

PHYTOCHEMICAL AND BIOLOGICAL STUDY OF GREVILLEA ROBUSTA A. CUNN CULTIVATED IN EGYPT

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تم هذا البحث تعريض الخلاصة الكحولية لأوراق وقلف نبات الجريفليا روبستا أ. كان المنزرع في مصر لفحص أولي لمعرفة تأثيره المضاد للإلتهابات لدى حيوانات التجارب باستخدام الكاراجينان وقد أظهرت النتائج الأولية أن كلا من الخلاصتين تثير واضح كمضادات للإلتهابات لدى حيوانات التجارب كما تم تعريض الخلاصة الكحولية والمائية للقف لفحص أولي لاختبار تأثيره على انزيم البروتياز وكذلك كل من خلاصات الكلوروفورم وخلات الإيثيل و ن البيوتانول لقف و ثمار النبات لفحص أولي لاختبار تأثيره على انزيم الريفيرس ترانسكريبيناز (الريفيرس ترانسكريبيناز والبروتياز انزيمات مرض نقص المناعة المكتسبة الأيدز) وقد أظهرت النتائج الأولية وجود فاعلية قوية لخلاصة خلات الإيثيل ومتوسطة ل ن بيوتانول ومنعدمة لخلاصة الكلوروفورم للقف على انزيم الريفيرس ترانسكريبيناز بينما أظهرت النتائج أن خلاصات الثمرة لها تأثير ضعيف على هذا الانزيم. وفي نفس الوقت أظهرت النتائج وجود متوسطة لكل من الخلاصة المائية والكحولية للقف على انزيم البروتياز. وقد تم فصل خمس مركبات من خلاصة الكلوروفورم للقف وهذه المركبات هي ثنائي ميثوكسي بينزوكينون والبيتا سيتوستيرول و (- هيدروكسي إيثيل) انيسول والروبستول والبيتا سيتوستيرول جلوكوزيد. كما تم فصل أربع مركبات من خلاصة ن بيوتانول وهي أربيوتين وأربيوتين ' - أ. - أبيوفورانوزيل و - هيدروكسي كحول فينيثيلي - - أ. - جلوكوبيرانوزيل (') - أبيوفورانوزيد و - بارا ترنس كومارويل (- جلوكوبيرانوزيل) - أ. - جلوكوبيرانوزيل (') - أبيوفورانوزيد وقد تم التعرف على هذه المركبات بدراسة خواصها الطبيعية والكيميائية والطيفية المختلفة.

The alcoholic extracts of the leaves and bark of Grevillea robusta A.Cunn cultivated in Egypt were subjected to preliminary anti-inflammatory screening. The results showed that, the alcoholic extracts showed a significant anti-inflammatory activity using carrageenan-induced edema. The alcoholic and aqueous extracts of the bark were tested as anti-HIV-1 protease, where they showed

a moderate HIV-1 protease (1 PR) inhibitory activity at conc. 200 µg/ml. The ethyl acetate fraction of the bark showed a significant HIV-RT inhibitory activity, the n-butanol fraction showed a moderate activity, while the chloroform fraction showed no activity at conc. 200 µg/ml. On the other hand the chloroform and ethyl acetate fractions of the fruit showed a weak HIV-RT inhibitory activity. From the chloroform-soluble fraction of the total alcohol extract of the bark 2,6-dimethoxy-1,4-benzoquinone, β-sitosterol, 4-(1-hydroxy ethyl) anisol, robustol and β-sitosterol glucoside were isolated. Four phenolic glycosides were isolated from the n-butanol-soluble fraction of the bark and identified as arbutin, arbutin 2'-O-β-apiofuranoside, 4-hydroxyphenethyl alcohol 8-O-β-apiofuranosyl(1"→2')- β-glucopyranoside and 4-O-trans-p-coumaroyl{β-glucopyranoside}8-O-β-apiofuranosyl-(1"→ 6')-O-β-glucopyranosyl. The identification of the isolated compounds was carried out using different methods including physical, chemical and spectral analysis.

INTRODUCTION

Genus *Grevillea* (F. Proteaceae) comprises about 190 species. Some of them yield useful timber. The natives of North-West Australia were observed to use the sap of two species of *Grevillea* to scarify their bodies. *G.robusta* A.Cunn (Australian silky - oak), also often called silver-oak, is used to line streets for shade purposes and in Los Angels "Grevillea poisoning" is well known to telephone linemen and pruners. It has been planted extensively in India and Sri Lanka as a shade for tea, and in Hawaii and Brazil as a shade for coffee.¹⁻³ Recently, *G.robusta* extract showed leishmanicidal activity.⁴

Previous study on the leaves, flowers and fruits of the plant resulted in isolation and identification of polyalkylphenols, cinnamic acid and arbutin derivatives, flavonoids, and

gallic acid derivatives.⁵⁻⁷ The presence of such biologically active compounds encourages the study of different extracts of the bark of the plant.

In this paper, upon fractionation of the total alcoholic extract of *Grevillea robusta* A.Cunn bark, nine compounds were isolated and identified.

EXPERIMENTAL

General

M.p and m.m.p on Stuart Scientific (SMP 1, England). IR spectra (KBr) were measured on JASCO FT/IR-5300 spectrophotometer. The ¹H and ¹³C-NMR spectra were obtained on JEOL, JMS (500, 300 MHz for ¹H and 125, 75 MHz for ¹³CNMR) using TMS as internal standard. EIMS spectra was obtained with a Jeol JMS- 500 T mass spectrometer. TLC analyses were

performed on silica gel 60 F₂₅₄ and RP-18 F₂₅₄ S (E. Merck, Darmstadt) precoated plates, detection was done by spraying with 10% H₂SO₄ followed by heating at 110°. For medium-pressure liquid chromatographic separation a LiChroprep C-18 (Merck) material was used. Silica gel 60 (60-120 mesh) and Diaion (Nippon Rensui Co.) were utilized for open column chromatography (CC). Sephadex LH-20 (Fluka) was used also for fine separations.

Analytical HPLC (for HIV-1 PR inhibitory activity): Shimadzu, Recorder: C-R6 Achromatopac, Pump: LC-9A. Detector: SPD-6A UV spectrophotometric. Column: RP col. (4.6x150 mm) (AP-302S-5 300A-ODS). Incubator: Bioshaker BR-30L. flow rate; 1ml/min. Solvent A: 1% TFA (trifluoroacetic acid in H₂O), Solvent B: CH₃CN. Range detector: 0.005.

