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Phytochemistry and bioactivity of *Pedicularis sibthorpii* growing in Iran

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Abstract: The methanol extract of the aerial parts of the medicinal plant *Pedicularis sibthorpii* Boiss., Scrophulariaceae, growing in the Azerbaijan province of Iran, was found to be active in the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and the antibacterial agar well diffusion assays, but no general toxicity was observed in the brine shrimp lethality assay. A combination of solid-phase extraction (SPE) and preparative reversed-phase high-performance liquid chromatography (prep-RP-HPLC) analyses of the methanolic extract afforded three phenylethanoids (verbascoside, martynoside and isomartynoside), an iridoid (aucubin), a flavonoid (luteolin 7-*O*-β-D-glucopyranoside) and mannitol, and the structures of these compounds were elucidated unambiguously by spectroscopic means. The distribution of the isolated compounds within the genus *Pedicularis* has also been discussed.

Introduction

The family Scrophulariaceae incorporates ca. 400 genera and 4500 species (Maia et al., 2000). The genus *Pedicularis* L., comprising about 500 medicinal herbs, mostly endemic to China, is one of those genera of this family (Zhang et al., 2011). Several species of this genus, *e.g., P. muscicola, P. oliveriana, P. kansuensis* and *P. rhinanthoides*, are used in Tibetan medicine system (Jiang et al., 2003; Zhang et al., 2011). Traditionally, many *Pedicularis* species have long been used in the Traditional Chinese Medicine (TCM) as a cardio-tonic, to improve blood circulation, and for the treatment of exhaustion, collapse, senility and digestive problems. It is effective in relieving uneasiness of body and mind (Jiang et al., 2003; Zhang et al., 2008). Some species of the *Pedicularis* are also used in the treatment of malignant sores (Shi et al., 1999). To the best of our knowledge, while the only previous phytochemical studies on *P. sibthorpii* revealed the presence of phenylpropanoid glycosides (Eribekyan et al., 1991), the volatile oil of this species has recently been investigated (Khodaei et al., 2012). However the extracts of *P. sibthorpii* have never been evaluated for their biological activities. As part of our on-going phytochemical and bioactivity studies on Iranian medicinal plants (Delazar et al., 2004, 2006, 2007, 2009, 2010a,b; 2011a,b; 2012; Babaei et al., 2008; Nazemiyeh et al., 2008a,b, 2011; Nazifi et al., 2008; Razavi et al., 2008, 2011; Modaressi et al., 2009; Asnaashari et al., 2010; Pasdaran et al., 2012), we now report on the bioactivity of the extracts of *P. sibthorpii* Boiss. growing in Iran, and the isolation and identification of three phenylethanoids [isomartynoside (**3**), martynoside (**2**), and verbascoside (**1**)], an iridoid (aucubin, **4**), a flavonoid (luteolin 7-*O*-β-D-glucopyranoside, **5**) and mannitol (**6**) from the methanolic extract of this species. The distribution of the isolated compounds within the genus *Pedicularis* has also been discussed.

Material and Methods

General

NMR spectra were obtained using a Bruker Spectrospin 200 NMR-spectrometer. Chemical shifts are given on δ (ppm) scale with TMS as the initial standard. UV-visible spectra were recorded using a Shimadzu-1600 spectrophotometer. Preparative HPLC was conducted on a Knauer-1800 prep-HPLC coupled with SPDM photo diode array detector (detection at 220 and 280 nm).

Plant material

Aerial parts of *Pedicularis sibthorpii* Boiss., Scrophulariaceae, were collected during the flowering stage from Lighvan region of the East Azerbaijan province in Iran in May 2008. A voucher specimen (TUM-ADE-0318) for this collection has been deposited at the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Extraction

Air-dried and ground aerial parts of *P. sibthorpii* (200 g) were successively Soxhlet-extracted using *n*-hexane, dichloromethane (DCM) and methanol (MeOH) (1.1 L each). All these extracts were separately concentrated under vacuum by rotary evaporator not exceeding the temperature of 50 ºC, yielding 2.2 g, 1.7 g and 13.8 g of the extracts, respectively.

