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Note

Pachycanthine: A new isoquinoline alkaloid and its antihepatotoxic activity from *Berberis pachycantha* Koehne

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The methanolic extract of whole plant of *Berberis pachycantha* Koehne (Berberidaceae) has afforded a new isoquinoline alkaloid, which was characterized as 8,9-dimethoxy-5,6,12*a*,6*a*-tetrahydro-2*H*-1,3-dioxoleno[4,5-*g*] isoquinolino [3,2-*a*] isoquinoline on the basis of spectral and chemical studies and has been designated as pachycanthine **1**. The pachycanthine **1** exhibited a significant antihepatotoxic activity by 25-53% (dose: 50 mg/kg) with respect to standard silybon-70 (36-60%) against CCl_4 induced toxicity in Albino Wistar rats.

Keywords: *Berberis pachycantha, a*ntihepatotoxic activity, isoquinoline alkaloids, pachycanthine, silymarin

The plant *Berberis pachycantha* Koehne (Berberidaceae) is distributed in Pakistan, North-West Himalayas and Kashmir region[1-4]. The plant is a shrub 2-3 meter tall, deciduous, glabrous; stem dark-red to pale-brownish. Leaves are usually 3-6 cm long. Roots and barks of various *Berberis* species are used as folk remedy for the treatment of various inflammatory diseases such as lumbago, rheumatism and to reduce fever[1]. The plants of genus *Berberis* have also been used as a folk medicine for the treatment of rheumatic and other chronic inflammatory disorders[5], antimicrobial properties[6], antiarrhythmic and sedative effects[7], antihypertensive activity[8], choleretic action, gallbladder stones[4], hepatoprotective activity[9] and many more biological properties.

Literature survey revealed that oxyacanthine, oxyberberine, magnoflorine iodide, berbamine, isotetrandrine, jatrorrhizine[2], pelargonidin-3-glucoside and cyanidin-3-glucoside[10]. Isoquinoline alkaloids[11], dimeric aporphine benzylisoquinoline[12], *seco*-bisbenzylisoquinolines[13], berbenine I, berbericine II, and berbericinine iodide III[11], 1,4-bis-(2′-hydroxy-5′-methylphenyl)-butane-1,4-dione[14] have also been reported from other species of *Berberis* genus.

We have isolated a new isoquinoline alkaloid from the methanol fraction of the rhizomes of *Berberis pachycantha*. The isolated isoquinoline alkaloid was characterized as 8,9-dimethoxy-5,6,12*a*,6*a*-tetrahydro-2*H*-1,3-dioxoleno[4,5-*g*] isoquinolino [3,2-*a*] isoquinoline, and has been designated as pachycanthine **1**. Pachycanthine **1** showed anti-hepatotoxic activity against CCl_4 induced toxicity in Albino Wistar rats, by about 25-53% with respect to standard drug silybon-70 (36-60%), a commercial product of Micro Labs Limited, prepared from the crude extract of *Silybum marianum*.

Results and Discussion

Phytochemical part

The compound **1**, named as pachycanthine obtained as yellow amorphous powder, had molecular formula $C_{20}H_{19}NO_4$ as established on the basis of HR-MS (337.1301), elemental analysis, ^{13}C NMR (**Table I**) and DEPT spectra. It gave a positive test for Dragendroff reagent indicating it to be an alkaloid. Its UV spectrum showed absorption peaks at 230, 265, 350, and 428 nm characteristic for isoquinoline ring containing alkaloids[13]. Its IR spectrum indicated the presence of C-O stretch (1058 cm^{-1}), aromatic ring =C-H stretch, (3050 cm^{-1}), tertiary amine (>N-, 1389 cm^{-1}) and phenolic linkage at (C-O, 1231 cm^{-1}). The ^{13}C and DEPT spectra[15] showed 20 carbon atoms for the molecule consisting of two methyls, three methylenes, seven methines and eight quaternary carbon atoms (in total $C_{20}H_{19}$). The sequential assignment of proton and carbon atoms were made with the help of ^1H-^1H-COSY and HMQC experiments[16] starting with the easily distinguishable proton at δ_H 8.10 (*d*, *J* = 8.8 Hz, δ_C 125.2) attributable to position-10, which showed a correlation in ^1H-^1H-COSY spectrum with another proton at δ_H 7.91 (*d*, *J* = 8.8 Hz, δ_C 128.4) assignable at position-11. The proton at position-10 showed a long range correlation in HMBC spectrum with carbon atoms at δ_C 145.3 and 139.6, which could be attributed to position 9 and 8 respectively (**Figure 1a**). The downfield shift of these carbon atoms indicated the linkage with oxygen atoms of the methoxyl groups. Further it was substantiated that methoxyl protons (δ_H 4.05, 3H, 14-OCH_3, δ_C 63.6; δ_H 4.10, 3H, 15-OCH_3, δ_C 56.7)

