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Polyphenolic constituents of *Callistemon lanceolatus* leaves

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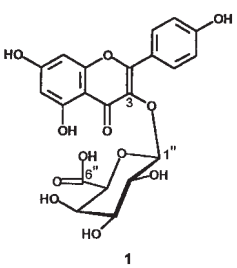
Two new flavonol glycosides, kaempferol 3-*O*-β-D-galacturonopyranoside and quercetin 3-*O*-(2″-*O*-galloyl)-β-D-glucoronopyranoside, were isolated, from leaves of *Callistemon lanceolatus* DC, as well as eighteen known polyphenols (phenolic acids, flavonoids and three tannins). All structures were established mainly on the basis of chemical and spectroscopic analysis (UV, 1D NMR and negative ESI-MS) and finally confirmed by 2D NMR experiments (HMQC and HMBC), in the case of flavonoid glycosides and tannins.

1. Introduction

The genus *Callistemon* (commonly named bottle brush) comprise about 25 species belonging to the family Myrtaceae, which are widely cultivated and much used as ornamental shrubs in California, and in warm countries and in green houses [1]. The genus *Callistemon* in known in folk medicine for its anticough, antibronchitis, and insecticidal effects [2] and its volatile oils have been used as an antimicrobial [3, 4] and antifungal [5]. A few phytochemical studies were done on *C. lanceolatus* and led to the isolation of ellagic acid, two of its methyl ether derivatives [6] and some methyl ether flavonoid aglycones and/or their glycosides [7, 8]. In the meantime, 2D-PC screening of the aqueous 70% MeOH extract of *C. lanceolatus* leaves showed a very complicated mixture of polyphenolic metabolites especially flavonoid glycosides, ellagic acid derivatives and tannins. In this paper we report on the isolation and identification of two new flavonol glycosides and eighteen known polyphenols.

2. Investigations, results and discussion

A mixture of polyphenols (flavonol 3-*O*-glycosides and tannins) was investigated in the total aqueous methanol extract of *C. lanceolatus* leaves, using 2D-PC and different spray reagents of polyphenols as well as direct flow infusion negative ESI-MS. The ethyl acetate soluble fraction yielded six known compounds by consecutive column chromatography and they were identified as gallic acid (**3**), methylgallate (**4**), kaempferol (**5**), quercetin (**6**), ellagic acid (**7**), and 3-*O*-methyl-ellagic acid (**8**). Fractionation of the residue on a polyamide column followed by successive separation on Sephadex LH-20 and cellulose columns yielded fourteen polyphenolic metabolites. On the basis of acid hydrolysis, comparative PC, UV, ESI-MS, ^{1}H-, ^{13}C NMR and in some cases 2D NMR spectroscopic analyses, the known compounds were identified as kaempferol 3-*O*-β-D-methylglucupyranuronate (**9**), hyperin (**10**), kaempferol 3-*O*-β-D-glucoronopyranoside (**11**), guaijaverin (**12**), kaempferol 7-*O*-β-D-galactopyranoside (**13**), afzelin (**14**), quercetin 3-*O*-β-D-methylglucupyranuronate (**15**), hyperin 6″-*O*-gallate (**16**), quercetin 3-*O*-β-D-glucoronopyranoside (**17**), a gallotannin 1,3,6-tri-*O*-galloyl-β-D-glucopyranose (**18**) and two ellagitannins pedunculagin (**19**) and casurinin (**20**). In addition, the new compounds kaempferol 3-*O*-β-D-galacturonopyranoside (**1**) and quercetin 3-*O*-(2″-*O*-galloyl)-β-D-glucuronopyranoside (**2**) were found.



