Pharmacognosy Magazine Vol 4, Issue 17, Jan-Mar, 2009 Page 28-36
(An Official Publication of Pharmacognosy Network Worldwide)
Received: 10/05/2008;
Modified: 07/07/2008
Accepted: 02/08/2008

PHCOG MAG.: Research Article

Chemical Composition and Hepato-protective activity of *Imperata cylindrica Beauv*.

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ABSTRACT
Phytochemical study of the aerial parts of *Imperata cylindrica* Beauv. (Graminae), growing in Egypt afforded four methoxylated flavonoids **1**-**4**, β-sitosterol-3-*O*-β-D-glucopyranosyl-6´-tetradecanoate **5**, 3-hydroxy-4-methoxy-benzaldehyde **6**, together with daucosterol, ß-sitosterol and α-amyrin **7-9**. To the best of our knowledge, this is the first isolation of compounds **1**-**5** from the genus *Imperata*. A significant hepato-protective activity had been observed upon co-administration of the methanolic extracts of *I. cylindrica* with CCl_4. The structures were determined using spectroscopic data; 1D (^1H and ^{13}C), 2D (HSQC, and HMBC) NMR; MS; UV and IR.
KEYWORDS: Methoxylated flavonoids, *Imperata cylindrica*, steroids, hepato-protective activity.

INTRODUCTION

Imperata cylindrica Beauv. is widely distributed in tropical regions. The Korean folk medicine has described the rhizomes as diuretic, anti-inflammatory, and antipyretic (1). Previous phytochemical studies of the rhizomes of *I. cylindrica* have resulted in the isolation of neuro-protective chromones (2), several arborane compounds such as arundoin, cylindrin and fernenol (3), in addition to cylindol (4), cylindrene (5), graminones (6) and imperarene (7).

This study is a phytochemical and biological evaluation of *I. cylindrica* Beauv. growing in Egypt, we describe the isolation and structural elucidation of several compounds: four methoxylated flavonoids **1**-**4**, tetradecanoyl ester of sitosterol glucoside **5**, and an aldehyde **6**. These compounds are reported for the first time from genus *Imperata*. In addition to daucosterol, ß-sitostrol and α-amyrin. More over, the total methanolic extract showed potent hepato-protective activity and the compounds **1**-**5** showed cytotoxic activity elicited by the brine shrimp lethality assay.

MATERIALS and METHODS

Plant Material

The fresh aerial parts of *I. cylindrica* Beauv. were collected in September 2006 from the wild plants around the campus of Al-Azhar University, Assiut, Egypt. The plant material was kindly identified by Prof. Dr. A. Fayed, Professor of Plant Taxonomy, Faculty of Science, Assiut University, Egypt. A voucher specimen was deposited in the Department of Pharmacognosy herbarium, Faculty of Pharmacy, Al-Azhar University, Assiut (Registration code WAz-006 IM).

Pre-coated silica gel 60 F_{254} plates (Merck) were used for TLC. Vacuum liquid chromatography (VLC) was carried out using silica gel 60, 0.04-0.063 mm mesh size (Merck). The solvent systems used for TLC analyses were *n*-hexane-EtOAc (95:5, solvent system I), $CHCl_3$-MeOH (90:10, solvent system II) and $CHCl_3$-MeOH (85:15, solvent system III). The TLC plates were visualized by UV light at λ_{max} 255 and 366 nm followed by spraying with *p*-anisaldehyde/H_2SO_4 reagent and heating at 110 ^0C for 1-2 min. HPLC was performed on

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semi-preparative RP-18 column (Cosmosil 5C18 ARII, 250 x 10 mm) with a UV detector at λ_{max} 220 nm and flow rate of 2.5 ml/min. Melting points were carried out in electrothermal 9100 Digital Melting Point (England). UV spectra were recorded on a Hitachi 300 spectrometer. ^1H and ^{13}C-NMR spectra were recorded on a JEOL-JNM-EX-400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C, respectively). EI-MS data were obtained with a JEOL JMS–700T mass spectrometer. All solvents were distilled prior to use. NMR grade solvents (Merck), were used for NMR analysis.

