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Antifungal Effects of *Silybum marianum* Extract Individually and in Combination with Fluconazole on Clinical *Candida* Isolates in Northern Iran

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Abstract

Background: Candidiasis is a spectrum of opportunistic fungal diseases. The resistance of *Candida* to antibiotics is unfortunately increasing. *Silybum marianum*, which belongs to the Asteraceae family, is a wild plant growing in most parts of Iran.
Objectives: The present study was conducted to investigate the effect of *Silybum marianum* extract, both individually and in combination with fluconazole, on the growth of drug-resistant clinical *Candida* isolates.
Methods: *Candida* species isolated from 85 patients suspected of Candidisis was identified and cultured on CHROMagar *Candida* and API 20CAUX system. The test of susceptibility to fluconazole was performed using broth microdilution. The minimum inhibitory concentration (MIC) of *Silybum marianum* extract and its antagonistic effects were determined using the microdilution assay.
Results: The highest resistance to fluconazole was reported in *Candida glabrata* (81.8%) and *Candida albicans* (72.9%). Variations in the MIC of the aqueous extract of *Silybum marianum* in a range of 4096 - 8 μL/mL showed that 77.8% of *C. glabrata* isolates and 88.6% of *C. albicans* isolates were resistant to fluconazole, and did not grow at a concentration of 2048 μL/mL; nevertheless, in the case of *Silybum marianum* extracts in combination with fluconazole, 89% of *C. glabreta* and 94.3% of *C. albicans* isolates were resistant to fluconazole, and stopped growing at concentrations of at least 128 μL/mL ($P < 0.01$).
Conclusions: The aqueous extract of *Silybum marianum* seeds found to present proper inhibitory effects on clinical fluconazole-resistant *Candida* isolates at high concentrations. *Silybum marianum* extract in combination with fluconazole was found to have a more potent in-vitro activity than the extract and drug individually.

Keywords: *Candida*, Fluconazole, *Silybum marianum* Extract

1. Background

The growing emergence of candidiasis and its epidemiological changes and drug resistance clearly justify the need for conducting studies on fungal infections. Among all the *Candida* species, *C. albicans* is considered the most frequent species that forms part of the natural flora of mucus and the skin. These yeasts are a major cause of mortality especially in immunocompromised patients (1).

An antifungal agent for treating candidiasis belongs to the group of azoles. Despite having a low toxicity, successful treatments with azoles such as fluconazole are rare due to the extensive resistance of different species of *Candida* (2). In recent years, the clinical isolates of fluconazole-resistant *Candida* have emerged in Iran. Using different antimicrobial compounds, including medicinal herbs, is therefore recommended for preventing or controlling this resistance. A long history of applying herbal medicine for treating diseases can be traced in Iran. *Silybum marianum*, commonly known as milk thistle, is an ancient medicinal plant that widely grows in Iran (3, 4) and has long been used to treat different diseases, including liver and gallbladder disorders. The active constituents of *Silybum marianum* are obtained from its dried seeds containing approximately 70% - 80% silymarin (5, 6), which is a mixture of flavonolignan isomers, including silybin, isosilybin, dehydrosilybin, silychristin, silydianin and a few flavonoids mainly taxifolin (7). Silymarin presents a wide range of biological and pharmacological activities, including antioxidative and anti-inflammatory metastatic effects (8).

2. Objectives

The present study was conducted to determine the effect of *Silybum marianum*, individually and in combination

with fluconazole, on clinical *Candida* species isolated from patients in- vitro using a community approach to traditional medicine and treatment with natural substances.

3. Methods

3.1. Phenotypic Identification of Candida Species

The present cross-sectional study was conducted on 85 clinical samples randomly collected from the skin and mucosa of patients, aged 15 - 60 years, suspected of candidiasis and presenting to four hospitals in Iran's northern city of Gorgan. A total of 55% of the study population were female.

To identify the isolated microorganisms, swab samples were cultured on Sabouraud dextrose agar (SDA, Merck, Germany) and incubated for 48 hours at 35°C. After performing direct microscopic checks using 10% KOH and subculturing on SDA, other tests were conducted, including germ tube test, culturing on Chromogenic CHROMagar (Hi Media, India) and the carbohydrate assimilation test using API 20CAUX kits (BioMe'rieux, France).

