevated lipid peroxide level (P<0.01) and restored the altered glutathione (P<0.05) and catalase (CAT) levels (P<0.01) in CCl_4 intoxicated rats. The enzymatic antioxidant such as glutathione (GSH) and CAT level was markedly decreased in CCl_4 treated rats, but treatment with EEMH restored the altered GSH and CAT level.

Histological observation of liver tissue in normal animal showed hepatic cells with well-preserved cytoplasm, nucleus, nucleolus, and central vein (Figure 3a). In CCl_4-induced group, histological observation showed fatty degeneration, damage of parenchymal cells, lymphocyte infiltration and hydropic degeneration of liver tissue (Figure 3b). Prominent damage of central lobular region appeared in the liver. Treatment with EEMH and Silymarin decreased the abnormality of liver architecture induced by CCl_4 (Figure 3c, 3d and 3e) and restored the altered histopathological changes significantly.

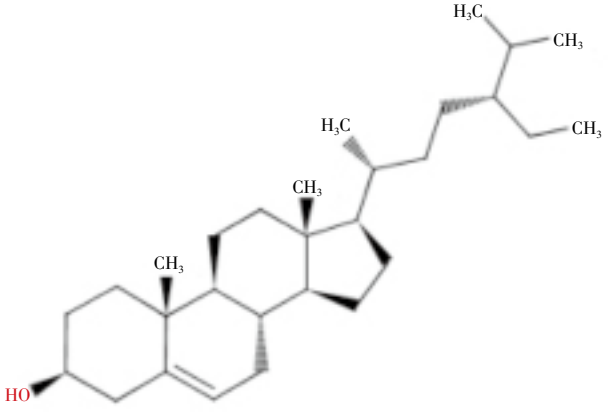

Figure 2. Structure of Stigmast-5-en-3 β-ol (β-sitosterol).

Table 1
Effect of EEMH and silymarin on serum biochemical parameters(mean±SEM, *n*=6).

Group	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total protein(mg/dL)	Total bilirubin(mg/dL)
Control	33.32 ±2.37	109.90±8.08	130.2 0±10.75	8.39 ±0.53	1.10±0.23
CCl_4(1 mL/kg)	97.59 ±5.76△	365.10±34.75△	407.90±11.77△	5.81±0.42△	4.70±1.23△
EEMH (200 mg/kg) + CCl_4	40.82 ±1.14*	168.90±21.36*	207.50±10.75*	7.12± 0.17	2.90±0.35
EEMH (400 mg/kg) + CCl_4	42.76 ±2.19*	151.80±15.85*	184.70±7.58*	7.49±0.59**	2.60±0.57**
Silymarin (25 mg/kg) + CCl_4	42.73 ±5.83*	187.90±25.99*	176.10±10.83*	6.68±0.32	2.30±0.34*

*:P<0.01, **: P<0.05 as compared with CCl_4; △: P<0.01 significant as compared with control.

Table 2
Effect of EEMH on lipid peroxidation and different antioxidant enzyme activity(mean±SEM, *n*=6).

Group	MDA(nM/gm tissue)	GSH(μM/mg tissue)	CAT(U/mg tissue)
Control	25.61 ±0.72	11.01 ±0.93	274.4 ±3.49
CCl_4(1 mL/kg)	65.35 ±0.79△	7.67 ±0.56△	152.1 ±4.34△
EEMH (200 mg/kg) + CCl_4	50.27 ±3.77*	9.87 ±0.53**	202.4 ±2.58*
EEMH (400 mg/kg) + CCl_4	45.35 ±1.38*	10.04 ±0.24**	205.4 ±6.29*
Silymarin (25 mg/kg) + CCl_4	44.15 ±0.85*	10.2 ±0.23**	251.4 ±3.72*

*:P<0.01, **: P<0.05 as compared with CCl_4; △: P<0.01 significant as compared with control.

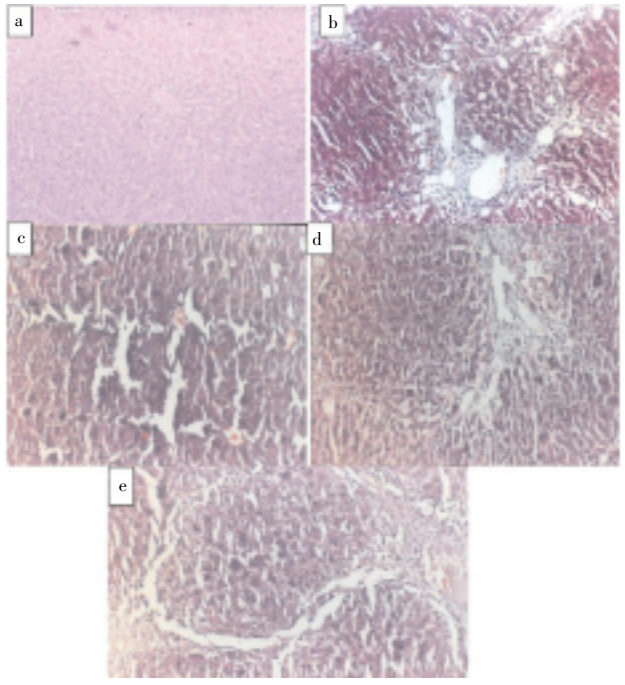

Figure 3. Photomicroscoph of liver sections from CCl_4 intoxicated rats (10×).
Histological observations of the liver tissue of the normal animals showed hepatic cells with well preserved cytoplasm, nucleus, nucleolus and central vein (Figure 3a). Treatment with CCl_4 caused fatty degeneration with severe necrosis of the parenchyma cells in the central lobular region of the liver. Furthermore hepatocytic necrosis was predominant surrounding the central vein, which formed a streak like appearance (Figure 3b). Figure 3c, 3d and 3e showed animals treated with EEMH (200 and 400 mg/kg, *po*.) and silymarin (25 mg/kg) and restored the altered histopathological changes, respectively.

4. Discussion

Carbon tetrachloride is one of the most commonly used hepatotoxin for inducing experimental hepatic damage[11], due to the formation of free radical during its metabolism by hepatic microsome, which in turn, cause lipid peroxidation of the cellular membrane leading to the necrosis of hepatocyte.

Lipid peroxidation has been postulated to be the destructive process of liver damage due to CCl_4 intoxication[12]. GSH constitutes the first line defense against free radicals, as it is one of the abundant tripeptide non-enzymatic biological antioxidants present in the liver[13-19]. Catalase is an enzymatic antioxidant widely distributed in all animal tissues[20]. Catalase decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals. Therefore, reduction in the activity of these enzymes may result in several deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide.

The observed hepatoprotective effect of the *M. heterophylla* extract against CCl_4-induced hepatotoxicity may be attributed to the presence of β-sitosterol[21]. Plant provides a source of inspiration for separation of novel drug compounds as plants derived medicines have made significant contribution as hepatoprotective agent, so it can be beneficial towards human health. Phytomedicine can be used for the treatment of diseases in Unani and Ayurvedic system of medicines. No synthetic drugs have been developed so far as effective hepatoprotectives, and some are hepatotoxic. Hence the treatment with plant derived drugs can be the base for the development of a natural hepatoprotective medicine.

The hepatoprotective effects of EEMH may be because of its antioxidant property. Phyto-chemical analysis showed that EEMH contain steroids, saponins and flavonoids. β-sitosterol, the major bioactive compound isolated from *M. heterophylla*. Results of this study demonstrate that ethanol extract of *M. heterophylla* has a potent hepatoprotective action on $CC1_4$ induced hepatic damage in rats.

Conflict of interest statement

We declare that we have no conflict of interest.

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