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Original Article

In vitro activities of garlic essential oil against *Candida species*

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Pathogenic Candida species are widely distributed in human and animal hosts. Accordingly, they account for 88% of nosocomial fungal infections and are the fourth cause of hospital-acquired blood infections. Fresh Garlic (*Allium sativum*) contains a sulfur compound that is composed of an amino acid (cysteine) called Alliin. The compounds in garlic are divided into two groups of sulfur and nonsulfur-containing types. In this study, the standard specimens of different Candida species were cultured on Sabouraud dextrose agar (Merck) and chromogen agar *Candida*. The extracted essential oil was stored in a sterile container with a lid at 4°C for laboratory analysis. The extraction was accomplished using hydrodistillation by means of Clevenger apparatus. Comparison of the minimum inhibitory concentrations of garlic essential oil showed that *C. albicans* was the most susceptible *Candida* species to this plant essential oil. In other words, garlic essential oil inhibited the fungal growth at the lowest concentration. *Candida species* has been well accepted as the most important etiologic agent of oral candidiasis. Therefore, the control of infections caused by *C. albicans* and *C. glabrata*, as well as the early diagnosis and prevention of candidiasis, is a matter of particular importance. The adoption of an accurate treatment requires the identification of Candida species and genotype analysis of clinical isolates, which also facilitate the evaluation and prevention of candidiasis, especially among inpatients.

Keywords: Garlic essential oil, *Candida species*, Medicinal allergy

Introduction

More than 150 Candida species have been identified today; however, only a few of them are known as pathogens. Based on the evidence, 65% of *Candida species* cannot grow at 37°C; meanwhile, growth potential at this temperature is necessary for the pathogenicity of an infectious agent [1]. The number of pathogenic *Candia species* agents is on a growing trend owing to the identification of new species and variation in their classification (e.g., isolation of *C. dubliniensis* and identification of *C. glabrata*).

Candida species exist as saprophytes in the environment and are often nonpathogenic for humans. Nonetheless, the pathogenic species for humans and animals, especially those with immunodeficiency, are on a rising trend [2]. Among medically valuable yeast species, *C. albicans* can grow in various forms and develop *chlamydoconidia* under suitable growth conditions. Moreover, this species produces spherical yeast, short and long pseudomycelium, true mycelium, and germinated blastoconidia; therefore, it is considered a pleomorphic organism [3]. Moreover, these species have the ability to change between yeast cells and hyphal cells in a laboratory culture medium under different conditions. Various genes have been identified to be involved in morphological processes [4].

Pathogenic Candida species are widely present in human and animal hosts. These species account for 88% of nosocomial fungal infections and are the fourth cause of blood infections among all infectious agents. *Candida* species are acquired at birth, during the voyage through the birth canal, and commonly colonize the gastrointestinal tract. Moreover, they are found in the vagina and urethra, on the skin, and under the fingernails as symbiotic organisms. *C. albicans*, as the most important pathogen in humans, have been isolated from other sources, including fresh water, air, seawater, and soil. Non-sanitary

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conditions sometimes cause the environment, food, and vegetables to be contaminated with this yeast [5]. The frequency of *C. albicans* and other blood-isolated species considerably varies based on the patient's age, as well as local, regional, and global conditions. *C. albicans* and *C. parapsilosis* as nosocomial infectious agents are prevalent among children. *C. glabrata* is more common among middle-aged individuals. *C. albicans* accounts for the majority of nosocomial blood infections in the countries of Asia-Pacific region. On the other hand, in Latin America, *C. tropicalis* and *C. parapsilosis* are more prevalent than other Candida species.

The number and types of *Candida* species causing infection may be affected by patient's age, immune system, exposure to antifungal drugs, and infection control methods. For instance, the use of fluconazole as antifungal prophylaxis may increase the infections caused by *C. glabrata* and *C. krusei*. Furthermore, the lack of attention to infection control precautions and improper care of vascular catheters can raise the prevalence of C. parapsilosis infection [5]. *C. albicans* are able to grow in a wide range of pH (<2 to about 8) under both anaerobic and anaerobic conditions. Glucose, galactose, and sucrose are essential for the growth of the fungus. Carbohydrate degradation occurs through glycolysis and the tricarboxylic acid cycle and affects the cyanide-resistant respiratory pathway [6]. Absorption of amino acids and peptides in *C. albicans* is carried out by low- and high-affinity permeases [7]. *Candida albicans* produces various enzymes, one of the most important of which is secreted aspartyl proteinase, which is also produced by *C. dubliniensis*, *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis*. Proteinases secreted by *Candida* species cause non-specific proteolysis of host proteins, which are involved in host defense against infection [8].

Garlic (*Allium sativum*) contains sulfur compounds, 97% and 0.7-0.15% of which are water- and oil-soluble, respectively. Garlic owes its properties mainly to the presence of sulfur compounds, namely allicin with the chemical name of S-Allyl-L-cysteine sulfoxide, as well as organic acids, carbohydrates, and vitamins.

