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Original Article

POM analyses, immunomodulatory, cytotoxic activities and polyphenolic constituents of *Callistemon viridiflorus* fruits

Mohamed I.S. Abdelhady[a], Amel M. Kamal[a,*], Mohamed F. Barghash[b]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Cairo 11795, Egypt
[b] Biochemistry Department, Genetic Engineering and Biotechnology Division, NRC, Cairo, Egypt

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ABSTRACT

Phytochemical study on 80% ethanolic extract of *Callistemon viridiflorus* Sims fruits (CVF) resulted in isolation of five phenolic compounds 1; Gallic acid, 2; Ellagic acid, 3; Reynoutrin, 4; Methoxy ellagic acid, 5; Quercetin. Petra/Osiris/Molinspiration (POM) analyses predicted that Reynoutrin and Quercetin have interesting potential activities, also Reynoutrin is found to be safer in relation to Quercetin. 80% ethanolic extract of CVF, Reynoutrin and Quercetin displayed remarkable immunomodulatory activity, as increased proliferation of RAW 264.7 macrophage cells by 1.48, 1.51 and 1.46 fold respectively. 80% ethanolic extract of the *C. viridiflorus* fruits showed significant cytotoxic activity against P388 leukemia cells (IC_{50} = 15.11 μg/ml), followed by Quercetin (IC_{50} = 21.5 μg/ml) then Reynoutrin (IC_{50} = 33.32 μg/ml).

1. Introduction

Genus *Callistemon* (family Myrtaceae) is known in folk medicine for its various biological activities. These activities may be related to phenolic compounds, which had been previously identified from different *Callistemon* species [1–7]. Our earlier work have described isolation of several polyphenolic contents in addition of performance of antioxidant, cytotoxic, antinociceptive, antimicrobial and immunomodulatory activities of *C. viridiflrous* leaves and flowers [4–7]. The current study is a continuation for further investigation on this interesting species using different organ (i.e. fruits). Since nothing was previously published concerning phytochemical constituents of fruits of *Callistemon viridiflorus* Sims, it was of interest to perform this study that dealt with an investigation of polyphenolic constituents of *Callistemon viridiflorus* Sims fruits (CVF) and evaluation of the immunomodulatory and cytotoxic activities of 80% ethanolic extract and the isolated compounds (3,5).

2. Materials and methods

2.1. Plant material

Dr. Trease Labeb, senior specialized of plant taxonomy, Orman garden, Giza, Egypt confirmed the identification of CVF and by comparison with reference herbarium specimens as mentioned before by Abdelhady et al. (2016) [7].

2.2. Extraction and isolation

Dry CVF (200 g) was extracted with hot 80% EtOH (5 × 1L), under reflux. The total dry residue obtained (15 g) followed by extraction with chloroform (3 × 300 ml) yielding (3g) chloroform extract and (11 g) remaining aqueous ethanolic residue. It was observed by 2D-PC that polyphenolic content was concentrated in the aqueous ethanolic extract. So the aqueous residue (11 g) was fractionated on a polyamide column and was eluted by water then water/methanol of decreasing polarity. Getting four main fractions (I–IV) according to detection on PC was performed by using S_1 and S_2 systems and visualization by UV. Fraction I eluted with 10%MeOH was subjected to Sephadex LH-20 column (MeOH eluent) to give pure compound 1 (41 mg). Fraction II (40% MeOH eluent) was applied on cellulose column using aqueous methanol solvent and further purification was achieved by Sephadex LH-20 (MeOH eluent) affording pure compounds 2 (18 mg) and 3 (16 mg). Fraction III (50% MeOH eluent) was applied on Sephadex LH-20 column (*n*-BuOH : H_2O saturated eluent), and finally purified by EtOH to give pure compound 4 (19 mg). Application of fraction IV (eluted with 60–70% MeOH) on Sephadex LH-20 and MeOH eluent resulted in pure compound 5 (27 mg).

