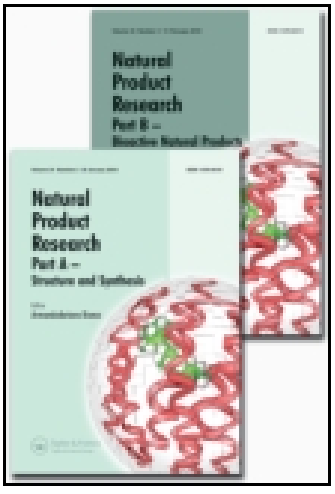

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A new flavonol glycoside and biological activities of Adenanthera pavonina L. leaves

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A new flavonol glycoside and biological activities of *Adenanthera pavonina* L. leaves

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Adenanthera pavonina is a plant belonging to family Fabaceae. The 95% ethanol extract (EtOH) of the dried powdered leaves of the plant and successive extracts with solvents of increasing polarities were prepared. Fractionation of the successive aqueous EtOH extract on polyamide column and purification of the isolated compounds on Sephadex LH20 led to the isolation of a new methoxy flavonol glycoside named as quercetin 3-*O*-α-dirhamnopyranosyl-(1‴ → 2″,1⁗ → 6″)-β-glucopyranoside-4′-methoxy (**1**), as well as kaempferol-3-*O*-α-dirhamnopyranosyl-(1‴ → 2″,1⁗ → 6″)-β-glucopyranoside (**2**), isovitexin (**3**), quercetin-3-*O*-rhamnopyranosyl(1‴ → 4″)-β-glucopyranoside (**4**), quercetin-3-*O*-β-glucopranoside-4′-*O*-rhamnopyranoside (**5**), kaempferol-3-*O*-α-rhamnopyranosyl(1‴ → 2″)-β-glucopyranoside (**6**), quercetin-3-*O*-rhamnopyranosyl (1‴ → 2″)-β-glucopyranoside (**7**), quercetin-3-*O*-β-glucopyranoside (**8**), kaempferol (**9**) and quercetin (**10**). Structures of the isolated compounds were established by spectroscopic analysis. Antioxidant activities of EtOH extract, successive extracts and compounds **1** and **2** were evaluated. The ethyl acetate (EtOAc) extract and EtOH extract showed 62.67% and 49.30% free radical scavenging activity, respectively. Cytotoxic activities of the EtOH extract and compounds (**1**) and (**2**) were evaluated. The EtOH extract showed a significant cytotoxic activity against Hep G-2 ($IC_{50} = 2.50\ \mu g$) as compared with cisplatin ($IC_{50} > 10\ \mu g$).

Keywords: *Adenanthera pavonina*; methoxy flavanol glycoside; antioxidant; cytotoxic activity

1. Introduction

Adenanthera pavonina L. is a plant belonging to family Fabaceae, its bark and leaves are used in Indian folk medicine as astringent, anthelmentic and aphrodisiac. The bark is also used in colonorrhea, ulcers, pharngopathy, gout and rheumatism (Vaidyaratnam 1994). The roots were reported to be emetic (Khare 2004). The seeds afforded a better protection against diabetes, hyperlipidemia (Krishnaveni et al. 2011) and had a blood pressure lowering effect (Adedapo et al. 2009). It was reported that the seeds and the leaves possessed a potent anti-inflammatory activity (Olajide et al. 2004; Ara et al. 2010; Abou Zeid et al. 2012; Jayakumari et al. 2012). Previous chemical literature survey of *A. pavonina* L. revealed the presence of triterpenes, sterols, hydrocarbons, fatty acids (Nigam et al. 1973; Yadav et al. 1976; Chandra et al. 1982; Sotheeswaran et al. 1994; Ahmad et al. 2002; Zarnowski et al. 2004; Enuo et al. 2007; Abou Zeid

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et al. 2012), flavonoids (Gennaro & Nasini 1972; Devi et al. 2007) and five-membered lactone ring, pavonin (Ali et al. 2005).

