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Original Article

In Vitro Assessment of Antibacterial, Antioxidant, and Cytotoxic Activities of Essential Oil and Methanolic Extract of *Satureja rechingeri* Jamzad

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Abstract

Satureja rechingeri Jamzad is a highly aromatic species of Lamiaceae. This species is one of the rare and endemic plants of Iran. This study aimed to assess the biological properties such as the antibacterial, antioxidant, and cytotoxic activity of essential oil (EO) and methanolic extract of *S. rechingeri* collected from the west of Iran and analyzed the plant EO constituent. Gas chromatography/mass spectrometry (GC/MS) was performed for analyzing the EO. The antioxidant activities of extract from *S. rechingeri* were evaluated using DPPH radical scavenging assay. The major component of *S. rechingeri* EO was carvacrol (86.45%). The minimum inhibitory concentration value was 15.625 µl/ml for the bacterial strains. The extract exhibited antioxidant activity in DPPH radical scavenging assay (EC50 = 47.56 µg/mL). In addition, the total phenolic content in the plant methanolic extract was 79.9 mg/g gallic acid. The highest toxicity for essential oil was found in the MCF7 cell line with an IC50 value of 47.71 µg/ml. Carvacrol was the main component of *S. rechingeri* EO. The plant extract and EO showed considerable antioxidant and antimicrobial activity. It seems that this plant could be a commercial source of carvacrol.

Keywords: *Satureja rechingeri*; Essential oil; Antimicrobial agents; Antioxidants; Cytotoxicity; Carvacrol.

1. Introduction

Iran is regarded as a head gene center of the Lamiaceae family. The *Satureja* genus is an aromatic plant belonging to this family which is growing wild in the northern, northwestern, and western parts of Iran. *Satureja rechingeri* Jamzad is recently identified as a new species from Iran [1, 2]. *Satureja hortensis* L. (summer savory) and *Satureja montana* L. (winter savory) are two prominent, well-known species of *Satureja* that are used as culinary herbs [3]. Traditionally, *S. hortensis* has been used as a

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stimulant, stomachic, carminative, expectorant, antidiarrheal, and aphrodisiac. The plant essential oil was reported to contain phenolics with antimicrobial and antidiarrheal activities [1].

The essential oil composition of *S. khuzistanica* Jamzad and *S. bachtiarica* Bunge, two endemic *Satureja* species of Iran, was analyzed. *P*-cymene (39.6%) and carvacrol were the major components of *S. khuzistanica*, while *S. bachtiarica* contains thymol (44.5%) and γ -terpinene (23.9%) [4]. It was reported that Carvacrol and γ –terpinene were the major components of essential oil obtained from *S. hortensis* cultivated in Iran [3].

It is known that reactive oxygen (ROS) is ceaselessly produced in the human body, and they are regulated by endogenous enzymes (superoxide dismutase, glutathione peroxidase, and catalase). Overproducing these species with exposure to external oxidant substances or a downfall in the defense mechanisms may damage some valuable biomolecules (DNA, lipids, proteins).

It was reported that antioxidants can prevent oxidative damage caused by ROS. Antioxidants can prevent certain diseases, such as cancer and aging. This process is achieved by reacting with free radicals and chelating metals [5]. Interest in the usage of natural antioxidants has increased dramatically in recent decades due to: (A) the possible carcinogenic effects of synthetic antioxidants in foods, (B) the antioxidative efficacy of a variety of phytochemicals, (C) the consensus that foods rich in certain phytochemicals can affect the etiology and pathology of chronic diseases and the aging process [6]. Nowadays, cancer is one of the significant causes of death worldwide, and plant-derived compounds are an essential source of anticancer drugs [7].

The main objectives of this study were to investigate the antimicrobial activity of the essential oil of *S. rechingeri* by disc diffusion method against some food pathogen bacteria and assessment of the in vitro cytotoxicity of essential oil and methanolic extract against some selected cancerous and non-cancerous cell lines. The chemical composition of the essential oil was determined by GC/MS. Besides, this study was also designed to evaluate the antioxidant capacity of the plant methanolic extract and its total phenolic content.

