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New acylated flavonol glycosides from the aerial parts of *Gouania longipetala*

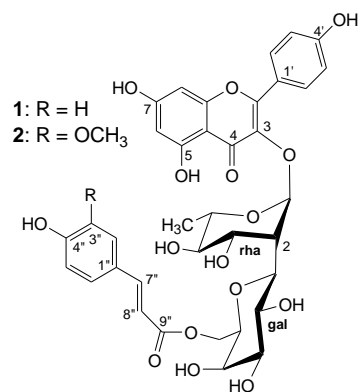
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Highlights

- ▶ Two new acylated flavonol glycosides were isolated from *Gouania longipetala*.
- ▶ Their structures were established on the basis of NMR spectroscopy and HRESIMS.
- ▶ Four flavonoids isolated from this plant showed significant antioxidant effects.

Abstract

Two new flavonol diglycosides kaempferol-3-*O*-(6-*O*-*trans*-*p*-coumaroyl)- β -D-galactopyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranoside and kaempferol-3-*O*-[6-*O*-*trans*-feruloyl]- β -D-galactopyranosyl-(1 \rightarrow 2)- α -L rhamnopyranoside were isolated from the aerial parts of *Gouania longipetala* in addition to seven known compounds. The structure elucidation of these compounds was based on analyses of spectroscopic data including 1D- and 2D-NMR and HR-ESI-MS techniques. The abilities of these compounds to scavenge the DPPH were evaluated. Results showed that compounds **2**, **3**, **8** and **9** have significant antioxidant potential with EC₅₀ values ranging from 13.8 to 47.4 μ M, compared with ascorbic acid (EC₅₀ 60 μ M) which was used as positive control.

Keywords: *Gouania longipetala*; Rhamnaceae; Flavonoids; DPPH free radical scavenging activity.

1. Introduction

Gouania is a plant genus comprising 70 tropical and subtropical species belonging to the family Rhamnaceae (Yao et al., 2011). Previous chemical investigation of this genus has demonstrated the presence of triterpenes and polyphenols (Giacomelli et al., 2007, Kennely et al., 1993, Li et al., 2007, Nair and Rao, 1993, Yao et al., 2011). *Gouania longipetala* (Hemsl.) is a scandent shrub or liana mainly present in closed forests, forest margins and in jungle regrowths. It occurs in Angola, Cameroun, Congo, Guinea, and Southwest of Ivory Coast (Burkill et al., 1985). Its salient characteristics are the watch spring tendrils, spike-like thyrsus, a more or less lobed disc, inferior ovary and longitudinally three-winged septicidal fruits (Buerki et al., 2011). *G. longipetala* has been used in traditional medicine for the treatment of different ailments such as edema, venomous stings, gout, abdominal pain and stomach troubles (Ezeja et al., 2014). It is also used for eye treatments, pain killers and for treating heart disease. The stem extract of the plant has been shown to possess antibacterial, anti-inflammatory, and antioxidant activities (Ekuadzi et al., 2012). This present study deals with the isolation and structure elucidation of nine flavonol glycosides from the methanol extract of the aerial parts including two new acylated flavonol diglycosides and seven known compounds. The methanol extract and isolated compounds were evaluated for their antioxidant activity by using a DPPH radical-scavenging assay.

2. Results and discussion

The methanolic extract of the aerial parts of *G. longipetala* was separated by combination of chromatographic methods to obtain two new flavonol glycosides **1** and **2** (Fig. 1) in addition to seven known compounds. The sugar composition was determined by comparative TLC after acid hydrolysis of the flavonoid mixture as xylose (xyl), glucose (glc), galactose (gal) and rhamnose (rha) and their common D-xyl, D-glc, D-gal, or L-rha configurations were verified by measurement of the optical rotation of each purified sugar. The known compounds were elucidated as kaempferol-3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-galactopyranoside (**3**) (Brasseur and Agenot, 1986), kaempferol-3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D -glucopyranoside (**4**) (Pawlowska et al., 2009), kaempferol-3-*O*- α -L-rhamnopyranoside (**5**) (Mok and Lee, 2013), kaempferol-3-*O*- β -D-xylopyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranoside (**6**) (Soicke et al., 1990), kaempferol-3-*O*- β -D-galactopyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranoside (**7**) (Szewczyk et al., 2014), quercetin-3-*O*- β -D-galactopyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranoside (**8**) (Manguero Arot and Williams, 1997), and quercetin-3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-galactopyranoside (**9**) (Brasseur and Agenot, 1986). Their structural assignments were made by HR-ESI-MS, 1D, and 2D NMR analysis. Their spectroscopic data were in good agreement with those reported in the literature.

