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Original Article

Effects of *Zataria Multiflora, Mentha Longifolia,* and *Origanum Vulgare* Plant Essential Oils on the Inhibition of *Candida Albicans*

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Abstract

Background and Aim: In recent years, yeast, particularly Candida species, has been the most common fungus separated from human infections. In this research, effects of essential oils of some plants on the clinical strains of *Candida albicans* were examined.

Materials and Methods: Forty clinical strains of Candida were obtained from the Infectious Diseases Research Center of Arak University of Medical Sciences. After preparing the essential oils of *Zataria multiflora, Mentha longifolia,* and *Origanum vulgare*, the minimum inhibitory concentration (MIC) of the essential oils against *Candida albicans* strains was determined. the Morphological changes at different times were investigated using the negative staining method and a transmission electron microscope (TEM).

Results: The results of the disc diffusion test indicated that the highest resistance rates in 0.625 mg/ml of *O. vulgare*, *M. longifolia*, and *Z. multiflora* essential oils were 31(77.5%), 15(37.5%), and 13(32.5%), respectively. The lowest MIC was related to *Z. Multiflora* essential oil (0.625 mg/ml). After treatment with the essential oils, the yeasts immediately decreased at zero hour. This decrease became more noticeable with the passage of time and reached the minimum number after 24 hours. Moreover, electron microscope images showed changes in the morphology of the yeasts.

Conclusion: *Zataria multiflora, Mentha longifolia,* and *Origanum vulgare* essential oils, particularly *Zataria multiflora,* had antimicrobial effects against *Candida albicans* that were isolated to be studied. Thus, *Zataria multiflora* essential oil can be used as an anti-candida agent in the preparation of antifungal compounds.

Keywords: *Candida albicans,* Anti-fungal, Essential oil, Minimum inhibitory concentration (MIC)

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Introduction

Candida species is the major cause of fungal infections in humans and animals. The genus Candida includes a heterogeneous group of organisms that grow as yeast, and most members of this genus produce pseudo

hyphae during their growth, but *Candida albicans* and *Candida daublinensis* form true hyphae and walls and thick-walled cells. They produce chlamydospores (1, 2). The spectrum of these infections varies from mucosal colonization to invasive and fatal infections. Among distinct clinical types of Candida infections, cutaneous and mucosal candidiasis are more prevalent (3). Clinical forms of candidiasis include cutaneous-mucosal candidiasis and systemic candidiasis, which cause oral, skin, genital, and vaginal candidiasis (2).

Today, various drugs such as azole drugs (clotrimazole, ketoconazole, fluconazole, etc.) and polyene drugs (nystatin, echinocandin, and caspofungin) are used in the treatment of these infections (4). However, due to the growing resistance of fungi to chemical compounds, the process of improving these infections has faced difficulties (5). Moreover, herbal treatments for diseases, particularly infectious diseases, has seen an increasing trend in recent years. Hence, researchers have a great tendency to investigate herbal compounds for the treatment of infections because they have lower side effects compared with chemical drugs. Approximately 500/000 plant species have been identified in the world of which less than a thousand species have been named as medicinal plants. In comparison with the progress achieved in the production and supply of medicinal substances, the trend and development of antifungal drugs are slow. Thus, the variety of anti-fungal drugs is much less, and more limited than antibacterial drugs (6, 7).

Zataria multiflora is a medicinal plant from the mint family. Thymol, carvacrol, para-cymene, and transcaryophyllin are the most important components in *Zataria multiflora* essential oil. This plant has a limited geographical distribution and grows in Iran and Afghanistan. Its distribution in Iran is in central, southern, and southeastern regions (8,9). *Mentha longifolia* belongs to the Lamiaceae family. As a herbaceous and perennial plant, *Mentha longifolia* is an important and common aromatic plant in different parts of Iran (10,11). *Origanum vulgare* is one of the plants of the mint family. It is considered one of the most significant medicinal and spice plants in the world. Its aerial parts are used as one of the most popular spices and flavorings in food, perfume, and cosmetic industries (12, 13). The aim of the present study was to investigate the effects of *Z. multiflora*, *M. longifolia*, and *O. vulgare* essential oils on the inhibition of *C. albicans* clinical strains.

