Study of Coumarin Content of *Pelargonium fragrans* - Willd. Root Grown in Egypt

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Abstract: Three coumarins viz; **C1** (Umbelliferone), **C2** (Umckalin) and novel coumarin glycoside **C3**, were isolated from *Pelargonium fragrans* Willd root. Isolated compounds were identified on the basis of their physico-chemical, UV spectral data, ^{1}H-NMR, ^{13}C-NMR and EIMS. These compounds were isolated for the first time from the plant. Significant antiwormal effect on fresh earthworms (*Allolobophora caliginosa*), remarkable antioxidant activities and strong antifungal activity were recorded on the extract, fractions and isolated compounds.
[K.M. Meselhy. **Study of Coumarin Content of *Pelargonium fragrans* - Willd. Root Grown in Egypt.** *Life Sci J* 2013;10(1):159-164] (ISSN:1097-8135). http://www.lifesciencesite.com. 23

Keywords: Coumarin, *Pelargonium fragrans*, antioxidant activities, antiwormal, antifungal.

1. Introduction

The genus Pelargonium Family (Geraniaceae) is a rather big one and comprises over 200 species (Bailey, 1953 and Leung and Foster, 1977). A paste made from Pelargonium leaves is used to treat wounds and abscesses. A decoction from the roots is used to wash feverish patients and extracts of the roots have found widespread usage against infections of the sinus, throat and respiratory tract. In aromatherapy, Pelargonium is used as sedative, in depression, confusion, panic and anxiety (Dweck ,1997 and Jacobs, 1997). The Pelargonium oil is of economic significance in food flavoring, mouth washes, soaps, and cosmetic industries (Bailey, 1953 and Kowalchik and Hylton, 1986).

Reviewing the available current literature, few Pelargonium species have been investigated for their coumarin content and their biological effects (Bladt, 1977; Baldt and Wagner, 1988; Kayser and Kolodziej, 1995, El-Sherei *et al.*, 2004; Trun *et al.*, 2006; Koch and Biber; 2007; Kolodziej, 2007 ; and Franco and Oliveira, 2010)

A literature survey indicated that no data are available on the presence of coumarin constituents of *Pelargonium fragrans* Willd. Preliminary phytochemical screening of the root of the title plant revealed the presence of coumarins in the roots. Therefore, it was deemed of interest to isolate and identify these constituents; as well as to evaluate certain biological activities of the plant.

2. Material and methods

2.1. Plant material

The roots of *Pelargonium fragrans* Willd. Were collected from plants cultivated in the Experimental Station of Medicinal and Aromatic Plants, Faculty of Pharmacy, Cairo University, Giza, Egypt. The identity of the plant was kindly confirmed by Prof. Dr. Mohamed El-Gebally, Prof. of Plant Taxonomy, NRC, Dokki, Giza. The plant was air-dried, reduced to fine powder and kept in tightly closed amber colored glass containers. Voucher specimens are kept in the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

2.2. Chemicals

2.2.1 Reference samples:

-Coumarins from Sigma (USA) and Aldrich (Germany).

-Glucose, galactose, xylose, rhamnose and glucouronic acid (E. Merck, Darmstadt, Germany).

2.2.2 Material for chromatography:

Silica gel G (60 mesh) for TLC, silica gel (70-230 mesh) for CC, precoated TLC plates (silica gel 60 GF_{254}) from E. Merck (Darmstadt, Germany), sephadex LH-20 from Pharmacia (Uppsala, Sweden).

Solvent systems:

Petroleum ether/ethyl acetate (in different ratios v/v)
Chloroform - methanol (in different ratios v/v).
Ethyl acetate - methanol -water (24:4:1)
Methanol:water (8:2)

Spray reagents;

Sulphuric acid (50%) and P-anisaldehyde were used (Stahl, 1969).

2.3. Material for biological evaluation:

2.3.1. Plant extracts:

The biological evaluation was performed on the three isolated compounds in addition to the acetone (95 %) extract of the air-dried powdered roots. The extract was prepared by defatting 200 g of air-dried powdered roots with petroleum ether and the dry defatted marc was exhaustively percolated with acetone: water (5:1) followed by distillation of the solvent. The solvent-free residue was kept for investigation. All samples were dissolved in water by the aid of few drops of tween80.