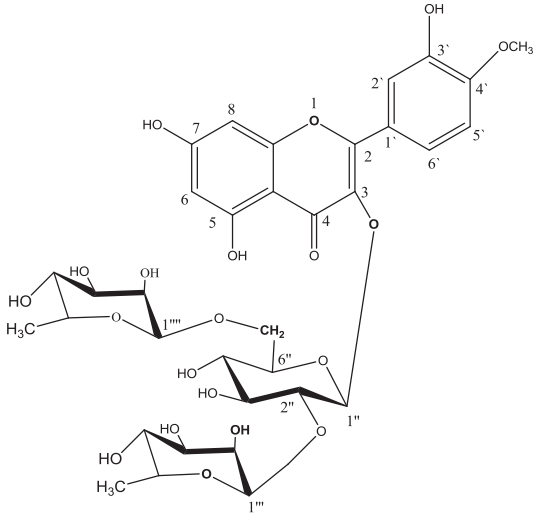
2. Results and discussion

2.1. *Identification of the isolated compounds*

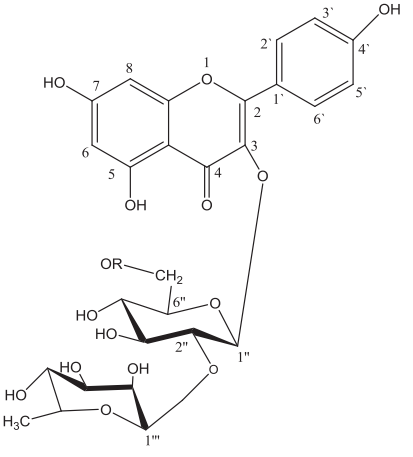
Phytochemical investigation of the defatted aqueous EtOH extract (DFAE) of *A. pavonina* L. leaves afforded 10 flavonoids (**1**–**10**). Compound **1** was isolated as yellow amorphous powder. The negative ESI-MS showed a molecular ion peak at *m/z* 769.73 $[M - H]^-$ with a molecular formula $C_{34}H_{42}O_{20}$. The UV spectral data using various shift reagents indicated a flavonol skeleton with free hydroxyl groups at C-5 and C-7, while those at C-3 and C-4′ are substituted (Mabry et al. 1970; Markham 1982). Complete acid hydrolysis (2 N HCl, 1 h, 100°C) yielded glucose and rhamnose as the sugar moieties (co-paper chromatography) and quercetin-4′methoxy [ESI-MS and UV]. ^{1}H NMR spectrum of **1** confirmed the presence of quercetin. It showed the three aromatic protons of ring B at δ 7.65 (dd, $J = 8.4$,2.3 Hz, H-6′), δ 7.74 (d, $J = 8.4$ Hz, H-2′) and δ 6.94 (d, $J = 8.4$ Hz, H-5′) with a down field shift of H-5′, indicating the substitution of the hydroxyl group at C-4′ (Ibrahim et al. 2012), two doublets at δ 6.32 and 6.11 ($J = 2.3$ Hz) assigned to H-8 and H-6, respectively. The sugar region showed three anomeric protons, each is doublet at δ 5.55 ($J = 7.65$ Hz, H-1″) corresponding to β-glucopyranose, and two doublets at δ 4.99 and δ 4.35 ($J = 1.5$ Hz) corresponding to H-1‴ and H-1⁗ of two α rhamnopryanose units. A singlet at δ 3.81 was assigned to three protons of the methoxy groups.^{13}C NMR spectrum of **1** revealed the presence of 18 carbon resonances in the aliphatic region which were assigned to glucose attached to two rhamnose moieties, among which the most downfield signals at δ 100.59, δ 100.02 and δ 99.06 ppm were assigned to the three anomeric carbons C-1″, C-1‴ and C-1⁗, respectively. Furthermore, the attachment of the two α-rhamnosyl moieties was confirmed by the downfield shift of C-2″ (δ 75.00) and C-6″ (δ 63.07) on the glucose moiety. HMBC correlation was between δ 5.55 (H-1″, glucose) and δ 133.12 (C-3), δ H 4.99 (H-1‴, rhamnose) and δ 75.00 (C-2″, glucose) and correlation between δ H-1⁗, rhamnose and δ 63.07(C-6″, glucose), these correlations revealed that glucose is substituting position 3 of the flavanol moiety and is substituted by two rhamnose moieties at C-2″ and C-6″. The position of OCH_3 group was assigned to C-4′ on the basis of HMBC correlation between OCH_3 protons signal at δ 3.81 and C-4′ at δ 149.89. From the above data, compound **1** is identified as quercetin 3-*O*-α-dirhamnopyranosyl-(1‴ → 2″,1⁗ → 6″)-β-glucopyranoside-4′-methoxy. This is the first report of isolation of this compound from natural source (SCI-Finder 2011). This new compound is considered to be the methoxylated derivative of quercetin glycoside that was previously isolated from *Gaura longiflora* (Xu et al. 2009). Compounds **2**–**10** were isolated for the first time from the investigated plant, their structure elucidation was carried out through R_f-values, colour reactions, chemical investigations (complete and mild acid hydrolysis) and spectral investigations (UV, NMR and MS) (Mabry et al. 1970; Markham 1982; Agrawal 1989). Spectral data of the known flavonoids were in good accordance with those previously published (Agrawal 1989; Markham & Geiger 1994). They were identified as kaempferol-3-*O*-α-dirhamnopyranosyl-(1‴ → 2″,1⁗ → 6″)-β-glucopyranoside (**2**), isovitexin (**3**), quercetin-3-*O*-rhamnopyranosyl(1‴ → 4″)-β-glucopyranoside (**4**), quercetin-3-*O*-β-glucopranoside-4′-*O*-rhamnopyranoside (**5**), kaempferol-3-*O*-α-rhamnopyranosyl(1‴ → 2″)-β-glucopyranoside (**6**), quercetin-3-*O*-rhamnopyranosyl(1‴ → 2″)-β-glucopyranoside (**7**), quercetin-3-*O*-β-glucopyranoside (**8**), kaempferol (**9**) and quercetin (**10**) (Figure 1).

