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Phytochemical constituents and hepatoprotective activity of *Viburnum tinus*

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Abstract

From the leaves of *Viburnum tinus* L. (Adoxaceae) two acylated iridoid glucosides (viburtinoside A and B), a coumarin diglucoside scopoletin 7-*O*-β-D-sophoroside and a natural occurred dinicotinic acid ester 2,6-di-*C*-methyl-nicotinic acid 3,5-diethyl ester were isolated. In addition to these, 10 known compounds were isolated, namely two bidesmosidic saponins, a hexamethoxy-flavone and five flavonol glycosides, as well as suspensolide A and oleanolic acid were isolated for the first time in this genus and species, respectively. The structures were determined mainly by spectroscopic methods (UV, IR, ESI-MS, ^{1}H-, ^{13}C NMR and DEPT). Toxicity of the investigated extract was determined ($LD_{50} = 500$ mg/kg). CCl_4-induced hepatotoxicity has been evaluated in terms of the determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lipid peroxide and nitric oxide levels in serum and compared using adult male rats weighing 150–180 g. Their highly elevated levels were significantly reduced by treatment with the investigated aqueous methanol extract in dose-dependant manner.

Keywords: *Viburnum tinus*; Adoxaceae; Acylated iridoid glucosides; Nicotinic acid diethyl ester; Coumarin sophoroside; Hepatoprotective activity; Lipid peroxidation; Nitric oxide

1. Introduction

Viburnum genus comprises more than 230 species distributed from South America (Peru) to South-East Asia (Philippines, Malaysia), the majority of them are being endemic (Lobstein et al., 1999). *Viburnum tinus* (Adoxaceae) is an evergreen shrub widely distributed in Southern Europe, especially in Mediterranean regions (Tomassini et al., 1995). *Viburnum* species are commonly used in folk medicine for their diuretic, antispasmodic and sedative properties, mainly as uterine excitability (British herbal Pharmacopoeia, 1983; Cometa et al., 1998) and the dry powder of *V. tinus* leaves was reported as an effective molluscicidal agent (Ibrahim et al., 1994). The genus *Viburnum* is known to be rich in iridoid glycosides (Bock et al., 1978; Hase et al., 1985; Jensen et al., 1985), mainly of the Valeriana type with a β-D-glucoside linked to C-11 (El-Naggar and Beal, 1980; Boros and Stermitz, 1991, 1990; Iwagawa et al., 1990; Iwagawa and Hase, 1989). Previous phytochemical studies on some *Viburnum* species have led to isolation of coumarins, flavonoids and biflavonoids (Glasby, 1991; Plouvier, 1992). Several oleanane-type triterpenes and diterpenes have been isolated from other *Viburnum* species (Kagawa et al., 1998; Iwagawa et al., 1993;

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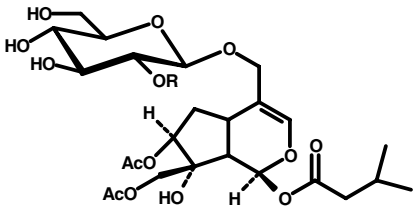
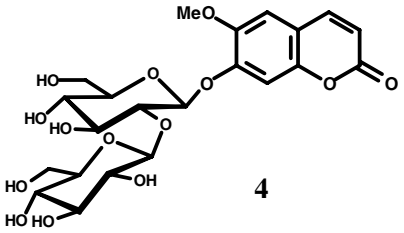
Fukuyama et al., 1999). Also from *V. tinus*, a group of five new iridoids were isolated (Tomassini et al., 1995). In this paper, we describe the isolation and structure elucidation of four new metabolites viz. two acylated iridoids (**2**, **3**), a coumarin sophoroside (**4**) and diethylester of nicotinic acid (**5**) along with 10 known compounds an iridoid, suspensolide A (**1**), two triterpenoidal saponins (**6**, **7**), nobiletin (**8**), five flavonol glycosides (**9**–**13**), and oleanolic acid (**14**). LD_{50}, lipid peroxidation and hepatoprotective activity were determined for the investigated extract, as well.