Solvent systems:

S_1: *n*-BuOH:AcOH:H_2O (4:1:5 v/v/v upper layer), S_2; AcOH:H_2O

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* Corresponding author.
E-mail address: kh.omran@yahoo.com (A.M. Kamal).

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(15:85 v/v). Specifications of instruments used; UV analyses, acquisition of ESI-MS, ^{1}H and ^{13}C NMR spectra according to Abdelhady et al. (2016) [7].

2.3. Immunomodulatory activity

80% ethanolic extract of CVF, compounds 3 and 5 were tested for their immunomodulatory activity on the proliferation of RAW 264.7 macrophage cells, estimated by an MTT assay [8]. Procedure of the assay was reported in our previous study [7].

2.4. Cytotoxic activity against P388 leukemia cells

Measurements of cytotoxic activity was performed according to the method illustrated by Knick et al. (1995) [9] using various concentrations of the extract (0.5–100 µg/ml).

Statistical analysis was carried out using ANOVA design.

3. Results and discussion

3.1. Identification of polyphenolic contents

CVF ethanolic extract was fractionated on a polyamide column. The resulted fractions were subjected for elution on cellulose and/or Sephadex LH-20 columns yielded five compounds. The isolated pure compounds were identified on the basis of acid hydrolysis, physical characters, comparative PC against standards, UV, ESI-MS, ^{1}H-, ^{13}C NMR spectroscopic analyses and comparing with previous reported data [2,3,10–13]. The known isolated compounds are identified as 1; Gallic acid, 2; Ellagic acid, 3; Reynoutrin, 4; Methoxy ellagic acid, 5; Quercetin.

Previous literatures showed that quercetin has significant immunomodulatory and cytotoxic activities [14–16], so it was interesting to determine certain biological activities of the related derivative of quercetin i.e. Reynoutrin (Quercetin 3-*O*-β-D-xylopyranoside) which is isolated from CVF in this study.

Compound 3: Reynoutrin (Quercetin 3-*O*-β-D-xylopyranoside): Isolated as yellow amorphous powder (16 mg). Chromatographic properties: R_f values; 0.33(S_1), 0.39 (S_2); observed dark purple spot under UV 365, then changed to yellow fluorescence when exposed to ammonia vapor. It showed deep green color and orange fluorescence with $FeCl_3$ and Naturstoff spray reagents, respectively. UV- spectral data λ_{max} (nm) (MeOH): 258, 355; (+NaOMe): 271, 325 (sh), 403; (+NaOAC): 272, 324 (sh), 373; (+$AlCl_3$): 276, 433; (+$AlCl_3$/HCl): 270, 362(sh), 405. D-xylose was resulted in aqueous phase and quercetin in organic phase upon acid hydrolysis (CoPC). ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 12.60 (1H, s, H-bonded OH-5), 7.52 (1H, dd, J = 8.1, 2.5 Hz, H-6′), 7.43 (1H, d, J = 2.5 Hz, H-2′), 6.81 (1H, d, J = 8.1 Hz, H-5′), 6.38 (1H,d, J = 2.4 Hz, H-8), 6.16 (1H,d, J = 2.4 Hz, H-6), 5.24 (1H, d, J = 7.2 Hz, H-1″), 4.12 –3.13 (5H, m, H-2″, 3″, 4″, 2H-5″). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 177.2 (C-4), 164.8 (C-7), 161.8 (C-5), 156.9 (C-2), 156.3 (C-9), 149.1 (C-4′), 145.7 (C-3′), 133.4 (C-3), 122.3 (C-6′), 121.5 (C-1′), 116.1 (C-5′),115.6 (C-2′), 101.5 (C-1″), 104.5 (C-10), 99.2 (C-6), 94.1 (C-8), 76.4 (C-3″), 72.7 (C-2″), 67.5 (C-4″), 64.8 (C-5″). Negative ESI-MS: m/z 867.07 $[2M-H]^-$, 433.44 $[M-H]^-$, 301.32 [quercetin-H]$^-$.

