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Evaluation of antifungal effect of grape seed extract (GSE) on *Candida glabrata* and *Candida krusei*: *in vitro* study.

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Abstract

Background: Recently, following the widespread use of broad-spectrum antibiotics and immunosuppressive treatments, the prevalence of mucosal and systemic infections caused by *glabrata* and *Candida krusei* has been increased significantly. On the other hand, in addition to the problems caused by side effects of common antifungal drugs, the conducted studies have been indicative of the failure of antifungal treatments on Candidiasis different clinical infections and drug resistance including triazoles group (e.g., fluconazole) in the long-term use of them, especially in patients with a weakened immune system problem.

Objective: Comparing the antifungal effect of grape seed extract with fluconazole and Nystatin on *glabrata* and *Candida krusei in vitro*

Material and methods: In the present study, to review the antifungal effect and to determine MIC (the Minimum Inhibitory Concentration of growth) of grape seed extract, CLSI (Clinical and Laboratory Standards Institute) has been used. To ensure the correctness of the findings of macro dilution method, agar well diffusion method has been used. Finally, the obtained results are analyzed using SPSS 16 software.

Findings: MIC of grape seed extract was 50 µg/ml for *glabrata* and *Candida krusei*. On the other hand, in evaluation of the effect of different concentrations of grape seed extract with well diffusion of *Candida krusei* method was almost equal to *Candida glabrata* in same concentrations. As a result, the results related to both well diffusion methods (agar well diffusion) and the maximum inhibitory concentration MIC (macro dilution) are confirming the same antimicrobial susceptibility of both *glabrata* and *Candida krusei* to GSE.

Conclusion: Grape seed extract having benefits like low price, being available, suitable taste rather than Nystatin, and fewer side effects can be a good Candidate as antifungal drug to against infections caused by *glabrata* and *Candida krusei*, after clinical trial.

Keywords: Grape seed extract, *Candida glabrata*, *Candida krusei*, Fungal infection.

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Introduction

Oral candidiasis is one of the most common fungal opportunistic infections in mouth that is made by a yeast-formed fungus microorganism called *Candida*, and almost all people naturally carry this species of fungi [1]. The main cause of *Candida* infection is *albicans* species. However, other species of *Candida* like *glabrata* and *krusei* can also cause oral and systemic infections [2].

Candida glabrata is a non-pathogenic saprophyte that has been considered as normal flora of healthy people. However, following the widespread use of broad-spectrum antibiotics and immunosuppressive treatments, the prevalence of mucosal and systemic infections caused by *glabrata* and *Candida krusei* has been increased significantly [2]. In fact, depending on the location of infection, the micro-organism has been proposed as the second most common microorganisms causing candidiasis, after *Candida albicans* [3].

The incidence of antifungal resistance in *Candida glabrata* against antifungal Triazoles group including fluconazole has made an increasing concern in the recent years [4]. This issue is mostly because of the ability to produce biofilm by this microorganism [5]. *Candida krusei* also occurs commonly in neutropenic patients, hematologic malignancies and patients

with bone marrow transplants that are resistant against antifungal treatments such as fluconazole and the other antifungal treatments. In addition, the survival probability of patients is very weak [6].

Some of anti-fungal drugs, based on the needs of patients, are used locally including Nystatin and clotrimazole, or systemically like Azole and amphotericin B to treat Candidiasis.

In the recent years, many studies have been conducted indicating the failure of antifungal treatments on Candidiasis different clinical infections and drug resistance including triazoles group in the long-term use of them, especially in patients with a weakened immune system problem [3,4,7].

The grape seed extract obtained from the Vitis vinifera plant is from Vitaceae family that is native of the Mediterranean region, central Europe and South West Asia [8]. In addition, it is a compound that has attracted lots of attention in medical treatments in the recent years. In traditional Indian medicine, this herb is used to cure cough, Catarrh of the respiratory tract, sub-acute liver, and spleen disease, as well as in the alcohol-based tonics (Aasaus) [9]. This compound is available in the form of 100 mg capsules in Iran pharmaceutical market. Moreover, the most important biochemical components of GSE include Epi catechin and Catechin [10].

