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Comparative evaluation of antioxidant and antimicrobial activity of crude extract and secondary metabolites isolated from *Artemisia kulbadica*

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ABSTRACT

Objective: To investigate the antimicrobial and antioxidant properties of Et_2O/MeOH/petrol extract and three isolated compounds from *Artemisia kulbadica* (*A. kulbadica*). **Methods:** The antimicrobial activity was tested by using the disc-diffusion method and determining the minimal inhibitory concentration (MIC) using the agar dilution method against Gram positive and Gram negative bacteria, and fungi. The antioxidant activities of crude extract and tree isolated compounds were evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assays. **Results:** The plant extract was showed moderate values DPPH radical scavenging activity (IC_{50}= (422.4 ± 2.4) μg/mL) while it was showed no considerable antimicrobial activity against tested microorganisms. Three isolated compounds tested for the first time, demonstrated antimicrobial and antioxidant activities. Two sesquiterpenes showed higher antimicrobial activity than the flavone while the later compound was better antioxidant than the sesquiterpens. **Conclusions:** The present study clearly demonstrated that *A. kulbadica* and some of its isolates each one separately possess antimicrobial or antioxidant properties and may act as potential antioxidant for biological systems susceptible to free radical-mediated reactions.

1. Introduction

Artemisia is a genus of small herbs or shrubs found in Northern temperate regions. It belongs to the important family compositae (Asteraceae), one of the most bulky vegetal groupings, which comprises about 1 000 genera and over 20 000 species. Within this family, Artemisia is included into the tribe Anthemideae and comprises itself over 500 species. The 500 species of Artemisia are mainly found in Asia, Europe and North America. They are mostly perennial herbs and dominating the vast steppe communities of Asia. Thirty-four species of the genus Artemisia are found in Iran, of which two are endemic: *Artemisia melanolepis* (A. melanolepis) and *Artemisia kulbadica* (*A. kermanensis*)[1].

Much interest has recently been focused on development of drugs from natural origins[2] and screening of plants can lead to the discovery of novel therapeutics. Many plants have shown considerable cytotoxic activities and many antitumoural agents are derived from plant origin[3]. Similarly, antioxidant and antimicrobial properties have also been frequently found in several plants from different regions[4,5]. For example *Artemisia annua* (*A. annua*) is a traditional medicinal herb of China. It is presently being cultivated on a commercial scale in China and Vietnam for its antimalarial sesquiterpene lactone artemisinin and its essential oil[6]. Since plants growing in Iran have not been extensively studied for their biological activities, they represent an invaluable source of potentially useful biologically active compounds. The genus Artemisia has always been of great botanical and pharmaceutical interest and is useful in traditional medicines for the treatment of a variety of diseases and complaints. Many species have been used since ancient times as folk remedies for some treatment purposes for example: reducing phlegm, relieving cough, invigorating blood circulation, stopping pain, inducing sweat, diuresis, anti-hypertension, anthelminthic, antitoxic and antiallergy[7].

A. kulbadica, a perennial, herbaceous and strongly aromatic plant, is grown widely in Iran and Afghanistan[8].

A literature review shows that there are a few reports on the phytochemical and biological investigation of

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A. kulbadica. The antimicrobial activity and chemical composition of volatile oil of this plant was investigated previously and twenty-seven compounds were identified. Sabinene (25.1%), Trans-thujone (18.7%) and γ-cadinene (16.0%) were the main components. The essential oil of *A. kulbadica* has a strong and broad spectrum of antibacterial activity compared with other Artemisia species[9]. We have recently reported from Et_2O/ MeOH/petrol extract of the aerial parts of *A. kulbadica* new germacranolide and aguaianolide type sesquiterpene lactones and penta methoxylated flavone[10]. In this study the crude extract and the aforementioned isolated compounds of *A. kulbadica* were comparatively screened for their antimicrobial and antioxidant properties.

