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Antifungal Effect of *Zataria multiflora* Essence on Experimentally Contaminated Acryl Resin Plates With *Candida albicans*

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Background: Adherence and colonization of *Candida* species particularly *C. albicans* on denture surfaces, forms a microbial biofilm, which may result denture stomatitis in complete denture users.
Objectives: The purpose of the present study was to evaluate the antifungal effect *Zataria multiflora* essence in removing of *Candida albicans* biofilms on experimentally contaminated resin acryl plates.
Materials and Methods: In the present experimental study, 160 resin acrylic plates (10 × 10 × 1 mm) were contaminated by immersion in 1×10^3 *C. albicans* suspension for 24 hours to prepare experimental *Candida* biofilms. The total number of *Candida* cells, which adhered to 20 randomly selected acryl resin plates was determined as the *Candia* load before cleaning. The remaining 140 plates were divided to seven groups of 20 and immersed in five concentrations of *Zataria multiflora* essence from 50 to 3.125 mg/mL as test, 100000 IU nystatin as the positive and sterile physiologic serum as the negative control. The remaining *Candida* cells on each acryl plate were also enumerated and data were analyzed using the SPSS 16 software with Kruskal-Wallis and Wilcoxon tests.
Results: *Zataria* essence at concentrations of 50 and 25 mg/mL removed 100% of attached *Candida* cells similar to nystatine (MFC), while weaker *Zataria* essence solutions cleaned 88%, 60.5% and 44.7% of attached *Candida* cells. Kruskal-wallis test showed a statistically significant difference between all test groups (P = 0.0001). In this study 12.5 mg/mL concentration of *Zataria multiflora* was considered as the minimum inhibitory concentration (MIC_{90}).
Conclusions: *Zataria* essence, at concentrations of 50 and 25 mg/mL, effectively removed *Candida* cells that had adhered to the denture surface, similar to the level of removal observed for 100000 IU nystatin.

*Keywords:*Disinfection; *Candida albicans*; Acrylic Resins; multiflorol

1. Background

Dentures as an indwelling medical device in an individual's mouth, prepare an optimal environment for adhesion and colonization of both pathogenic and non-pathogenic organisms, and can cause inflammatory lesion of the oral mucosa (1). Denture stomatitis known as a chronic inflammatory condition of the palatal and alveolar mucosa underlying removable dental prostheses is seen in 15% to 65% of individuals (2) and is even more significant in the institutionalized denture wearing population with a rate of up to 72% (3, 4). However denture stomatitis is usually asymptomatic and can be associated with burning, bleeding and unpleasant taste in denture users (5). Candida species, particularly *C. albicans*, which are a part of the human oral microbiota, have been reported as the main etiological agents responsible for the development of this inflammatory infection (6). Adherence of *C. albicans*to the host mucosal tissues (7) and also on the acrylic denture surfaces prepares reservoirs that produce proteolytic enzymes and damage mucosal tissues resulting in denture stomatitis (8).

In addition to *Candida*, most cariogenic bacteria especially *Streptococcus* mutants are also known as major etiological agents of dental caries, which adhere and accumulate on teeth surfaces, lead to plaque formation (9). It is believed that the attachment between microorganisms and the denture surface is due to electrostatic and hydrophobic interactions. These interactions may be disrupted by mechanical and chemical removal of microbial biofilms. Poor oral hygiene in patients with denture especially in case of badly fitted dentures promotes denture stomatitis. Regular cleaning and removal of microbial biofilm from denture surfaces is necessary for prevention and control of denture stomatitis in edentulous patients (10). There are numerous investigations in the dentistry literature advising the use of chemical solutions for denture disinfection (11-13); unfortunately these products cause mucosal allergic reactions, damage the acrylic resin and metal alloys of the dentures (14, 15).

The use of natural antimicrobials such as herbal mouthwashes has been recently increased as investigations

have indicated their potential to prevent oral diseases such as plaque-related diseases, particularly dental caries (16-18). Natural substances have been shown to possess antibacterial action mainly because most plants used in alternative medicine are composed of carvacrol and thymol, which act on bacterial cells disrupting their cytoplasmatic membrane and inhibiting their enzymatic activity (19, 20).