On the basis of the obtained chromatographic properties and UV-spectral data, compound 3 was expected to be Quercetin 3-*O*-glycoside. From UV-spectral data, it gave a typical MeOH spectrum of a 3-*O*-substituted flavonol. The bathochromic shift of band II in NaOAc and $AlCl_3$ spectra together with the bathochromic shift in band I in $AlCl_3$-HCl indicated the presence of free7-OH and 5-OH groups. The bathochromic shift without decrease in intensity of band I was confirmative to a free 4′–OH. The acid hydrolysis of compound 3 afforded Quercetin as an aglycone and the sugar moiety was identified as xylose (CoPC).

Negative ESI-MS spectrum exhibits a molecular ion peak at m/z 433.44 $[M-H]^-$, corresponding to M.wt 434 and molecular formula $C_{20}H_{18}O_{11}$ for quercetin pentoside. As well as, this evidence was further supported by the fragment ions at m/z 867.07 $[2M-H]^-$, for the dimeric adduct ion and at 301.32 [quercetin-H]$^-$, for quercetin aglycone (i.e. loss of pentose, 132 m/z). ^{1}H NMR spectra showed the two characteristic spin coupling systems in aromatic region, the ABX (H-2′d, H-6′dd, H-5′d) and AM (H-8 d, H-6 d) for 3′, 4′ dihydroxy B-ring and 5,7-dihydroxy A-ring protons respectively. In the aliphatic region, a doublet at δ ppm 5.24 with J = 7.2 Hz was characteristic for the anomeric proton of β-D sugar moiety [17]. ^{13}C NMR spectrum showed 15 carbon signals among which the key signals at δ ppm 177.2 (C-4), 149.1 (C-4′), 145.7 (C-3′), and 133.4 (C-3) for a 3-*O*-glycosyl-quercetin [13]. In addition, five ^{13}C resonances 101.5 (C-1″), 76.4 (C-3″), 72.7 (C-2″), 67.5 (C-4″), 64.8 (C-5″) assignable to xylopyranoside moiety by comparison with previous published data [2,17,18]. Accordingly compound 3 was identified as Quercetin 3-*O*-β-D-xylopyranoside which is isolated for the first time from the genus of Callistemon (See Fig. 1).

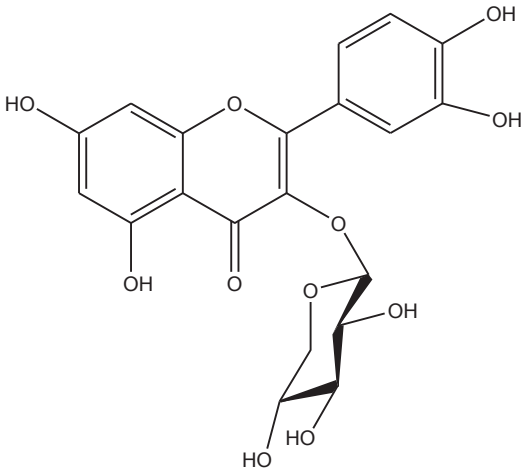

Fig. 1. Reynoutrin: Quercetin 3-*O*-β-D-xylopyranoside.

It is well documented that Gallic, Ellagic and Methoxy ellagic acids are known for their immunomodulatory and cytotoxicity activities. So it was interesting to compare between the aglycone (i.e. Quercetin) and its glycoside (i.e. Quercetin 3-*O*-β-D-xylopyranoside). As reynoutrin cytotoxic and immunomodulators activities of this specific glycoside was not previously studied.

3.2. POM analyses of Reynoutrin and Quercetin

The main problems of the synthetic drugs are manifested by their side effects. The potential drug should have good biological activity with good pharmacokinetic properties. The well established *in silico* tools such as POM represent a good tool to access the pharmacokinetic profile of the detected molecules [19–23].

Table 1 represents results of theoretical toxicity risks of Reynoutrin and Quercetin calculated with the aid of the Osiris program. Reynoutrin was found to be not toxic in relation with Quercetin, so Reynoutrin may be utilized as safe therapeutic agent. As well as these compounds showed good pharmacokinetic properties, which in accordance with previous studies on similar compounds [24,25]. Furthermore, Table 2 shows drug-likeness of Reynoutrin and Quercetin, in the comparable zone with standard known drugs.

