Isoflavonoid Glycosides and Rotenoids from *Pongamia pinnata* Leaves

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Chromatographic separation of a 70% aqueous methanol extract (AME) of *Pongamia pinnata* (Linn.) Pierre (Leguminosae) leaves has led to the isolation of two new isoflavonoid diglycosides, 4′-*O*-methyl-genistein 7-*O*-*β*-D-rutinoside (**2**) and 2′,5′-dimethoxy-genistein 7-*O*-*β*-D-apiofuranosyl-(1″→6″)-*O*-*β*-D-glucopyranoside (**6**), and a new rotenoid, 12a-hydroxy-*α*-toxicarol (**5**), together with nine known metabolites, vecinin-2 (**1**), kaempferol 3-*O*-*β*-D-rutinoside (**3**), rutin (**4**), vitexin (**7**), isoquercitrin (**8**), kaempferol 3-*O*-*β*-D-glucopyranoside (**9**), 11,12a-dihydroxy-munduserone (**10**), kaempferol (**11**), and quercetin (**12**). Their structures were elucidated on the basis of chemical and spectroscopic analyses.

Key words: Pongamia pinnata, Rotenoids, Methoxy-Isoflavonoid Diglycosides

Introduction

Pongamia pinnata (Linn.) Pierre (Leguminosae, about 700 genera, 17000 species) (Heywood, 1978) is commonly known as Bennet, Pongam, Karum tree, Poonga oil tree or Karanja syn. *Derris indica* Bennet, *Pongamia glabra* Vent., *Cytisus pinnatus* L., *Galedupa indica* (Lam). This Indomalaysian species is a medium-sized subevergreen tree, and common on alluvial and coastal situations from India to Fiji, from sea level to 1200 m, now found in Australia, Florida, Hawaii, India, Malaysia, Oceania, Philippines, and Seychelles (Allen and Allen, 1981). Different parts of *P. pinnata* have been used in traditional medicines for the treatment of tumours (Gandhidasan *et al*., 1987), skin diseases, wounds, ulcers (Tanaka *et al*., 1991), as antiparasitic, insecticide, emetics, anthelmintics, anti-inflammatory (Pathak *et al*., 1983), and for the treatment of bronchitis, whooping cough, rheumatic joints and to quench dipsia in diabetes (Kirtikar and Basu, 1995). Accordingly, considerable attention has been directed to the phytochemical examination of different plant parts, that led to the isolation of furanodiketones, furano-, chromeno- and prenylflavones and flavonols (Do Nascimento *et al*., 1976; Kirtikar and Basu, 1995). Several anti-dermatophyte-active isoflavones and anti-inflammatory and cancer chemo-preventive rotenoids have been also reported from *Derris scandens* (Sekine *et al*., 1999; Laupattarakasem *et al*., 2004) and *Derris trifoliata* (Ito *et al*., 2004). Recently, furanoflavonoid aglycones and glucosides have been reported from *P. pinnata* fruits, and antihyperglycemic (Yadav *et al*., 2004; Ahmed *et al*., 2004) and antilipid peroxidative effects of the ethanolic flowers extract have been studied (Punitha and Manoharan, 2006). The present report aims at the isolation and structure elucidation of further flavonoids from *P. pinnata* leaves.

Materials and Methods

Equipment

The NMR spectra were recorded on Varian Mercury VX-300 or JEOL GX 500 and GX-270 spectrometers. ^{1}H NMR spectra were run at 300 or 270 MHz and ^{13}C NMR spectra at 125 or 75.46 MHz in DMSO-d_6, $CDCl_3$ or $(CD_3)_2CO$. Chemical shifts are quoted in δ (ppm) and were relative to those of the solvents. ESI-MS analyses were run on a double focusing sector field Finnigan MAT 90 mass spectrometer (Finnigan, Bremen, Germany). UV analyses for pure samples were recorded, separately, as MeOH solutions and with different diagnostic UV shift reagents on a Shimadzu UV 240

(P/N 240–58000) spectrophotometer. For column chromatography, Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck, Darmstadt, Germany) and polyamide S (Fluka, St. Louis, Missouri, USA) were used. For paper chromatography (2D-PC and Co-PC) Whatmann No. 1 sheets (Whatmann Ltd., England) were used, while silica F_{254} and cellulose plates (20 × 20 cm, 0.2 mm thickness, Merck) were used for TLC.