According to the conducted studies, the effects of GSE include the following items:

The effect of GSE in increasing re-mineralization of the tooth root surface caries, its local use to facilitate and accelerate skin wound healing, GSE antioxidant effect by inhibiting free radicals, antimicrobial and antiviral effects of GSE (by having compounds such as hydroxy cinamic acids, trans reveratol flavanols, Tannins), and anti-cancer effects particularly substantial protection against the development of carcinogenic stimuli in the skin [11-14]. GSE with amphotericin B has a synergistic effect against fungal infections as well [15]. In addition, GSE effect on improving liver function, reducing infarct size and cardiac arrhythmias, lipid profile and lipid peroxidation in patients with type II diabetes has been reported [16-19].

The conducted toxicological studies show very low toxicity of GSE, so that the lethal dose, Median lethal dose (LD50), of this medicine reaches to more than 5000 mg/kg in mice, as a result, the doses used for medical treatment does not cause any harmful effects [12].

In a case-control study on 0.2 ml of *Candida albicans* yeasts in 20 samples of rats in 2007, Han showed that GSE compound and amphotericin B has a synergistic effect against fungal samples [15].

About the antioxidant properties of grape seed extract, the study of Katsuda et al. in 2015 can be mentioned as one of the most important studies that its results showed the GSE extract can have protective effect on Gingival fibroblasts cells due to its anti- oxidant potential [20].

The significant study conducted by Furiga et al. in 2014 showed that the combination of grape seed extract and amine fluoride (Fluorinol) plays an important role in preventing the formation of dental plaque biofilm [21].

In the case of anti-inflammatory and antimicrobial properties of grape seed extract, a double blind and clinical trial study was done by Hemmati et al. in 2014. In this study, a cream containing 2% of grape seed extract was used to improve surgical wounds on the eighth day for the subject group, and to improve the fourteenth day of the placebo group, and this difference shows the important role of this extract in the process of healing the wound [22].

In the case of anti-viral properties of grape seed extract, a study was done by Joshi et al. in 2015 that is indicative of the anti-viral activity of this extract against hepatitis A [23].

The study results of Benjamin et al. in 2012 showed that grape seed extract decreases demineralization and increases re-mineralization of tooth, and as result, it causes inhibition or regression of dental caries [24].

No studies have been conducted regarding the anti-fungal effect of GSE and its comparison with the fluconazole and Nystatin antifungal drugs. Due to the above reasons and mentioned side effects for antifungal drugs used in local and systemic treatment, the use of anti-fungal drug with fewer side effects in treatments seems necessary.

The objective of the present study is to survey the antifungal effect of GSE on *krusei* and *Candida glabrata*, and a comparison of its effect with fluconazole and Nystatin antifungal drugs. Grape seed extract has important biochemical compounds. In various studies, it has reported that grape seed extract's polyphenolic part has anti-*Candida albicans* property. However, since a similar study has not been conducted on the antifungal effect of this extract against *Candida krusei* and *Candida glabrata*, and the responsible substance has not been identified, an overview of the extract was made in the present study. If the antifungal property of the whole grape seed extract is proven, we will examine the exact components of each of these components by extracting the effective components in the future.

Materials and Methods

Preparation of fungal strains

The fungi used in the present study were standard *Candida glabrata* (BSM 11226) and *Candida krusei* (BSM 70079) standard strains that were provided from Tehran Pastor Institute. To calculate the sample size, estimation for average method was used (according to CLSI protocol). In this study, which was conducted in microbiology lab of Medicine College, fungal strains were passaged in the agar Sabouraud dextrose environment from 24 h ago in order to have live and fresh strains for the test.

