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Anti-Proliferative and Antimicrobial Activity of Methanolic Extract and SPE Fractions of *Artemisia spicigera*

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Abstract

Objectives and Methods: This study was conducted to investigate in vitro, methanolic extract and methanol/water fractions obtained from the arial parts of *Artemisia spicigera* C. Koch (*Asteraceae* family) and for evaluation of their antiproliferative effects against HT-29, L-929 and A 549 cell lines by MTT assay at different concentrations (1, 10, 100, 1000 μg/mL). Furthermore, this study aimed to detect antimicrobial activities of the mentioned samples: two Gram-positive (*Staphylococcus epidermidis* and *Staphylococcus aureus*), two Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) and a fungi (*Candida albicanse*), using agar well diffusion method.

Results: The IC50 values for antiproliferative activity of the methanolic extract, 20%, 40% and 60% SPE (solid phase extraction) fractions, found to be 345.91 $\pm$ 28.77, 442.44 $\pm$ 83.22, 220.19 $\pm$ 43.13 and 579.90 $\pm$ 153.19 μg/mL, respectively; in this case, the maximum inhibition percentage belonged to 40% SPE fraction (220.19 $\pm$ 43.13 μg/mL). The total methanolic extract of *A. spicigera* indicated inhibitory activity against Gram- positive strains, *S. epidermidis* and *S. aureus*, with MIC values of 150 mg/mL for both bacteria. Among SPE fractions, 20% was the only active one against Gram possitive species with MIC values of 35 mg/mL for both strains.

Conclusions: This study revealed that methanolic extract of *A. spicigera* and its SPE fractions might be regarded as bioactive natural products, which deserves to be further identification and isolation of cytotoxic and antibacterial phytochemicals from them.

Keywords: Artemisia, Antiproliferative, Antimicrobial, *Asteraceae*, Flavonoid, MTT

1. Background and Objectives

Recently, researchers have shown an increasing interest in the biological activities of medicinal plants. Over 60% of the antibacterial and cytotoxic agents are either natural substances, or derivatives of them (1). Genus *Artemisia* with the common Persian name of "Dermane" belonging to *Asteraceae* (Compositeae) family contains 34 species wildly growing in Iran (2, 3). Isolated biologically active secondary metabolites from plants of this genus showed a variety of pharmacological activities, including anti-microbial, anti-viral, anti-tumoral, anti-pyretic, anti-malarial, anti-haemorrhagic, anti-coagulant, and anti-inflammatory, anti-oxidant, anti-hepatitis, anti-ulcerogenic and anti-spasmodic effects (4-10). *Artemisia spicigera* known as "Dermane ye sonbolei" in Persian language, is an aromatic herb growing in the North and Northwestern parts of Iran (5). According to the previous studies, many biological activities assayed on different extracts of this plant showed the antioxidant (11), antimalarial and insecticidal activities of the tested samples (12). Although several studies have been conducted to assess the antimicrobial and antiproliferative activities of the extracts from different species of *Artemisia*, to our knowledge, no data were available on the antibacterial and antiproliferative effects of methanolic extract and SPE fractions of *A. spicigera*. Hence, the aim of this study was to determine the mentioned biological activities of the tested samples.

2. Methods

2.1. Plant Material and Preparing the Extracts

Aerial parts of *Artemisia spicigera* C. Koch were collected from Julfa, border of Aras river in East Azarbaijan province, Iran in 2009. A Voucher specimen of this collection (Tbz-FPH 716) has been deposited at the herbarium of the pharmacy faculty, Tabriz University of Medical Sciences, Tabriz, Iran.

Air-dried and ground aerial parts of *A. spicigera* (100 g) were successively Soxhlet-extracted, using n-hexane, dichloromethane (DCM) and methanol (MeOH) (1.1 L each). All the extracts were separately concentrated under vacuum by rotary evaporator, not exceeding the temperature of 50°C, yielding 6.41 g, 1.45 g and 8.63 g of the extracts, respectively; 8 g of the dried MeOH extract was fractionated by solid-phase-extraction (SPE) on Sep-Pak (C18, 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. Then all SPE fractions were dried, using a rotary evaporator at a temperature not exceeding 50°C yielding 1570, 444, 794, 422, 65 and 670 mg of 10%, 20%, 40%, 60%, 80% and 100% SPE fractions, respectively.