Extraction and Isolation

The air-dried powdered aerial parts of *I. cylindrica* (1.2 kg) were extracted with 70 % MeOH (6 x 5 L) at room temperature; evaporation of the methanol extract under reduced pressure affords a dark brown residue (34.1 g). The residue was suspended in 500 ml water and was successively partitioned with *n*-hexane, $CHCl_3$ and ethyl acetate to yield residues of 4.15, 8.74 and 8.4 g respectively, and an aqueous residue of 12.3 g. Preliminary cytotoxicity assay on the crude fractions showed that the *n*-hexane and ethyl acetate fractions possess cytotoxic activity as elicited by brine shrimp assay. The ethyl acetate residue (8.4 g) was subjected to VLC on silica gel using $CHCl_3$: MeOH gradients and afforded 8 fractions. Fraction 4 (225 mg) was chromatographed on silica gel and eluted with EtOAc: MeOH gradient followed by final purification on semi-preparative RP-18 HPLC column using 25% MeCN in H_2O to afford compounds **1** (20.2 mg) and **2** (13.7 mg). Meanwhile, fraction 5 (238 mg) was chromatographed on silica gel and eluted with EtOAc: MeOH gradient, followed by semi-preparative RP-18 HPLC [MeOH: H_2O (40:60)], to afford **3** (8.3 mg) and **4** (6.5 mg). On the other hand, the *n*-hexane fraction was subjected to VLC on silica gel column eluted with *n*-hexane:EtOAc gradient to yield 7 fractions. Fractions 4 (112 mg), 5 (188 mg), 6 (224 mg), and 7 (118 mg) were separately chromatographed on silica gel eluted with CH_2Cl_2: EtOAc gradient to afford compounds **5** (38.2 mg), **6** (7.3 mg), **7** (12.4 mg), **8** (15.9 mg) and **9** (21.0 mg), respectively.

Alkaline treatment of compound 5 (8)

Alkaline treatment of compound **5** described by Nakano *et al*, afforded sitosteryl-ß-glucopyranoside (**5a**) and a methyl ester of fatty acid which was identified as tetradecanoic acid methyl ester by EI-MS analysis. Aliquot of **5a** was subjected to acid hydrolysis to afford D-glucose that was identified by comparison with authentic sugar samples [TLC paper-chromatography using n-butanol: acetic acid: water (4:1:5)], in addition to β-sitosteryl moiety by direct comparison with authentic sample (IR, TLC and mixed melting point).

Tricin 1 (9). Yellow crystals (20.2 mg); R_f = 0.62 (solvent system II); HRESI-MS (Neg. mode) *m/z* 329.0661 $[M^+- H]^-$, calculated for $C_{17}H_{13}O_7$. UV λ_{max} (MeOH): 270, 352; +NaOMe 279, 413; +$AlCl_3$ 276, 368 sh, 394; +$AlCl_3$/HCl 277, 389; +NaOAc 268, 411.

Jaceidin 2 (10): Yellow sticky residue (13.7 mg); R_f = 0.71 (solvent system II); HRESI-MS (Neg. mode) *m/z* 359.0767 $[M^+- H]^-$, calculated for $C_{18}H_{15}O_8$. UV λ_{max} nm (MeOH): 272, 354; +NaOMe 274, 412; +$AlCl_3$ 285 sh, 391; +$AlCl_3$/HCl 283 sh, 390; +NaOAc 274, 330 sh, 362.

Quercetagetin-3,5,6,3`-tetramethyl ether 3 (11)**:** Yellow amorphous powder (8.3 mg); R_f = 0.79 (solvent system II); HRESI-MS (Neg. mode) *m/z* 373.0923 $[M^+-H]^-$, calculated for $C_{19}H_{17}O_8$. UV λ_{max} nm (MeOH): 269, 345; +NaOMe 270, 405; +$AlCl_3$ 269, 344; +$AlCl_3$/HCl 269, 344; +NaOAc 275, 388.