Materials and Methods

Preparation of the Samples

In our study, 40 strains of clinical *C. albicans* from clinical purified samples were taken from the Infectious Diseases Research Center of Arak University of Medical Sciences. These strains that were isolated from patients who suffered from vaginitis were detected and evaluated.

The production of tube masses and chlamydoconidia on cornmeal agar medium (High Media, India) was examined to detect *C. albicans*.

After preparing the plants, essential oil extraction was done with a Clevenger device (Model 85000-10 from Sina Glass) (14) .In this way, the powdered plant sample was placed completely under water in the distiller. The heat from boiling water caused the essential oil in the plant to turn into vapor. These vapors were emitted to the upper part of the tank and entered the refrigerant through the pipe, which cooled the vapors and accelerated their condensation to create liquid forms. The water saturated with essential oil accumulated in the pipes, rose to the top, and was separated. The essential oil was collected in colored glasses and placed in the refrigerator until use.

Determining the Minimum Inhibitory Concentration (MIC) of the Essential Oils

In order to determine the minimum inhibitory concentration (MIC) by the micro dilution method in accordance with NCCL M27 A standard method, a the 96-well plate was first prepared from the 24-hour cultured yeast on Saburo dextrose agar medium (HiMedia, India) containing chloramphenicol (15). After preparing the McFarland half of the yeast in Sabouraud Dextrose Broth (SDB), 100 μL of the prepared suspension was added to the wells of the 96-well plate, and then 100 μL of the extract was added to the first well and 100 μL was added to the next well. In the same way, dilution was done up to the 10^{th} well, and finally, 100 μL of the solution was discarded from the 10^{th} well to create different concentrations of the extract in the first to 10^{th} wells. Yeast suspension was added to the 11^{th} well along with the culture medium as a positive control. After 18-24 hours of incubation at 35°C, the

amount of turbidity created was read using an ELISA reader (Molecular Devices Emax, CA, USA) at a wavelength of 550 nm. Finally, the MIC was measured for the studied essential oils. In this study, all the experiments were repeated three times.

Investigation of the Antifungal Activity of the Essential Oils Using Disc Diffusion Methods

In the Kirby-Bauer disc diffusion method, *C. albicans* with a turbidity equal to half McFarland ($1x10^6$ CFU/m*L*) was cultured using sterile swabs on Potato Dextrose Agar (PDA) (Difco, USA) (16). Then, it was inoculated and 20 *µL* of each tested essential oil was poured on the blank discs, and the plates were incubated for 24 to 48 hours at 35 °C. After the desired time, the formation of the non-growth zone around the discs was investigated and the diameter of the yeast non-growth halo was measured with a ruler and reported in millimeters.

Morphological Changes of *C. Albicans* Strains after the Treatment

Alterations in the morphology and number of yeasts exposed to essential oils were examined in order to investigate the effects of the essential oils on the morphology of the yeasts. First, 1 mL of Sabouraud Dextrose Broth medium and 1 mL of yeast suspension with a concentration equal to half McFarland were added to the test tube, and then the dissolved essential oil was added to the tubes, which resulted in different MIC concentrations (17). Subsequently, the cultures were incubated at 30 ºC. To investigate the effects of the essential oils on the number of yeast cells, a modified neobar slide was used. Moreover, the crystal violet staining method and an optical microscope were used to examine the morphological changes. Based on the results obtained from the light microscope, it was determined at what concentration and at what time the alterations in the yeast morphology would occur. Based on the results of light microscopy and accurate determination of the time of the effects of the essential oil on the yeast, the yeast exposed to essential oil is gradually affected. Before destruction, the sample was centrifuged at a speed of 3000 rpm for 4 minutes. The sediment obtained by centrifugation was separated after washing three times with distilled water. Finally, the stabilizing buffer (glutaraldehyde) was poured on the remaining sediment. By observing with the negative staining method, the overall morphology was checked. Moreover, after using the cross-section method, the investigations were carried out with the help of a transmission electron microscope (TEM).