2.2. Antiproliferative Activity

2.2.1. Cell Culture

HT-29 (colon carcinoma cell line), L-929 (normal cell line) and A549 (adenocarcinoma human alveolar basal epithelial cells) cell lines were obtained from Pasture Institute, Tehran, Iran. All cell lines were grown in RPMI 1640 as a cell culture medium supplemented, with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 units/mL penicillin G. They were incubated in a humidified air/carbon dioxide (95:5) atmosphere at 37°C. At 75% confluence, phosphate buffered saline (PBS)/0.5% ethylenediamine tetraacetate (EDTA) and 0.25% trypsin/ EDTA solution were used to rinse and harvest the cells from the flasks. Finally, cells were seeded in 96-well plate (Nunc, Denmark).

2.2.2. MTT Assay

MTT, a colorimetric, in vitro cell growth inhibition assay was used to evaluate the antiproliferative activity of *A. spicigera* MeOH extract and its fractions (13). For this purpose, 1×10^4 cells/well were seeded in to 96-well plate and incubated for 24 hours. Afterwards, different dilutions of MeOH extract and its fractions (1, 10, 100, 1000 μg/mL) were prepared in dimethylsulfoxide (DMSO) and were diluted with cell culture medium. They were added to wells and then transferred into the incubator. After 48 hours of incubation, the medium was replaced with a fresh one, containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (MTT), which was dissolved in PBS to obtain 5 mg/mL solution. The medium was removed after four hours of incubation in the air /carbon dioxide (95:5) atmosphere at 37°C, and 100 μL of DMSO was added to dissolve formazan crystals completely. Microplate reader (ELISA plate reader, Bio Teck, Bad Friedrichshall, Germany) was used at 570 nm wavelength to measure the optical density of the wells. Each assay was performed in triplicate. To compare the antiproliferative activity of the extract and its fractions, DMSO was considered as a negative control. Moreover, inhibitory rate was calculated by the following equation:

Relative viability (%) = (optical density of sample/optical density of control) $\times$ 100. IC_{50} value was defined as the concentration of the MeOH extract to produce a 50% reduction in the viability of the cells relative to the negative control, calculated from the dose-response curve plotted in the Sigma Plot 10 software.

2.2.3. Antimicrobial Activity

Bacterial cultures included Gram- negative species, including *Pseudomonas aeruginosa* (ATCC 9027) and *Escherichia coli* (ATCC 8739), as well as Gram- positive species *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 6538), and a fungi (*Candida albicans*) (ATCC) were used to evaluate antimicrobial properties of the MeOH extract of *A. spicigera* and its SPE fractions. Lyophilized form of the microbial strains was purchased from Pasture institute, Tehran, Iran. Centrifuged pellets of bacteria from a 24- hour culture were mixed with saline solution. Turbidity was corrected as per the standard of 0.5 McFarland [108 colony forming units (CFUs) per mL] by adding sterile distilled water. Then the mentioned inoculums were used for seeding the Muller Hinton agar medium (MERCK). About 10 mL of the prepared inoculums (10^6 CFUs per mL) was seeded. The antimicrobial activity of the samples was monitored, using agar diffusion method, a highly recommended test for a routine assessment of preliminary antimicrobial screening. Each Muller Hinton petri plate was inoculated with a 0.5 McFarland's standard of selected bacteria, including five wells for test samples, two for solutions of the samples, and one for vehicle control (DMSO). Finally, 100 μL of test solutions were poured in respective wells, and subsequently petri plates were incubated at 37°C. After 24 hours of incubation, the diameter of the clear zones, indicating no bacterial growth, around each well (excluding well diameter) was measured, using the vernier caliper. Triplet plates were prepared for each sample. Samples showing significant antimicrobial activity were further assayed for their minimum inhibitory concentration (MIC), which is the lowest concentration of a fraction and has the ability to completely inhibit the growth of each bacterial strain. At First, serial two-fold dilutions of MeOH extract and its fractions were prepared in a nutrient broth. Second, control cultures, which did not influence bacterial growth were also prepared, containing sterile nutrient broth. Third, an equal volume of the adjusted inoculums were added to each test tube. Finally, MIC was read after 24 hours of incubation at 37°C (14).