Compound **1**, $[\alpha]_D^{20}$ -19.7, was isolated as a yellow amorphous powder. The positive HR-ESI-MS showed a molecular ion peak at m/z 763.1856 $[M + Na]^+$, (calcd for $C_{36}H_{36}O_{17}Na$, 763.1850) enabling us to determine the molecular formula $C_{36}H_{36}O_{17}$. The UV spectrum displayed maximum absorption bands of a flavonol skeleton at λ_{max} 267 and 314 nm. The 1H and ^{13}C NMR spectra of **1** comprised resonances corresponding to aromatic and glycosidic protons and carbons. The A-ring of the flavonol was represented by two *meta*-coupled resonances at δ_H 6.17 (d, $J = 2.2$ Hz, δ_C 99.8) and δ_H 6.18 (d, $J = 2.2$ Hz, δ_C 94.9), assigned to H-6 and H-8, respectively. The 1H and COSY NMR spectra of **1** (Table 1) exhibited two *ortho*-coupled doublet signals at δ_H 7.70 and 6.93 (each 2H, d, $J = 8.4$ Hz) revealing the presence of an AA'BB' system of ring B of the aglycone assignable to H-2'/6' and H-3'/5' respectively, which were correlated in the HSQC spectrum with their aromatic carbon atoms at 131.9 and 116.4 ppm, respectively. Complete assignment of the remaining resonances of the aglycone in the ^{13}C NMR spectrum of **1** was achieved by analysis of the HSQC and HMBC data, which confirmed the presence of kaempferol (3,5,7,4'-tetrahydroxy-flavone). A full list of the corresponding assignments is given in Table 1 (Sahakitpichan et al., 2014, Tang et al., 2001). The COSY and 1H NMR spectra of **1** also showed four aromatic protons at δ_H 7.23 and 6.70 (each 2H, d, $J = 8.3$ Hz) assignable to H-2''/6'' and H-3''/5'' respectively and two olefinic protons at δ_H 7.47 and 6.04 (each 1H, d, $J = 16.0$ Hz) assignable to H-7'' and H-8''. These data were consistent with the presence of an *E*-coumaroyl moiety (Table 1) (Bendaikha et al., 2014) and its 1H and ^{13}C resonances were assigned using 2D NMR experiments (COSY, HSQC, and HMBC). Furthermore, two anomeric proton resonances corresponding to *O*-linked sugars were displayed in the 1H NMR spectrum as two doublets at δ_H 4.44 ($J = 7.7$ Hz) and 5.85 ($J = 1.2$ Hz). Based on the results of the acid hydrolysis of flavonoids mixture, the magnitudes of their $J_{1,2}$ coupling constants and the analysis of 1D and 2D NMR data, the sugar units were elucidated as β -D-galactopyranose (δ_{H-1} 4.44 and δ_{C-1} 107.6) and α -L-rhamnopyranose (δ_{H-1} 5.85 and δ_{C-1} 102.3) (Table 1) (Agrawal, 1992). The β -D-gal was characterized by the large coupling constants $J_{H-1,H-2}$ and $J_{H-2,H-3}$ (> 7.7 Hz) and the small coupling constant $J_{H-3,H-4}$ (3.3 Hz) whereas the α -L-rha was characterized by the small coupling constants $J_{H-1,H-2}$ (1.2 Hz) and its methyl group at δ_{H-6} 1.07 (d, $J = 6.2$ Hz) and δ_{C-6} 17.8 as summarized in Table 1. A correlation between H-1-rha and δ_C 136.7 in the HMBC spectrum of **1** defined C-3 of kaempferol as the site of *O*-glycosylation. The interglycosidic linkage of the disaccharide moiety was also determined from HMBC data. Correlations from H-1-gal to the downfield-shifted C-2-rha (δ_C 83.6) indicated that the gal residue was 2-*O*-linked to rha. Furthermore, the downfield shifted methylene protons H-6-gal (δ_H 4.24 and 4.40) and the HMBC correlation observed between these protons and the ester carbonyl at δ_C 168.8 indicated the

