Evaluation of the Cytotoxicity of Satureja spicigera and Its Main Compounds

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Satureja spicigera (Lamiaceae) grows wildly in Northwest of Iran. In this study, bioassay-guided isolation and identification of the main compounds has been reported using various chromatographic methods and comparison of their spectral data with those reported in the literature. Brine shrimp lethality and four cancerous cell lines HT29/219, Caco2, NIH-3T3, and T47D were used for cytotoxicity evaluations. From the aerial parts of S. spicigera, nine known compounds including two flavanones, 5,7,3′,5′-tetrahydroxy flavanone (8) and 5,4′-dihydroxy-3′-methoxyflavanone-7-(6′-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside (9), one dihydrochalcone, nubigenol (7), together with thymoquinone (1), thymol (2), carvacrol (3), β-sitosterol (4), ursolic acid (5) and oleanolic acid (6) were identified. Among the isolated chalcone and flavanones, compound 8 was effective against Artemia salina larva (LC50 = 2 μg/mL) and only the compound 9 demonstrated IC50 value of 98.7 μg/mL on the T47D (human, breast, ductal carcinoma). Other compounds did not show significant inhibition of the cell growth.

1. Introduction

The genus Satureja (Lamiaceae) has 13 species in Iran and is called Marzeh. One of these species is S. spicigera that grows wildly in Northwest of Iran [1, 2]. Recent phytochemical studies indicated the presence of flavanones (narigenin, aristodendrin, eriodictyol, taxifolin, and flavanone trimethyl ether) and flavones (apigenin, luteolin, diosmetin, genkwanin, ladanein, thymosin, thymonin, cirsimaritin, and xanthomicrol) in some species of Satureja [3–5]. So far, antimicrobial [6], spasmodic [7], anti-HIV [8], antiviral [9], antioxidant [10] and cytotoxic activities [11, 12] have been reported from several species of this genus. Volatile composition of some Iranian species of Satureja has been investigated [13]. Recently, we have reported the chemical composition of the volatile oil of S. spicigera [14]. The oil of S. spicigera was rich in monoterpenes (89.9%) with thymol (37.3%) as the major compound [14]. Comparison of some Satureja species by phylogenetic and chemotaxonomic analysis showed that the genetic distance between S. spicigera and S. mutica is close [15]. Antibacterial activity of the oil of S. spicigera has been reported against B. subtilis, S. aureus, E. coli, and K. pneumonia [16]. Also, we reported the trypanocidal activities of the several extracts of S. spicigera and other species of this genus [17–19]. A literature survey has shown that the phytochemical constituents of S. spicigera were not previously published. Therefore, we aim to report the isolation, structural elucidation, and cytotoxicity of the main constituents of S. spicigera for the first time.
in δ (ppm). HR-ESITOFMS were obtained on a Bruker MicroTOFI mass spectrometer. The FT-IR spectra were recorded on a Nicolet 550 instrument. Silica gel 60F254 precoated plates (Merck TM) were used for TLC. The spots were detected by spraying anisaldehyde-H2SO4 reagent followed by heating (120°C for 5 minutes).

2.2. Plant Material. Aerial parts of Satureja spicigera (C. Koch) Boiss, at flowering stage, were collected from Astara in the Northwest of Iran, in September 2004. Voucher specimen (78410 TARI) was deposited at the Herbarium of the Institute of Forests and Rangelands Researches. Plant specimen was identified by Dr. Vali-allah Mozaffarian from the same institute.

