



Comparison of cytotoxic activity of some Iranian *Stachys* spp. extracts on different cancer cell lines

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Abstract

The genus *Stachys* belongs to Lamiaceae family with about 300 species and worldwide distribution. In the present study, the cytotoxic activity of four fractions of different *Stachys* species (*S. byzantina* C. Koch., *S. inflata* Benth., *S. setifera* Ten. and *S. persica* Gmel.), has been investigated against HT-29 (colon carcinoma), Caco-2 (colorectal adenocarcinoma), T-47D (breast ductal carcinoma) and NIH-3T3 (Swiss mouse embryo fibroblast) cell lines by MTT test. The samples were extracted by percolation method with four solvents; petroleum ether (60-80 °C), chloroform, ethyl acetate and 80% aqueous methanol, successively. All cell lines were cultured in proper medium. Concentrations of 62.5-750 µg/mL from partition fractions of all samples, dissolved in 1% (v/v) DMSO were tested on each cell line. Cells with no treatment and methotrexate were examined as negative and positive controls, respectively. Cell viability was determined by MTT assay. Some fractions showed good cell inhibitory activity with IC₅₀<100 µg/mL, but most of them were considered not potent compared to methotrexate. The chloroform fraction of *S. setifera* was the most cytotoxic sample with high selectivity toward cancerous cell lines, and it could be investigated for its pharmaceutical active components in future studies.

Keywords: Cytotoxic activity, Lamiaceae, MTT assay, *Stachys* spp.

Introduction

Cancer is known as a chronic disease that is described by the uncontrolled development and spread of irregular cells causing masses of tissues called tumors [1]. In general, cancer develops due to the particular effects of environmental agents on a certain genetic background [2]. Tumors of

lung, colon, prostate and breast are the most common types of cancer cases [1]. Some plant secondary metabolites are known as potential anticancer drugs through direct cytotoxicity toward cancer cells or inhibition of tumor development [2].

There are 300 species of genus *Stachys* (Lamiaceae) distributed worldwide [3]. Iran is particularly rich of this genus [4]. A variety of secondary metabolites such as flavonoids, iridoids, diterpenes, triterpenoids and sterols have been reported from different species of the genus [5,6]. In addition, previous studies have elucidated pharmacological and biological effects of some species of *Stachys* [7-17]. Antioxidant, anti-inflammatory and anti-nociceptive activities of some *Stachys* species were previously assessed [18,19], while the oil of *S. byzantina* and the extract of *S. striatinux* have demonstrated potential inhibitory activity against *Candida albicans* and *Helicobacter pylori*, respectively [20,21]. Antimicrobial effect of the essential oil of *S. inflata* was considerably attributed to alpha terpineol [22]. In addition, chloroform and ethyl acetate fractions of four *Stachys* spp. were effective against colon and breast ductal cancer cell lines due to their non-polar compounds [23]. Literature survey has shown that some species including *S. byzantina*, *S. inflata*, *S. setifera* and *S. persica* have not been studied for their cytotoxic activity against the cancerous cell lines HT-29, Caco-2, T-47D and the normal cell line NIH-3T3. In this study, cytotoxic effect of the above species has been evaluated against the mentioned cell lines by MTT assay.

Experimental

Plant material

Flowering aerial parts of all species were collected from Khalkhal, Ardabil province in the North-West of Iran (1800 m) in July 2007. Voucher specimens have been deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (*S. byzantina*: 6513 TEH, *S. setifera*: 6528 TEH, *S. persica*: 6529 TEH, *S. inflata*: 6527 TEH).

Extraction

Plant materials were dried at room temperature in shade. They were powdered finely and extracted by percolation method. 100 g of each sample was

extracted three times with petroleum ether (60-80 °C), CHCl₃, EtOAc and 80% aq. MeOH, successively. Removal of the solvents with rotary evaporator gave the corresponding extracts. Subsequently, all extracts were stored at 4 °C until cytotoxic examination.

Cell culture

All cancerous cell lines were preserved in RPMI 1640 cell culture medium (PAA, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) for HT-29 cells and 15% FBS for Caco-2 and T-47D cells. The normal cell line (NIH-3T3) was grown in Dulbecco's Modified Eagle's Medium (DMEM; PAA, Germany) supplemented with 10% FBS. 100 IU/mL penicillin and 100 µg/mL streptomycin (Boehringer, Germany) was added to the media. All cell lines were cultured at 37 °C in atmosphere of 5% CO₂.