attachment of the *E*-coumaroyl group to C-6-gal. Therefore, the structure of compound **1** was determined as kaempferol-3-*O*-(6-*O-E-p*-coumaroyl)- β -D-galactopyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranoside.

Compound **2**, $[\alpha]_D^{20}$ -23.2, was obtained as a yellow amorphous powder and displayed a molecular ion peak $[M + Na]^+$ at m/z 793.1966 in the positive HR-ESI-MS, (calcd for $C_{37}H_{38}O_{18}Na$, 793.1956) in agreement with a molecular formula of $C_{37}H_{38}O_{18}N$ and indicating an additional methoxy group by comparison with **1**. It exhibited an absorption band at λ_{max} 266 and 326 nm suggesting a flavonol skeleton. Analysis of 2D NMR experiments of **2** and comparison of its 1H and ^{13}C NMR data with those of **1** showed that **2** was kaempferol glycoside acylated with cinnamic acid derivative and contained the same disaccharide chain linked to C-3 of kaempferol (Table 1). This disaccharide was elucidated as above as β -D-galactopyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranoside. Thus the additional methoxy group [δ_H 3.78 (3H, s); δ_C 56.2] should be located at the cinnamoyl derivative. This latter was represented on the 1H NMR spectrum by two *trans*-coupled double-bond protons at δ_H 7.44 (H-7'') and 6.06 (H-8'') (each H, d, $J = 15.9$ Hz), and three coupled aromatic protons δ_H 6.88 (d, $J = 1.6$ Hz, H-2''), 6.69 (d, $J = 8.2$ Hz, H-5''), and 6.83 (dd, $J = 8.2, 1.6$ Hz, H-6''). The presence of an *E*-feruloyl moiety was evident by the connectivities observed in the HMBC spectrum between the *trans* double-bond and the 1,3,4-substituted aromatic ring as well as to an ester carbonyl (δ_C 168.7), and from the protons of the methoxy group to the C-3'' (δ_C 149.1) of the feruloyl moiety (Bendaikha et al., 2014). Full assignments of the proton and carbon resonances of **2** were achieved by analysis of the COSY, HSQC and HMBC spectra (Table 1). The site of glycosylation was established at the 3-OH position of kaempferol, as demonstrated by the HMBC spectrum showing correlations from H-1-rha to C-3 kaempferol and from H-1-gal to C-2-rha. The *E*-feruloyl was linked to OH-6-gal as indicated by the downfield shifts of H-6-gal (δ_H 4.23 and 4.47) and was confirmed by the cross peak between H-6-gal and the carbonyl C-9'' in the HMBC experiment. The structure of **2** was therefore identified as kaempferol-3-*O*-(6-*O-E-feruloyl*)- β -D-galactopyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranoside.

