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Original article

Phytochemical constituents, antioxidant activity and toxicity potential of *Phlomis olivieri* Benth.

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Abstract

Background and objectives: *Phlomis olivieri* Benth. (Lamiaceae) is a medicinal plant widely distributed in Iran. In the present study, we have investigated the phytochemical constituents, antioxidant activity and general toxicity potential of the aerial parts of this species. **Methods:** Silica gel (normal and reversed phases) and Sephadex LH-20 column chromatographies were used for isolation of compounds from methanol-soluble portion (MSP) of the total extract obtained from *P. olivieri* aerial parts. The structures of isolated compounds were elucidated using ^{1}H-NMR, ^{13}C-NMR and UV spectral analyses. Antioxidant activity and general toxicity potential of MSP were also evaluated in DPPH free radical-scavenging assay and brine shrimp lethality test (BSLT), respectively. **Results:** One caffeoylquinic acid derivative, chlorogenic acid (**1**), one iridoid glycoside, ipolamiide (**2**), two phenylethanoid glycosides, phlinoside C (**3**) and verbascoside (**5**), along with two flavonoids, isoquercetin (**4**) and naringenin (**6**) were isolated and identified from MSP. The MSP exhibited considerable antioxidant activity in DPPH method (IC_{50}; 50.4 ± 4.6 µg/mL), compared to BHT (IC_{50}; 18.7 ± 2.1 µg/mL), without any toxic effect in BSLT at the highest tested dose (1000 µg/mL). **Conclusion:** the results of the present study introduce *P. olivieri* as a medicinal plant with valuable biological and pharmacological potentials.

Keywords: brine shrimp lethality test, chromatography, DPPH, Lamiaceae, *Phlomis olivieri* Benth.

Introduction

Phlomis olivieri Benth. belonging to the Lamiaceae family, is a perennial herbaceous plant distributed in south-western Asia [1]. In Iranian Traditional Medicine, the leaves of this species have been mentioned useful for alleviation of pains and its aerial parts have also been used as carminative [2,3].

So far, a number of biological and phytochemical studies have been conducted on the various extracts obtained from the aerial parts of *P. olivieri* [4-13]. In 2003, Sarkhail *et al*. reported significant antinociceptive effects from the total extract of *P. olivieri* aerial parts at the dose of 150 mg/kg in visceral writhing test model in mice [4]. The methanol extract of the aerial parts has shown to possess a concentration-dependent antibacterial activity against *Staphylococcus aureus*, *Streptococcus sanguis*, *Escherichia coli*,

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Pseudomonas aeruginosa and *Klebsiella pneumoniae*, as well as antioxidant effect when used in sunflower oil [5,6]. Two flavonoid derivatives including chrysoeriol-7-O-β-D-glucopyranoside and 6,7-dimethoxy-5-hydroxy flavanone, together with one phenylethanoid glycoside, verbascoside have been isolated from the aerial parts of *P. olivieri* during previous phytochemical investigations [7,8]. Moreover, there are some reports on essential oil compositions of this species indicating to the presence of sesquiterpene hydrocarbones (mainly germacrene D), as the main group of its chemical constituents [9-13].

In the present study, phytochemical constituents of *P. olivieri* aerial parts were investigated and its antioxidant and general toxicity potentials were evaluated in DPPH method and brine shrimp lethality test, respectively.

Experimental

General procedures

^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Bruker Avance DRX 500 spectrometer. UV spectra were recorded on a CECIL 7250 spectrophotometer in methanol and after the addition of shift reagents.

Silica gel (230-400 mesh, Merck), RP-C18 (230-400 mesh, Fluka, Switzerland) and Sephadex LH-20 (Fluka, Switzerland) were used as solid phases for column chromatographies. Pre-coated Silica gel GF_{254} sheets (Merck, Germany) were applied for the thin layer chromatography (TLC) and the spots were monitored under UV (254 and 366 nm) and by spraying anisaldehyde/H_2SO_4 reagent. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and *Artemia salina* eggs were obtained from Sigma-Aldrich (Germany) and Ocean nutrition (Belgium) companies, respectively. Other chemicals and all of the used solvents were also purchased from Merck chemical company.

Plant material

The flowering aerial parts of *P. olivieri* were gathered in July 2013 from the southern slopes of Mishu-dagh Mountains, East-Azerbaijan province, Northwest of Iran. The plant specimen was then authenticated by botanist Dr. M. Aghaahmadi from University of Isfahan, Isfahan, Iran.

