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Anti-Candida activity of ethanolic extracts of Iranian endemic medicinal herbs against *Candida albicans*

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It has long been known that herbs and their extracts have antimicrobial activities. *Heracleum lasiopetalum* Boiss., *Satureja bachtiarica* Bunge., *Thymus daenensis* Celak., *Echiophora platyloba* L., *Dracocephalum multicaule* Benth., *Kelussia odoratissima* Mozaff. and *Achillea kellalensis* Boiss. are Iranian endemic plant species that have been traditionally used as medicinal herbs and spices in different regions of Iran especially Central Zagross. Seven ethanolic extracts of endemic medicinal herbs and one extract of native medicinal herb (*Stachys lavandulifolia* Vahl.) collected from Chaharmahal va Bakhtiari province of Iran were assayed for the *in vitro* antifungal activity against *Candida albicans* (ATCC1023), using agar dilution methods. Most of the extracts showed relatively high anti-Candida activity against the tested fungi with the diameter of inhibition zone ranging between 8 and 17 mm. The extracts of *S. bachtiarica* and *T. daenensis* exhibited high inhibitory effect against *C. albicans*. The extracts of *S. bachtiarica* and *T. daenensis* were characterized using HPLC, the major components of *S. bachtiarica* and *T. daenensis* were carvacrol and thymol, respectively. The minimum inhibitory concentration (MIC) values for active extract range between 25 and 50 µg/ml. In conclusion, it can be said that the extract of some of the Iranian endemic medicinal plants (*S. bachtiarica* and *T. daenensis*) could be used as natural anti-Candida.

Key words: *Candida albicans*, endemic medicinal herbs, antifungal activity, *Satureja bachtiarica*, *Thymus daenensis*.

INTRODUCTION

Infectious diseases especially of fungal origin are major health hazard all over the world and in some cases they cause premature deaths, that is, almost 50,000 people per day (Mulligen et al., 1993) and they have increased markedly during the last decade (Terell, 1999; Meis and Verweji, 2001). Candidal infection represents one of the most rapidly increasing healthcare infections with a significant mortality rate in hospitalized patients (Jarvis, 1995; Wey et al., 1989). These fungal infections are becoming more prevalent worldwide because the size of the immunocompromised patient population is rising, and despite appropriate anti-fungal therapy, mortality from candidemia is over 30% (Morgan et al., 2005). *Candida*

species are now recognized as major agents of hospital acquired infection (Douglas, 2003). *Candida* species are the most common fungal pathogens of humans and are the causative agents of oral and vaginal candidiasis, giving rise to severe morbidity in millions of individuals worldwide (Calderone and Fonzi, 2001; Ruhnke, 2002). *Candida albicans* is the organism most often associated with serious fungal infection and it is showing increased resistance to traditional antifungal agents (Hawser and Douglas, 1995; Jarvis, 1995). *C. albicans* is a diploid fungus that grows both as yeast and filamentous cells, and is a causal agent of opportunistic oral and genital infections in humans (Ryan and Ray, 2004; Enfert and Hube, 2007). *C. albicans* is one of the leading causes of opportunistic fungal infections in immunocompromised individuals, including AIDS patients, transplant recipients, and cancer patients (Scherer and Magee, 1990;

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Table 1. Iranian medicinal plants used in this study.

