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Evaluation of the antifungal activities of various extracts from *Pistacia atlantica* Desf

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Abstract

Background and Purpose: Despite the availability of various treatments for fungal diseases, there are some limitations in the management of these conditions due to multiple treatment-related side-effects. The present study was designed to investigate the antifungal properties of different extracts from *Pistacia atlantica* Desf.

Materials and Methods: Different parts of *P. atlantica* (i.e., dried fruit, fresh fruit and dried leaf) were separately extracted via percolation method with 80% methanol and water. Gas chromatography/mass spectrometry (GC/MS) analysis was performed to determine the main constituents of leaf and fruit extracts from *P. atlantica*. *In vitro* anti-*Candida* activities of the extracts against *Candida albicans*, *Candida glabrata* and *Saccharomyces cerevisiae* were studied. For this purpose, the minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) were determined, using broth microdilution method, according to the modified M27-A3 protocol on yeasts, proposed by the Clinical and Laboratory Standards Institute (CLSI).

Results: Based on GC/MS analysis, the main constituents of *P. atlantica* fruit extracts were β-myrcene (41.4%), α-pinene (32.48%) and limonene (4.66%), respectively, whereas the major constituents of *P. atlantica* leaf extracts were trans-caryophyllene (15.18%), α-amorphene (8.1%) and neo-allo-ocimene (6.21%), respectively. As the findings indicated, all the constituents exhibited both fungistatic and fungicidal activities, with MICs ranging from 6.66 to 26.66 mg/mL and MFCs ranging from 13.3 to 37.3 mg/mL, respectively. Among the evaluated extracts, the methanolic fresh fruit extract of *P. atlantica* was significantly more effective than other extracts (P<0.05).

Conclusion: Based on the findings of the present study, novel antifungal agents need to be developed, and use of *P. atlantica* should be promoted in the traditional treatment of *Candida* infections.

Keywords: *Pistacia*, *Candida albicans*, *Candida glabrata*, *Saccharomyces cerevisiae*, *In vitro*

Introduction

Today, the incidence of fungal infections is rising, considering the increased vulnerability of immunocompromised individuals, including patients with organ transplants, cancer and HIV/AIDS [1]. Candidiasis is a fungal infection caused by yeasts belonging to the genus *Candida*. Over the past three decades, *Candida* species have emerged as an important cause of opportunistic and healthcare-associated infections [2].

Candida albicans has been introduced as the most prevalent pathogen in systemic fungal infections [3]. On the other hand, other *Candida* species account for more than 50% of fungal infections [3]. *Saccharomyces cerevisiae* is being increasingly reported as an agent of invasive infection, particularly in immune-compromised or critically ill patients. Therefore, this organism should be included in the growing list of emerging fungal pathogens [4].

The available treatments for fungal diseases are diverse and numerous. However, only a few classes of antifungal agents are currently available for the treatment of yeast infections due to their high toxicity, emergence of drug resistance, pharmacokinetic deficiencies and/or

insufficient antifungal activities [5, 6]. Therefore, there is an urgent need for the development of novel, effective treatment alternatives.

Due to limited side-effects, low cost and extensive availability, plant extracts and plant-derived compounds are valuable sources, which are commonly used for the treatment of a variety of conditions including infectious diseases [7]. The genus *Pistacia* belongs to the family *Anacardiaceae*. Among 15 known species of pistachios, only three grow in Iran, including *P. vera*, *P. khinjuk* and *P. atlantica* [8].

For the past 3000 years, *P. atlantica* Desf. has commonly grown in the Mediterranean and Middle Eastern countries. This plant, regionally known as "*Baneh*", grows in the central, Western and Eastern areas of Iran [9]. Different parts of this plant, including the resin, leaf, bark, fruit and aerial parts, have been widely used as traditional medicines for the treatment of various conditions such as gastrointestinal, respiratory, cutaneous, renal and infectious diseases. Moreover, previous studies have indicated the anti-inflammatory, antioxidant, anti-tumor, anti-asthmatic and antimicrobial properties of this plant [9].

To the best of our knowledge, no previous research has focused on the anti-*Candida* activities of different parts of *P. atlantica*. Therefore, in this study, we aimed to evaluate the chemical composition and *in vitro* antifungal activities of various leaf and fruit extracts from *P. atlantica* against *C. albicans*, *C. glabrata* and *S. cerevisiae*.

