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Sphinx Journal of Pharmaceutical and Medical Sciences

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SPHINX UNIVERSITY

CHEMICAL INVESTIGATION OF *GREVILLEA ROBUSTA* A. CUNN FLOWERS

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The ethyl acetate soluble fraction of the air-dried flowers of *Grevillea robusta* was fractionated and separated to obtain eight compounds by different chromatographic techniques. Structures of the isolated compounds were determined on the basis of the extensive spectroscopic analysis, including 1D and 2D NMR and compared with the literature data. These compounds were identified as 3,5-dihydroxy cinnamate, 6-hydroxy coumarin, *p*-hydroxy benzeldahyde, methyl gallate, ethyl gallate arbutin 6"-*O*-3,5-dihydroxycinnamic acid ester, robustaside D, together with cyanogenic glycoside named dhurin (s). This study aims to further phytochemical investigation of *G. robusta*' flower to discover more secondary metabolites with wide range of therapeutic values.

Keywords: *Grevillea robusta*, Proteaceae, phenolic compounds, cyanogenic glycoside.

INTRODUCTION

Silk-oak (*Grevillea robusta*), also often called silver-oak, is a medium to large tree commonly planted as an ornamental in many warm-temperate and semitropical climates. It is a flowering plant in the family Proteaceae. It is a tree, the largest species in its genus but is not closely related to the true oaks, Quercus[1].

Previous work has shown the presence of several 5-/7-alkyl resorcinol and arbutin derivatives in the wood and leaves of *Grevillea robusta*[2-5].

Kaempferol-5-*O*-*β*-glucoside a rare flavonol glycoside has been isolated from the fresh flowers of *Grevillea robusta*[6].

The alcoholic extracts of the leaves and bark of *Grevillea robusta* A. Cunn showed a significant anti-inflammatory activity using carrageenan-induced edema[7].

Grevillea flowers were used as a food source by Australian Aborigines. The flowers were sucked for their sweet nectar or used to make sweet drinks. They were also used by Australian Aborigines as traditional bush medicines to treat wounds and sores, skin diseases, diarrhea and dysentery and as bactericidal preparations[8].

MATERIALS AND METHODS

General experimental procedure ^{1}H NMR (500 MHz, $CDCl_3$ and CD_3OD), ^{13}C NMR (150 MHz) Varian JMNGX 500 MHz spectrometer, with tetramethylsilane (TMS) as an internal standard. Mass analyses were recorded on a JEOL JMS-DX 200L and FAB-MS mass spectrometer. Column chromatography was carried out on silica gel 60 (Merck; 230-400 mesh) and octadecylsilyl (ODS) (Pharmacia Co. Tokyo, Japan). TLC was performed on silica gel 60 F_{254} plates (0.25 mm, Merck Co.) and on Rp -18 plates (40-63 µm, Merck Co.), and spots were detected under UV light and colored by spraying with 10% H_2SO_4 solution followed by heating.

Plant material

Received in 12/9/2021 & Accepted in 11/10/2021

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The flowers of *Grevillea robusta* A. Cunn were air-dried, powdered and kept in closed containers. The plant material was collected in Assiut area.

Extraction and isolation

The powdered flowers (750 g) of *Grevillea robusta* A. Cunn were extracted with methanol at room temperature. The extract was concentrated *in vacuo* to obtain a gummy residue (139 g). The concentrated crude extract was suspended in water and shacked in order with 1L *n*- Hexane, 5L $CHCl_3$, 6L EtOAc and 5 L *n*-BuOH.

EtOAc soluble fraction (17 g) was fractionated on silica gel Column chromatography (500 g silica, 5×60 cm). Elution was performed starting with $CHCl_3$-MeOH (9.5:0.5), the polarity is gradually increased till $CHCl_3$-MeOH (8:2). Fractions (200 ml, each) were collected and monitored by TLC using four solvent systems, $CHCl_3$-MeOH, 8:2 v/v, $CHCl_3$-MeOH, 6:4 Acetonitril-H_2O 8:2 v/v and MeOH:H_2O 2:1 v/v. Sulphuric acid (10%) spray reagent was used for compound spots detection. Similar fractions were pooled according to their chromatographic properties to yield six collected fractions as the following: I (2.0 g), II (3.5 g), III (7.0 g), IV (6.0 g), V (5 g) and VI (7.5 g). Fractions II-VI were selected for further chromatographic processing, according to TLC investigations. Fractions II and III were separately fractionated by silica gel column, elution was started with $CHCl_3$-EtOAc (2:1) till $CHCl_3$-EtOAc (1:2) to give 3,5-dihydroxy cinnamate (**1**, 5 mg), 6-hydroxy coumarin (**2**, 5 mg) and *p*-hydroxy benzeldehyde (**3**, 5 mg).