2.2. *Antioxidant activity*

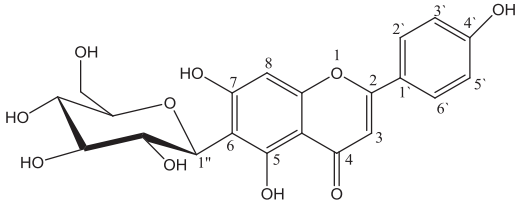
The EtOH extract of *A. pavonina* leaves was subjected to LD_{50} determination in a previous work (Abou Zeid et al. 2012). It was found to be 5.8 g/kg bwt. *In vivo* antioxidant activity of the DFAE



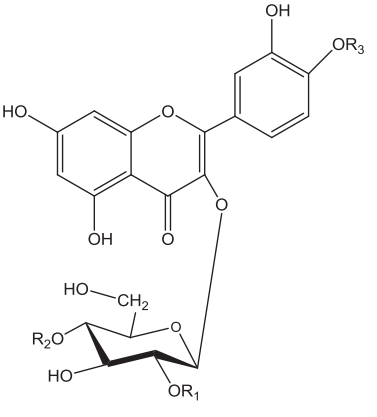
1



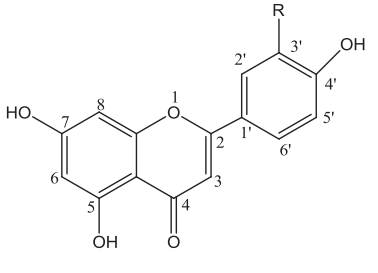
2: R=α–Rhamnopyranose
6: R=H



3



4: R1=R3=H, R2= α–Rhamnopyranose
5: R1=R2=H, R3= α–Rhamnopyranose
7: R2=R3=H, R1= α–Rhamnopyranose
8: R1=R2=R3=H



9: R = H
10: R=OH

Figure 1. Chemical structure of compounds **1**–**10**.

and successive extracts of *A. pavonina* L. leaves (Table 1) were evaluated as indicated by the increase in glutathione level as compared with diabetic control. The result showed that the EtOAc extract exhibited the highest free radical scavenging activity (95.78% potency) followed by the EtOH extract at 100 mg/kg bwt (75.35% potency) and chloroform extract (ChE) (60.57% potency) as compared with the standard antioxidant (vitamin E) (100% potency). *In vitro* antioxidant activity of **1** and **2** revealed that they have no antioxidant activity. This result is explained by what was reported about the structure–activity relationships. It was reported that the differences in antioxidant activity between polyhydroxylated and polymethoxylated

Table 1. *In vivo* antioxidant activity of EtOH extract and successive extracts of *A. pavonina* L. leaves.