1: R = H, 2: R = *E*-*p*-coumaroyl
3: R = *Z*-*p*-coumaroyl



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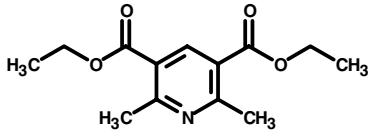

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2. Results and discussion

Total 80% methanol extract of air-dried leaves of *V. tinus* was defatted with pet. ether and then subjected to successive fractional partition by chloroform and ethyl acetate. Oleanolic acid **14** and the new 2,6-di-*C*-methyl-dinicotinic acid 3,5-diethyl ester (**5**) were purified from the chloroform extract. The ethyl acetate soluble fraction was chromatographed on a silica gel column followed by repeated CC on sephadex LH-20 or cellulose and precipitation or crystallization in some cases, to obtain the pure samples of the other 12 isolates. Their structures have been established as two new iridoids viburtinoside A and B (**2** & **3**), a new coumarin diglucoside scopoletin 7-*O*-β-D-sophoroside (**4**) along with suspensolide A (**1**) (Iwagawa et al., 1990), two bidesmosidic saponins 3-*O*-β-D-$^4C_1$-galactopyranosyl-(1″ → 2′)-*O*-β-D-$^4C_1$-glucuronopyranoside-oleanolic acid 28-*O*-β-D-$^4C_1$-glucopyranosyl ester (**6**) (Takabe et al., 1985) and 3-*O*-β-$^4C_1$-glucuronopyranoside-oleanolic acid 28-*O*-β-D-$^4C_1$-glucopyranosyl ester (**7**) (Nie et al., 1984), and six flavonoids (nobiletin **8**, afzelin **9**, quercitrin **10**, isoquercitrin **11**, hyperin **12**, and rutin **13**), (Gonzalez et al., 1991; Agrawal, 1989; Harborne and Mabry, 1982).

Iridoids (**1**), (**2**) and (**3**) were obtained, individually, as creamy-white amorphous powder and as blue fluorescent spots changed into greenish yellow with Naturstoff reagent, in case of **2** and **3**, to indicate the presence of a cinnamic acid derivative functionality in their structures. They emitted an odour of isovaleric acid on standing and turned black with hydrochloric acid, suggesting that they are Valeriana iridoids (Iwagawa et al., 1990) and gave brown colour with vanilline-hydrochloride spray reagent. Acid hydrolysis of each led to the identification of glucose in the aqueous phase (R_f-values, Comp-PC against authentic sample and response with aniline hydrogen phthalate spray reagent, see Section 3) with *p*-coumaric acid in the organic phase (R_f-values, Comp-PC against authentic sample and its UV spectral data, see Section 3) in case of both **2** and **3** only, which was indicative to the presence of glucoside moiety in the molecules of the three compounds together with a *p*-coumaroyl in case of **2** and **3**. Structure of **1** was confirmed as the known iridoid suspensolide A, through a comparison study of its ^1H and ^{13}C spectral data Table 1, with those published before from *V. suspensum* (Iwagawa and Hase, 1989).

The molecular formula of compound **2** or **3** was deduced as $C_{34}H_{44}O_{16}$ from negative HR ESI-MS. Compound **2** showed in its IR spectrum absorption bands at 3405, 1733 and 1630 for OH, CO-ester and an olefinic double bond, respectively. Its ^1H and ^{13}C NMR spectral data were very similar to those of **1**, except for more one *p*-*E*-coumaroyl group on the basis of its extra characteristic two AX spin coupling systems, each of two doublets, in the aromatic region. The first one was located at 7.55 *d* (8.7), 6.90 (8.7) for H-2″/6″ and H-3″/5″, respectively, for the 1,4-disubstituted phenyl moiety, while the second was observed at 7.63 (15.9) and 6.40 (15.9) assigned to H-7″ and H-8″, respectively, for the two *E*-olefinic protons. This was confirmed by its characteristic seven resonances in its ^{13}C NMR spectrum (Table 1). The attachment of the *E*-*p*-coumaroyl group to C-2′ was deduced from the characteristic downfield