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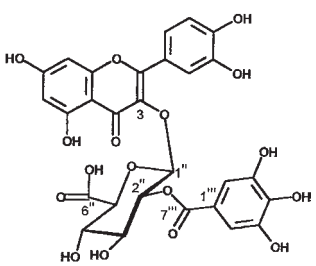

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Compound **1** was expected to be a kaempferol 3-*O*-glycoside on the basis of its chromatographic properties. UV-spectra in methanol and after addition of the diagnostic shift reagents provided evidence for a 5,7,4′-trihydroxy-flavonol with a substituted 3-OH group [9]. Acid hydrolysis of **1** gave D-galacturonide and kaempferol (comparative PC). The negative ESI-MS spectrum indicated quasi-molecular ions at *m/z* 461.2 $[M-H]^-$ and 483.3 $[M+Na-2H]^-$ in agreement with a molecular formula of $C_{21}H_{18}O_{12}$. With high in-source CID potential, a fragment ion peak at *m/z* 285 $[M-H-176]^-$ due to the loss of an uronic acid residue was observed. ^{1}H NMR spectrum showed two *ortho*-doublets (J = 8.8 Hz) each integrated to two protons of an AX system at δ_H ppm 8.0 (H-2′/6′) and δ_H 6.85 (H-3′/5′), as well as two *meta*-doublets (J = 1.6 Hz) each integrated to one proton of another AX system at δ_H 6.25 (H-8) and 6.04 (H-6), which was indicative of a kaempferol moiety as an aglycone. The ^{1}H resonances in the sugar region, especially that of H-1″ at δ_H 5.6 (J = 7.4 Hz), showed typical δ_H and J-values for a β-galacturonopyranoside unit [10]. All the sugar proton resonances were assigned by means of the cross peaks in the ^{1}H, ^{1}H-COSY spectrum. The corresponding C-resonances for the sugar unit were thereafter assigned by the cross peaks at δ 5.6/100.9, 3.21/74.2, 3.37/74.7, 3.28/72.2 and 3.28/76.4 in the HMQC spectrum for H-1″/C-1″, H-2″/C-2″, H-3″/C-3″, H-4″/C-4″, and H-5″/C-5″, respectively. A resonance of a carboxyl group (C-6″) was observed at δ 172.9 in the ^{13}C NMR spectrum and correlated with H-5″ in the HMBC spetrum. The identity of the sugar moiety was at least confirmed as a galacturonide due to the upfield shift of C-3″ resonance (δ 74.7) with respect to that of glucuronide (ca. 76.5–77.5), [11–13] and change of H-3″ signal from *t* in to *dd* in galacturonide. Finally, the cross peaks in the HMBC spectrum confirmed the assignment of all ^{1}H and ^{13}C resonances (Table 1) and structure of **1** as the new natural product kaempferol 3-*O*-β-D-$^4C_1$-galacturonopyranoside.

Table 1: ^{1}H, ^{13}C NMR assignments and correlations in HMBC for compound 1 (DMSO-d_6)

Position	1		
	^{13}C, δ	^{1}H, δ m (J)	Cross peaks in HMBC
2	156.38	–	–
3	133.01	–	–
4	177.40	–	–
5	161.06	–	–
6	98.74	6.04 d (1.6)	C-5, 7, 8, 10
7	164.90	–	–
8	93.89	6.25 d (1.6)	C-6, 7, 9, 10
9	156.31	–	–
10	103.62	–	–
1′	120.98	–	–
2′/6′	131.04	8.0 d (8.8, H-2′/6′)	C-2, 4′, 2′/6′, 3′/5′
3′/5′	115.14	6.85 d (8.8, H-3′/5′)	C-1′, 4′, 3′/5′, 2′/6′
4′	160.14	–	–
5′	–	–	–
6′	–	–	–
1″	100.86	5.6 d (7.4)	C-3, 2″, 3″
2″	74.19	3.21 brt (7.5)	C-1″, 3″, 4″
3″	74.69	3.37 dd (9.2, 3.4)	C-2″, 4″
4″	72.17	3.32 m (H-4″/5″)	C-5″, 6″
5″	76.39	–	–
6″	172.95	–	–

m = multiplicity, J-values are given in parentheses in Hz

Table 2: ^{1}H, ^{13}C NMR assignments and correlations in HMBC for compound 2 (DMSO-d_6)

