Bioassay-guided isolation and POM analyses of a new immunomodulatory polyphenolic constituent from *Callistemon viridiflorus*

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Chromatographic separation of 80\% EtOH extract of *Callistemon viridiflorus* leaves led to the isolation of six known constituents (1–6) along with a new polyphenolic compound 7 identified as apigenin 4'-O-\beta-D-glucopyranosyl-(1\'\rightarrow 4\')-O-\beta-D-glucopyranoside. The ethanolic extract of *C. viridiflorus* leaves and isolated compounds were evaluated for in vitro immunomodulatory activity by means of RAW 264.7 macrophages proliferation (MTT) assay. Ethanolic extract of leaves and compounds 1, 3, 4, 6 and 7 caused a significant increase in macrophage proliferation; these findings may suggest that this medicinal plant could be utilised as an excellent source of compounds for immunomodulatory activity.

**Keywords:** *Callistemon viridiflorus*; flavonoids; immunomodulatory; POM analyses

1. Introduction

The family Myrtaceae (Myrtle family) consists of approximately 130–150 genera and more than 5000 species of evergreen shrubs and trees. The genus *Callistemon* (family: Myrtaceae) contains 34 species which are widely distributed in warm-temperate regions (Kanjilal & Das 1992). The genus *Callistemon*, commonly named bottle brush plant, is known in folk medicine for its antitussive, antibronchitis, antifungal, antibacterial, anti-inflammatory, analgesic, anticonvulsant, antidiabetic, anti-hemorrhoidal and antinociceptive activities (Ji 2009).

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Due to the biological and medicinal importance of plant polyphenols, particularly tannins and flavonoids content, the present study deals with the isolation and identification of some polyphenolic constituents of \textit{C. viridiflorus} species grown in Egypt and to evaluate the immunomodulatory activity of its ethanol extract and that of the isolated polyphenolic compounds.

2. Results and discussion

2.1. Characterisation and identification of the isolated compounds

The aqueous ethanol leaf extract of \textit{C. viridiflorus} was subjected to 2D-Pc and different spray reagents as well as to direct flow infusion ESI-MS. The methanol-soluble fraction yielded six polyphenolic compounds by successive columns chromatography, five of them, compounds 1–5, were previously isolated and identified from this species (Seikel & Hillis 1970; Haddock et al. 1982; Barakat et al. 1987; Agrawal & Bansal 1989; Mahmoud et al. 2002; Marzouk et al. 2006; Cao et al. 2010; Abdelhady et al. 2012).

Compounds 1–5 were identified by comparing their NMR and other properties with those of authentic samples; these compounds are as follows: 1, isoquercetin 2, hyperin 3, 1,2,3,4-(bis(s)-hexahydroxy diphenol)-\(\beta\)-D-glucopyranose 4 and quercetin-3-O-\(\alpha\)-D-glucuronopyranoside 5 as shown in Figure 1. In addition, a new compound 6 was isolated for the first time from \textit{C. viridiflorus}. It was obtained as a yellow amorphous powder (19 mg), and its UV spectrum in methanol displayed two major absorption bands with \(\lambda_{\text{max}}\) 265 nm (band II) and \(\lambda_{\text{max}}\) 367 nm (band I). In addition, compound 6 has chromatographic \(R_f\) values of 0.71 (S1), 0.19 (S2); dull yellow spot under UV-light with no change on exposure to ammonia vapours and gave a greenish yellow colour upon treatment with \(\text{FeCl}_3\) and Naturstoff spray reagents. Negative ESI-MS spectrum exhibited a molecular ion peak at \(m/z\) 299.1 \([\text{M} - \text{H}]^{-}\). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)): \(\delta\) (ppm); 12.54 (1H, s, OH-5), 7.82 (2H, d, \(J = 9.1\) Hz, H-2\(^{1/2}\)), 7.32 (2H, d, \(J = 9.1\) Hz, H-3\(^{1/2}\)), 6.39 (1H, d, \(J = 2.1\) Hz, H-8), 6.21 (1H, d, \(J = 2.1\) Hz, H-6), 3.85 (3H, s, OCH\(_3\)-4\(^{0}\)). \(^1\)C NMR (75 MHz, DMSO-\(d_6\)): \(\delta\) ppm 177.1 (C-4), 165.1 (C-7), 162.1 (C-5), 159.4 (C-4\(^{0}\)), 154.3 (C-9), 146.3 (C-2), 135.9 (C-3), 131.1 (C-2\(^{1/2}\)), 121.2 (C-1\(^{1}\)), 117.1 (C-3\(^{1/2}\)), 104.3 (C-10), 98.6 (C-6), 94.1 (C-8), 57.1 (OCH\(_3\)-4\(^{0}\)). Methylation of the hydroxyl group at 4\(^{0}\) was confirmed by the down field shift of the 3\(^{1/2}\)/5\(^{1/2}\) protons (\(\delta\) 7.32 ppm) and their carbons (\(\delta\) 117.0 ppm), compared to that of kaempferol (\(\delta\) 6.84 and 115.0 ppm, respectively) and by the

