Polyphenolic constituents of the methanolic extract of *Callistemon viridiflorous* leaves and its antimicrobial activity

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**ABSTRACT**

**Introduction:** The genus *Callistemon* (family: Myrtaceae) contains 34 species which are widely distributed in the temperate regions and used in folk medicine. **Methods:** Chromatographic separation of 80% MeOH extract of the leaves of *Callistemon viridiflorus* (sims) Sweet (Cv) was performed. **Results:** Seven known polyphenolic compounds were isolated for the first time from this species: gallic acid, ellagic acid, isoquercetin, hyperin, 1,2:3,4-(bis(s)-hexahydroxy diphenoyl-β-D-glucopyranose, nilocitin and quercetin-3-O-α-L-glucuronopyranoside. The methanolic extract of Cv leaves exhibited a significant anti-microbial activity against the tested microorganisms.

**Keywords:** Myrtaceae, *Callistemon viridiflorous*, polyphenols, anti-microbial activity.

**INTRODUCTION**

Family Myrtaceae (Myrtle family) comprises about 130–150 genera and about 5000 species of evergreen shrubs and trees. The genus *Callistemon* (family: Myrtaceae) contains 34 species which are widely distributed in the temperate regions of Australia, and are widely distributed in warm-temperate regions.1–5 The genus *Callistemon* (commonly named bottle brush plant) is known in folk medicine for its anticough, antibronchitis, antifungal, antibacterial, anti-inflammatory, analgesic, anticonvulsant, anti-diabetic, anti-hemorrhoidal and antinociceptive activities.6–13 Previous phytochemical investigations of some species of *Callistemon* genus resulted in the identification of C-methyl flavonoids, falvonol glycosides, phenolic acids, hydrolysable ellagitannins, triterpenoids and phloroglucinol derivatives.4–19 Because of the biological importance of plant polyphenols, particularly tannins and flavonoids, a phytochemical study was of interest to investigate the constitutive polyphenols in the extract of leaves for *Callistemon viridiflorus*. Synonyms of the plant include *Callistemon viridiflorus* (Sieber ex Sims) Sweet, *Callistemon salignus* var. *viridiflorus* (Sieber ex Sims) F. Muell., *Melaleuca virens* Craven, *Metrosideros viridiflora* Sieber ex Sims.20

The present study deals with the isolation and identification of some polyphenolic constituents of *Callistemon viridiflorous* species growing in Egypt and to explore the antimicrobial activity of its methanolic extracts.

**MATERIALS**

**Plant material**

Leaves of *Callistemon viridiflorus* (Sims) Sweet were collected from Alexandria-Cairo Road, Egypt, in July, 2008. The plant was identified by Dr. Trease Label, Lecturer of Taxonomy, Department of Floral and Taxonomy, Orman Garden, Cairo, Egypt.

**Microorganisms**

Gram-positive bacteria (*Staphylococcus aureus* ATCC4175), Gram-negative bacteria (*E. coli*) and yeast (*candida albicans*)
ATCC60193) were supplied by Microbiology Department, Faculty of Pharmacy, Helwan University.

**Culture media**

Trypticase soy agar (Difco) was used as a culture media.

**Standard antimicrobial agents**

Ofloxacin and Ketoconazol were used as positive control.