Table 1
^{1}H and ^{13}C NMR spectral data of 1–3 (300, 75 MHz, acetone-d_6)

C, H-No.	1		2		3	
	C	^{1}H	C	H	C	H
1	90.51		90.34	6.17 *d* (5.4)	90.30	6.15 *d* (5.4)
3	139.94	6.42 *brs*	139.78	6.37 *brs*	139.68	6.35 *brs*
4	115.54		115.44		115.30	
5	33.37	3.07 *brq*	32.67	2.95 *brq*	32.90	2.92 *brq*
6	35.73	2.05 *m*	35.55	2.06 *m*	35.76	2.03 *m*
7	80.34	5.01 *t*-like (3.9)	80.25	5.07 *t*-like (3.6)	80.19	5.01 *t*-like (3.6)
8	81.79		81.64		81.56	
9	45.20	2.39 *dd* (9.9,5.4)	45.09	2.42 *dd* (9.9, 5.4)	45.10	2.35 *dd* (9.9, 5.4)
10	67.30	4.20 *brd* AA′ (12.5)	67.16	4.16 *brd* AA′(11.3)	67.20	4.14 *brd* AA′ (11.5)
11	68.90	4.09 *m*	68.67	4.07(12.5, 1.3)	68.70	4.07 *dd* (12.5, 1.2)
1′	102.93	4.36 *d* (7.8)	100.54	4.62 *d* (8.1)	100.47	4.56 *d* (8.1)
2′	74.44	3.70–3.1 *m* (H-2′-6′)[a]	74.70	4.87 *dd* (9.3, 8.1)	74.67	4.85 *dd* (9.3, 8.1)
3′	77.35		76.00	3.53 *t*-like (9.3)	75.92	3.50 *t*-like (9.1)
4′	71.55		71.75	3.42–3.29 *m* (H-4′/5′)[a]	71.78	3.40–3.27 *m* (H-4′/5′)[a]
5′	77.81		77.15		77.18	
6′	62.59		62.81	3.85 *brd* (12.9, H6_b)	62.83	3.83 *brd* (12.5, H6_b)
				3.70 *brd* (12.5, 5.1 H6_a)		3.69 *brd* (12.5, 5.1 H6_a)
1″			126.82		126.87	
2″/6″			130.79	7.55 *d* (8.7)	133.57	7.76 *d* (8.7)
3″/5″			116.53	6.90 *d* (8.7)	115.16	6.82 *d* (8.7)
4″			160.40		159.60	
7″			145.48	7.63 *d* (15.9)	144.24	6.88 *d* (12.9)
8″			115.55	6.40 *d* (15.9)	114.67	5.78 *d* (12.9)
9″			166.44		166.36	
2× *CH*$_3$CO–	20.89	2.00 *s*, 1.99 *s*	20.56	1.95 *s*, 1.89 *s*	20.46	1.98 *s*, 95
	20.79		20.52		20.22	
2× CH$_3$*CO*–	170.79		170.74		170.24	
	170.29		170.11		170.09	
Isovaleroyl						
–CH–	26.03	2.14 *m*	26.04	2.10 *m*	26.04	2.12 *m*
–CH$_2$–	43.50	2.20 *d* (7)	43.55	2.23 *d* (7)	43.55	2.19 *d* (7)
2× Me	22.37	0.97 *d* (6.6)	22.42	0.95 *d* (6.0)	22.43	0.93 *d* (6.0)
–*CO*O–	171.24		171.24		171.26	

δ in ppm and J values (Hz), were given in parentheses.
[a] Unresolved proton resonances.

shifts of both H-2′ and C-2′ and upfield of C-1′ relative to those of **1**, which were consistent with the corresponding values in case of the similar compounds viburtinosides II and III. The presence of extra acetyl group on C-7 in case of **2** more than that of viburtinoside II was evidenced from the presence of two resonances at 1.95 and 1.89 (two CH_3-acetyl) rather than only one in case of the last compound. Location of this extra acetyl on the C-7 of **2**, also followed from the downfield shift of H-7 at 5.07 relative to its normal position in case of viburtinosides II and III (~3.9 ppm), (Tomassini et al., 1995). Therefore, **2** was identified as 7-*O*-acetyl derivative of viburtinosid II, which was empirically named as viburtinoside-A.