Scientific name	Family name	Local name	Type	Parts used	Uses/ailments treated
<i>Satureja bachtiarica</i> Bung.	Lamiaceae	Marzeh-e- Koohi	Endemic	Aerial parts	Edible as vegetable, flavoring, indigestion, cough, anti-bacterial
<i>Thymus daenensis</i> Celak.	Lamiaceae	Avishan-e- Koohi	Endemic	Aerial parts	Green tea, spice, culinary, cough, anti-bacterial, carminative
<i>Dracocephalum multicaule</i> Montbr and Auch.	Lamiaceae	Zarrin giah, Zeravihi	Endemic	Aerial parts	Sedative, analgesia, inflammatory, anti-bacterial, anti-septic, foot pain
<i>Stachy slavandulifolia</i> Vahl.	Lamiaceae	Lolopashmak, Chay-e- Koohi	Native	Aerial parts	Green tea, anti-bacterial, skin diseases, menorrhagia
<i>Achillea kellalensis</i> Boiss. and Hausskn.	Asteraceae	Golberenjaz	Endemic	Flowers	Wound, carminative, indigestion
<i>Kelussia odoratissima</i> Mozaff.	Apiaceae	Kelus	Endemic	Leaves	Edible as vegetable, flavoring, indigestion, rheumatism
<i>Heracleum lasiopetalum</i> Boiss	Apiaceae	Goolpar, Keresom	Endemic	Fruit	Anti-septic, spice and condiment
<i>Echinophora platyloba</i> DC.	Apiaceae	Khosharizeh	Endemic	Aerial plant	Anti fungal, spice and culinary

Shepherd et al., 1985). However, this fungus frequently causes a range of mucosal infections such as oral thrush and vaginitis (Ruhnke, 2002). The remedial uses of commercially available antifungal drugs have induced varieties of toxic side effects (Chotomongakol and Sukeepaisarncharoen, 1997; Rukayadi et al., 2008); therefore, there is a distinct need for the discovery of new, safer, and more effective antifungal agents (Frontling and Rathway, 1987). Plant derived medicines have been part of traditional health care in most parts of the world for thousands of years, and nowadays there is increasing interest in plants as sources of agents to fight microbial diseases (Portillo et al., 2001; Natarajan et al., 2003).

Numerous Iranian folklore herbs for example: *Heracleum lasiopetalum*, *Satureja bachtiarica*,

Thymus daenensis, *Echiophora platyloba*, *Dracocephalum multicaule*, *Kelussia odoratissima*, *Achillea kellalensis* and *Stachys lavandulifolia* have been utilized as traditional medicines by the indigenous people of Chaharmahal va Bakhtiari, Southwest Iran (Ghasemi Pirbalouti, 2009). There is no information or report on the anti-Candida properties of these plants. Hence, the present first-time investigation was carried out to test the antifungal activity of ethanolic extracts against *C. albicans*, which can cause candidiasis in human beings. It is necessary to establish the scientific basis for the therapeutic actions of traditional plant medicines as these may serve as the source for the development of more effective drugs. This study aimed to determine the anti-Candida activity of the extracts of eight plant species, which are endemic

Iranian plants.

MATERIALS AND METHODS

Plant material

The seven Iranian endemic plants and one native herb were collected from mountain areas of Central Zagross, Chaharmahal va Bakhtiari district, during May to September, 2010 (Table 1). Their identity was confirmed and voucher specimens were deposited at the Research Centre of Medicinal Plants, Islamic Azad University, Shahrekord Branch, Iran.

Sample preparation

Harvested flowering aerial parts (leaves and flowers) were dried at room temperature for one week. The extracts were obtained by stirring 100 mg of ground samples with 30 ml

Table 2. Anti-Candida activity of Iranian endemic medicinal herbs.

Plants	Concentrations (µg)				
	50	25	12.5	6.25	3.12
<i>Satureja bachtiarica</i> Bung.	17.6 ^a	13.1	10.7	-	-
<i>Thymus daenensis</i> Celak.	16.3	12.4	10.5	-	-
<i>Dracocephalum multicaule</i> Montbr and Auch.	15.8	12	9.8	-	-
<i>Stachys lavandulifolia</i> Vahl.	15.6	11.9	9.6	-	-
<i>Echinophora platyloba</i> DC.	15.4	12.3	10.6	-	-
<i>Achillea kellalensis</i> Boiss. and Hausskn.	13.8	12.1	8	-	-
<i>Kelussia odoratissima</i> Mozaff.	15.6	12	8	-	-
<i>Heracleum lasiopetalum</i> Boiss.	13	10	-	-	-

a: The inhibition zone diameter (mm) for all extracts of plants.

of pure ethanol (analytical grade; Merck, Germany) for 30 min. Samples were filtered by a Whatman no 4. filter paper.