Plant material

The fresh leaves of *P. pinnata* (Linn.) Pierre were collected in April 2002 from the Zoo Botanical Garden, Giza, Egypt. The late Dr. Nabil El-Hadidi, Professor of Botany, Faculty of Science, Cairo University, Cairo, Egypt, performed the identification of the plant. A voucher sample is kept in the Pharmacognosy Department, Faculty of Pharmacy, Al-Azhar University (Girls), Nasr City, Egypt.

Extraction and isolation

Powdered air-dried leaves of *P. pinnata* (1.5 kg) were preliminary extracted with hot $CHCl_3$ (3 × 5 L, under reflux, 50 °C) to give 50 g dry $CHCl_3$ extract and the marc was then treated with hot 70% MeOH (6 × 4 L, under reflux, 70 °C). The dry extract was desalted with hot MeOH (3 × 2 L, under reflux, 50 °C), affording a dry MeOH-soluble portion (240 g). The dry extract (150 g in H_2O) was fractionated on a polyamide column (10 × 110 cm, 300 g) eluted with a step gradient: H_2O, H_2O/MeOH mixtures up to pure MeOH. Based on comparative paper chromatography (Co-PC) with the use of UV light, 1% $FeCl_3$, or naturstoff spray reagents for detection, the individual 64 fractions (each 1 L) were pooled in 8 collective fractions (I–VIII). Fraction I (H_2O, 10 g) was phenolic-free (two-dimensional paper chromatography, 2D-PC, $FeCl_3$ spray reagent), while dry fraction II (10–20% MeOH, 290 mg) was precipitated with excess MeOH from its conc. aqueous solution to get red off the free sugars and salts. The concentrated filtrate was fractionated on cellulose with 40% EtOH as an eluent, followed by a Sephadex column using BIW (*n*-BuOH/2-propanol/H_2O, 4:1:5 v/v/v, organic layer) to afford pure **1** (17 mg) and **2** (8 mg). Fraction III (30% MeOH, 1.1 g) was precipitated as in case of fraction II, then the filtrate was subjected to CC on cellulose (20–40% EtOH) followed by Sephadex (BIW), and each of the two major subfractions were separately purified on Sephadex, also with BIW, to give pure **3** (47 mg) and **4** (60 mg). The dry powder of fraction IV (40% MeOH, 230 mg) was treated with diethyl ether (1 × 50 mL, under reflux, 30 °C) to give pure **5** (50 mg) and a residue, which was fractionated on Sephadex twice with 50% EtOH and then with EtOH to yield pure **6** (17 mg). Fraction V (50% MeOH, 280 mg) was twice applied on a Sephadex column using BIW as eluent and finally purified on Sephadex with EtOH affording pure **7** (28 mg). The dry fraction VI (60–65% MeOH, 1.9 g) was preliminary mixed with hot diethyl ether (75 mL, under reflux, 30 °C, 15 min) to remove the non-phenolic impurities, and the residue, thus obtained, was isolated on a cellulose column with BIW as eluent to give **8** (43 mg) and **9** (32 mg). The dry sample of fraction VII (70–80%, 174 mg) was dissolved in EtOH and left for spontaneous precipitation and the precipitate was then recrystallized from EtOH to give yellow needles of **10** (29 mg). Fraction VIII (95% EtOH, 240 mg) was twice chromatographed on a Sephadex column using BIW as an eluent for the first column and EtOH for the second one, giving pure **11** (12 mg) and **12** (7 mg). All separation processes were followed by Co-TLC with the solvent systems: MeOH/$CHCl_3$ (2:8 v/v), EtOAc/$CHCl_3$ (7:3 v/v), MeOH/EtOAc/$CHCl_3$/H_2O (35:32:28:7 v/v/v/v) and *n*-BuOH/MeOH/H_2O (4:1:1 v/v/v) or 2D-PC and Co-PC with S_1: *n*-BuOH/HOAc/H_2O (4:1:5 v/v/v, top layer), and S_2: 15% HOAc.