In order to assess the antioxidative properties of **1-9**, the DPPH radical scavenging activity of these compounds was measured. According to the results, summarized in Table 2, compounds **2**, **3**, **8**, and **9** had significant antioxidant potential (EC_{50} values ranging from 13.8 to 47.4 μM) compared with ascorbic acid, which was used as a positive control (EC_{50} 60 μM). Generally, substitution patterns on the B-ring especially affected antioxidant potencies of the flavonoids (Arora et al., 1998). The di-OH substitution at 3' and 4' of the B-ring is particularly important to the antiradical activity of a flavonoid. These trends are consistent with less active flavonoids (**1**, **4**, **6**, and **7**) that possess mono-OH substitution in the B-ring. Compounds **1-4**, **6**, and **7** shared a common aglycone mono-OH

substituted in the B-ring (kaempferol). Comparison of the antioxidant activity of **1** and **2** indicated that acylation with feruloyl moiety (**2**) was more favorable for the antioxidant activity than acylation with coumaroyl (**1**) residue.

Comparison of the antioxidant activity of kaempferol diglycosides **3**, **4**, **6**, and **7** indicated that compounds **3** and **7** (EC₅₀ 40.4 and 90.4 μ M, respectively) with a disaccharide chain composed of rhamnose-galactose were more active than compounds **4** and **6** whose disaccharide chains were composed of rhamnose-glucose or rhamnose-xylose. These results indicated that galactose residue in **3** and **7** was more favorable for the activity than glucose (**4**) or xylose (**6**). Comparison of the antioxidant activity of **8** and **9** (EC₅₀ 13.8 and 41.5 μ M, respectively) showed that **8** possessing the saccharide chain gal-(1 \rightarrow 2)-rha- was 3 times more active than **9** possessing the saccharide chain rha-(1 \rightarrow 6)-gal-. These findings suggested that the saccharide chain linked at C-3 of the aglycone might contribute to the antioxidant activity.

3. Experimental

3.1. General experimental procedures

NMR spectra were carried in MeOH-*d*₄ on Bruker Avance DRX III 500 instruments (¹H at 500 MHz and ¹³C at 125 MHz). Standard pulse sequences and parameters were used to obtain 1D (¹H and ¹³C) and 2D (COSY, HSQC, and HMBC) spectra. Chemical shift referencing was carried out using the internal solvent resonances at δ_{H} 3.31 and δ_{C} 49.0. HR-ESI-MS experiments were performed using a Micromass Q-TOF micro instrument (Manchester, UK). Optical rotations were determined in MeOH with a Perkin-Elmer 341 polarimeter. TLC was performed on pre-coated silica-gel 60 F₂₅₄ Merck and compounds were observed under UV light at 254 and 365 nm or visualized by spraying the dried plates with 50% H₂SO₄, followed by heating. CC was carried out on Kieselgel 60 (63-200 mesh), or LiChroprep RP-18 (40-63 μ m) Merck. HPLC was performed on a Dionex apparatus equipped with an ASI-100 autosampler, an Ultimate 3000 pump, a diode array detector UVD 340S and Chromeleon software. C18 reversed phase column (Phenomenex 250x10 mm, Luna 5 μ) was used for semi preparative HPLC with binary gradient eluent (H₂O (pH 2.4 with TFA); MeOH) and a flow rate of 3 ml/min; the chromatogram was monitored at 205, 225, 250, and 350 nm. Absorbance (A) values in the DPPH free radical scavenging assay were read on a Fluostar omega microplate reader (BMG labtech). UV spectra were recorded on Shimadzu UV-2450 spectrophotometer in MeOH.

3.2. Plant material

The aerial parts of *G. longipetala* were collected in Gagnoa-Bayota, Abidjan, Ivory Coast, in September 2009. A voucher specimen (No Aké Assi S.N-02) has been deposited in the herbarium of the National Center of Floristic of FHB University of Cocody (Ivory Coast).