Table I — 1D-and 2D NMR data of pachycanthine **1**

Position	^{1}H NMR[a]	^{13}C NMR / DEPT[b]	^{1}H-^{1}H COSY	HMQC	HMBC[c]	
					$2J_{CH}$	$3J_{CH}$
2	5.85*s* (2H,$CH_{2)}$	103.8*t*	-	103.8	-	C-3*a* C-13*a*
3*a*	-	153.1*s*	-	153.1	-	-
4	7.05*d* (1.5)	110.18*d*	--	110.1	C-3*a*, C-4*a*	C-12*b*
4*a*	-	132.4*s*	-	132.4	-	-
5	3.18*d* (2H, 5.6)	28.0*t*	H_2-6	28.0	C-4*a*, C-6	C-12*b*
6	4.89*d* (2H, 5.6)	56.8*t*	H_2-5, $^3J_{HH}$ H-7	56.8	C-5	C-12*a*
6*a*	-	-	-	-	-	-
7	9.58*s*	149.4*d*	$^3J_{HH}$ H_2-6	149.4	C-7	C-12*a*
7*a*	-	123.1*s*	-	123.1	-	-
8	-	139.6*s*	-	139.6	-	-
9	-	145.3*s*	-	145.5	-	-
10	8.10*d* (8.8)	125.2*d*	C-11	125.2	C-9, C-11	C-8, C-11*a*
11	7.91*d* (8.8)	128.4*d*	C-10	128.4	C-10, C-11*a*	C-7*a*
11*a*	-	134.7*s*	-	134.7	-	-
12	8.85 *d* (8.3)	121.8*d*	-	121.8	C-11*a*, C-12*a*	C-7*a*
12*a*	4.22 *d* (8.3)	44.4*d*	-	44.4	C-12, C-12*b*	-
12*b*	-	122.0*s*	-	122.0	-	-
13	7.75*d* (1.5)	107.1*d*	-	107.1	C-13*a*	C-12*b*, C-3*a*
13*a*	-	147.2*s*	-	147.2	-	-
14	4.05*s* (3H, OCH_3)	63.6*q*	-	63.6	C-8	-
15	4.10*s* (3H, OCH_3)	56.7*q*	-	56.7	C-9	-

[a]Assignments were based on 1H-1H COSY, and HMQC experiments; coupling constants in Hertz are given in parentheses; *s*: singlet, *d*: doublet, *t*: triplet

[b]DEPT chemical shifts are presented at $\theta = 3\pi/4$ when methylene groups reaches negative maximum. C-multiplicities were established by DEPT experiments; *s* = C, *d* = CH, *t* = CH_2, *q* = CH_3.

[c] The correlations in HMBC have been shown from protons to carbons.

exhibited long range correlation in HMBC spectrum with C-9 and C-8 respectively. Moreover, it had also been confirmed that the methoxyl groups were linked with C-9 and C-8 respectively. Moreover, H-10 displayed long range correlations in HMBC spectrum with C-11 (δ_C 128.4), and C-11*a* (δ_C 134.7), while C-11 showed correlation with C-10 (δ_C 125.2), C-11*a*, and C-7*a* (δ_C 123.1), which supported the above assignment of ring A.

