SUPPLEMENTARY MATERIAL

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

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Abstract

Chromatographic separation of 80% EtOH extract of *Callistemon viridiflorus* leaves led to the isolation of five known polyphenolic compounds (**1–5**) in addition, to Kaempferide (**6**), which was isolated for the first time from this species along with a new polyphenolic compound **7** identified as apigenin 4'-*O*- β -D-glucopyranosyl-(1'' $\rightarrow$ 4'')-*O*- β -D-glucopyranoside. In addition to the isolation and identification of these compounds, the aim of the present work was to evaluate, *in vitro*, the immunomodulatory activity of the isolated polyphenols and the ethanol extract of *C. viridiflorus* leaves by means of RAW 264.7 macrophages proliferation (MTT) assay. Ethanol 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 utilized as an excellent source of compounds for immunomodulatory activity and possibly for Ebola virus inhibition. However, more detailed studies are required to establish the safety and efficacy of the active constituents of the plant.

Keywords: *Callistemon viridiflorus*, Myrtaceae, flavonoids, cannabinoids, immunomodulatory activity, RAW 264.7 macrophage, hypothetical anti-Ebola virus agents, Petra/Osiris/Molinspiration (POM) analyses.

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Experimental

Instrumentation

We acquired ^1H and ^{13}C NMR spectra with the aid of a Varian Mercury 300-MHz spectrometer with $\text{DMSO-}d_6$ as solvent and TMS as the internal standard; chemical shifts (δ) are expressed in ppm whereas coupling constants (J -values) for ^1H - ^1H are given in Hertz. ESI-MS spectra were obtained with a Finnigan LCQ Deca Advantage MAX LC/MS System, with Surveyor LC pump at the Department of Biological Genetics, National Research Center, Cairo, Egypt. An ELISA reader (BioRad, München, Germany) was employed for measuring the absorbance of viable cells in the proliferation assay. Extracts were concentrated at low temperature, under reduced pressure, by means of rotary evaporation. UV analyses were performed on a Shimadzu UV 240 spectrophotometer, whereas a UV-VS spectrophotometer (Milton Roy 601) was used for the determination of total phenolic content. Chromatographic fractionation was monitored with a UV lamp (VL-215 LC, Marna, La Vallee, France).

Plant material

Leaves from *Callistemon viridiflorus* (Sims) were collected from Alexandria-Cairo Road, Egypt. The plant was identified and authenticated by Dr. Trease Labebe, a Lecturer of Taxonomy at the Department of Floral and Taxonomy, Orman Garden, Cairo, Egypt. A voucher specimen (Reg. no. R.s-6) was deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Cairo, Egypt. The leaves were then air-dried in the shade and reduced to a coarse powder.

Chemicals

The following chemicals and materials were obtained from commercial sources (in parentheses) and were used as received without further purification: polyamide 6S (Riedel-De Hën Ag, Seelze Hannover, Germany), cellulose (Pharmacia, Uppsala, Sweden), and Sephadex (Fluka,

Switzerland) were used in chromatographic experiments. Sugars, reagents, and solvents of analytical grade (Sigma-Aldrich Co., St. Louis, MO, USA), Chemicals used in biological activity, MTT and paclitaxel standards (Sigma-Aldrich Co. (St. Louis, MO, USA), Cell culture materials (Cambrex Bio-Science, Copenhagen, Denmark). Authentic samples of flavonoids were obtained from Phytochemistry Laboratory, Department of Pharmacognosy department, Faculty of Pharmacy, Helwan University.

Extraction and isolation of phenolics

The dried ground leaves of *Callistemon viridiflorus* (1 kg) was defatted with petroleum ether and then extracted four times with 4 L of 80% aqueous ethanol at 70 °C. The combined extract was concentrated and dried under reduced pressure to afford 150 g of dried residue. The residue was fractionated on a polyamide 6S column (Ø 5.5 × 120 cm) eluted with water and then with a gradient of H₂O/MeOH mixtures of decreasing polarity. Separation processes were continued with 2D-PC and CoPC using Whatmann No. 1 paper using the solvent systems (S₁) *n*-BuOH–AcOH–H₂O (4:1:5, top layer) and (S₂) 15% aqueous AcOH (Mabry, 1970); spots were visualized with UV lamp and by spraying with FeCl₃ and Naturostoff reagent (Diphenylboryl oxyethylamine in MeOH / 5% polyethylene glycol 400 in EtOH) (Brasseur and Angento, 1986). Pure compounds were obtained by successive column chromatography on cellulose and Sephadex using different solvent systems of H₂O/MeOH mixtures and (S₃) *n*-butanol–isopropanol–water (BIW) (4:1:5, v/v upper layer) as outlined by previously (Mohamed *et al.*, 2012).