The following solvent systems were used: CHCl₃-MeOH (4:1, 9:1), n-BuOH-Acetic acid-H₂O (4:1:2) and MeOH-H₂O (1:1, 3:2).

Chemicals

- A- Authentics: β-sitosterol, β-sitosterol glucoside, glucose, apiose, robustol, *p*-coumaric acid, hydro-quinone and arbutin, were obtained from Pharmacognosy Dept., Faculty of Pharmacy, Assiut University, Assiut, Egypt.
- B- HIV-RT (Eiken Chemicals Co.Ltd., Osaka, Japan), Peptide (Poly(rA)_n-p(dT)₁₂₋₁₈) (Pharmacia, Uppsala, Sweden), [methyl-3H]

thymidine 5'-triphosphate (dTTP) (Amersherdan, Tokyo, Japan), Adriamycin (Sigma Chemical Co., St. Louis, USA), Aquasol-2 (NEN Research Products, Boston, USA). HIV-1PR: As in reference [5].

Plant material

The bark, leaves and fruits of *Grevillea robusta* A.Cunn were collected from Assiut University garden on March 2003, and the plant was kindly identified by Prof. Dr. Ibrahim Hassan, Department of Horticulture, Faculty of Agriculture, Assiut University.

Materials for phytochemical study

1 kg of the air-dried bark of *Grevillea robusta* A. Cunn was powdered and extracted with methanol by maceration for 48 hours. This process was repeated for three successive times and the combined alcoholic extract was concentrated under reduced pressure till afforded a syrupy dark residue (100 g).

Fractionation and isolation

The dried alcohol extract (70 g) was mixed with water and partitioned between n-hexane (1Lx3), CHCl₃ (1Lx3), EtOAc (1Lx3) and n-butanol (1Lx3). The combined extract of each solvent was evaporated to dryness under vacuum to give hexane (5 g), CHCl₃ (20 g), EtOAc (13 g) and n-butanol (25 g).

Ten grams of the dried chloroform-soluble fraction was fractionated over silica gel column chromatography (400 g) using gradient elution with CHCl₃-EtOAc

mixture as a solvent system. Fractions (100 ml, each) were collected and monitored with TLC, similar fractions were combined. Fraction **C-1** (50 mg) eluted with CHCl_3 , where an orange crystals was obtained on repeated crystallisation from CHCl_3 (compound **1**, 20 mg). Fraction **C-2** (400 mg) eluted with CHCl_3 -EtOAc (95:5) upon repeated crystallization from CHCl_3 , afforded β -sitosterol (compound **2**, 300 mg). Fraction **C-3** (200 mg) eluted with CHCl_3 -EtOAc (9:1) when subjected to silica gel column chromatography using CHCl_3 -MeOH (9:1) gave compound **3** (30 mg). Fraction **C-4** (100 mg, CHCl_3 -EtOAc, (85:15) when subjected to sephadex LH-20 column chromatography afforded robustol^{5&6} (compound **4**, 20 mg) and other spots. These spots (minors) which attained orange colour after spraying with H_2SO_4 (10%), have the same R_f value as polyalkyl phenols previously isolated from the leaves.^{5&6} Fraction **C-5** eluted with CHCl_3 -EtOAc (8:2) afforded β -sitosterol glucoside (compound **5**, 100 mg) upon rechromatography on silica gel column using benzene-acetone mixture in a gradient manner.

Part of the dried n-butanol-soluble fraction (15 g) was subjected to a column of Diaion-HP-20 and eluted with water, 50% MeOH, 100% MeOH and acetone respectively. The 50% MeOH fraction (7 g) was chromatographed on silica gel using CHCl_3 -EtOAc gradient as a solvent system to give two groups of fractions. Fraction **B-1** (100 mg) was

eluted with CHCl_3 -EtOAc (8:2) subjected to LiChroprep RP-18 using MeOH 50%, to afford compound **6** (20 mg). Fraction **B-2** (900 mg) was eluted with CHCl_3 -EtOAc (2:8) and chromatographed on silica gel column using CHCl_3 -MeOH gradient as eluent to give three sub-fractions (F **B-2-1**, F **B-2-2** and F **B-2-3**). F **B-2-1** [CHCl_3 -MeOH (85:15), 100 mg] was subjected to sephadex column chromatography using MeOH, followed by silica gel column chromatography using CHCl_3 -MeOH (4:1) to give compound **7** (15 mg). F **B-2-2** [CHCl_3 -MeOH (85:15), 500 mg] was subjected to sephadex column chromatography using MeOH, followed by silica gel column chromatography using benzene-MeOH (3:1) to afford compound **8** (50 mg). F **B-2-3** [CHCl_3 -MeOH (8:2), 300 mg] gave brown gummy residue which signed as compound **9** (70 mg).

TLC of the EtOAc-soluble fraction revealed the presence of many inseparable spots most of them are similar to those present in the leaves.^{5&6} The isolation of these compounds is in progress now.

Compound 1 obtained as an orange crystals (20 mg), m.p 248-250°. Molecular formula $\text{C}_8\text{H}_8\text{O}_4$ from the EIMS m/z 168 $[\text{M}]^+$. Other peaks at m/z (% rel.int.), 140 (2), 137 (2.3), 109 (3.6), 108 (1), 80 (3.3) and 28 (100). Possible fragmentation pattern was shown in (Fig. 2).

IR (KBr): 3050, 2945, 1690, 1640, 1452, 1385, 1338, 1251, 1215, 1102 and 869 cm^{-1} . $^1\text{H-NMR}$ (CDCl_3 , 500

MHz): 3.8 (6H, s, OCH₃ X 2) and 5.8 (2H, s, H-3,5). ¹³C-NMR (CDCl₃, 125 MHz): δ 56.5 (OCH₃ X 2), 107.5 (C-3,5), 157.4 (C-2,6), 176.9 (C-1) and 186.9 (C-4).

Compound 2 obtained as white needle crystals (methanol), m.p 135-137°. IR (KBr): 3440, 3050, 1636 and 1075 cm⁻¹. It gave positive Libermann-Burchards test.