**Chemicals, sugars, drugs and authentic reference materials for chromatography**

Galactose, glucuronic acid, glucose, quercetin, hyperin, isoquercetin, kaempferol, ellagic acid, gallic acid and nilocitinin were obtained from Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Helwan, Egypt. Polyamide S6 (50–160 μm, Fluka chemie AG, Switzerland) for column chromatography. Microcrystalline cellulose (E. Merck, Darmstadt, Germany) for column chromatography. Sephadex LH-20 (25–100 μm, Pharmacia, Uppsala, Sweden) for column chromatography. Silica gel 60 F254 precoated aluminum sheets (20 × 20, 0.2 mm thickness) and cellulose precoated aluminum sheets (20 × 20, 0.2 mm thickness), (E. Merck, Darmstadt, Germany) for thin layer chromatography. Whatmann No.1 paper chromatography (Whatmann Ltd., Maidstone, Kent, England). Diphenyl borinic acid ethanolamine complex (Naturstoff reagent for flavonoids). Aluminum chloride reagent (1% in ethanol) for flavonoids. Ferric chloride reagent (1% in ethanol) for phenolic compounds. Aniline hydrogen phthalate reagent for sugars. Nitrous acid spray reagent for ellagitannins. Potassium iodate spray reagent for gallotannins. Reagents for UV spectroscopic analysis of flavonoids such as aluminium methoxide, Aluminium chloride, HCl, Boric acid and sodium acetate.

**Solvent systems**

S1: n-Butanol – Acetic acid – Water (4:1:5 v/v/v, top layer)
S2: Acetic acid – Water 15:85 v/v
S3: n-Butanol – Isopropyl alcohol – Water

**Apparatus**

NMR spectrometers: JOEL GX-500 (500 and 125 MHz for 1H and 13C NMR), NMR Department, National Research Center (NRC) were used. The results were reported as δppm values relative to TMS as internal reference. All samples have been prepared in DMSO-d6 solvent. MS Spectrometer: Finnigan LCQ-deca or Finnigan LTQ for HRESI-MS, Strathclyde Institute of Pharmacy and Biomedical Science, Glasgow, United Kingdom. UV-visible spectrophotometer: JASCO (V-630) UV spectrophotometer was used for UV spectroscopic analysis of isolated compounds in UV range (200–450 nm) in different diagnostic shift reagents, Faculty of Pharmacy, Helwan University. Rotary evaporator (Büchi, G, Switzerland). Ultraviolet lamp (VL-215 LC, Marne La Vallee, France) was used for visualization of spots on paper and thin layer chromatograms and follow up the columns fractionation on columns at 254 and/or 365 nm.

**METHODS**

**Extraction and isolation of polyphenolic constituents from Callistemon viridiflorous leaves**

The air dried ground leaves (1500 g) were subjected to exhaustive extraction with hot 80% aq. methanol under reflux (70° C). The collective extract was dried under vacuum (45° C) and resulted in 240 g dry extract. The dried extract was treated by petroleum ether under reflux (60° C). The pet-ether insoluble portion was suspended in water and preliminary fractionated on polyamide column using a step gradient H2O/MeOH mixture with decreasing polarity from 100% water to MeOH 100% for elution to yield 35 individual fractions. Those fractions were collected into five major fractions, which were subjected to successive column chromatography on cellulose, sephadex LH-20. The eluted fractions were tested by spotting using two dimensional paper technique (2D-PC), S1 used for the first run and S2 for second run. After air drying, the spots were visualized under UV-light before and after spraying with a suitable reagent. Glass columns of different sizes were packed with required adsorbent. Methanol soluble part of fraction 1 was applied on Sephadex-LH20 column and eluted by methanol resulted in compound F1. Fraction 2 was applied on Sephadex-LH20 column and eluted by 20% H2O/80% methanol followed by ethanol resulted in compound F2. Fraction 3 was applied on cellulose (20% MeOH/H2O) followed by sephadex LH-20 (eluted by S1 solvent followed by 30% EtOH/H2O) resulted in compound F3 and F4. Fraction 4 was applied on cellulose column and eluted by S1 solvent followed by 20–60% MeOH/H2O then subfractions applied on Sephadex-L20 column and eluted by MeOH which resulted in compound F5a and F5b. Fraction 5 will be discussed later. Bands formed during the elution process were traced in both visible and UV light.
Each fraction eluted from the column was evaporated under reduced pressure and analyzed by PC or TLC; similar fractions were collected and separately investigated. About 2–3 mg of each glycoside was treated with 0.1 N HCl in methanol for 60 min at 100°C. The reaction mixture was examined every 10 min by CoPC to detect any intermediate or sugars that may be formed against the available authentic samples.[27] A complete acid hydrolysis was carried out by treating few mgs of each glycoside with 1.5N HCl in aqueous 50% methanol for 2 hr at 100°C, then evaporated and extracted with ethyl acetate. Aglycones were traced in ethyl acetate fraction by CoPC alongside authentic aglycones or by using different spectroscopic analysis. The aqueous phase was neutralized with sodium bicarbonate (5% aqueous solution) and used for investigation of the sugar moiety on CoPC against authentic sugar samples and spraying with anilone hydrogen phthalate reagent.[27] Few spicks of each pure hydrolysable tannin were heated at 100°C under reflux for about 8 hr with 2 N H₂SO₄ in 50% aqueous methanol. After cooling the aqueous phase was extracted with ethyl acetate, then filtered on anhydrous sodium sulphate and concentrated under vacuum. This was used for identification of phenolic acids on PC against authentic samples and using S₁ and S₂ as solvent systems, whereas the neutralized aqueous phase with 0.5% NaHCO₃, was examined for the identification of the present sugars, using CoPC.[27]