The ^{1}H NMR spectrum exhibited a one proton singlet at δ_H 9.58 (δ_C 149.4) that could be attributed to position 7 with the help of HMBC spectrum, which showed long range correlation of H-7 with carbon atoms at δ_C 123.1 and 44.4 assignable to carbon atoms at C-7*a* and C-12*a* respectively. The downfield shift of C-7 indicated that it was linked with nitrogen atom at position 6*a* as the nitrogen atom had withdrawn the electron density of C-7 causing the downfield shift. A one proton doublet in ^{1}H NMR spectrum δ_H 8.85 (δ_C 121.8) was attributed to position 12, which showed long range correlations in HMBC spectrum with C-11*a*, C-7*a* and C-12*a* of the ring B. A two proton doublet at δ_H 4.89 (J = 5.6 Hz, δ_C 56.8) due to a methylene group exhibited correlation in ^{1}H-^{1}H-COSY spectrum with another methylene proton at 3.18 (2H, *d, J* = 5.6 Hz, δ_C 28.0) attributable to the

position 6 and 5 of the ring C respectively. Further the downfield shift of the methylene group at position 6 indicated that it was linked with nitrogen atom. The assignment of CH_2-6 and CH_2-5 were further substantiated with the help of long-range correlation in HMBC spectrum, wherein H_2-6 displayed correlation with C-12*a* (δ_C 44.4) and C-5 (δ_C 28.5), while H_2-5 showed with 12*b* (δ_C 122.0), C-5 and 4*a* (δ_C 132.4). A one proton doublet at δ_H 4.22 (J = 8.3 Hz, δ_C 44.4) due to a methine proton was also attributed to position 12*a* of the ring C with the help of long range correlations in HMBC spectrum.

A two proton singlet in ^{1}H NMR spectrum has also appeared at δ_H 5.85 (δ_C 103.8), which indicated a dioxymethylene group attributable at position 2, which displayed long range correlations with carbon atoms at δ_C 147.2 attributable to position 13*a* and δ_C 153.1 assignable to C-3*a*. The proton at δ_H 7.75 (1H, *d, J* = 1.5 Hz, δ_C 107.1) attributable to position-13 showed also long range correlation with C-13*a* (δ_C 147.2) and C-12*b* (δ_C 122.0) of ring D and C. It also showed a long range correlation, with a carbon at δ_C 153.1 assignable at position 3*a*. The downfield shift of C-3*a* indicated its linkage with oxygen atom of dioxymethylene group. The downfield shift of methylene group was also another evidence it to be dioxymethylene group, which is also found in other alkaloids of *Berberidaceae*. The proton at δ_H 7.05 (*d, J* = 1.5 Hz, δ_C 110.18) showed long range correlations in HMBC spectrum with C-3*a*, C-4*a* (δ_C 132.4) and C-12*b* of the ring D. The downfield shift of C-3*a* (δ_C 153.1) indicated further its linkage with oxygen atom of dioxymethylene group. The spectral data of compound **1** were also compared with other isoquinoline alkaloids, indicating it to be an isoquinoline alkaloid which had close resemblance with oxyberberine and neo-oxyberberine[2].

The mass spectrum fragmentation pattern also supported the proposed structure of the compound **1**, which exhibited prominent peaks at *m/z* 337.1 (M^+) due to molecular ion peak, *m/z* 322 (base peak), 306 and 393 respectively due to elimination of methyl, methoxyl and dioxymethylene from the molecule. The prominent peaks at *m/z* 204 due to species-a, (**Scheme I**) also supported the proposed structure of the compound.

Thus on the basis of above chemical and spectral evidences, the structure of the compound **1** was elucidated as 8,9-dimethoxy-5,6,12*a*,6*a*-tetrahydro-2*H*-1,3-dioxoleno[4,5-*g*] isoquinolino [3,2-*a*] isoquinoline, and has been designated as pachycanthine **1**.