2. Material and methods

2.1. Reagents, chemicals and microorganisms

Trolox (water soluble equivalent of vitamin E) was from Acros Organics (Geel). Acetic acid glacial, dimethyl sulphoxide, hexane, methanol, sodium acetate and sodium carbonate were purchased from Merck (Darmstadt). 2,2-diphenyl-1-picrylhydrazyl (DPPH), and hydrochloric acid 32% were obtained from Sigma-Aldrich (St. Louis). The bacteria that were used in this study were *Bacillus pumilus (B. pumilus)* (PTCC 1319), *Escherichia coli (E. coli)* (PTCC 1533), *Kocuria varians (K. varians)* (PTCC 1484), *Pseudomonas aeruginosa (P. aeruginosa)* (PTCC 1310), *Salmonella typhi (S. typhi)* (PTCC 1609), and *Listeria monocytogenes (L. monocytogenes)* (PTCC 1298). The fungal strains that were used in this study were *Aspergillus niger (A. niger)* (PTCC 5154), *Aspergillus flavus (A. flavus)* (PTCC 5006) and *Candida glabrata (C. glabrata)* (PTCC 5297). All microorganisms were obtained from the Persian type culture collection (PTCC), Tehran, Iran.

2.2. Plant Materials

The aerial parts of the wild-growing *A. kulbadica* were collected during the full flowering stage in September 2009 from their natural habitats in North East of Iran province of Khorasan. It was identified by Dr. Valiollah Mozaffarian and a voucher specimen (no. AC-23-11) was deposited at the Herbarium of the Research Instituted of Forests and Rangelands (TARI), Tehran, Iran. Ground aerial parts of plant (500 g) were extracted with Et_2O/MeOH (1:1) (2×5 L) at room temperature for 3 d to give 45 g (8.3% yield) of the crude extract which was suspended in EtOH (300 mL) at 55 ℃, diluted with H_2O (259 mL) and extracted successively with n-hexane (3×650 mL) and $CHCl_3$(3×450 mL).

2.3. Antioxidant activity measured by DPPH radical scavenging activity

Radical scavenging activity of natural samples against the stable free radical DPPH was measured as described previously[11], with some modifications. Briefly, 4 different concentrations of the plant extract or isolated compounds dissolved in methanol were incubated with a methanolic solution of DPPH (100 μM) in 96-well microplates. Concentrations were carefully chosen according to the activity of this plant, in order to produce an appropriate dose-response curve. Plant extract concentrations used in this study ranged from 1.6 to 100 μg/mL. After 30 min of incubation at room temperature in the dark, the absorbance at 490 nm was measured by a microplate reader (Bio-Tek, model 680). The % inhibition (%I) for each concentration was calculated by using the absorbance (A) values according to the following formula:

$$\%I = [(A_{DPPH} - A_{PS})/A_{DPPH}] \times 100$$

Where A_{DPPH} and A_{PS} are the absorbance of the DPPH solutions containing methanol and plant samples, respectively. The dose-response curve was plotted by using the software SigmaPlot for Windows version 8.0 and IC_{50} values for extract was calculated. These values were divided by the extraction yield (Y) to calculate the IC_{50} value for the dry plant.

2.4. Assessment of antimicrobial activity by disc diffusion assay

The antimicrobial activity was tested by using the disc-diffusion method[12] with minor modifications. The dried compounds or extract isolated from *A. kulbadica* was dissolved in DMSO to a final concentration of 30 mg/mL and filtered by 0.45 lm Millipore filters for sterilization. Using 100 μL of suspension containing 108 CFU/mL of bacteria and 104 spore/mL of fungi spread on the nutrient agar (NA) and potato dextrose (PD) agar mediums, respectively. The discs (6 mm in diameter) impregnated with 10 μL of the essential oil solution (300 μg/disc) and DMSO (as negative control) were placed on the inoculated agar. The inoculated plates were incubated for 24 h at 37 ℃for bacterial strains and 48 h and 72 h at 30 ℃for mold isolates, respectively. Gentamicin (10 μg/disc) and ampicillin (5 μg/disc) were used as positive controls for bacteria and ketoconazole (100 IU) for fungi. The diameters of inhibition zones were used as a measure of antimicrobial activity and each assay was repeated twice.