The iridoid **3** showed also more or less the same spectroscopic data as those of **2**, with only one difference in its ^{1}H and ^{13}C spectra (Table 1). In its ^{1}H NMR, relatively slight upfield location of H-2″/6″ and H-3″/5″ resonances with characteristic upfield AX system of two olefinic protons (H-7″ & H-8″) at 6.88 and 5.78 with *J*-value of 12.9 Hz resulted in presence of *Z*-coumaroyl group in the structure of **3** instead of the *E*-form one in case of **2**. Also from ^{13}C NMR data, this difference was confirmed as in case of viburtinoside II, which is also *E*-isomer of viburtinoside III (Tomassini et al., 1995). Consequently, iridoid **3** was finally identified as the *Z*-isomer of **2** or 7-*O*-acetyl derivative of viburtinosid III and named as viburtinoside-B.

Compound **4** was expected to be coumarin *O*-glycoside, on the basis of its chromatographic properties (R_f-values, blue fluorescent under UV-light changed to greenish blue with ammonia vapors) and UV spectral data (Sibanda et al., 1989). Its molecular formula was inferred as $C_{22}H_{28}O_{14}$ from negative HR ESI-MS. On complete acid hydrolysis, it gave scopoletin (R_f-values, Comp-PC against authentic sample and its UV spectral

data, see Section 3) and glucose (R_f-values, Comp-PC against authentic sample and response with aniline hydrogen phthalate spray reagent, see Section 3) in the organic and aqueous phases, respectively. According to the above documents, compound **4** was tentatively identified as scopoletin 7-*O*-glucoside. The identity of the aglycone moiety was proved to be scopoletin 7-*O*-substituted by a comparison study with the previous published ^{1}H and ^{13}C NMR data of scopoletin 7-*O*-β-D-glucopyranoside (scopolin) and scopolin 6′-β-D-apiofuranoside (Sibanda et al., 1989; Reisch and Achenbach, 1992). The presence of a sophoroside moiety in the molecule of **4** has been clearly deduced from the two-anomeric proton doublets at 5.28 and 5.07 with *J*-values of 7.8 and 7.5, which were characteristic for the two β-glucopyranosides. Downfield location of H-1″ at the previous value was indicative to 1″ → 2′ interglucosidic linkage. Confirmation of its structure was followed from the ^{13}C NMR spectrum of 22 carbon resonances, 10 of which were typical for a scopoletin moiety and 12 carbon resonances were assigned to the diglucoside moiety in the aliphatic region. The location of the terminal glucosyl moiety on C-2′ of the inner one was evidenced from the downfield shift of its resonance to 81.25 (+Δ ~ 7 ppm) relative to that of C-2″ (73.77) and the β-upfield shift of both C-1′ and C-3′ to 100.37 and 74.13, respectively, relative to the corresponding carbon resonances of the terminal glucosyl, C-1″ and C-3″ at 103.60 and 77.23. Thus, compound **4** was identified as the new scopoletin 7-*O*-β-D-sophoroside.