3.2. Antifungal Susceptibility Test

The drug susceptibility test was performed using the broth microdilution test according to the CLSI-M27-A3 guideline (9). After preparing the fluconazole solution (Gibco, Germany) in water and diluting it at a range of 1024 - 2 μg/mL, dilutions were poured into the 96-well ELISA microplates containing RPMI-1640 (with glutamines, without bicarbonate and with a PH indicator) and MOPS buffer (Sigma, USA). Yeast suspensions (1×10^3 cfu/mL) were then inoculated in microplate wells and incubated for 48 hours at 35°C. The ELISA Reader was used to confirm the results when reporting MIC. To determine the minimum fungicidal concentration (MFC), 100 μL of the wells' content with a concentration higher than the MIC together with 100 μL of the positive control were separately inoculated on SDA and incubated for 48 hours at 35°C.

According to the instructions of this committee, *Candida* strains with MIC $\leq$ 8 μg/mL were considered susceptible, ones with MIC = 16 - 32 μg/mL were considered susceptible-dose dependent, and ones with MIC $\geq$ 64 μg/mL were considered resistant to fluconazole. The present study used *Candida albicans* ATCC90028 as the control strain.

3.3. Preparing Silybum marianum Extract

To obtain the plant extract, *Silybum marianum* was collected from Gorgan's plains. After extracting the seeds from the *Silybum marianum* flower, they were was mixed and soaked in 70% ethanol at a ratio of 1:10 (each 100 cc of the extract was soaked in 1000 cc of ethanol), and placed in a shaker. After 24 hours, the mixture was filtered and placed in a rotary container to evaporate the solvent (ethanol). The aqueous extract was obtained through maceration, and the filtrate solution was therefore heated for 15 minutes up to boiling. The mixture was then stored in a container at room temperature to be cooled. Afterwards, the mixture was passed through Whatman grade 1 filter paper. The solution was placed in a closed glass at 4°C during usage.

3.4. Evaluating the Inhibitory Ability of Silybum marianum Extract

Broth microdilution was performed with concentrations of 4096 - 8 μL/mL to investigate the anti-candidiasis effects of *Silybum marianum* extract. A microbial suspension with a concentration of 10^3 cfu/mL was therefore prepared first and the plant stock was made by dissolving *Silybum marianum* extract in dimethyl sulfoxide (DMSO). Afterwards, 100 μL of the stock was inoculated in the microplate wells containing 100 μL of RPMI medium, and was serially diluted. One hundred μL of the yeast suspension was also added to each well. The minimum concentration inhibiting fungal growth up to 90% compared to positive controls is considered MIC_{90}. The negative control well contained the stock with RPMI, and the positive control well contained RPMI with yeast suspensions. MFC was ultimately determined.

3.5. Susceptibility Test of Fluconazole in Combination with Silybum marianum Extract

To make the stock solution and reach a concentration of 1024 μg/mL, 1 mg of fluconazole powder was dissolved in water and 1 mL of *Silybum marianum* extract in DMSo. To prepare serial dilutions, 25 μL of the drug and 25 μL of the extract were added to the first well of the 96-well microplate containing 50 μL of RPMI medium. After adding 50 μL of yeast suspensions at a concentration of 10^3 cfu/mL and performing a 48-h incubation at 35°C, MIC and MFC were evaluated and interpreted.

4. Results

Direct microscopic tests revealed 73 cases of yeast cells in 85 study patients, 90.4% of which were identified as *Candida* species through phenotypic tests. Most of the isolated species of *Candida* strains were *C. albicans* (72.7%) (Table 1). The present study found the highest resistance to fluconazole to be associated with *C. glabrata* (81.8%) and *C. albicans* (72.9%) (Table 2).

The mean MIC of fluconazole against *C. albicans* and *C. glabrata* was also determined at 210 μg/ mL, and most growth fluctuations were observed at concentrations of 128 and 64.