Fractions IV and V were separately fractionated by reversed phase chromatography on octadecylsilyl (ODS) column. Elution was done with 10:90 v/v (CH_3CN:H_2O), it yielded methyl gallate (**4**, 4 mg), ethyl gallate (**5**, 10 mg), arbutin 6”-*O*-3,5-dihydroxycinnamic acid ester (**6**, 4 mg).

Fraction VI was fractionated by reversed phase chromatography with 10:90 v/v (CH_3CN:H_2O), it yielded two subfractions A and B, SubVI-B was fractionated by reversed phase chromatography with 5:95 v/v (CH_3CN:H_2O), it yielded robustaside D (**7**, 6 mg), together with cyanogenic glycoside named dhurin (s) (**8**, 5 mg).

RESULTS AND DISCUSSION

Identification of purified compounds (Fig. 1)

Compound 1

Yellow powder; ^{1}H NMR (500 MHz, CD_3OD) δ: 3.77 (3H, s), 6.46 (IH, d, *J*= 16.2 Hz, H-8), 6.65 (2H, br. s, H-2, H-6), 6.96 (IH, br. s, H-4), 7.93 (1H, d, *J*= 16.2 Hz, H-7). ^{13}C NMR (125 MHz, CD_3OD) δ: 122.83 (C-1), 117.93 (C-2), 151.31 (C-3), 114.65 (C-4), 117.68 (C-5), 151.50 (C-6), 142.18 (C-7), 120.21 (C-8), 169.91 (C-9), 52.02 (OCH_3); EI-MS *m/z* 194 $[M]^+$, $C_{10}H_{10}O_4$.

Compound 2

Yellow crystals, m.p. 177-179°C; ^{1}H NMR (500 MHz, $CDCl_3$-CD_3OD) δ: 6.49 (IH, d *J*= 9.6 Hz, H-3), 7.78 (1H, d, *J*= 9.6 Hz, H-4), 6.95 (1H, d, *J*= 2.9 Hz, H-5), 7.09 (1H, dd, *J*= 2.9, 8.9 Hz, H-7), 7.21 (1H, d, *J*= 8.9 Hz, H-8). ^{13}C NMR (125 MHz, $CDCl_3$-CD_3OD, 2:1) δ: 163.0 (C-2), 116.6 (C-3), 144.9 (C-4), 112.9 (C-5), 154.6 (C-6), 120.1 (C-7), 118.0 (C-8), 120.8 (C-9), 148.0 (C-10); EI-MS *m/z* 162 $[M]^+$, $C_9H_6O_3$.

Compound 3

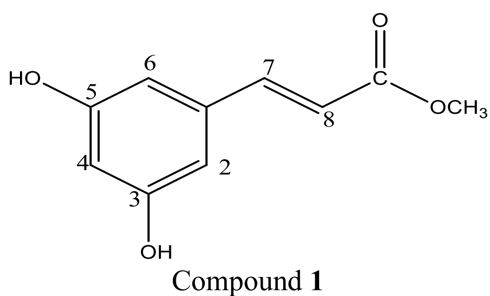
White powder; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.77 (2H, d, *J*= 8.9 Hz, H-2, H-6), 6.91 (2H, d, *J*= 8.9 Hz, H-3, H-5), 9.76 (1H, s, CHO). ^{13}C NMR (125 MHz, CD_3OD) δ: 138.9 (C-1), 133.4 (C-2, 6), 116.8 (C-3,5), 165.2 (C-4), 192.8 (CHO). FABMS 123 $[M+H]^+$, $C_7H_6O_2$.