3,5-Di-*O*-methyl-kaempferol 4 (12-15)**:** Yellow amorphous powder (6.5 mg); R_f = 0.65 (solvent system II); UV λ_{max} nm (MeOH): 275, 339; +NaOMe 282, 391; +$AlCl_3$ 275, 341; +$AlCl_3$/HCl 276, 340; +NaOAc 290, 372; HRESI-MS (Neg. mode) *m/z* 313.0712 $[M^+- H]^-$, calculated for $C_{17}H_{13}O_6$.

β-Sitosterol-3-*O*-β-D-glucopyranosyl-6´-tetradecanoate 5 (16)**:** White greasy substance (38.2 mg); R_f = 0.78 (solvent system III); HRESI-MS (Neg. mode) *m/z* 785.6295 calculated for $C_{49}H_{85}O_7$.

3-Hydroxy-4-methoxy benzaldehyde 6 (17)**:** White needles (7.3 mg); R_f = 0.52 (solvent system II); mp 117-118 °C; HR ESI-MS, *m/z* 152 (M^+, 100%). HRESI-MS (Neg. mode) *m/z* 151.0395 $[M^+- H]$, caculated for $C_8H_7O_3$.

BIOLOGICAL STUDY

Brine shrimp lethality test

The test was performed using 5 and 10 µg of the obtained fractions and isolated compounds as published by Mayer and Edrada *et al*. (18,19). The test was done in triplicate.

Hepato-protective activity

1. Experimental animals: Adult male albino rats of Charles River strain weighing 120-150g were obtained from Assiut University animal house.

2. CCl_4-induced hepatotoxicity (20): The animals were divided into four groups of six animals each.

Group I: Normal control received distilled water (1 ml/kg) daily for 5 days and olive oil (1 ml/kg, intraperitoneal) on days 2 and 3.

Group II: CCl_4 control received distilled water (1 ml/kg) daily for 5 days and CCl_4: olive oil (1:1, 1ml/kg, intraperitoneal) on days 2 and 3.

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Group III: Treated with *I. cylindrica* extract orally through intragastric feeding tube at dose of 100 mg/kg, the extract dose was fixed after trying out different doses (25, 50, 100 and 150 mg/kg).

Groups IV: Treated with *I. cylindrica* extract doses of 100 mg/kg, for 5 days and 30 mins after administration of extract the rats received CCl_4: olive oil (1:1, 1ml/kg, intraperitoneal) on days 2 and 3.

3. Biochemical estimations: The rats were sacrificed on the sixth day and blood was collected from orbital sinus in plain tubes. The serum was obtained by centrifugation and serum samples were taken for biochemical assays; namely glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) (21).

RESULTS and DISCUSSION

Compound **1**.

The HR ESI-MS identified a molecular formula of $C_{17}H_{14}O_7$. ^{1}H NMR spectrum showed the presence of two methoxyl groups as a singlet peak at δ_H 3.91 (6H, s). A singlet proton at δ_H 6.96 (1H, s) which showed HSQC correlation to the carbon at δ_C 103.5 indicated a flavone structure (15). Two doublet signals at δ_H 6.21 (1H, d, *J* = 1.7 Hz, H-6) and 6.55 (1H, d, *J* = 1.7 Hz, H-8) revealed meta-disubstituted ring A as confirmed by the carbon resonances at δ_C 98.7 and 94.0, respectively. Furthermore, a singlet signal at δ_H 7.31 (2H, s, H-2`, 6`) revealed trisubstituted ring B which was confirmed by the carbon resonances at δ_C 104.2, 147.9 and 139.6 (22,23). The downfield proton at δ_H 12.95 afforded the presence of a hydrogen bonded hydroxyl group at C-5 and confirmed by the carbon atom resonated at δ_C 161.0. The HMBC experiment afforded the attachment of two methoxyl groups at C-3` and C-5`, which identified a tricin structure (9). It is the first isolation of this compound from the genus *Imperata*.