Compound 3 obtained as yellowish powder. ¹H-NMR (CD₃OD, 500 MHz): δ 6.9 (2H, *d*, J= 8.6 Hz, H-2,6), 6.7 (2H, *d*, J= 8.6 Hz, H-3,5), 4.6 (1H, *m*, H-7), 0.9 (3H, *d*, J= 6.8 Hz, CH₃), OCH₃ (obscured under solvent peak). ¹³C-NMR (CD₃ OD, 125 MHz) (Table 1).

Compound 4 obtained as white needle crystals, m.p 143-144°. IR (KBr): 3440, 1615, 1228, 1201 and 1135 cm⁻¹.

Compound 5 obtained as white needle crystals, m.p 270-280°. IR (KBr): 3415, 2960, 1636 and 1075 cm⁻¹. It gave positive Libermann-Burchards test.

Compound 6 obtained as white needle crystals, m.p 199-200°. ¹H-NMR (CD₃OD, 500 MHz) 6.9 (2H, *d*, J=9.5 Hz, H-2,6), 6.6 (2H, *d*, J=9.5 Hz, H-3,5), 4.7 (1H, *d*, J=8 Hz, H-1' glc). The other sugar protons appear at δ 3.3-4.4.

Compound (7) obtained as brownish residue. ¹H-NMR (CD₃OD, 500 MHz): δ 6.9 (2H, *d*, J= 8.7 Hz, H-2,6), 6.6 (2H, *d*, J= 8.7 Hz, 3,5), 5.4 (1H, *d*, J= 1.2 Hz, H-1" apiose), 4.7 (1H, *d*, J= 8.2 Hz, H-1' glc), 3.98 (1H, *d*, J= 9.5 Hz, H-4" a apiose), 3.70 (1H, *d*, J= 9.5 Hz, H-4" b apiose). The

other sugar protons appear at δ 3.3-4.5. ¹³C-NMR (CD₃OD, 75 MHz) (Table 1).

Compound 8 obtained as brownish residue. ¹H-NMR (CD₃OD, 500 MHz): δ 6.9 (2H, *d*, J= 8.7 Hz, H-3,5), 6.6 (2H, *d*, J= 8.7 Hz, H-2,6), 5.4 (1H, *s*, H-1" apiose), 4.5 (1H, *d*, J= 7.2 Hz, H-1' glc.), 4.2 (2H, *m*, H-8), 3.3 (2H, *m*, H-7). The other sugar protons appear at 3.3-4.4 ¹³C-NMR (CD₃OD, 125 MHz) (Table 1).

Compound 9 obtained as brownish yellow residue, ¹H-NMR (CD₃OD, 500 MHz) 8.1 (1H, *d*, J= 16.8 Hz, H-7), 7.1 (2H, *d*, J= 9.2 Hz, H-2,6), 6.6 (2H, *d*, J= 9.2 Hz, H-3,5), 6.4 (1H, *d*, J= 16.8 Hz, H-8), 5.4 (1H, *s*, H-1" apiose), 4.64 (1H, *d*, J= 7.5 Hz, H-1' glc.), 4.52 (1H, *d*, J= 7.8 Hz, H-1" glc.), 4.05 (1H, *d*, J= 9.5 Hz, H-4" a apiose), 3.76 (1H, *d*, J= 9.5 Hz, H-4" b apiose). The other sugar protons appear at 3.3-4.5 ¹³C-NMR (CD₃OD, 125 MHz) (Table 1).

Acid hydrolysis

Compound 7 was subjected to partial acid hydrolysis using 5% methanolic HCl for 3 hours, small samples were withdrawn at 10 min. intervals from the reaction mixture and spotted on TLC and PC alongside with authentic samples.

For complete acid hydrolysis five mg of each glycoside was refluxed with 2N methanolic HCl for 3 hours. The reaction mixture was diluted with water and the aglycone was extracted with ether. The aqueous layer was neutralized with barium carbonate, filtered and evaporated under

vaccum. The residue was dissolved in MeOH for identification.

Preliminary biological screening

Preparation of the extract for anti-inflammatory study

Twenty grams of each of the powdered leaves and bark were separately extracted with 90% MeOH till exhaustion. The alcohol extract of each organ was separately evaporated under reduced pressure yielding 3 g and 2 g respectively. The residue of both leaves and bark were separately dissolved in 2% Tween-80 to obtain 400 and 200 mg/kg concentration of each.

The anti-inflammatory activity of the alcoholic extracts of *Grevillea robusta* A. Cunn. bark and leaves was performed. Six groups, each of four male albino rats (150-170 g) were used. At the beginning of the experiment edema was induced in rats by backpaw sub-planter injection of 0.4 ml of 1% carrageenan suspension⁸ by injection into right paw. The volume displacement was measured in mm by using Varinier calibrator.

The plant extracts, indomethacin (8 mg/kg, as a positive control) and 2% tween-80 (in saline, as a negative control) were taken orally 30 minutes before testing of paw swelling. Paw volumes were assessed just after carrageenan injection and 30, 60, 120, 180 and 240 min. afterwards. The inflammatory response is represented as the percentage of paw edema inhibition, calculated for each group at each time point, using the following equation:

$$\% \text{ Inhibition} = \frac{(V_t - V_o)_{\text{control}} - (V_t - V_o)_{\text{treated}} \times 100}{(V_t - V_o)_{\text{control}}}$$

Where V_t is the average volume at each time point and V_o is the average volume just after carrageenan injection. The results are illustrated in Tables (4 and 5).

Preparation of the extract for HIV-1PR and RT inhibitory test⁵

50 g of each of the powdered fruits and bark was separately extracted with water and methanol under reflux for 3 hr. The extracts were filtered and evaporated under reduced pressure. The dried extracts were separately prepared to give solution of 2 mg/ml. The sample solution was used in the reaction mixture for determination of HIV, HIV-1PR and HIV-RT inhibitory activity.

The reaction mixture for HIV-1PR inhibitory activity

- PR enzyme: dissolved in (50 mM NaOAc, pH: 5.02, 1 mM EDTA.2Na, 2 mM mercaptoethanol): glycerol [75:25].
- Substrate: peptide dissolved in 50 mM NaOAc (pH: 5.02).
- Buffer solution: 50 mM NaOAc (pH: 5.02).

