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Chemical Composition, and Antibacterial (Against *Staphylococcus aureus*) and Free-Radical-Scavenging Activities of the Essential Oil of *Scrophularia amplexicaulis* Benth.

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Abstract: Chemical composition of the essential oil obtained from the aerial parts of *Scrophularia amplexicaulis* Benth. was analyzed, for the first time, by the gas chromatography/mass spectrometry (GC-MS) and gas chromatography/flame ionization detection (GC-FID). A total yield of 3 mg of essential oil per100 g of plant dry mass was obtained, and 27 compounds were identified, representing 97.7% of total oil. The essential oil were characterized by a high content of oxygenated monoterpenes and phenolic derivatives. The main constituents were eugenol (53.8%), eugenol acetate (24.5%), β-caryophyllene (5.7%), caryophyllene oxide (6.4%) and aromadendrene oxide II (2.1%). The antimicrobial activity of the essential oil was tested against *Staphylococcus aureus* using the well diffusion method, and the free-radical-scavenging activity was assessed by the 2,2-diphenyl-picryl-hydrazyl (DPPH) assay .

Keywords: *Scrophularia amplexicaulis;* Scrophulariaceae; essential oil; eugenol; eugenol acetate; caryophyllene oxide; antimicrobial activity; DPPH; *Staphylococcus aureus*

1. Introduction

The genus *Scrophularia* L. (Scrophulariaceae) comprises about 200 species of herbaceous flowering plants, commonly known as ‘figwort’ [1], and *Scrophularia amplexicaulis* Benth. is one such species that is endemic to western and central regions of Iran [2]. Species of this genus, which are distributed throughout the Northern Hemisphere, but concentrated in Asia with only a few species in Europe and North America, all share square stems, opposite leaves and open two-lipped flowers forming clusters at the end of their stems. Plants from this genus have long been used in traditional medicines around the world, *e.g.,* Ningpo figwort or Chinese figwort (*S. ningpoensis*), for the treatment of a form of tuberculosis. These species have also been found to possess antibacterial, antiprotozoal, antitumor, anti-inflammatory, and diuretic activities and have been used in the treatment of mental, nervous and gastrointestinal conditions [3-9]. Numerous species of the *Scrophularia* have been subjected to extensive studies aiming at identification of biologically active compounds, *e.g.,* phenylethanoids, phenylpropanoids, flavonoids, iridoids, iridoid glycosides and terpenoids [3-14]. To the best of our knowledge, no research has been conducted on *S. amplexicaulis.*

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Therefore, as part of our on-going phytochemical and bioactivity studies on plants from the Iranian flora [15-26], we now report on the GC-MS and GC-FID analyses, and the antimicrobial (against *Staphylococcus aureus*) and free-radical-scavenging activities of the essential oil obtained from the aerial parts of *S. amplexicaulis*.

2. Materials and Methods

2.1. Plant material

The aerial parts of *Scrophularia amplexicaulis* Benth. were collected at the flowering stage from wild population from Sahand Mountain, Gharegol village, East Azerbaijan, Iran, in April 2010. A voucher specimen (TUM-ADE-0367) was deposited at the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences.

2.2. Distillation of plant materials

The air-dried ground aerial parts of *Scrophularia amplexicaulis* was subjected to hydro-distillation for 4h using a Clevenger-type apparatus. The resulting oil was subsequently dried over anhydrous sodium sulfate and dissolved in *n*-hexane (0.3%) for analysis.

2.3. GC-MS and GC-FID analyses

The essential oil was analyzed using a Shimadzu GCMS-QP5050A gas chromatograph-mass spectrometer (GC-MS) fitted with a fused methyl silicon DB-5 column (60 m x 0.25 mm i.d., 0.25 µm film thickness). Helium was used as carrier gas at a flow rate of 1.3 mL/min. The column temperature was kept three min at 50°C, increased to 260°C at a rate of 3°C/min, and finally kept at 260°C for 5 min. The injector temperature was 240ºC and split ratio was adjusted at 1:33. The injection volume was 1 µL. The mass spectral (MS) data were obtained at the following conditions: ionization potential 70 eV; ion source temperature 200ºC; quadrupole temperature 100ºC; solvent delay 2 min; resolution 2000amu/s and scan range 30-600 *m/z*; EM voltage 3000 volts. Identification of compounds was based on direct comparison of the Kovats indices and MS data with those for standard compounds, and computer matching with the NIST NBS54K Library, as well as by comparison with respective literature data [27, 28].

For quantitation (area %), the GC analysis was also performed on the same sample on an Agilent 6890 series apparatus fitted with a FID detector. The FID detector temperature was 300 ºC. To obtain the same elution order as with GC-MS, simultaneous auto-injection was performed on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

2.4. The DPPH assay

The free-radical-scavenging property of the essential oil was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay as described in the literature [29, 30]. DPPH was purchased from Fluka Chemie AG, Bucks. The essential oil was dissolved in $CHCl_3$ to obtain the stock concentration of 1 mg/mL. 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 (5 mL each) were mixed with DPPH (5 mL; 0.08 mg/mL) and allowed to stand for 30 min for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive control, quercetin. Data were processed using EXCEL and the concentration that caused a 50% reduction in absorbance (RC_{50}) was calculated.