Compound **2**.

The HR ESI-MS identified a molecular formula of $C_{18}H_{16}O_8$. The ^{1}H NMR spectrum, showed resonances for three methoxyl groups at δ_H 3.92, 3.87, and 3.78 which were correlated to the carbons resonated at δ_C 54.3, 58.3 and 58.7, respectively as shown by the HSQC experiment. More over, three proton signals at δ_H 6.91 (1H, d, *J* = 8.3 Hz), 7.61 (1H, dd, *J* = 8.3, 1.7 Hz) and 7.69 (1H, d, *J* = 1.7 Hz) were assigned to H-5`, H-6` and H-2`, respectively. On comparison to **1**, absence of the singlet proton signal at $\delta_H$ 6.96 and its carbon at $\delta_C$ 103.5, suggested a flavonol structure (9,15). The HMBC experiment afforded the attachment of the methoxyl groups at C-3`, 3 and 6 (Figure 2). The compound was identified as jaceidin (10), which was isolated for the first time from the genus *Imperata*.

Compound **3**.

The HR ESI-MS spectrum revealed a molecular formula of $C_{19}H_{18}O_8$. The ^{1}H and ^{13}C NMR spectral data of **3** (Tables 1 and 2) afforded a similar structure to **2** except in presence of additional singlet proton signal at δ_H 3.35 assigned to another methoxyl group. This was confirmed by the carbon resonance at δ_C 61.5. The HMBC experiment afforded the attachment of the additional methoxyl group at C-5 (Figure 2). Accordingly, compound **3** was identified as quercetagetin-3,5,6,3`-tetramethyl ether (11), it is isolated for the first time from the genus *Imperata*.

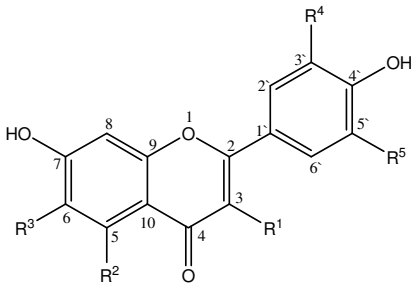
Compound **4**.

HR ESI-MS revealed a molecular formula of $C_{17}H_{14}O_6$. The ^{1}H and ^{13}C-NMR indicated a similar structure to **2**. The ^{1}H and ^{13}C -NMR spectra of **4** (tables 1 and 2), showed the presence of a meta-disubstituted ring A and *para*-disubstituted ring-B. The HMBC experiment afforded the attachment of the methoxyl groups at δ_H 3.4 and 3.96 (3H, s), to C-3 and C-5, respectively (Figure 2). Compound **4** was identified as 3,5-di-*O*-methyl-kaempferol (12-16), it is isolated for the first time from the genus *Imperata*.

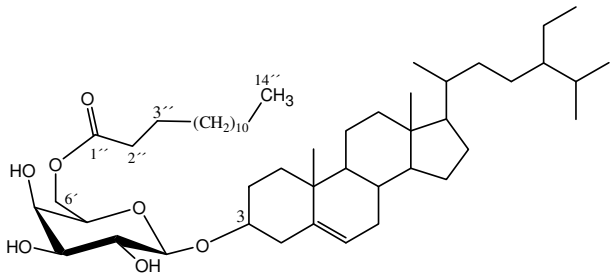
Compound **5**.