Microbiological study

The disc agar diffusion method

The antimicrobial study was determined using modified Kirby-Bauer disc diffusion method.[28] One hundred ml of the test bacteria or fungi were grown in 10 μl of fresh media until they reached a count of approximately 108 cells/ml. 100 ml of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained. Disc diffusion method for yeasts developed by using approved standard method (M44-P) by the NCCLS.[29] Plates with Gram +ve bacteria as Staphylococcus aureus, Gram -ve bacteria as Escherichia coli were incubated at 35–37°C for 24–48 hours. Yeast as Candida albicans was incubated at 30°C for 24–48 hours then the diameters of the inhibition zones were measured in millimeters. The extract of C. viridiflorus leaves were diluted with DMSO, 1:5 w/v, then 20 μl (4 μl of diluted extract/disc) was aseptically transferred onto sterile discs of Whatman filter paper (5 mm diameter). Standard discs of ofloxacin and fluconazole served as positive controls for antimicrobial activity.

RESULTS AND DISCUSSION

Characterization and identification of isolated compounds

**Compound F₁** Yellowish white amorphous powder (40 mg). chromatographic properties: Rf value: 0.71 (S₁), 0.56 (S₂); shine violet fluorescent under UV-light turned to deep blue color with FeCl₃ spray reagent. ¹HNMR (500 MHz, DMSO-d₆), δppm 6.87 (2H, S, H-2/6). ¹³CNMR (125 MHz, DMSO-d₆), δppm 168.13 (C-7), 145.86 (C-3/5), 138.52 (C-4), 120.99 (C-1), 109.24 (C-2/6). From the previous data and by comparison with previous reported data,[29,39] Compound F₁ was confirmed as gallic acid which was isolated for the first time from C. viridiflorus and identified earlier from the leaves of C. lanceolatus.

**Compound F₂** Brown amorphous powder (35 mg). Chromatographic properties: Rf values; 0.46 (S₁), 0.11 (S₂); buff fluorescent under long and short UV-light turned to dull yellow with ammonia vapors and greenish yellow with Naturstoff reagent. It gave faint blue color with FeCl₃ reagent. ¹HNMR (500 MHz, DMSO-d₆), δppm 7.49 (2H, s, H-5/’). ¹³CNMR (125 MHz, DMSO-d₆), δppm 159.73 (C-6/6’), 148.72 (C-4/4’), 140.48 (C-3/3’), 136.89 (C-2/2’), 112.93 (C-1/1’), 110.71 (C-5/5’). According to the previous data and by comparison with the previous reported data,[39,40] Compound F₂ was confirmed as ellagic acid which was identified earlier from C. lanceolatus and first time from C. viridiflorus.

