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Evaluation of Antifungal Effects of Ethanolic and Aqueous Extracts of *Zataria multiflora* Herb in the Pathogenic Yeast *Candida albicans* Biofilm Inhibition

Ghasem Rahimi[1], Alireza Khodavandi[2]*, Ramin Jannesar[3], Fahimeh Alizadeh[2], Ramin Yaghobi[4] and Abdolla Sadri[5]

[1]Young Researchers and Elite Club, Marvdasht Branch, Islamic Azad University, Marvdasht, Iran.
[2]Department of Microbiology, Yasouj Branch, Islamic Azad University, Yasouj, Iran.
[3]Department of Pathology, School of Medicine, Yasouj University of Medical Sciences, Yasuj, Iran.
[4]Shiraz Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[5]Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom, Iran.

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Infections caused by the opportunistic yeast *Candida albicans* has been increased dramatically in recent decades. Currently, the antifungal agents derived from plants have attracted considerable attention. *Zataria multiflora* is an herb *that* has antimicrobial and antifungal properties. In the present research, the antifungal effects of ethanolic and aqueous extracts of *Zataria Multiflora* herb evaluated against pathogenic yeast *Candida albicans*. The minimum inhibitory concentrations value (MIC) of ethanolic and aqueous extracts of *Zataria multiflora* was evaluated using broth microdilution test (CLSI-protocol). Time kill study was also performed to show the effect of antifungals tested to inhibit the growth of *Candida* by increasing-time. Eventually, MTT assay was carried out to investigate the biofilm-inhibitory potential of ethanolic and aqueous extracts of *Zataria multiflora* tested against *Candida albicans*. Our results showed that all extracts tested could be able to inhibit the growth of sessile *Candida* cells as well as biofilm formation, significantly ($p \leq 0.05$). Indeed MICs were ranged from 0.85-1.50 mg/ml for ethanolic and aqueous extracts of *Zataria multiflora*, respectively. MTT results also showed the significant reduction of biofilm in *Candida* after treatment by extracts tested at level $p \leq 0.05$. The results of this study revealed the fact that *Candida albicans* is sensitive to the *Zataria multiflora* and in the presence of this plant, the expression level of effective genes in biofilm synthesis could be reduced. Additionally, the ethanolic extracts of the *Zataria multiflora* was stronger than the aqueous extract in terms of antifungal activity.

Key words: *Candida albicans*, biofilm, *Zataria multiflora*, aqueous extract, ethanol extract.

Fungal infections are estimated to occur in over a billion people each year, and recent evidence suggests the rate is increasing. Although several species of fungi are potentially pathogenic in humans, *Candida* (esp. *Candida albicans*) is the organism responsible for most fungal infections[1,2]. These fungi have a balance with other microorganisms in the body, and several factors such as continuous treatment with antibiotics, the using of dental prosthesis, the use of immune suppressive drugs dietary factors and hospital factors were predisposing factors for fungal infection and the infection has spread to other organs in the body. Clearly, binding ability and also biofilm formation could be resistant to the common antifungals such as nystatin, amphotripcin B, fluconazole, and ketoconazole[3,4,5]. On the other hand, *Candida* species could be able to growth in the low concentrations of azoles and

* To whom all correspondence should be addressed.
TeleFax: +987432331103; +987432333533;
E-mail: khodavandi@iaug.ac.ir

the other antifngals. However, because of long-term using of antifungal drugs, incompatibility of synthetic drugs show side effects due to continuous usage and also resistance, especially in immunocompromised patients[6,7]. Recently, the use of medicinal plants and their derivatives have been raised and many researchers evaluate the antifungal activities of herbal medicines. *Zataria multiflora* Boiss is a plant belonging to the family *Labiateae* and has global dispersion. This plant is a native herbal originated from south of Iran[8]. The main chemical compounds in this plant are including linalool, thymol, paracemen and carvacrol that all of these materials have antimicrobial and antifungal properties[9]. The antifungal properties of this plant have been proven in various studies. For example, in Akbari's *et al* study, *Candida albicans* was susceptible to fluconazole and resistant strains were evaluated. The results indicated the effectiveness of antifungal plant *multiflora* against yeast tested[10]. Indeed, the present study aimed to evaluate aqueous and ethanol extracts of *multiflora* plants against pathogenic yeast *Candida albicans* and its biofilm activity.

MATERIALS AND METHODS

Candida albicans strain PTCC (5027) was earned from Scientific and Industrial Research of Iran and then cultured on sabouraud dextrose media and cornmeal agar, and finally incubated at 37° C for 24 h.