2. Materials and Methods

2.1. Chemicals

Folin–Ciocalteu’s reagent and methanol were obtained from Merck (Germany). Dimethylsulphoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma–Aldrich GmbH. (Steinheim, Germany). Trypsin 0.5% in EDTA, Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), streptomycin, and penicillin was purchased from Gibco-BRL (Germany).

2.2. Plant Materials

The aerial parts of plant were collected at the flowering stage from Ilam province (Iran) In August. The identification of plant materials (voucher no. THE-6625) was performed by, Dr. Gholamreza Amin (Tehran University of Medical Sciences, Iran.).

2.3. Isolation of The Essential Oil

The plant material was dried in the shade and ground in a grinder with a 2 mm diameter mesh. The ground aerial parts were submitted, for 3 h, to hydro-distillation, using a Clevenger apparatus to produce the essential oil. The essential oil was dried over anhydrous sodium sulfate and kept at 4˚C [8]. The GC/MS analysis was used to evaluate the essential oil composition.

2.4. Preparation of The Methanolic Extract

Eighty grams of the dried and powdered plant materials were extracted with methanol (HPLC grade) using the maceration method (3×48h), then concentrated by a rotary evaporator (Heidolph, Laborota 4000, Germany). Finally, the extract was lyophilized and stored at 4˚C.

2.5. Microorganisms

E. coli O157:H7, *Staphylococcus aureus* ATCC 25923 were used as test bacteria. Nutrient broth (NB) and Tryptic soy broth (TSB) were used to culture test bacteria. All strains were stored at -20 ˚C in the appropriate medium containing 10% glycerol and regenerated twice before use in the manipulations.

2.6. Determination of Minimum Inhibitory Concentrations (MIC)

The broth microdilution susceptibility assay was used for MIC assessment [9]. A stock solution of the essential oil was prepared in 10% DMSO, and then serial dilutions of the essential oil were made in a concentration range from 600 to 1400 µg/mL. The 96-well plates were dispensed into each well 95 µl of NB, 100µl of the oil (dissolved in 10% DMSO), and 5 µL of the inoculum. The inoculum of microorganisms was prepared using 24 h cultures, and suspensions were adjusted to 5.0 McFarland standard turbidity (final volume, 200 µL/well). A positive control (containing 5 µl inoculum and 195 µL NB) and negative control (containing 100 µL of essential oil dissolved in 10% DMSO, 100 µl NB without inoculum) were included on each microplate. The wells were shaken, and the microplates were incubated at 37˚C for 24 h. The lowest concentration of the compounds which inhibit the microorganism's growth was defined as MIC. The experiment was carried out in triplicate.

2.7. Gas Chromatography/Mass Spectrometry (GC/MS) Analysis Conditions

GC–MS was used for analyzing the chemical composition of essential oil. The Agilent 6890 N GC/5973MSD-SCAN (CA, USA) mass spectrometer was used in the electron impact (EI) ionization mode (70 eV), and HP-5MS (bonded and cross-linked 5% phenyl-methylpolysiloxane,30 mm × 0.25 mm, coating thickness 0.25 µm) capillary column (Restek, Bellefonte, PA). The temperatures of the injector and detector were set at 220 ˚C. The oven temperature was held at 50 ˚C for 30 min, then programmed to 240˚C at a rate of 3 ˚C /min. The carrier gas was Helium (99.99%) with a 1 mL/min flow rate. The samples were diluted by Hexane (1/100, v/v) and injected manually (1.0 µL). The components were identified by comparing the mass spectra and retention times with Wiley 7 N, Nist 2002, and Flavor libraries.