Material and Methods
Chemical substances

The crude powder of miconazole (MZ) as the control agent was purchased from Sigma-Aldrich, St Louis, MO, USA. RPMI-1640 medium, l-glutamine and Sabouraud dextrose agar (SDA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Also, potato dextrose agar (PDA) was obtained from Oxoid Ltd. (Basingstoke, Hampshire, UK). In addition, chloramphenicol and cycloheximide were purchased from Merck, Germany. All other chemicals and solvents were of an analytical grade.

Fungal strains

Standard strains of *C. albicans* (PTCC 5012), *C. glabrata* (CBS 138) and *S. cerevisiae* (PTCC 5177) were obtained at the Department of Medical Mycology, Iran University of Medical Sciences, Tehran, Iran. The strains were incubated in SDA at 30 ˚C for 2-3 days.

Plant materials

The plant materials (i.e., fruit and leaves), investigated in this study, were collected from rural regions of Lorestan province, situated in West of Iran during May-September 2013. The plant parts were identified by a botanist at the Department of Botanical Sciences at Lorestan University, Lorestan, Iran. Voucher specimens were deposited in the herbarium of Research Center for Agricultural Sciences at Lorestan University of Medical Sciences, Khorramabad, Iran (No: 2522).

Extract preparation

For extract preparation, 10 g of the powdered plant materials (i.e., dried fruit, fresh fruit and dried leaf) was separately and successively extracted through the percolation method with 80% methanol and water for 72 h at room temperature. The extracts were passed through a filter paper (Whatman No. 3, Sigma-Aldrich Co., Germany) to remove the plant debris. The extracts were finally vacuum-concentrated at 50 °C, using a rotary evaporator (Heidolph, Germany) and stored at -20 °C until use [10].

Gas chromatography/mass spectrometry (GC/MS) analysis of the extracts

The chemical components of *P. atlantica* extracts were identified via extraction, using the solid-phase microextraction (SPME) technique. Initially, the samples were ground into a fine powder, using a household mill. Two grams of the samples were weighed and transferred to a 20 ml vial. Afterwards, the vial containing the sample was transferred to an ultrasonic device for extracting the volatile substances. The temperature of the ultrasonic device was set at 50 °C for 15 min.

In the next step, the SPME fiber was placed

on the upper surface of the sample for 40 min to extract the volatile compounds. Immediately after the extraction, the SPME fiber was injected into the GC-MS device for desorbing and identifying the composition of the samples. Desorption was performed in the GC column for 2 min. The SPME fiber holder was applied for manual use, and polydimethylsiloxane (PDMS, 100 µm) fibers were obtained from Supelco Inc. (Bellefonte, PA, USA).

GC/MS analysis was performed, using the Agilent 6890N, coupled with the HP-5MS column (30m×0.25 mm, film thickness: 0.25 mm). The column temperature was maintained at 40 °C for 12 min. Afterwards, the temperature was programmed to increase to 180 °C at a rate of 10 °C per min and remained at 180 °C for 4 min. Also, the temperature of the injector and interface was set at 250 °C and 280 °C, respectively.

The flow rate of helium as the carrier gas was 1 mL/min CF. The percentages of components were calculated by electronic integration of peak areas in flame ionization detector (FID), without using response factor corrections. Linear retention indices for all the components were determined by co-injection of the samples with a solution containing homologous C8-C24 series of n-alkanes.

Identification of the extract constituents

The constituents of the extracts were identified by comparing their relative retention time and mass spectra with the Standard Wiley Library (2001) data on the GC/MS system or the data reported in the literature [11].

Extract dilutions

For the preparation of extract dilutions, 1,280 mg of the extracts was dissolved in 1 mL of normal saline. Serial dilutions were subsequently prepared to obtain 1-128 mg/mL concentrations of the extracts (Table 1). To prepare the MZ dilutions, 0.01280 g of the crude powder was dissolved in 10 mL of dimethyl sulfoxide (DMSO 1%), and the serial dilution was subsequently prepared to obtain MZ at 0.0009-64 µg/mL (Table 1).

Selection of the dilutions of the extracts and MZ was based on the initial experiments, which showed that DMSO below 1.5% could exert no effects on the growth of *Candida* species. In the present study, the concentration of DMSO in various dilutions was 1% or less [12].

In vitro antifungal activity

Anti-*Candida* effects of fruit and leaf extracts from *P. atlantica* against the tested fungi were determined via broth microdilution method, according to the modified M27-A3 protocol on yeasts by the Clinical and Laboratory Standards Institute (CLSI) [13].