Pharmacological section

Antihepatotoxic activity

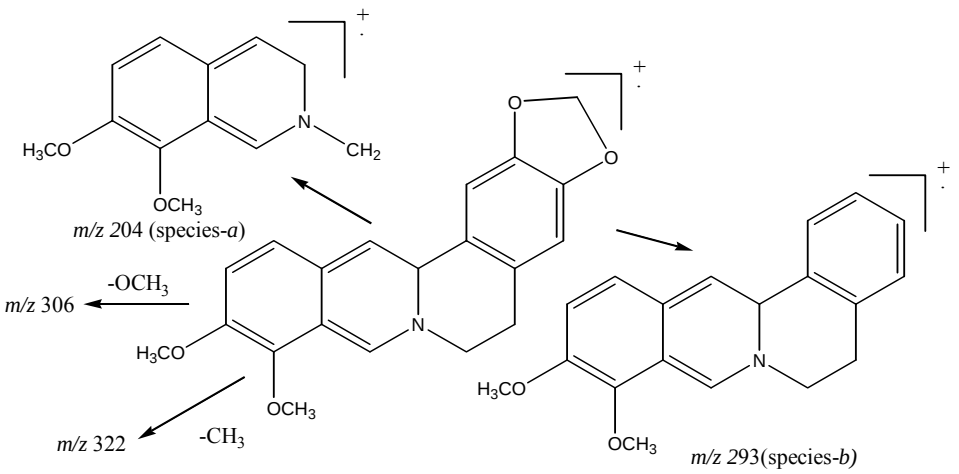
As shown in **Table II**, activities of liver enzymes SGPT, SGOT and alkaline phosphatase were markedly elevated from normal values 36.47, 25.52 and 27.58 units/mL to 119.19, 94.70 and 55.08 units/mL in SGOT, SGPT and ALKP respectively, whereas the level of total proteins was decreased from normal values 7.36 to 4.49 gm/dL in CCl_4 treated animals in comparison to normal values. Administration of silymarin (standard drug-10 mg/kg), and pachycanthine (50 mg /kg) body weight, had decreased CCl_4 induced elevation of serums SGPT, SGOT, alkaline phosphatase and decrease in total proteins. The silymarin (Slybon-70) had significantly decreased the levels of SGOT, SGPT and alkaline phosphatase by 55.97, 41.70 and 35.32 units/mL and increased the levels of total proteins by 7.15 gm/dL respectively. Whereas, pachycanthine **1** had a considerable decrease by 69.13 units/mL in SGOT, 55.23 units/mL in SGPT, 41.53 units/mL in alkaline phosphatase and increased total proteins by 6.83 gm/dL.

It was observed that pachycanthine **1** decreased the levels of SGOT and SGPT ($p<0.05$) comparable with that of standard drug silymarin, exhibiting 42% and 41% decrease in SGOT and SGPT comparable with that of standard drug silymarin, which decreased by 53% and 55.96% respectively against intoxicated control, (**Graph I**) while the levels of alkaline phosphatase were also decreased by 25% and that of total proteins increased by 52% considerably in comparison to standard and intoxicated control (**Table II**).

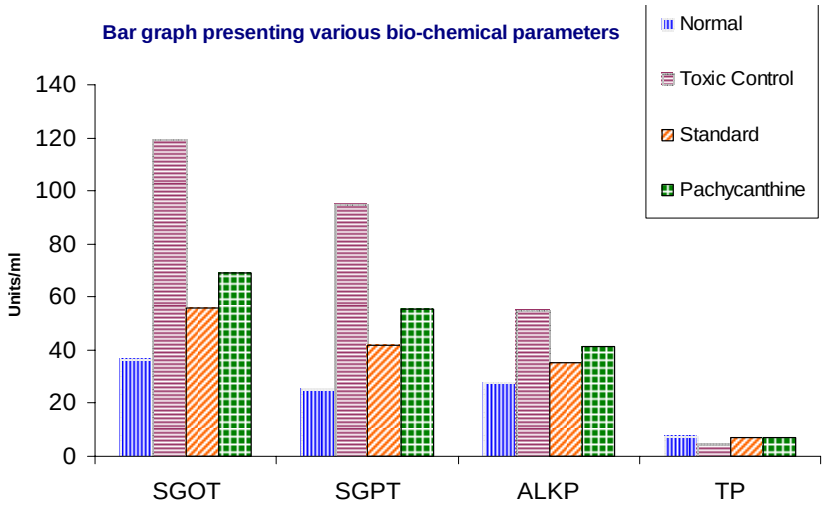
Histopathological studies have also revealed that rats treated with pachycanthine had almost normal architecture of hepatocytes indicating significant recovery as compared to the standard silymarin (**Table III, Figure 1b**).