2.3. Extraction, Isolation, and Structural Elucidation. The flowered aerial parts of S. spicigera (1 kg) was cut into small pieces and extracted with ethyl acetate and methanol, consequently, at room temperature. The ethyl acetate extract (23 g) was subjected to silica gel column chromatography (CC) with hexane : CHCl3 : AcOEt (8 : 2), CHCl3 : AcOEt (2 : 8), and AcOEt as eluent to give six fractions (A–F). The fraction C (7 g) was subjected to silica gel CC with hexane : AcOEt (9 : 1) to obtain five fractions C1–C5. The fraction C2 (97 mg) was chromatographed on sephadex LH20 with MeOH to result compound M1 (14 mg). The fraction C3 (1 g) was subjected to CC with hexane : AcOEt (9 : 1) to give four fractions C31–C34. The compounds 2 (35 mg) and 3 (15 mg) were given from the fractions C31 and C34, respectively, using sephadex LH20 eluted with AcOEt : MeOH (3 : 1). The fraction C5 (400 mg) was submitted to silica gel CC with hexane : AcOEt (9 : 1) to obtain compound 4 (26 mg).

The MeOH extract (70 g) was successively subjected to silica gel CC with hexane : AcOEt (8 : 2), CHCl3 : AcOEt (8 : 2), AcOEt, and MeOH as eluent to give eight fractions (M1–M8). Fraction M3 (5.3 g) was fractionated on CC with CHCl3 : AcOEt (8 : 2) to yield compounds 5 (320 mg) and 6 (45 mg). The fraction M4 (690 mg) was chromatographed on sephadex LH20 with MeOH to result M41–M43. Fraction M43 (44 mg) was purified on sephadex LH20 with MeOH to yield compound 7 (15 mg). Compound 8 (26 mg) was yielded from fraction M5 (1.9 g) using twice sephadex LH20 with MeOH. The fraction M8 (9 g) was submitted to reverse-phase (C8) CC with aqueous MeOH (30%, 50%, and 100%) to give 7 fractions (M81–M87). Compound 9 (90 mg) was obtained from fraction M87 (3.3 g) using sephadex LH20 with MeOH.

2.4. Brine Shrimp Lethality Assay (BSA). Gohari et al. reported the method which was adopted to study the cytotoxic activity of the compounds [11]. Water life brand brine shrimp (Artemia salina) eggs were purchased from the Shalat Center (Tehran). The eggs were hatched in a flask containing 300 mL artificial seawater made by dissolving distilled water. The flask was well aerated with 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 extracts and pure compounds were dissolved in normal saline. Different concentrations were obtained by serial dilution. Solution of each concentration (500 μL) was transferred into clean 24-well plates via a pipette, and aerated seawater having 10–20 nauplii (500 μL) was added. A check count was performed and the number alive noted after 24 h. The mortality end point of the bioassay was determined as the absence of controlled forward motion during 30 seconds of observation. The controls used were seawater and berberine hydrochloride (LC50 = 26 μg/mL). Lethality percentage was determined and LC50 calculated based on probit analysis with 95% of confidence interval [11].

2.5. Cell Cultures and Cytotoxicity Assay. Four cancerous cell lines HT29/219 (human, colon, epithelial-like, carcinoma), Caco2 (human, colon, adenocarcinoma), NIH-3T3 (Swiss NIH mouse, embryo fibroblast), and T47D (human, breast, ductal carcinoma) were purchased from the Pasteur Institute, Tehran, Iran. The cells were maintained in RPMI 1640, supplemented with 10% fetal bovine serum, 0.28 units/mL insulin, 100 μg/mL streptomycin, 100 units/mL penicillin, and 0.3 mg/mL glutamine. The cells were grown at 37°C in a humidified atmosphere of 5% CO2, in air.

The cytotoxicity of the compounds isolated from S. spicigera was assayed using the MTT cytotoxicity assay. The cells (3 × 104) were plated in 500 μL of medium/well in 48-well plates (NUNC Cell Culture Flasks, Denmark). After an overnight incubation at 37°C, in 5% CO2, and a humidified atmosphere, the samples were added to the cells to a final concentration of 500 μg/mL. “Methotrexate” (positive control) and pure compounds were examined at concentrations ranging from 5, 10, 20, 40, 80, and 100 μg/mL. The plates were incubated at 37°C, in 5% CO2, humidified atmosphere, for 48 hours. After 48 hours, 50 μL of 5 mg/mL MTT (dissolved in PBS) was added per well. After three hours of incubation, the MTT solution was removed and the cells were washed with 100 μL of PBS, twice. One hundred and fifty microlitres of DMSO was added per well, to solubilize the formazan crystals. The optical densities of the wells were then measured at 570 nm (690 nm reference wavelength). By referring to the control (medium with DMSO), the cell survival was assessed [20].