2.3.2. Animals:

Male albino rats of Sprague Dawely Strain (120-150g) were used. Animals were obtained from

the animal house, of the National Research Center, Dokki, Giza, Egypt. The animals were fed on a standard laboratory diet composed of vitamin mix (1 %), mineral mix (4 %), corn oil (10 %), sucrose (20 %), cellulose (0.2 %), casein (10.5 %) and starch (54.3 %). Fresh earthworms *(Allolobophora caliginosa)* were used for testing the antiwormal activity.

2.3.3. Fungi:

The following fungi available in stock culture in Microbiology Department, Faculty of Pharmacy, Cairo University were used: yeast (*Candida albicans, Candida tropicalis and Torulepsis glabrara) and* Mycelial fungi *(Aspergillus niger, Aspergillus fumigatus, Penicillium vermiculatum* and *Rhizopus sp*. (ATCC).

2.3.4. Reference drugs and kits:

Glutathione Kit (Wak, company – Germany), and Vitamin E (Pharco Pharmaceutical Co, Egypt) were used in the pharmacological screening

2.3.5. Solid medium for antifungal testing:

Trypticase soy agar medium. Fungal suspension was applied as spots on the dried agar surface using inoculum size of nearly 10^5 yeast cells or fungal spores.

2.4. Apparatus:

UV-visible spectrophotometer, Shimadzu UV 240 (P/N 204-58000); Mass spectrometer: Varian 90 NMR spectrophotometer; NMR Jeol GLM, Jeol TMS Route instrument (^{1}H-NMR, 300 MHZ, ^{13}C, 75 MHZ, Japan); Koffler's heating stage microscope.

2.5. Phytochemical study:

2.5.1 Investigation of coumarin content:

2.5.1.1. Extraction, isolation and Identification:

The air-dried powdered roots (2 Kg) were exhaustively defatted with petroleum ether and the dry defatted marc was exhaustively percolated with acetone: water (5:1). After stripping- off the solvent under reduced pressure, the residue (40 g) was suspended in water and successively partitioned with methylene chloride and ethyl acetate. The solvent of each fraction was evaporated, separately, under reduced pressure to give Methylene chloride fraction (12 g) and ethyl acetate fraction (9 g). Fractions obtained were investigated on TLC using system Chloroform-Methanol (9:1 v/v) and the chromatograms were examined under UV before and after exposure to ammonia vapours.

Methylene chloride fraction (12 g) was separated over a silica gel CC eluted with a petroleum ether/EtOAc solvent system with increasing polarity to afford compound C1 (petroleum ether/EtOAc, 4:2) and C2 (petroleum ether/EtOAc, 1:1). Combined fractions (10-19) were purified on silica gel 60 CC (20 g) and re-crystallized from chloroform to afford compounds **C1** as a white powder (18 mg) & **C2** as a yellow powder (15 mg).

Ethyl acetate fraction (9g) was chromatographed over Sephadex LH-20 column (l x 40cm) with methanol to give three fractions. The major fraction (9-15) was rechromatographed over silica gel using ethyl acetate-methanol-water (24: 4: 1) to afforded compound **C3** as 19 mg, yellow powder.

Structures of compounds (**C1-C3**) were established by a combination of physico-chemical data, UV, ^{1}H-NMR, ^{13}C-NMR and EIMS studies (Table 1).