2.8. DPPH Radical Scavenging Assay

Radical scavenging activity was determined by a spectrophotometric method based on the reduction of a methanol solution of DPPH using the method of Blois (1958) [10]. One milliliter of various concentrations of the extract or the essential oil was added to 1 mL of a 0.004% methanol solution of DPPH, then shaken vigorously and left for 30 min at room temperature in the dark. Then the absorbance was measured at 517 nm against a blank by a spectrophotometer (Optizen 2120 UV PLUS, Mecasys, Korea). The percentage of free radical inhibition (I%) was calculated according to the formula:

$$I\% = (A_{blank} - A_{sample}) / A_{blank} \times 100$$

A_{blank} is the absorbance of the control (which contains all of the reagents without the sample), and A_{sample} is the absorbance of the sample. The concentration of the extract that scavenge 50% of DPPH (EC50) was calculated by plotting the inhibition percentage against extract concentration. Tests were carried out in triplicate.

2.9. Determination of Total Phenolic Content

The total phenolic content of the extract were analyzed using the Folin–Ciocalteu's reagent using gallic acid as standard. The 0.1 mL extract solution was mixed with 0.2 mL of 50% Folin–Ciocalteu's reagent. The mixture was allowed to react for 5 min, and 1 ml of aqueous solution of 2% Na2CO3 was added. Then, the mixture was vortexed vigorously. At the end of incubation for 45 min at room temperature, the absorbance of each mixture was measured at 725 nm. The total phenol content were expressed as l g gallic acid equivalents per mg of the extract [11].

2.10. Cytotoxicity Assay

Vero (African monkey green kidney cell), A549 (Human Caucasian lung carcinoma), and MCF7 (Human breast carcinoma) were provided by Pasteur Institute, Iran. The cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 1.2 g/L $NaHCO_3$, 100mM Sodium pyruvate, 100U/ml penicillin and 100µg/ml streptomycin, and 1% (w/v) glutamine. The cells were incubated at 37°C under 5% CO2 in a humidified incubator. The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide] test was used for the determination of the essential oil and methanolic extract cytotoxicity. The tetrazolium dye affected by the mitochondrial enzyme reduces to a blue formazan product which reflects cell viability. Briefly, the cell suspension was dispensed into 96-well culture plates (5000 cells per well) with 100 µl culture media. After 24 h, the cells were treated with the essential oil (1, 10, 50, 100µg/mL) and methanolic extract (10, 100, 500, 1000 µg/mL) and kept for incubation for 24h, then the medium in wells were flicked off, and 20µl of MTT dye (5mg/mL) was added to each well. The plate was incubated for four h at 37°C, and the medium in each well was aspirated and replaced with 20µl of DMSO to dissolve the formed formazan crystals. The culture plate was shacked for 15 min at room temperature, and the absorbance of each well read at 570 nm [12].

2.11. Statistical Analysis

All data are presented as Means ±S.D. of at least three independent measurements and statistically analyzed by one-way ANOVA test with statistical significance determined at $P<0.05$. The Pearson rank order correlation test was used to compare agar well diffusion and the antimicrobial activity of the essential oil.

3. Results and Discussion

3.1. Phytochemical Screening

The yield of the extract was 18.20% w/w. The total phenolic content of the plant extract was tested using the diluted Folin-Ciocalteu reagent. The standard curve equation was y = 0.0104 x– 0.0811, R^2 = 0.99. *S. rechingeri* methanolic extract total phenolic content was obtained at 71.93mg/100g, expressed as gallic acid equivalents.

The essential oil was isolated by hydro-distillation method from the aerial parts of a plant. Based on dry weight, essential oil yield was calculated at 6.5% w/w. Its content was analyzed by GC-MS. Twenty-two components were identified in *S. rechingeri* at the complete flowering stage (99.95% of the oil) (**Table 1**). Carvacrol (86.45%), *p* -cymene (2.2%), and γ-terpinene (2.38%) were found as the major compounds. The essential oil was characterized by a high content of monoterpenoids (98.95%), of which carvacrol (86.45%) was the primary compound.

