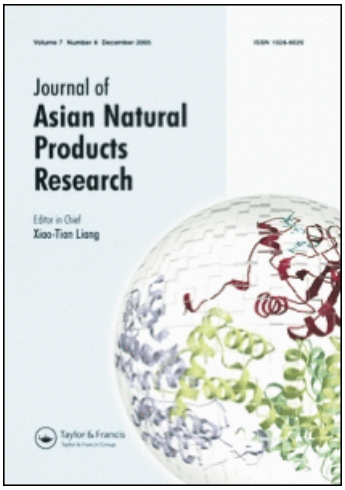

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Anti-hepatotoxic activity of cichotyboside, a sesquiterpene glycoside from the seeds of *Cichorium intybus*

Bahar Ahmed[a]; Shamshir Khan[a]; Mubashir H. Masood[a]; Anwarul H. Siddique[a]
[a] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Hamdard University, New Delhi, India

To cite this Article Ahmed, Bahar , Khan, Shamshir , Masood, Mubashir H. and Siddique, Anwarul H.(2008) 'Anti-hepatotoxic activity of cichotyboside, a sesquiterpene glycoside from the seeds of *Cichorium intybus*', Journal of Asian Natural Products Research, 10: 3, 218 — 223
To link to this Article: DOI: 10.1080/10286020701590764
URL: http://dx.doi.org/10.1080/10286020701590764

Journal of Asian Natural Products Research
Vol. 10, No. 3, March 2008, 218–223

Taylor & Francis
Taylor & Francis Group

Anti-hepatotoxic activity of cichotyboside, a sesquiterpene glycoside from the seeds of *Cichorium intybus*

Bahar Ahmed*, Shamshir Khan, Mubashir H. Masood and Anwarul H. Siddique

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Hamdard University, Hamdard Nagar, New Delhi 110062, India

(*Received 29 August 2006; final version received 27 April 2007*)

The seeds of *Cichorium intybus* L. (Asteraceae) afforded a new guaianolide sesquiterpene glycoside, cichotyboside, which was characterized as 2α, 6β, 7β, 15-tetrahydroxy-1 (10), 4 (5)-diene-guaian-9α, 12-olide-7-O-β-caffoyl-15-O-β-D-glucoside (**1**) by means of spectral methods. Cichotyboside (**1**) exhibited a significant anti-hepatotoxic activity against CCl_4 induced toxicity in Wistar rats, wherein it reduced the elevated levels of liver enzymes such as serum glutamate oxaloacetate transaminase (SGOT) by 52 units/ml; SGPT 38 units/ml; ALKP 24.97 units/ml and 7.54 g/dl, 5.48 g/dl increase in total protein and albumin, respectively. It was observed that cichotyboside (**1**) decreased the level of ALKP comparable with that of standard drug silymarin, exhibiting an 88% decrease in comparison to silymarin (92%) and increased the level of total albumin 85% in comparison to silymarin (89%) against intoxicated control. Whereas, the levels of SGOT and SGPT were also decreased considerably in comparison to standard and intoxicated control.

Keywords: anti-hepatotoxic activity; carbon tetrachloride; *Cichorium intybus*; silymarin

1. Introduction

The plant *Cichorium intybus Linn* (Asteraceae), commonly known as Kasni or Chicory, is native to the temperate parts of the old world and found wildly in Punjab and Andhra Pradesh. It is cultivated in Bihar, Himachal Pradesh, Assam, Maharashtra, Gujarat, Tamil Nadu, Orissa, and Kerala.[1] The plant is an erect perennial herb with a fleshy taproot. The leaves are crowded at the base forming a rosette arranged spirally on the stem.[2] It has been reported to possess antimicrobial, anti-inflammatory, and anticoagulant activity. It has also been used in hepatic disorders.[3]

The literature survey has shown that some sesquiterpenes have been reported from the plant.[4–5] The crude extract has also been reported to possess antihepatotoxic activity.[3] We have isolated a new sesquiterpene glycoside from the methanolic extract of the seeds of the plant, which has been characterized as 2α, 6β, 7β, 15-tetrahydroxy-1 (10), 4 (5)-diene-guaian-9α, 12-olide-7-O-β-caffoyl-15-O-β-D-glucoside, designated as cichotyboside (**1**). The compound has also showed anti-hepatotoxic activity on Wistar rats.