*4′-O-Methyl-genistein 7-O-β-D-rutinoside (**2**)*: Yellowish-white amorphous powder. – R_f-values: 0.27 (S_1), 0.90 (S_2). – Under UV-light, it appeared as pink-purple spot, turned to light green colour with $FeCl_3$, and showed a weak and unclear change with ammonia vapours and naturstoff spray reagent on the paper chromatogram. – UV (MeOH): λ_{max} = 261, 293sh; (+NaOMe) 264, 295sh; (+NaOAc) 260, 292sh; (+NaOAc/H_3BO_3) 260, 292sh; (+$AlCl_3$) 262, 295sh; (+$AlCl_3$/HCl) 260, 295sh nm. – ^{1}H NMR (300 MHz, DMSO-d_6): δ_H = 8.23 (1H, s, H-2), 7.66 (2H, d, J = 9 Hz, H-2′/6′), 7.00 (2H, d, J = 9 Hz, H-3′/5′), 6.72 (1H, d, J = 2.4 Hz, H-8), 6.47 (1H, d, J = 2.4 Hz, H-6), 5.02 (1H, d, J = 7.5 Hz, H-1″), 4.36 (1H, br s, H-1‴), 3.72 (3H, s, OCH_3-4′), 3.70–3.10 (10H, m, remaining rutinoside protons), 0.99 (3H, d, J = 6.3 Hz, CH_3-6‴).

Table I. 1D and 2D NMR spectral data of **5** ($CDCl_3$, 500 MHz) and **10** (DMSO-d_6, 300 MHz).

No.	**5**				**10**	
	δ_H m (*J* in Hz)	δ_C	^{1}H,^{1}H-COSY	HMBC	δ_H m (*J* in Hz)	δ_C
1	6.70 s	109.44	–	2,3,4a,12a,12b	6.72 s	109.14
2	–	143.99	–		–	143.81
3	–	151.75	–		–	151.74
4	6.45 s	101.19	–	2,3,4a,12b	6.52 s	101.43
4a	–	148.93	–		–	149.01
6*α*	4.57 dd (12.3, 1.6)	63.72	6*β*,6a	12a,6a,4a	4.47 dd (12.3, 2.1)	63.96
β	4.44 d (12.3)		6*α*,6a		4.35 d (12.3)	
6a	4.52 br s	75.71	6*α*,6*β*	6,12a,12b	4.68 br s	76.14
7a	–	155.57	–	–	–	165.01
8	–	102.10	–		6.06 d (2.1)	95.89
9	–	163.58	–	–	–	168.64
10	5.94 s	97.99	–	8,9,11,11a	6.00 d (2.1)	94.35
11	–	164.09	–		–	164.28
11a	–	100.05	–		–	101.88
12	–	195.10	–		–	196.96
12a	–	66.90	–		–	67.43
12b	–	109.44	–		–	112.18
14	6.49 d (9.9)	115.18	15	7a,8,9,15,16,	–	–
15	5.46 d (9.9)	126.74	14	8,14,16,17,18	–	–
16	–	78.70	–	–	–	–
17	1.35 s	28.45	–	15,16,18	–	–
18	1.41 s	28.69	–	15,16,17	–	–
OMe-2	3.71 s	56.47	–	2	3.75 s	56.94
OMe-9	–	–	–	–	3.72	56.66
OMe-3	3.78 s	56.36	–	3	3.60 s	56.34
OH-11	11.66 s	–	–	11	11.98 s	–

*12a-Hydroxy-α-toxicarol (**5**)*: Creamy-white amorphous powder. – R_f-values: 0.70 (S_1), 0.59 (S_2). – Under short UV-light, it appeared as pink-purple spot, did not change under ammonia vapours or naturstoff spray reagent, but turned to brown colour with $FeCl_3$ spray reagent on the paper chromatogram. – UV (MeOH): λ_{max} = 273, 296sh, 319 nm. – ^{1}H NMR (500 MHz, $CDCl_3$): see Table I. – ^{13}C NMR (125 MHz, $CDCl_3$): see Table I. – Positive ESI-MS: *m/z* = 449.3 $[M+Na]^+$.