Inoculum preparation for antifungal susceptibility tests

In broth microdilution method, standardized inocula ($2.5–5 \times 10^3$ CFU/mL) for *Candida* species were prepared by turbidimetry. The stock inocula were prepared on day two for culturing *Candida* species, grown on SDA at 30 °C. Sterile normal saline solution (0.9%, 3 mL) was added to the agar slant, and the cultures were gently swabbed to dislodge the blastoconidia from *Candida* species.

The blastoconidia suspensions of *Candida* species were transferred to sterile tubes, and the volume of suspensions was adjusted to 4 mL, using sterile saline solution. The resulting suspensions were allowed to settle for 5 min at 28 °C. The density of suspensions was read at 530 nm and adjusted to 95% transmittance. The suspensions were diluted to 1:2000 in RPMI-1640 medium, supplemented with l-glutamine (without sodium bicarbonate). The suspensions were buffered (pH=7.0) with 0.165 mol/L of morpholinepropanesulfonic acid to obtain an inoculum size of $2.5–5 \times 10^3$ CFU/Ml.

Table 1. Concentrations of *P. atlantica* extracts used in the present study

Tested samples	Abbreviations	Concentrations
Dried fruit methanolic extract	DFM	1-128 mg/mL
Fresh fruit methanolic extract	FFM	1-128 mg/mL
Leaf methanolic extract	LM	1-128 mg/mL
Leaf aqueous extract	LA	1-128 mg/mL
Miconazole	MZ	0.0009–64 µg/mL

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Table 2. The composition of *P. atlantica* fruit extracts identified by gas chromatography/mass spectrometry (GC/MS) analysis

No.	Compounds	Retention time	Components (%)
1.	α-pinene	6.68	32.48
2.	Sabinene	7.85	3.066
3.	β-myrcene	8.33	41.04
4.	α-terpinene	8.72	0.377
5.	Delta-3-carene	8.89	1.337
6.	Limonene	9.51	4.66
7.	Cis-ocimene	9.78	1.621
8.	Trans-ocimene	10.13	1.100
9.	γ-terpinene	10.49	0.478
10.	α-terpinolene	11.47	0.807
11.	Farnesyl acetone	11.69	0.121
12.	Linalool	12.01	1.019
13.	(E)-4,8-dimethyl-1,3,7-nonatriene	12.40	2.390
14.	Alloocimene	12.88	1.363
15.	Trans-pinocarveol	13.34	0.179
16.	Verbenol	13.56	0.582
17.	Pinocarvone	14.11	0.135
18.	L-menthol	14.54	0.133
19.	4-terpineol	14.65	0.165
20.	Piperitone	17.25	0.392
21.	Heneicosane	17.88	0.118
22.	Trans-carane	18.47	0.130
23.	Camphene	19.84	0.085
24.	α-terpinenylacetat	20.30	0.422
25.	α-ylangene	20.96	0.189
26.	Copaene	21.11	0.069
27.	Trans-caryophyllene	22.47	1.368
28.	α-caryophyllene	23.47	0.388
29.	β-santalene	24.15	0.403
30.	Eicosane	24.48	0.140
31.	Isoseychellene	24.64	0.258
32.	α-muurolene	24.75	0.097
33.	Delta-cadinene	25.37	0.228
34.	Nerolidol	26.46	0.683
35.	Verbenyl ethyl ether	26.99	0.292
36.	Mayuron	28.46	0.172
37.	11-n-decyldocosane	30.74	1.339
38.	1-Hexacosene	32.54	0.234
	Total		98.7

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The MICs were determined by broth microdilution method in accordance with the CLSI M27-A3 protocol. The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for each tested extract. The microplates were incubated at 37 ˚C and read visually after 48 h.

The assays were run in duplicate for all the extracts and repeated at least twice. MIC was defined as the lowest oil concentration, causing 100% inhibition of visible fungal growth. The results were read visually, as recommended by CLSI. MFCs were determined by subculturing 100 µL of the solution from the wells without turbidity on PDA at 28 ˚C. MFC was defined as the lowest concentration resulting in no growth on the subculture after two days.

Statistical analysis

SPSS version 17 (SPSS Inc., Chicago, USA) was used for data entry and statistical analysis. Differences between the extracts were determined, using one-way analysis of variance (ANOVA). P-value less than 0.05 was considered statistically significant.