Compound **5** was observed under short UV-light as dark purple spot. Its CI-MS spectrum exhibited a molecular ion peak at *m*/*z* 251.9 $[M + H]^+$ corresponding to an odd M_r of 251 and a MF of $C_{13}H_{17}NO_4$, which is confirmed from its HR ESI-MS. In its simple ^{1}H NMR spectrum, four resonances were observed; the most downfield located one was a singlet at 8.66 of H-4 of a pyridine nucleus. In the aliphatic region, two typical resonances of two equivalent ethyl ester groups were described at 4.39 (*q*, *J* = 7.2) and 1.41 (*t*, *J* = 7.2) for two CH_2 and CH_3 groups. A singlet signal, integrated to six protons at 2.84, was assigned to two equivalent C-alkyl methyl groups on C-2 and C-6 of the pyridine residue. Therefore, **5** was expected to be 2,6-di-*C*-methyl-dinicotinic acid diethyl ester. This structure was confirmed by ^{13}C NMR analysis. Seven carbon resonances were assigned as it was given in Section 3, one of which at 140.83 assigned to a methine carbon and two at 61.35 and 14.27 assigned to the two equivalent ethyl ester groups. As well as, an intrinsic carbon resonance of the two equivalent *C*-alkyl methyl was assigned at 24.89. The carbonyl carbon resonance of the two equivalent esters observed at 165.95 to confirm finally the diethyl ester form of **5**. Accordingly, it was confirmed as 2,6-di-*C*-methyl-dinicotinic acid 3,5-diethyl ester.

Toxicity of the investigated extract was studied (LD_{50} = 500 mg/kg). The results given in Table 2 showed that, when CC_4 was injected (*s.c.*), the levels of ALT and AST enzymes were significantly elevated compared with the normal control, which can be considered due to the oxidative damage in the structural integrity of the liver (Brent and Rumack, 1993). Administration of 25 mg/kg (*i.p.*) of Silymarin (Sigma, USA) induced a significant hepatoprotection as a reduction in serum ALT and AST compared with CCl_4 group. Treatment with 25 mg/kg (*i.p.*) of *V. tinus* extract showed no significant change in serum ALT and AST levels, while its high dose, i.e., 50 mg/kg caused a significant hepatoprotection, evidenced by improvement of ALT and AST values (Table 2).

Administration of CCl_4 exerted a significant increase in serum lipid peroxides and nitrite production. Treatment with Silymarin significantly reversed these elevated parameters. Similarly, as in case of ALT and AST the treatment with the low dose (25 mg/kg) of *V. tinus* extract exhibited no significant effect on either serum lipid peroxides or nitric oxide levels, while their levels were significantly reduced with the high dose 50 mg/kg of the tested extract (Table 2).

On the basis of these findings in this study, it can be observed that the investigated extract has shown significant activity in preventing changes mediated by CCl_4, which is meanly responsible for elevation the serum levels of liver enzymes, especially ALT and AST (Anand et al., 1992). The high dose of the extract seem to preserve the integrity of liver cell membrane as documented by the significant reduction of CCl_4-induced rise in ALT and AST levels. As well as, the damaging effect of CCL_4

Table 2
Effect of treatment on serum ALT, AST, lipid peroxide and nitric oxide production levels in CCl_4-induced hepatotoxicity in rats[a]

Treatment dose (mg/kg)	ALT (units/ml)	AST (units/ml)	Lipid peroxide (nmole/ml)	Nitric oxide (μg/L)
Control normal (saline)	42.5 ± 1.92	93.8 ± 1.57	2.99 ± 0.11	1.01 ± 0.08
CCl_4 (saline)	140.0 ± 3.61[b]	216.4 ± 3.53[b]	5.62 ± 0.30 [b]	2.80 ± 0.17[b]
Silymarin (25)	78.2 ± 3.10[c]	130.7 ± 3.20[c]	3.70 ± 0.16[c]	1.06 ± 0.11[c]
V. tinus extract (25)	136.1 ± 2.40	213.9 ± 2.57	5.20 ± 0.17	2.70 ± 0.19
V. tinus extract (50)	118.6 ± 3.67[c]	192.4 ± 4.73[c]	4.6 ± 0.18[c]	2.38 ± 0.25

[a] Values are means ± s.e. of *n* = 6 rats.
[b] Significantly different from the corresponding control normal value at $p < 0.05$.
[c] Significantly different from the corresponding CCl_4 group value at $p < 0.05$.

on liver is dependant on the peroxidative decomposition of structural membrane lipid (Recknagel, 1983); thus one possible mechanism of the hepatoprotective effect of the examined extract against CCl_4-induced hepatotoxicity would be its antioxidative properties. In addition, the production of NO free radicals, as another marker for cytotoxicity and tissue damage, was significantly inhibited by treatment with the *V. tinus* extract. Accordingly, the scavenging of excessive NO radicals induced by extract can prevented excessive peroxy nitrite production leading to hepatoprotection, which is indicated by the reduction of ALT and AST levels.