*2′,5′-Dimethoxy-genistein 7-O-β-D-apiofuranosyl-(1‴→6″)-O-β-D-glucopyranoside (**6**)*: Yellowish-white amorphous powder. – R_f-values: 0.27 (S_1), 0.90 (S_2). – Under UV-light, it appeared as purple spot, turned to brownish-yellow with naturstoff spray reagent and pale-green with $FeCl_3$ spray reagent on the paper chromatogram. – UV (MeOH): λ_{max} = 230, 260, 290, 326sh; (+NaOMe) 295sh, 380sh; (+NaOAc) 260, 288, 326sh; (+NaOAc/H_3BO_3) 260, 289, 326; (+$AlCl_3$) 270, 297, 389sh; (+$AlCl_3$/HCl) 270, 299, 388sh nm. – ^{1}H NMR (300 MHz, DMSO-d_6): δ_H = 8.21 (1H, s, H-2), 6.88 (1H, s, H-6′), 6.72 (1H, d, *J* = 2.1 Hz, H-8), 6.59 (1H, s, H-3′), 6.47 (1H, d, *J* = 2.1 Hz, H-6), 5.02 (1H, d, *J* = 7.2 Hz, H-1″), 4.82 (1H, d, *J* = 3 Hz, H-1‴), 3.95–3.10 (11H, m, remaining sugar protons). – ^{13}C NMR (75.46 MHz, DMSO-d_6): δ_C = 180.00 (C-4), 162.90 (C-5), 161.50 (C-7), 157.30 (C-9), 155.96 (C-2), 152.11 (C-2′), 148.30 (C-5′), 148.02 (C-4′), 120.63 (C-3), 116.67 (C-6′), 109.49 (C-1‴)*, 109.08 (C-1′)*, 106.09 (C-10), 101.96 (C-1″), 99.93 (C-6), 99.64 (C-3′), 94.68 (C-8), 78.73 (C-3‴), 76.46 (C-2‴), 76.02 (C-3″), 75.64 (C-5″), 73.40 (C-4‴), 73.10 (C-2″), 70.00 (C-4″), 67.80 (C-6″), 63.40 (C-5‴), 56.80, 56.10 (2 X OCH_3) (* exchangeable signals). – Negative ESI-MS (at tl-voltage of 120 V): *m/z* = 623.2 $[M-H]^-$, 489.2 [M–deoxyapiosyl–2H]$^-$, 329.1 [M–deoxyapiosylglucoside–H]$^-$, 299.1 [aglycone–2 × Me–2H]$^-$.

Results

Two new methoxy-isoflavone di-*O*-glycosides, **2**, and **6**, and a new rotenoid, **5**, have been isolated among twelve flavonoidal metabolites from the aqueous methanol extract of *Pongamia pinnata* (Linn.) Pierre (Leguminosae) leaves by consecu-

tive column chromatographic separations. The structures of the known metabolites were established as vecinin-2 (**1**), kaempferol 3-*O*-β-D-rutinoside (**3**), rutin (**4**), vitexin (**7**), isoquercitrin (**8**), kaempferol 3-*O*-β-D-glucopyranoside (**9**), 11,12a-dihydroxy-munduserone (**10**), kaempferol (**11**), and quercetin (**12**) on the basis of chemical and physicochemical analyses as well as comparison with published data (Agrawal and Bansal, 1989; Markham and Geiger, 1994; Laupattarakasem *et al.*, 2004; Ito *et al.*, 2004; Silva and Parente, 2002; Rao *et al.*, 1991; Lin and Kuo, 1993; Hui Wang *et al.*, 1997; Sekine *et al.*, 1999).