Inoculum Preparation

The yeast *Candida albicans* suspensions were prepared from fresh culture media. Five colonies grown of yeast on SDA was removed and placed in 5 ml of sterile normal saline and mixed well using a spectrophotometer at a wavelength of 530 nm, and then the amount of OD (optical density) was earned to achieve 0.5 McFarland standards. The suspension obtained from 1×10^6 to 5×10^6 cells/ml. Subsequently, the working suspension was diluted to achieve inoculum containing between 5×10^2 and 2.5×10^3 yeast cells/ml in a 96-well microplate (Brand 781660, Wertheim, Germany). The microplates including drugs and cells were incubated at 35°C and MICs were measured at 530 nm from two independent experiments in three separate technical replicates[11].

Preparation of the aqueous extract

Fifty g of the plant was added to 200 ml of sterile distilled water with a gentle boil took place and then used Whatman paper No.1. To ensure with no contamination, the extract obtained was passed directly through filtered number (0.22 µm) to the desired dilution. The extract was kept at -20°C until use.

Preparation of ethanolic extract

Fifty g of plant extracts were poured in to the container. Then the ethanol solvent (80%) was added and shaked at 37°C for 48 h. After this time the solution is passed through a No. 42 whatman paper in a 50°C water bath. Finally, the extract was filtered again, using 0.22 µm milipore filter. The extract was kept at -20°C until use.

Preparation of working solutions

To evaluate the ethanolic and aqueous extracts, *multiflora* herb was diluted 10 times and then was added to fresh media and pH was adjusted to 7. Indeed, 100 ml of sterilized medium (SDB) was poured in the sterile flask and then the 0.008, 0.01, 0.021, 0.042, 0.085, 0.150, 0.350, 0.625, 1.25 and 2.50 ml of extract was added. The final dilutions were including 0.080, 0.10, 0.21, 0.42, 0.85, 1.50, 3.50, 6.25, 12.50, and 25 mg/ml. The solutions were kept in -20°C until use[12].

Determination of the MIC of aqueous and ethanolic extract of *multiflora* herb using broth microdilution test (CLSI-modified):

The amount of 50 µl of aqueous and ethanolic *multiflora* concentrations (0.080, 0.10, 0.21, 0.42, 0.85, 1.50, 3.50, 6.25, 12.50, and 25) mg/ml was added to the wells. The well number 11 was considered as positive controls with no extract and for the number 12 which has 50 µl concentrations of the extracts was considered as the negative control. Hundred µl of fungal suspension was added to each wells and then 100 µl of sterilized medium (SDB) added and incubated at 35°C for 24 h. Indeed, the MICs were measured at 530 nm from two independent experiments in three separate technical replicates using EMax® microplate reader after 24 h.

Effect of aqueous and ethanolic extracts of *multiflora* on *Candida albicans* biofilms

Candida albicans biofilm developed in 96-well microplate. Hundred µl of fungal suspension containing 1×10^6 cells/ml was poured in to the 96-well microplate and then placed in a

shaking incubator with75 rpm (rate per minute) for 90 min at 37°C until cells attached to the bottom of the wells. The wells were rinsed with 100 µl of phosphate buffer saline with pH 7.4 in three times to remove of no attached cells. Then, 100 µl of culture medium (SDB) was added to the wells and microplate was maintained in shaking incubator for 48 h at 37°C. After this period, the biofilm was rinsed with 100 µl of phosphate buffer saline. Then the prepared dilutions (0.080, 0.10, 0.21, 0.42, 0.85, 1.50, 3.50, 6.25, 12.50 and 25 mg/ml) from the extract with 100µl of medium were added to the wells and incubated for 24 h. For measurement the number of viable cells in biofilms, vital tetrazolium dye (MTT) was used. Twenty µl of tetrazolium solution was added to 50 µl of cell suspension and 50 µl of culture medium and the mixture was kept at 37°C for 4 h. After washing with phosphate-buffered saline, 100 ml of 0.5% solution of dimethyl sulfoxide was added to all wells and incubated at 37°C for 10 min. The absorbance microplate investment in equipment microplate-reader at a wavelength of 490 nm and percentage of biofilm growth inhibition was calculated by the following formula[13,14].

Percentage of growth inhibition= 100 - (extract in the wave-length of OD 490) / (Control in the wave-length of OD 490) × 100

Microscopic observations

The concentrations of extracts based on MIC was mixed with yeast and incubated for 0, 12 and 24 h and then spread on sterile slides and observed via a stereo-microscope. To compare with the control group, the extract was carried out without adding any extracts [15].