3. Experimental

3.1. General

The NMR (^1H and ^{13}C) spectra were recorded at 300, 270 (^1H) and 75, 67.5 (^{13}C) MHz, on Varian Mercury 300 and JEOL-270 GX. The δ-values are reported as ppm relative to TMS in DMSO-d_6, $CDCl_3$, Pyridine-d_5 or Acetone-d_6 and *J*-values are in Hz. CI-MS analysis was recorded on a SSQ 7000 Finnigan MAT mass spectrometer and negative HR ESI-MS analyses were measured on a API Q-STAR PULSA of Applied Bio-system. IR spectra were recorded on a Bruker Vector 22 instrument. UV analyses for pure samples were recorded separately as solutions in methanol and with different diagnostic UV shift reagents (Mabry et al., 1970), in case of the flavonoids on a Shimadzu UV 240 spectrophotometer.

3.2. Plant material

Leaves of *V. tinus* L. were collected from the plant grown in El-Zoharia Botanical Garden, Cairo, Egypt in January 2003. Identification of the plant confirmed by Dr. Amal A. Hagag Lecturer of Taxonomy, Ornamental Research Institute, El-Dokki, Cairo, Egypt. Voucher specimens (Reg. No.: V-20) are kept in herbarium, Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Egypt.

3.3. Extraction and isolation

Powdered, air-dried leaves of *V. tinus* (1 kg) were exhaustively extracted with 80% MeOH (7×4L, on hot, 70 °C), under reflux. The dry residue obtained (105 g) was defatted with pet. ether (60–80 °C) under reflux at 60 °C (5×750 ml), followed by successive partition with chloroform and ethyl acetate from water. Compound **14** (430 mg) was spontaneously precipitated from chloroform soluble portion. The supernatant was concentrated on rotary evaporator and subjected to prep.TLC (Silica gel 60G F254, 20×20 cm glass plate) with toluene to give 30 mg and finally crystallized from MeOH to give 20 mg of pure **5**. The dry ethyl acetate soluble fraction (77 g) was fractionated on a Silica gel 60 (Sigma, 28-200 mesh) column ($\emptyset 4.0 \times 110$ cm, 250 g) and eluted with pet. ether (40–60 °C), pet. ether–$CHCl_3$ and then $CHCl_3$–MeOH mixtures to increase gradually the polarity up to pure MeOH. A pure sample of component **8** (35 mg) was spontaneously precipitated in the concentrated collective fraction eluted with 10% MeOH. Consequative CC on sephadex LH-20 (*n*-BuOH satd. with water for elution) of the collective fraction eluted by 20–30% MeOH gave rise pure samples of both compounds **9** (22 mg) and **10** (18 mg). Collective fraction obtained by 35–40% MeOH was subjected to consequative CC on sephadex LH-20 (eluent: *n*-BuOH–*iso*-propanol–H_2O, 4:1:5, top layer) to give a pure sample of the coumarin derivative **4** (15 mg), and then the major subfraction was fractionated on cellulose (aq EtOH for elution) to give pure sample of **11** (25 mg) and **12** (55 mg). A mixture of major three iridoids (1–3) from 50% to 60% MeOH collective fraction was obtained. This fraction was chromatographed on cellulose, with 40% EtOH, to give two major subfractions. A dry pure sample of compound **1** (28 mg) was obtained by repeated CC of the first subfraction on sephadex and MeOH for elution. The second one was subjected to successive CC on sephadex LH-20 with EtOH as an eluent to afford pure samples of **2** (47 mg) and **3** (33 mg). A pure sample of compound **13** was obtained by CC fractionation of the collective fraction, eluted by 70–75%, on sephadex (for twice) and EtOH–H_2O (1:1) for elution. Component **7** was detected as a major compound in the collective fraction with 75–80% MeOH, which was purified by precipitation of its conc. MeOH soln. with excess EtOAc to give 42 mg. Finally, elution with 95% MeOH gave a mixture of a major saponin among other minor constituents. From this last fraction, a pure sample of **6** (30 mg) was purified as in case of the previous fraction. All separation processes were followed up by Comp-TLC with different solvent systems, 2D-PC and Comp. PC using Whatmann No. l paper with (S_1) *n*-BuOH–HOAc–H_2O (4:1:5, top layer) and (S_2) 15% aqueous HOAc as solvent systems. Acid hydrolysis products: glucose [R_f-values, 0.12 (S_1), 0.24 (BTPW *n*-BuOH–toluene–water, 5:1:3:3), 0.34 PhOH satd. with H_2O, brown colour with aniline hydrogen phthalate reagent]; *p*-coumaric acid [R_f-values, 0.92 (S_1), 0.88 (BEW *n*-BuOH–EtOH–H_2O, 4:1:2.2), 0.43 (H_2O), mauve fluorescence/UV-light, UV λ_{max}(MeOH) 227, 310 nm]; scopoletin [R_f-values, 0.83 (S_1), 0.51 (S_2), 0.29 (H_2O), blue-violet fluorescence/UV-light, UV λ_{max}-(MeOH) 229, 253, 300, 346 nm], (Harborne, 1984). All other aglycones and free sugars were also identified by Comp-TLC and PC with authentic samples using the previous convenient solvent systems and specific spray reagents (e.g., vanilline–HCl, aniline hydrogen phthalate).