3.3. Extraction and isolation

The powdered dry aerial parts of *G. longipetala* (950 g) were sequentially extracted with 19 l of petroleum ether, ethyl acetate and methanol at room temperature for 24 h. After evaporation of the solvents, 7.9 g of petroleum ether extract, 4.7 g of ethyl acetate extract and 26.5 g of methanol extract were obtained. The methanol extract was subjected to VLC over RP-18 (9 x 5 cm) eluted successively with 20, 40, 60, 80, and 100% MeOH in H₂O, to give five fractions (A₁-A₅, respectively). Fraction A₂ (2.3 g) was applied to a RP-18 CC (2 x 16 cm) eluted with a gradient of MeOH:H₂O (2:8-5:5) to afford 130 fractions, each 30 ml. Frs [48-50] (96 mg), eluted with MeOH:H₂O (35:65), were purified by silica gel CC eluted with a gradient of CHCl₃:MeOH (9:1-7:3) yielding compound **9** (12 mg). Frs [55-80] (367 mg), eluted with MeOH:H₂O (35:65), were purified by silica gel CC eluted with a gradient of CHCl₃:MeOH (9:1-6:4) to yield compounds **3** (14 mg) and **8** (14 mg). Fraction A₃ (5.5 g) was applied to a silica gel VLC (9 x 5 cm) eluted with CHCl₃:MeOH:H₂O (9:1:0-60:40:7) to give six fractions (B₁-B₆, respectively), each 280 ml. Fraction B₅ (1.8 g), eluted with CHCl₃:MeOH:H₂O (14:6:1) was further purified over silica gel CC (2.8 x 24 cm, 40 fractions, each 100 ml). Frs [5-9] (83 mg), eluted with CHCl₃:MeOH (9:1) were purified by semi-prep HPLC using 50% of MeOH for 30 min yielding compound **5** (*rt* 17.5 min, 16 mg). Frs [13-17] (331 mg), eluted with CHCl₃:MeOH (85:15) were applied to a silica gel CC eluted with a gradient of CHCl₃:MeOH (9:1-7:3) to afford 64 mg of compound **7** (eluted with 85:15 of CHCl₃:MeOH) and fraction eluted with CHCl₃:MeOH (8:2) were further purified by semi-prep HPLC using 50% of MeOH for 35 min yielding compounds **6** (*rt* 12.5 min, 9 mg), **4** (*rt* 13.5 min, 8 mg), **2** (*rt* 21.1 min, 10 mg), and **1** (*rt* 32.5 min, 24 mg).

3.4. Acid hydrolysis

A part of fractions A₂ and A₃ (500 mg each) was refluxed (90 °C) with 50 ml of 2M TFA for 4 h. After extraction with ethyl acetate (3 x 30 ml), the aqueous layer was evaporated to furnish the monosaccharide residue (300 mg). Four sugars were identified as xylose, glucose, galactose and rhamnose by comparison with authentic samples on TLC in MeCOEt:*iso*-PrOH:acetone:H₂O (20:10:7:6). A part of the monosaccharide residue (50 mg) was subjected to a preparative TLC using the same solvent. The optical rotation of each purified sugar was measured to reveal rhamnose ($[\alpha]_{\text{D}}^{20} + 2.4$, (*c* 1, H₂O)), glucose ($[\alpha]_{\text{D}}^{20} + 27.6$, (*c* 0.75, H₂O)), xylose ($[\alpha]_{\text{D}}^{20} + 15.1$, (*c* 0.95, H₂O)) and galactose ($[\alpha]_{\text{D}}^{20} + 41.2$, (*c* 1, H₂O)).

3.5. kaempferol-3-O-(6-O-E-p-coumaroyl)-β-D-galactopyranosyl-(1→2)-α-L-rhamnopyranoside (1)
 $[\alpha]_{\text{D}}^{20} - 19.7$ (*c* 0.3, MeOH); UV (MeOH) λ_{max} nm (A): 314 (1.54), 295 (1.41), 267 (1.23); ¹H and ¹³C NMR (MeOH-*d*₄) spectroscopic data, see Table 1; HR-ESI-MS *m/z*: 763.1856 [M + Na]⁺ (calcd for C₃₆H₃₆O₁₇Na, 763.1850).