Statistical Analysis

The results were evaluated using SPSS version 20 statistical software. The statistical comparison of the data was carried out based on the t-test and at the probability level of 5% ($P<0.05$).

Results and Discussion

Antifungal Activities of the Essential Oils Evaluated by Disc Diffusion Methods

The results of the disc diffusion test in the presence of the studied essential oils indicated that the highest resistance rates in 0.625 mg/mL of *O. vulgare*, *M. longifolia*, and *Z. multiflora* essential oils were 31(77.5%), 15(37.5%), and 13(32.5%), respectively; while the lowest resistance rates were observed in dilutions of 10 and 5 with zero frequency (Figure 1).

The Results of Antifungal Activities of the Essential Oils Obtained Using the Micro Dilution Method

MIC results obtained from the microplate dilution of the essential oils tested against 40 *C. albicans* have been shown in Table 1. Based on these results, the lowest MIC was related to *Z. Multiflora* essential oil.

Table 1: Minimum inhibitory concentration (MIC) of the essential oils on *C. albicans* strains.

	***C. albicans* strains**
MIC (mg/ml) of *O. vulgare* essential oil	0.3125
MIC (mg/ml) of *M. longifolia* essential oil	0.3125
MIC (mg/ml) of *Z. multiflora* essential oil	0.625

The Results of the Morphological Changes of C. Albicans Strains after Treatment by *Z. Multiflora* Essential Oil

Due to the fact that the lowest MIC was related to *Z. Multiflora* essential oil, the morphological changes of the studied yeasts were investigated after treatment with *Z. Multiflora* essential oil.