2. Results and discussion

Compound **1**, cichotyboside, was obtained as viscous brown solid, which gave a single spot on TLC. The positive Molish's and ferric chloride tests indicated it to be a phenolic glycoside. It was hydrolyzed with dilute HCl, which afforded an aglycone **2** and a sugar in the filtrate. The aglycone **2** had a molecular formula $C_{24}H_{24}O_{10}$ as established on the basis of ^{13}C NMR, DEPT and MS spectra. The ^{13}C and DEPT spectra showed 24 carbon atoms for the molecule consisting of two methyls, one methylene, eleven methines, three carbonyl and seven quaternary carbon atoms. The NMR spectra established the presence of a caffoyl moiety consisting of $C_9H_7O_4$, and thus, the remaining part $C_{15}H_{17}O_6$ of the aglycone **2** was assigned to the basic skeleton of sesquiterpene.[6] The IR spectrum indicated the presence of γ-lactone (1750 cm^{-1}), carbonyl group (1700 cm^{-1}), alcoholic group (3550 cm^{-1}), —COOH group (3550-3000 cm^{-1}), ester linkage (1280 cm^{-1}), alcoholic linkage (1060 cm^{-1}), and double bonds (1600, 690 cm^{-1}) in the molecule. The sequential assignments of protons and carbon atoms were made with the help of ^1H-^1H COSY and HMQC experiments starting with the easily distinguishable carbinolic protons at δ 4.98 (d, $J = 7.5$ Hz, δ 63.1) attributable to position-9 and 4.61 (dd, $J = 8.0$, 7.5 Hz, δ 69.9) assignable at position-8, respectively. Furthermore, in HMBC spectrum, H-9 displayed long-range correlations with C-10 and C-8; and H-8 with C-9 and C-7 (Figure 2). The carbinolic proton at position-8 also showed HMBC correlation with C-9′ of caffeic acid moiety. The double doublet at δ 6.21 ($J = 16.0$, 3.0 Hz) assignable at position-7′, and another doublet at δ 7.46 ($J = 16.0$ Hz) attributable to position-8′ showed correlation in COSY spectrum, which indicated a

*Corresponding author. Email: drbahmed@rediffmail.com

ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020701590764
http://www.informaworld.com

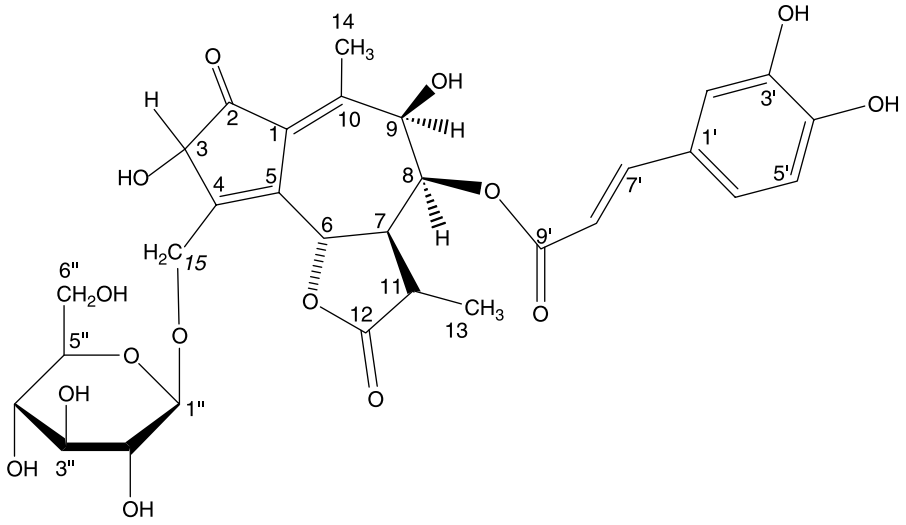

Figure 1. The structure of **1**.