Preparation of the extract

To prepare grape seed extract, the grape seeds (*Vitis vinifera*) were washed with water, then chopped. The crude extract between H_2O and n-hexane, were divided to separate the lipoid components. Then, GSE was provided using ethanol 95% and water (water/ethanol, 30/70) as the solvent [25-28]. In order to extract better, the resulting suspension of Erlenmryer for 12 h was placed completely randomized in a shaker incubator. The temperature was set at 27°C and the speed of stirring was 120 rpm. The extracts were first filtered using filter fabric and then using Buchner funnel and a Whatman No. 1 filter paper. Prior to filtration using the filter paper, the extracted oil was allowed to dissolve the solvent based on the difference in density with the solvents used. Most solvents were removed using a vaporizer. The condensate extract was propagated at the surface of the glass plate and then transferred to an oven under vacuum at 40°C. After drying, the extract was scraped off the surface of the plate using a metal blade and placed in a desiccator to achieve a solid dry weight, and then the extraction efficiency was calculated. The powder was stored at -18°C until further experiments were carried out [25].

Macro dilution

To examine the effect of grape seed extract, fluconazole, and Nystatin drugs on these fungi, and to determine MIC (Minimum Inhibitory Concentration), Macro dilution method was used [26]. Next to the flame of oven, and under the hood to prevent contamination of saprophytic fungi in test tubes containing sterile saline, we took an amount of fungal colony, and provided a suspension in physiology serum with the concentration equivalent to 5.0 of McFarland standard. Then, to provide grape seed extract, the provided powder was solved in ethanol solution, and according to the CLSI protocol, we provided ten different concentrations of GSE extract for each *Candida* (20 tubes in total). Moreover, for each *Candida*, we set two positive and negative control tubes (24 tubes, in total) as a witness.

After provision of suspensions, their mixture, and acquisition of the desired concentration of the extract, with all the levels being done under hood, and beside the oven flame, the lids of test tubes were closed (to avoid evaporation), and placed them in incubator at 35°C for 48 h. After passing the mentioned time, transparency and opacity of each of them were studied. In addition, to ensure the growth or lack of growth of fungi in each tube, we cultivated them (Figure 1). It is reminded that this work was done for both *krusei* and *Candida glabrata* with 24 h strain.

Agar well diffusion

In the present study, to review the antifungal effect of grape seed extract, well diffusion method was used. In the method, after preparing the concentration 0.5 McFarland concentration of fungal solution, we diluted it until the 1.5 × 166 cfu/ml concentration. Then, we transferred 500 µl of the provided suspension to the Mueller-Hinton Agar environment (MHA), and cultivated them in three directions using a sterile swab. Then, we created 6 mm diameter wells with the approximate distance of 2.5 cm from each other, in the number of concentrations of grape seed extract tubes 2 to 9 (A total of 8 wells), at the agar surface. In addition, we injected 100 µl of each of the provided concentrations of grape seed extracts into each well. Then, the plates were incubated for 24 h at 37°C. Finally, growth inhibitory zone was examined [29] (Figures 2 and 3). This action was repeated twice [30].

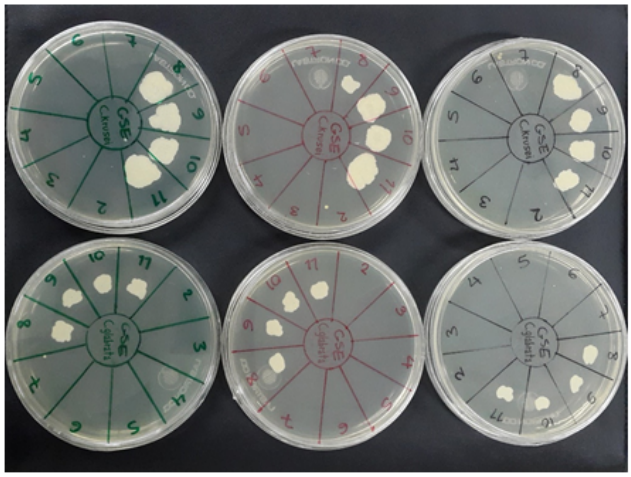

Figure 1. *Determination of the growth or lack of growth of fungi (glabrata and krusei) in different concentration of grape seed at the agar plates*