Position	2		
	^{13}C, δ	^{1}H, δ m (J)	Cross peaks in HMBC
2	156.55	–	–
3	132.67	–	–
4	176.86	–	–
5	161.17	–	–
6	98.73	6.15 d (1.9)	C-5, 7, 8, 10
7	164.26	–	–
8	93.58	6.34 d (1.9)	C-6, 7, 9, 10
9	156.25	–	–
10	103.88	–	–
1′	120.69	–	–
2′	116.28	7.61 d (2.0)	C-2, 1′, 3′, 4′, 6′
3′	144.98	–	–
4′	148.62	–	–
5′	115.25	6.81 d (8.46)	C-1′, 3′, 4′, 6′
6′	121.75	7.55 dd (8.46, 2.0)	C-2, 2′, 3′, 4′
1″	98.65	5.76 d (8.0)	C-3, 3″, 5″
2″	73.86	4.98 t (8.7)	C-1″, 3″, 4″, 7‴
3″	73.86	3.42 t (9.1)	C-2″, 4″, 5″
4″	71.85	3.52 t (10.1)	C-3″, 5″, 6″
5″	75.38	3.65 d (9.5)	C-1″, 4″, 3″, 6″
6″	170.30	–	–
1‴	119.63	–	–
2‴/6‴	109.03	7.04, s	C-1‴, 2‴/6‴, 4‴, 3‴/5‴, 7‴
3‴/5‴	145.46	–	–
4‴	138.39	–	–
7‴	165.12	–	–

m = multiplicity, J-values are given in parentheses in Hz

Compound **2** yielded quercetin, gallic acid and D-glucuronic acid after complete acid hydrolysis. In addition, its chromatographic properties as well as its UV spectra in methanol and after addition of diagnostic reagents were nearly identical to those of a quercetin 3-*O*-(galloyl)-glucuroniode. Negative ion ESI-MS spectrum of **2** gave a pseudomolecular ion at *m/z* 629.2 $[M-H]^-$ corresponding to a molecular formula of $C_{28}H_{22}O_{17}$. High CID potential gave fragment ions at *m/z* 477 $[M-153]^-$ and 301 $[M-329]^-$, attributable to the loss of galloyl and galloyl-deoxyuronide moieties, respectively. The ^{1}H NMR spectrum of **2** showed an ABX at δ_H 7.61 (H-2′), 7.55 (H-6′) and 6.81 (H-5′) and a 2 H AX system at 6.34 (H-8) and 6.15 (H-6), in good accordance with a quercetin 3-*O*-substituted aglycone. The anomeric proton was recorded as a doublet at δ_H 5.76 with $J_{1''/2''} = 8$ Hz, which, with the other δ- and J-values of the sugar protons, indicated a β-glucuronopyranoside moiety [14]. The exact position of glycosidation on C-3 was determined by the typical upfield shift of C-2 (ca. 5 ppm) and downfield shift of C-3 (ca. 3.5 ppm) with respect to the aglycone quercetin [15] and was supported by the cross peak correlated H-1″ and C-3 in the HMBC. The signal at δ_H 7.04 (H-2‴/6‴) in the ^{1}H NMR spectrum, which was correlated to a carbon at δ 109.03 (C-2‴/6‴) in the HMQC spectrum and with C-7‴ at δ 165.12 in HMBC, confirmed the presence of a gallic acid residue [10]. The pronounced downfield shift of the H-2″ signal (δ_H 4.98 ppm) compared to the analogous H-2″ of unsubstituted glucuronide (δ_H 3.27 ppm, see Experimental), indicated that the galloyl moiety was connected to C-2″. This evidence was supported by the upfield shifts of both C-3″ and C-1″ resonances of about 2.18 and 2.74, respectively relative to those of quercetin 3-*O*-glucuronide [10, 16–18]. Finally, the position of acylation (on C-2″) and assignment of all other ^{1}H and ^{13}C resonances were confirmed by the cross peaks in the HMBC spectrum as given in Table 2. Accordingly, **2** was identified as the new natural product quercetin 3-*O*-(2″-*O*-galloyl)-β-D-glucuronopyranoside.