trans olefinic side chain of caffeoyl moiety. H-7′ also exhibited long range correlations with C-1′, C-8′, and C-9′, while H-8′ showed correlations with C-7′ and C-9′, substantiating further the presence of caffeoyl moiety in the molecule. Other protons at δ 7.06 (dd, $J = 2.0$, 3.0 Hz) attributable to position-2′, δ 6.78 (d, $J = 8.0$ Hz) and 6.99 (d, $J = 8.0$ Hz) due to aromatic protons at positions 5′ and 6′, respectively, were also obtained by the aromatic ring of caffeic acid. The ^{13}C NMR spectrum exhibited two peaks in the downfield region at δ 145.2 and 145.6, which indicated that the hydroxyl groups are present at positions 3′ and 4′, respectively, in the caffeoyl moiety. The long-range couplings in the HMBC spectrum further confirmed that the hydroxyl groups were present at position-3′ and 4′, wherein H-2′ correlated with C-3′ and C-4′, while H-5′ showed correlations with C-4′, C-3′, and C-6′ (Table 1, Figure 2). The ^{13}C NMR spectrum exhibited peaks at δ 172.6 and 166.1 for ketonic groups, which were assigned at positions 2 and 12, respectively, with the help of long-range correlations in HMBC spectrum, wherein H-3 correlated with the carbonyl group at position-2, while H-11 correlated with carbonyl group at position-12.

The ^{1}H NMR spectrum showed peaks at δ 1.24 (3H, d, $J = 6.0$ Hz) and a singlet at δ 2.30 (3H) due to two methyl groups attributable to positions 13 and 14, respectively. Further, the assignments were confirmed with the help of correlations in the HMBC spectrum, wherein H-11 showed correlations with Me-13, and Me-13 with C-11 and Me-14 displayed correlation with C-10 (δ 145.6). The ^{1}H NMR spectrum indicated two doublets at δ 3.61 ($J = 15.0$ Hz) and 3.74 ($J = 15.0$ Hz, δ 63.1) attributable

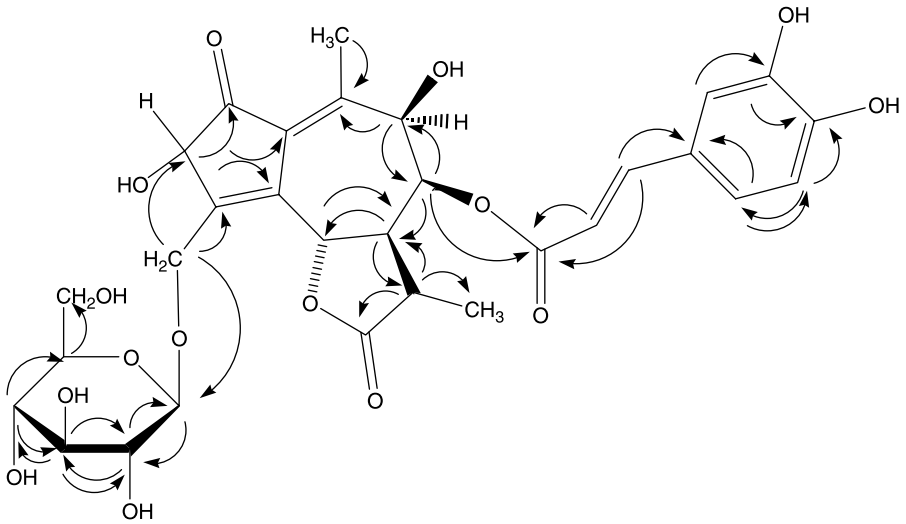

Figure 2. Significant HMBC correlations for **1**.

Table 1. 1D and 2D NMR spectral data of cichotyboside (**1**).