Additionally, this multi-biological and important activities of flavonoids (anti-inflammatory, antiviral, antitumoural and antibacterial) encouraged us to look on chemical stability of flavonoids in media of different pH and try to understand the origin of the complex therapeutic behavior of their metabolites. POM theory of identification of pharmacophoric sites [22,26] has certainly become as one of the recent well-known methods that are regularly used [27,28] to detect structure

Table 1
Osiris calculations of toxicity risks of Quercetin and Reynoutrin.

Compounds	MW	Toxicity Risks[a]				Osiris calculations[b]			
		MUT	TUM	IRRIT	REP	cLog*P*	Sol	DL	DS
Reynoutrin	434	+ + +	+ + +	+ + +	+ + +	0.76	−2.53	3.14	0.79
Quercetin	302	− − −	− − −	+ + +	+ + +	1.49	−2.49	1.6	0.3

Higly toxic: (− − −), Slightly toxic: (+), Not toxic (+ + +).
[a] MUT: Mutagenic, TUM: Tumorigenic, IRRIT: Irritant, REP: Reproductive effectiveness.
[b] Sol: Solubility, DL: Drug likeness, DS: Drug score.

Table 2
Molinspiration calculations of Quercetin and Reynoutrin.

Compounds	Molinspiration calculations[a]				Drug-likeness[b]					
	TPSA	NONH	NV	VOL	GPCRL	ICM	KI	NRL	PI	EI
Reynoutrin	190.28	7	2	347.36	0.07	−0.03	0.12	0.26	0.09	0.56
Quercetin	131.35	5	0	240	−0.06	−0.19	0.28	0.36	−0.25	0.28

[a] TPSA: Total molecular polar surface area, NONH: number of hydrogen bond interaction, NV: Liponosky rules, VOL: volume.
[b] GPCRL: GPCR ligand, ICM: Ion channel modulator, KI: Kinase inhibitor, NRL: Nuclear receptor ligand, PI: Protease inhibitor, EI: Enzyme inhibitor.

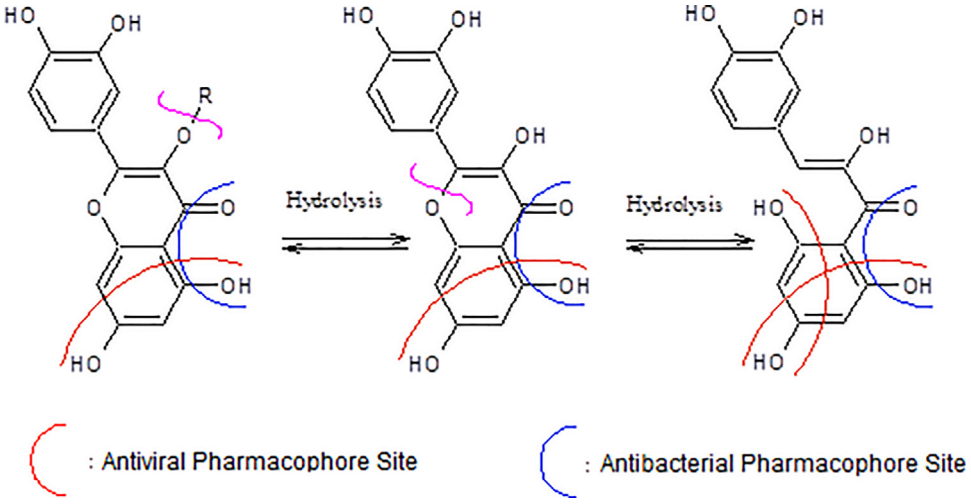

Fig. 2. Possible mechanism of synergistic effect of three pharmacophoric sites resulting from opening/closing [29,30] of the central ring of flavonoids such as Reynoutrin and quercetin.