2.6. Biological study:

2.6.1. Antioxidant activity:

The antioxidant activity of the tested samples (acetone extract and isolated compounds) was assessed by measuring the glutathione level in blood samples collected from alloxan-induced diabetic rats as compared to Vitamin E (12 mg / kg b.wt., positive control) and adopting the procedure described by Beutler (Beutler *et al.*, 1963). The restoration of blood glutathione levels (reduced due to induction of diabetes) was taken as a measure of antioxidant activity. The percentage change observed after dose administration was, in each case, calculated according to the following equation: % of change= $(G_c–G_t)\times100/G_c$ and results are listed in (Table 2, Figure 2)

2.6.2. Antiwormal activity:

The antiwormal effect of the acetone extract and the isolated compounds was evaluated using earth worms (*Allolobophora caliginosa*) not less than 10 cm long as experimental animals (Jain and Jain, 1972). Different dilutions viz., 0.1 and 0.2 % of each sample, prepared in 1 % Tween 80, were tested. A group of three worms was dipped in 5 ml of each of the tested solutions in addition to a negative control consisting of 1 % Tween 80. The activity of the treated worms was observed and the time required for complete inhibition of the response of the worms to external stimuli (indicating paralysis or death) was recorded and taken as a measure for antiwormal activity. Results are displayed in (Table 3, Figure 3).

2.6.3. Antifungal activity:

The antifungal activity of acetone extract and different fractions prepared thereof (methylene chloride and ethyl acetate fractions) were tested against representatives of yeasts (*Candida albicans , Candida tropicalis and Torulepsis glabrara) and* mycelial fungi *(Aspergillus niger, Aspergillus fumigatus, Penicillium vermiculatum* and *Rhizopus sp)* as described (Lorian, 1980).

Serial dilutions from 80 to 5 mg/mL of Trypticase soy agar medium were used. 20 µl of each dilution was transferred to cups preformed in

Trypticase soy agar innoculated with suspension of 105 /ml yeast cells or fungal spores at the surface of agar plates and incubated at 30°C for 4-5 days. After incubation, the lowest concentration producing inhibition was recorded as the minimum effective concentration. Results are recorded in (Table 4).

3. Results and Discussion

3.1. Investigation of coumarin content:

Acetone extract of the dried powdered roots was partitioned with methylene chloride and ethyl acetate. Methylene chloride fraction afforded compounds **C1** and **C2**, while ethyl acetate fraction afforded compound **C3**. The structure elucidation was established on the basis of physicochemical data, UV data, ^{1}H-NMR, ^{13}C-NMR and EIMS (Table. 1).

Compound C1: 18 mg; white powder; soluble in chloroform, The UV spectral data are similar to those reported by Murray and Kayser for 7-hydroxy-coumarin. ^{1}H-NMR spectrum revealed the characteristic chemical shifts and coupling patterns for a 7-hydroxy-coumarin (Murray *et al*.,1982 and Kayser and Kolodziej, 1995) The molecular formula was deduced as $C_9H_6O_3$ from the molecular ion peak observed at m/z 162 $(M)^+$ in the mass spectrometry, ascribed to umbelliferone (**Figure 1**).

Compound C2: 15 mg; yellowish white crystals; soluble in chloroform, The UV spectral data are similar to those reported for 7-hydroxy-5,6-dimethoxycoumarin compounds with free OH at position 7. ^{1}H-NMR spectrum showed the signals characteristic for 7-hydroxy-5,6-dimethoxycoumarin (Murray *et al*.,1982 and Kayser and Kolodziej, 1995) , being different from C1, in protons 5 and 6, where both protons were substituted by 2 methoxyl groups and this was verified by absence of H-5 and H-6 proton signals, and appearance of H-8 as a singlet peak; in addition to the presence of, 2 sharp singlets at δ 4.25 and 4.2 ppm; each integrated for 3 protons ascribed for 2 methoxy protons. The mass spectrum of **C2** showed a molecular ion peak at m/z = 222 compatible with the molecular formula $C_1H_{10}O_5$ and ascribed to umckalin (**Figure 1**), Other fragment ion peaks at m/z 193, indicating a loss of one methoxy group, and *m/z* 163, indicating a loss of two methoxy groups.

Compound C3: 19 mg; yellow powder; soluble in methanol. Bands characteristic for 5,8-dimethoxy-coumarin glycoside compounds were observed by UV spectrum (Murray *et al.*, 1982). The EIMS of compound **C3** exhibited molecular-ion peaks at m/z 397 $(M-H)^+$, and 235 $(M - H - glucose)^+$, allowed the establishment of the molecular formula $C_{18}H_{22}O_{10}$.