**Compound F₃** Light yellow amorphous powder (60 mg). chromatographic properties: Rf values; 0.42 (S₁), 0.81 (S₂), dark purple spot under short and long UV light, it gave blue color with FeCl₃ reagent and rose red colour, colour changed to dark brown with HNO₃ reagent (specific spray reagent for ellagitannins).[43] On complete acid hydrolysis it afforded ellagic acid in organic phase and glucose in aqueous phase (CoPC). ¹HNMR (500 MHz, DMSO-d₆), δppm 6.62, 6.50, 6.48, 6.33 (each 1H, s, H-6/6’/6’’/6’’’), 2 x HHDGP, 5.45 (1H, dd, J= 9.5, 10.0 Hz, H-2), 5.35 (1H, d, J= 9.5Hz, H-1), 4.84 (1H, t, J= 10.0 Hz, H-3), 4.74(1H, t, J=10.0 Hz, H-4), 3.91(1H, brd, J = 11.85 Hz, H-6). ¹³CNMR (125 MHz, DMSO-d₆), δppm 168.66, 166.30, 165.86, 163.38 (C-7/7’/7’’/7’’’), 4 x carbonyl HHDGP, 146.96, 145.41, 145.21, 145.01 (4 x C-3 HHDGP), 144.94, 144.60, 144.18, 144.17 (4 x C-5 HHDGP), 137.57, 136.16, 136.02, 135.49 (4 x C-4 HHDGP), 126.32, 125.30, 125.20, 123.96(4 x C-1HHDGP), 115.63, 115.59, 114.89, 114.75(4 x C-2 HHDGP), 107.32, 107.22,
Compound $F_4$ Off white amorphous powder (22 mg). Chromatographic properties: $R_f$ values; 0.32 ($S_0$), 0.71 ($S_2$); dark purple spot under short and long UV-light, it gave deep blue color with $FeCl_3$ reagent and positive response toward specific spray reagents.

On complete acid hydrolysis, it produces ellagic acid in organic phase and glucose in aq. phase (COPC).

1HNMR (500 MHz, DMSO-d6): δ ppm 6.31, 6.30 (each 1H, s, H-6α/β), 6.20, 6.19 (each 1H, s, H-6`α/β), 5.20 (1H, d, $J=3.8$ Hz, H-1z), 5.09 (1H, t, $J=9.9$ Hz, H-3z), 4.83 (1H, t, $J=9.9$ Hz, H-3β), 4.81 (1H, d, $J=9$ Hz, H-1β), 4.70 (1H, dd, $J=9.9$, 3.8 Hz, H-2α), 4.47 (1H, t, $J=9.9$, H-2β). Negative ESI-MS m/z 481.41 [M-H], 301.51 [ellagic acid=H]. Accordingly, compound $F_4$ was tentatively identified as ellagitannin on the basis of its chromatographic properties and the products of complete acid hydrolysis. In addition, Negative ESI-MS gave a molecular ion peak and fragment ion peak at m/z 481 and 301 which assigned to [M-H] and [ellagic acid=H]. This indicates a hexahydroxydiphenoyl-D-glucose structure.

The location of HHDP group on C-2 and C-3 as a biester was indicated from the downfield shift of H-2 and H-3 resonances. The duplication of the sugar protons resonances together with δppm and $J$-values confirmed the sugar moiety as α/β glucopyranose. The presence of one HHDP group was evidenced from the aromatic signals at 6.31, 6.3 and 6.20, 6.19 of H6` and H6``` in both anomers. So according to the previous data and by comparison of the NMR data with the published ones and also CoPC with authentic sample, compound $F_4$ was identified as 2,3-O-hexahydroxydiphenoyl-(α/β)-glucopyranose (Nilocitin) which is isolated for the first time from genus Callistemon.