Positions	^{1}H NMR	^{13}C & HMQC	DEPT	COSY	HMBC ($3J_{CH}$)
1	–	114.8 s	C	–	–
2	–	172.6 s	C	–	–
3	5.21 s	70.1 d	CH	–	C-1, C-5
4	–	148.5 s	C	–	–
5	–	121.3 s	C	–	–
6	4.07 d (8.0)	79.2 d	CH	H-7	C-11
7	1.93 ddd (6.0, 8.0. 7.5)	48.6 d	CH	H-8, H-6, H-11	C-5
8α	4.61 dd (8.0, 7.5)	69.9 d	CH	H-9, H-7	C-6
9α	4.98 d (7.5)	63.1 d	CH	H-8	C-7
10	–	145.6 s	C	–	–
11	3.41 dd (8.0, 6.0)	48.6 d	CH	Me-13, H-7	–
12	–	166.1 s	C	–	–
13	1.24 d (6.0)	38.4 q	CH_3	H-11	H-7
14	2.30 s	39.3 q	CH_3	–	–
15a	3.61 d (15.0)	63.1 t	CH_2	H-15b	C-3
15b	3.74 d (15.0)	–	–	H-15a	–
1′	–	121.5 s	C	–	–
2′	7.06 dd (2.0, 3.0)	121.5 s	C	–	C-4′
3′	–	145.2 s	C	–	–
4′	–	145.6 s	C	–	–
5′	6.78 d (8.0)	115.9 d	CH	H-6′	C-3′
6′	6.99 d (8.0)	125.6 s	CH	H-5′	C-4′
7′	6.21 dd (16.0, 3.0)	148.5 d	CH	H-8′	C-9′
8′	7.46 d (16.0)	115.8 d	CH	H-7′	–
9′	–	165.3 s	C	–	–
1″	4.66 d (8.0)	100.4 d	C	H-2″	C-2″
2″	3.27 dd (8.0, 8.5)	74.9 d	CH	H-1″, H-3″	C-4″
3″	3.46 dd (8.5, 9.0)	78.0 d	CH	H-4″, H-2″	C-1″
4″	3.36 dd (9.0, 8.5)	73.6 d	CH	H-3″, H-5″	C-2″
5″	3.39 m	78.5 d	CH	H-4″, H_2-6″	C-3″
6″a	3.75 dd (12.0, 5.5)	63.4 t	CH_2	H-5′, H-6b	C-4″
6″b	3.98 dd (12.0, 2.8)	–	–	H-5′, H-6a	C-4″
OH	9.2 brs	–	–	–	–
OH	9.8 brs	–	–	–	–

∗Assignments were based on ^{1}H NMR, COSY, and HMQC experiments; coupling constants in Hertz are given in parentheses; s: singlet, d: doublet, m: multiplet, t: triplet, brs: broad singlet;
∗∗DEPT chemical shifts are presented at $\theta = 3\pi/4$ when methylene groups reach negative maximum;
∗∗∗The correlations in HMBC were shown from protons to carbons.

to a methylene group at position-15. The downfield shift of the methylene group showed that the group contains a hydroxyl group, which was linked with the sugar moiety in compound **1**. The HMBC spectrum of compound **1** showed correlation between C-15 and the anomeric proton at δ 4.66 (d, $J = 8.0$ Hz, δ 100.4) of the sugar moiety, substantiating strongly the linkage of the sugar unit with CH_2—O-group of the molecule. The paper chromatography of the aqueous fraction of the hydrolysed product along with an authentic sample of glucose showed a single spot indicating the sugar to be glucose.

The ^{1}H-NMR spectrum displayed a singlet at δ 5.21 (1H, H-3) and its corresponding carbon peak in ^{13}C NMR spectrum at δ 70.1 indicating a third hydroxyl group placeable at position-3 with the help of long-range correlation in HMBC spectrum, wherein H-3 displayed correlations with C-4 (δ 148.5), C-1 (δ 114.8), and C-2 (δ 172.6), and H_2-15 with C-3 (δ 70.1).

The high values of coupling constant ($J = 7.5$ Hz) for carbinolic proton at positions 9 and 8 indicated the α-orientation of hydrogens and β-orientation of hydroxyl groups.[7] The spectral data of aglycone **2** were compared with lactucin, and other related sesquiterpenes, which indicated that compound **2** had a guaianolide skeleton of sesquiterpene,[4] and was found to be a new compound.

The mass spectrum fragmentation pattern of aglycone **2** exhibited prominent peaks at *m/z* 472 $[M]^+$, 455, 308, 280, 264, 262, 220, 202, and 163, which further strongly supported the proposed structure of compound **1**.

Thus, based on the above chemical and spectral evidences, the structure of compound **1** was elucidated as 2α, 6β, 7β, 15 - tetrahydroxy -1 (10), 4 (5) - diene–guaian - 9α, 12 - olide - 7 - O - β - caffoyl - 15 - O- β - D - glucopyranoside and has been designated as cichotyboside (**1**). The aglycone **2** obtained on hydrolysis of

Table 2. Effect of cichotyboside on liver enzymes in CCl_4 induced liver damage in rats.