2.5. MIC agar dilution assay

Antimicrobial activity of plant samples was tested also by determining the minimal inhibitory concentration (MIC) using the agar dilution method[13]. The lowest concentration of the compounds that prevented visible growth was considered to be the minimal inhibitory concentration (MIC). In antifungal activity evaluation, appropriate amounts of the natural compounds or extract of *A. kulbadica* were added aseptically to sterile molted sabouraud dextrose agar (SDA) medium containing Tween 20 (0.5%, v/v) to produce the concentration range of 8-512 μg/mL. The resulting SDA agar solutions were immediately mixed and poured into petri plates. The plates were spot inoculated with 5 μL (104 spore/ mL) of fungus isolate. At the end of incubation period, the plates were evaluated for the presence or absence of growth. The antibacterial activity was carried out similarly through the aforementioned protocol. The only difference is using 5 μL of suspension containing 108 CFU/mL of bacteria Instead of fungus isolate. The MIC was defined as the lowest concentration of the oil to inhibit the growth of

microorganisms. Ampicillin, Tetracycline and Fluconazole were used as references for Gram-positive, Gram-negative bacteria and fungus, respectively. Each test was repeated at least twice.

3. Results

3.1. Isolated compounds

Recently, we isolated and reported three compounds from *A. kulbadica* by phytochemical analysis[10].

The isolated compounds were identified as 4α-hyroxy-guaia-1(10), 5-dien-12,8α-olide 1, 3-Oxogermacr-1(10)-(E)-en-12,6α-olide 2 and 5-hydroxy-6,7,8,2′,4′-pentamethoxyflavone3. The structures were elucidated by spectropic methods, including 1D and 2D NMR analysis.

Table 1.
Antioxidant activity of the crude extract of *A. kulbadica* and its major components and Troloxc in DPPH free radical scavenging activity.

Sample	DPPH IC_{50} (μg/mL)
Crude extract	422.40 ± 2.40
Sesquiterpen lactone 1	ND[a]
Sesquiterpen lactone 2	ND[a]
Flavonoide 3	89.50 ± 0.65
Trolox	19.72 ± 0.82

[a]Less than 40% inhibition for the compound 1 and no inhibition for compound 2 for concentrations up to 2 mg/mL, ND (Not determined).

3.2. Antioxidant activity

Extract of the aerial parts of *A. kulbadica* and three isolated compounds of this extract were subjected to screening for their possible antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay method. DPPH is a stable free radical which can readily experience reduction in the presence of an antioxidant. It shows a maximum ultraviolet and visible (UV-Vis) absorbance at 517 nm. The reduction in the intensity of absorption at 517 nm of methanol solutions of DPPH radical in the presence of antioxidants is usually taken as a measure of their antioxidant activity. In this study, the ability of samples to scavenge DPPH radical was determined on the bases of their concentrations providing 50% inhibition (IC_{50}). Plant extract, its three isolated components and positive control (Trolox) IC_{50} values are given in Table 1. The isolated flavonoid 3 showed the best radical scavenging activity with an IC50 value of (89.50 ± 0.65) μg/mL, about 22% of the potency of standard Trolox. The plant extract was showed moderate values DPPH radical scavenging activity while two sesquiterpene lactones did not demonstrated considerable antioxidant activities.

3.3. Antimicrobial activity

The antimicrobial activity of *A. kulbadica* extract and three isolated components of this extract were evaluated against a set of 9 microorganisms and their potency were assessed qualitatively and quantitatively by the presence or absence of inhibition zones, zone diameters and MIC values. The results are given in Table 2 and indicate that, at tested concentrations, the plant has no considerable antimicrobial activity against tested microorganisms. However, germacranolide, and guaianolide type sesquiterpene lactones (Compounds 1 and 2) showed moderately good antibacterial activities against *E. coli* and *S. typhi* and the penta methoxylated flavone 3 exhibited week anticandidal activity.

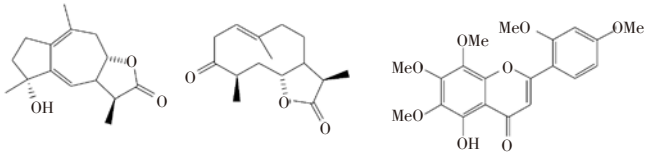

Figure 1. Isolated compounds from *A. kulbadica*.

Table 2.
Antimicrobial activity of the crude extract of *A. kulbadica* and its major components.