Extraction

The shade-dried aerial parts (0.8 kg) were powdered and macerated with methanol (6×4 L) at room temperature. The obtained total methanol extract was concentrated using a rotary evaporator at 40 °C and dried completely by a freeze dryer. The freeze dried extract was then defatted by eluting with enough volumes of petroleum ether and chloroform, respectively. Finally, the residual methanol-soluble portion (MSP) was subjected to phytochemical and biological studies.

Isolation and purification of the compounds

Thirty five grams of the MSP was moved to a Sephadex LH-20 column and eluted with MeOH-H_2O (9:1) to get three fractions (A-C). Reversed-phase (C_{18}) column chromatography of the fraction B (10 g) with a gradient mixture of ACN-H_2O (0.5:9.5-2:8) yielded six fractions (B1-B6). Compound **1** (23 mg) was isolated from the fraction B1 (1.2 g) on a Sephadex LH-20 column (MeOH-H_2O, 8:2) and its impurities were removed over a RP-18 column (ACN-H_2O, 1:9). Faction B2 (169 mg) was subjected to RP-18 column chromatography with ACN-H_2O (0.2:9.8-1:9) to get four fractions (B2a-B2d). Compound **2** (25 mg) was obtained from the fraction B2c (43 mg) over the Sephadex LH-20 column eluted with MeOH-H_2O (8:2). Fraction B3 (215 mg) was eluted on a Sephadex LH-20 column with MeOH-H_2O (8:2) to get compound **3** (17 mg). RP-18 column chromatography of the fraction B4 (720 mg) with ACN-H_2O (1:9-2:8) resulted in five fractions (B4a-B4e). Compounds **4** (21 mg) and **5** (36 mg) were isolated from the fractions B4b and B4d, respectively, over the Silica gel columns with EtOAc-CH_3COOH-HCOOH-H_2O (36:1:1:2.4) as the eluent. Silica gel column chromatography of the fraction B6 (276 mg) with $CHCl_3$-EtOAc (7:3-3:7) resulted

in five fractions (B6a-B6e). Compound **6** (15 mg) was achieved from the fraction B6a (83 mg) on a RP-18 column eluted with ACN-H_2O (7:3).

Free radical-scavenging assay

The free radical-scavenging effect of MSP was assessed in 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [14]. The stock of DPPH solution was prepared at the concentration of 8.0×10^{-2} mg/mL in methanol. MSP dilutions were also made in methanol to get concentrations of 5.0×10^{-1}, 2.5×10^{-1}, 1.2×10^{-1}, 6.2×10^{-2}, 3.1×10^{-2} and 1.6×10^{-2} mg/mL. MSP prepared solutions (2.0 mL each) were mixed with DPPH solution (2.0 mL). After thirty minutes, UV absorbances of the solutions were recorded at 517 nm. Butylated hydroxytoluene (BHT), a synthetic antioxidant, was used as the positive control. The experiments were repeated tree times and the IC_{50} values were expressed as Mean ± SD.

Brine shrimp lethality test

Brine shrimp lethality test (BSLT) was used for evaluation of general toxicity potential of MSP [15]. The *Artemia salina* L. eggs were hatched in sterile artificial seawater (38 g/L, pH 9) under constant aeration for 48 hours at 30 °C. MSP (50 mg) was dissolved in freshly prepared artificial sea water and then diluted to obtain the solutions with 1000, 700, 500, 300, 100 and 10 µg/mL concentrations in a series of tubes containing about 20 active nauplii in each. Following the incubation of the tubes at 30 °C for 24 hours under light, the surviving nauplii were counted to achieve the LC_{50} value (the concentration causing 50% lethality). Podophyllotoxin, a known cytotoxic natural compound, was applied as the positive control. The assays were performed in triplicate and the LC_{50} values were reported as Mean ± SD.

Results and discussion

Phytochemical analyses of the methanol-soluble portion (MSP) of *P. olivieri* total extract on Silica gel (normal and reversed phases) and Sephadex LH-20 columns resulted in isolation of one caffeoylquinic acid derivative, chlorogenic acid (**1**), one iridoid glycoside, ipolamiide (**2**), two phenylethanoid glycosides, phlinoside C (**3**) and verbascoside (acteoside) (**5**), together with two flavonoids, quercetin-3-O-β-D-glucopyranoside (isoquercetin) (**4**) and naringenin (**6**) (figure 1).