Compound 4

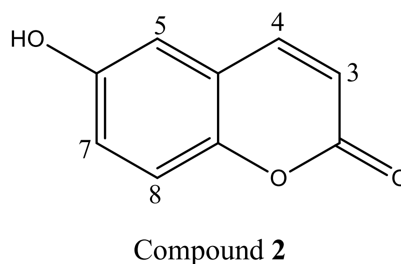
White powder; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.15 (2H, s, H-2,H-6), 3.81 (3H, s, OCH_3). FABMS 184 $[M]^+$, $C_8H_8O_5$.

Compound 5

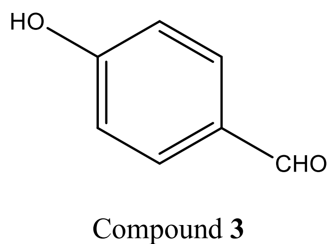
White powder; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.06 (2H, s, H-2,H-6), 4.28 (2H, q, $COOCH_2CH_3$), 1.35 (3H, t, $COOCH_2CH_3$). ^{13}C NMR (125 MHz, CD_3OD) δ: 121.7 (C-1), 109.9 (C-2, 6), 146.4 (C-3,5), 139.6 (C-4), 168.5 (C=O), 61.6 (CH_2), 14.6 (CH_3). FABMS 198 $[M]^+$.



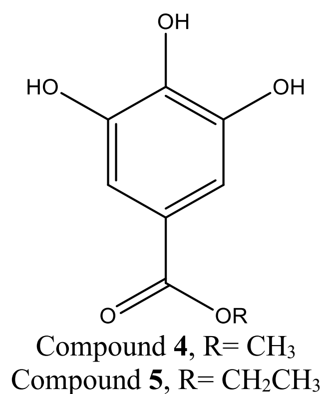
Compound **1**



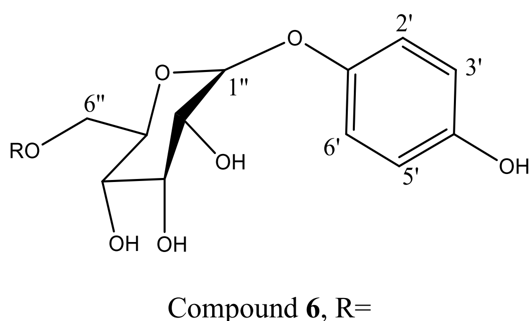
Compound **2**



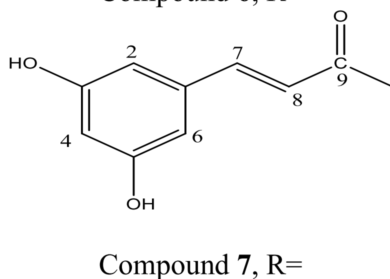
Compound **3**

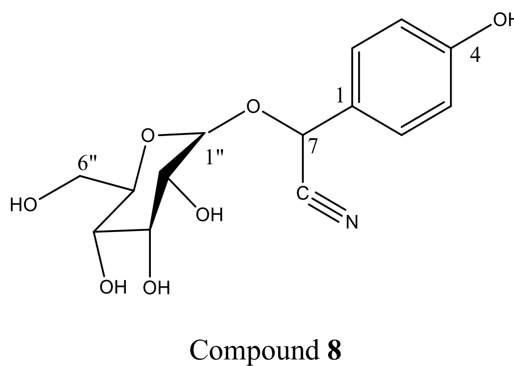


Compound **4**, R= CH_3
Compound **5**, R= CH_2CH_3



Compound **6**, R=





Compound **7**, R=

Compound **8**

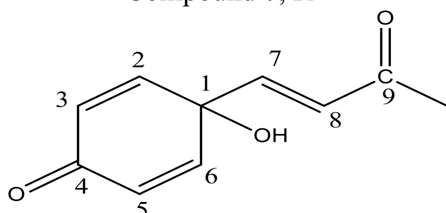


Fig. 1: Structures of the isolated compounds.

Compound 6 Amorphous powder; see tables 1&2, ^{1}H and ^{13}C NMR (500, 125 MHz, CD_3OD).

FABMS (pos.) m/z 435 $[M+H]^+$, FABMS (neg.) m/z 433 $[M-H]^-$.