Test microorganisms		Crude extract		Sesquiterpene lactone 1		Sesquiterpene lactone 2		Flavonoide 3		Antibioticsc	
		MIC[a]	DD[b]	MIC	DD	MIC	DD	MIC	DD	MIC	DD
Gram-negative bacteria	*E. coli*	256	10.1 ± 0.2	64	13.00± 0.82	32	16.1 ± 1.1	512	7.1 ± 0.1	16	18.1 ± 0.1
	P. aeroginosa	–	–	128	12.50 ± 0.69	256	10.1 ± 0.5	512	8.3 ± 0.5	8	15.3 ± 0.5
	S. typhi	512	6.7±0.7	64	14.20± 0.86	64	14.6 ± 1.5	256	11.6 ± 1.1	32	18.6 ± 1.1
Gram-positive bacteria	*B. pumilus*	–	–	128	14.30 ± 1.02	128	13.5 ± 0.5	256	10.1 ± 0.3	64	16.1 ± 0.3
	K. varians	–	–	256	11.50 ± 0.30	256	–	512	7.5 ± 0.5	16	15.5 ± 0.5
	L. monocytogenes	512	7.1±0.7	128	13.10 ± 2.37	128	12.9 ± 0.5	512	7.3 ± 0.1	16	17.3 ± 0.1
Fungi	*A. flavus*	–	–	512	–	512	–	–	–	64	25.5 ± 0.7
	C. glabrata	–	–	256	8.30 ± 1.80	512	–	–	–	128	19.1 ± 0.9
	A. niger	–	–	512	–	512	–	–	–	64	27.0 ± 0.1

aMinimum Inhibitory Concentration (range of concentration: 8-512 μg/mL); [b]DD (Disk diffusion method), Inhibition zones in diameter (mm) around the impregnated disks (Mean ± SD); cAmpicillin(5 μg/disc), gentamicin(10 μg/disc) and ketoconazole (100 IU) were used as references for Gram-positive, Gram-negative bacteria and fungus, respectively for disc diffusion method. In MIC assessment, ampicillin, tetracycline and fluconazole were used as references for Gram-positive, Gram-negative bacteria and fungus, respectively. A dash (-) indicate no antimicrobial activity.

4. Discussion

Comparatively screen of the crude extract and the aforementioned isolated compounds of *A. kulbadica* for their antimicrobial and antioxidant properties lead to the interesting results. In the case of antioxidant activity, as expected for all phenolic compounds, isolated flavonoid 3 was showed higher antioxidant activates in comparison with the non-phenolic sesquiterpenes 1 and 2.

At the same time the crude extract was showed moderate values DPPH radical scavenging activity. The relations reported between phenolic compounds content such as flavonoids and antioxidant activities in the literature are somewhat confusing. Some investigators have proposed close correlations between antioxidant activity and phenolic compounds content of the extracts obtained from various natural sources[14,15] while others did not correlate them merely to each other and contributed a wide range of compounds such as phenolic, peptides, organic acids and other components in antioxidant activity[16-18].

Results of antimicrobial tests showed that two isolated sesquiterpenes 1 and 2 remarkably inhibited the growth of all tested bacteria (especially against *E. coli* and *S. typhi)* in terms of minimal inhibitory concentration (MIC) and zone of inhibition around the disc, while flavonoid 3 was showed mildly activity against these microorganisms. This is particularly interesting from a medical point of view because these microbial agents are responsible for severe opportunistic infections. Inconsiderable antimicrobial activity of the whole extract of the plant despite the activity of its isolated components suggests a possible antagonistic relationship between these components and the rest of plant extract. We also screened the antifungal activity of the plant extract and isolated compounds. All tested plant samples has no significant activity against fungal.

Plant secondary metabolites generally display remarkable biological activities such as antioxidant and antimicrobial properties which are useful for preserving foods from decay and contamination and/or preventing living tissues from various diseases. According to literature data, this is the first study on the antioxidant and antimicrobial activity of the extract of *A. kulbadica* and some of its isolates indicating good to moderate antioxidant activity for the plant. These results encourage complementary and more in-depth studies on the chemical composition of the other plant extracts with the aim of separation and structure elucidation of their active components and evaluation of biological activity of each compound separately.

Conflict of interest statement

We declare that we have no conflict of interest.

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