Discussion

Compound **2** showed the chromatographic behaviour and UV spectral data of a 5,7,4′-oxygenated isoflavone 7-*O*-glycoside-like structure (Mabry *et al.*, 1970). On complete acid hydrolysis, it gave glucose and rhamnose in the aqueous phase (Co-PC with authentic samples). Negative ESI-MS of **2** exhibited a pseudo-molecular ion peak at 607.1 $[M-H]^-$, corresponding to a methoxy-isoflavone 7-*O*-rhamnosylglucoside. The ^{1}H NMR spectrum showed in the aromatic region an A_2X_2-spin coupling system of two *ortho* doublets at δ 7.66 and 7.00 (J = 9 Hz), two protons each, characteristic for H-2′/6′ and H-3′/5′ of a 1,4-disubstituted B-ring. The relative downfield location of H-3′/5′ ($\Delta \sim +$ 0.2 ppm) was indicative to a 4′-*O*-methoxy function, which was assigned as a singlet at 3.72. Additionally, an intrinsic 1H singlet at 8.28 of H-2 further confirmed the isoflavone identity of the aglycone. Also, the downfield location ($\Delta \sim +$ 0.25 ppm) of the two *meta* doublets, 1H each, of H-6 and H-8 at 6.47 and 6.72 (J = 2.4 Hz) together with the β-anomeric doublet at 5.02 (J = 7.5 Hz) were two evidences for the attachment of a β-glucopyranoside moiety to OH-7 (Markham and Geiger, 1994). The characteristic anomeric proton at 4.36 (br s) with the doublet at 0.99 (J = 6.3 Hz, CH_3-6‴) assigned for a terminal α-rhamnopyranosyl on OH-6″ of the glucoside moiety. Therefore, on comparison of the complete ^{1}H NMR data of **2** with those of 4′-demethylated ether (4′-free OH) and 5-deoxy (derriscanoside A) analogues (Laupattarakasem *et al.*, 2004; Sekine *et al.*, 1999), **2** was identified as 4′-*O*-methyl-genistein 7-*O*-β-D-rutinoside (Fig. 1).

Compound **5** exhibited more or less the same chromatographic behaviour and UV spectral data like **10** (Fig. 1). The positive ESI-MS spectrum showed a pseudo-adduct molecular ion peak at *m/z* 449.3 assigned to $[M+Na]^+$ and corresponding to a M_r of 426 with C_4H_4 or 52 mu (extra chromene group) more than that of **10**. On comparison with **10**, the ^{1}H NMR spectrum of **5** showed two differences in the aromatic region; the first one was the disappearance of the H-8 resonance, and the second difference was the appearance of the characteristic resonances of a chromene group as an AX-spin coupling system of two *endo Z*-olefinic protons at 6.49 and 5.46 (J = 9.9 Hz) assigned to H-14 and H-15 (Table I, Fig. 1) and the two geminal methyl group (Me-17 and 18) resonances as two singlets at their typical δ-values of 1.35 and 1.41, respectively (Lin and Kuo, 1993; Rao *et al.*, 1991; Hui Wang *et al.*, 1997; Sekine *et al.*, 1999). This was diagnostic for the location of the chromene group at C-8 and C-9. Furthermore, two singlets, three protons each, have been assigned at 3.71 and 3.78 for only two methoxy groups instead of three ones in case of **10** (Table I, Fig. 1). The presence of the chromene group was further confirmed from the ^{13}C NMR spectrum that showed the characteristic five resonances for C-14 to C-18 at 115.18, 126.74, 78.70, 28.45 and 28.69, respectively (Lin and Kuo, 1993; Rao *et al.*, 1991; Hui Wang *et al.*, 1997; Sekine *et al.*, 1999). The attachment of this group to C-8 and C-9 was also proved due to the downfield shift of C-8 to 102.10 (Δ + 6.21 ppm) and the upfield shift of C-9 to 163.58 (Δ– 5.06 ppm) relative to those of **10** (Table I). The characteristic J-values of the geminal CH_2-6 protons with H-6a were an evidence for the β-configuration of the second one. All assigned ^{1}H- and ^{13}C-resonances were confirmed by ^{1}H,^{1}H-COSY and HMQC experiments through the vicinal correlation cross-peaks in the fist spectrum and the direct ^1J-connectivities in the second spectrum. The HMBC spectrum of **5** (Table I) indicated a cross-peak connected between the hydrogen-bound OH-singlet at δ_H 11.66 and C-11 at δ_C 164.09 to confirm the attachment of this OH-group to C-11. Also, it exhibited long-range peaks between H-14 (δ_H 6.49) and C-7a, C-8 and C-9 and between H-15 (δ_H 5.46) and C-8, proving the position of the chromene group at C-8 and C-9. Similarly, the differentiation between the position of the two methoxy groups was achieved through the two three-bond correlation peaks between Me-2 (δ_H 3.71) and C-2 (δ_C 143.99) and Me-3 (δ_H 3.78) and C-3 (δ_C 151.75). Moreover, this was further confirmed