Reagents and chemicals

Methanol (HPLC grade), ethanol (analytical grade), acetonitrile (analytical grade) and water (HPLC grade) were purchased from Merck Co. (Darmstadt, Germany). The standard of thymol and carvacrol acid were purchased from ROTH (Karlsruhe, Germany).

Preparation of standard solution

Stock standard solutions were prepared by accurately weighing 22.3 mg thymol reference standard and 16.4 mg carvacrol into separate 50 ml volumetric flasks, and dissolving in acetonitrile/water (50:50, v/v). Working standard solutions (1, 2.5 and 5 ml) were prepared by dilution from the stock standard solution. The mixture was stirred carefully and refluxed in a water bath at 90°C for 1 h.

Identification of phenolic compounds using HPLC

The isolation and analysis method for thymol and carvacrol were conducted according to previously published protocols (Hajimehdipoor et al., 2010; Shekarchi et al., 2010). The obtained mixture was injected to HPLC system (Kanauer, Germany). An HP 1000 series liquid chromatography system comprising vacuum degasser, quaternary pump, autosampler, thermostatted column compartment and diode array detector was used. Column Machery-NAGEL, Nucleosin-100-5 C18, Loop 20 µl was maintained at 30°C. Solvents used for separation were water (eluent A) and acetonitrile (eluent B).

The gradient program was as follows: 70% A/30% B, 0 to 5 min; 42% A/58% B, 5 to 18 min; 70% A/ 30% B, 18 to 30 min. The calibration curves (correlation coefficient) for thymol and carvacrol were $Y = 89322x - 382440$ ($r^2 = 0.998$) and $Y = 74919x - 247838$ ($r^2 = 0.994$), respectively. Samples were filtered through a 0.45 µm membrane filter before injection.

The flow rate was kept at 1 ml min⁻¹. The injection volume was 20 µl, and peaks were monitored at 330 nm. The chromatographic peaks of thymol and carvacrol were confirmed by comparing their retention times and UV spectra with that of their reference standard. Working standard solutions were injected into the HPLC and peak area responses were obtained. Standard graphs were prepared by plotting concentration versus area. Quantification was carried out

from integrated peak areas of the samples using the corresponding standard graph.

Fungal strain

The activity of extracts was assayed against isolate of *C. albicans* (ATCC1023). The *Candida* grown overnight at 36°C in RPMI 1640 with 1-gluamin without bicarbonate sodium with MOPS (0.165 µ, pH = 7.5) plates, and inoculums for the assays was prepared by diluting scraped cell mass in solution, adjusted to McFarland scale 0.5 and confirmed by spectrophotometer reading at 600 nm. Cell suspensions were finally diluted to 10⁶ colony forming units (CFU)/ml for use in the assays (Table 1).

Antifungal test

The disc diffusion method of Iennette (1985) was used with some modification to determine the rate of growth inhibition of fungi by the examined plant extracts. Sabouraud dextrose agar (Merck, Germany) was used to prepare the culture medium and autoclaved at 121°C for 15 min. Plates (8 cm diameter) were prepared with 10 ml agar inoculated with 1 ml of each microbial suspension. The extracts were dissolved in dimethyl sulfoxide (DMSO, 20 µl) before testing for antifungal activity. Sterile paper discs (6 mm in diameter) were impregnated with 60 µl of dilutions of known extracts concentrations (3.12 to 50 µg/disc) and incubated at 37°C for 48 h. Microbial growth inhibition was determined as the diameter of the inhibition zones around the discs (mm). The growth inhibition diameter was the average of three measurements, taken at three different directions. All tests were performed in triplicate. The minimum inhibitory concentration (MIC) value was determined using serial dilution assay. The MIC was defined as the lowest concentration of the compound to inhibit the growth of 50% of microorganisms. Each tube was inoculated with 5 ml of microbial suspension at a density of 10⁶ CFU/ml and incubated at 37°C for 48 h. The growth of microorganisms was observed as turbidity determined by the measure of optical density at 600 nm (Eppendorf spectrophotometer, AG, Germany). Extract-free solution was used as a negative control. Control tubes were incubated under the same condition. All assays were carried out in triplicate.