Fractionation of the methanolic extract

A portion of the dried MeOH extract (2 g) was fractionated by solid-phase-extraction (SPE) on Sep-Pak (C_{18}, 10 g cartridge) using a step gradient of MeOH-water mixture (10:90, 20:80, 40:60, 60:40, 80:20 and 100:0), 200 mL each. All SPE fractions were dried using a rotary evaporator at a temperature not exceeding 50 ºC.

Isolation of compounds

The SPE fractions (10, 20, 40 and 60% MeOH in water) were subjected to repeated preparative reversed-phase HPLC (prep-HPLC), conducted on a Knauer HPLC (preparative pump 1800), fitted with a Reprosil 100 C18 (250 mm length, 20 mm i.d, particle size 10 µm, Dr. Maisch, Germany) column. The mobile phase consisted of (A) MeOH and (B) water. The following mobile phase programme was used over 32 min to isolate aucubin (**4**, 3.9 mg, t_R = 18.12 min) and mannitol (**6**, 169.2 mg, t_R = 7.1 min) from the 10% SPE fraction: 10% A initially, changed to 40% A in 20 min, ran for 5 min, changed back to 10% A in 2 min and maintained there for 5 min. A similar programme over a run time of 55 min was applied for separating verbascoside (**1**, 8.0 mg, t_R = 27.7 min) from the 20% SPE fraction: 10% A, changed to 80% A in 45 min, changed back to 10% A in 5 min, and stayed there for another 5 min. Similarly, the following programme was applied to isolate luteolin 7-*O*-β-D-glucopyranoside (**5**, 6.2 mg, t_R = 29.0 min) from the 40% SPE fraction: 35% A initially, changed to 50% A in 50 min, maintained there for 5 min, changed to 100% A in 3 min, maintained there for 7 min, changed back to 35% in 3 min, and kept there for 7 min. The 60% SPE fraction was analysed by the following mobile phase programme: 40% A initially, changed to 100% A in 38 min, maintained there for 20 min, changed back to 40% A in 7 min, and maintained there for 5 min, to isolate martynoside (**2**, 3.5 mg, t_R = 22.0 min) and isomartynoside (**3**, 3.6 mg, t_R = 23.0). Photodiode array (PDA) detector was used to monitor the chromatogram, and the HPLC separation was carried out at room temperature. The flow rate was 8 mL/min and the injection volume was 1 mL. The structures of the compounds were determined by spectroscopic means as well as by comparison with the literature data of respective compounds.

Verbascoside (**1**): Brown amorphous solid. UV, ^{1}H NMR (200 MHz, CD_3OD) and ^{13}C NMR (50 MHz, CD_3OD) data were in agreement with the published data (Nazemiyeh et al., 2008c; Delazar et al., 2005, 2012).

Martynoside (**2**): Pale brown amorphous solid. UV, 1H NMR (200 MHz, CD_3OD) and ^{13}C NMR (50 MHz, CD_3OD) data were in agreement with the published data (Toth et al., 2007).

Isomartynoside (**3**): Brown amorphous solid. UV, ^{1}H NMR (200 MHz, CD_3OD) and ^{13}C NMR (50 MHz, CD_3OD) data were in agreement with the published data (Calis et al., 1984).

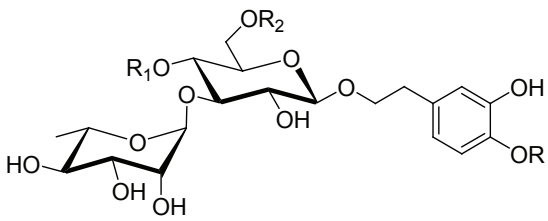
Aucubin (**4**): White amorphous solid. UV, ^{1}H NMR (200 MHz, CD_3OD) and ^{13}C NMR (50 MHz, CD_3OD) data were in agreement with the published data (Bernini et al., 1984; Rønsted et al., 2000).