The specie *Zataria multiflora* (ZM) with the Persian name of Avishane Shirazi, has been used in traditional medicine for treatment of respiratory tract infections and managing irritable bowel syndrome (21), and as an antispasmodic, anesthetic, antinociceptive agent (22, 23). More recently its antibacterial and antifungal activities have also been demonstrated (19, 24, 25). The antifungal activity of *Zataria multiflora* essence against several dermatophytes such as *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and saprophytes like *Aspergillus flavus* was reported by Effatpanah et al. (26). The purpose of the present study was to evaluate the antimicrobial effect of *Zataria multiflora* essence on experimentally produced *C. albicans* biofilms on resin acryl plates.

2. Objectives

This study was conducted to evaluate the antifungal effect *Zataria multiflora* essence in removing *Candida albicans* biofilm from experimentally contaminated resin acryl plates.

3. Materials and Methods

3.1. Sample Size

In order to reach a minimum of six unit difference in the average Candida colony counts between tested groups, a significance level of 5%, $\alpha = 5\%$, $\beta = 2\%$, $S = 80\%$ and based on the following formula, 20 samples were chosen for each test and control groups.

$$n = \frac{\left(Z_{\beta}+Z_{\frac{\alpha}{2}}\right)^{2} 2s^{2}}{\left(x_{1}-x_{2}\right)^{2}}$$

3.2. Preparation of Acrylic Resin Plates

In the present experimental study, 160 square shaped (10 × 10 × 1 mm) resin acrylic plates (Acropars, Marlic, Iran) were prepared (20 samples for each group) using thermally activated resin acrylic according to the manufacturer's instructions (Figure 1). The specimens were kept in a flask containing physiological serum (NaCl 0.85%), sterilized in an autoclave (Labtron, Iran) at 121°C for 15 minutes (was calibrated based on manufactured instruction) and incubated at 4°C for further adherence testing.

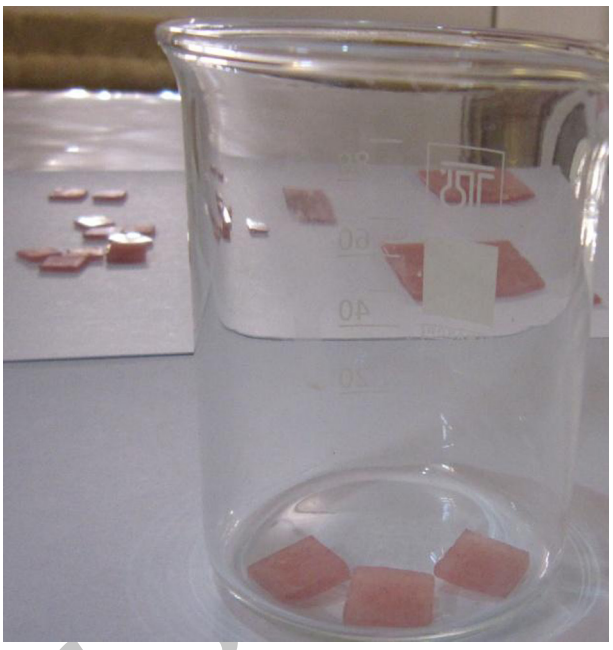

Figure 1. Acrylic Resin Plates Used for Experimental Contamination With *C. albicans* (10 × 10 × 1 mm)

3.3. Preparation of Fungal Suspension and Experimental Biofilm Formation

Clinical isolates of *C. albicans* (ATCC 10231), cultured on Sabouraud dextrose agar plates (Merck, Germany) and incubated at 37°C for 24 hours, were used as test organisms for the current experimental study. An isolated fresh single colony was used for preparing Candida suspensions containing 1×10^3 viable cells per milliliter in sterile saline solution (NaCl 0.85%) using a hemocytometer. The experimental biofilm was created by immersing all acrylic resin plates in *C. albicans* suspension and incubating on a reciprocal shaker (100 RPM) at 37°C for 24 hours. Twenty resin plates were randomly selected, washed three times with sterile PBS, and transferred separately to a 50 mL sterile Falcon tube containing 5 mL of sterile PBS and glass pearls; the tubes were then agitated in a sonicator (Elma, Germany) for five minutes (45 KH/5 minutes) to remove viable attached cells. Next, 10 µL of each suspension was added to 90 µL of sterile physiological solution and inoculated on Sabouraud dextrose agar (Merck, Germany) plates to evaluate attached viable cells before initiation of the disinfection protocol as a variable in the present study.