The HRESI-MS spectrum identified a molecular formula of $C_{49}H_{86}O_7$. ^{1}H NMR (400 MHz, C_5D_5N): showed the presence of a triplet methyl group at δ_H 1.1 (3H, t), in addition to six methyl groups at δ_H 0.66 (3H,s) , 0.91 (3H,s), 0.97 (3H,d, *J* = 6.4 Hz), 0.89 (3H,d, *J* = 6.4 Hz), 0.93 (3H,d, *J* = 6.5 Hz) and 0.87 (3H, t). More over, a ß-anomeric sugar proton at δ_H 4.75 (1H, d, *J* = 7.2 Hz), indicated a gluco-steroidal structure. ^{1}H and ^{13}C-NMR spectral data of **5** indicated signals for sitosteryl (24) and glucopyranosyl moieties (25) in addition to an ester carbonyl carbon at δ_C 174.1, a methyl group at δ_C 14.1 in addition to a cluster of methylene groups at δ_C 22.7–34.3. The downfield shift of C-6´ (63.7) and an upfield shift of C-5´ (73.8) in the ^{13}C-NMR spectrum revealed the attachment of the fatty acid at C-6´ of the glucose moiety (25) that was confirmed by the HMBC experiment. Alkaline treatment of compound **5** afforded sitosteryl-D-glucopyranoside and tetradecanoic acid methyl ester that was identified by EI-MS analysis. Acid hydrolysis revealed the presence of D-glucose and sitosterol. Accordingly, compound **5** was identified as β-sitosterol-3-*O*-β-D-glucopyranosyl-6´-tetradecanoate (16), it is isolated for the first time from the genus *Imperata*.

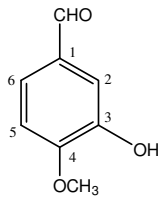
Figure 1: *Structures of the isolated compounds* ***1-9***



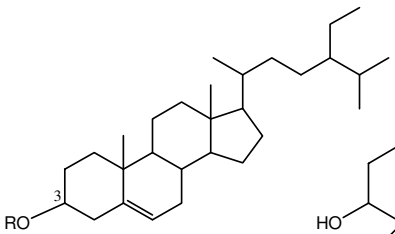
	R_1	R_2	R_3	R_4	R_5
1	H	OH	H	OCH3	OCH3
2	OCH3	OH	OCH3	OCH3	H
3	OCH3	OCH3	OCH3	OCH3	H
4	OCH3	OCH3	H	H	H



5



6



7 R= Glucose
8 R= H

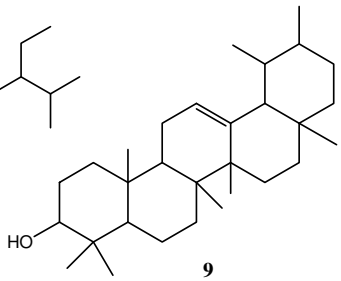


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Figure 2: *Important HMBC correlations of compounds* ***1-4****.*