Method of assay of HIV-1PR inhibitory activity⁵

1 μ l of buffer was placed in a tube and centrifuge. 1 μ l of the substrate and 1 μ l of the sample were transferred in the same tube (centrifuge and mix after each addition). 2 μ l of the enzyme was added on the wall of

the tube (without mixing), until finishing the addition of the enzyme to all tubes, centrifuge, mix and recentrifuge. All the tubes were incubated at 37° for 1-2 hours. The reaction was stopped by heating the tubes in water bath at 90° for 1 min. 35 µl of distilled water was added, centrifuge, mix and centrifuge again, transfer 40 µl of the reaction mixture to HPLC glass tubes and put them in HPLC for analysis as in reference 5 and references cited herein.

The reaction mixture for HIV-RT inhibitory activity

50 mM Tris-HCl (pH 8.3), 30 mM NaCl, 10 mM MgCl₂, 5 mM DTT, 5 µg/ml template-primer (rA)_n P (dT)₁₂₋₁₈. 0.1 mM methyl-[³H]dTTP (18.5 MBq/ml) and 0.5 U of RT enzyme in a final volume of 20 µl.

Method of assay of HIV-1RT inhibitory activity⁵

The reaction mixture was prepared (except the addition of the enzyme). The plant extract was dissolved in DMSO and 1 µl of it was added to the reaction mixture, which was incubated for 30 min at 37°. The reaction was stopped by immersion in ice and addition of 0.2 M EDTA. 10 µl of the reaction mixture was applied to DEAE-cellulose discs. Wash the discs with 5% Na₂HPO₄ (5x3 ml), distilled water (2x3 ml), 99% ethanol (2x3 ml) and finally with ether (1x3 ml).

% inhibition was calculated through the equation

% inhibition =

$$[1 - (\text{dpm test}/\text{dpm control})] \times 100$$
 dpm= disintegration per min

Control assay is performed by adding DMSO (final conc. 5%) containing no sample. 0.5 mM Adriamycin is used as a positive control, which inhibited the enzyme activity by 99.0%.

Direct HIV-1 inhibitory activity reagent and method of screening as mentioned in literatures^{5&9}

The results for HIV-1, HIV-1 PR and RT inhibitory activities were represented in Tables (2 and 3).

Table 1: ¹³C-NMR data of compounds **3,8,9** (CD₃OD, 125 MHz) and **7** (CD₃OD, 75 MHz).

C	3	7	8	9
1	154.2	132.40	131.0	125.0
2,6	117.0	119.42	132.1	135.3
3,5	128.1	116.69	117.7	117.1
4	133.0	152.34	151.1	152.7
7	70.4		37.8	140.5
8	14.2		71.6	115.0
C=O				167.6
Glc-1				
1'		102.24	100.9	102.4
2'		77.94	77.3	74.09
3'		78.12	76.7	77.3
4'		71.52	70.1	70.1
5'		78.77	77.3	76.6
6'		62.56	61.1	63.4
Apiose				
1''		110.76	109.0	109.4
2''		78.64	76.6	76.5
3''		80.74	79.5	79.4
4''		75.43	74.1	73.6
5''		66.70	64.7	64.7
Glc-2				
1'''				100.8
2'''				74.03
3'''				76.73
4'''				70.2
5'''				76.75
6'''				61.1
OCH ₃	59.5			

Table 2: HIV-1 RT inhibitory activity of different fractions of the methanol extract of *Grevillea robusta* A. Cunn. bark and fruit.

Fraction	% inhibition		
	Bark		Fruit
	(100 µg /ml)	(200 µg /ml)	(200 µg /ml)
CHCl ₃	NI	NI	6.8±2.3
EtOAc	NI	86.1± 6.9	22.7±8.2
BuOH	NI	47.7 ± 5.8	NT

NI= no inhibition

NT= not tested

Table 3: HIV-1 PR inhibitory activity and anti-HIV activity of methanol and aqueous extracts of *Grevillea robusta* A. Cunn. bark.

Extract	% inhibition		Anti-HIV	
	200 µg/ml	100 µg/ml	IC	CC
MeOH	47.3±1.2	10.5±1.2		
H ₂ O	46.7±7.6	Not tested	500	1000

IC= the minimum concentration for complete inhibition of HIV-1 induced CEP in MT-4 cells by microscopic observation.

CC= the minimum concentration for the appearance of MT-4 cell toxicity by microscopic observation.

Table 4: Anti-inflammatory effect of the alcoholic extracts of *G. robusta* A.Cunn bark and leaves.

Group	Thickness of the right paw (mm)(mean ±S.E.)					
	zero	½ hr	1 hr	2 hr	3hr	4hr
-ve control	7.5±0.016	7.5±0.03	7.5±0.016	8.2±0.04	8.4±0.016	8.5±0.016
Indomethacin	7.0±0.01	6.5±0.012	5.8±0.016**	5.3±0.028**	5.3±0.033**	4.6±0.033**
Bark						
200 mg/kg	7.0±0.028	6.6±0.016	6.5±0.013	6.5±0.028	5.7±0.028**	6.6±0.033
400 mg/kg	6.8±0.016	6.3±0.033	6.3±0.025*	6.3±0.016*	5.6±0.033**	5.6±0.014**
Leaves						
200 mg/kg	6.8±0.016	6.8±0.017	6.3±0.016*	5.6±0.016**	5.5±0.018**	6.0±0.017**
400 mg/kg	6.2±0.016	6.1±0.017*	6.1±0.017*	5.5±0.019**	5.56±0.014**	5.5±0.016**

T-test (*p<0.05, **p<0.01)

Table 5: % of edema inhibition of the alcoholic extracts of *G. robusta* A. Cunn bark and leaves.

Group	% of inhibition					
	zero	½ hr	1 hr	2 hr	3 hr	4 hr
-ve control	-	-	-	-	-	-
Indomethacin	7	13	23	35	37	46
Bark						
200 mg/kg	7	12	13	21	32	22
400 mg/kg	9	16	16	23	33	33
Leaves						
200 mg/kg	9	9	16	32	35	29
400 mg/kg	17	17	19	33	35	35

RESULTS AND DISCUSSION

The dried bark of *Grevillea robusta* A.Cunn was extracted with MeOH, and the extract was fractionated with n-hexane, CHCl₃, EtOAc and n-butanol. From the CHCl₃-soluble fraction five compounds were isolated using different types of chromatography. The n-butanol-soluble fraction was applied on a column of Diaion-HP 20 and eluted with water, 50% MeOH, 100% MeOH and acetone. From the 50% MeOH, four compounds were isolated.