^{1}H and ^{13}C NMR spectra of **C3**, revealed the characteristic chemical shifts and coupling patterns for4-methyl-5,8-dimethoxy-coumarin-7-*O*-glucoside (Murray *et al*., 1982), which indicated by the absence of 5,7 and 8 proton signals. Long-range coupling was observed between H-3 (δ 6.05, d, J = 0.6 Hz), and methyl protons at C-4, which has been reported in the spectra of 4-methylcoumarin (Murray *et al*., 1982), 5,8-dimethoxy protons appear at 4.05 and 4.19, respectively, with integration of 3 protons each.

Signals for five oxymethine protons in *trans* diaxial confirmations (J = 8.4 - 9.5 Hz) and one oxymethylene protons at δ 3.60 (dd, J = 10.4/ 5.7 Hz, H-6_a) and δ 3.74 (dd, J = 10.4/ 4.2 Hz, H-6_b) indicated the presence of a β-D-glucopyranoside moiety. The six carbon signals observed at (δ 60.7 - 99.7) in the ^{13}C NMR spectrum also confirmed the presence of an *O*-glucosyl unit in **C3**. The β-anomeric configuration for the glucose moiety was determined from the $^{3}J_{H\text{-}1',\ H\text{-}2'}$ coupling constant (5.35, d, J = 8.4 Hz) corresponding to the axial proton of β-D-glucopyranosyl. Furthermore, a loss of 162 mass units from the molecular ion in the EIMS spectrum at m/e 235 $(M - H - glc)^+$ clearly suggested the presence of *O*-glucose moiety in **C3**.

^{13}C-NMR showed a signal at 166 of (C-2), due to effect of carbonyl and hetero oxygen. Oxygenated aromatic carbons appear at 165.1 (C-5), 154.3 (C-7), 144.4 (C-8a) and 142.3 (C-8) as well as signals at 104.5 (C-4a) and 100.2 (C-6) ascribed for aromatic carbons. $(8\text{-}OCH_3)$, $(5\text{-}OCH_3)$ and (CH_3) appear upfield at δ 62.5, 61.6 and 20, respectively;

From the previously mentioned physicochemical and spectral data, as well as, comparison with the published data (Devon. and Scott ,1975; Murray *et al*.,1982 and The Merck Index, 2001)**,** compounds **C1-C3** (**Table 1, Figure 1**) could be identified as **umbelliferone** (7-Hydroxycoumarin), **umkalin** (7-Hydroxy-5,6-dimethoxy- coumarin) and 5,8-dimethoxy-4-methyl-7-*O*-glucoside coumarin, respectively. Although Compounds **C1** and **C2** were previously reported in other species of *Pelargonium* (Bladt ,1977; Bladt and Wagner, 1988, Kayserand Kolodziej, 1995 and Franco and Oliveira., 2010), they were isolated for the first time from *P. fragrans* Willd., while compound **C3** was isolated for the first time from the genus.

3.2. Biological evaluation:

The different biological activities evaluated (antioxidant, antiwormal and antifungal) for extract, fractions and isolated compounds, as represented in (Tables 2-4), revealed variable although significant efficacy & potency for all the samples when

compared to standard and suggest their incorporation in herbal formulations after necessary clinical trials.

3.2.1. Antioxidant effect:

The variation in biological activities of the tested samples may be attributed to the difference in chemical nature of tested sample. As a matter of fact, acetone extract, **C1 and C2** samples exerted high potency as antioxidant **(Table 2**, **Figure 2)**; which may be due to synergetic effect of acetone extract content and phenolic nature of **C1 and C2** (Stephen and Duke, 1996).

3.2.2. Antiwormal effect:

On the other hand compounds **C1, C2 & C3** recorded higher activity as antiwormal **(Table 3**, **Figure 3)**; where paralysis was induced in few minutes & dose dependent when tested in 0.1% and 0.2% concentrations of tested samples. This response was of irreversible nature, even after leaving the worms into recovery bath (fresh water bath). This observation offers a support to recommend the possible utility of isolates as antiwormal agents.