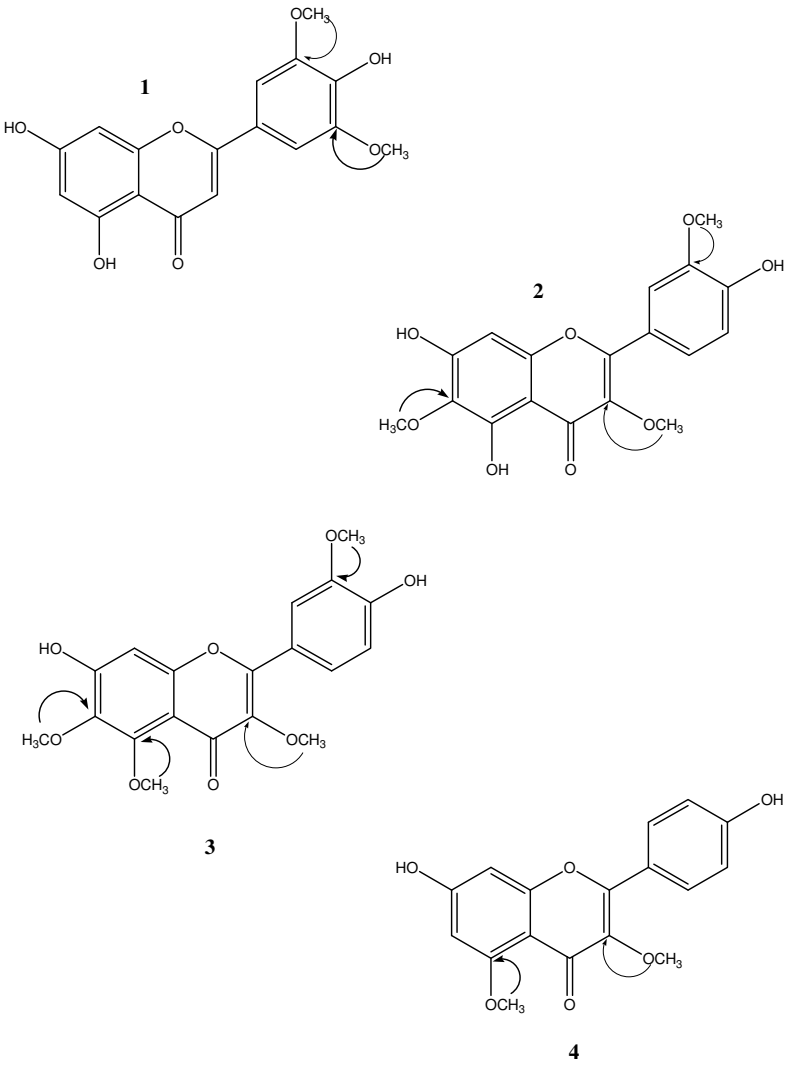

Table 1: ^{1}H NMR [400 MHz] spectral data for compounds 1-4.

	δ_H (J in Hz)			
Position	**1**	**2**	**3**	**4**
3	6.96, 1H, s	-	-	-
6	6.21, 1H, d, 1.7	-	-	6.70, 1H, s
8	6.55, 1H, d, 1.7	6.50, 1H, s	6.78, 1H, s	6.86, 1H, s
2`	7.31, 1H, s	7.69, 1H, d, 1.7	7.71, 1H, br s	7.86, d, 8.5
3`	-	-	-	7.18, 1H, d, 8.5
5`	-	6.91, 1H, d, 8.3	6.92, 1H, d, 8.3	7.18, 1H, d, 8.5
6`	7.31, 1H, s	7.61, 1H, dd, 1.7, 8.3	7.65, 1H, br d, 8.3	7.86, 1H, d, 8.5
5-OH	12.95, 1H, s	-	-	-
3`-OCH_3	3.91, 3H, s	3.92, 3H, s	3.81, 3H, s	-
5`-OCH_3	3.91, 3H, s	-	-	-
3-OCH_3	-	3.78, 3H, s	3.78, 3H, s	3.40, 3H, s
5-OCH_3	-	-	3.35, 3H, s	3.96, 3H, s
6-OCH_3	-	3.87, 3H, s	3.38, 3H, s	-

Compounds **2** and **3** were measured in CD_3OD, **1**and **4** in DMSO-d_6.

Table 2: ^{13}C NMR [100 MHz] spectral data for compounds 1-4.

	δ_C			
Position	**1**	**2**	**3**	**4**
2	163.3	155.4	158.4	150.9
3	103.5	136.8	139.9	132.5
4	181.4	177.7	180.4	180.5
5	161.0	151.2	160.8	162.2
6	98.7	130.1	133.6	101.3
7	163.9	156.1	154.1	165.0
8	94.0	92.7	92.5	89.2
9	157.0	151.2	153.6	156.8
10	103.4	103.9	107.6	101.3
1`	120.2	120.4	124.1	119.7
2`	104.2	110.4	113.2	126.5
3`	147.9	146.4	151.5	114.5
4`	139.6	148.6	149.2	160.3
5`	147.9	114.0	116.8	114.5
6`	104.2	121.3	123.1	126.5
3`-OCH_3	56.3	54.3	57.1	-
5`-OCH_3	56.3	-	-	58.3
3-OCH_3	-	58.7	57.5	54.1
5-OCH_3	-	-	61.5	-
6-OCH_3	-	58.3	61.0	-