3. Results

Table 1 demonstrated the antiproliferative activity of the MeOH extract and SPE fractions of *A. spicigera* tested on HT29, A549 and L929 cell lines, which was evaluated using MTT assay. The IC 50 values extracted from the plots of cytotoxicity percentages, versus sample concentrations, presented the rate of antiproliferative activity of different samples. As displayed in Table 1, MeOH extract and SPE fractions were not active against L929 and HT29, whereas MeOH extract indicated inhibitory activity against A549 cell line (345.91 $\pm$ 28.77 μg/mL). Furthermore, the antiproliferative activity of MeOH extract, 20%, 40% and 60% SPE fractions, were found to be 345.91 $\pm$ 28.7, 442.44 $\pm$ 83.22, 220.19 $\pm$ 43.13 and 579.9 $\pm$ 153.19 μg/mL, respectively. In this case, the maximum inhibition percentage belonged to 40% SPE fraction (220.19 $\pm$ 43.13). The results of the antimicrobial activity of the MeOH extract and SPE fractions of *A. spicigera* are presented in Table 2. The mean inhibition zone diameters (MIZD) as well as MIC values demonstrated the rate of activity against the susceptible strains. As demonstrated in Table 2, MeOH extract of *A. spicigera* indicated inhibitory activity against Gram-positive strains, *S. epidermidis* and *S. aureus*, with MIC value of 150 mg/mL for both susceptable species. Furthermore, among SPE fractions, 20% one was the only active sample against susceptible strains, with MIC values of 35 mg/mL for both species. Neither MeOH extract, nor SPE fractions showed activity against Gram- negative strains as well as *Candida albicans*.

Table 1. The Antiproliferative Activity of the MeOH Extract and SPE Fractions of *A. Spicigera* Tested by MTT Assay[a]

	Cell Lines		
Samples	**HT 29**	**A 549**	**L 929**
MeOH	> 1000	345.91 $\pm$ 28.77	> 1000
10%	> 1000	> 1000	> 1000
20%	> 1000	579.9 $\pm$ 153.19	> 1000
40%	> 1000	220.19 $\pm$ 43.13	> 1000
60%	> 1000	442.44 $\pm$ 83.22	> 1000
80%	> 1000	> 1000	> 1000
100%	> 1000	> 1000	> 1000

Abbreviations: MeOH, Methanolic extract; 10%, SPE fraction 10% MeOH-water; 20%, SPE faction 20% MeOH-water; 40%, SPE fraction 40% MeOH-water; 60%, SPE fraction 60% MeOH-water; 80%, SPE fraction 80% MeOH-water; 100%, SPE fraction 100% MeOH.
[a]Values are expressed as IC_{50} (μg/mL) $\pm$ SD (n = 3).

4. Discussion and Conclusion

Plants are the valuable source for searching potential anticancer and antimicrobial agents (15). Moreover, the side effects of the current chemotherapeutical and antimicrobial drugs cause a severe reduction in the quality of life, leading to the development of novel agents (16). Prior studies have noted the importance of plants belonging to *Artemisia* genus to contain phyto constituents indicating anti proliferative activities which have potential of being used as therapeutic agents. For instance, DCM and MeOH extracts of *A. ciniformis* with IC_{50} values of 35 and 60 μg/mL showed antiproliferative activity against AGS cells. Moreover, HeLa cells were sensitive to both DCM extract of *A. diffusa* and ethyl acetate extract of *A. ciniformis*, with IC_{50} values of 71 and 73 μg/mL, respectively. In addition, DCM extracts of *A. diffusa*, A. *santolina Schrenk* and *A. ciniformis* indicated inhibitory activity against HT-29 cells, with IC50 values of 42, 91 and 94 μg/mL, respectively. Furthermore, the growth of MCF-7 cells were best inhibited by *A. ciniformis* DCM (IC_{50} value: 29 μg/mL) and *A. vulgaris* L. ethyl acetate (IC_{50} value: 57 μg/mL) extracts (17). Moreover, the flower MeOH extract of *A. absinthium* and *A. vulgaris* were found to have cytotoxic effect on MCF-7 cell line, with an IC_{50} values of 221.5 and > 500 μg/mL, respectively (18). According to IC_{50} values, displayed in Table 1, antiproliferative activity of tested samples might be connected to active phytochemicals, purified from mentioned fractions reported in our previous study (19). Either luteolin, an abundant dietary component or chrysoeriol as well as their glycosides, are widely distributed in plant kingdom and possess a variety of pharmacological activities, including antiproliferative effects (20, 21). Thus, the antiproliferative activity of 40% SPE fraction might be discussed by the presence of 5-methoxyluteolin 7-O-β-D-glucopyranoside, Luteolin and chrysoeriol 7-O-β-D-glucopyranoside purified from 40% SPE fraction (19). Furthermore, the antiproliferative activity of 60% SPE fraction might be due to the presence of 5-methoxy Luteolin in this fraction, noted in our previous study (19). Moreover, 20% SPE fraction demonstrated antiproliferative activity, which might be due to the presence of 4, 6-di-methoxy acetophenone-2-O-β-D- glucopyranoside. The cytotoxicity of acetophenone derivatives have been found in some preceding researches (22, 23). However, further investigations are needed to separate and identify more potent phytochemicals, which play an important role in the antiproliferative activity of 40% and other active SPE fractions.