Results
GC/MS analysis of the extracts

Tables 2 and 3 present the identified constituents of fruit and leaf extracts from *P. atlantica* and the percentages obtained by GC/MS analysis. The main constituents of fruit extracts were β-myrcene (41.4%), α-pinene (32.48%) and limonene (4.66%), respectively. In addition, the major constituents of leaf extracts from *P. atlantica* included trans-caryophyllene (15.18%), α-amorphene (8.1%) and neo-allo-ocimene (6.21%), respectively.

In vitro antifungal activities of the extracts

As presented in Table 4, various extracts of *P. atlantica* demonstrated both fungistatic and fungicidal activities, with MICs ranging from 6.66 to 26.66 mg/mL and MFCs ranging from 13.3 to 37.3 mg/mL. Among the tested extracts, the methanolic extract of fresh *P. atlantica* fruit was significantly more effective than other evaluated extracts, as it exhibited lower MICs (range: 6.66-10.66 mg/mL) and MFCs (range: 13.3-21.3 mg/mL) for all the tested fungal strains (*P*<0.05).

Table 3. The composition of *P. atlantica* leaf extracts identified by gas chromatography/mass spectrometry (GC/MS) analysis

No.	Compounds	Retention time	Components (%)
1.	α-pinene	6.67	3.345
2.	β-pinene	7.89	0.801
3.	β-myrcene	8.30	2.097
4.	Tetradecane	10.36	0.480
5.	γ-terpinene	10.48	0.715
6.	Nonanal	12.03	0.554
7.	L-camphor	13.44	0.134
8.	Menthone	13.76	1.298
9.	Borneol	14.30	0.662
10.	L-menthol	14.54	1.725
11.	Dodecane	15.19	0.369
12.	Bornyl acetate	18.18	0.725
13.	Thymol	19.04	0.180
14.	Dodecane	19.41	0.633
15.	Camphene	20.30	0.837
16.	Aromadendrene	20.98	3.137
17.	Copaene	21.12	1.818
18.	α-cadinene	21.55	0.417
19.	Tetradecane	21.77	1.113
20.	α-gurjunene	22.15	1.826
21.	trans-caryophyllene	22.48	15.18
22.	Calarene	22.70	1.290
23.	Germacrene D	22.91	1.149
24.	Neo-allo-ocimene	23.05	6.21
25.	β-selinene	23.15	1.493
26.	α-humulene	23.47	3.000
27.	Heptacosane	23.56	1.100
28.	Valencene	23.65	1.537
29.	α-amorphene	24.11	8.1
30.	Dodecane	24.48	2.451
31.	Isoledene	24.61	2.989
32.	α-muurolene	24.76	2.803
33.	β-cadinene	25.16	3.669
34.	Delta-cadinene	25.38	5.915
35.	β-cadinene	25.69	0.607
36.	α-muurolene	25.77	1.143
37.	α-calacorene	25.95	1.118
38.	Nerolidol	26.47	1.422
39.	Caryophyllene oxide	27.00	1.620
40.	1,2-dihydro-2,2,3-trimethyl-1-quinoxaline-4-dioxide	28.35	3.436
41.	Eudesmol	28.79	1.718
42.	Tritetracontane	30.68	0.901
43.	Tetracosane	32.75	1.699
	Total		85.7

In contrast, the aqueous extract of *P. atlantica* leaf exhibited the least significant antifungal effects, with the highest MIC (21.33 mg/mL) and MFC (37.3 mg/mL) values. Moreover, the MICs and MFCs for MZ as the control agent against the tested yeasts ranged from 0.0013 to 0.0026 mg/mL and 0.002 to 0.004 mg/mL, respectively. The negative controls also did not show any inhibitory effects against the tested yeast strains.

The difference in the antifungal effects between the extracts and the standard drugs was not statistically significant, whereas a significant difference was detected between the methanolic fruit extract and others ($P<0.05$). Based on the findings, among the tested fungi, *C. albicans* was most sensitive to *P. atlantica* extracts, while *C. glabrata* was the most resistant yeast.

Discussion

Natural products such as plant extracts, either as pure or standardized compounds, provide unlimited opportunities for new drug discoveries, considering the unmatched availability of chemical diversity [14]. According to World Health Organization (WHO), more than 80% of the world's population relies on traditional medicines for their primary healthcare needs.