Compound $F_5$ Yellow amorphous powder (40 mg). Chromatographic properties: $R_f$ values; 0.56 ($S_0$), 0.65 ($S_2$); dark purple spot under UV-light turned to dark yellow fluorescence on exposure to ammonia vapors; it gave deep green color with FeCl$_3$ and orange fluorescence with Naturalstoff reagent. UV spectral data: $\lambda_{max}$ (nm) (MeOH): 232,259,354; (+NaOME): 236,271,405; (+NaOAc): 237,272,320 (sh),395; (+NaOAc/ H$_2$BO$_3$): 238,260,300 (sh),378; (+AlCl$_3$): 236,268,300 (sh),415; (+AlCl$_3$/ HCl): 235,267,305 (sh),400. Complete acid hydrolysis producing glucouronic acid in aq. phase and quercetin in organic phase (COPC). 1HNMR (500 MHz, DMSO-d6): δ ppm 8.14 (1H, d, $J=2.0$ Hz, H-2`), 7.33 (1H, dd, $J=8.4$, 2 Hz, H-6`), 6.79 (1H, d, $J=8.4$ Hz, H-5`), 6.35 (1H, d, $J=2.0$ Hz, H-8), 6.15 (1H, d, $J=2.0$ Hz, H-6), 5.22 (1H, d, $J=3.4$ Hz, H-1``). 13CNMR (125 MHz, DMSO-d6), δ ppm 178 (C-4), 173.05 (C-6``), 165.32 (C-7), 157.31 (C-9), 157.08 (C-8), 148.97 (C-4`), 145.33 (C-3`), 134.52 (C-3), 121.21 (C-6`), 121.22 (C-1`), 118.31 (C-5`), 115.97 (C-2`), 103.29 (C-10), 100.03 (C-1``), 94.54 (C-6`), 90.06 (C-8), 77.16 (C-3``), 74.64 (C-5``), 72.85 (C-2`), 72.27 (C-4`). Negative ESI-MS Figure 22: m/z 477.32 [M-H], 378.30 [quercetin- H], 955.16 [2M-H].

Compound $F_5$ was expected to be quercetin 3-O-glycoside on the basis of its chromatographic properties and UV spectral data. The UV spectra in MeOH showed the two characteristic absorption maxima at $\lambda_{max}$ 259 (band II) and 354 (band I) for a quercetin aglycone.
The bathochromic shift in band I without decrease in the intensity observed with NaOMe indicated the presence of 4`-OH group. The bathochromic shift in band II observed on the addition of NaOAC indicated a free 7-OH group while the bathochromatic shift resulted in band I after addition of H₃BO₃ revealed the presence of an ortho-di hydroxy function in ring B. Moreover the bathochromatic shift of band I in AlCl₃ together with hypsochromic shift observed after addition HCl confirmed the presence of ortho dihydroxy B ring, however there is still a bathochromatic shift relative to MeOH indicating the presence of a free 5-OH group. On complete acid hydrolysis, F₅ gave glucuronic acid in aq. phase and quercetin in organic phase, suggesting quercetin 3-O-glucuronide structure. This finding was in accordance with the M.wt. at 478, which was given from molecular ion peak at m/z 477.32 [M-H]. Moreover the aglycone was identified as quercetin due to the fragment ion peak at 301.51 [quercetin-H]. ¹H NMR spectra, showed an ABX spin coupling system at 8 ppm 8.14, 7.33 and 6.79 for H-2`, H-6` and H-5` of 3`,4`-dihydroxy B ring. Additionally the AM coupling system of two meta coupled protons at 6.35 and 6.15 for H-8, H-6, respectively to indicate 5, 7-dihydroxy ring A. The identification evidence of sugar moiety as α-glucuronic acid was in turn reinforced from the presence of α-anomeric protons signal at 8 5.22 (3.4 Hz) for glucuronyl moiety attached at C-3[36]. The structure of the compound F₅ was finally confirmed by the complete assignment of all C-resonances in its ¹³C NMR spectrum. It showed a typical 15 ¹³C resonances for 3-O-substituted quercetin[41]. The sugar moiety was confirmed as glucuronic acid due to resonance of C-6'' at 173.05 ppm and also the rest of carbon resonance at 77.16, 74.64, 72.85 and 72.27 for C-3`', C-5`', C-2`' and C-4`', respectively for the glucuronol moiety. All ¹H and ¹³C resonances were assigned by comparison with the corresponding published data of structural related compounds[33]. Thus compound F₆ was identified as Quercetin 3-O-α-L-glucuronopyranoside, which is isolated for first time from genus *Callistemon*.