Compound 1 was obtained as an orange crystals. Its molecular formula was established as C₈H₈O₄ on the basis of EIMS which showed molecular ion peak [M⁺] at m/z 168. ¹³C-NMR data of compound **1** showed five signals equivalent to eight carbon atoms including four quaternary at δ_C 186.9, 157.4 (2C) and 176.9, two methine carbon atoms

at δ_C 107.5 (2C) and two methoxy at δ_C 56.5 (2C). The ¹H-NMR spectral data, showed only two signals at δ_H 5.8 (2H, s, H-3,5), and at δ_H 3.4 (6H, s, OCH₃ X 2).

The above mentioned data indicated the presence of 1,4 benzoquinone derivatives where the signal at δ_C 186.9 is characterized for the carbonyl carbon at C-4, while the signal at δ_C 176.9 corresponding to another carbonyl carbon at C-1. The possible fragmentation pattern of compound **1** (Fig. 2) was in a good agreement with the proposed structure. From the above mentioned data, the compound was identified as 2,6-dimethoxy-1,4-benzoquinone. This compound was reported for the first time from the family Proteaceae, and it is previously isolated from the wood of *Kielmeyera rupestris* A.P.Duarte¹⁰ and from the root of *Heliicteres angustifolia* L.¹¹

1,4 benzoquinone is harmful to skin and eyes, causing dermatitis and conjunctivitis. It is used as an oxidizing agent in photography and in manufacture of dyestuff. 2,6-dimethoxy benzoquinone has been reported as an anticancer agent.¹²

Compound 2 was obtained as white needle crystals, and it was identified as β -sitosterol by direct comparison with authentic sample (m.m.p and co-chromatography).

Compound 3 was obtained as yellowish powder. The ¹H-NMR spectrum of **3** exhibited the characteristic signals belong to the 1,4- disubstituted benzene ring as an AB system at δ 6.9 (2H, *d*, *J*= 8.6 Hz, H-2,6), 6.7 (2H, *d*, *J*= 8.6 Hz, H-3,5), one methine proton at δ 4.6 (1H, *m*) and one methyl group at δ 0.9 (3H, *d*, *J*= 6.8 Hz, CH₃). The ¹³C-NMR spectral data showed signals for AB-spin system at δ 128.1 and 117.0, methine carbon at δ 70.4, methyl carbon at 14.2 and aromatic methoxy group at δ 59.5. Usually the connection of benzene ring to oxygen atom results in a downfield shift of the connected carbon,⁶ in compound **3** one of the substituted carbons appears at δ 133.0 indicating its connection to another carbon not to oxygen atom, while the second substituted carbon appear at δ 154.2 indicating its connection to an oxygen atom. Comparing these data to that of anisol and *p*-cresol,¹³ compound **3** was suggested to has the structure showed in Fig. 1 and was supposed to be 4-(1-hydroxy ethyl) anisol.

Compound 4 was obtained as white needle crystals, The IR spectrum showed signals for hydroxy group at 3440 cm⁻¹, aromatic ring at 1615 cm⁻¹ and ether linkage at 1228, 1201 and 1135 cm⁻¹. It was identified as robustol by direct comparison with authentic sample (m.m.p and co-chromatography).

Compound 5 was obtained as white needle crystals, and it was identified as β -sitosterol glucoside by direct comparison with authentic sample (m.m.p and co-chromatography).

Compound 6 was isolated as white needle crystals. The ¹H-NMR spectral data, showed signals at δ _H 6.9 (2H, *d*, *J*= 9.5 Hz, H-2,6) and 6.6 (2H, *d*, *J*= 9.5 Hz, H-3,5) suggesting the presence of aromatic protons as an AB-spin system. The presence of signal at δ _H 4.7 (1H, *d*, *J*= 8 Hz, H-1' glc) with the other signals at δ _H 3.3-4.4, indicating the glycosidic nature of this compound. The large *J* value of the anomeric proton indicating the β -configuration of the sugar. The ¹H-NMR spectrum was similar to that of arbutin.^{5&6} Acid hydrolysis of compound **6** afforded aglycone identified as hydroquinone (m.p 171°, co-chromatography, system 2) and one sugar identified as glucose (PC, system 3). The structure of compound **6** was confirmed by co-chromatography with authentic sample of arbutin using system 1. Therefore, compound **6** was presumed to be arbutin.

Compound 7 was isolated as brownish residue. The ¹H-NMR

spectral data were similar to those of arbutin δ_{H} 6.9 (2H, *d*, $J= 8.7$ Hz, H-2,6), 6.6 (2H, *d*, $J= 8.7$ Hz, 3,5), with an additional anomeric proton at δ_{H} 5.4 (1H, *d*, $J=1.2$ Hz, H-1" apiose). ^{13}C -NMR spectrum showed the presence of 15 carbon signals for 17 carbons, four of them were assigned for *p*-hydroquinone. The rest of the carbons were assigned to pentose (apiose) and hexose (glucose) sugars (Table 1). The attachment of apiose to glucose was deduced to be in C-2' from the downfield shift of C-2' (3.5 ppm). Mild acid hydrolysis of **7** yielded a sugar identified as apiose (PC, system 3) and arbutin (co-chromatography, system 1). Complete acid hydrolysis gave aglycone identified as hydroquinone (co-chromatography, system 2) and two sugars identified as glucose and apiose (PC, system 3). Finally, comparing the chemical shift of anomeric proton of apiose with that reported for arbutin 2'-*O*- β -apiofuranoside and arbutin 6'-*O*- β -apiofuranoside,¹⁴ the structure of compound **7** was elucidated to be 2' -*O*- β -apiofuranosyl arbutin. This is the first report for the isolation of this compound from family Proteaceae.

Compound 8 was obtained as brownish residue. The NMR data showed signals at δ_{C} 100.9 with δ_{H} 4.5 and δ_{C} 109.0 with δ_{H} 5.4 corresponding to anomeric carbons and protons of β -glucopyranosyl and β -apiofuranosyl moieties respectively.

In the ^1H -NMR spectra, the signals due to the two methylene protons displayed at δ 3.3 and 4.2,

besides, signals at δ 6.6 and 6.9 (AB-spin system, each 2H, *d*, $J= 8.7$ Hz, H-2, 6 and H-3, 5) suggesting the presence of *p*-hydroxyphenethyl alcohol moiety.

