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Original Article

In vitro antifungal activities of *Euphorbia macroclada* and fluconazole against pathogenic *Candida* species

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Abstract

Background and Purpose: *Candida* species constitute an important group of opportunistic fungi, which cause various clinical diseases. Considering the resistance of some *Candida* species to conventional antifungal agents, treatment of such cases may be challenging and complicated. The purpose of this study was to evaluate and compare the antifungal activities of *Euphorbia macroclada* latex and fluconazole against different *Candida* species.

Materials and Methods: A total of 150 *Candida* isolates including *C. albicans* (n=77), *C. glabrata (n*=28), *C. parapsilosis* (n=23), *C. tropicalis* (n=15), *C. krusei* (n=4), *C. famata* (n=1), *C. kefyr* (n=1) and *C. inconspicua* (n=1) were included in this study. In vitro antifungal activities of *Euphorbia macroclada* latex and fluconazole against these *Candida* species were evaluated, according to M27-A2 protocol on broth macrodilution method by the Clinical and Laboratory Standards Institute (CLSI).

Results: Among 150 *Candida* isolates, 98 isolates (65.33%), i.e., *C. albicans* (n=41), *C. glabrata* (n=23), *C. tropicalis* (n=12) and *C. parapsilosis* (n=22) with minimal inhibitory concentration (MIC) ≤ 8 µg/ml were susceptible to fluconazole. Resistance to fluconazole was noted in 15 isolates, i.e., *C. albicans* (n=10), *C. glabrata* (n=2), *C. krusei* (n=1), *C. kefyr* (n=1), and *C. inconspicua* (n=1), with MICs of 64 µg/ml. The remaining isolates (n=37) including *C. albicans* (n=26), *C. glabrata* (n=3), *C. tropicalis* (n=3), *C. parapsilosis* (n=1), *C. krusei* (n=3) and *C. famata* (n=1) with MIC= 16-32 µg/ml showed dose-dependent susceptibility. The latex of *Euphorbia macroclada* was able to inhibit the growth of 30 out of 150 tested *Candida* isolates with MIC range of 128-512 µg/ml. These isolates were as follows: *C. albicans* (n=2), *C. glabrata* (n=4), *C. parapsilosis* (n=19), *C. krusei* (n=2) and *C. tropicalis* (n=3). Compared to other isolates, higher MIC values were noted for *C. albicans* and *C. glabrata* (512 µg/ml), respectively.

Conclusion: The latex of *Euphorbia macroclada* showed notable antifungal activities against some pathogenic *Candida* species. Therefore, it can be potentially used as an alternative antifungal agent in future. However, further research is required to identify its active components.

Keywords: Antifungal agents, *Candida*, *Euphorbia*, Fluconazole

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Introduction

Candida species are opportunistic pathogens, which give rise to a wide spectrum of clinical manifestations [1]. These opportunistic fungi can cause acute or chronic invasive infections in immunocompromised or debilitated individuals, leading to high morbidity and mortality rates [2]. Moreover, these fungal species are the fourth leading cause of bloodstream infections in hospitalized patients with a mortality rate of 40% [3].

Treatment of candidiasis can be quite challenging. In fact, high cost, toxic side-effects and recurrent infections caused by isolates' resistance to antifungal agents encumber the treatment process. Generally, long-term prophylactic therapy and some genetic factors lead to the emergence of resistance in *Candida* isolates [4-6].

According to World Health Organization (WHO), approximately 80% of populations in developed countries use traditional medicine [7]. Therefore, several studies have been conducted in order to determine the antifungal activities of different herbal medicines such as *Allium sativum, Azadirachta indica, Allium cepa, Zataria multiflora, Boswellia, Zingiber officinale, Allium cepa var. aggregatum, Petroselinum crispum, Cuminum cyminum, Bunium persicum* and *Euphorbia macroclada (abbreviated as E. macroclada)* [8-14]. *E.*

macroclada is a member of *Euphorbiaceae* family, which is the sixth largest family among flowering plants with more than 3000 genera and over 5000 species [15].