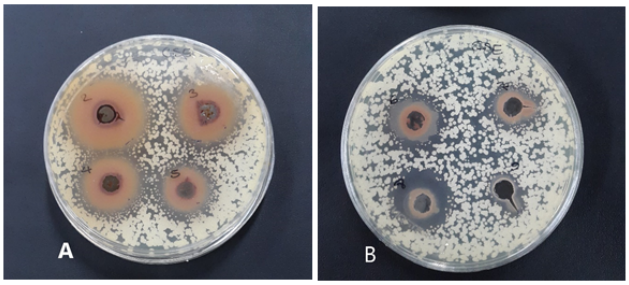

Figure 2. *Agar well diffusion method of Candida krusei. (A) Wells 2 to 5 with concentrations of 1600, 800, 400, 200 µg/ml of grape seed, respectively; (B) Wells 6 to 9 with concentrations of 100, 50, 25, 12.5 µg/ml of grape seed, respectively.*

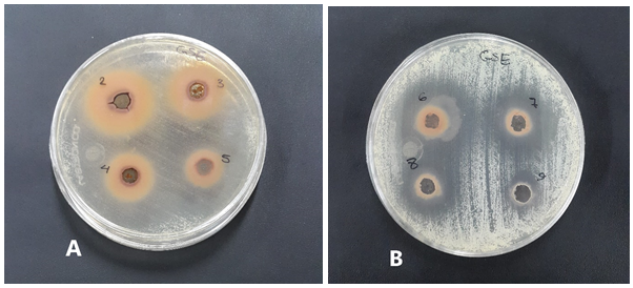

Figure 3. *Agar well diffusion method of Candida glabrata. (A) Wells 2 to 5 with concentrations of 1600, 800, 400, 200 µg/ml of grape seed, respectively; (B) Wells 6 to 9 with concentrations of 100, 50, 25, 12.5 µg/ml of grape seed, respectively.*

Statistical analysis

After recording the mentioned data for each sample, statistical analysis of data was done using version 16 of SPSS statistical software. The data obtained from well diffusion method were analyzed using descriptive statistical (mean ± standard deviation) and variance analysis methods.

Results

Findings

According to the obtained results, it can be concluded that the growth of *krusei* and *Candida glabrata* has been stopped at the concentration of 50 µg/ml (Table 1).

According to Table 1, MIC (the Minimum Inhibitory Concentration) of grape seed extract is 50 µg/ml for both *krusei* and *Candida glabrata* and it is indicative of the same sensitivity of both species of fungi toward grape seed extract.

Table 1. *Growth results of both studied species of Candida, according to the growth status, in various concentrations of grape seed extract.*

Species	Candida growth in different concentrations of GSE (µg/ml)									
	3.12	**6.25**	**12.5**	**25**	**50**	**100**	**200**	**400**	**800**	**1600**
Candida krusei	+	+	+	+	-	-	-	-	-	-
Candida glabrata	+	+	+	+	-	-	-	-	-	-

Note: ⁺*Candida* growth; ⁻*Candida* growth inhibition. The experiments were repeated 3 times and the results were similar.

According to the obtained results, antifungal effect of grape seed extract on both species of *glabrata* and *Candida krusei* is less than Fluconazole and Nystatin standard drugs (Table 2).

Table 2. *A comparison of MIC (based on µg/ml) of grape seed extract with Fluconazole and Nystatin Standard drugs on both species of krusei and Candida glabrata [30].*

Antifungal/agents species	Nistatin	Fluconazole	Grape seed
glabrata	1	32	50
krusei	1	32	50

A comparison of different concentrations of grape seed extract with well diffusion method on two strains of *glabrata* and *Candida krusei* showed that both strains for the studied *Candida* have almost identical bacterial susceptibility compared to grape seed extract due to their similar growth inhibitory zone (Tables 3 and 4). The results related to the maximum inhibitory concentration of MIC (Table 1) confirm the above-mentioned results (Figure 4).