The ^{13}C -NMR spectral data showed signals for two methylenes at δ 37.8 and 71.6 and *p*-hydroxyphenyl at δ 131.0, 132.1, 117.7 and 151.1 confirmed the presence of *p*-hydroxyphenethyl alcohol moiety as aglycone part of this compound. The β -configuration of apiose was confirmed from the chemical shift of C-1" in ^{13}C -NMR spectra.¹⁵

These data indicated the presence of salidroside (*p*-hydroxy phenethyl- β -glucopyranosyl) in addition to apiosyl moiety. The attachment of apiose to C-2' of glucose was determined by downfield shift (δ 2.2 ppm) of C-2' of glucose (appeared at δ 77.3 ppm) when compared with salidroside.¹⁶

So, the structure of compound **8** was supposed to be 4-hydroxy phenethyl alcohol 8-*O*- β -apiofuranosyl (1" 2') - β -glucopyranoside. This is the first report for the isolation of this compound from family Proteaceae.

Compound 9 was obtained as brownish yellow residue. The ^1H -NMR spectrum of **9** exhibited the characteristic signals belong to the *trans p*-coumaroyl four aromatic protons as an AB system at δ 7.1 (2H, *d*, $J= 9.2$ Hz, H-2,6), 6.6 (2H, *d*, $J= 9.2$ Hz, H-3,5), and two olefinic protons signals at δ 8.1 (1H, *d*, $J= 16.8$ Hz, H-7), 6.4 (1H, *d*, $J= 16.8$ Hz, H-8) indicating the *trans*

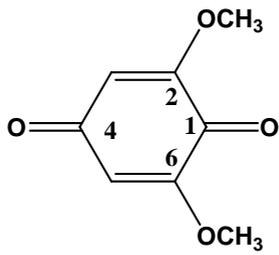
configuration of the *p*-coumaroyl moiety.

Additionally, the three anomeric proton resonances appeared at δ_{H} 4.64 (1H, *d*, $J = 7.5$, H-1' glc.), 4.52 (1H, *d*, $J = 7.8$, H-1'' apiose) and 5.4 (1H, *s*, H-1''' apiose), indicating the triglycosidic structure. The ^{13}C -NMR spectroscopic data confirmed the triglycosidic structure of **9** exhibiting three anomeric carbon resonances at δ_{C} 109.4, 102.4 and 100.8. The connection of sugar moieties was proved to be as in Fig. 1, these results were supported by a mild and complete acid hydrolysis of **9** yielded two sugar moieties, and aglycone. The sugars were identified as glucose and apiose (PC, system 3) using authentic sugars and the aglycone identified as *p*-coumaric acid (co-chromatography, system 2). The relatively large *J* values of anomeric protons indicated β -configurations for both glucopyranosyl moieties. The proton and carbon chemical shifts due to the apiose unit indicated that, it has to be in the terminal position.¹⁵ The carbon resonances arising from the two glucose moieties revealed that one glucose unit was glycosylated at C-1' (δ_{C} 102.4) and C-6' (δ_{C} 63.4), while the second glucose unit at C-1''' (δ_{C} 100.8). The attachment of the second glucose unit to C-4 of *p*-coumaroyl moiety was supported by the slight upfield shift of C-4 (δ_{C} 152.7) and the down field shift of C-3,5 (δ_{C} 117.1).⁶

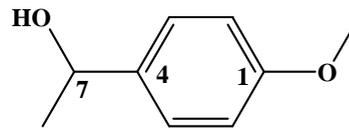
The ^{13}C signals assigned to the aglycone moiety were almost the same as that reported for the *trans p*-coumaroyl moiety.⁶ The ^{13}C -NMR shift of the anomeric carbon of the apiofuranosyl unit at (δ_{C} 109.4) indicating β configuration.¹⁵

HMBC experiment permitted the determination of all the relevant interfragmental connectivities. Thus cross peaks were observed between carbonyl carbon (δ_{C} 167.6) of the *p*-coumaroyl moiety and the H-1' (δ_{H} 4.57) of the first glucose unit, C-4 of *p*-coumaroyl moiety (δ_{C} 152.7) and the H-1''' (δ_{H} 4.64) of the second glucose unit, C-6' (δ_{C} 63.4) of the first glucose unit and H-1'' of the apiose (δ_{H} 5.4). Finally, alkaline hydrolysis of compound **9** yielded two sugars identified as glucose and apiose. Based on these results, the structure of compound **9** was established as 4-*O-p*-coumaroyl{ β -glucopyranoside}-8-*O*- β -apiofuranosyl-(1'' 6')}-*O*- β -glucopyranosyl. This is the first report for the isolation of this compound from family Proteaceae.

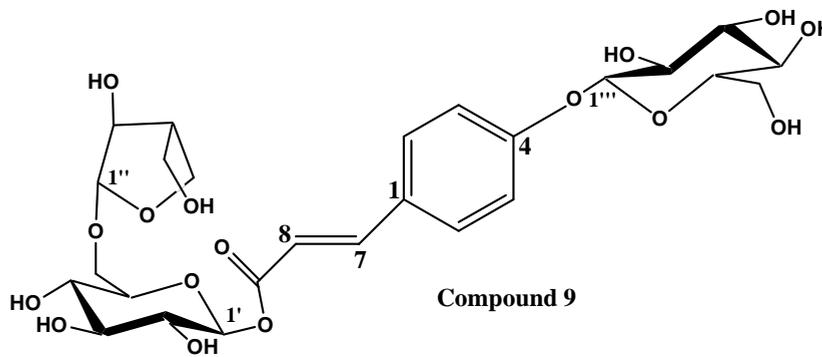
The isolated compounds having a phenolic nucleus were previously isolated from many plants belonging to family Proteaceae, from the taxonomic point of view it is interesting to make a scheme for the possible biogenetic pathway of the isolated compounds as illustrated in Fig. 3.