Group n = 6	Treatment	dose (p. o.)	SGOT (units/ml)	SGPT (units/ml)	ALKP (units/ml)	T.P (g/dl)	T.A (g/dl)
1	normal	Nil	34 ± 1.41	21.75 ± 0.85	21.94 ± 0.47	10.45 ± 0.27	6.4 ± 0.04
2	CCl_4	1.5 ml/kg	179.25 ± 6.87*	117 ± 1.29*	62 ± 0.35*	5.92 ± 0.1*	3.47 ± 0.12*
3	silymarin	10 mg/kg	44.33 ± 1.74**	29 ± 0.85**	23.17 ± 0.45**	8.57 ± 0.17**	5.72 ± 0.03**
4	ethanol extract	500 mg/kg	78.67 ± 2.29**	63.67 ± 0.95**	44.53 ± 0.43**	7.15 ± 0.07**	4.23 ± 0.09**
5	methanol fraction	500 mg/kg	70.67 ± 1.61**	53.67 ± 0.61**	36.48 ± 0.28**	6.9 ± 0.04**	5.29 ± 0.07**
6	cichotyboside	50 mg/kg	52 ± 0.73**	38 ± 0.73**	24.97 ± 0.34**	7.54 ± 0.17**	5.48 ± 0.1**

All data were expressed as Mean ± SEM, n = 6
*$p < 0.01$, when compared to Normal group i.e. Group I, ANOVA followed by Dunnett t-test
**$p < 0.01$, when compared to Toxic group i.e. Group I, followed by Dunnett t-test

compound **1**, a new compound, has been designated as cichotucin.

2.1 Anti-hepatotoxic activity

As shown in Table 2, the activities of liver enzymes SGPT (117.0 units/ml), SGOT (179.25 units/ml) and alkaline phosphatase (62.0 units/ml), were markedly elevated, whereas the levels of total protein (5.92 g/dl) and total albumin (3.47 g/dl) were markedly decreased in CCl_4 treated animals in comparison to normal values (21.75, 34.0, 21.94, 10.45 and 6.40, respectively). Administration of silymarin (standard drug silybon-70), ethanolic extract, methanolic fraction, and cichotyboside at the dose level 10, 500, 500, and 50 mg/kg body weight, respectively, had prevented CCl_4 induced elevation of serums GPT, GOT, and ALKP. However, it also prevented the decrease in total protein and albumin. The silymarin (10 mg/kg) had significantly decreased the levels of SGOT, SGPT, and ALKP, and increased the levels of total protein and total albumin by 44.33, 29.00, and 23.17 units/ml and 8.57, 5.72 g/dl, respectively. The ethanolic extract and methanolic fraction exhibited decreases by 78.67, 70.67 in SGOT, 63.67, 53.67 in SGPT, and 44.53 and 36.48 units/ml in ALKP, and increase in total protein and total albumin by 7.15, 6.90 units/ml and 4.23, 5.29 g/dl, respectively. While, cichotyboside (**1**) had also decreased significantly the levels of enzymes by 52 units/ml in SGOT; 38 units/ml in SGPT; 24.97 units/ml in ALKP and decreased the levels by 7.54 g/dl, 5.48 g/dl in total protein and albumin, respectively.

It was observed that cichotyboside (**1**) decreased the level of ALKP comparable with that of standard drug silymarin, exhibiting an 88% decrease in comparison to silymarin (92%) and increase in the levels of total albumin 85% in comparison to silymarin (89%) against intoxicated control. In addition, the levels of SGOT and SGPT were also decreased significantly (63 and 55%) in comparison to silymarin (77 and 73%) against intoxicated control (Table 2) indicating a potent antihepatotoxic activity.

2.2 Histopathological studies

The results of the liver histopathological studies have been presented in Table 3, which, showed hepatocytes swelling and necrosis in CCl_4 treated rats in comparison with normal control rats. Administration of cichotyboside exhibited a significant protection of hepatocytes injury and showed complete normalization of the tissues as neither fatty accumulation nor necrosis was observed. The central vein appeared clearly, indicating a potent antihepatotoxic activity. The methanolic fraction of the

Table 3. Histopathological changes in liver of Wistar rats.