3.4. Disinfection of Contaminated Acrylic Resin Plates

The remaining 140 contaminated resin plates were randomly divided to seven groups of 20. Each group of plates were separately immersed in *Zataria* essence dilutions of 50 to 3.125 mg/mL (Baridge-essence, Kashan, Iran)

Table 1. Effectiveness of *Zataria multiflora* Essence and Percentage Removal of *C. albicans* From Twenty Experimentally Contaminated Resin Acryl Plates [a]

Disinfectants	Initial Culture [b]	After Disinfection [b]	Removing Ability %	P Value
Nystatin (gold)	721.5 ± 67.3	0	100	0.00001
DW (neg. control)	721.5 ± 67.3	567 ± 54	20.1	0.062
50 mg/mL *Zataria*	721.5 ± 67.3	0	100	0.00001
25 mg/mL *Zataria*	721.5 ± 67.3	0	100	0.00001
12.5 mg/mL *Zataria*	721.5 ± 67.3	81.5 ± 20.8	90	0.0001
6.25 mg/mL *Zataria*	721.5 ± 67.3	285 ± 38	60.5	0.001
3.125 mg/mL *Zataria*	721.5 ± 67.3	399.5 ± 45	44.7	0.015

[a] Abbreviation: DW, distilled water.
[b] Data are presented as mean ± SD.

Table 2. Susceptibility Profile of *Candida albicans* to *Zataria multiflora* Essence Solution [a]

Variable	Value, mg/mL
MFC	< 25
MIC 90	12.5
MIC 50	> 6.25

[a] Abbreviation: MFC, minimum fungicidal concentrations; MIC, minimum inhibitory concentration, Candida viable cells.

and 100000 IU nystatin solutions (gold standard) as test groups, as well as sterile physiologic solution (NaCl 0.85%) as the negative control group, in sterile Falcon tubes. All Falcon tubes were incubated on a reciprocal shaker (100 RPM) at 37°C for two hours, washed three times as explained previously. Each single resin plate from all groups were then transferred to another sterile Falcon tube containing 5 mL of sterile PBS and glass pearls, agitated in a sonicator (Elma, Germany) for five minutes (45 KH/5 minutes calibrated based on the manufactured instructions) to remove the adhered viable *Candida* cells. Finally, 10 μL of each washed solution was mixed with 90 μL of sterile physiological solution and inoculated on Sabouraud dextrose agar plates in order to enumerate the attached viable *Candida* cells after the disinfection procedures.

3.5. Statistical Tests

The mean isolated Candida colonies (CFU/mL) showed mean attached viable *Candida* cells in the seven groups, which were compared using the Kruskal-Wallis test. Wilcoxon statistical test was also used to compare the average counts of isolated *Candida* colonies before and after disinfection. Differences in the *Candida* removing ability of the tested solutions were considered significant if $P < 0.05$. All statistical calculations were performed using the SPSS 15 software.

4. Results

The average number of viable *C. albicans* cells (CFU/mL ± SD), which adhered to the acrylic resin plates before and after disinfection, as well as the removing percentage are illustrated in Table 1. *Zataria* essence solutions at concentrations of 50 and 25 mg/mL as well as 100000 IU nystatin completely removed all adhered *Candida* cells from resin acryl plates as there were no isolated *C. albicans* colonies in the culture of their washing solutions. Concentration of 25 mg/mL of *Zataria* essence was determined as the minimum fungicidal concentration (MFC) in the present study. Concentration of 12.5 mg/mL of *Zataria* essence removed about 90% of attached *Candida* viable cells and this concentration was considered as the minimum inhibitory concentration (MIC_{90}) in the present study (Table 2). There was no statistically significant difference between the average *Candida* colonies isolated from cultures of resin acryl plates incubated with 50 and 25 mg/mL *Zataria,* and 100000 nystatin solutions ($P = 0.00001$).