3. Results

Dried aerial parts of S. spicigera, collected during full flowering stage, were successively extracted with ethyl acetate and methanol. These extracts were concentrated and examined with brine shrimp lethality assay (BSA). Both extracts showed cytotoxic activity against Artemia salina larvae, so were used for further isolation on silica gel and sephadex column chromatography to obtain compounds 1–9 (Figure 1). Isolated compounds, 1–7, were identified as thymoquinone (1), thymol (2), carvacrol (3), β-sitosterol (4), ursolic acid (5), oleanolic acid (6), and nubigenol (7) by comparison of their spectral data with those reported in literature [11, 21–23]. The results have a good agreement with references. Full assignments of the compounds 8 and 9, performed by 2D-NMR and HR-ESITOFMS techniques, have been reported in
Figure 1: Chemical structures of the isolated dihydrochalcone and flavanones from *Satureja spicigera*.

Table 1: NMR spectra of the compound 8 in DMSO-$d_6$.

| No. | $^{13}$C-NMR | $^1$H-NMR | HMBC          |
|-----|--------------|-----------|---------------|
| 2   | 78.4         | 5.37 ($dd, J = 12.4, 2.9$ Hz, 1H) | H-3b, H-2$'$ or H-6$'$ |
| 3   | 42.1         | 2.69 ($dd, J = 17.0, 2.9$ Hz, 1H) | 3.18 ($dd, J = 17.0, 12.5$ Hz, 1H) |
| 4   | 196.2        |           | H-3a, H3b     |
| 5   | 162.9        |           | H-6           |
| 6   | 95.8         | 5.88 ($d, J = 1.2$ Hz, 1H) | 5-OH          |
| 7   | 166.7        |           | H-6 or H-8    |
| 8   | 95.0         | 5.88 ($d, J = 1.2$ Hz, 1H) |               |
| 9   | 163.4        |           | H-8, 5-OH     |
| 10  | 101.7        |           | H-6 or H-8, 5-OH |
| 1$'$| 129.4        |           | H-3b, H-2$'$ or H-6$'$ |
| 2$'$| 117.9        | 6.75 ($brs, 1H$) | H-4$'$        |
| 3$'$| 145.7        |           | H-4$'$        |
| 4$'$| 114.3        | 6.88 ($brs, 1H$) | H-2$'$ or H-6$'$ |
| 5$'$| 145.2        |           | H-4$'$, H-2$'$ or H-6$'$ |
| 6$'$| 115.3        | 6.75 ($brs, 1H$) |               |
| 5-OH| 12.14 (s, 1H) |           |               |
| 7-OH| 10.76 (s, 1H) |           |               |
| 3'-OH| 9.00 (s, 1H) |           |               |
| 5'-OH| 9.05 (s, 1H) |           |               |
Table 2: NMR spectra of the compound 9 in DMSO-d$_6$.