Table 3. *Mean and SD of growth inhibitory zone in millimetres of two fungal strains of grape seed extract in different concentrations. Note: Values=mean ± SD. (The values obtained from the results of two times repeat of experiment).*

Antifungal agent	GSE concentration (µg/ml)								
	6.25	**12.5**	**25**	**50**	**100**	**200**	**400**	**800**	**1600**
Candida krusei	8 ± 0.8	6 ± 0.14	8 ± 0.14	9 ± 0.84	15	16 ± 0.7	17	23 ± 0.07	25 ± 0.08
Candida glabrata	8 ± 1.41	6 ± 0.8	9	10 ± 1.41	11 ± 0.98	14	18 ± 0.8	21 ± 0.42	26

Table 4. *Assessment of antifungal susceptibility in strains of glabrata and Candida krusei in well diffusion method [29].*

Antifungal agent	Well diffusion		
	Resistant (mm)	**Susceptible dose dependent (mm)**	**Susceptible (mm)**
Candida krusei	<10	10-20	24 (>20)
Candida glabrata	<10	11	14 (>11)

Discussion

Oral candidiasis is one of the most common fungal opportunistic infections in mouth. On the other hand, in addition to the problems caused by side effects of common antifungal drugs, the conducted studies have been indicative of the failure of antifungal treatments on Candidiasis different clinical infections and drug resistance including triazoles group (e.g., fluconazole) in the long-term use of them, especially in patients with a weakened immune system problem [1,2,4].

Therefore, today, plants and herbal compounds in the treatment of diseases including fungal infections are used as a potential source for new drugs production [12]. *Vitis vinifera* plant is from Vitaceae family. The grape seed extract obtained from this plant has attracted medical studies and treatments in the recent years [11].

In the present study, anti-bacterial effects of grape seed extract on two species of *glabrata* and *Candida krusei* were studied. The results indicated that grape seed extract has the same effect on both fungal strains of *glabrata* and *Candida krusei* (diameter of inhibition zone of *glabrata* and *Candida krusei*

was almost same, and the minimum inhibitory concentration for both *glabrata* and *Candida krusei* was same). So far, few studies have been conducted about antifungal effects of this plant.

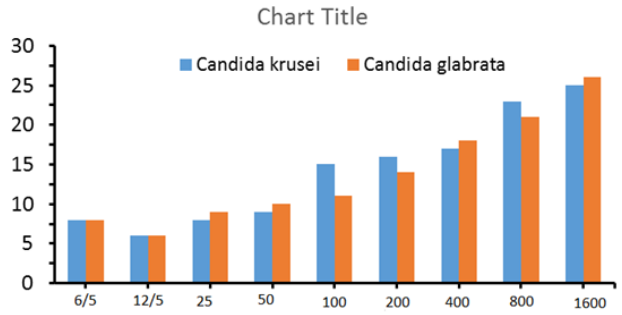

Figure 4. *Candida diameter of inhibition zone (krusei and glabrata) in different concentrations of grape seed extract.*

Some studies have surveyed the anti-bacterial effect of grape seed extract on bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Porphyromonas gingivalis*, *Salmonella typhimurium*, *Streptococcus pyogenes*, *Staphylococcus epidermidis*, *Haemophilus influenza*, as well as fungal strains like *Candida albicans*. The results of these studies indicate that grape seed extract has more inhibitory effect on Gram-positive cocci (especially *Staphylococcus aureus*) rather than gram-negative cocci, so that in 1 mg/ml of this extract, 99% inhibitory effect without return is observed [15,31-36].

The antibacterial effect of grape seed extract on *Streptococcus* mutans in the areas of enamel caries is also investigated. The results of this study showed that grape seed extract has an inhibitory effect on the development of caries in enamel [37].

The results of another study showed that grape seed extract might inhibit the bacteria *Enterococcus faecalis*, one of the main bacteria in root canal of tooth [38]. Proanthocyanidins effect in the extract on *Enterococcus faecalis* resistant to vancomycin has been verified [35].

Anti-viral properties of grape seed extract against viruses hepatitis A, norovirus and Kelsey Flynn virus are also found [39,40].

Unlike previous studies, the study of Sherestha et al. in 2012 showed that grape seed extract is ineffective, or has the least antibacterial effect on bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Candida albicans* and *Candida parapsilosis* [31].