Carvacrol and its isomer, thymol, are the main components of essential oil of some genera of the Lamiaceae family, such as *Satureja*, *Origanum*, *Thymus*, and *Thymbra* [13]. The essential oil component of *S. rechingeri* has been determined by other researchers. Sefidkon and coworkers reported that carvacrol (86.6%) was the major component of *S. rechingeri* essential oil at the entire flowering stage [1]. Recently, Alizadeh et al. analyzed the essential oil composition of *S. rechingeri* in several stages of the growth by hydro-distillation method. It was reported that carvacrol amounts in the pre-flowering, full flowering, and post-flowering stages were 83.58 %, 90.35%, and 88.25%, respectively [14].

Table 1: Chemical composition of *S. rechingeri* essential oil.

N.	Name	Retention Time	Percentage (%)
1	Nonane(n)	9.67	0.52
2	Alpha-Thujene	10.92	0.53
3	Alpha-Pinene	11.22	0.31
4	Beta-Pinene	13.31	0.08
5	Myrcene	14.16	0.75
6	Alpha-Phellandrene	14.74	0.12
7	Alpha-Terpinene	15.39	0.57
8	Para-Cymene	15.81	2.20
9	Beta-Phellandrene	16	0.19
10	Gamma-Terpinene	17.61	2.38
11	Sabinene hydrate	17.97	0.32
12	Undecane	19.79	2.08
13	Terpinene-4-ol	23.48	0.42
14	Carvacrol methylether	26.65	0.12
15	Thymol	29.08	0.22
16	Carvacrol	30.25	86.45
17	Eugenol	31.92	0.08
18	Carvacryl acetate	32.55	0.19
19	B-Funebrene	34.30	0.10
20	Caryophyllene	34.59	0.35
21	B-Bisabolene	38.25	0.78
22	Caryophyllene oxide	41.15	0.19

The literature reviewed showed that carvacrol is the main component of several species of *Satureja*. However, its content in *S. rechingeri* was higher (**Table 2**) [1, 14-19]. The essential oil composition is influenced by genetic factors, climate, geographical conditions, and stages of development [20]. The highest carvacrol content was observed in the full-flowering stage, up to 90 percent. Also, the results showed that the highest yield was obtained in Iranian savory (*S. rechingeri*). It seems that Iranian savory could be a commercial source of carvacrol.

Recently, there has been considerable interest in evaluating the antimicrobial and antioxidant properties of the essential oils and extracts from aromatic plants for controlling pathogens and toxin-producing oxidants in human life and relating to health [21, 22].

Agar dilution assay was used to determine the antibacterial activity of the *S. rechingeri* essential oil against *S. aureus* and *E. coli*. The minimal inhibitory concentration (MIC) value of essential oil for both microorganisms was obtained at 15.625µg/mL.

Based on the result, *S. rechingeri* essential oil exhibited high antimicrobial activities against *Staphylococcus aureus* and *Escherichia coli*. The highest antimicrobial activity of essential oil components was reported for phenolics [23]. It was previously reported that *Satureja* and carvacrol-bearing plant's essential oil inhibited molds, yeasts, and bacteria. Phenolic monoterpenes such as carvacrol and thymol exhibit antibacterial properties by destabilization of the bacterial cell membrane and, as a result, disrupt cellular proton and potassium ion balance. That ultimately leads to cell death. In addition, it has to be considered that the other constituents of volatile oil act through different mechanisms [20].