Determination of cell viability by MTT assay

Concentrations of 62.5-750 µg/mL of all samples were tested against each cell line. Samples were dissolved in DMSO and further diluted with the cell culture medium. The final DMSO concentration was 1% of total volume of medium in all treatments including the blank. Cells with no treatment or methotrexate were examined as the negative and positive controls, respectively. 1×10⁴ cells/well were plated into 96-well plates (Nunc, Denmark) and incubated for 24 h before addition of the extracts. After 72 h of incubation for HT-29 cells, 96 h for T-47D and NIH-3T3 cells and 120 h for Caco-2 cells, 20 µL of 5 mg/mL MTT reagent (Merck, Germany) in phosphate buffered saline (PBS, pH 7.4) was added to each well. The plates were incubated at 37 °C for 4 h. Then the medium was removed and 100 µL DMSO was added to each well. The metabolized MTT product was quantified by reading the absorbance at 550 nm on a micro plate reader (Anthos, Austria) [24]. The cell viability in MTT assay was calculated corresponding to the control value (untreated cells). Moreover, the selectivity index (SI) was

also calculated from the IC₅₀ ratio in NIH-3T3 cells versus cancerous cell lines. Any sample which demonstrated SI value higher than 3 was considered to show selectivity for cancerous cell line.

Statistical analysis

All tests were examined in triplicate and data were represented as mean ± standard deviation (SD). The cytotoxic activity was presented as IC₅₀ of treatments compared to the control. IC₅₀ values were calculated by Sigma plot (version 10) software.

Results and Discussion

Four different extracts of *Satchys byzantina*, *S. inflata*, *S. setifera*, and *S. persica*, were subjected to cytotoxic evaluation using MTT assay. The investigated *Stachys* yields were 0.41-21.48 w/w, based on plant dry weight. The yields of 80% aq. MeOH extracts of the plants were higher than non-polar fractions (table 1).

Table 1. Extraction ratios of four different fractions of *Stachys* species

Plant name	Extraction ratio (%)			
	petroleum ether	chloroform	ethyl acetate	80%aq. methanol
<i>S. inflata</i>	0.73	0.59	0.41	15.52
<i>S. byzantina</i>	0.67	1.03	0.59	18.49
<i>S. setifera</i>	0.86	1.70	0.60	17.27
<i>S. persica</i>	0.46	0.66	0.46	21.48

The results of MTT assay are presented in table 2. CHCl₃ fraction of *S. setifera* exhibited cytotoxic activity toward T-47D cell line with IC₅₀ value of 2.44 µg/mL and the highest tumor specific cytotoxicity (SI=161.83). All petroleum ether and CHCl₃ fractions of the plants, and the EtOAc fraction of *S. byzantina* in the second place, were more cytotoxic against T-47D compared to other fractions (IC₅₀<100 µg/mL), whilst other fractions demonstrated weak cytotoxic activity on cancerous cell lines (IC₅₀>100 µg/mL).

Most of the fractions showed lower cytotoxicity on colon carcinoma cell lines compared to the breast carcinoma cells. The most important

constituents of *S. inflata* have been previously identified as phenolic compounds [12], flavonoids such as stachyflaside, isostachyflaside and 4'-methoxyiso-scutellarin and iridoids like ajugoside, ajugol and harpagide [25-27].

Flavonoids and phenols are mostly extracted by polar solvents and because of poor cytotoxicity of the polar fractions of *S. inflata*, no correlation between phenolics and cytotoxic activity of this plant could be established. In previous reports, harpagide was introduced as a potent cytotoxic agent on different tumor cell lines [9]. Whereas, iridoids could be released in petroleum ether and chloroform fractions in our study and higher cytotoxicity was observed. Therefore, harpagide could be suggested as the active component of *S. inflata* extract. Phytosterols, unsaturated fatty acids, phytyl nonadecanoate and phenolics were demonstrated as major compounds in *S. byzantina* extract [11,12]. In the present study, cytotoxic effects of CHCl₃ fraction of *S. byzantina* which could contain phytosterols and unsaturated fatty acids, was reported more cytotoxic than other extracts (table 2). In addition, some terpenoids of the *Stachys* species have showed strong cytotoxic activity in previous studies [28]. For instance, germacrene D has been reported as one of the major constituents of the essential oil of *Stachys* species, and it was known to be a potentially cytotoxic agent [29,30]. The results suggested that 80% aq. MeOH extract of *S. byzantina*, the petroleum ether and CHCl₃ fractions of *S. persica* and EtOAc fraction of *S. persica* demonstrated selective cytotoxicity (SI>3) in (Caco-2 and T-47D), (T-47D) and Caco-2 cells, respectively. The CHCl₃ fraction of *S. setifera* greatly inhibited the proliferation of T-47D cell line (IC₅₀ 2.44 µg/mL) in comparison to normal cells (IC₅₀ 394.88 µg/mL). Previous studies have revealed the major compounds of *S. setifera* as terpenoids and flavonoids [31]. Also, eugenol was characterized as the main component of its volatile oil [32]. Eugenol has showed to be cytotoxic due to the formation of a reactive intermediate, possibly a quinone methide