Time-kill study

To evaluate the dilution factor, 2 × MIC concentrations of the ethanolic and aqueous extracts of *multiflora* were mixed with prepared yeast cells and 100 µl of each dilution was picked and cultured on SDA medium. The plates were incubated at different time intervals and colonies grown were counted on CFU (Colony Forming Unit)[15].

Basically, colonies ranged from 30-300, were valid and must be counted.

The following formula was used for colony counting.

Number of yeasts (yeast cell/ml) = conversely dilution × number of colonies

All experiments performed in triplicates. All data were examined in terms of normality and then one way analysis of variance (ANOVA) was carried out.

RESULTS

The obtained results indicated the significant differences in the average data and control at level $p \leq 0.05$.

Minimum inhibitory concentration, MIC

Absolute MIC represents the lowest concentration of extract that has not grown yeast in to the positive control. The results showed that the aqueous and ethanolic extracts of *multiflora* herb were very strong and significant effect in preventing the growth of the yeast *Candida albicans*. Available data indicated that the MIC of ethanolic extracts of *multiflora* was equivalent to 0.840 mg/ml while the MIC of the aqueous extract was equal to 1.50 mg/ml.

Effect of aqueous and ethanol extracts of *multiflora* herb on the growth of *Candida albicans* biofilm

Figures 1 and 2 showed the absorbance of microplate containing *Candida albicans* biofilm at a wavelength of 490 nm in different aqueous and ethanolic extracts of *multiflora* after 24 h. Interestingly, ethanolic extract is stronger than the

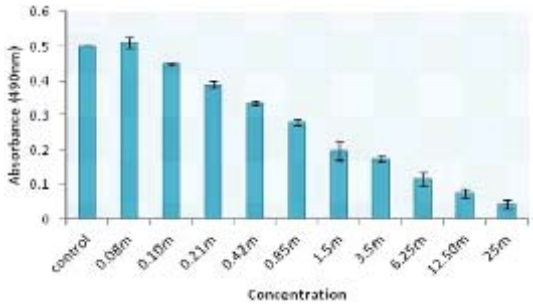

Fig. 1. Absorbance of aqueouse xtract of *multiflora* on *Candida albicans*.

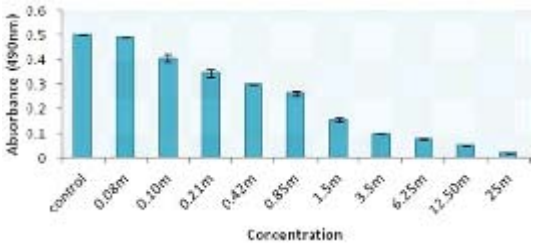

Fig. 2. Absorbance of the ethanol extract of *multiflora* on *Candida albicans*.

aqueous extracts. Indeed, the ethanolic extract was able to decrease 97% of growth, while the aqueous extracts was only 87% ($p \leq 0.05$).

Time-kill study

Figure 3 represents the effect of aqueous and ethanolic extracts of *multiflora* herb on the number of *Candida albicans* yeast cells and ranged from 0 to 48 h. Indeed, the plot was a significant reduction ($p \leq 0.05$) in the number of yeast cells treated with the different extracts after 24 h incubation.

Microscopic observations

These results indicate a biofilm-reduction after treatment with extracts in the presence of *Candida albicans* after 0-24 h incubation. Figure 4 shows *Candida* biofilm reduced in the presence of aqueous and ethanolic extracts of *multiflora* in 1× MIC concentrations after incubation. The reduction of biofilm was significant at level $p \leq 0.05$.

DISCUSSION

Candidiasis is the most common fungal infection in healthy people and in people with impaired immune systems. The most important virulence factor of *Candida albicans* is biofilm formation, phenotypic changes and the production of extracellular hydrolytic enzymes such as

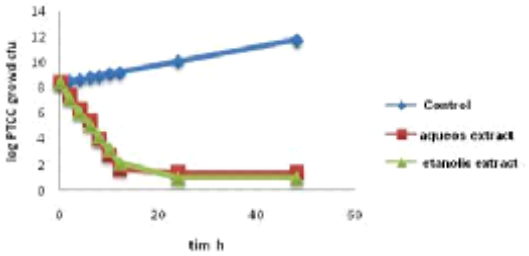

Fig. 3. Time Kill CURVE in the presence of aqueous and ethanol extracts of *multiflora*