RESULTS AND DISCUSSION

The growth inhibition value of extracts on fungal strains is

Table 3. Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of extracts against *C. albicans*.

Plants	MIC ($\mu\text{g/ml}$)	MFC ($\mu\text{g/ml}$)
<i>Satureja bachtiarica</i> Bung.	25	50
<i>Thymus daenensis</i> Celak.	25	>50
<i>Dracocephalum multicaule</i> Montbr and Auch.	50	>50
<i>Stachys lavandulifolia</i> Vahl.	>50	>50
<i>Echinophora platyloba</i> DC.	50	>50
<i>Achillea kallelensis</i> Boiss. and Hauskn.	>50	>50
<i>Kelussia odoratissima</i> Mozaff.	>50	>50
<i>Heracleum lasiopetalum</i> Boiss.	>50	>50

shown in Table 2. The extracts from the different plant species studied showed antifungal activities, with the diameters of the inhibition zone ranging from 8 to 17 mm. There were significant differences ($p \leq 0.05$) in the antifungal activities of the plant extracts. Among the plants tested, the extracts of *Satureja bachtiarica* and *Thymus daenensis* showed the best antifungal activity. These were followed by the ethanol extracts of *Dracocephalum multicaule*, *Kelussia odoratissima* and *Stachys lavandulifolia* (Table 2). The results showed that most of the extracts could effectively inhibit the growth of *C. albicans*.

Subsequent experiments were conducted to determine the minimal inhibitory concentration of all selected plant extracts. The MIC values for active extracts ranged between 25 and 50 $\mu\text{g/ml}$. Among the plants tested, *T. daenensis* and *S. bachtiarica* showed the best antifungal activities (Table 3). Also, the ethanol extract of *Echinophora platyloba* and *Dracocephalum multicaule* showed promising antifungal activities against *C. albicans* (Table 3). The results obtained appeared to confirm the antifungal potential of the plants investigated. The extracts of *T. daenensis* and *S. bachtiarica* showed the best MIC value and activity against yeast used.

In this study, the extracts from *S. bachtiarica* and *T. daenensis* exhibited inhibitory effect on fungal growth, suggesting that the studied plant extracts are potentially a safe and natural source of antifungal agents. All the extracts showed varying degrees of antifungal activity on the yeast tested. Some of these plants were more effective than traditional antimicrobial to combat the pathogenic microorganisms studied.

The result of identification of phenolic compounds using HPLC showed that the major components of *S. bachtiarica* and *T. daenensis* were carvacrol and thymol, respectively. Some studies claim that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (Hara-Kudo et al., 2004). Previous studies (Shan et al., 2005) showed that a highly positive linear relationship exists between antioxidant and antimicrobial activity and total phenolic content in some spices and herbs. Many herb and spice

extracts for example *S. bachtiarica* and *T. daenensis* contained high levels of phenolics and exhibited antimicrobial activity (Sefidkon and Jamzad, 2000; Sajjadi and Khatamsaz, 2003). Previous report (Rasooli et al., 2006) on the antimicrobial activity of the essential oils of some *Thymus* spp., most of them possessing large quantities of phenolic monoterpenes, have shown activity against viruses, bacteria, food-derived microbial strains and fungi. The essential oil and extract of some aromatic plants (for example mint family, Lamiaceae) with a higher percentage of carvacrol and thymol have a higher efficacy against microbial (Rasooli et al., 2006). The results obtained represent a worthwhile expressive contribution to the characterization of the anti-Candida activity of plant extracts of traditional medicinal plants from the Iranian flora. The extracts of *T. daenensis* and *S. bachtiarica* leaves and flowers had antifungal activities. The present study suggests that the extracts of these plants are a potential source of natural antifungal agents. Evaluations of the extracts against other important human pathogens are also being conducted. After this screening experiment, further work should be performed to describe the antifungal activities in more detail as well as their activity *in vivo*. In addition, phytochemical studies will be necessary to isolate the active constituents and evaluate the antifungal activities against a wide range of fungi population.

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