Compound 7

Amorphous powder; see tables 1&2, ^1H and ^{13}C NMR (500, 125 MHz, CD_3OD). FABMS (pos.) m/z 457 $[M+Na]^+$, FABMS (neg.) m/z 433 $[M-H]^-$.

Table 1: ^1HNMR data of compounds **6** and **7** (500 MHz, CD_3OD).

H	Compound **6**	Compound **7**
2, 6	6.72, d (1.2 Hz)	6.25, d (8.9 Hz)
3, 5		6.80, d (8.9 Hz)
4	6.93, t (1.2 Hz)	
7	7.97, d (16.0 Hz)	6.67, d (15.6 Hz)
8	6.54, d (16.0 Hz)	6.27, d (15.6 Hz)
2’, 6’	6.95, d (8.9 Hz)	6.9, d (8.95 Hz)
3’, 5’	6.67, d (8.9 Hz)	6.68, d (8.95 Hz)
Glc.1’’	4.72(7.3 Hz)	4.71(7.2 Hz)
2’’	3.45, m	3.43, m
3’’	3.47, m	3.45, m
4’’	3.41, m	3.37, m
5’’	3.65, m	3.61, m
6’’ a	4.5 (dd, 11.9, 2.1 Hz)	4.47 (dd, 11.9, 2.1 Hz)
6’’b	4.4 (dd, 11.9, 6.6 Hz)	4.32 (dd, 11.9, 6.8 Hz)

Table 2: ^{13}CNMR data of compounds **6** and **7** (125 MHz, CD_3OD).

C	Compound **6**	Compound **7**
1	122.6	70.8
2	120.8	128.5
3	151.4	151.1
4	114.7	185.8
5	151.4	151.1
6	120.8	128.5
7	142.5	148.0
8	117.6	122.7
9	169.2	167.1
1’	153.7	153.7
2’, 6’	119.2	119.5
3’, 5’	116.5	116.5
4’	152.3	152.1
Glc.1’’	103.8	103.1
2’’	74.2	74.3
3’’	77.8	77.7
4’’	71.8	71.5
5’’	75.2	75.1
6’’	64.7	64.9

Compound 8

Yellow powder; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.41 (2H, d, *J*= 8.5 Hz, H-2,6), 6.85 (2H, d, *J*= 8.5 Hz, 3,5), 5.91 (1H, s, CHCN), 4.63 (1H, d, *J*= 7.6, H-1'), 3.93 (1H, dd, *J*= 12.0, 2.0, Gluc/H-6'a), 3.89 (1H,dd, *J*= 12.0, 6.5 Hz, H-6'b). ^{13}C NMR (125 MHz, CD_3OD) δ: 125.0 (C-1), 130.5 (C-2,6), 116.6 (C-3, 5), 160.1 (C-4), 68.3 (CHCN), 118.7 (CN), 101.8 (C-1'), 74.7 (C-2'), 77.8 (C-3'), 71.5 (C-4'), 78.5 (C-5'), 62.8 (C-6').

Compound 1 showed two doublets for trans olefinic protons δ: 7.93 and 6.46 (*J*= 16.2 Hz), a signal for methyl ester group at δ: 3.77 (3H, s), and three aromatic protons at δ: 6.65 (2H, br s, H-2, H-6) and 6.96 (1H, br s, H-4).

The ^{13}C NMR spectrum showed signals characteristic for conjugated ester carbonyl at δ: 169.91 and methyl ester at δ: 52.02.

The EI-MS spectrum indicated a molecular weight of 194, consistent with the molecular formula $C_{10}H_{10}O_4$. In addition a fragment ion peak at m/z 163 due to the loss of OCH_3 supported the structure of compound **1** to be 3,5-dihydroxy cinnamate.

Compound 2, the mass spectrum showed a molecular ion peak at 162 with characteristic fragments at 118 and 74 for the successive loss of CO_2, a phenomenon characteristic for coumarin[9].

