

## Antifungal Activity of the Native Essential oil of *Thymus vulgaris* on *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus* from Iran

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The composition of the native essential oil of *Thymus vulgaris* 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 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 *Thymus vulgaris* essential oil exhibited a significant activity against fungi, and its MIC on *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus* were respectively 0.30, 0.15 and 0.25  $\mu\text{g ml}^{-1}$  (ppm). The present study indicates that *Thymus vulgaris* essential oil has considerable antifungal activity, deserving further investigation for clinical applications.

**Key words:** *Thymus vulgaris*, MIC, Antifungal activity.

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

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antifungal activity (Kalemba & Kunicka, 2003), but only limited information exists about activity towards human fungal pathogens. They have been empirically used as antimicrobial agents, but the mechanisms of action are still unknown.

According to our preliminary results (Pina-Vaz *et al.*, 2004; Salgueiro *et al.*, 2003, 2004), some essential oils show important antifungal activity, which could predict therapeutic benefits, mainly for diseases with mucosal, cutaneous and respiratory tract involvement.

Several studies have shown that thyme oils, particularly those of *Thymus vulgaris* and *Thymus zygis* possess antimicrobial activity, those of the phenol type being the most active. The limited occurrence of these phenols in nature is one of the reasons why *Thymus* oils containing thymol and carvacrol have been of great interest for some time (Bruneton, 1999; Pina-Vaz *et al.*, 2004; Stahl-Biskup & Sáez, 2002).

The objective of the present research is to evaluate the antifungal activity and investigate the mechanism of action of *Thymus vulgaris* essential oil from Iran.

## MATERIAL AND METHODS

### Plant material and chemicals.

Aerial parts of the plants were collected at the flowering stage from Tokestan, 11 km. Gorgan-Mashhad Road, Gorgan, Iran.

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

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).

### Analysis of Essential oil

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 carvacrol and thymol.

### 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  $\mu$ m. Chromatographic conditions were as follows: helium as carrier gas at 1.0 ml min<sup>-1</sup>; 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<sup>-1</sup> and held isothermal for 15 min. Volume injected 1  $\mu$ 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  $\mu$ m) and an HP-20M column (Carbowax 20M, Hewlett Packard; 50 m  $\times$  0.2 mm i.d., film thickness 0.2  $\mu$ 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<sup>-1</sup> 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<sup>-1</sup> and held isothermal for 15 min (for HP-101 column). Carrier gas was helium, flow rate: 1 ml min<sup>-1</sup>; injector temperature: 250°C; volume injected: 1  $\mu$ 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 the 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  $-70^{\circ}\text{C}$ .

Fungi were plated on Sabouraud 2% (w/v) glucose agar (SGA), and incubated at  $25 \pm 2^{\circ}\text{C}$  for the 5–7 days.

For the antifungal activity testing, thymol and thyme essential oil were 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 thymol and thyme essential oil were 2% (w/v) and 2% (w/v), respectively.

#### Determination of antifungal activity

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined by using the serial broth dilution method described by Pepelnjak *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.  $10\ \mu\text{l}$  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). *Thymus vulgaris* essential oil exhibited significant antifungal activity. MIC values ranged from 0.15 to  $0.25\ \mu\text{l ml}^{-1}$  against *Aspergillus* strains. *Candida* showed the highest MIC values,  $0.30\ \mu\text{l ml}^{-1}$ . 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 carvacrol and thymol. The importance of the phenolic hydroxyl groups for the antimicrobial activity of the monoterpenoids has previously been reported (Adam *et al.*, 1998; Aligiannis *et al.*, 2001; Dorman & Deans, 2000; Nostro *et al.*, 2004; Sivropoulou *et al.*, 1996). Other species of the genus *Thymus*, such as *T. zygis* and *T. pulegioides*, with high amounts of phenols, also show a broad spectrum of activity against a variety

**Table 1.** Antimicrobial activity (MIC) and (MFC) of the essential oil of *Thymus vulgaris* for *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus*.

<i>Candida albicans</i>	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Thymus vulgaris</i> effects
0.30	0.15	0.25	MIC in $\mu\text{g ml}^{-1}$
0.30	0.25	0.30	MFC in $\mu\text{g ml}^{-1}$

Results were obtained from three independent experiments performed in duplicate

of pathogenic yeasts and filamentous fungi, including fungi with decreased susceptibility to fluconazole (Dorman & Deans, 2000; Nguefack *et al.*, 2004; Pina-Vaz *et al.*, 2004).

In conclusion, the findings of the present study indicate that *T. vulgaris* 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 inhibits the growth of *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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