| No. | $^{13}$C-NMR | $^1$H-NMR | HMBC |
|-----|-------------|-----------|------|
| 2   | 78.4        | 5.50 $(dd, J = 12.5, 3.0$ Hz, 1H) | H-3b, H-2’ |
| 3   | 42.1        | 2.77 $(dd, J = 17.5, 3.2$ Hz, 1H) | 3.14 $(m, 1H)$ |
| 4   | 197.1       |           |      |
| 5   | 162.5       |           |      |
| 6   | 96.4        | 6.13 $(d, J = 2.5$ Hz, 1H) | H-8 |
| 7   | 165.2       |           | H-6, H-8 |
| 8   | 95.6        | 6.14 $(d, J = 2.5$ Hz, 1H) | H-8 |
| 9   | 163.1       |           |      |
| 10  | 103.4       |           |      |
| 1’  | 131.9       |           |      |
| 2’  | 114.2       | 6.94 $(m, 1H)$ |      |
| 3’  | 146.5       |           | H-5’, OCH$_3$ |
| 4’  | 148.0       |           |      |
| 5’  | 112.1       | 6.94 $(m, 1H)$ |      |
| 6’  | 118.0       | 6.89 $(dd, J = 8.0, 2.0$ Hz, 1H) | H-2, H-2’ |
| –OCH$_3$ | 55.7 | 3.79 $(s, 3H)$ |      |
| 5-OH |           | 12.08 |      |
| Glc-1” | 99.5 | 4.97 $(d, J = 7.5$ Hz, 1H) |      |
| 2”  | 73.0        | 3.21 $(m, 1H)$ |      |
| 3”  | 76.3        | 3.26 $(m, 1H)$ |      |
| 4”  | 69.6        | 3.15 $(m, 1H)$ |      |
| 5”  | 75.5        | 3.54 $(m, 1H)$ |      |
| 6”  | 66.1        | 3.37 $(m, 1H)$ |      |
|     |             | 3.80 $(m, 1H)$ |      |
| Rha-1”’ | 100.6 | 4.51 $(d, J = 1.0$ Hz, 1H) |      |
| 2”’ | 70.7        | 3.62 $(m, 1H)$ | H-1”’ |
| 3”’ | 70.3        | 3.42 $(m, 1H)$ |      |
| 4”’ | 72.1        | 3.17 $(m, 1H)$ |      |
| 5”’ | 68.4        | 3.42 $(m, 1H)$ |      |
| 6”’ | 17.9        | 1.08 $(d, J = 6.0$ Hz, 3H) | H-1”’ |

Table 3: The effects of dihydrochalcone and flavanones isolated from S. spicigera on the viability of various cell lines using MTT assay.

| Sample | T47D | Caco-2 | HT-29 | NIH 3T3 |
|--------|------|--------|-------|---------|
| 7      | 132.0| 143.0  | >150  | 112.0   |
| 8      | >150 | >150   | >150  | 100.7   |
| 9      | 98.7 | >150   | >150  | 140.4   |

*Results are expressed as IC$_{50}$ values (μg/mL). Key to cell Lines employed: HT-29 and Caco-2 (colon adenocarcinoma); T47D (breast carcinoma); NIH 3T3 (Swiss embryo fibroblast).

The results obtained from BSA showed that the compound 8 (LC$_{50} = 2$ μg/mL) was effective against larvae of Artemia salina. This is a toxic compound compared to berberine hydrochloride (LC$_{50} = 26$ μg/mL) which was used as a positive control. Among the three various compounds of S. spicigera, the compound 9 demonstrated IC$_{50}$ value of 98.7 μg/mL on the T47D (human, breast, ductal carcinoma) ($P < 0.01$). The other compounds did not show significant inhibition of the cell growth or proliferation, even compound 8 which showed toxicity against brine shrimp larvae (Table 3).

5,7,3’,5’-Tetrahydroxy Flavanone (8). Pale yellow crystal; melting point 264–268°C; IR (CHCl$_3$) ν$_{max}$ 3353 (O–H), 2974, 2924, 2855 (C–H), 1627, 1598 (C=O), 1435,
1114, 695 cm$^{-1}$; HR-ESI-TOFMS m/z 311.0526 [M + Na]$^+$ (calcd for C$_{15}$H$_{12}$O$_6$Na, 311.0532). NMR spectra are shown in Table 1.