The ^{1}H NMR spectrum of compound **2** showed two signals at δ: 7.78 and 6.49 (each 1H, d, *J*= 9.6 Hz) assigned to H4 and H-3 respectively. It also showed ABC aromatic protons at δ: 7.21 (1H, d, *J*= 8.9 Hz, H-8), 7.09 (1H, dd, *J*= 2.9, 8.9 Hz, H-7) and 6.95 (1H, d, *J*= 2.9 Hz, H-5) of the coumarin moiety, respectively.^{13}C NMR spectral analysis displayed nine carbon atom of which one carbonyl carbon has been recognized at δ: 163.0 and one oxygenated carbon at δ: 154.6.

Comparison with the known compound, authentic umbelliferone showed significant differences in the ^{1}H NMR concerning signals at δ: 7.41 (1H, d, *J*= 8.3 Hz), 6.76 (1H, dd, *J*= 2.3, 8.3Hz) and 6.73 (1H, d, *J*= 2.3 Hz) for H-5, H-6 and H-8, respectively. The ^{13}C NMR showed also some differences[10]. These data suggest that compound **2** should be 6-hydroxy coumarin.

Compound 3, the ^{1}H, ^{13}C NMR spectral data showed aromatic protons of the A_2B_2 type at δ: 7.77 (δ_C 133.4) and 6.91 (δ_C 116.8), and singlet at δ: 9.76 suggesting the presence of aldehyde group. This was confirmed by the presence of a carbon signal at δ_C: 192.8. FABMS spectrum showed a molecular ion peak at m/z 123 $[M+H]^+$, which agreed with the molecular formula $C_7H_6O_2$. These data confirmed that compound **3** is *p*-hydroxy benzeldahyde.

Compound 4. It gives blue colour with $FeCl_3$ reagent, confirmed its phenolic character. The ^{1}H NMR spectrum of compound **4** showed singlet signal for two equivalent aromatic protons at δ: 7.15 (2H, s, H-2,H-6), and a singlet signal at δ: 3.81 (3H, s, OCH_3) for aliphatic methyl group.

Cochromatography with authentic sample of methyl gallate confirmed the identity.

Compound 5. It gives blue colour with $FeCl_3$ reagent, confirmed its phenolic character. The ^{1}H NMR spectrum showed a singlet signal at δ: 7.06 integrated for two equivalent aromatic protons, a signal at δ: 4.28 (q) for an aliphatic methylene neighboring to oxygen and a triplet at δ: 1.35 for an aliphatic methyl group.

^{13}C NMR spectrum showed carbon signals at δ: 14.6 and 61.6 for $–CH_2-CH_3$ system. The other signals are very close to those reported for galloyl moiety[11]. So, compound **5** was identifies as ethyl gallate.

Compounds 6 and 7. ^{1}H and ^{13}C NMR spectra showed an arbutin moiety which is acylated in C-6 of the sugar with a phenyl propanoid moiety.

In compound **6,** the phenyl propanoid moiety having α, β unsaturated carbonyl, with A_3–substituted ring (3,5-disubstituted benzene), showing signals at δ: 6.72 (2H, d, *J*= 1.2 Hz) and 6.93 (1H, t, *J*= 1.2 Hz). The presence of trans olefinic system was characterized through signals at δ: 7.97 and 6.54 (each 1H, d, J= 16.0 Hz, H-7 and H-8), respectively. The β-configuration of the sugar was confirmed from the *J* value of the anomeric proton (*J*= 7.5 Hz). The downfield shift of H-6 of the sugar at δ: 4.4 and 4.5 suggesting the possible acylation of C-6.

^{13}C NMR spectrum revealed the downfield shift of C-6 of the sugar part at δ: 64.7 and the upfield shift of C-5 at δ: 75.2 confirmed the acylation of C-6.

^{13}C NMR signals assigned for the sugar part and the aromatic carbons are similar to those of arbutin[2].

Compound 6 was identified as arbutin 6”-*O*-3,5-dihydroxycinnamic acid ester, which is isolated for the first time from the flowers of *Grevillea robusta* A. Cunn.

Compound 7 showed an arbutin moiety acylated at C-6 of the sugar part with a phenyl propanoid moiety as compound **6**. ^{1}H NMR spectrum showed that the trans olefinic protons of the phenyl propanoid moiety are more upfield at δ: 6.67 and 6.27 than the corresponding ones in compound **6**.