Table 1. Frequency of the *Candida* Species Isolated from Hospitalized Patients

Candida spp.	Absolute Frequency	Relative Frequency
C. albicans	48	72.7
C. glabrata	11	16.7
C. tropicalis	3	4.5
C. parapsilosis	4	6.1
Total	66	100

Table 2. Frequency Distribution of Drug- Resistance Among *Candida* Isolates[a]

Species	Resistant	S-DD	Suseptibility
C. albicans	35 (72.9)	9 (18.8)	4 (8.3)
C. glabrata	9 (81.8)	0 (0)	2 (18.2)
C. tropicalis	1 (33.3)	0 (0)	2 (66.7)
C. parapsilosis	0 (0)	0 (0)	4 (100)

Abbreviations: R, resistant; S, sensitive; SDD, susceptible- dose dependent.
[a] Values are expressed as No. (%).

The changes in the MIC of *Silybum marianum* against *C. albicans* and *C. glabrata* indicated significant differences between the growth and non-growth of fluconazole-resistant *Candida* (Table 3). The MFC value of *Silybum marianum* extract determined at a concentration of 4096 μL/mL for 86% of fluconazole-resistant *Candida* isolates suggested fungicidal properties in this extract at high concentrations.

The mean MIC of *Silybum marianum* extract in combination with fluconazole against *C. albicans* and *C. glabrata* was reported at 60 μg/mL, and the most growth fluctuations were observed at concentrations of 32 and 64. Significant differences were also observed between growth and non-growth of the drug-resistant isolates (Table 4). The concentration of *Silybum marianum* extract in combination with fluconazole that inhibited 90% of *C. glabrata* isolates (MIC_{90}) was found to be 128 μg/mL, four times lower than that of fluconazole (MIC_{90} = 512 μg/mL) ($P < 0.01$). This rate was also fourfold for *C. albicans* isolates. The concentration of *Silybum marianum* extract in combination with fluconazole that inhibited 90% of *Candida* isolates was 16 times lower than that of the extract individually. Comparing the in-vitro antifungal susceptibility of *C. albicans* and *C. glabrata* revealed differences in MIC_{50} and MIC_{90} between the isolates.

The MFC of *Silybum marianum* extract in combination with fluconazole against fluconazole-resistant *C. glabrata* was found to be four times lower and against *C. albicans* isolates to be 16 times lower than the MFC of the single extract.

5. Discussion

Research suggests a significant increase in the prevalence of opportunistic infections caused by *Candida* species. Although *C. albicans* is the predominant yeast, Candidiasis has been reported to be caused by other *Candida* species, including *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. paraposilosis*, *C. guilliermondii*, *C. famata* and *C. lusitaniae* (10, 11). Prolonged or frequent exposure to antifungal drugs can be associated with the risk of antifungal resistance among *Candida* strains (12).

The present study found 73% of *C. albicans* isolates to be resistant to fluconazole. A study conducted by Diba et al. using the disk-diffusion method found *C. albicans* species to present low sensitivity to fluconazole (13). Matar et al. showed that 4% of the isolates were resistant to fluconazole. A study conducted in South Africa reported 100% susceptibility to fluconazole among *C. albicans* isolates (14). Badiee et al. assessed the susceptibility of 178 *C. albicans* isolates to fluconazole using broth microdilution and the frequency of isolated fluconazole-resistant *C. albicans* was estimated at 4.6% (12). Kabli found 26.1% of 107 *C. albicans* isolates to be resistant to fluconazole using the disk-diffusion method (15). Bagg et al. reported a resistance to fluconazole of about 3% in 93 *C. albicans* isolated from patients with advanced cancer (16).

These figures are less than those found in the present study. Different levels of antibiotic resistance reported in different regions of Iran and across the word can be associated to genetic changes in the causative strains, differences in the amount of antibiotics used and changes in the availability of new antibiotics in different regions.

The present study found high resistance to fluconazole in *C. albicans*. Future research is therefore strongly recommended to focus on drug resistance in *Candida* species, especially to azole agents.