5. Discussion

There are different chemical mouth washes, which are commonly used for controlling various plaque formations on teeth and dentures; they may cause an allergic response and probable mucosal alterations (27). There are many reports that discourage patients to use these mouthwashes as a result of their several side effects such as undesirable tooth and denture discoloration, unpleasant taste, dryness and burning sensation in the mouth (14, 28). However in the general worldwide population, herbal medicines are popular, many modern medicines are still derived from herbs (29). *Zataria multiflora*, which grows naturally in central and southern parts of Iran, is used in traditional herbal medicines for its antiseptic, analgesic and carminative properties (22, 30).

The antimicrobial activity of *Z. multiflora* essence against *C. albicans* on experimentally contaminated acryl resin plates was evaluated in the present study. A standard broth macrodilution method introduced by the clinical and laboratory standard institute (CLSI) was employed in the current study (31). As indicated in Table 1, concentrations of 50 and 25 mg/mL of *Zataria* essence completely cleaned contaminated acryl resin plates (100%) since there were no viable *Candida* cells, in the culture of their

washing solution. Concentration of 25 mg/mL of *Zataria* essence was also determined as the minimum fungicidal concentration (MFC) in the present study. Results of the current study was supported by the study of Zia et al.which showed the anti-*Candida* properties of *Zataria* extract against *C. albicans* isolated from patients with oral candidiasis (32). Akbari also reported antifungal ranges of 0.5 to 125 mg/mL for aqueous extracts of *Zataria* against different *Candida* species (33).

This wide range of effective Zataria concentrations in Akbari's study unlike the outcome of present study may results from difference in methods, as they used disk diffusion, whereas the broth dilution method was used in the current study.

Mahmoudabadi et al. in an in vitro study for anti-Candida activity of methanolic *Z. multiflora* Boiss reported the concentration of 70.7 mg/mL as the MIC, which is more than the MIC of the present study (24).

Different chemical compounds including thymol, carvacrol, p-cymene and linalool were isolated from the aerial parts of this plant (28, 34) and the anti-Candida activity of aqueous essence of *Z. multiflora* on contaminated resin acryl is probably due to the above-mentioned essential oils. Anti-erythema effect of *Z. multiflora* essence in comparison with miconazole gel in denture stomatitis was reported by Amanlou et al. (28), which supported the results of the current study. However in their study *Z. multiflora* extracts did not reduce the colony count of *Candida albicans* on denture surface as efficiently as miconazole; concentrations of 50 and 12.5 mg/mL of *Zataria* essence in the current study showed reduction in the colony counts of *C. albicans* on resin acryl plates as efficiently as nystatin.

Besides antifungal effects, asignificant antibacterial efficiency for *Z. multiflora* essential oils against clinical isolates of *S. aureus*, especially MRSA was also reported (25). Antiviral activity of *Z. multiflora* extracts was also reported against Herpes simplex type 1 virus at concentrations of 800 and 1000 μg/mL by Arabzadeh et al. (35). Since *Z. multiflora* increased IFN-γ, decreased IL-4, and enhanced the ratio of IFN-γ to IL-4 (Th1/Th2 balance), it may have a therapeutic value in inflammatory responses such as allergies, autoimmunity and infectious diseases associated with Th1/Th2 imbalance (36). The immunomodulatory activity of the *Z. multiflora* on dendritic cells and T cell responses was also shown, which resulted from decreasing proliferation of mitogen-stimulated lymphocytes (37).

In the present in vitro study, concentrations of 50 and 25 mg/mL of *Zataria* essence effectively removed Candida cells from experimentally contaminated resin acryl plates as efficiently as 100000 IU nystatin.

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Authors' Contributions

Abbas Falah Tafti and Abbas Ali Jafari supervised the study, participated in designing and conducting the study and prepared the manuscript. Seyed Mehdi Hosseiny carried out the study and collected the data. Abdolhossein Kazemei performed the statistical analysis. All authors have studied and approved the content of the present manuscript.

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