The ^{13}C NMR spectrum showed the presence of conjugated ester group at δ: 167.1, conjugated ketone group at δ: 185.8 and quaternary carbon at δ: 70.8.

The ^{13}C-^{1}H COSY spectrum showed that the quaternary carbon at δ: 70.8 was correlated to H-7, H-2 and H-6, so this signal is assigned to C-1 which bears also a hydroxyl group. It also showed that the signal for the carbonyl carbon at δ: 185.8 is correlated to H-3 and H-5 at δ: 6.80, so it is assigned for C-4.

FABMAS (pos.) analysis showed m/z at 457 $[M+Na]^+$ and 434 $[M]^+$, FABMAS (neg.) mode showed m/z at 433 $[M-H]^-$ and 417 $[M-OH]^-$. From these data compound **7** was assigned to be formed of two moieties, one is arbutin and the other is a phenyl propanoid, which has ketone group at C-4 and quaternary carbon at C-1.

The downfield shift of H-6 of the sugar at δ: 4.47 and 4.32 suggesting the possible acylation of C-6. The downfield shift of C-6 of the sugar part at δ: 64.9 and the upfield shift of C-5 at δ: 75.1 confirmed the acylation of C-6.

Compound 7 was identified as Robustaside D, which is previously isolated from the leaves of *Grevillea robusta* A. Cunn[2].

Compound 8 showed a typical A_2B_2 pattern at δ: 7.41 and 6.84 for *p*-substituted benzene ring, sharp singlet at δ: 5.90 for oxygen bearing methine proton, and a doublet of the anomeric proton at δ: 4.63 in the β configuration (J= 7.5 Hz). HSQC and HMBC were measured (Table 3). All these spectral data are similar to those reported for dhurin (s)[12], isolated previously from the leaves of *Grevillea robusta* A. Cunn.

Table 3: HMQC and HMBC data of compounds **8**.

^{1}H	^{13}C	Long range correlation
7.41	130.5	C-4, CHCN
6.85	116.5	C-4
5.90	68.3	C2,6, C-1, CN, C-1’

Conclusion

Eight secondary metabolites (**1-8**) (Fig. 1) were characterized from the ethyl acetate extract of the flowers of *Grevillea robusta* A. Cunn, including seven phenolic compounds (**1-7**) and a cyanogenic glycoside (**8**). Further phytochemical investigation and biological evaluations on *Grevillea robusta* A. Cunn are required for discovery new bioactive secondary metabolites that may be used as phytotherapeutic agents in treatment of different diseases.

Conflict of interests

Declared none.

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الفحص الكيميائي لمستخلص اسيتات الإيثيل لزهرة نبات الجريفليا روبستا أ.كان

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[١]قسم العقاقير ، كلية الصيدلة ، جامعة أسيوط ، أسيوط ٧١٥٢٦ ، جمهورية مصر العربية
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تـم تجزئـة مسـتخلص أسـيتات الإيثيـل القابـل للـذوبان مـن أزهـار *Grevillea robusta* A.Cunn المجففة بالهواء وتم الحصول على ثمانية مركبات نقية باستخدام تقنيات كروماتوغرافية مختلفة. تم تعريف التركيب الكيميائي للمركبات المفصولة بواسطة تحاليل الطيف النووي المغناطيسي ذات البعد الواحد وثنائي البعد والطرق الطيفية الأخرى ومقارنتها مع بيانات المركبات المفصولة سابقاً عن طريق المسح المرجعي . تـم تحديـد هـذه المركبـات علـى أنهـا ٣،٥-ثنـائي هيدروكسـي سـينامات (١) ، ٦-هيدروكسـي كومارين (٢) ، بارا هيدروكسي بنزالديهايد (٣) ، ميثيل جلات (٤) ، إيثيل جلات (٥) ، اربيوتين ٦"-*O*-٥،٣-ثنائي هيدروكسي سيناميات (٦) ، روبوستاسيد D (٧) ، مع جليكوسيد السيانوجين المسمى dhurin (s) (٨).

تهدف هذه الدراسة إلى مزيد من الاستقصاء الكيميائي لزهرة نبات *Grevillea robusta* لاكتشـاف المزيد من المركبات الثانوية الفعالة تتميز بمدى واسع من الأهمية الطبية.