5,4′′-Dihydroxy-3′′-methoxyflavonone-7-(6′′-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside (9). White amorphous crystal; melting point 256°C; HR-ESI-TOFMS m/z 633.1790 [M + Na]$^+$ (calcd for C$_{28}$H$_{34}$O$_{15}$Na, 633.1795). NMR data is shown in Table 2.

References

[1] K. H. Rechinger, Flora Iranica, Labiatae, vol. 150, Academische Druck-U-Verlaganstalt, Graz, Austria, 1986.
[2] V. Mozaffarian, A Dictionary of Iranian Plant Names, Farhang Moaser, Tehran, Iran, 1996.
[3] M. Skoula, R. J. Grayer, and G. C. Kite, “Surface flavonoids in Satureja thymbra and Satureja spina (Lamiaceae),” Biochemical Systematics and Ecology, vol. 33, no. 5, pp. 541–547, 2005.
[4] V. R. S. de Rojas, B. Somozoa, T. Ortega, and A. M. Villar, “Isolation of vasodilatory active flavonoids from the traditional remedy Satureja obovata,” Planta Medica, vol. 62, no. 3, pp. 272–274, 1996.
[5] S. Saeidnia, M. S. Nourbakhsh, A. R. Gohari, and A. Davood, “Isolation and identification of the main compounds of Satureja sahendica Bornm,” Australian Journal of Basic and Applied Sciences, vol. 5, pp. 1450–1453, 2011.
[6] G. E. Ferresin, A. A. Tapia, and D. A. Bustos, “Antibacterial activity of some medicinal plants from San Juan, Argentina,” Fitoterapia, vol. 71, no. 4, pp. 429–432, 2000.
[7] V. Hajhashemi, H. Sadraei, A. R. Ghannadi, and M. Mohseni, “Antispasmodic and anti-diarrhoeal effect of Satureja hortensis L. essential oil,” Journal of Ethnopharmacology, vol. 71, no. 1-2, pp. 187–192, 2000.
[8] L. M. Bedoya, S. Sanchez-Palomino, M. J. Abad, P. Bermejo, and I. Alcamí, “Anti-HIV activity of medicinal plant extracts,” Journal of Ethnopharmacology, vol. 77, no. 1, pp. 113–116, 2001.
[9] M. J. Abad, P. Bermejo, E. Gonzales, I. Iglesias, A. Iruzun, and L. Carrasco, “Antiviral activity of Bolivian plant extracts,” General Pharmacology, vol. 32, no. 4, pp. 499–503, 1999.
[10] A. Radonic and M. Milos, “Chemical composition and in vitro evaluation of antioxidant effect of free volatile compounds from Satureja montana L,” Free Radical Research, vol. 37, no. 6, pp. 673–679, 2003.
[11] A. R. Gohari, A. Hadjiakhoondi, S. E. Sadat-Ebrahimi, S. Saeidnia, and A. Shafiee, “Cytotoxic terpenoids from Satureja macrantha C. A. Mey,” Daru, vol. 13, no. 4, pp. 177–181, 2005.
[12] A. R. Gohari, S. Saeidnia, M. R. Gohari, F. Moradi-Afrapoli, M. Malmir, and A. Hadjiakhoondi, “Bioactive flavonoids from Satureja atropatana Bunge,” Natural Product Research, vol. 23, no. 17, pp. 1609–1614, 2009.
[13] A. R. Gohari, A. Hadjiakhoondi, A. Shafiee, E. S. Ebrahimi, and V. A. Mozaffarian, “Chemical composition of the essential oils of Satureja atropatana and Satureja mutica growing wild in Iran,” Journal of Essential Oil Research, vol. 17, no. 1, pp. 17–18, 2005.
[14] A. R. Gohari, A. Hadjiakhoondi, E. Sadat-Ebrahimi, S. Saeidnia, and A. Shafiee, “Composition of the volatile oils of Satureja spicigera and Satureja macrantha from Iran,” Flavour and Fragrance Journal, vol. 21, no. 2, pp. 348–350, 2005.