

Antifungal activity of the native essential oil of *Zataria multiflora* on *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus* from Iran

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ABSTRACT

The composition of the native essential oil of *Zataria multiflora* and its antifungal activity on *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus* fungal strains were studied in Iran. Essential oil from the aerial parts of the plant was obtained by hydrodistillation and analysed by GC and GC-MS. The oil showed high contents of p-Cymene, carvacrol and thymol. The MIC was used to evaluate the antifungal activity against *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 9642 and *Aspergillus flavus* ATCC 9643. Antifungal activity was evaluated for the essential oil and simultaneously for Amphotericin B. Results showed that *Zataria multiflora* essential oil exhibited a significant activity against fungi, and its MIC on *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus* were respectively 0.125, 0.125 and 0.125 $\mu\text{g ml}^{-1}$ (ppm). The present study indicates that *Zataria multiflora* essential oil has considerable antifungal activity, deserving further investigation for clinical applications.

Key words: *Zataria multiflora*, MIC(minimal inhibitory concentration), Antifungal

INTRODUCTION

Fungal infections have been increasing in recent years due to a growing number of high-risk patients, particularly immunocompromised hosts. *Candida* is the third- or fourth-most-common isolate in nosocomial bloodstream infections in the USA. In addition, The mortality rate due to invasive aspergillosis increased by 357 % between 1980 and 1997 in the USA. In spite of the introduction of new antifungal drugs, they are limited in number. The increase of fungal resistance to classical drugs, the treatment costs, and the fact that most available antifungal drugs have only fungistatic activity, justify the search for new strategies (Eugenia pinto, 2006). Aromatic plants have been widely used in folk

medicine. It is known that most of their properties are due to their volatile oils. Essential oils from many plants are known to possess antifungal activity, but only limited information exists about activity toward human fungal pathogens. They have been empirically used as antimicrobial agents, but the mechanisms of action are still unknown.

Some essential oils show an important antifungal activity against yeasts, dermatophyte fungi and *Aspergillus* strains, which could predict therapeutic benefits, mainly for diseases with mucosal, cutaneous and respiratory tract involvement. (Pina-Vaz *et al.*, 2004; Salgueiro *et al.*, 2003, 2004),

The objective of our present research was to evaluate the antifungal activity and investigate the mechanism of action of *Zataria multiflora* essential oil from Iran.

METHODS

Essential oil analysis

Essential oil from the aerial parts of the plant was obtained by hydrodistillation and analysed by GC and GC-MS. Gas chromatography (GC) and GC-mass spectrometry (MS) analysis of essential oil of thyme were performed. The oil showed high contents of *p*-cymene, carvacrol and thymol.

Plant material and chemicals. Aerial parts of the plants were collected at the flowering stage from Tokestan, 11th Km. Gorgan-Mashhad Road, Gorgan, Iran.

Thymol (99.5 %) was purchased from DBH, and carvacrol, γ -terpinene and *p*-cymene (all 99.5 %) from Fluka.

Essential oil analysis

Essential oil was isolated by water distillation for 3 h from air-dried material, using a Clevenger-type apparatus, according to the procedure described in the European Pharmacopoeia (Council of Europe, 1997).

Gas chromatography (GC-FID)

Gas chromatography analysis was performed on a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with flame ionization detector and capillary column HP-101 (Methyl silicone fluid), 25 m \times 0.2 mm i.d., coating thickness 0.2 μ m. Chromatographic conditions were as follows: helium as carrier gas at 1.0 ml min⁻¹; injector and detector temperatures, 250°C and 300°C. Oven temperature was isothermal at 70°C for 2 min, then increased to 200°C, at a rate of 3°C min⁻¹ and held isothermal for 15 min. Volume injected 1 μ l. Split ratio 1 : 50.

Gas chromatography-mass spectrometry

Essential oil was also analysed by Hewlett Packard GC-MS (model 5890 series II) with mass selective detector (model 5971A). Two columns of different polarity were used: an HP-101 column

(Methyl silicone fluid, Hewlett Packard; 25 m \times 0.2 mm i.d., film thickness 0.2 μ m) and an HP-20M column (Carbowax 20M, Hewlett Packard; 50 m \times 0.2 mm i.d., film thickness 0.2 μ m). Oven temperature was programmed as follows: isothermal at 70°C for 4 min, then increased to 180°C, at a rate of 4°C min⁻¹ and subsequently held isothermal for 15 min (for HP-20M column); isothermal at 70°C for 2 min, then increased to 200°C, at a rate of 3°C min⁻¹ and held isothermal for 15 min (for HP-101 column). Carrier gas was helium, flow rate: 1 ml min⁻¹; injector temperature: 250°C; volume injected: 1 μ l; split ratio: 1 : 50. MS conditions: ionization voltage: 70 eV; ion source temperature: 280°C; mass range: 30-300 mass units.