Euphorbia has a worldwide distribution, except in polar regions and high mountain peaks [16]. Some species of genus *Euphorbia* have been used for the treatment of various conditions such as asthma, leukemia, cancer, skin diseases and intestinal parasitic infections. A number of these species also possess antiviral, antibacterial, antifungal and cytotoxic properties [17-19]. The stems and leaves of *E. macroclada* contain some quantities of polyphenols, flavonoids, tannins, alkaloids, saponins and terpenoid compounds [7, 20].

Seventy species of genus *Euphorbia* can be found in Iran, although only 17 species are endemic [21]. Since no previous study has investigated the latex antifungal activities of this plant in Iran, the present study was designed to evaluate the antifungal activities of the latex of *E. macroclada* (LEM) and fluconazole, as a conventional antimycotic agent, against 150 pathogenic *Candida* isolates.

Material and Methods

Fungal isolates

A total of 150 *Candida* isolates including *C. albicans (*n=77), *C. glabrata (*n=28), *C. parapsilosis (*n=23), *C. tropicalis (*n=15), *C. krusei (*n=4), *C. famata (*n=1), *C. kefyr (*n=1) and *C. inconspicua (*n=1) were evaluated in this study. All samples were oropharyngeal isolates from high-risk patients, which were previously identified, based on colony color on CHROMagar Candida medium (CHROMagar, Paris, France), germ tube test, chlamydospore formation on corn meal agar medium (Merck, Germany) and carbohydrate assimilation pattern, detected by API 20C auxanographic kit (API Laboratory Products Limited, France). *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as quality-control strains.

Collection of latex from E. macroclada

The latex was collected early in the morning from the stems of *E. macroclada* and maintained in cold ice until being freeze-dried. The plants were grown in Malayer city, located in southeast of Hamadan province, Iran. The taxonomic identity of plants (specimen No. 97511) was determined at National Botanical Garden of Iran [20].

After cleaning the plant stems with 70% ethanol and making incisions on plant stems, the exuded LEM was collected in sterile tubes. A loopful of latex from each tube was inoculated on Sabouraud dextrose agar (Merck, Germany) and RPMI-1640 medium (Sigma-Aldrich, USA) and incubated at 35ºC for 48 hours to ensure the absence of microbial contaminants in the samples. Sterile latex samples were lyophilized, using a freeze dryer, which enabled the latex to be reconstituted to a given dry weight (per milliliter) in an appropriate solvent or medium and be preserved.

Antifungal susceptibility testing

M27-A2 protocol on broth macrodilution method by the Clinical Laboratory Standards Institute (CLSI) was applied to evaluate the susceptibility of *Candida* isolates to LEM and fluconazole [22]. Briefly, the isolates were tested against fluconazole and LEM in RPMI-1640 medium (Sigma-Aldrich, USA), buffered with 0.075M 3- (N-morpholino) propanesulfonic acid (MOPS) (Sigma-Aldrich, USA); pH was adjusted to 7.0.

Fluconazole stock solution (100x, Sigma-Aldrich, USA) was prepared in sterile distilled water, following the CLSI M27-A27 guidelines. Afterwards, serial dilutions (2x) of fluconazole were performed in RPMI medium, and 100 µl of the dilutions was dispensed on multiwell macrodilution plates (48 U-shaped wells, Nunc, Denmark). The plates were frozen at -70ºC before being used.

In order to find the best solvent for LEM, distilled water, ethanol, methanol and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) were tested; finally, DMSO was found to be the only LEM solvent. LEM stock solution with a concentration of 40960 µg/ml was prepared by dissolving sterile LEM powder in DMSO via shaking (30 min). Then, two-fold serial dilutions of LEM were prepared in RPMI, and 100 µl of the solution was dispensed on macrodilution plates. The plates were frozen at -70ºC before being used.

For each isolate, the inoculum was prepared by picking three colonies from an overnight culture on Sabouraud Dextrose Agar (Merck, Germany) at 35ºC and suspended in sterile normal saline. The fungal inocula were adjusted to yield a 2x inoculum ($1\text{-}5\times10^6$ CFU/ml). Then, a working suspension was prepared with a 1:100 dilution, followed by a 1:20 dilution of stock suspension with RPMI-1640 broth medium, which resulted in concentrations of $0.5x10^2$-$2.5x10^3$ CFU/ml.