The evaluation of the changes of the strain exposed to

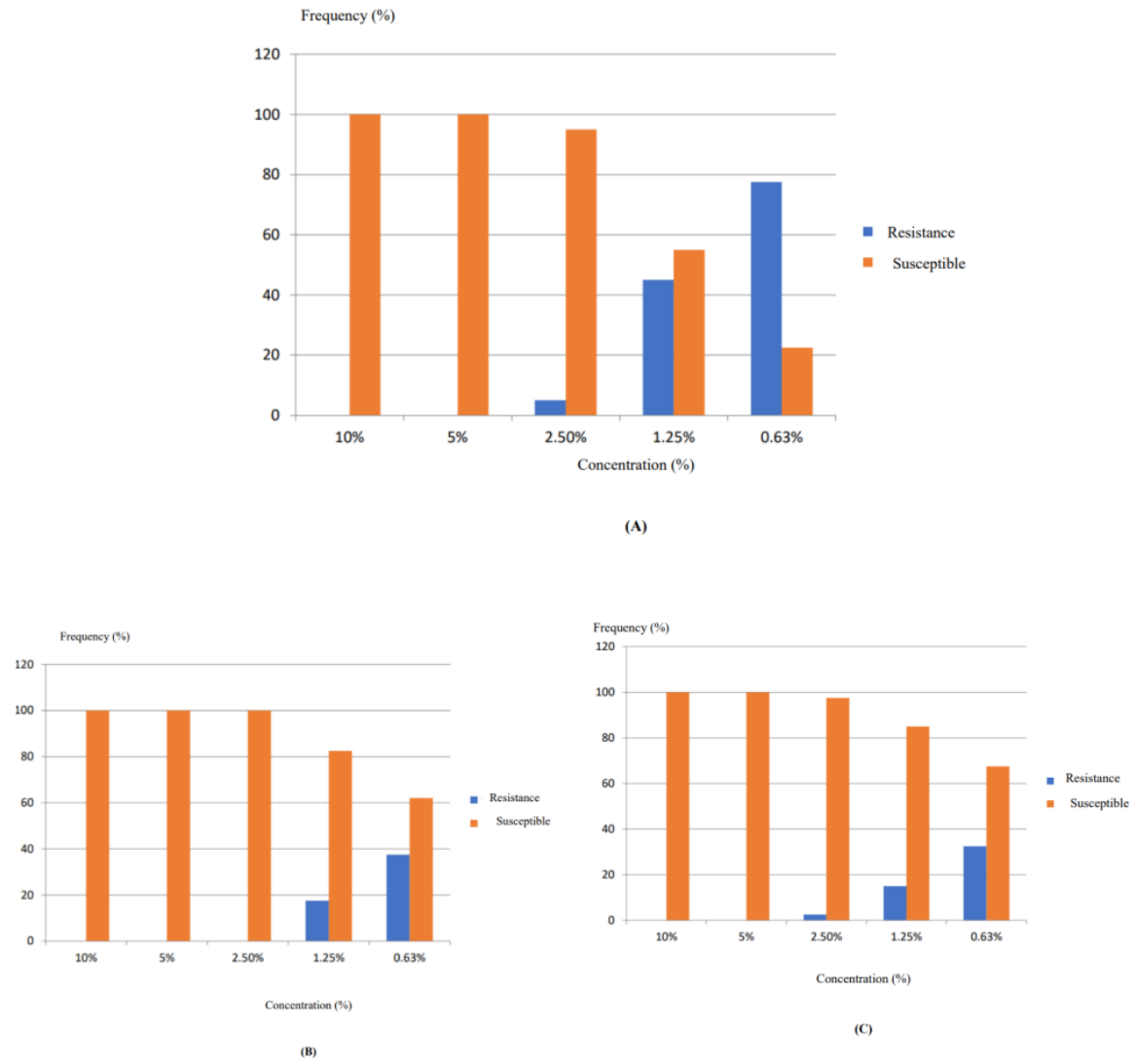

Figure 1. Resistance percentage of the yeasts using the disk diffusion test in the presence of the tested essential oils. (A): Diffusion disc related to *O. vulgare* essential oil. (B): Diffusion disc related to *M. longifolia* essential oil. (C): Diffusion disc related to *Z. multiflora* essential oil.

10% essential oil in 0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 24 hours showed that as soon as the essential oil came in contact with the yeasts, an immediate decrease in the number was observed at the zero hour. The decrease increased with the passage of time and reached the minimum number after 24 hours. At the same time, the sample exhibited an increase in number (Figures 2A and 2B).

At the 0 hour, treatment with essential oils reduced the average yeast count from 34 ± 5.292 to 25.33 ± 2.517. At 0.5 hour the average yeast count was reduced from 42.67 ± 6.429 to 22.33 ± 1.528. In 1 hour, the average number of the yeasts decreased from 47.67 ± 6.429 to 20.33 ± 1.528. In 2 hours, the average number of the yeasts decreased from 7.638 ± 56.67 to 1.155 ± 19.33. In 3 hours, the average number of the yeasts decreased from 70 ± 5 to 1.528 ± 16.67. In 4 hours, the average number of the yeasts decreased from 5.033 ± 95.33 to 1.528 ± 14.67. In 5 hours, the average number of the yeasts decreased from 115 ± 5 to 13 ± 1. In 6 hours, it decreased the average number of the yeasts from 130.33 ± 4.509 to 11.67 ± 1.155. In 7 hours, it decreased the average number of the yeasts from 140 ± 5 to 1.155 ± 9.33. In 24 hours, it decreased the average number of