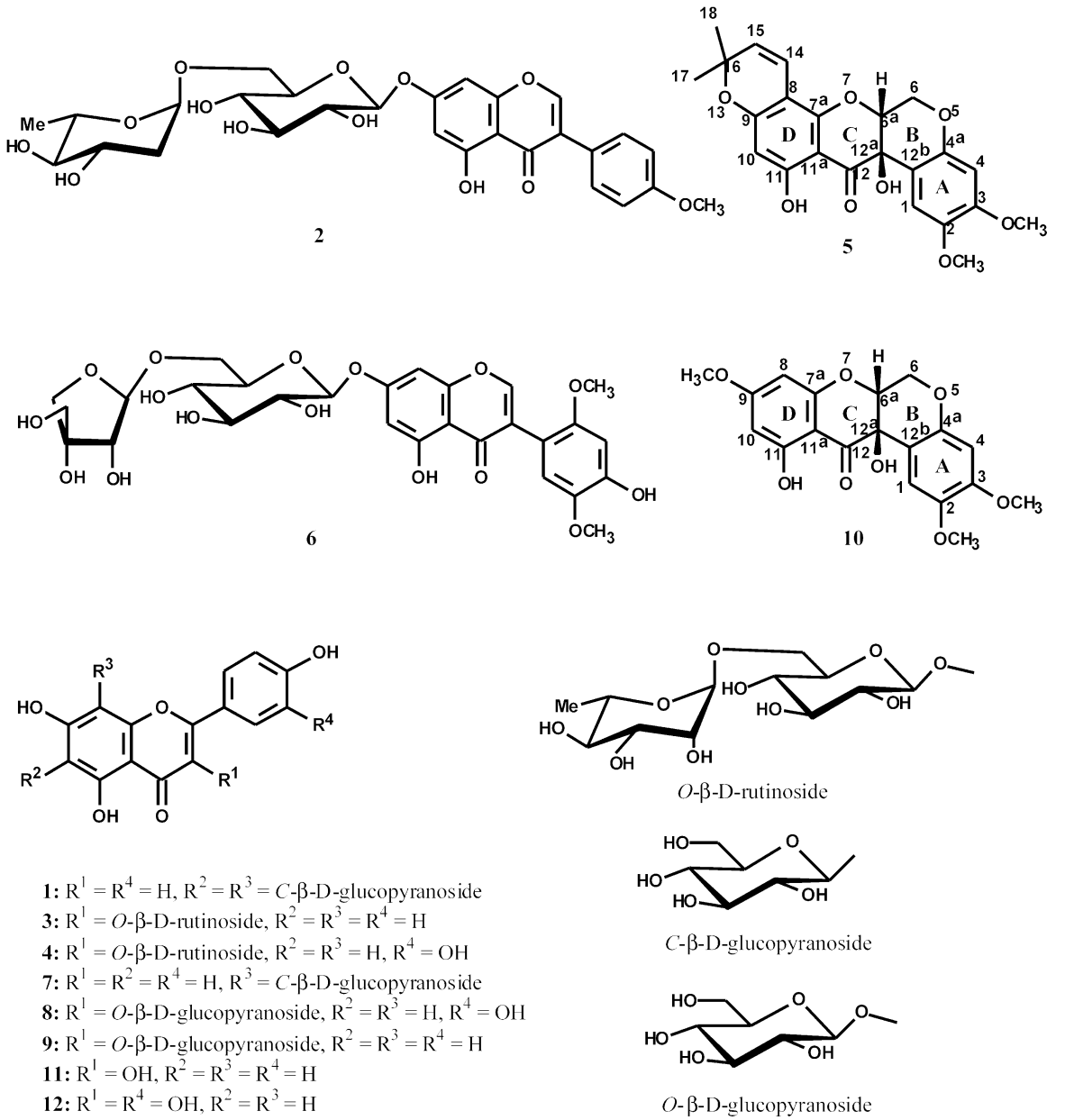

Fig. 1. Chemical structures of the isolates **1**–**12** from *P. pinnata* leaves.

by the correlations of H-1 (δ_H 6.70) with C-2, C-3, C-4a, C-12a and C-12b and H-4 (δ_H 6.45) with C-2, C-3, C-4a and C-12b. The remaining C-resonances were finally established by the comparison with those of **10** and other structural related compounds (Ito *et al.*, 2004; Silva and Parente, 2002; Agrawal and Bansal, 1989) and the full analysis of the 2D NMR spectra of **5** (Table I). Thus, **5** was identified as 12a-hydroxy-α-toxicarol or 11-hydroxy-tephrosin (Fig. 1).