Qualitative and quantitative determination

The individual peaks were identified by comparison of their retention indices to those of authentic samples, as well as by comparing their mass spectra with the Wiley 6-0 library (Wiley, New York, NY, USA) and NIST98 (National Institute of Standards and Technology, Gaithersburg, MD, USA) mass spectral database and literature (Adams 1995).

The percentage composition of the samples was computed from the GC peak areas by using the normalization method (without correction factors). Quantitative results are mean of data derived from duplicate GC-FID analyses.

Isolation and detection of Fungi

The antifungal activity of *Zataria multiflora* essential oil was evaluated against *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 9642, and *Aspergillus flavus* ATCC 9643. The fungal isolates were identified by standard microbiology methods and stored in Sabouraud dextrose broth with glycerol at -70 °C.

Fungi were plated on Sabouraud 2% (w/v) glucose agar (SGA), and incubated at 25 \pm 2°C for the 5-7 days.

For the antifungal activity testing, *Zataria multiflora* essential oil was dissolved in 96% (v/v) ethanol and then diluted with 30% (v/v) ethanol in distilled water with 0.1% (w/v) Tween 80. Final concentrations of the essential oil was 2% (w/v).

Antifungal activity testing by dilution method: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined by using the serial broth dilution method described by Pepeljnjak *et al.* (2003). Serial twofold dilutions in DMSO, ranging from 0.02 to 20 $\mu\text{l ml}^{-1}$, were tested for essential oil. In addition, the reference antifungal compounds, fluconazole (Pfizer) for *Candida albicans* or amphotericin B (Sigma) for *Aspergillus*, were used as standard antifungal drugs. Twofold serial dilutions ranging from 0.25 to 128 $\mu\text{g ml}^{-1}$ for fluconazole and 0.016 to 16 $\mu\text{g ml}^{-1}$ for amphotericin B were used. Quality control determinations of the MICs of fluconazole and amphotericin B were performed by testing *C. parapsilosis* ATCC 90018 and *C. krusei* ATCC 6258. The results obtained were within the recommended limits.

MIC is defined as the lowest concentration of extract or essential oil that allows no more than 20% growth of the fungus, visualized as a reduced number of colonies after removing the loop with approx. 10 μl of each dilution, and then inoculated on SGA and incubated at $25 \pm 2^\circ\text{C}$ for 7 days. MFC is defined as the lowest concentration of essential oil that completely inhibited the growth of fungi.

These experiments performed in duplicate were repeated independently three times and yielded essentially the same results. A range of values is presented where different results were obtained. Two growth controls, RPMI medium and RPMI with 2.0 % (v/v) DMSO, were included for each strain.

Statistics

The data obtained as MIC and MFC of essential oil, expressed in $\mu\text{g ml}^{-1}$, were statistically analysed by using the Wilcoxon matched pairs test. The level of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The oil was obtained from air-dried plant material in a yield of 1.8 % (v/w)

Evaluation of MIC showed that the oil was active against all the tested strains (Table 1). *Zataria multiflora* essential oil exhibited significant antifungal activity. MIC value 0.125 $\mu\text{l ml}^{-1}$ against *Aspergillus* strains and *Candida*. It is difficult to attribute the activity of a complex mixture to particular constituents. Nevertheless, it is reasonable to speculate that the activity of this oil can be related to the presence of *p*-cymene, carvacrol and thymol.

Table 1: Antimicrobial activity (MIC) and (MFC) of the essential oil of the *Zataria multiflora* for *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus*

<i>Candida albicans</i> ,	<i>Aspergillums niger</i>	<i>Aspergillus flavus</i>	<i>Zataria multiflora</i> effects
0.125	0.125	0.125	MIC in $\mu\text{g ml}^{-1}$
0.25	0.25	0.25	MFC in $\mu\text{g ml}^{-1}$

The importance of the phenolic hydroxyl groups for the antimicrobial activity of the monoterpenoids has previously been reported (Adam *et al.*, 1998; Aliannis *et al.*, 2001; Dorman & Deans, 2000; Nostro *et al.*, 2004; Sivropoulou *et al.*, 1996).

In conclusion, the findings of the present study indicate that *Zataria multiflora* essential oil has potential as a topical antifungal agent against fungi that are pathogenic to humans. This essential oil is a broad-spectrum agent that inhibites *Aspergillus* and *Candida* species,

Given the results described above, particularly the possible mechanisms of action, which might induce side-effects in humans, these antifungals require further investigation.

The results presented should stimulate studies on toxicity, improved formulations and the determination of optimal concentrations for clinical applications, as well as comparative studies alongside currently used drugs of the therapeutic efficacy of essential oils to control infections.

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