Luteolin 7-O-β-D-glucopyranoside (**5**): Yellow amorphous solid. UV, ^{1}H NMR (200 MHz, CD_3OD) and ^{13}C NMR (50 MHz, CD_3OD) data were in agreement with the published data (Mabry et al., 1970; Suntar et al., 2012).

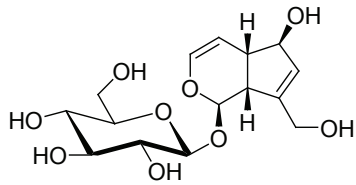
Mannitol (**6**): While amorphous solid. ^{1}H NMR (200 MHz, CD_3OD) and ^{13}C NMR (50 MHz, CD_3OD) data were in agreement with the published data (Bock & Pedersen, 1983; SDBS database, 2012).

Free-radical-scavenging activity: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The free-radical-scavenging effect of the extracts and fractions was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Kumarasamy et al., 2002, 2007). DPPH was obtained from Fluka Chemie AG, Bucks and a solution of DPPH (0.08 mg/mL) in MeOH was used. Dilutions were made to obtain concentrations of 5 × 10-1, 2.5×10^{-1}, 1.25×10^{-1}, 6.25×10^{-2}, 3.13×10^{-2} and 1.56×10^{-2} mg/mL. Diluted solutions (1 mL each) were mixed with DPPH solution (1 mL) and allowed to stand for 30 min for any reaction to take place. The UV absorbance was recorded at 517 nm. The experiment was performed



1 R=R$_2$=H; R$_1$=caffeoyl
2 R=CH$_3$; R$_1$=feruloyl; R$_2$=H
3 R=CH$_3$; R$_1$=H; R$_2$=feruloyl



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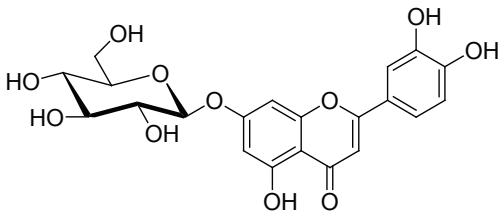

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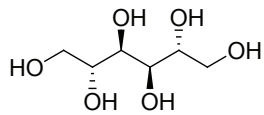

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in triplicate and the average absorption was noted for each concentration.

The agar well diffusion assay

Bacterial cultures of Gram-negative species *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Salmonella paratyphii* (ATCC 4420), as well as Gram-positive species namely *Staphylococcus epidermidis* (ATCC 12228), *Bacillus cereus* (ATCC 9372), *Staphylococcus aureus* (ATCC 6538), *Micrococcus luteus* (ATCC 10240), and a fungus (*Candida albicans*) strain were used to evaluate antimicrobial properties of the methanolic extract. The bacterial strains in lyophilized form were purchased from the Institute of Pasteur, Tehran, Iran. Centrifuged pellets of bacteria and fungus from 24 h cultures were mixed with sterile distilled water, and the turbidity was corrected by adding sterile distilled water until 0.5 McFarland's turbidity standard [10^8 colony forming units (CFU) per mL] was obtained. Then these inocula were used for seeding the Muller Hinton agar (Merck). Autoclaved Muller Hinton agar medium was allowed to cool down. Then it was seeded with 10 mL of prepared inocula (10^6 CFU per mL). The antimicrobial activity of test samples was monitored using the agar well diffusion method (Perumal et al., 1998; Essawi & Srour, 2000; Aqil & Ahmad , 2007), which is a highly recommended method for routine assessment of preliminary antimicrobial screening. Using the Muller Hinton plates, inoculated with a 0.5 McFarland's standard of selected bacteria, five wells for test samples, two for solutions of extract and different fractions, and one for vehicle control (DMSO), were applied to each Petri dish. For incubation and analysis, 100 µL of test solution was poured by micropipette into respective wells (200 mg/mL). Petri dish was incubated at 37 ˚C. After 24 h of incubation, diameter of the clear zones, showing no bacterial growth, around each well (excluding well diameter) was measured with the help of venire callipers. Plates were prepared in triplicate for each sample. Extracts and fractions that showed significant antibacterial activity at this concentration were further assessed for determination of their minimum inhibitory concentration (MIC). Serial two-fold dilutions of fractions were prepared in broth. Cultures containing only sterile nutrient broth, which did not influence bacterial growth, were used as controls. To each test tube an equal volume of the adjusted inocula was added. After incubation at 37 ºC for 24 h the MIC was read. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of a fraction which was able to completely inhibit the growth of each microbial strain (Bussmann et al., 2010).