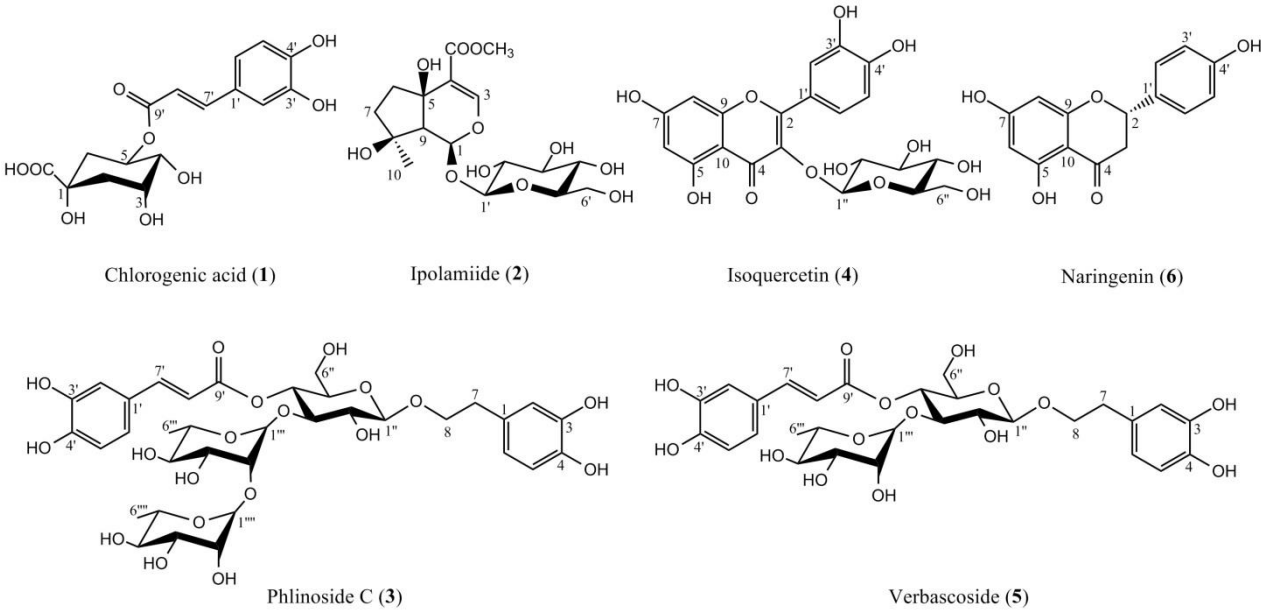

Figure 1. Structures of the isolated compounds (**1-6**) form the aerial parts of *Phlomis olivieri*

The structures of the isolated compounds were established using ^{1}H-NMR, ^{13}C-NMR and UV spectral analyses, as well as by comparison with published data [7,16-20].

Compound **1**: *Chlorogenic acid*; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 7.45 (1H, *d*, *J*= 15.9 Hz, H-7'), 7.04 (1H, *d*, *J*= 1.8 Hz, H-2'), 6.98 (1H, *dd*, *J*= 8.0, 1.8 Hz, H-6'), 6.77 (1H, *d*, *J*= 8.0 Hz, H-5'), 6.22 (1H, *d*, *J*=15.9 Hz, H-8'), 5.16 (1H, *m*, H-5), 3.93 (1H, *m*, H-3), 3.50 (1H, *dd*, *J*= 9.7, 2.7 Hz, H-4), 1.6-2.0 (4H, *m*, H-2,6); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 176.28 (C-7), 166.35 (C-9'), 148.61 (C-4'), 145.77 (C-3'), 144.64 (C-7'), 125.38 (C-1'), 121.22 (C-6'), 115.80 (C-5'), 114.64 (C-2'), 114.49 (C-8'), 75.18 (C-1), 73.33 (C-4), 71.62 (C-3), 71.48 (C-5), 39.7 (C-2), 38.11 (C-6) [16].

Compound **2**: *Ipolamiide*; ^{1}H-NMR (DMSO-d_6, 500 MHz): 7.44 (3H, *s*, H-3), 5.81 (1H, *s*, H-1), 4.58 (1H, *d*, *J*= 7.8 Hz, H-1'), 3.90 (1H, *br d*, *J*= 11.6 Hz, H-6'b), 3.73 (3H, *s*, $COOCH_3$), 3.65 (1H, *dd*, *J*= 11.6, 6.0 Hz, H-6'a), 3.0-3.4 (4H, H-2'-5'), 2.47 (1H, *s*, H-9), 2.26 (1H, *m*, H-6b), 2.07 (1H, *m*, H-7b), 1.93 (1H, *m*, H-6a), 1.56 (1H, *m*, H-7a), 1.15 (3H, *s*, C-10); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 168.03 (COOCH3), 153.40 (C-3), 115.81 (C-4), 100.20 (C-1'), 94.80 (C-1), 78.90 (C-8), 77.82 (C-5'), 76.84 (C-3'), 74.93 (C-2'), 71.09 (C-5,4'), 62.80 (C-6'), 61.13 (C-9), 51.06 (COOCH3), 40.3 (C-7), 38.83 (C-6), 23.72 (C-10) [17].