Group (dose mg/kg bwt)	Blood glutathione (mg%) (mean ± SE)	Change from diabetic control (%)	Potency (%)
Control (1 ml saline)	36.3 ± 1.4	–	–
Diabetic control	21.7 ± 0.3	–	–
Diabetic + EtOH (50)	29.1 ± 0.6*	34.10	52.11
Diabetic + EtOH (100)	32.4 ± 0.8*	49.30	75.35
Diabetic + PE (100)	29.8 ± 0.6*	37.33	57.05
Diabetic + Ch (100)	30.3 ± 0.9*	39.63	60.57
Diabetic + EtOAc (100)	35.3 ± 0.7*	62.67	95.78
Diabetic + DFAE (100)	29.2 ± 0.5*	34.56	52.82
Diabetic + vitamin E (7.5)	35.9 ± 0.9*	65.43	100

Significantly different from the diabetic control at $p < 0.01$.

flavonoids are most likely due to differences in both hydrophobicity and molecular planarity. Suppression of antioxidant activity by *O*-methylation may reflect steric effects that perturb planarity. Also aglycones are more potent antioxidants than their corresponding glycosides (Heim et al. 2002). Presence of free 3′,4′-dihydroxy group in the B ring as in quercetin increases the antioxidant activity as compared with the free mono-hydroxy as in kaempferol (Williamson et al. 1996). The inactivity of **1** and **2** may be due to the presence of three sugar moieties, in addition to the presence of the methoxy group in compound **1**.

2.3. *Cytotoxic activity*

The EtOH extract showed a potent cytotoxic activity against HepG2 ($IC_{50} = 2.50$ μg/ml) as compared with cisplatin ($IC_{50} > 10$ μg/ml) (Table 2), moderate effects towards MCF7 (40% of dead cells), HeLa (37%), HEp-2 (44%) and HCT116 cancer lines (45%) at 10 μg/ml of the extract. Compound **1** caused moderate cytotoxic activity on MCF7 and HepG2cell lines (59.7% and 47.16% death, respectively) and a weak cytotoxic activity on HCT116 cancer cells (23.7% death), while compound **2** caused death to 39.7% of MCF7cancer cells and no cytotoxic activity against HCT116 and HepG2 cancer cell lines at 100 μg/ml (Table 3).

Table 2. Cytotoxic activity of EtOH extract of *A. pavonina* L. leaves.

	Dead cells (%)				
	Cell lines				
Concentration (μg/ml)	Hep G2	MCF7	HeLa	HEp-2	HCT116
EtOH extract					
1	38	8	5	16	12
2.5	50	27	22	37	25
5	42	44	0	46	37
10	33	40	37	44	45
Cisplatin					
1	40	89	10	80	75
2.5	41	87	58	77	70
5	48	87	83	69	67
10	48	86	94	73	63

Table 3. Cytotoxic activity of compounds **1** and **2**.

	Dead cells (%)		
	Cell lines		
Concentration (μg/ml)	Hep G2	MCF7	HCT116
Compound **1**			
12.5	0	9.4	0
25	7.2	25.2	0
50	19.5	37.6	13.6
100	47.16	59.7	23.7
Compound **2**			
12.5	0	5.9	0
25	0	10.5	0
50	0	21.02	0
100	5.3	39.7	0