Materials and Methods

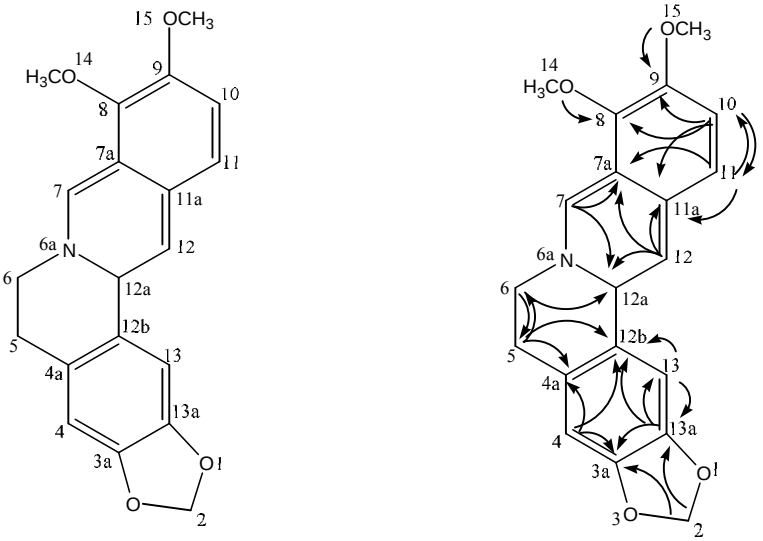
Melting point was determined on Metier 9100 Electrothermal apparatus by open capillary method and is uncorrected. The IR spectra were recorded as KBr pellets on PYE UNICAM spectrophotometer; mass spectra on a Finnegan MAT 300 mass spectrophotometer; 1D and 2D NMR on Bruker DRX 400 spectrometer in DMSO using TMS as internal standard reference, chemical shift in δ (ppm) and *J* values in Hz.



Scheme I: Pachycanthine **1**: $C_{20}H_{19}NO_4$ (M^+ 337.1)



Graph 1 — Bar graph representation of various bio-chemical parameters. SGOT, SGPT and ALKP in units/mL and TP in g/dl.



Pachycanthine **1**

Figure 1a—Significant heteronuclear multiple bond correlations in (HMBC) for Pachycanthine **1**. Arrows point from proton to carbon

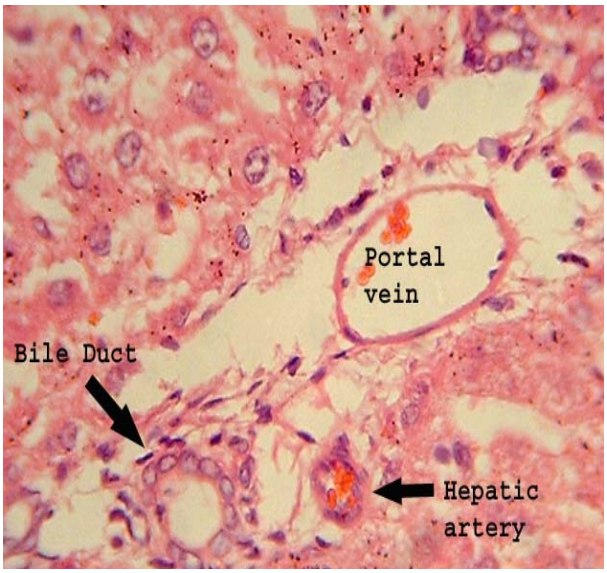


Plate I. High power photomicrograph of Normal control rat liver on day 8 (HE x 400).

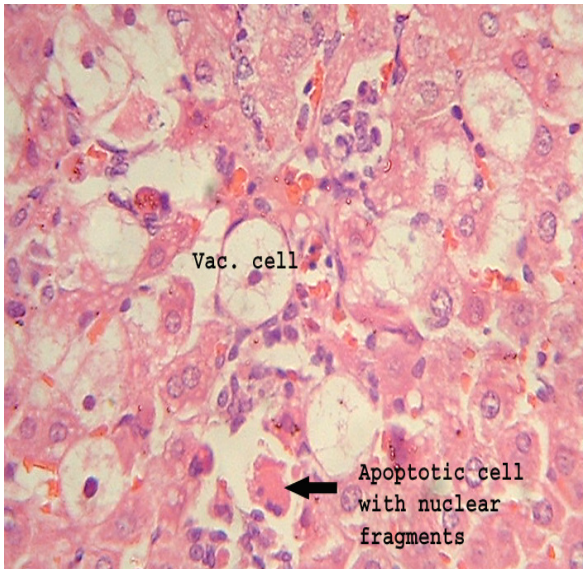


Plate II. High power photomicrograph of Toxicity Control rat liver on day 8 (HE x 400X).