Group n = 6	Treatment	Microscopic observations
1	normal	Liver samples showed normal architecture without any degeneration, necrosis or inflammation seen.
2	CCl_4	Prominent centrilobular fatty change with prominent and enlarged central vein. There is significant periportal inflammation. Fatty deposition was also seen, reflecting liver damage.
3	silymarin (Standard drug)	Liver samples showed a significant reduction in portal inflammation and in the sinusoidal dilatation. The central vein was clearly visible. Liver samples also showed good recovery with absence of necrosis and fatty depositions.
4	ethanolic extract	The liver section showed collection of inflammatory cells within the portal triad. Vacuolated hepatocytes are seen in the periportal zone.
5	methanolic fraction	Liver histology was almost normal with only very little sinusoidal dilatation seen in some hepatic lobules. Central vein appeared clearly with the disappearance of fatty depositions and necrosis thus indicating a potent anti-hepatotoxic activity.
6	cichotyboside	The liver section showed the structures of the portal triad and a normal liver parenchyma. There was no lymphocytic infiltration and fatty deposition indicating a potent antihepatotoxic activity.

extract has shown a potent activity, as the central vein appeared clearly with complete disappearance of fatty depositions and necrosis. The cichotyboside was found to exhibit most potent antihepatotoxic activity comparable to standard drug silymarin. The liver section showed the structures of the portal triad and a normal liver parenchyma, and the central vein appeared clearly. There was no lymphocytic infiltration and fatty deposition indicating a potent antihepatotoxic activity, which has established a correlation that the active constituent cichotyboside is present in the methanolic fraction responsible for antihepatotoxic activity of the ethanolic extract.

3. Experimental

3.1 General experimental procedures

The IR spectra were recorded as KBr pellets on PYE UNICAM spectrophotometer, UV spectra on Shimadzu UV-1601 UV-Vis Spectrophotometer, optical rotation on Perkin-Elmer 241 MC Polarimeter and mass spectra on a Finnegan MAT 300 mass spectrometer. The ^{1}H (500 MHz) and ^{13}C & DEPT 90 and 135 NMR (125 MHz) and 2D NMR (COSY, TOCSY, HMBC, HMQC & NOESY) were recorded on Bruker DRX 500 spectrometer in $CDCl_3$ using TMS as internal standard reference, chemical shift in δ (ppm) and coupling constants (J values) are in Hz. Column chromatography was performed using silica gel (0.04-0.063 mm, 230-400 mesh) as an adsorbent. TLC were performed on silica gel 60 F-254 Merck plates and sprayed with vanillin-H_2SO_4 reagents for visualization of the spots.

3.2 Plant material

The seeds of *Cichorium intybus* were collected from in the month of October, Khari Bawli market, Old Delhi, India and authenticated by a taxonomist, Department of Botany, Jamia Hamdard, New Delhi. The voucher specimen (No. 524) has been kept in the herbarium of Jamia Hamdard for future reference.

3.3 Extraction and isolation

The plant materials were crushed to a coarse powder (6.0 kg). The powder was exhaustively extracted with ethanol by cold percolation. The crude alcoholic extract was concentrated to a small volume under reduced pressure to obtain a viscous mass (800 g). It was then fractionated into petroleum ether, chloroform, and methanol soluble portions. The petroleum ether and chloroform fractions were investigated earlier and did not

show antihepatotoxic activity.[3] The methanolic fraction was taken and the solution thus obtained was concentrated under reduced pressure on water bath to get a viscous mass (225 g). It was chromatographed over silica gel column, and eluted with petroleum ether, benzene and methanol, and their mixtures in increasing order of polarity. The eluent methanol: benzene (1:4) afforded compound **1** (2.0 g) as viscous solid.

3.3.1 *Cichotyboside (**1**)*

Obtained as brown viscous mass from methanol; $[\alpha]_D^{25} + 33.5$ (c = 0.03, MeOH); IR (KBr): ν_{max} 3550 (OH), 3550-3000 (COOH), 1700 (C═O), 1280 (ester linkage), 1060 (alcoholic linkage), 1750 (γ-lactone) and 1600 and 690 (double bonds) cm^{-1}. NMR spectral data (see Table 1); EIMS: *m/z* 472, 455, 308, 280, 264, 262, 220, 202, 163. HRMS: *m/z* 634.1902 $[M]^+$ (calcd for $C_{30}H_{34}O_{15}$, 634.1901).

3.4 *Experimental animals*

Male Albino rats (Wistar strain), weighing 150–200 g, were employed for the anti-hepatotoxic activity. They were procured from Central Animal House Facility, Hamdard University, New Delhi (173/CPCSEA), after approval under the project proposal number-236. The animals were kept in polypropylene cages (6 in each cage) under standard laboratory conditions (12 hr light and 12 hr dark: day: night cycle) and had a free access to commercial pellet diet (Amrut rat feed, manufactured by: Nav Maharastra Chakan Oil Mills Ltd, Delhi, India) and tap water *ad libitum*. The animal house temperature was maintained at 25 ± 2°C and relative humidity was maintained at (50 ± 15%).