The DPPH radical scavenging activity of the methanolic extract was measured by the spectrophotometric method, and the EC_{50} value was calculated using the regression line equation. The result showed that *S. rechingeri* extract contained high DPPH radical scavenging activity (EC_{50}=47.56 ± 0.7µg/ml). This method determines the ability of natural compounds to act as free radical scavenging or hydrogen donors. The extract of *S. rechingeri* exhibited remarkable antioxidant activities. It is reported that phenolic compounds have potent free radical scavenging and antioxidant activities. They act as free radical oxidation terminators and are used to prevent various diseases mainly associated with free radicals [24]. In this study, the total phenolic content of the methanolic extract of *S. rechingeri* was determined by the Folin-Ciocalteu method. The total amount was expressed as gallic acid equivalents. Based on the result, it was very high in the methanolic extract. A comparison of the DPPH scavenging activity of methanolic extract and the essential oil of *S. rechingeri* in another study showed that the extract possessed a more potent antioxidant effect than the essential oil.

The viability of Vero, A549, and MCF7 cells exposed to methanolic extract and essential oil was evaluated by MTT assay. IC50 value for essential oil was measured at 87.57, 47.71, and 53.44 µg/mL for the Vero, MCF7, and A549 cells, respectively. Also, the methanolic extract IC50 values were estimated to be 852.5, 1086.99, and 713.55 µg/ml, respectively (**Table 3**). EC50 value of carvacrol (79.75 µg/mL), the main constituent of the essential oil, was previously determined [25].

Table 2: Carvacrol and Thymol content of some Satureja species (Harvesting time was in flowering stage).

Species	Preparation method	Yield (%)	Thymol (%)	Carvacrol (%)	References
***S. cuneifolia* Ten**	Steam distillation	2.2	9.01	44.99	[15]
	Hydrodistillation	-	0.1	53.3	[16]
S. montana	Hydro-distillation	-	14.1	30.6	[17]
	Hydrodistillation	0.5-1.7	3.8-31.7	10.6-23.3	[18]
S. hortensis	Hydrodistillation	1.28-4.75	29-43	42-63	[19]
S. rechingeri	Various distillation methods	2.46-4.24	Trace	84.0-89.3	[1]
	Hydrodistillation	3.84-4.65	0.15-1.12	83.58-88.25	[14]

Table 3: Cytotoxity assay of essential oil and methanolic extract of *S. rechingeri*.

No	Cell lines	Essential oil IC50 ± SD (µg/ml)	Methanolic extract IC50 ± SD (µg/ml)
1	Vero	87.57±1.03	852.5±2.29
2	MCF7	47.71±5.32	1086.99±7.53
3	A549	53.44±3.68	713.55±1.92

The radical scavenging activity of *S. rechingeri* was higher than carvacrol [16], which exhibited a synergistic scavenging effect of essential oil constituents. Our main observation in this study demonstrated that the growth of all cell lines was inhibited after exposure to the essential oil and methanolic extract. The cytotoxic activity of *S. rechingeri* essential oil and extract has not been reported. The IC_{50} of essential oil was lower than the extract. In the case of MCF7 cells, the IC_{50} of the essential oil was 47.71 µg/ml. The results showed low cytotoxic activity of the *S. rechingeri* methanolic extract (IC50=1086.99 µg/mL) on MCF7 cells, although it was not statistically significant. Yousefzadi et al. investigated the cytotoxicity of *S. khuzistanica* with high carvacrol content (93%) on the human colon adenocarcinoma (SW480), breast adenocarcinoma (MCF7), and choriocarcinoma (JET 3) cell lines, as well as a monkey kidney cell line (Vero). The essential oil significantly reduced cell viability dose-dependently [26]. Carvacrol exhibited potent cytotoxic activity against human non-small cell lung cell line (A549), human cervical cancer, HeLa, and SiHa, cells (27). Moreover, it caused the induction of apoptosis in the human metastatic breast cancer cell line, MDA-MB 231 [28].

4. Conclusion

As a result of the present study, the essential oil composition of *S. rechingeri* showed the highest percentage of Carvacrol among the other *Satureja* species. Moreover, the essential oil was an effective antibacterial and cytotoxic agent in the in vitro assays.

Conflict of interest

The authors declare to have no conflict of interest.

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