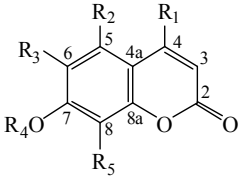
3.2.3. Antifungal activity

The antifungal activity of acetone extract and different fractions prepared thereof (methylene chloride and ethyl acetate fractions) were tested against representatives yeasts and mycelial fungi **(Table 4)**.

The acetone extract and fractions thereof exhibited a significant antifungal activity with MIC between 20 - 80 mg/mL; methylene chloride and ethyl acetate fractions rich with coumarins being the most active. In general, yeasts were more sensitive than mycelial fungi. Coumarins have been found to stimulate macrophages (Casley-Smith and J. R. 1997), which could have an indirect negative effect on infections. More specifically hydroxylated derivatives of coumarins, can be presumed to have strong antifungal activity (Hoult and Paya, 1996).

Table 1. Data of compounds C1, C2 & C3 isolated from acetone extract of *Pelargonium fragrans* - Willd. root

Data	Carbon number	C1	C2	C3	
		δ_H	δ_C	δ_H	δ_C
^{1}HNMR(300 MHz, DMSO-d6) & ^{13}CNMR (75 MHz DMSO-d6)	2	-			166
	3	*6.2 (1 H, d, J =9.5 Hz)*	*6.36 (1 H, d, J= 9.5Hz)*	*6.05 (1 H, d, J = 0.6 Hz)*	111.3
	4	*7.93 (1H, d, J = 9.6 Hz)*	*7.75 (1 H, d, J= 9.5 Hz)*	-	152.1
	5	*7.65 (1 H, d, J =8.4 Hz)*	-	-	165.1
	6	*6.79 (1 H, dd, J = 8.6 Hz, J = 2.6 Hz)*	-	*6.25 (1 H, s)*	100.2
	7	-		-	154.3
	8	*6.74 (1 H, d, J =2.4 Hz)*	*6.83 (1 H, s)*	-	142.3
	4a	-	-	-	104.5
	8a	-	-	-	144.4
	4 CH_3	-	-	*2.48 (3 H, d, J = 0.6 Hz)*	20
	5 OCH_3	-	*4.2 (3 H, s)*	*4.05 (3 H, s)*	61.6
	6 OCH_3	-	*4.25 (3 H, s)*	-	-
	8 OCH_3	-	-	*4.19 (3 H, s)*	62.5
	7 glucose	-	-	*-5.35 (1H, d, J = 8.4 Hz, H-1'anomeric)* *-3.15-3.73 (remain of sugar protons)*	99.7(C-1') 73.1 (C-2') 76.5 (C-3') 69.5 (C-4') 77.0 (C-5') 60.7 (C-6')
MS (EI, 70 eV) m/z		162 (M^+, 90%), 134 (100%), 105 (28%), 78 (37%)	222 (M^+, 10%), 193 (11%), 163 (12%), 111 (34%), 83 (75%)	397 (M, 95%)$^+$, 235 ((M- glucose)$^+$, 13%)	
UV/Vis λmax (MeOH) nm (log ε)		222, 243 sh, 259 sh, 326	220, 243, 261 sh, 322	223, 245, 261 sh, 323	
physicochemical data		18 mg, white powder MP: 224-225 ºC. Rf : 0.72 (CHCl3-MeOH, 9:1)	15 mg, yellowish white powder MP: 148-149 ºC. Rf : 0.83 (CHCl3-MeOH, 9:1)	19 mg, yellow powder MP: 150-152 ºC. Rf : 0.99 (CHCl3-MeOH, 9:1)	



	R_1	R_2	R_3	R_4	R_5
C1	H	H	H	H	H
C2	H	OCH_3	OCH_3	H	H
C3	CH_3	OCH_3	H	glc	OCH_3

Figure 1: **Isolated coumarins from acetone extract of *Pelargonium fragrans* - Willd. root**