2.5. Antibacterial assay

The bacterial culture of *Staphylococcus aureus* (ATCC 6538) was used to evaluate the antibacterial properties of the essential oil using the well-diffusion method [31]. Bacterial cells used in this project were purchased in lyophilized form from the Institute of Pasture, Iran. These were cultured in LB agar medium after dissolving in sterile distilled water. The plates were incubated at 37°C for 24h. Single colony from the plate was transferred into 4 mL fluid LB medium and incubated over night at 37°C and 200 rpm in shaking incubator. The cells were harvested by centrifugation at 3000 rpm for 15 min and 4˚C. Subsequently, they were washed twice and re-suspended in Ringer solution to provide bacterial concentrations between 10^6–10^7 cfu/mL [33].

The medium was inoculated with the microorganism. Once the agar was solidified, it was punched with a six millimeters diameter wells and filled with 25 μL of the essential oil of various concentrations (40-100 μg/mL) and blank (hexane). Simultaneously ampicillin was used as a positive control at a concentration of 10 μg/mL. The dilution medium for the positive controls was sterile distilled water. The test was carried out in triplicate. The plaques were incubated at 35 ± 2°C for 24 h. The antimicrobial activity was calculated as the zones of inhibition in mm.

3. Results and Discussion

The ground aerial parts of the flowering plant *S. amplexicaulis* was subjected to hydro-distillation for 4h using a Clevenger-type apparatus to provide odorous pale yellow oil with a yield of 0.03 % (v/w). The chemical compositions of these essential oil, as determined by the GC-MS analysis and identified based on direct comparison of Kovats indices and MS data with those for standard compounds, and computer matching with the NIST NBS54K Library, as well as by comparison with respective literature data [27, 28], are listed in Table 1. This is the first report on the analysis of the essential oil of *S. amplexicaulis.*

A total of 27 compounds, representing about 97.9% of the total oil, were identified in the essential oil of *S. amplexicaulis*. Eugenol (53.8%) and its acetate (24.5%) constituted over two third of the oil. Among the others β-caryophyllene (5.7%), caryophyllene oxide (6.4%) and aromadendrene oxide II (2.1%) were notable (Table 1). The essential oil of this plant, as analyzed in the current study, was clearly rich in phenolic derivatives and oxygenated monoterpenes. According to the literature search, secondary metabolites such as cucurbitacins, stearic acid derivatives and flavonoids, β-caryophyllene, caryophyllene oxide, 6α-acetoxymanoyl oxide were previously detected in other species of the Scrophulariaceae [34-37].

In the antibacterial assay, the essential oil (100 μg/mL) showed comparable antibacterial activity of that of the positive control ampicillin (10 μg/mL) against *Staphylococcus aureus* (Table 2). This is the first report on the antibacterial activity of the essential oil of this species. However, other species of the genus *Scrophularia* have previously been shown to have antibacterial activity against *Staphylococcys aureus* [38-40]. Therefore, the current finding is in line with the previous results on other species of the genus *Scrophularia.*

In the DPPH assay, the essential oil of *S. amplexicaulis* displayed considerable free-radical-scavenging activity comparable to that of the positive control Trolox (Table 2). The free-radical-scavenging activity of the essential oil was mainly owing to the presence of a number of phenolic components as identified by the GC-MS (Table 1). The DPPH antioxidant assay is based on the principle that 2,2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical, is decolorized in the presence of free radical scavengers (antioxidants). The odd electron in the DPPH radical is responsible for the absorbance at 517 nm, and also for visible deep purple color [30, 41]. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorised which can be quantitatively measured from the changes in absorbance.

Table 1. GC-MS and GC-FID data of the components of the essential oil of *Scrophularia amplexicaulis* Benth.

Compounds	K. I.	Real % area	Compounds	K. I.	Real % area
trans-2-Hexenal	854	tr	*trans*-Nerolidol	1562	0.1
β-Hexen-1-ol	857	tr	Caryophyllenyl alcohol	1565	0.1
1-Octen-3-ol	942	0.1	Caryophyllene oxide	1573	6.4
Methyl salicylate	1206	tr	Humulene oxide	1607	0.8
trans-Anethole	1283	0.1	Allo-aromadendrene epoxide	1623	1.1
Eugenol	1351	53.8	Aromadendrene oxide II	1678	2.1
α-Copaene	1376	0.1	Benzyl benzoate	1762	0.3
β-Caryophyllene	1404	5.7	6,10.14-Trimethyl-2-pentadecanone	1843	0.1
α–Guaiene	1442	0.1	Methyl palmitate	1927	0.2
β-Selinene	1485	1.1	*n*-Hexadecanoic acid	1984	0.2
α-Amorphene	1506	0.1	Methyl linoleate	2092	0.1
Eugenol acetate	1524	24.5	Methyl stearate	2130	tr
δ-Cadinene	1530	0.2	Phytol	2148	0.3
α-Calacorene	1548	0.1			
Total identified			**97.7%**		

Kovats Indice (K. I.) was calculated according to the method described by Kovats [42].

Table 2. Amntimicrobial and free-radical-scavenging activities of the essential oil of *S. amplexicaulis*

Test samples	DPPH assay RC_{50} value in mg/mL	Antimicrobial assay (zone of inhibition in mm) 10 mg/mL	40 mg/mL	60 mg/mL	80 mg/mL	100 mg/mL
Essential oil	4.41×10^{-3}	N/A	-	-	8	13
Ampicillin*	N/A	13	N/A	N/A	N/A	N/A
Quercetin*	2.78×10^{-5}	N/A	N/A	N/A	N/A	N/A
Trolox*	3.07×10^{-3}	N/A	N/A	N/A	N/A	N/A

*Positive controls; N/A = Not applicable

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