3.6. kaempferol-3-O-(6-O-E-feruloyl)-β-D-galactopyranosyl-(1→2)-α-L-rhamnopyranoside (2)

$[\alpha]_D^{20}$ -23.2 (c 0.25, MeOH); UV (MeOH) λ_{\max} nm (A): 326 (1.05), 305 (0.80), 266 (0.85); ^1H and ^{13}C NMR (MeOH- d_4) spectroscopic data, see Table 1; HR-ESI-MS m/z : 793.1966 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{37}\text{H}_{38}\text{O}_{18}\text{Na}$, 793.1956).

4.6. DPPH free radical scavenging assay

DPPH (1,1-diphenyl-2-picrylhydrazyl radical) and ascorbic acid used for the bioassay were purchased from Sigma-Aldrich, Chemical Co. (Germany). The scavenging activity of isolated compounds against DPPH was investigated by spectrophotometric methodology as described by Lee et al. (1998) with slight modification. Briefly, 5 μl of the standard or sample solutions (dissolved in DMSO) was mixed with 95 μl of DPPH solution (158 μM , dissolved in absolute EtOH). After mixing gently and incubating for 30 min at 37 °C, the optical density was measured at λ 515 nm using a Fluostar omega microplate reader (BMG labtech). The percentage of absorbance inhibition at λ 515 nm was calculated using the following equation: % inhibition $[(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$. DPPH solution in EtOH was used as a control. The curve of the % scavenging activity against the concentration of sample was prepared by an MS Excel based program to obtain the EC_{50} (concentration required to obtain a 50% antioxidant effect). All the tests were conducted in triplicate. The experimental data were expressed as mean \pm standard deviation.

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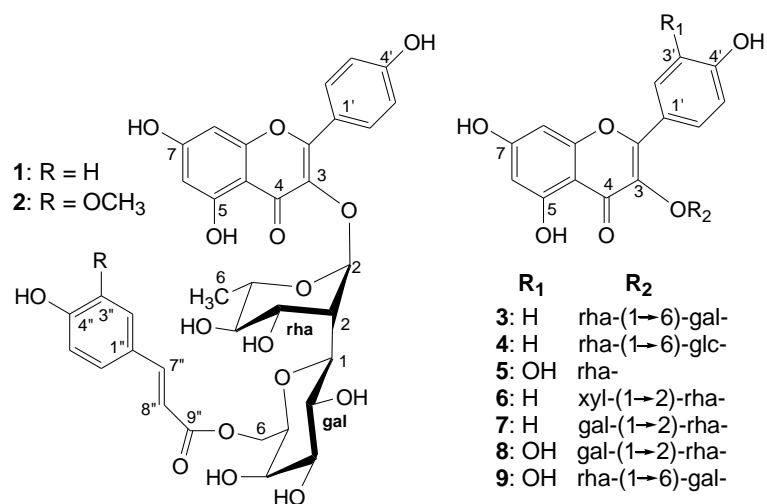


Fig.1. Chemical structures of flavonoids 1- 9, isolated from *G. longipetala*.

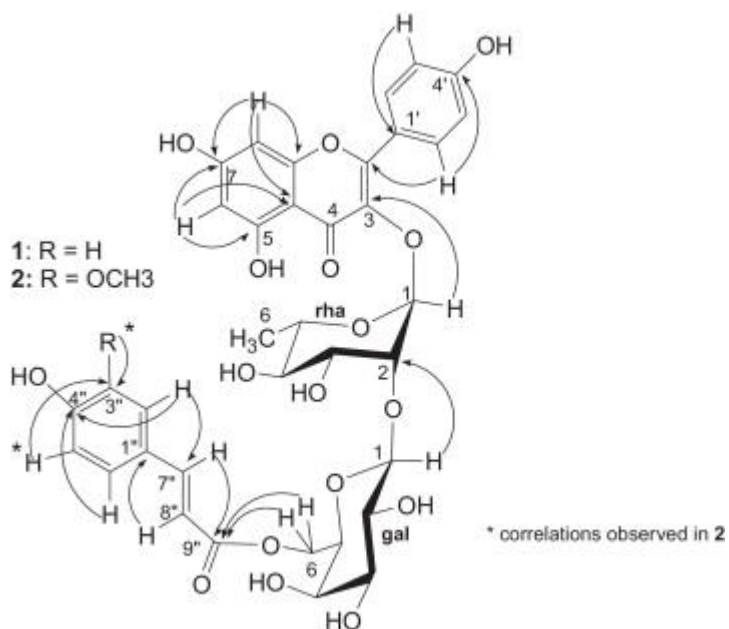


Fig. 2. Key HMBC correlations of compounds 1 and 2 from *G. longipetala*.