Table 2 & Figure 2: **Antioxidant effect of acetone extract and isolated coumarins obtained from the root of *Pelargonium fragrans* - Willd. grown in Egypt:**

Test	Blood glutathione (mg%)		
	Mean ± S.E.[1]	% of change[2]	Potency[3]
Control Saline)	36.2±0.8	-	-
Diabetic	24.9±0.5*	31.2	-
Acetone extract	33.2±0.3	8.3	20.5
C1	32.7±0.8	9.6	17.7
C2	33.4±1.4	9.1	18.7
C3	30.6±1.1·	15.5	11.0
Diabetic + Vit E.	35.6±0.7	1.7	100.0

1: Blood glutathion level expressed in mg/kg as mean ± S.E.
2: Percentage of change as compared to control.
3: Percentage of Potency as compared to standard.
* Significantly different from the control group at $p < 0.01$.

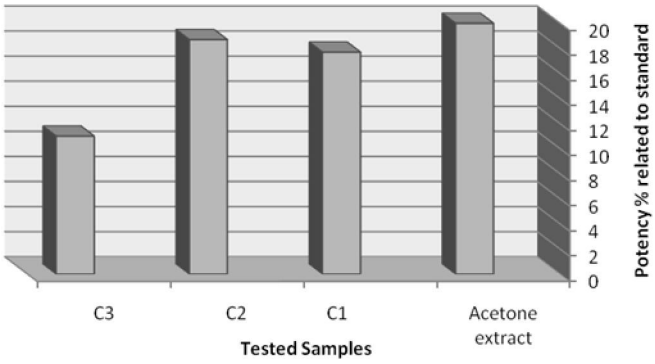


Table 3 and Figure 3: **Antiwormal effect of acetone extract and isolated coumarins obtained from the root of *Pelargonium fragrans* - Willd. grown in Egypt:**

Test	Time elapsed before worm death (min.) after treatment	
	0.1%[1]	0.2%[2]
Control 1% Tween80)	-	-
Acetone extract	12	8
C1	7	4
C2	8	5
C3	9	7

1: Time elapsed before worm death in minutes after treatment by 0.1 % of tested samples.
2: Time elapsed before worm death in minutes after treatment by 0.2 % of tested samples.

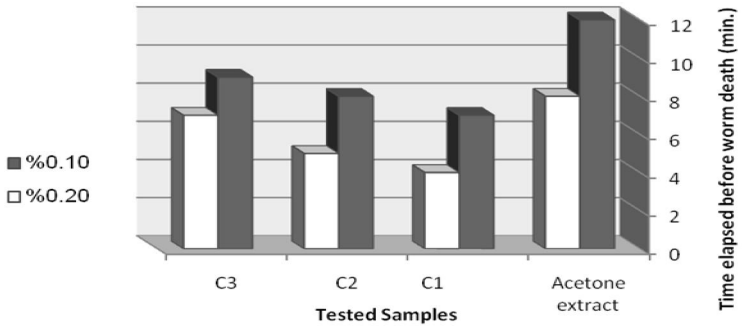


Table 4: **Antifungal effect of acetone extract and fractions obtained from the root of *Pelargonium fragrans* - Willd. grown in Egypt:**

Tested solution	MIC(mg/mL)						
	Mycelia fungi				*Yeast*		
	Aspergillus niger	*Aspergillus fumigatus*	*Penicillium vermiculat.*	*Rhizopus sp*	*Candida albicans*	*Candida tropicalis*	*Torulepsis glabrara*
Amphotericine B µg/mL)	2	2	1	2	1	0.5	1
Acetone extract	80	40	80	80	40	80	80
Methylene chloride fraction	80	80	40	80	40	80	80
Ethyl acetate fraction	40	40	20	40	20	40	20

Acknowledgement

The author is thankful to Dr. Amani, Sleem, Prof. of Pharmacology, NRC for her help in the study of antioxidant effect and to Dr. Aly Ahmedy, Ass. Prof of Microbiology, Microbiology Department, Faculty of Pharmacy, Cairo University in testing the antifungal activity.

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11/22/2012