According to previous studies, different species of *Artemisia* genus not only demonstrated antiproliferative effects, but also antibacterial activities. MeOH extract of *A. nilagirica* was active against *Escherichia coli*, *Bacillus subtilis*,

Table 2. The Antimicrobial Activity of the MeOH Extract and Fractions of *A. spicigera* as the Mean Inhibition Zone Diameter ± SD (MIZD) and Minimum Inhibitory Concentration (MIC) of the Samples Against Different Strains (n = 3)

Samples	MO					
		E. coli	*P. aeruginosa*	*S. epidermidis*	*S. aureus*	*Candida albicans*
MeOH	MIZD ± SD, mm	-	-	11 ± 0.00	11 ± 1.40	-
	MIC, mg/mL	-	-	150	150	-
10%	MIZD ± SD, mm	-	-	-	-	-
	MIC, mg/mL	-	-	-	-	-
20%	MIZD ± SD, mm	-	-	75 ± 7.00	80 ± 0.00	-
	MIC, mg/mL	-	-	35	35	-
40%	MIZD ± SD, mm	-	-	-	-	-
	MIC, mg/mL	-	-	-	-	-
60%	MIZD ± SD, mm	-	-	-	-	-
	MIC, mg/mL	-	-	-	-	-
80%	MIZD ± SD, mm	-	-	-	-	-
	MIC, mg/mL	-	-	-	-	-
100%	MIZD ± SD, mm	-	-	-	-	-
	MIC, mg/mL	-	-	-	-	-

Abbreviations: MO, microorganisms; MeOH, methanolic extract; 10%, SPE fraction 10% MeOH-water; 20%, SPE fraction 20% MeOH-water; 40%, SPE fraction 40% MeOH-water; 60%, SPE fraction 60% MeOH-water; 80%; SPE fraction 80% MeOH-water; 100%, SPE fraction 100% MeOH.

Yersinia enterocolitica, *Salmonella typhi*, *Enterobacter aerogenes*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* (24). Buffered methanol (80% methanol and 20% PBS) and acetone extracted substances from *A. absinthium* showed inhibitory activity against *E. coli*, *S. infantis*, *S. aureus* and *L. monocytogenes* (25). *A. capillaris* Thunb and *A. caruifolia* Buch were active against *B. cereus* and *L. monocytogenes* (26). Furthermore, *A. annua* and *A. vulgaris* L. var indica maxim possessed antimicrobial activity against *streptococcus mutans* (27). To the best of our knowledge, in reviewing the literature, there was no report about the antimicrobial activity of *A. spicigera* MeOH extract. The findings of this study revealed that MeOH extract and 20% SPE fraction were active against *S. epidermidis* and *S. aureus*, which are both Gram- positive strains. The obtained results were consistant with the findings of previous studies and suggested that due to the several possible reasons mentioned in our previous study, the most susceptible strains were Gram-positive microorganisms (28). The antibacterial effects of acetophenone derivatives have been previously shown against *Staphylococcus aureus* (29, 30). Therefore, the existence of 4, 6-di-methoxy acetophenone-2-O-β-D- glucopyranoside in 20% fraction and its antibacterial activity might be considered as a possible explanation for the inhibitory effect of the mentioned SPE fractions. Although flavonoids which have indicated antibacterial activities were purified from 40% and 60% SPE fractions (31-35), contrary to the expectations, these two fractions did not demonstrate any antibacterial activity against the tested strains. Further investigations are needed to separate and identify phytoconstituents, with the antibacterial activities in 40% and 60% SPE fractions.

Focusing on the biological activities of the herbs could lead to the discovery and development of new pharmaceuticals. Acetophenones derivatives may serve as a novel group of useful therapeutics against Gram- positive strains. In light of the present findings, MeOH extract of *A. spicigera* and its SPE fractions might be regarded as bioactive natural products and deserve to be further investigated for their potential therapeutic effects in both experimental models of tumor and infection. Future studies are encouraged to separate and identify the principal phytochemicals, with lower IC_{50} values responsible for the observed biological activities of the tested MeOH extract and SPE fractions. Another possible area of future research would be to investigate the mechanisms underlying the cytotoxic and antibacterial effects of MeOH extract and fractions of *A. spicigera* as well as isolation and identification of principal phytoconstituents.

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