Over the past decades, advent of synthetic antimicrobials has resulted in an increasing aversion against medicinal plants as a rich resource of antimicrobial agents [15]. However, in recent years, some limitations in the use of these agents have led to some changes and attracted interest in the field of ethnobotanical research [16].

Based on the findings of the present study, all the tested extracts indicated both fungistatic and fungicidal activities with MIC and MFC values ranging from 6.66 to 26.66 mg/mL and 13.3 to 37.3 mg/mL, respectively. Each of the evaluated extracts in the present study exhibited antifungal activities against at least one of the tested fungi. However, differences in the antifungal activities of the plant extracts indicated a broad antifungal spectrum. These variations in antifungal activity could be related to differences in the chemical composition of

Table 4. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) (mg/ml) of *P. atlantica* extracts against *C. albicans*, *C. glabrata* and *S. cerevisiae*

Tested samples	Yeast strains					
	C. albicans		*C. glabrata*		*S. cerevisiae*	
	MIC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)
Dried fruit methanolic extract	10.66	32	10.66	32	13.33	21.33
Fresh fruit methanolic extract	6.66[*]	13.3[*]	10.66[*]	32	10.66[*]	21.3
Leaf methanolic extract	21.33	32	26.66	32	21.33	32
Leaf aqueous extract	21.33	37.3	21.33	37.3	21.33	37.3
Miconazole (μg/mL)	0.0026[**]	0.004[**]	0.0013[**]	0.002[**]	0.0013[**]	0.002[**]

[*]The difference in the anti-fungal effects with other P. atlantica extracts was statistically significant (P<0.05)
[**]The difference in the anti-fungal effects with other P. atlantica extracts was statistically significant (P<0.05)
Data are expressed as mean values (n=3)

different parts of the plant as secondary metabolites, affecting the antimicrobial properties of these parts [15].

The activity of the plant extracts could be influenced by the nature of the plant material or its origin, climatic conditions in which the plant grows, the used plant parts and the extraction solvent [17]. In our study, the methanolic extract of fresh *P. atlantica* fruit significantly inhibited the growth of all tested fungi, while other extracts demonstrated weak to moderate antifungal activities.

Similarly, various studies have demonstrated the remarkable antifungal activities of various *P. atlantica* extracts against some pathogenic fungi including *Fusarium solani*, *Rhizoctonia solani* and *Geotrichum candidum* [18, 19]. The results of the present study were in agreement with previous findings, indicating the antimicrobial properties of commonly used medicinal plants, which can be used in the traditional treatment of some conditions [20].

In this study, the major constituents of *P. atlantica* fruit extracts included β-myrcene (41.4%), α-pinene (32.48%) and limonene (4.66%), respectively, while the main constituents of leaf extracts were trans-caryophyllene (15.18%), α-amorphene (8.1%) and neo-allo-ocimene (6.21%), respectively. However, as previously indicated, the chemical composition of the extracts depends on their species, climatic conditions, time of collection and growth stage, which alter their biological activities [21].

The phytochemical screening of crude extracts showed the presence of terpenoids, phenols, flavonoids, fatty acids and sterols in *P. atlantica* [9]. The individual activities of these compounds have been previously demonstrated [15]. In addition, antifungal activities of these compounds and their derivates such as α-pinene, limonene, thymol and carvacrol against some pathogenic fungal strains have been confirmed [22-25].

Ismail et al. [26] reported the correlation between antifungal activity and percentage of some compounds such as α-pinene, limonene and α-terpinene in *P. atlantica*. Therefore, phytoconstituents in this plant could be responsible for the anti-*Candida* activities, since the exact mode of action is poorly understood. Regarding the antimicrobial mechanism of some terpenoid compounds such as monoterpenes, Sikkema et al., have reported that compounds diffuse into pathogens and damage the structure of cell membranes [27].

Other studies have related antimicrobial activities to the ability of terpenes to affect not only permeability, but also other functions of cell membranes. These compounds might cross the cell membranes, penetrate into the cells and interact with critical intracellular sites [26, 28].

In conclusion, the findings of the present study revealed the antifungal effects of various *P. atlantica* extracts, particularly the fresh fruit extracts. Our results also provided scientific evidence on the use of natural plants in traditional medicine for the prevention and treatment of *Candida* infections.

Authors' Contributions

M.A. designed the study, A.S. was responsible for data collection and analysis, H.M. performed the critical revision of the manuscript, PB and SJ collected the data, A.G. performed data analysis and M.Y. wrote the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest in this study.

Financial Disclosure

No financial interests declared.

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