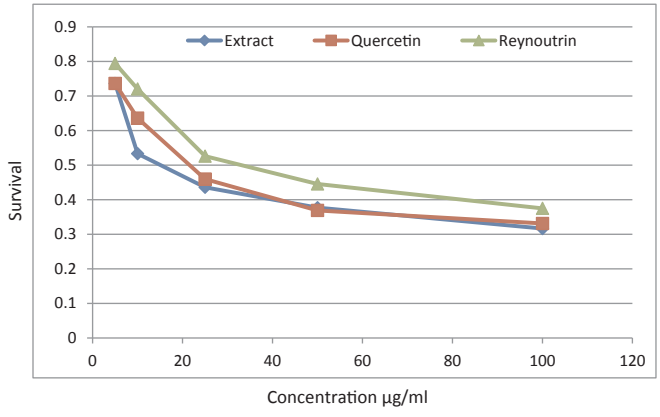

Fig. 3. Cytotoxic activity against P388 leukemia cells.

activity relationship. POM analysis represents a good tool to predict the biological activity of the studied compounds according to their pharmacophoric sites [29,30].

It is well established that flavonoids are subjected to important chemical processes of opening/closing of the central ring, leading to regeneration of supplementary pharmacophoric sites as shown in Fig. 2. Possible mechanism of synergistic effect of three pharmacophoric sites resulting from opening/closing of the central ring of flavonoids such as Reynoutrin and Quercetin may give opportunity to use these compounds as antibacterial and antiviral agents. This may give a benefit to use these compounds to enhance immunity. Most importantly, the molinspiration calculations pertaining to Reynoutrin and Quercetin showed that they are good candidates to interact with various enzymatic targets (GPCR ligand, ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease inhibitor and enzyme inhibitor) as indicated in Table 2. These findings may give us the opportunity to test the isolated Quercetin derivative (i.e Reynoutrin) to estimate its immunomodulatory and cytotoxic activities in comparison with Quercetin and the 80% ethanolic extracts of CVF as it will be discussed later.

3.3. Immunomodulatory activity

Results revealed that the incubation of macrophages with 80% of ethanolic extract, Quercetin and Reynoutrin cause a significant increase ($P < 0.05$) in the cells proliferation at the tested dose (100 µg/ml). In

addition, the extract, Reynoutrin and Quercetin increased cell proliferations by 1.48, 1.51 and 1.46 fold of the control, respectively, indicating immunomodulatory activity [31].

3.4. Biological assay against P388 leukemia cells

The United State National Cancer Institute (USNCI) plant screening program consider crude extract possess an in vitro cytotoxic activity only if its IC50 value is less than 20 μg/ml [32], the CVF ethanolic extract is considered active against P388 leukemia cells (IC_{50} = 15.11 μg/ml), followed by Quercetin (IC_{50} = 21.5 μg/ml) then its derivative i.e. Reynoutrin (IC_{50} = 33.32 μg/ml) in this aspect (Fig. 3). Polyphenolic compounds are expected to be suppressive and chemo-preventive active agents against cancer cells. Its mechanism of action is by inhibition of metabolic enzymes involved in the activation of potential carcinogens or arresting the cell cycle [33,34].

4. Conclusion

In summary, findings from this study showed that 80% ethanolic extract from *C. viridiflorus* fruits contain phenolic compounds like Gallic acid, Ellagic acid, Reynoutrin, Methoxy ellagic acid and Quercetin. Reynoutrin isolated for the first time from the genus *Callistemon*. POM analyses predicted that Reynoutrin and Quercetin have interesting potential activities, also Reynoutrin is found to be safer in relation to quercetin. The extract, Reynoutrin and Quercetin displayed remarkable immunomodulatory activity, and may be used in immune compromised patients to improve their immunity against various bacterial infections, giving *C. viridiflorus* great potential as a natural health care product. Moreover, findings from this study show that Reynoutrin and Quercetin have an interesting potential to inhibit certain enzymes as predicted by POM Analyses. However, more work is required to establish the safety and efficacy of these compounds.

Conflict of interest

The authors declare no conflicts of interest.

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