A review of literature shows that the MIC of fluconazole on *Candida* species such as *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsylosis* are 0.06 - 128, 0.03 - 8 and 0.06 - 16 μg/mL (17), which are comparable with the present findings. The present study found *C. parapsylosis* to show the lowest resistance (0%) to fluconazole at a concentration of 16 μg/mL, as 99% of them were eliminated. Fluconazole has been shown to exert its maximum antifungal effect on C. parapsylosis, and its MIC to be about 0.5 - 16 μg/mL. Moreover, a study conducted in China reported reductions in azole susceptibility among *C. tropicalis* isolates (18).

Scientists therefore seek modern therapeutic approaches and alternative compounds with antimicrobial properties. Given the diversity of plant species in Iran and the stress placed by the country's top-level authorities on food and medicine, including the vice president, herbal compounds have been particularly emphasized in Iran in recent years.

Table 3. Comparison of Growth and No-Growth of Fluconazole-Resistant *Candida albicans* and *Candida glabrata* in Concentration of 2048 μL/mL of *Silybum marianum* Extract[a]

Extract/Density, μL/mL	Strains 1 $\times$ 10^3 cfu/mL	No Growth	Growth	Comparison
2048	*C. albicans*	31 (88.6)	4 (11.4)	$x^2 = 0$, Significant
2048	*C. glabrata*	7 (77.8)	2 (22.2)	$x^2 = 0$, Significant

[a] Values are expressed as No. (%).

Table 4. Comparison of Growth or No-growth of *Candida albicans* and *Candida glabrata* Strains in the Presence of the Combination of *Silybum marianum* Extract and Fluconazole[a]

Extract + Fluconazole, μL/mL	Strains 1 $\times$ 10^3 cfu/mL	No Growth	Growth	Comparison
128	*C. albicans*	33 (94.3)	2 (5.7)	$x^2 = 0$, Significant
128	*C. glabrata*	8 (89)	1 (11)	$x^2 = 0$, Significant

[a] Values are expressed as No. (%).

Research confirms the inhibitory effects of *marjoram* essential oil on *Aspergillus*, *Honsonella* and Dermatophyte fungi, including *Trichophyton rubrum* and *C. albicans* (19). The present research also confirmed the inhibitory effect of *Silybum marianum* extract on *Candida* species at high concentrations.

Anti-*Candida* activities caused by high levels of thymol and carvacrol have been associated to marjoram essential oil (20). Karaman et al. observed the inhibitory effect of the essential oil obtained from the aerial parts of Thyme on the growth of *C. albicans*, *C. tropicalis* and *Saccharomyces cerevisiae* (21). The antifungal effect of *Silybum marianum* on Dermatophytes has been reported to be higher compared to other plants such as *Allium*, *Juglans regia*, *Sativum*, *Piper betle*, *Azadirachta* (22, 23). The present study therefore sought to inspect the antifungal effect of this plant on fluconazole-resistant *Candida* species. In certain types of fungi, the combination of two drugs may weaken the effects of individual drugs (24). Researchers have therefore focused on the effects of antibiotics combined with other antimicrobial compounds.

The antifungal activity of fluconazole combined with lovastatin and its effect on gene expression in the pathway of ergosterol biosynthesis in *C. albicans* have been evaluated. Studies reporting an MIC of growth suggest that lovastatin acts synergistically with fluconazole in-vitro (25). The present findings suggest that *Silybum marianum* extract combined with fluconazole significantly affects fluconazole-resistant *Candida*. After combining fluconazole with the extract, the levels of MIC_{50}, MIC_{90} and MFC decreased in yeasts.

The present study found silybin or silidianin to be a potential inhibitor of *Candida* species. The observed antifungal effects can therefore be associated with one of the compounds cited.

5.1. Conclusions

The results of the present study showed that a combination of fluconazole and aqueous extracts of *Silybum marianum* exerts greater antifungal effects on the resistant strains of *C. albicans* and *C. glabrata* compared to fluconazole or the plant extract individually.

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Footnotes

Authors' Contribution: Leila Fozouni contributed to study concept, and designed, supervised, and edited the final manuscript. Mahdis Palang performed sample collection and laboratory examinations and interpreted the data. All authors discussed the results and implications and provided their comments during all stages.

Conflict of Interests: The authors declare no conflict of interest and they haven't it.

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