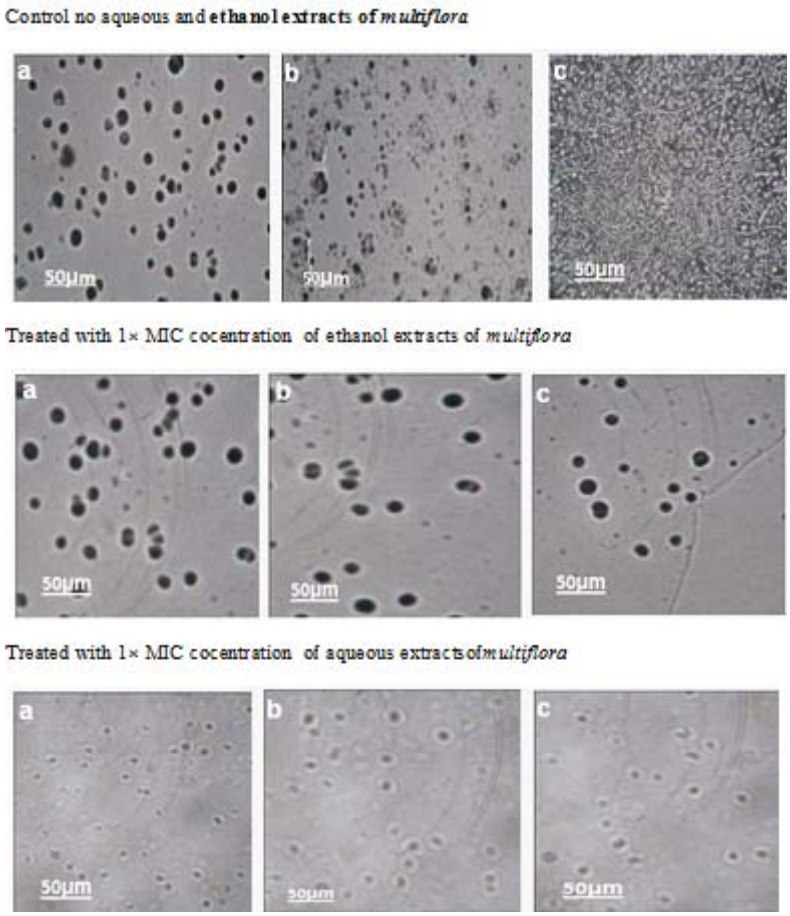

Fig. 4. Microscopic observation of biofilm formation *Candida albicans* PTCC 5027 after 0 h (a), 12 h (b) and 24 h (c).

proteinase. Indeed, the first step in the infection of *Candida* yeast-like fungus is colonization on the surface of the body tissues[16]. On the other hand, due to a phenomen on known as drug resistance, treatment of fungal infections, especially candidiasis has been failed. Therefore, the frequently of drug resistance could be probably due to increased expression of genes involved in the synthesis of the ergosterol by *candida*[17]. Nowadays, the antifungal agents originated from plants are more noticed by researchers due to increasing of side effects and also resistance phenomena with usage of chemical drugs. Therefore the use of medicinal plants with no or less side effects and resistance will be necessary. In this study, the aqueous and ethanolic extracts of *multiflora* herb against pathogenic *Candida albicans* were investigated. Our data showed that the aqueous and ethanolic extracts of *multiflora* herb dilutions inhibited the growth of *Candida* at 1.50, 0.85 mg/ml, respectively. The use of aqueous and ethanolic extract of the plant was ranged from 0 to 48 h, indicating a significant reduction in the number of yeast cells. More researches about the impact of antimicrobial and antifungal activity of *multiflora* have ever been done. In the one of these studies, the effect of the plantin relation to *Aspergillus flavus* has been studied. The results indicated a strong inhibitory effect of the extracts and the minimal inhibitory concentrations were started from 400 ppm[18]. Moreover, Giordani *et al*. measured the minimal inhibitory concentration of *multiflora*. The MIC was 0.016 mg/ml and was less in compare of our data. This may be due to differences in the extraction methods[19]. The study conducted by Ziai and his colleague demonstrated that our data were similar to their findings. For example, The MIC of aqueous and ethanolic extracts of our plant against *Candida* was 0.85 and 1.5 mg/ml, respectively and was compatible to their MIC (0.97-0.195)[20]. Interestingly, this study demonstrated the antibiofilm activity of *multiflora* against *Candida* for the first time. Both of results conducted from that study and also our research showed the significant effect of aqueous and ethanolic of *multiflora* extracts against growth and also biofilm formation of *Candida albicans* resulting in reduction of pathogenesity of *Candida*. These encouraging results demonstrated that *multiflora* and its related bioactive compounds could be further developed into an alternative or supplementary therapeutic arsenal against *Candida* infections in humans. Further work would be also performed in order to investigate the molecular mechanisms of action of *multiflora* against *C. albicans*.

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