The brine shrimp lethality assay

The method described by Meyer et al. (1982) was adopted to study the general toxicity of the plant. Brine shrimp (*Artemia salina*) was purchased from Water Life, Middlesex, UK. The eggs were hatched in a conical flask containing 300 mL of artificial seawater made by dissolving NaCl in distilled water. The flasks were well aerated by the aid of an air pump, and kept in a water bath at 29-30 °C. A bright light was left on. The nauplii hatched within 48 h. The *n*-hexane, DCM and MeOH extracts and SPE fractions were dissolved in 5% DMSO to obtain a concentration of 1 mg/mL each. These were serially diluted two times and seven different concentrations were obtained. A solution of each concentration (1 mL)

was transferred into clean sterile universal vials with a pipette and aerated sea water (10 mL) was added. About ten nauplii were transferred into each vial by the aid of pipette. A check count was performed. The number of alive nauplii after 24 h was noted. The mortality, end point of this bioassay was defined as the absence of controlled forward motion of nauplii during 30 s of observation. The controls used were 5% DMSO, saline and podophylotoxin (Huang et al., 2002; Lee et al., 2002; Padmaja et al., 2002; Verdi et al., 2004; Sarker et al., 2012).

Results and Discussion

Solid-phase extraction (SPE) of the MeOH extract of the aerial parts of *Pedicularis sibthorpii* Boiss., Scrophulariaceae, followed by reversed-phase prep-HPLC analyses of the SPE fractions (10, 20, 40 and 60% aqueous MeOH fractions) afforded three phenylethanoids, verbascoside (**1**, Nazemiyeh et al., 2008c; Delazar et al., 2005, 2012), martynoside (**2**, Toth et al., 2007) and isomartynoside (**3**, Calis et al., 1984), an iridoid, aucubin (**4**, Bernini et al., 1984; Rønsted et al., 2000), a flavonoid glycoside, luteolin 7-*O*-β-D-glucopyranoside (**5**, (Mabry et al., 1970; Suntar et al., 2012), and a monosaccharide mannitol (**6**, Bock & Pedersen, 1983; SDBS, 2012). All isolated compounds were identified unequivocally by UV and NMR analyses. All spectroscopic data were in agreement with respective published data. While verbascoside (**1**) was previously reported from *P. sibthorpii* (Eribekyan et al., 1991), to the best of our knowledge, this is the first report on the occurrence of compounds **2-6** in this species. Within the genus *Pedicularis*, the distribution of phenylethanoids glycosides, especially verbascoside (**1**), appears to be widespread (Table 1). Iridoids, e.g., aucubin (**4**), also occur in many *Pedicularis* species (Chu et al., 2011).

The free-radical-scavenging activity of the extracts and SPE fractions were determined by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free-radical-scavenging assay (Kumarasamy et al., 2002, 2007) (Table 2). This assay is based on the ability of DPPH, a stable free-radical, to decolorize in the presence of free-radical-scavengers (antioxidants). The absorption maximum (λ_{max} in MeOH) of DPPH at 517 nm and its visible deep purple colour are because of its odd electron (Kumarasamy et al., 2007). When DPPH accepts an electron, donated by a free-radical scavenger, DPPH is decolorized, and the extent of decolorization can be quantitatively measured from the changes in absorbance. The MeOH extract showed the highest level of free-radical scavenging activity with

Table 1. Distribution of compounds **1-6** within the genus *Pedicularis*.