### Compounds F₆α, F₆β

Yellow amorphous powder (45 mg). Chromatographic properties: Rₘ values; 0.43 (S₂), 0.52 (S₂); dark purple spot under UV-light turned to dark yellow fluorescence on exposure to ammonia vapors; it gave deep green color with FeCl₃ and orange fluorescence with Naturstoff reagent. UV spectral data: λ_max (nm) (MeOH): 256,355; (±NaOme): 270,408; (±NaOAC): 264,379; (±NaOAC/H₃BO₃): 258,377; (+AlCl₃): 270, 300,356,430; (+AlCl₃/ HCl): 269,300,370 ¹H NMR, ¹³CNMR, Negative ESI-MS (Table 1). Based on the chromatographic properties and UV- spectral data[36] as described before in F₅, compound F₆ was expected to be quercetin-3-O-glycoside. Complete acid hydrolysis of compound F₆ resulted in quercetin in organic phase, glucose and galactose in aqueous phase, suggesting the presence of either quercetin 3-O-diglycoside or two compounds which are quercetin 3-O-glucoside and quercetin 3-O-galactoside. The Negative ESI-MS, showed a molecular ion peak at m/z 463.31 [M-H] as the base peak, together with an adduct dimeric ion peak at m/z 927.08 [2M-H] which were consistent with the M.wt 464 for either quercetin 3-O-glucoside or galactoside. This finding was more confirmed with ¹H NMR spectrum that showed a duplication of the signals with the same ratio indicating the presence of two compounds. It showed a typical proton signals for quercetin aglycone (Table 1)[36] as described before

<table>
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<th>C. No.</th>
<th>¹³CNMR</th>
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<td>156.85</td>
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<td>161.59</td>
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<td>6`</td>
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<td>7.52 (2.3)</td>
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<td>7.51 (2.3)</td>
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[¹CNMR (125 MHz, DMSO-d₆), ¹HNMR (500 MHz, DMSO-d₆) Values between Parenthesis represent the J-value in Hz]
in compound F₆. The sugar moieties were identified as glucoside and galactoside from the presence of two β-anomeric proton signals for glucoside at δ 5.40 (7.5 Hz) and for galactoside at δ 5.31 (7.6 Hz). This evidence was confirmed by $^{13}$CNMR exhibited also a duplication of typical 15 $^{13}$C- resonance for 3-O-substituted quercetin.$^{[41]}$ And the two sugar moieties were proved to be β-glucoside and β-galactoside from their $^{13}$C resonance of 2`, 3`, 4`, 5` and 6` and the two β-anomeric carbon resonance at 101.34 for glucoside moiety and 102.38 for galactoside moiety. According to the above described data (UV, ESI-MS, NMR) as well as comparing with previous reported data$^{[35–36]}$ and with authentic samples, CoPC, so F₆ was confirmed to be two compounds F₆A and F₆B. F₆A was identified as quercetin 3-O-β-D-glucopyranoside (Isoquercetin) and F₆B was identified as quercetin 3-O-β-D-galactopyranoside (Hyperin), which is isolated once before from the leaves of C. lanceolatus.

The antimicrobial activity of the alcoholic leaves extract exhibited broad spectrum effect against Gram-positive, Gram-negative and yeasts (Table 2).

### Table 2. Results of antimicrobial activity of the alcoholic extract of leaves of C. viridiflorus.

<table>
<thead>
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<th>Tested microorganisms</th>
<th>Extract of leaves</th>
<th>Ofloxacine</th>
<th>Fluconazole</th>
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<tr>
<td>Staphylococcus aureus (gram +ve)</td>
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<td>30</td>
<td>–</td>
</tr>
<tr>
<td>E. coli (gram –ve)</td>
<td>14</td>
<td>26</td>
<td>–</td>
</tr>
<tr>
<td>Candida albicans (yeast)</td>
<td>12</td>
<td>–</td>
<td>23</td>
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</table>

*No inhibition zone.*
REFERENCES