Finally, 900 µl of the final product was dispensed in each well of the macroplate for testing; this process produced an appropriate concentration of the media, drugs and yeasts in each well. The plates were incubated at 35 ºC. Two drug-free control wells were included in each macroplate. One medium contained fungal suspension as positive control and the other medium contained DMSO as the control medium. All tests were performed twice and the end-points were determined visually using a reading mirror in comparison to drug free controls after 24-48 hours of incubation.

The MICs of fluconazole and LEM were determined as the lowest concentrations, which inhibited 80% of fungal growth, compared to the control. According to CLSI interpretive breakpoints for *Candida* species and fluconazole, MIC ≤ 8 µg/ml, MIC=16-32 µg/ml and MIC ≥ 64 µg/ml were considered to show susceptibility, dose-dependent susceptibility and resistance, respec-tively. However, no interpretive breakpoints were established for LEM.

Results

The results revealed that 63.33% of all species with MIC≤ 8 µg/ml were susceptible to fluconazole. Resistance to fluconazole was reported in 24.66% of species with MIC≥ 64 µg/ml. Also, 10% of the samples showed dose-dependent susceptibility to fluconazole with MIC= 16-32 µg/ml.

C. parapsilosis showed the greatest susceptibility to fluconazole (22 out of 23 isolates), followed by *C. glabrata* (23 out of 28 isolates) and *C. tropicalis* (12 out of 15 isolates) (Table 1). No interpretive breakpoints were established for LEM, and MICs were mostly in the range of 128-≥512 µg/ml, with higher MICs noted for *C. albicans and C. glabrata* (512 µg/ml), respectively (Table 1).

Discussion

Plant-based antifungals have remarkable therapeutic potentials as they have fewer side-effects, which are often associated with synthetic therapeutic agents. The first step towards using these potentials is testing the in vitro antifungal activities. Considering the global scarcity of studies on the antifungal effects of LEM, we aimed to evaluate the antifungal activity of the latex of endemic

Table 1. MIC and geometric mean values of *E. macroclada* and fluconazole against clinical *Candida* species

Candida species (n)	MIC (µg/ml)								Susceptibility to fluconazole		
	LEM				Fluconazole				S n	S-DD n	R n
	MIC 50	MIC 90	MIC range	GM	MIC 50	MIC 90	MIC range	GM			
C. albicans (77)	512	512	512	512	8	64	2-64	11.89	41	26	10
C. glabrata (28)	256	512	128-512	256	4	32	2-64	6.09	23	3	2
C. parapsilosis (23)	256	512	128-512	342.76	4	8	2-16	3.65	22	1	0
C. tropicalis (15)	256	256	128-256	203.19	4	8	2-32	5.33	12	3	0
C. krusei (4)	128	256	128-256	121.02	16	64	16-64	26.91	0	3	1
C. famata (1)	*-	-	-	-	-	-	-	-	0	1	0
C. kefyr (1)	-	-	-	-	-	-	-	-	0	0	1
C. inconspicua (1)	-	-	-	-	-	-	-	-	0	0	1

*LEM: Latex of E. macroclada, S: Susceptibility, SDD: Dose-dependent susceptibility, R: Resistance, MIC: Minimum inhibitory concentration, MIC50: Minimum concentration inhibiting 50% of isolates, MIC90: Minimum concentration inhibiting 90% of isolates, GM: Geometric mean. *The ranges of MIC50, MIC90, MIC and GM were not calculated, as the number of isolates was insufficient*.

Iranian *E. macroclada* and fluconazole against 150 clinical *Candida* isolates in hospitalized patients.

Darwish et al. [23] by applying microdilution method showed that the antifungal activities of fresh LEM against *C. albicans* ATCC10231, *C. glabrata* ATCC1615 and *C. krusei* ATCC6958 were more significant than its activities against clinical isolates with MICs of 12.5 and 25 mg/ml, respectively. Our results indicated the inhibitory effect of LEM on *C. albicans* (n=2, 2.59%), *C. glabrata* (n=4, 14.28%), *C. parapsilosis* (n=19, 82.59%), *C. tropicalis* (n=3, 19.99%) and *C. krusei* (n=2, 50%) isolates with MIC values of 128-512 µg/ml. In consistence with the results reported by Darwish et al. [23], the clinical *Candida* isolates showed high MICs, as well.