3. Experimental

3.1. *General*

The structure of the compounds was identified by spectroscopic methods including: ultraviolet and visible absorption spectrometer (UV–VIS, Labomed Inc., USA) for measuring UV spectral data of the isolated compounds, in the range of 200–500 nm in methanol and with different diagnostic shift reagents. Nuclear magnetic resonance spectrophotometer (NMR, JEOL EX, 500 MHz for determination of ^{1}H NMR and 125 MHz for determination of ^{13}C NMR), while ESI-MS was measured on LCQ Advanced Thermo Finnigan, San Gose, CA, USA. Column chromatography was carried out on polyamide 6S (Riedel-De-Haen AG, Seelze Haen AG, D-30926 Seelze Hanver, Germany) and Sephadex LH-20 (Pharmazia, Sweden). Paper chromatography (PC, descending) Whatman No. 1 and 3 mm papers, using solvent systems 15% HOAc (H_2O–HOAc, 85:15), BAW (*n*-BuOH–HOAc–H_2O, 4:1:5, upper layer). Complete acid hydrolysis for *O*-glycosides(1,2,4,5,6,7,8) was carried out and followed by co-chromatograph with authentic samples to identify the aglycone and sugar moieties, the sugar unit of **3** was determined using ferric chloride degradation (Mabry et al. 1970). Source of solvents used for plant extraction: SDFCL (Industrial Estate, 248 Worli Road, Mumbai, 30, India).

3.2. *Plant material*

Fresh leaves of *A. pavonina* L. were obtained from the Zoo garden in May 2008. The plant was identified by Mrs Terase Labib, Taxonomist of Orman Garden, Giza. A voucher specimen (M95) has been deposited by Dr Mona Marzouk in the Herbarium of National Research Centre (CAIRC).

3.3. *Experimental animals*

Adult albino rats of Sprauge Dawely Strain weighing 130–150 g were used. Animals were obtained from the animal house colony of NRC, Egypt. They were kept under the same hygienic conditions with well-balanced diet and water. Medical Research Ethical Committee (MREC) in NRC has approved the work (09/143)

3.4. *Material for antioxidant activity*

Vitamin E (α-tocopheryl acetate) (Pharco Pharmaceutical Co., Alexandria, Egypt). It is available in the form of gelatinous capsules, each contains 400 mg, it was used as a reference antioxidant drug. Alloxane (Sigma Co., Aldrich, USA) was used for induction of diabetes in rats. 1,1-diphenyl-2-picryl-hydrazil (DPPH) is a relatively stable free radical.

3.5. *Material for cytotoxic activity*

The carcinoma cell lines were HepG2 (Liver), MCF7 (breast), HeLa (cervix), HEp-2 (larynx) and HCT116 (colon) and were obtained from the American Type Culture Collection, University Boulevard, Manassas, VI, USA. Cisplatin (Glaxo-Wellcome, London, UK) was used as a reference cytotoxic agent.

3.6. *Extraction and isolation*

Dried powdered leaves of *A. pavonina* L. (900 g) were extracted with petroleum ether (PE, 48 g), chloroform (Ch, 51.3 g), EtOAc (19.3 g); the residue was dried and extracted four times with 70% EtOH at room temperature to give DAEE (120 g). The previous extracts were dried under reduced pressure and subjected to *in vivo* antioxidant assay. DAEE was dissolved in water, and then applied to the top of a polyamide 6S column chromatography (120 × 5 cm) eluting with MeOH–H_2O mixture of decreasing polarities. Six main fractions (A → F) were obtained by combining similar fractions according to their PC (1 mm) properties using H_2O, 15% HOAc and BAW as eluent. Fraction A was chromatographed on preparative paper chromatography (PPC) (3 mm) to give two main subfractions A1 and A2. A1 was chromatographed over Sephadex using saturated butanol to yield compounds **1** (30 mg) and **2** (30 mg). A2 was also chromatographed over Sephadex using MeOH to afford compound **3** (10 mg). B was subjected to PPC then Sephadex using MeOH–H_2O (10%) to give compounds **4** (28 mg) and **5** (20 mg). C was chromatographed using PPC using acetic acid–water (15:85) followed by Sephadex using MeOH as an eluent to give compound **6** (20 mg). D was purified on Sephadex with saturated butanol as eluent to give compound **7** (15 mg). Fraction E was purified on Sephadex using MeOH–H_2O (10%) to give compound **8** (10 mg). F was subjected to Sephadex using MeOH–H_2O (1:1) to yield compounds **9** (12 mg) and **10** (15 mg). Final purification of all compounds was achieved by Sephadex LH20 column using MeOH as eluent.