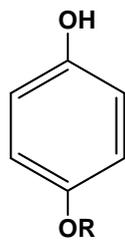
Compound 1



Compound 3

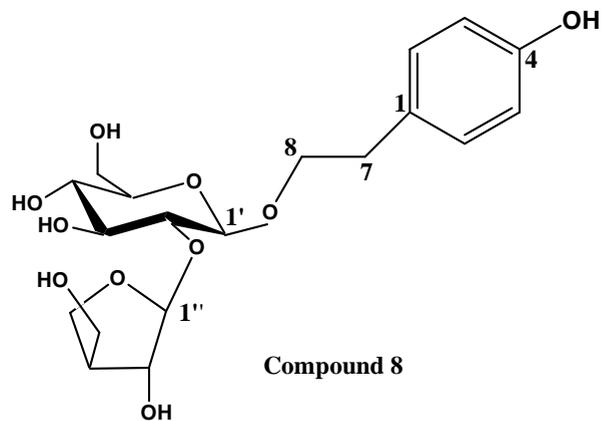


Compound 9



Compound 6, R= Glucose

Compound 7, R=Apiose (1''-2')-glucose



Compound 8

Fig. 1: List of the isolated phenolic compounds from *Grevillea robusta* A. Cunn bark.

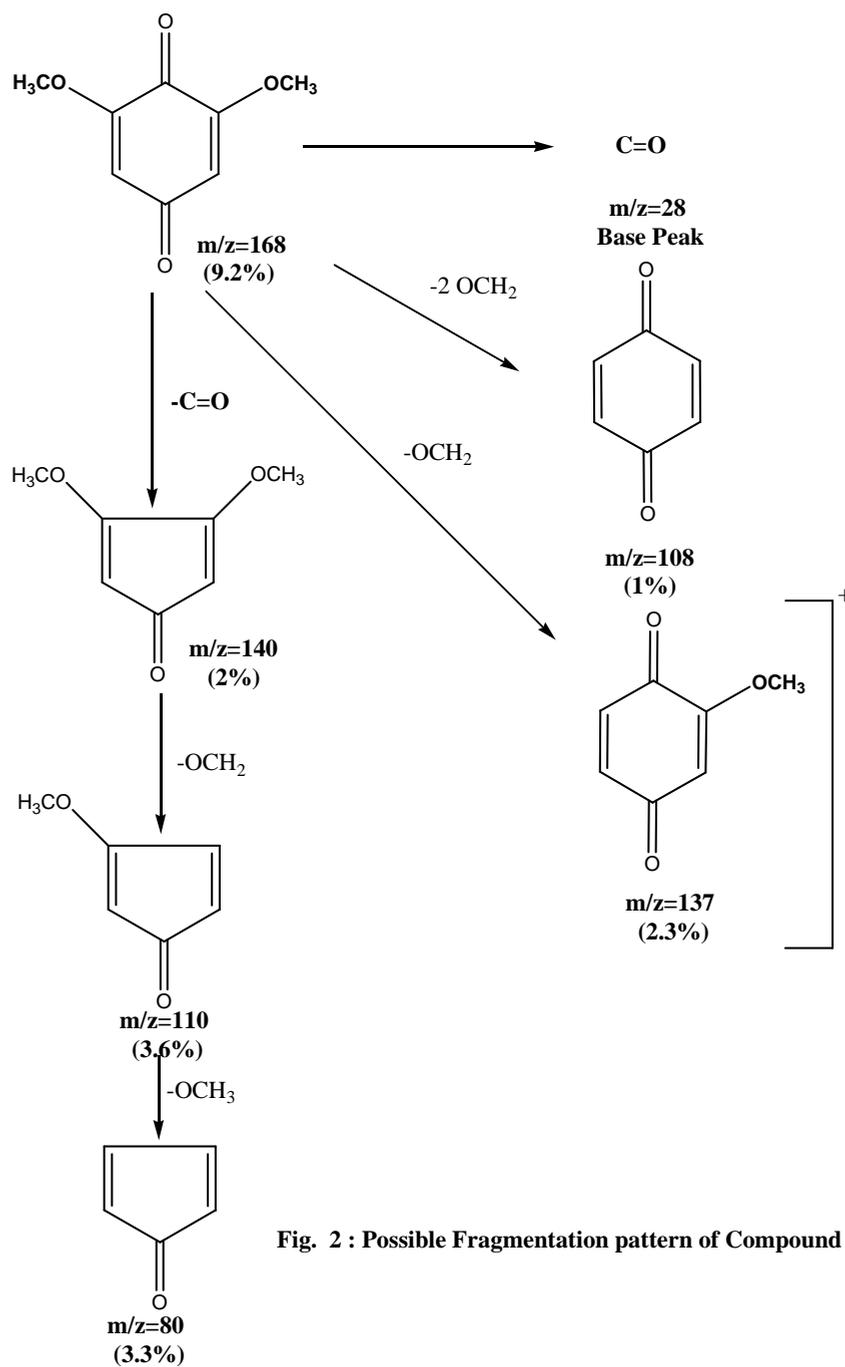


Fig. 2 : Possible Fragmentation pattern of Compound 1

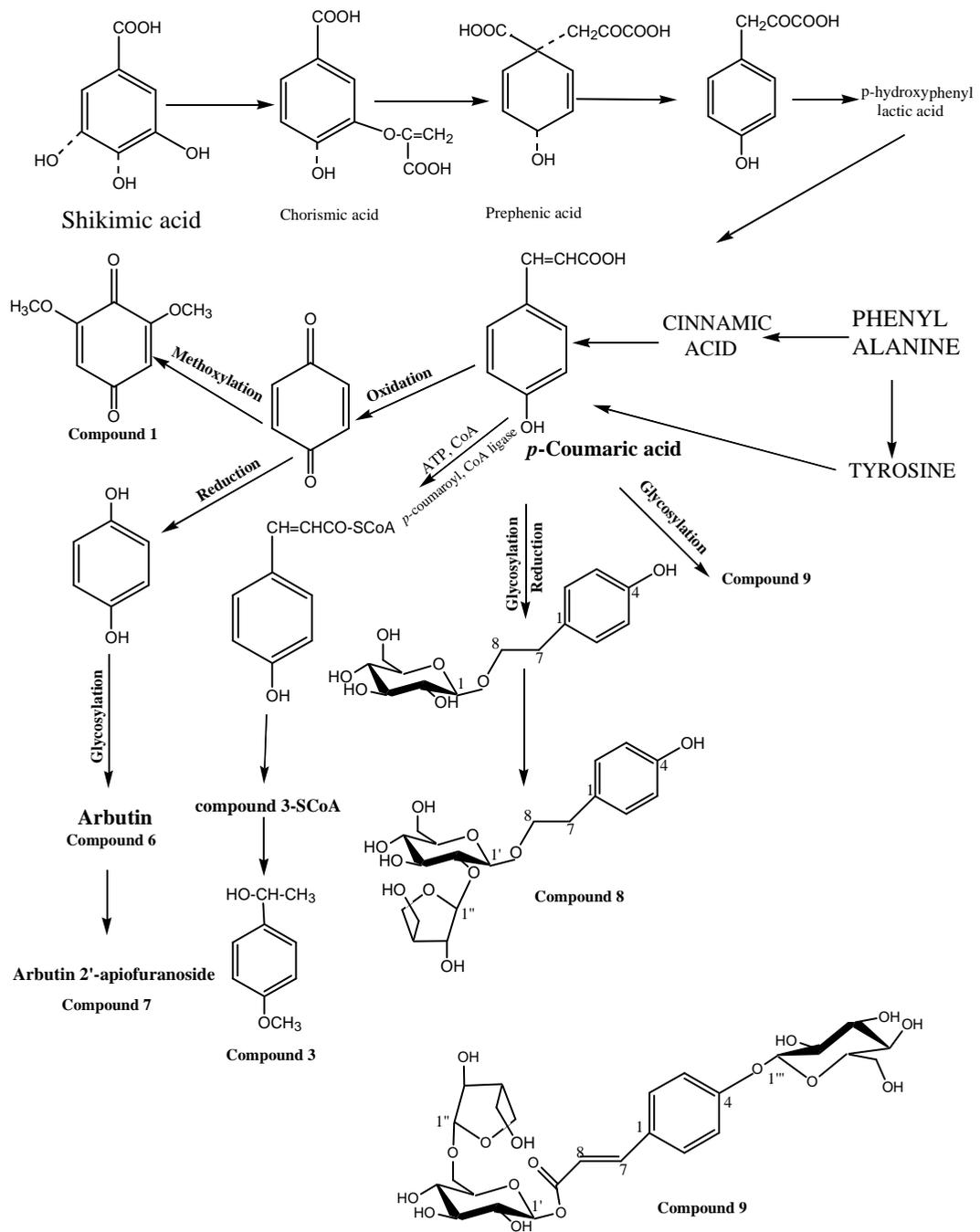


Fig. 3: Possible biogenic pathway of the isolated compounds.

Antiinflammatory effect

Effects of *Grevillea robusta* A.Cunn alcoholic extracts of the leaves and bark and indomethacin on carrageenan induced inflammation were represented as time course of edema (Table 4). At 400 mg/kg – dose level, results indicated that the tested extracts caused a marked decrease in edema when compared to control group. The anti-inflammatory activity of the alcoholic extract of the bark (400 mg/kg) reach its maximum after 2 hours, and be approximately similar to that of indomethacin. While the alcoholic extract of the leaf (400 mg/kg) has a maximum activity after 3 hours.

Inhibitory effects of the extracts and fractions on HIV and its enzymes

Investigation of anti HIV, 1 PR and RT inhibitory activities of the extracts and fractions of *Grevillea robusta* A. Cunn. bark and fruits indicated that some with high activities, while others with moderate activities (Tables 2 and 3). In the primary screening test for anti HIV of the aqueous extract of *Grevillea robusta* A. Cunn. bark, it was found that the extract inhibit HIV-1 induced cytopathic effect (CPE) in MT-4 cells with a 100% inhibitory concentration (IC) value 500 µg/ml, and the IC value was half of the respective cytotoxic concentration (CC) value 1000 µg/ml. As for HIV-1 PR inhibitory effects, the PR activity was determined by analyzing the reaction mixture in the presence or absence of