![Figure 1. Structures of isolated compounds from \textit{C. viridiflorus} leaves (1–7).](image-url)
slight upfield shift of C-4 (δ 159.43 ppm) compared to that of kaempferol (δ 160.0 ppm) (Otake & Walle 2002). Thus, compound 6 has been identified as kaempferol 4′-O-methyl ether (kaempferide) (Marzouk 2008; Park et al. 2013), which was obtained for the first time from C. viridiflorus.

Moreover, compound 7 has been isolated for the first time from a natural source. Compound 7 was obtained as pale yellow amorphous powder (18 mg) with the following chromatographic properties: Rf values; 0.34 (S1), 0.49 (S2); dark purple spot under UV-light turned to green colour with FeCl3 and greenish yellow with Naturstoff spray reagents. UV-spectral data: λmax (nm) (MeOH): 270, 300, 333; (NaOMe): 284, 301, 340; (NaOAC): 284, 303, 337; (AlCl3): 277, 303(sh), 347, 388; (AlCl3/HCl): 278, 303(sh), 345, 388. 1H NMR (500 MHz, DMSO-d6): δ ppm 13.11 (1H, s, H-bonded OH-5), 7.96 (2H, d, J = 8.4 Hz, H-2′/6′), 6.86 (2H, d, J = 8.4 Hz, H-3′/5′), 6.69 (1H, s, H-3), 6.46 (1H, d, J = 2.1 Hz, H-8), 6.2 (1H, d, J = 2.1 Hz, H-6), 4.78 (1H, d, J = 6.1 Hz, H-1″), 4.61 (1H, d, J = 7.4 Hz, H-1‴), 4.04-3.13 (remaining sugar protons). 13C NMR (125 MHz, DMSO-d6): δ ppm 182.6 (C-4), 164.3 (C-2), 163.1 (C-7), 161.6 (C-4′), 161.2 (C-5), 156.8 (C-9), 129.5 (C-2′/6′), 122.3 (C-1′), 116.7 (C-3′/5′), 104.4 (C-6), 104.2 (C-10), 103.2 (C-1‴), 99.1 (C-1″), 98.7 (C-6), 93.7 (C-8), 82.3 (C-5″), 80.17 (C-2″), 79.0 (C-3″), 76.8 (C-5″), 76.6 (C-3″), 74.9 (C-2″), 70.7 (C-4″), 70.0 (C-4‴), 61.5 (C-6″), 61.0 (C-6‴). Negative ESI-MS: m/z 593.60 [M – H]−, 431.91 [M–glucosyl]−. The UV spectrum in MeOH exhibited the two characteristic absorption bands at λmax, 270 nm (band II) and 333 nm (band I) of the apigenin nucleus. Upon addition of NaOAc, a bathochromic shift of band II (= + 7) was observed which is diagnostic of a free 7-OH group. The remaining diagnostic shift reagents were in complete agreement with the 5,7-dihydroxy-4′-glucosyl flavones structure (Mabry 1970). Negative ESI-MS spectrum exhibited the molecular ion peak at m/z 593 [M – H]− which corresponds to a MW of 594 and a molecular formula of C27H30O15. 1H NMR spectrum showed an AX coupling system of two ortho doublets at δ 7.96 and 6.86 ppm, each accounted for two protons; these were assigned to H2′/6′ and H-3′/5′, respectively of 1′,4′-disubstituted ring-B. In addition, the singlet signal at δ 6.69 assigned to H-3 and the two doublets at 6.46 and 6.20 ppm assigned to H-8 and H-6, respectively, are characteristics of an apigenin moiety. Moreover, the two anomic protons appeared as doublets at δ ppm 4.78 with a J value 6.1 Hz and 4.61 with a J value 7.4 Hz suggest the presence of O-glucoside moieties with a β-linkage, respectively. HMBC-NMR experiment confirmed the linkage positions of the two sugars with each other and with the aglycone moiety and is in full agreement with the proposed structure of compound 7. All 1H and 13C chemical shifts were assigned by comparison with the corresponding values of structurally related compounds of previously published data (Harborne 1986; Agrawal & Bansal 1989; Kim et al. 2004). Therefore, compound 7 was identified as apigenin 4′-O-β-d-glucopyranosyl-(1″→4″)-O-β-d-glucopyranoside as shown in Figure 1.