*3.3.1. Viburtioside A (**2**)*

Creamy-white amorphous powder. R_f-values: 0.74 (S_2), 0.56 (S_1). $[\alpha]_D = -59$ (MeOH, $c = 0.26$). UV λ_{max} nm ($\log\epsilon$): 225 (3.7), 297 (3.9), 309 (4.0). IR ν_{max} (KBr) cm^{-1}: 3405, 1733, 1700, 1630, 1600, 1515, 1240 and 830. HR ESI-MS (negative): m/z 707.6980 $[M-H]^-$ for $C_{34}H_{43}O_{16}$, ca. 707.7003. ^1H NMR (Acetone-d_6, 300 MHz) and ^{13}C NMR (Acetone-d_6, 75 MHz) were given in Table 1.

*3.3.2. Viburtinoside B (**3**)*

Creamy-white amorphous powder. R_f-values: 0.73 (S_2), 0.58 (S_1). $[\alpha]_D = -61$ (MeOH, $c = 0.23$). UV λ_{max}, nm ($\log\epsilon$): 226 (3.7), 299 (3.9), 311 (4.1). IR ν_{max}(KBr) cm^{-1}: 3400, 1735, 1705, 1625, 1600, 1510, 1235 and 835. HR ESI-MS (negative): m/z 707.6980 $[M-H]^-$ for $C_{34}H_{43}O_{16}$, ca. 707.7003. ^1H NMR (Acetone-d_6, 300 MHz) and ^{13}C NMR (Acetone-d_6, 75 MHz) were given in Table 1.

*3.3.3. Scopoletin 7-O-β-D-sophoroside (**4**)*

Off-white amorphous powder. R_f-values: 0.48 (S_1), 0.65 (S_2). UV spectral data λ_{max} nm: (MeOH): 338, 285, 256, 224. HR ESI-MS (negative): m/z 515.5421 $[M-H]^-$ for $C_{22}H_{27}O_{14}$, ca. 515.5444. ^1H NMR (DMSO-d_6, 300 MHz): δ ppm 7.96 (1H, *d*, $J = 9.3$ Hz, H-4), 7.30 (1H, *s*, H-5), 7.14 (1H, *s*, H-8), 6.31 (1H, *d*, $J = 9.3$ Hz, H-3), 5.28 (1H, *d*, $J = 7.8$ Hz, H-1′), 5.07 (1H, *d*, $J = 7.5$ Hz, H-1″), 3.81 (3H, *s*, O*Me*), 4.0–3.1 (m, remaining sugar protons hidden by solvent signal). ^{13}C NMR (DMSO-d_6, 75 MHz): δ ppm 161.20 (C-2), 150.59 (C-7), 149.59 (C-10), 146.69 (C-6), 144.89 (C-4), 113.96 (C-3), 112.97 (C-9), 110.46 (C-5), 103.60 (C-8), 103.72 (C-1″), 100.37 (C-1′), 81.25 (C-2′), 77.75 (C-5″), 77.45 (C-5′), 77.23 (C-3″), 74.13 (C-3′), 73.77 (C-2″), 71.00 (C-4″), 70.31 (C-4′), 61.79 (C-6″), 61.34 (C-6′), 56.78 (C-O*Me*).