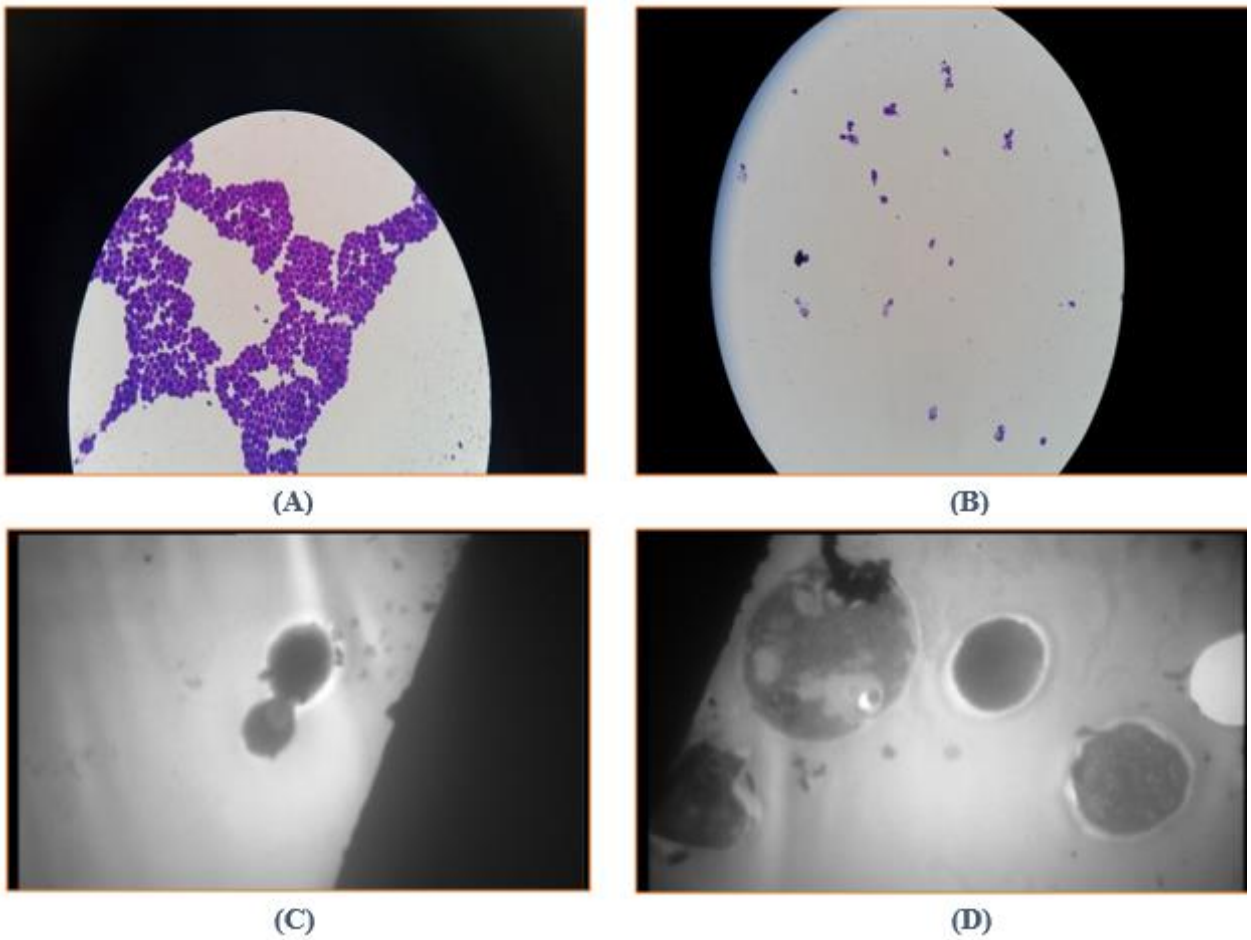

Figure 2. A and B: Light microscope images. C and D: TEM images. (A) Examining the morphology of control yeast cells in 24 hours with 100X magnification. (B) Examining the morphology of yeast cells exposed to the essential oil in 24 hours with 100X magnification. (C) Electron microscope examination of control yeast cells with 30.000 × magnification .(D).

the yeasts from 3.606 ± 151 to It was 8 ± 2.