Pedicularis species	Compounds						References
	1	**2**	**3**	**4**	**5**	**6**	
P. alaschanica	+	+	-	-	-	-	Gao & Jia, 1995; Wang et al., 1996
P. chinensis	-	-	-	+	+	-	Yang et al., 1995
P. condensata	+	-	-	+	-	-	Akdemir et al., 1991
P. densispica	+	+	+	-	-	-	Chu et al., 2011
P. dolichocymba	+	+	-	-	-	-	Zhu et al., 2010
P. striata	+	-	-	-	-	-	Mu et al., 2008
P. kansuensis	+	-	-	-		-	Di et al., 2004
P. lasiophrys	+	-	-	-	-	-	Jia et al., 1992
P. longiflora	+	-	-	-	-	-	Di et al., 2004
P. longiflora var tubiformis	+	+	-	+	+	-	Fujii et al., 1995; Di et al., 2004
P. plicata	+	+	+	+	-	-	Jia et al., 1994
P. punctata	+	-	-	+	-	-	Schneider & Romero, 1995
P. rex	+	+	+	+	+	-	Chu et al., 2007
P. semitorta	+	-	-	+	-	-	Wang et al., 1997
P. sibthorpii	+	+	+	+	+	+	Eribekyan et al., 1991; Present study
P. spicata	+	-	-	-	-	-	Jia et al., 1991
P. striata	+	-	-	-	-	-	Li et al., 1997
P. striata ssp. arachnoides	+	-	-	+	-	-	Jia & Gao, 1993; Gao et al., 1997
P. sylvatica	-	-	-	-	+	-	Carron et al., 1988
P. torta	+	-	-	-	-	-	Wang & Jia, 1997
P. verticillata	+	-	-	+	-	-	BaoNing et al., 1997

a RC50 value of 3.36 $\times 10^{-2}$ mg/mL among all extracts, whereas the dichloromethane extract did not show any activity at test concentrations. The activity of the MeOH extract was mostly contributed by the SPE fractions of 20, 40 and 60% aq. MeOH fractions (Table 2). Of the six isolated compounds (**1-6**) from various SPE fractions, four were phenolic compounds (**1-3**, **5**), and are well known for their free-radical scavenging properties (Delazar et al., 2012; Suntar et al., 2012). This is the first report on the free-radical-scavenging property of *P. sibthorpii* growing in Iran. However, free-radical scavenging or antioxidant property was also documented in several other species of the genus *Pedicularis, e.g*., *P. alaschanica* (Wang et al., 1996), *P. decora* (Gao et al., 2011), *P. lasiophrys* (Li et al., 1992) *P. mexicana* (Moreno-Escobar et al., 2011), *P. striata* (Li et al., 1992, 1997; Mu et al., 2008) and almost in all cases, the activity was found to be associated with phenylpropanoid glycosides, *e.g*., **1-3** (Li et al., 1992; Miao et al., 2003; Mu et al., 2008; Shi et al., 2011).

In the *in vitro* antimicrobial assay, only the MeOH extract was found to be active against three bacterial strains, *P. aeruginosa*, *S. aureus* and *S. epidermidis*, but not against the fungal strain *C. albicans* (Table 3), and the MIC was 200 µg/mL. Similar antimicrobial properties were also previously observed with the MeOH extracts of many other species of the family Scrophulariaceae, *e.g*., *Verbascum vacillance*, *V. chianophyllum*, *V. cilicium*, *V. trapifolium*, *V. meinkianum*, *Scrophulariaea tricopoda* and *Scrophulariaea candelabrum* (Dulger, 2006; Dulgar & Hagioglu, 2008). Earlier studies on the antimicrobial activity of the genus *Pedicularis* revealed that compounds isolated from *P. armata* were active against *S. aureus*, and *P. olympica* was active against Gram-positive strains (*S. aureus* and *M. luteus*) (Dulger & Ugurlu, 2005; Yuan et al., 2007). Thus, the findings of the current study are in agreement with previous reports. SPE fractions of the antimicrobial MeOH extract were also tested against susceptible strains (Table 3). Among the SPE fractions,