Acid hydrolysis for glycosides

Complete acid hydrolysis was carried out by treating 4–5 mg of each isolated compound with 1.5 N HCl in aqueous methanol (50%) for 2 hours at 100°C. The mixture was then extracted with ethyl acetate and the extract was subjected to CoPC investigation along with authentic aglycones. Mild acid hydrolysis was done using 0.2 N HCl in aqueous methanol (50%). The mother liquor was neutralized with sodium carbonate and used for the identification of the sugars by CoPC against standard samples according to published procedures (Mabry, 1970).

Proliferation of immune cells

Effect of the isolated compounds on the growth of Raw macrophage 264.7 was evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Hansen, 1989). The yellow tetrazolium salt of MTT is reduced by mitochondrial dehydrogenase in metabolically active cells to form insoluble purple formazan crystals which are solubilized by the addition of a detergent. Cells (5×10^4 cells/well) were incubated with various concentrations of the investigated compounds at 37°C in a FBS-free medium, before being subjected to MTT assay. The absorbance was measured with an ELISA reader at 570 nm and the relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. Results obtained were expressed as the mean percentage of viable cells as compared to DMSO-treated cells. Treatment of macrophage with 1000 U/mL recombinant macrophage colony-stimulating factor (M-CSF, Pierce, USA) was used as positive control.

Statistical analysis

Data were subjected to two-factorial analysis of variance (ANOVA), including first-order interactions (two-way ANOVA), followed by the Tukey *post hoc* test for multiple comparisons. Differences were considered significant at $p \leq 0.05$.

Figure S2: The effect of different doses of 80% EtOH leaf extract of *C. viridiflorus* and of compounds **1-7** on the growth of RAW 264.7 macrophages, as measured by an MTT assay (only the alc. Extract and compounds 1,4,5-7 showed significant immunomodulatory activity, $p \leq 0.05$)

Figure S3. Plausible mechanism synergic effect of three pharmacophoric sites resulting from opening/closing of the central ring of flavonoids **2, 3, 5, 6,** and **7**.

Figure S4. Identification of joint pharmacophoric sites of patented anti-Ebola agents (**AEB-01**, **AEB-02** and **CBD**).

Figure S5. Attribution of bioactivity of Cannabinoids against the Ebola virus.

Table S1: Osiris calculations of toxicity risks of compounds **1-7**.

Compd.	MW	Toxicity Risks ^[a]				Osiris calculations ^[b]			
		MUT	TUM	IRRIT	REP	cLogP	Sol	DL	DS
1	482	+++	+++	+++	+++	-0.84	-2.13	-0.68	0.50
2	464	+++	+++	+++	+++	-0.35	-2.19	-2.17	0.43
3	464	+++	+++	+++	+++	-0.35	-2.19	-2.17	0.43
4	784	+++	+++	+++	+++	0.94	-4.52	-0.66	0.28
5	492	+++	+++	+++	+++	-0.24	-2.44	2.82	0.71
6	300	---	+++	+++	---	2.11	-3.11	-1.76	0.18
7	594	---	+++	+++	---	-1.49	-2.44	-9.96	0.11

Hgily toxic: (---), Slightly toxic: (+), Not toxic (+++).

^[a] MUT: Mutagenic, TUM: Tumorigenic, IRRIT: Irritant, REP: Reproductive effective.

^[b] Sol: Solubility, DL: Drug likeness.

Table S2: Molinspiration calculations of compounds (**1-7**).

Compd.	Molinspiration calculations ^[a]				Drug-likeness ^[b]					
	TPSA	NONH	NV	VOL	GPCRL	ICM	KI	NRL	PI	EI
1	244	9	2	372	0.17	0.01	-0.06	0.04	0.12	0.37
2	211	8	2	372	0.06	-0.04	0.13	0.20	-0.06	0.42
3	211	8	2	372	0.06	-0.04	0.13	0.20	-0.06	0.42
4	377	13	3	592	0.04	-1.47	-2.72	-2.25	-1.05	-1.83
5	228	8	2	391	0.18	0.07	0.14	0.39	0.14	0.55
6	100	3	0	250	-0.12	-0.28	0.19	0.30	-0.28	0.20
7	249	9	3	488	-0.05	-0.38	-0.04	0.03	0.02	0.19

^[a] TPSA: Total molecular polar surface area; NONH: number of OH---N or O---NH interaction, NV: number of violation of five Lipinsky rules; VOL: volume.

^[b] GPCRL: GPCR ligand; ICM: Ion channel modulator; KI: Kinase inhibitor; NRL: Nuclear receptor ligand; PI: Protease inhibitor; EI: Enzyme inhibitor.

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Figure S6 : HMBC

Figure S7 : HNMR

Figure S8 : CNMR

Figure S9 : ESI-MS