Table 2. Cytotoxic activity of different fractions of *Stachys* species by MTT assay

Fractions	Cell lines			
	HT-29	Caco-2	T-47D	NIH-3T3
<i>S. inflata</i>				
petroleum ether	123.27±2.03 (0.95)	228.60±8.5 (0.5)	84.70±9.45 (1.4)	118.07±3.57
chloroform	203.35±1.23 (0.66)	134.77±4.12 (1)	55.66±5.6(2.4)	135.37±12.42
ethyl acetate	396.14±4.3 (0.4)	162.87±5.34 (0.96)	121.83±2.84 (1.3)	157.06±10.02
80% aq. methanol	>750	248.40±3.5(1.23)	126.45±11.2 (2.4)	306.02±7.42
<i>S. byzantina</i>				
petroleum ether	299.10±5.1(0.4)	109.52±10.1(1.13)	62.41±13.4(2)	124.30±5.1
chloroform	113.84±6.7(0.72)	126.06±1.5(0.65)	57.94±7.64 (1.4)	82.00±6.34
ethyl acetate	-	128.53±6.79 (1.04)	73.35±6.5(1.8)	133.68±5.56
80% aq. methanol	468.49±11.4 (1.1)	115.80±3.85 (4.4)	148.29±2.3(3.5)	520.62±2.51
<i>S. setifera</i>				
petroleum ether	>750	129.52±6.84	54.84±12.62	-
chloroform	191.71±5.8(2.06)	127.97±13.4 (3.08)	2.44±7.42 (161.83)	394.88±3.3
ethyl acetate	430.55±4.46	99.74±4.9	349.12±9.77	>750
80% aq. methanol	207.18±11.8	476.45±9.47	123.099±2.67	-
<i>S. persica</i>				
petroleum ether	294.07±3.5 (0.63)	185.41±9.47 (1.01)	52.42±7.1(3.6)	187.85±8.3
chloroform	291.10±8.29 (0.6)	127.97±5.67 (1.37)	47.39±3.85 (3.7)	176.03±2.9
ethyl acetate	>750	63.77±4.7 (3.2)	115.99±6.34 (1.76)	204.19±11.9
80% aq. methanol	515.76±6.49	>750	134.24±1.8	>750
methotrexate	0.23±0.02	0.32±0.04	0.16±0.09	0.24±0.01

SI (The selectivity index): IC₅₀ for normal cell line/IC₅₀ for cancerous cell line.

Key to cell Lines employed: HT-29 (colon carcinoma) and Caco-2 (colorectal adenocarcinoma), T-47D (breast ductal carcinoma), and NIH-3T3 (Swiss embryo fibroblast).

-: not determined.

[33] and presence of eugenol in CHCl₃ fraction of *S. setifera* could be a reason for the high potency of the extract to prevent breast ductal carcinoma.

Previous evaluations on *S. persica* have assessed presence of carvacrol, saturated fatty acids and phenolic compounds [12,34]. The essential oils which contain carvacrol as a major compound have exhibited cytotoxicity against tumor cell lines [35]. Based on the results of the present investigation, it was discovered that the non-polar fractions of these plants have mostly affected the breast and colon cancerous cell lines. The non-polar fractions were more cytotoxic compared to the polar ones, but most of them showed less potency in comparison to methotrexate. Further researches are needed for defining potential component as cytotoxic constituents. The chloroform fraction of *S. setifera* (the most potent fraction with high selectivity) could be

investigated for its main pharmaceutical active components in future studies.

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