This study showed that leaves of *C. lanceolatus* are rich in kaempferol and quercetin 3-*O*-glycosides and some of their galloylated esters (**9**–**17**). They also contain some gallo- and HHDP ellagitannins, based on glucopyranose (**18** and **19**) or open chain C-glycosidic glucose (**20**). All the identified compounds were isolated for the first time from the genus *Callistemon* except for ellagic acid.

3. Experimental

3.1. Equipment

3.1.1. UV analysis

Pure samples were measured separately as MeOH solutions and with various diagnostic shift reagents [9] on an IKON 940 UV spectrophotometer.

3.1.2. NMR analyses

NMR analyses were run on Bruker 300, JEOL EX-270 and 400 MHz spectrometers relative to TMS in different deuterated solvents (at 25 °C).

3.1.3. Negative ESI-MS

The diluted solution of each sample, in 1 : 1 MeOH–H_2O; was directly infused using a Harvard syringe infusion pump to a Finnigan MAT 95 double focusing sector field mass spectrometer according to previously published conditions [19] or to a Finnigan LC-MS LCQ^{deca} spectrometer (Finnigan, Bremen, Germany).

3.2. Plant material

Leaves of *C. lanceolatus* DC. were collected from a plant grown in the Zoological Garden, Giza, Cairo, Egypt in March 1999. Dr. Mohamed El-Gebaly, Lecturer in Plant Flora Taxonomy, Chemistry and Taxonomy Department, NRC, Giza, Egypt, confirmed identification of the plant. It was also compared to reference herbarium specimens. Voucher specimens are kept in the herbarium (No.: C-II), Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Egypt.

3.3. Extraction and isolation

Powdered, air-dried leaves of *C. lanceolatus* (3.5 kg) were exhaustively extracted with hot 70% MeOH (5 × 6 l), under reflux. The dry residue obtained was extracted with EtOAc (6 × 2 l) to give 135 g dry extract and 198 g residue. The EtOAc extract was defatted with hot pet. ether (60–80), under reflux (6 × 1 l) and then fractionated on microcrystalline cellulose for CC (E. Merck, Darmstadt, Germany) using aqueous EtOH mixtures with decreasing polarity to give three major fractions A, B and C eluted by 30, 85 and 90% EtOH, respectively. Each subfraction was further chromatographed on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and eluted with EtOH (twice) to afford pure samples of **3** (12 mg) and **4** (16 mg) from subfraction A; **5** (13 mg) and **6** (16 mg) from B; **7** (14 mg) and **8** (22 mg) from C. The residue was subjected to CC on polyamide 6S (Riedel De Haen AG, Seelze, Hannover, Germany) using H_2O, then with gradual addition of EtOH up to pure EtOH yielding 7 collective phenolic fractions. Fraction 1 was fractioned by CC (twice) on Sephadex LH-20 with EtOH for elution to give a pure sample of compound **1** (58 mg). CC of fraction 2 on Cellulose with equeous EtOH for elution gave two subfractions A (by 30% EtOH) and B (by 60%). Subfraction A gave **9** (60 mg), while B gave **10** (20 mg), after further purification on Sephadex LH-20 with MeOH as eluent. **11** (34 mg) and **12** (19 mg) were obtained by consecutive fractionation of fraction 3 on Sephadex LH-20 with *n*-BuOH-*iso*-propanol-H_2O (4 : 1 : 5, top layer) for elution. Fraction 4 was chromatographed on cellulose with 40% equeous EtOH to give two subfractions. Each was subjected to repeated CC on Sephadex LH-20/EtOH (for elution) yielding **13** (11 mg), **14** (9 mg), and **15** (36 mg), respectively. CC of fraction 5 on cellulose with *n*-BuOH (saturated with H_2O) followed by purification on Sephadex with EtOH, yielded **16** (44 mg). Compounds **17** (42 mg) and **2** (55 mg) were isolated from fraction 6 by CC on cellulose with 50% aqueous EtOH and then each of the two subfractions obtained was further purified on Sephadex LH-20 with EtOH. Fraction 7 was chromatographed on cellulose with aqueous EtOH. The first major subfraction gave **18** (38 mg) by CC on Sephadex LH-20/EtOH (3 times), while the second subfraction was subjected to successive CC on Sephadex LH-20/*n*-BuOH-*iso*-propanol-H_2O (4 : 1 : 5, top layer) to give **19** (37 mg) and **20** (15 mg). All separation processes were followed up by 2D-PC and CoPC using Whatman No. 1 paper with (S_1) *n*-BuOH–AcOH–H_2O (4 : 1 : 5, top layer) and (S_2) 15% aqueous AcOH as solvent systems.