Table 2. Free-radical-scavenging activity and brine shrimp lethality of the extracts and fractions of the aerial parts of *Pedicularis sibthorpii.*

Extracts/fractions	RC50 value (mg/mL)[a]	LD50 value (mg/mL)[b]
n-hexane extract	20.9×10^{-1}	>1.00
dichloromethane extract	>10.0	>1.00
methanol extract	3.36×10^{-2}	>1.00
SPE fraction 10% MeOH-water	8.2×10^{-1}	>1.00
SPE fraction 20% MeOH-water	7.0×10^{-2}	>1.00
SPE fraction 40% MeOH-water	1.2×10^{-2}	>1.00
SPE fraction 60% MeOH-water	10.7×10^{-2}	>1.00
SPE fraction 80% MeOH-water	3.37×10^{-1}	>1.00
SPE fraction 100% MeOH-water	6.16×10^{-1}	>1.00
quercetin (positive control)	2.50×10^{-3}	ND
podophylotoxin	ND	2.80×10^{-3}

[a]Determined by the DPPH assay; [b]Determined by the brine shrimp lethality assay; ND: Not determined.

Table 3. Antimicrobial activity of the extracts and SPE fractions of the aerial parts of *Pedicularis sibthorpii* in the agar well diffusion assay.

Test samples	Zones of inhibition (in mm)[a]							
	[MIC in mg/mL]	EC	ML	PA	SA	SE	SP	CA
n-Hexane extract	-	-	-	-	-	-	-	-
DCM extract	-	-	-	-	-	-	-	-
MeOH extract	-	-	-	11.6 [200]	9.0 [200]	15.6 [200]	-	-
SPE fraction 10% MeOH-water	NT	NT	NT	-	-	-	NT	NT
SPE fraction 20% MeOH-water	NT	NT	NT	9.0 [100]	-	-	NT	NT
SPE fraction 40% MeOH-water	NT	NT	NT	9.0 [100]	14.5 [100]	16.5 [100]	NT	NT
SPE fraction 60% MeOH-water	NT	NT	NT	15.0 [100]	15.5 [100]	24.5 [100]	NT	NT
SPE fraction 80% MeOH-water	NT	NT	NT	16.0 [100]	-	12.0 [100]	NT	NT
SPE fraction 100% MeOH	NT	NT	NT	-	-	-	NT	NT

[a]Zones of inhibition at the concentration of 200 mg/mL (applied volume 100 µL); BC: *Bacillus cereus*; CA: *Candida albicans*; EC: *Escherichia coli*; ML: *Micrococcus luteus*; PA: *Pseudomonas aeruginosa*; SA: *Staphylococcus aureus*; SE: *Staphylococcus epidermidis*; SP: *Salmonella paratyphii*; MIC: Minimum inhibitory concentration; -: No activity at test concentration; NT: Not tested.

the 40 and 60% SPE fractions were active against *P. aeruginosa, S. aureus* and *S. epidermidis*. It is noteworthy, that neither the most polar (10% MeOH-water), nor the least polar (100% MeOH) SPE fractions displayed any activity against any of the above strains. The most prominent antibacterial activity was exhibited by the 60% MeOH-water SPE fraction against *S. epidermidi*s with a zone of inhibition of 24.5 mm.

In the brine shrimp lethality assay (Meyer et al., 1982), none of the extracts or fractions showed any toxicity at the highest test concentration (1 mg/mL) (Table 2).

The phytochemical investigation of the aerial parts of *P. sibthorpii* has demonstrated that this plant is a good source of phenolic glycosides, and the MeOH extract has significant free-radical scavenging and antibacterial properties, with no general toxicity.

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