Al-Mughrabi et al. [14] showed the inhibitory properties of leaves, flowers and stems of *E. macroclada* with other solvents against phytopathogenic filamentous fungal species. They found that the butanolic extract from the stems had stronger antifungal activities. Moreover, Kirbag et al. [24] performed a disc diffusion study on the antifungal activities of the latex of eight *Euphorbia* species (i.e., *E. szovitsi, E. aleppica, E. falcata, E. denticulata, E. macroclada, E. cheiradenia, E. virgata* and *E. petiolata*) against *C. albicans, C. tropicalis,* and *C. glabrata.*

As Kirbag and colleagues indicated, LEM showed antifungal activities against *C. albicans* with an inhibition zone of 21 mm and against *C. glabrata* and *C. tropicalis* with an inhibition zone of 15 mm. However, they could not determine MIC in the latex due to the insufficient amount of latex.

Goyal et al. [25] studied the inhibitory properties of *E. caducifolia* latex on *Aspergillus niger* and *C. albicans.* MICs against *Aspergillus niger and C. albicans* were found to be 237 and 225 µg/ml, respectively. In another study, Sumathi et al. [26] reported the strong inhibitory activities of *E. antiquorum* latex against *C. albicans*, *A. flavus* and *A. fumigatus*. However, no such activities were reported against *Rhizopus stolonifer* or *Mucor indicus*, according to agar plug assay and disk diffusion method. According to the results, the inhibition zones were 10, 5-6, and 12 mm for *C. albicans*, *A. flavus* and *A. fumigatus,* respectively.

In a similar study, Ganpati et al. [20] showed the maximum activity of 100 µl of fresh latex in comparison with diluted latex (1:10, 1:50 and 1:100 dilutions), dried latex (10 mg/ml) and *Euphorbia thymifolia* extracts in organic solvents against *A. niger* ATCC 16404, *C. albicans* ATCC 10231 and *Penicillium chrysogenum,* using the cup-plate method. The inhibition zones of latex in this study were 25, 12 and 17 mm for fresh, dried and 1:10 dilution, respectively. However, 1:50 and 1:100 dilutions did not show such activities.

In a recent study by Hussain et al. [27], the fresh latex of *E. thymifolia* showed poor inhibitory response against *C. albicans* and *A. niger* with MICs of 600 and 1200 µg/ml, respectively; disc diffusion method was applied in the mentioned study. In the present study, we tested 1-512 µg/ml dilutions of freeze-dried LEM by CLSI macrodilution method against clinical *Candida* isolates and found LEM antifungal activity at an MIC range of 128-512 µg/ml. Differences in the activities of LEM and latex of other *Euphorbia* species in our study suggest that the isolation and identification of pure antifungal fractions from latex need to be performed with regard to their use in the development of new phytotherapeutic agents.

According to literature review, in vitro evaluation of the antifungal effectiveness of latex from *Euphorbia* species has been performed majorly by disc diffusion or agar plug method [14, 23, 26, 27], whereas CLSI dilution methods have been rarely applied [22, 24]. Dissimilar to Goyal et al. and Darwish et al., we used CLSI macrodilution method in the present study.

The results of the above mentioned studies indicated incomparable differences in the antifungal activities of the latex of various species. However, variations in the antifungal effectiveness of different extracts or compounds against different fungi were most likely due to differences in the nature of inhibitory materials they contained. These characteristics may arise from the genetic structure of plant species and their physical, bioactive/biochemical constituents, chemical

differences of plant extracts, solvents and tested fungi, and variations in susceptibility testing methods.

Conclusion

The results of the present study were indicative of the antifungal activity of LEM against some pathogenic *Candida* species, particularly *C. parapsilosis*. Further studies are required to determine the effective components of LEM as a natural antifungal agent.

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Authors' contributions

F.Z. designed, reviewed and edited the final version of the manuscript. P.K. and M.S were scientific and practical advisors of the study, respectively. M.R performed LEM specimen collection and antifungal susceptibility tests and S.M. wrote the manuscript.

Conflicts of interest

None.

Financial disclosure

The authors declare no financial interests related to the materials of the study.

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