3.4. Kaempferol 3-O-β-D-$^4C_1$-galacturonopyranoside (1)

Compound **1** was isolated as a yellow amorphous powder; R_f-values: 0.61 (S_1) and 0.36 (S_2); dark purple fluorescence under UV changing to dull yellow with NH_3. It gave a green color with $FeCl_3$ and greenish yellow fluorescence with NA/PE under UV (360 nm). Complete acid hydrolysis: A small amount of **1** (10 mg) was hydrolyzed with 2 N HCl in MeOH-H_2O (1 : 1), under reflux at 100 °C for 2 h to yield D-galacturonic acid and kaempferol (CoPC & spray reagents). UV λ_{max}, nm, (MeOH): 217, 266, 352; (+NaOMe): 215, 274, 326, 399; (+NaOAc): 224, 270, 305 sh, 361; (+NaOAc/H_3BO_3): 223, 266, 352; (+$AlCl_3$): 216, 275, 305, 350, 399; (+$AlCl_3$/HCl): 213, 275, 300 sh, 346, 395. Negative ESI-MS: *m/z* 483.3 $[M + Na - 2H]^-$, 461.2 $[M-H]^-$, 285 $[M-H-176]^- = [M-H\text{-deoxygalacturonide}]^-$. ^1H NMR (300 MHz, DMSO-d6), ^{13}C NMR (75 MHz, DMSO-d6) and HMBC data: Table 1.

3.5. Quercetin 3-O-(2″-O-galloyl)-β-D-$^4C_1$-glucuronopyranoside (2)

Compound **2** was isolated as a yellowish white amorphous powder; R_f-values: 0.29 (S_1) and 0.25 (S_2); dark purple fluorescence under UV changing to bright yellow with NH_3. It gave a deep green color with $FeCl_3$ and orange fluorescence with NA/PE under UV (360 nm). Acid hydrolysis: 8 mg of **2** were hydrolyzed under the same conditions as for **1** to yield D-glucuronic acid, gallic acid and quercetin (CoPC & spray reagents). UV λ_{max}, nm, (MeOH): 217, 266, 352; (+NaOMe): 215, 274, 326, 399; (+NaOAc): 224, 270, 305 sh, 361; (+NaOAc/H_3BO_3): 223, 266, 352; (+$AlCl_3$): 216, 275, 305, 350, 399; (+$AlCl_3$/HCl): 213, 275, 300 sh, 346, 395. Negative ESI-MS: *m/z* 629.2 $[M-H]^-$, 477 [M-galloyl]$^-$, 301 $[M-H\text{-galloyldeoxygalacturonide}]^-$. ^1H NMR (300 MHz, DMSO-d6), ^{13}C NMR (75 MHz, DMSO-d6) and HMBC data: Table 2.

Acknowledgements: We thank Dr. J. Mügge and his coworkers and Prof. Dr. M. Linscheid's coworkers (Chemie institute, Humboldt Universität) for their efforts and helpful cooperation in the NMR and ESI-MS measurements.

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Received October 15, 2001
Accepted January 25, 2002

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