3.5 *Anti-hepatotoxic activity*

The animals were divided into four groups, 6 rats in each group. Group I served as normal control, which received normal saline only. Group II as toxic control received CCl_4 diluted with liquid paraffin in a ratio of (1:1) (1.5 ml/kg b. w, p. o) on the first day.[8–9] Group III was given a single dose of CCl_4 on the first day (1.5 ml/kg b. w, p. o) and then silymarin (Slybon-70, 10 mg/kg. p. o.) once a day for 6 days. Group IV received a single dose of CCl_4 (1.5 ml/kg b. w, p. o) on the first day and then compound **1** at the dose of 50 mg/kg b. w, p. o for six days.[3] On the day 8 the blood samples were withdrawn by puncturing the orbital plexus first then the rats were sacrificed by decapitation. The blood samples were allowed to clot for 30–40 min at room temperature. Serum was separated by centrifugation at 2500 rpm for 15 min. Serum biochemical parameters were determined by assay kits.

3.6 *Assessment of the liver function*

Serum SGOT, SGPT was determined by the method of Reitman & Frankel,[10] ALKP and TP were determined by reported methods of Kind and King[11] and Wooton.[12]

3.7 *Statistical analysis*

The data of biochemical estimations were reported as ± S.E, where $n = 6$. The statistical significance one way analysis of variance (ANOVA) and Dunnett's test were employed for determining *P*-values of less than 0.05 were considered significant.[13]

3.8 *Histopathological studies of the liver*

Rat livers were quickly removed after autopsy and fixed in 10% formalin. The sections were cut and then stained by haemotoxylin and eosin. These were observed under microscope.[14]

Acknowledgements

The authors are thankful to the Head Department of Pharmaceutical Chemistry, Faculty of Pharmacy, and Jamia Hamdard for providing necessary research facilities and to the in-charge, Central Animal Facility, Jamia Hamdard, and New Delhi for providing rats and other related facilities for pharmacological activities.

References

[1] The Wealth of India, PID, Vol. III, pp. 555–557, CSIR, New Delhi (revised) (1992).
[2] K.R. Kirtikar and B.D. Basu. *Indian Medicinal Plants*, 2nd ed., Vol. II (Lalit Mohan Basu, Allahabad, India 1975), pp. 1433–1436.
[3] B. Ahmed, T.A. Al-Howiriny, and A.B. Siddique. *J. Ethnopharmacol.* **87**, 237 (2003).
[4] J. Dolzel. *Gartebbauwissencheaft* **41**, 160 (1976).
[5] M. Seto, T. Miyase, K. Umehara, A. Ueno, Y. Hirano, and N. Otani. *Chem. Pharm. Bull.* **36**, 2423 (1988).
[6] D.J. Pegg, D.M. Dedrell, and M.E. Bendal. *J. Chem. Phys.* **25**, 2745 (1982).
[7] R.M. Silverstein, G.C. Bassler, and T.C. Morill. *Spectrometric Identification of Organic Compounds*, 5th ed. (John Willey & Sons, New York 1999), pp. 267–284.
[8] B. Ahmed, T. Alam, and S.A. Khan. *J. Ethnopharmacol.* **76**, 187 (2001).
[9] H.J. Vogel. *Drug Discovery and Evaluation*, Pharmacological Assays, 2nd ed. (Springer-Verlag, Berlin, Heidel berg, New York, 2002), p. 924.
[10] S. Reitman and S.A. Frankel. *Am. J. Clin. Pathol.* **28**, 56 (1957).
[11] P.R.N. Kind and E.J. King. *J. Clin. Pathol.* **7**, 332 (1954).
[12] I.D.P. Wooton. *Microanalysis in Medical Biochemistry*, 4th ed. (Churchill, London, 1964), pp. 138–140.
[13] C.W. Dunnett. *Biometrics*, **20**, 482 (1964).
[14] L.G. Luna. *Manual of Histology*, Staining methods of Armed Forces. Institute of Pathology, 3rd ed. (Mc Graw Hill Book Co., New York, 1968), pp. 117–119.