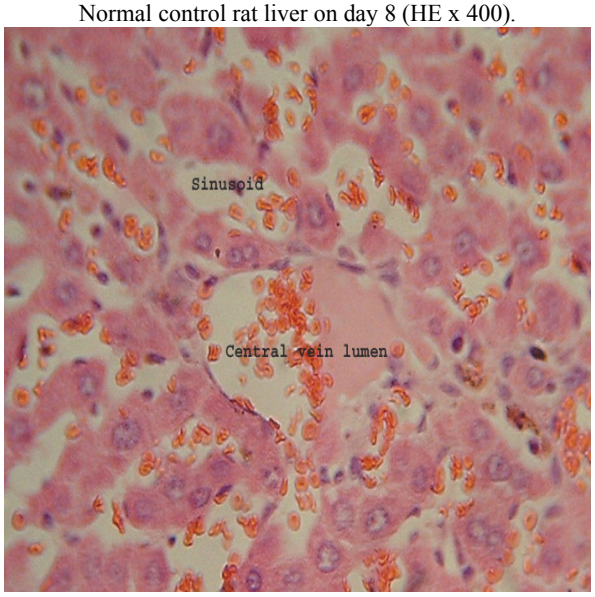


Plate III. High power photomicrograph of Standard control rat liver on day 8 (HE x 400).

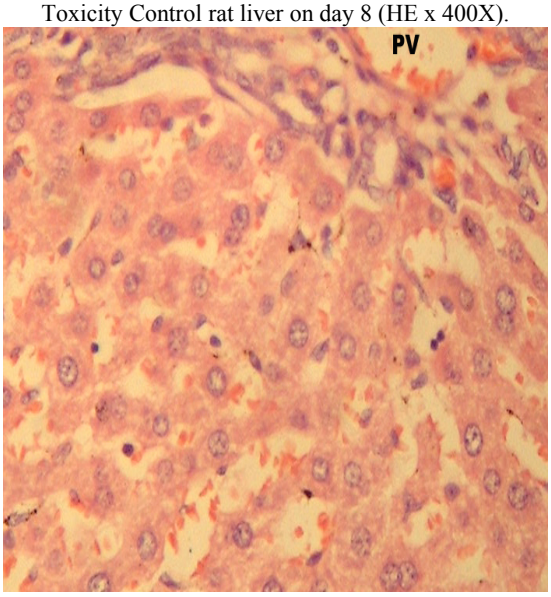


Plate IV. High power photomicrograph of Pachycanthine **1** rat liver on day 8 (HE x 400).

Figure 1b — Slides showing the histopathological changes in liver of Wistar rats.

Table II — Effect of pachycanthine **1** on liver enzymes in CCl_4 induced live damage in Wistar rats

Groups (n=5)	Treatment	Dose	SGOT units/Ml	SGPT units/ Ml	ALKP units/ Ml	TP gm/dl
I	Normal (control)	---	36.47 ± 1.45	25.52 ± 0.70	27.58 ± 0.56	7.36 ± 0.16
II	Toxic (control)	0.7 Ml/kg (i.p.)	119.19 ± 4.05	94.70 ± 4.03	55.08 ± 2.39	4.49 ± 0.16
III	Silymarin (standard drug)	10 mg/kg (p.o.)	55.97 ± 1.61**	41.70 ± 2.90**	35.32 ± 1.05**	7.15 ± 0.12**
VI	Pachycanthine	50 mg/kg (p.o.)	69.13 ± 3.74**	55.23 ± 3.05**	41.53 ± 2.35**	6.83±0.12**

SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvate transaminase; ALKP, alkaline phosphatase: TP, total protein; i.p. intraperitonally; p.o. per-oral

** $P < 0.01$; *$P < 0.05$ vs. CCl_4.

Values are mean ± S.EM of five animals. One way analysis and Student's *t*-test.

Table III — Histopathological changes in liver of Wistar rats

Groups	Treatment	Microscopic observations
I	Normal control	Liver samples showed normal architecture without any degeneration, necrosis or inflammation seen.
II	Toxic control	Prominent centrilobular necrosis with prominent and enlarged central vein. There is significant periportal inflammation. Fatty deposition also seen reflecting liver damage.
III	Standard control	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.
VI	Pachycanthine	Liver histology was almost normal with only very little sinusoidal dilatation seen in some hepatic lobules. Portal vein appeared clearly with the disappearance of fatty depositions and necrosis thus indicating a potent anti-hepatotoxic activity.