The reason for this difference in results might be due to differences in the following items:

Type and concentration of the used solvent to provide grape seed extract: The type of used solvent can be effective in various properties of the studied extract. The results of previous studies show that although solvents such as methanol, acetone, and ethyl acetate are effective solvents for preparation of grape seed extract, they are not suitable in terms of food and human health applications [41]. Therefore, solvents like water and different concentrations of ethanol were studied and compared. The results of them showed that ethanol solvent in the concentration of 50% has showed the best solubility rather than higher concentrations and water. In addition, it reveals anti-bacterial and anti-oxidant features of grape seed extract better [42].

The temperature used in the preparation of grape seed extract: Besides the type of solvent, the temperature of the applied solvent plays an important role in preparation of this extract; so that the highest amount of distillation was obtained at 80°C, and the lowest amount of distillation was obtained at 25°C [41,43].

Color of grape seed extract: The color of prepared extract can be the result of both color (like Antosianidin) and non-color combinations (like Catecin and Epicatechin) in grape seed extract. In addition, the color of extract is affected by the temperature applied for preparation of this extract; so that with increasing the temperature, color of extract will change from yellow to yellow/red. The results show that by increasing the amount of phenolic compounds present in the extract, the color tone of the extract increases [41].

The method used to investigate the antimicrobial property: There are different types of protocols for assay of antimicrobial drugs and extracts. Moreover, suggested methods for each of them (e.g. CLSI protocol that includes various methods based on the type of microorganisms or the surveyed drug, including agar well diffusion and MIC determination by macro-dilution) is different [44].

Type of the studied microorganism and differences in their drug resistance: For example, there are several species of *Candida* and drug resistance among existing *Candida* of a same species is different in various conditions [45].

In the present study, although we did not review the type of polyphenolic compound found in grape seed that is in charge of anti-fungal property against *Candida glabrata* and *Candida krusei*, other studies like the study of Maeta et al. in 2007, and the study of Okubo et al. in 1991 showed that polyphenolic compound of Epigallocatechin gallate has anti-*Candida* property [46,47].

Similar to results of previous studies, this study also demonstrates antimicrobial and antifungal effects of grape seed extract. Nevertheless, about the effect of grape seed extract, previous studies had been performed on the fungi *albicans Candida*, and there was not any study about other species of *Candida* except *albicans*. Therefore, in this study, we investigated the antifungal effects of the extract on *glabrata* and *krusei* Candia (species with outbreak and drug resistance more than *albicans* especially in patients with immunosuppressive diseases). In the present study, to survey the antifungal effects of the extract, two methods of MIC determination and agar well diffusion were used according to the protocol determined by CLSI (Clinical and Laboratory Standard Institute). The results of both experiments are consistent and indicative of the similar sensitivity of *glabrata* and *Candida krusei* to grape seed extract.

Due to the low side effects and low prices of grape seed extract, and taking into account the results of the study on the effects of the extract on *glabrata* and *Candida krusei*, and considering that *Candida* infections involve most areas of the skin and the oral mucosa; It is hoped that the extract could be used as an alternative to chemical drugs in the treatment of fungal infections in the coming years. Of course, for clinical use of this extract as an antifungal agent, the need to more studies, including clinical trial is inevitable.

Conclusion

The results obtained from the present study showed that both well diffusion method (Agar well diffusion) and the maximum inhibitory concentration of MIC (Macro dilution) are confirming the same antimicrobial susceptibility of both *glabrata* and *Candida krusei* to GSE.

The results of the present study are promising, and it is hoped that in future and by more laboratory and clinical studies, the extract can be used in treatment of fungal infections and as a suitable substitution for chemical antifungal drugs that have many side effects.

Recommendations

1. Although *Vitis Vinifera* is a medical plant that is used in traditional medicine for a long time and it has many remedial applications, to discover other remedial potentials of this drug, wider studies are required.
2. On the other hand, because using organic solvents and sometimes combination of polar and non-polar solvents can make attractive results in the field of antimicrobial properties of plants, using different solvents as well as investigating synergistic effects of grape seed extracts with other drugs and extracts is recommended for further studies.
3. Clinical trial to evaluate the effect of grape seed extract on the treatment of fungal infections in the mouth is recommended.

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Declaration of Conflicting Interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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