Compound **3**: *Phlinoside C*; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 7.67 (1H, *d*, *J*= 15.8 Hz, H-7'), 7.04 (1H, *brs*, H-2'), 6.93 (1H, *br d*, *J*= 8.3 Hz, H-6'), 6.76 (1H, *d*, *J*= 8.3 Hz, H-5'), 6.67 (1H, *brs*, H-2), 6.63 (1H, *d*, *J*= 8.3 Hz, H-5), 6.55 (1H, *d*, *J*= 8.3 Hz, H-6), 6.25 (1H, *d*, *J*= 15.8 Hz, H-8'), 5.33 (1H, *br s*, H-1'''), 4.90 (1H, *br s*, H-1''''), 4.36 (1H, *d*, *J*= 7.8 Hz, H-1''), 3.1-4.1 (16H, H-8, 2''-6'', 2'''-5''', 2''''-5''''), 2.77 (2H, *t*, *J*= 7.1 Hz, H-7), 1.24 (1H, *d*, *J*= 6.0 Hz, H-6''''), 1.05 (1H, *d*, *J*= 6.0 Hz, H-6'''); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 168.80 (C-9'), 148.82 (C-4'), 146.47 (C-7'), 145.97 (C-3'), 145.22 (C-3), 142.70 (C-4), 131.33 (C-1), 128.98 (C-1'), 124.28 (C-6'), 118.86 (C-6), 117.25 (C-5), 116.85 (C-2), 116.23 (C-5'), 115.13 (C-2'), 114.36 (C-8'), 104.12 (C-1''), 103.46 (C-1''''), 99.72 (C-1'''), 80.91 (C-2'''), 79.34 (C-3''), 77.36 (C-5''), 75.31 (C-2''), 73.37 (C-4'''), 73.31 (C-4''''), 71.62 (C-8), 71.24 (C-3'''), 71.04 (C-2''''), 69.67 (C-4'', 3''''), 69.40 (C-5''''), 69.71 (C-5'''), 61.60 (C-6''), 32.11 (C-7), 14.85 (C-6'''), 14.42 (C-6'''') [18].

Compound **4**: *Quercetin-3-O-β-D-glucopyranoside (Isoquercetin)*; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 7.60 (1H, *d*, *J*= 2.0 Hz, H-2'); 7.57 (1H, *dd*, *J*= 8.4, 2.0 Hz, H-6'); 6.84 (1H, *d*, *J*= 8.4 Hz, H-5'); 6.39 (1H, *d*, *J*= 2.0 Hz, H-8); 6.18 (1H, *d*, *J*= 2.0 Hz, H-6); 5.60 (1H, *d*, *J*= 6.9 Hz, H-1''); 3.0-3.7 (6H, H2''-6''); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 177.37 (C-4), 164.51 (C-7), 161.23 (C-5), 156.34 (C-2), 156.10 (C-9), 148.57 (C-4'), 144.83 (C-3'), 133.26 (C-3), 121.53 (C-6'), 121.18 (C-1'), 116.14 (C-5'), 115.24 (C-2'), 103.80 (C-10), 100.86 (C-1''), 98.74 (C-6), 93.59 (C-8), 77.55 (C-5''), 76.42 (C-3''), 74.08 (C-2''), 69.95 (C-4''), 60.91 (C-6''); UV (MeOH) λmax: 256, 358., + NaOMe: 272, 411., +NaOAc: 272, 378 [19].