the two extracts using HPLC method. Both extracts were found to have moderate HIV-1 PR inhibitory effect (Table 3). From Table (2) it is concluded that the EtOAc-soluble fraction of *Grevillea robusta* bark showed the highest HIV-1 RT inhibitory activity (86.1 ± 6.9) at conc. 200 µg/ml, while the butanol fraction showed a moderate activity (47.7 ± 5.8).

From Table (3) it can be concluded that the methanol and aqueous extracts of the bark have a moderate HIV-1PR inhibitory activity.

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REFERENCES

- 1- Y. M. L. Sharma, Myforest, Forest Department Mysore, 3, 35 (1966).
- 2- R. J. Streets, "Exotic Forest Trees in British Commonwealth". Clarendon Press, Oxford., 1962, p. 765.
- 3- Y. R. A. Rao, Bangalore, 25, 329 (1961).
- 4- M. Takahashi, H. Fuchino, M. Satake, Y. Agatsuma and S. Sekita, Biol. Pharm. Bull, Jun 27, 921 (2004).
- 5- A. S. Ahmed, Ph. D. thesis, Faculty of Pharmacy, Assiut University (1997).

- 6- A. S. Ahmed, N. Norio, R. M. Meselhey, A. M. Makboul, El. E. Nasr. and H. Masao, *Phytochemistry*, 53, 149 (2000).
- 7- A. S. Ahmed, *Bull. Fac. Pharm. Cairo Uni.*, 40, 245 (2002).
- 8- V. Emir, F. Manuel, G. D. Jesús, G. Antonio and B. Jaime, *Planta Med.*, 60, 395 (1994).
- 9- O. Torku, M. Haruyo, M. Motoko, U. Nboru, T. Ines, A. Yasmina, M. Hirotsugu, H. Masao, N. Tsuneo, G. Mahabir and C. Mireya, *J. Tradi. Med.*, 11, 188 (1994).
- 10- C. D. Barros, E. L. G. Fonseca, O. R. Gottlieb and S. J. Goncalves, *Phytochemistry*, 9, 447 (1970).
- 11- T. Higuchi, C. Satake and M. Hirobe, *J. Am. Chem. Soc.*, 117, 8879 (1995). (Supplemental Page 2).
- 12- J. Ellery, E. Olusegun and D. G. I. Kingston, *J. Nat. Prod.*, 44, 493 (1981).
- 13- E. Breitmaier and W. Voelter, "Carbon-13 NMR Spectroscopy" 3rd Ed., New York NY: VCH, 1987, pp. 257, 261.
- 14- Z. Xi-Ning, O. Hideaki, I. Toshinori, H. Eiji, T. Anki and T. Yoshio, *Phytochemistry*, 49, 2149 (1998).
- 15- Ç. Ihsan and K. Hasan, *ibid.*, 65, 2619 (2004).
- 16- N. Hiroaki, S. Hiroshi, M. Takashi, C. Masao and M. Hiroshi, *ibid.*, 29, 3303 (1990).