*3.3.4. 2,6-Di-C-methyl-nicotinic acid 3,5-diethyl ester (**5**)*

Colourless needles. R_f-values: 0.45 (toluene). CI-MS: m/z: 252 (100%, $[M+H]^+$). HR ESI-MS (negative): m/z 250.2700 $[M-H]^-$ for $C_{13}H_{17}NO_4$, ca. 250.2723. ^1H NMR ($CDCl_3$, 300 MHz): δ ppm 8.66 (1H, *s*, H-4), 4.39 (4H, *q*, $J = 7.2$ Hz, 2× –*CH*$_2$CH$_3$), 2.84 (6H, *s*, 2× alkyl-CH$_3$), 1.41 (6H, *t*, $J = 7.2$ Hz, 2× –CH$_2$*CH*$_3$). ^{13}C NMR ($CDCl_3$, 75 MHz),: δ ppm 165.95 (2× –*CO*O–), 162.15 (C-2/6), 140.83 (C-4), 123.09 (C-3/5), 61.35 (2× –*CH*$_2$CH$_3$), 24.89 (2× alkyl-CH$_3$), 14.27 (2× –CH$_2$*CH*$_3$).

3.4. Animals

Adult male pathogen free albino rats weighing 150–180 g purchased from of the animal house of Faculty of Pharmacy-Helwan University, Cairo were used. The animals were housed in standard metal cages in an air-conditioned room at 22 ± 3 °C, 55 ± 5% humidity, 12 h light and provided with standard laboratory diet and water ad libitum.

3.5. Determination of median lethal dose (LD_{50})

LD_{50} was determined according to Weil, 1952. The toxic symptoms and mortality rate were recorded after 24 h of *i.p.* administration of the tested drug. After a pilot experiment was performed on four groups, six animals each, several doses were chosen at equal logarithmic intervals.

3.6. Design of experiments

Rats were divided into five groups; each of six, one group served as control and received normal saline (10 ml/kg, imp.). CCl_4 (2.5 ml/kg, 50% in olive oil) was given to the animals of other four groups by back subcutaneous injection. Tested extract was administered to the 2nd and 3rd groups' *i.p.* at two dose levels 25 and 50 mg/kg in normal saline, while the reference drug Silymarin (25 mg kg^{-1} as a 2.5 mg/ml soln in 1% Tween 80 *i.p.*) was administered to the 4th group. Treatments were given 2 before and 2, 24 and 48 h after CCl_4-administration. All animals were killed 72 h after CCl_4 administration. Blood was drawn from the retero-orbital plexus venous and the serum was separated for the different assays. The animal experiments were carried out according to the animal care regulation in Egypt.

3.7. Assay of serum ALT and AST activities

Serum ALT and AST were determined colorimetrically by using kits of BioMérieux, France (Reitman and Frankel, 1957).

3.8. Measurement of serum lipid peroxidation level

Lipid peroxidation was quantified by measurement of thiobarbituric acid-reactive substance by spectrophotometrically assay (Ohkawa et al., 1979).

3.9. Measurement of serum nitric oxide production

It was assayed by measuring the accumulation of nitrate by using microplate assay method based on Griess reaction (Green et al., 1982).

3.10. Statistical analysis

All data are expressed as mean ± s.e. and the statistical significance was evaluated by the Student's *t*-test (Sendecor and Cochran, 1971). The values were considered to be significantly different when P values were less than 0.05.

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