Compound **5**: *Verbascoside* (*acteoside*); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 7.45 (1H, *d*, *J*= 15.9 Hz, H-7'), 7.04 (1H, *d*, *J*= 1.6 Hz, H-2'), 6.97 (1H, *dd*, *J*= 8.0, 1.6 Hz, H-6'), 6.77 (1H, *d*, *J*= 8.0 Hz, H-5'), 6.64 (1H, *d*, *J*= 1.6 Hz, H-2), 6.63 (1H, *d*, *J*= 8.0 Hz, H-5), 6.49 (1H, *dd*, *J*= 8.0, 1.6 Hz, H-6), 3.89 (2H, *m*, H-8), 2.70 (2H, *m*, H-7), 6.19 (1H, *d*, *J*= 15.9 Hz, H-8'), 5.03 (1H, *br s*, H-1'''), 4.35 (1H, *d*, *J*= 7.8 Hz, H-1''), 3.1-3.8 (10H, H-2''-6'', 2'''-5'''), 0.96 (3H, *d*, *J*= 6.2 Hz, H-6'''); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 165.84 (C-9'), 148.37 (C-4'), 145.51 (C-7'), 145.66 (C-3'), 144.85 (C-3), 143.43 (C-4), 129.32 (C-1), 125.63 (C-1'), 121.61 (C-6'), 119.70 (C-6), 116.33 (C-2), 115.83 (C-8'), 115.50 (C-5), 114.57 (C-2'), 113.71 (C-5'), 102.36 (C-1''), 101.31 (C-1'''), 79.31 (C-3''), 74.55 (C-2''), 74.54 (C-5''), 71.66 (C-4'''), 70.36 (C-8), 70.46 (C-2'''), 70.37 (C-3'''), 69.25 (C-4''), 68.80 (5'''), 61.71 (C-6''), 35.10 (C-7), 18.23 (C-6''') [7].

Compound **6**; *Naringenin*; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 7.32 (2H, *d*, *J*= 8.5 Hz, H-2', 6'); 6.80 (2H, *d*, *J*= 8.5 Hz, H-3', 5'); 5.88 (2H, *br s*,

H-6, 8); 5.43 (1H, *dd*, *J*= 12.6, 3.1 Hz, H-2); 2.67 (1H, *dd*, *J*= 17.0, 3.1 Hz, H-3eq), 3.20 (1H, dd, *J*= 17.0, 12.8 Hz, H-3ax); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 194.90 (C-4), 166.86 (C-7), 163.26 (C-5), 163.06 (C-9), 157.83 (C-4'), 129.04 (C-1'), 127.81 (C-2', 6'), 114.58 (C-3', 5'), 101.71 (C-10), 96.67 (C-6), 94.38 (C-8), 77.85 (C-2), 46.21 (C-3) [20].

Phenylethanoid glycoside verbascoside (**5**) has been previously reported from this species [7], however, this is the first report on isolation of the compounds **1**-**4** and **6** from the aerial parts of *P. olivieri*. Within the genus *Phlomis*, phenylethanoid glycosides, iridoids and flavonoids, are the main phytochemicals and the isolated compounds **1**-**6**, have been previously reported from other *Phlomis* species [21].
In DPPH test, the MSP exhibited a considerable free radical-scavenging activity with the IC_{50} value of 50.4 ± 4.6 µg/mL, compared to BHT (IC_{50}; 18.7 ± 2.1 µg/mL) (table 1). However, it did not show any lethality effect on the brine shrimps larva at the highest tested concentration (2000 µg/mL) (table 1).

Table 1. The results of brine shrimp lethality test and DPPH free radical-scavenging assay of the methanol-soluble portion (MSP) of *Phlomis olivieri* total extract

Samples	Brine shrimp lethality activity LD_{50} (µg/mL)	DPPH free radical scavenging activity IC_{50} (µg/mL)
Methanol-soluble portion (MSP)	> 1000	50.4 ± 4.6
Podophyllotoxin	2.6 ± 0.5	-
Butylated hydroxytoluene (BHT)	-	18.7 ± 2.1

A literature review revealed that some *in vitro* and *in vivo* biological activities such as antioxidant, antimicrobial and analgesic effects have been documented for the compounds **1** and **4**-**6** [22-30]. Accordingly, isolated phenolic compounds (**1**,**3**-**6**) could be considered responsible for the observed antioxidant, antimicrobial and analgesic effects of *P. olivieri* [4-6]. Verbascoside, a main phenylethanoid glycoside present in *Phlomis* spp., has been considered for its beneficial health effects such as antioxidant, neuroprotective, hepatoprotective, analgesic and anti-inflammatory properties [21,31]. Ipolamiide, an iridoid glycoside isolated from *Stachytarpheta mutabilis* (Verbenaceae), has also been reported as an antifeedant agent for two generalist insect species: *Schistocerca gregaria* and *Locusta migratoria* [32].
The results of the present study about the occurrence of these biologically active principles in *P. olivieri*, as well as its observed considerable antioxidant without toxicity effects, introduce it as a medicinal plant with valuable biological and pharmacological potentials. Moreover, the present study suggests *P. olivieri* as a safe carminative and antinociceptive remedy.

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Declaration of interest
The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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