Plant material

The rhizomes of *Berberis pachycantha* Koehne were collected on July 20, 2005 from Aharbal, Srinagar, (J&K), India, and by authenticated by taxonomist Prof. A.R. Naqshi (Dept. of Botany, University of Kashmir, Srinagar, India). The voucher specimen (LC-FP-19) of the plant has been deposited in the herbarium of Jamia Hamdard for future reference.

Experimental Section

Extraction and isolation

Air dried rhizomes (2.2 kg) were crushed to coarse powder and extracted exhaustively in a Soxhlet apparatus with methanol. The extracts were concentrated under reduced pressure to yield viscous mass (96.0 g). The extract was extracted with petroleum ether and thus defatted, the residue left was dried to yield a mass of 80.0 g. It was chromatographed on a column of silica gel using chloroform-methanol mixture as the eluent with increasing polarity. The eluent chloroform-methanol (95:5) afforded the compound **1** (425 mg).

Pachycanthine 1

It is a bright yellow amorphous solid (425.0 mg); m. p. 145-47°C, eluent: 5% MeOH in $CHCl_3$; R_f 0.53 ($CHCl_3$-MeOH 60:40v/v); IR (KBr): 3050 (C=C, aromatic), 2911 (CH_3), 2850 (CH_2), 1601, 1567, 1506 (C=C), 1479, 1389 (>N^+<), 1365, 1334, 1277, 1231 (C-O, phenolic), 1103, 1058 (C-O, alcoholic), 931, 871 cm^{-1}; 1D and 2D NMR data: see **Table I**; UV: 215 (*sh*), 230, 265, 350, 428 nm; EIMS (probe) 70 eV, *m/z* (rel. int): 337.1 [M^+ +1, 10%], 336.1 [M^+ 18%] 322.0 (100%, base peak), 306.0 (10), (8) 293.0 (60), 204.0 (5), 276.2 (5%); HRMS: *m/z* 337.1301 [$M.^+$]$^+$ (Calcd. for $C_{20}H_{19}NO_4$, 337.1300); Elemental analysis: Found: C, 71.20; H, 5.68; N, 4.15; O, 18.97; required for $C_{20}H_{19}NO_4$: C, 71.10; H, 5.67; N, 4.15; O, 18.98%.

Experimental animals

The animal experiments were performed in accordance with the ethical standards formulated in the Helsinki Declaration of 1964 (revised 2000), and guidelines issued by the Jamia Hamdard Animal Ethical Committee (JHAEC), registered (No. 173/CPCSEA, 22.03.2002) with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai (Madras), India.

Male Wistar rats weighing 150-200 gm were employed for assessing the anti-hepatotoxic activity. They were fed with a standard pellet diet and water *ad libitum*. The animals were maintained at 25 to 28°C with 40-70% RH and 12 hr light/dark cycles and were fastened for 12 hrs prior to the experiment.

Testing of anti-hepatotoxic activity

The animals were divided into four groups of five rats in each and were treated as follows: Group-1 (normal control without any treatment); Group-2 (toxic control) was given CCl_4 diluted with liquid paraffin in a ratio of (1:1) (0.7 mL/kg b.w, i.p.) on the first day to produce toxicity in the liver[17, 18]; Group-3 (standard control) was given 0.7 mL CCl_4/kg b.w, i.p. on the first day followed by treatment with silymarin (Slybon-70, 10 mg/kg b.w, p.o.) for 7 days. Group-4 (pachycanthine) received a single dose of CCl_4 on the first day (0.7 mL/kg b.w, i.p.) and then pachycanthine at the dose of (50 mg/kg b.w, p.o.) for 7 days in the same way as reported[19].

After the treatment, the blood was obtained from all groups of rats by puncturing retro-orbital plexus. The blood samples were allowed to clot for 30 to 40

min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30°C for 15 min and analyzed for various biochemical parameters. The livers were separated from the each group and histopathological studies were performed.

Assessment of liver function

Biochemical parameters such as serum glutamic oxaloacetic transaminase (SGOT) (**Graph I**) and serum glutamic pyruvate transaminase (SGPT) (**Graph I**) were estimated by reported method[20]. The alkaline phosphatase and total proteins were also measured by reported methods[21, 22].

Statistical analysis

The results of biochemical estimations are reported as mean ±SEM. Total variation, present in a set of data was estimated by one way analysis of variance (ANOVA) and student's *t* test was employed for determining the significance. *P*-values of less than 0.05 were considered significant[23].

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