2.2. Immunomodulatory activity (proliferation of immune cells)

Compounds 1–7 and the ethanol (80%) extract of C. viridiflorus leaves were tested in vitro for their immunomodulatory activity on the proliferation of RAW 264.7 macrophage cells, estimated by an MTT assay. Results revealed that the incubation of macrophages with the ethanol extract and with compounds 1, 4, 5 and 6 causes a significant increase (P < 0.05) in the cells proliferation at the highest tested dose and that this increase is dose-dependent. In addition, the extract and compounds 1, 4–7 increased cell proliferation by 1.53-, 1.41-, 1.49-, 1.46- and 1.43-fold of the control, respectively, at the highest tested dose, indicating immunomodulatory activity (Smith et al. 1985). Furthermore, treatment of macrophages with compounds 2 and 3 caused no significant increase (P > 0.05) in the macrophage proliferation at any of the tested doses as depicted in Figure S1 (See Figure S1 online only).
2.3. POM analyses of compounds (1–7)
We employed *in silico* tools such as Osiris, Petra and Molinspiration to assess the pharmacokinetic profile of the isolated compounds (Ben Hadda et al. 2014). These are well-established *in silico* tools validated with about 7000 drug molecules available on the market. Shown in Table S1 (See Tables S1, S2 and Figures S2–S4 online only) are the results of theoretical toxicity risks of compounds 1–7 calculated with the aid of the Osiris program. Our findings reveal that compounds 1–6, contrary to compound 7, are not toxic and can be utilised as therapeutic agents (Tables S1 and S2).

3. Conclusions
In summary, findings from this investigation suggest that leaves extract (ethanolic) from *C. viridiflorus* contains phenolic compounds. The ethanolic extract and most of the isolated compounds displayed remarkable immunomodulatory activity and may be used in immune compromised patients to improve their immunity against various bacterial infections. This *C. viridiflorus* is a great potential as a natural health care product. Moreover, findings from this study showed that compounds 4 and 7 have the potential as kinase inhibitors whereas 6 and 7 represent an interesting potential to inhibit other enzymes as predicted by POM Analyses.

Supplementary material
Supplementary material relating to this article is available online, alongside Figures S1-S9 and Tables S1-S2 at http://dx.doi.org/10.1080/14786419.2015.1045508.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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