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Table 3: ^{13}C NMR (C_5D_5N, 100 MHz) spectral data for compound 5

Position	δ_C	Position	δ_C
1	37.3	21	18.8
2	29.4	22	34.0
3	79.7	23	26.3
4	39.8	24	45.9
5	140.4	25	29.2
6	122.0	26	19.0
7	31.9	27	19.8
8	31.9	28	23.1
9	50.2	29	12.0
10	36.7	1´	101.3
11	21.1	2´	73.4
12	39.0	3´	76.4
13	42.3	4´	70.5
14	56.8	5´	73.8
15	25.0	6´	63.7
16	28.2	1´´	174.1
17	56.2	2``	34.3
18	11.9	3´´	24.3
19	19.4	$-(CH_2)_n-$	22.7-29.8
20	36.2	31´´	14.1

Table 4: Toxicity effect of the obtained fractions and the isolated compounds of I. cylindrica in Brine shrimp assay

	% Mortality (24 hr.)		% Mortality (48 hr.)	
Sample	5	10	5	10 (µg)
n-Hexane fr.	25	60	35	70
Ethyl acetate fr.	40	72	53	80
Comp**. 1**	30	45	40	50
Comp**. 2**	50	70	55	80
Comp**. 3**	60	75	70	80
Comp**. 4**	25	40	35	53
Comp**. 5**	70	85	75	95

Table 5: Hepato-protective effect of methanolic extract of I. cylindrica

Animal Groups	GOT*	GPT*
Control	21.56 ± 2.17	35.39± 3.36
CCl_4 treated	41.2 ± 3.16	85 ± 4.09
I. cylindrica extract	23 ± 2.4	35.9 ± 2.9
I. cylindrica and CCl_4	27 ± 1.5	40.2 ± 6

**Results are expressed as mean ± SD (n = 6). p < 0.01. ; *Activity is expressed as mmol of pyruvate liberated per mg of protein/hour for GOT, and GPT; Comparisons are made between group 1 (control) with group 2 (CCl_4, induced injury), and group 2 with group 4 (I. cylindrica and CCl_4).*

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Compounds (**6-9)** were identified as 3-hydroxy-4-methoxy-benzaldehyde (17), daucosterol (26,27), ß-sitoststerol (28) and α-amyrin (29), respectively on comparing their physical and spectral data with literatures. These compounds were isolated for the first time from *I. cylindrical*.

All the isolated compounds were evaluated for their cytoxic activity using brine shrimp assay (Table 4). Compounds **2**, **3** and **5** showed strong activity while compounds **1** and **4** showed moderate activity.

Liver injury in rats was induced through intraperitoneal administration of CCl_4 that was manifested by significant elevation in the level of serum hepatic marker enzymes; namely glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) as shown in table 5. The activities of the GOT and GPT enzymes in the serum of control and experimental groups showed marked elevation ($p < 0.01$) in the activities of these enzymes in group 2 (CCl_4 administered rats) when compared with group 1 (Control rats). Activities of these enzymes in serum were maintained at near normal levels ($p < 0.01$) in group 4 (*I. cylindrica* and CCl_4 co-administrated group). Group 3, rats treated with *I. cylindrica* extract alone, show minor changes when compared with group 1 (control rats), which revealed the non toxic effect of the *I. cylindrica* extract.

Acknowledgment

The authors are grateful to Professor A. Fayed (Plant Taxonomy Department, Faculty of Science Assuit University, Egypt) for the identification of the plant material and Mr. Hiroshi Matsuura, Faculty of Graduate School of Environmental Science, Hokkaido University, for his help in measuring the NMR and mass spectra. This work is a part of the DAAD project (Code 134.104401.212).

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