The results of the examination by an electron microscope: changes of the nature and morphology of the fungus, and cases of the destruction and decay of the fungus were observed (Figures 2C and 2D).

Treatment with antibiotics always brings the concern of drug side effects. At present, about 25-50% of common medicinal plants are used in the world. Since the number of pathogenic microbes resistant to common antibiotics is progressively increasing, the discovery of new therapeutic agents is an essential necessity in order to control and eliminate resistant microbial infections, particularly hospital infections (18). Medicinal plants have several advantages such as low price and accessibility. These plants are used in the treatment of different diseases, including infectious diseases. Plant essential oils are volatile and aromatic compounds that are found in plant organs. Since these essential oils simultaneously affect various parts of bacteria, they are of high significance in treatments of diseases without being challenged by serious resistances (19, 20). Among the uses of herbal essential oils, we can mention their uses in foods, complementary medicine, and pharmaceutical as well as herbal treatment industries (21, 22). In this study, the anti-candida effects of *O. vulgare*, *M. longifolia*, and *Z. multiflora* essential oils were investigated, and the results indicated the existence of anti-candida properties of the studied essential oils. There are many reports of the antibacterial and antifungal effects of these essential oils on other microorganisms in different conditions.

In two studies conducted by Javanmard *et al*., (23) and Viuda *et al*., (24) the antifungal effects of *Z. multiflora* essential oil and extract against Aspergillus flavus were reported. Moreover, Lahooji *et al*., showed that 16 μL

in 100 ml of *Z. multiflora* essential oil inhibited the growth of *Aspergillus parasiticus* isolates (25). Niczad *et al*. studied the essential oil compounds, and the antioxidant as well as antimicrobial activities of *Z. multiflora* were investigated. The results indicated that *Z. multiflora* essential oil has remarkable antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Candida albicans*. Moreover, the results supported the use of *Z. multiflora* essential oil and extract in food, pharmaceutical, and cosmetic industries (26).

The composition of plant essential oils is different based on the plant variety, the age of the plant when preparing the essential oil, the geographical region where the plant grows, the method of drying and extracting the essential oil, and the distinction in the different methods of checking the antimicrobial properties of the essential oils. Furthermore, there are differences in the microbial strains used in the sources. Thus, it is very difficult to compare the results of different studies on the antimicrobial properties of essential oils (27, 28).

In this study, the results of the TEM examination of *C. albicans* isolates after treatment with *Z. multiflora* showed a change in the morphology and even the destruction of the Candida isolates. The appearance of these changes in the wall and internal organs in the essence also showed the antimicrobial effect of this plant. This essential oil is probably able to change the cell wall and internal organelles by creating a hole in the cell membrane of the fungus. In the study conducted by Mohseni *et al*., the investigation of the antifungal effect of *Fumaria vaillantii* essential oil showed that *F. vaillantii* essential oil could affect the cellular structure of the *Aspergillus spp* filament, cause the walls to break, inhibit the growth, and induce the death of the fungus (29). The results of this study indicated that the antifungal effects of *O. vulgare* and *M. longifolia* essential oils were less significant than that of *Z. multiflora*. Furthermore, it was shown that *Z. multiflora* essential oil had significant antifungal effects. Hence, it seems that considering the problems of the medical community in treating and controlling various types of infections, it is essential to carry out further investigations on these valuable, inexpensive, easily available, and less harmful resources.

Conclusion

Fortunately, in recent years, extensive research has been conducted in the field of evaluating the antimicrobial impacts of plant compounds, including essential oils and various extracts, which indicates the ability of these compounds to inhibit the growth of a wide range of pathogenic microorganisms. In this research, the anti-candida effects of *O.vulgare*, *M. longifolia*, and *Z. multiflora* essential oils were evaluated. The results of the present study indicated that these essential oils, particularly *Z. multiflora*, have potential anti-candida properties that can be used to prepare antifungal compounds after further research in the field of pharmacology and pharmacokinetics.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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