Table 1
¹H and ¹³C NMR spectroscopic data for compounds **1** and **2** (in CD₃OD).

Aglycone	1		2	
	δ_{H} (<i>m</i> , <i>J</i> en Hz)	δ_{C}	δ_{H} (<i>m</i> , <i>J</i> en Hz)	δ_{C}
2		158.3		158.1
3		136.7		136.7
4		179.5		179.6
5		163.1		163.1
6	6.17 d (2.2)	99.8	6.17 d (2.0)	99.7
7		165.6		165.5
8	6.18 d (2.2)	94.9	6.13 d (2.0)	94.8
9		158.3		158.2
10		106.0		106.0
1'		122.6		122.6
2'	7.70 d (8.4)	131.9	7.69 d (8.8)	131.9
3'	6.93 d (8.4)	116.4	6.94 d (8.8)	116.4
4'		161.4		161.5
5'	6.93 d (8.4)	116.4	6.94 d (8.8)	116.4
6'	7.70 d (8.4)	131.9	7.69 d (8.8)	131.9
3-O-rha				
1	5.85 d (1.2)	102.3	5.88 d (1.2)	102.3
2	4.41 dd (3.5-1.2)	83.6	4.41 dd (3.6-1.2)	83.7
3	3.85 dd (9.3-3.5)	71.8	3.85 dd (9.7-3.6)	71.8
4	3.40 t (9.3)	73.5	3.41 t (9.7)	73.5
5	3.57 m	72.0	3.61 m	72.0
6	1.07 d (6.2)	17.8	1.11 d (6.2)	17.9
2_{rha}-O-gal				
1	4.44 d (7.7)	107.6	4.43 d (7.8)	107.5
2	3.64 dd (9.5-7.7)	72.5	3.64 dd (9.7-7.8)	72.5
3	3.57 dd (9.5- 3.3)	74.6	3.57 dd (9.7-3.2)	74.6
4	3.86 d (3.3)	70.1	3.86 d (3.2)	70.2
5	3.75 m	74.2	3.74 m	74.3
6	4.24 dd (11.4-8.0)	64.0	4.23dd (11.4-8.3)	64.1
	4.40 dd (11.4-4.0)		4.47 dd (11.4-4.5)	
6_{gal}-Coum			6_{gal}-O-Fer	
1''		126.9		127.4
2''	7.23 d (8.3)	131.0	6.88 d (1.6)	111.2
3''	6.70 d (8.3)	116.6		149.1
4''		161.1		150.5
5''	6.70 d (8.3)	116.6	6.69 d (8.2)	116.2
6''	7.23 d (8.3)	131.0	6.83 dd (8.2-1.6)	124.0
7''	7.47 d (16.0)	146.7	7.44 d (15.9)	146.6
8''	6.04 d (16.0)	114.6	6.06 d (15.9)	115.0
9''		168.8		168.7
O-CH₃			3.78 s	56.2

Table 2Antiradical potential of compounds **1-9** isolated from the aerial parts of *G. longipetala*.^a

Compounds	DPPH radical scavenging activity EC ₅₀ ± S.D. (µM) ^b
1	- ^b
2	47.4 ± 1.3
3	40.4 ± 1.5
4	- ^b
5	- ^b
6	- ^b
7	90.4 ± 2.5
8	13.8 ± 0.2
9	41.5 ± 0.4
Ascorbic acid ^c	60 ± 5.1

^a Values are presented as the mean ± S.D. (n = 3).^b 50% inhibition not achieved at the concentration of 100 µg/ml .^c Used as a positive control.