Compound **6** showed chromatographic properties and UV spectral data of an isoflavone 7-*O*-glycoside (Mabry *et al.*, 1970). The presence of free 4′-OH was deduced from the strong bathochromic

shift accompanied with an increase in the intensity of the shoulder at λ_{max} 326 nm (MeOH UV spectrum) to 380 (NaOMe UV spectrum), while a free OH-5 was expected from a total characteristic bathochromic shift in the $AlCl_3$ UV spectrum, that remained on addition of HCl (Mabry *et al*., 1970). On addition of NaOAc or NaOAc/H_3BO_3, **6** exhibited more or less the same UV spectrum as in MeOH, confirming the substitution of 7-OH and expecting a polysubstituted B-ring. Based on Co-PC, sugar moieties were identified in this compound like apiose and glucose linked as *O*-glycosides due to their detection in the aqueous phase against authentic sugar samples. Negative ESI-mass spectrum showed a molecular ion peak at *m/z* 623.2 $[M-H]^-$ together with three fragment ions at 489.2, 329.1 and 299.1 corresponding to the oxidative loss of apiosyl, apiosylglucoside and two methoxy groups confirming the main units of the total molecule of **6**. ^{1}H NMR spectrum showed a characteristic singlet signal at 8.21 for H-2 of an isoflavone structure. Additionally, two singlets (1H each) were assignable to H-6′ and H-3′ at 6.88 and 6.59 for a 1,2,4,5-tetra-substituted B-ring of isoflavone. Location of the two *meta* doublets at 6.72 (H-8) and 6.47 (H-6), relatively downfield by about 0.25 ppm, was an evidence for the *O*-glycosidation at OH-7, which was further confirmed by the typical position of the β-anomeric proton doublet at 5.02 (J = 7.2 Hz, H-1″) (Markham and Geiger, 1994) of a glucopyranoside residue. A connection of a terminal β-apiofuranosyl moiety to the glucoside was also deduced from its anomeric signal at 4.82 (J = 3 Hz, H-1‴). On the basis of the above-mentioned documents and the assignment of two singlets (each of three protons of methoxy groups at 3.64 and 3.73) **6** was tentatively identified as 5,4′-dihydroxy-2′,5′-dimethoxy-isoflavone 7-*O*-apiofuranosyl-glucopyranoside (Markham and Geiger, 1994). ^{13}C NMR spectrum exhibited 15 signals of the aglycone and 11 of the diglycoside moiety identified by comparison with published data of similar structures (Agrawal and Bansal, 1989; Ahmed *et al*., 2000). As a conclusion for the application of α/β-substituent additive rules on the ^{13}C-resonances of analogous compounds containing a 3,4-dihydroxy-B-ring (Agrawal and Bansal, 1989), the two methoxy groups (δ_C 56.10 and 56.80) should be attached to C-2′ and C-5′ according to the characteristic downfield shifts of C-2′ to 152.11 ($\Delta \sim +30$ ppm) and C-5′ to 148.30 ($\Delta \sim +4$ ppm) accompanied with the upfield shift of C-1′ to 109.08 ($\Delta \sim +10$ ppm). Characteristic signals were established for C-1‴ (~ 109), C-4‴ (73.4) and C-5‴ (63.4) of the β-apiosyl and interpreted to connect to OH-6″-glucose due to the downfield location of C-6″ at 67.80 ($\Delta \sim +7$ ppm). Thus, **6** was finally identified as 2′,5′-dimethoxy-genistein 7-*O*-β-D-apiofuranosyl-(1‴→6″)-*O*-β-D-glucopyranoside (Fig. 1).

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