3.7. *Quercetin 3-O-α-dirhamnopyranosyl-(1‴ → 2″, 1⁗ → 6″)-β-glucopyranoside-4′-methoxy*

A yellow amorphous powder R_f 0.37(BAW), negative ESI-MS: *m/z* 769.73 $(M-1)^-$, UV λ max (nm): MeOH: 254, 264, 355, NaOMe: 272, 376, $AlCl_3$: 270, 300, 354, 402, $AlCl_3$/HCl: 270, 300, 354, 400, NaOAc: 271, 358, NaOAc/H_3BO_3: 255, 266, 355. ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm, *J*/Hz,): 6.11 (1H, d, $J = 2.3$ Hz, H-6), 6.32 (1H, d, $J = 2.3$ Hz, H-8), 7.74 (1H, d, $J = 2.3$ Hz, H-2′), 6.94 (1H, d, $J = 8.4$ Hz, H-5′), 7.65 (1H, dd, $J = 8.4, 2.3$ Hz, H-6′), 3.81(3H,s, OCH_3). Sugar moieties: 5.55 (1H, d, $J = 7.65$ Hz, H-1″ glucose), 3.7–3.1 (m, overlapped by OH proton, H-2″–H-6″), 4.99 (1H, d, $J = 1.5$ Hz, H-1‴), 4.35 (1H, d, $J = 1.5$ Hz, H-1⁗),1.01 (1H, d, $J = 6.1$ Hz), H6‴, 0.82 (1H, d, $J = 6.9$ Hz, H-6⁗). ^{13}C NMR (125 MHz DMSO-d_6, ppm): 155.57 (C-2), 133.12 (C-3), 177.09 (C-4), 161.18 (C-5), 99.06 (C-6), 167.01(C-7), 94.14 (C-8), 156.43 (C-9), 102.00 (C-10), 121.59 (C-1′), 111.25 (C-2′), 145.98 (C-3′), 149.89 (C-4′), 115.32 (C-5′), 122.73 (C-6′), 55.61 (OCH_3), 100.59 (C-1″), 75.00 (C-2″), 73.88 (C-3″), 65.00 (C-4″), 73.32 (C-5″), 63.07 (C-6″), 100.02 (C-1‴), 68.16 (C-5‴), 17.92 (C-6‴), 99.06 (C-1⁗), 17.24 (C-6⁗).

3.8. *Evaluation of antioxidant activity*

3.8.1. In vivo *antioxidant activity*

Glutathione (GSH) is the body's most abundant natural antioxidant. Diabetes is usually accompanied by increased production of free radicals and impaired antioxidant defences. A simultaneous fall in blood GSH was observed following the injection of diabetogenic doses of alloxan into rats. Therefore, blood glutathione was estimated in alloxan-induced diabetic rats for evaluating the antioxidant activity (Oberley 1988; Maritim et al. 2003). *In vivo* antioxidant activities were carried out according to Eliasson & Samet (1969). At the end of the experiment, blood glutathione was estimated using bio-diagnostic kits (Beutler et al. 1963). The results were expressed as mean ± SE. The data were statistically analysed using the Student's '*t*' test (Sendecor & Cohran 1982). Results are considered statistically significant with $p > 0.01$.

3.8.2. In vitro *antioxidant activity (free radical scavenging activity)*

In vitro antioxidant activity by DPPH is used to evaluate the free radical scavenging activity of compounds **1** and **2,** measured by DPPH following the method of Shimada et al. (1992).

3.9. *Evaluation of cytotoxic activity*

Cytotoxic activity of EtOH, against five human cancer cell lines (HepG2, MCF7, HeLa, HEp-2 and HCT116), was carried out following the method of (Skehan et al. 1990). Cytotoxic activity of **1** and **2** against three cancer cell lines (HepG2, MCF7 and HCT116) was carried out according to the method of Mosmann (1983).

4. Conclusion

Compound **1** was isolated for the first time from natural sources and nine known flavonoids were isolated for the first time from *A. pavonina* leaves; the plant is safe and could be used as a good antioxidant and as an effective cytotoxic agent against liver and breast cancer cell lines.

Supplementary material

Supplementary Figures S1–S10 are available online.

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