Allicin is one of the main components of fresh and crushed garlic with a wide range of effects, including genotoxic, antiapoptotic, antimicrobial, antiviral, antifungal, and antiparasitic properties. Accordingly, this compound entails antifungal properties against C. albicans. Garlic compounds are divided into two major groups of sulfur-containing and nonsulfur-containing. The main constituents of garlic essential oil include diallyl sulfide, methyl allyl disulfide, and dimethyl trisulfide.

These substances can split the outer layer of fungal and bacterial liposaccharides, followed by outer membrane degradation and cytoplasmic leakage [16]. Azoles (e.g., fluconazole, ketoconazole, and itraconazole) have selective toxicity against fungi because they interfere with the synthesis of ergosterol, a sterol uniquely found in the fungal membrane, and do not affect the cell membrane. These antifungal agents disturb the permeability of the fungal cell membrane through the inhibition of ergosterol production.

Among these drugs, fluconazole exerts its antifungal effect by the inhibition of the cytochrome P-450 enzyme, which leads to the disruption of ergosterol production, as a vital component in the fungal cell membrane [16]. Based on the evidence, it seems that garlic essential oil can inhibit the growth of *C. albicans*, *C. glabrata*, and *C. tropicalis* through the mentioned mechanism, and therefore be used as an effective antifungal agent.

Materials and Methods

In this study, the effect of garlic essential oil was evaluated by two methods, namely disk diffusion and broth macrodilution. The investigated Candida species included *C. albicans* (PTCC 5027), *C. glabrata* (PTCC 5297), and *C. tropicalis* (ATCC 13803).

The samples were obtained from the Department of Microbiology of Islamic Azad University of Arak, Iran. The standard specimens were cultured on Sabouraud dextrose agar (Merck) [17]. After purchasing the garlic, they were examined in terms of purity. To prepare the essential oil of this plant, 50 g of powdered garlic was added to 600 ml distilled water in a 2-liter flask and then subjected to hydrodistillation for 4 h in a Clevenger apparatus. Subsequently, the extracted essential oil was kept in a sterile container with a lid (to preventing escaping) at 4°C.

In the next step, 100 g of the crushed plant was poured into a volumetric flask, followed by the addition of 700 ml distilled water. Given the lack of minerals in distilled water, it was used in the present study to prevent any effect on essential oil when exposed to heat. The determination of minimum inhibitory concentration (MIC) was accomplished according to the National Committee on Clinical Laboratory Standards and M44-A protocol [10]. As stated by the Clinical and Laboratory Standards Institute, the results of

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this method are 95% similar to those obtained by broth dilution and Etest.

Mueller-Hinton agar with 2% glucose was used to increase Candida growth. A yeast standard suspension of 106 CFU/ml was prepared in sterile tubes containing physiological serum using the spectrophotometric method. Then, swabs were immersed into the suspension, and transferred onto the culture medium. The yeast cells were cultured in completely homogeneous form and in all directions.

In the next stage, 30 µl of essential oil was added to the sterile blank paper disc (Padtan Teb Company, Iran). After the absorption of essential oil into the paper disc, it was placed at the center of the plate by means of a sterile forceps and then incubated for 24-48 h at 35°C. Following the incubation, the diameter of the growth inhibition zone was measured in millimeter by means of a ruler [11].

This experiment was performed on all three *Candida* species, and the results were recorded. To prepare the essential oil dilution, based on previous studies, two-fold dilutions were used with a concentration range of 250-1000 µg/ml using the Sabouraud dextrose broth. The essential oil was dissolved and homogenized in this medium by means of dimethyl sulfoxide with a proportion of 1:10. Furthermore, the yeast susceptibility was evaluated by means of a dilution of essential oil with standard yeast suspension (1:1).

Candida species were cultured again in Sabouraud dextrose agar and incubated at 35°C for 18-24 h. The inoculated suspension was prepared by harvesting five colonies with a diameter of about 1 mm from fresh cultures in 10 cc physiological saline (0.85%). The suspension was vortexed for 15 Sec, and cell density was determined by 0.5MacFarland technique or spectrophotometric method at 530 NM. The number of yeast cells was 1×106-5×106 cells/ml.

In the next step, the dilutions of 1:100 and 1:20 were respectively prepared from the above suspension; therefore, the final density of yeast cells per ml was obtained at 0.5-2.5×103CFU/ml. To determine the minimum fungicidal concentration (MFC), the contents of the MIC dilution, as well as those of ±1 dilution of the reference MIC were cultured on the Sabouraud dextrose agar medium and then incubated at 37°C for 24 h. The highest dilution of the essential oil that inhibited the growth of the Candida species was considered as MFC. The obtained data were analyzed separately in SPSS (18) using one-way ANOVA. The charts were also drawn by means of the SPSS version 16.

Results

One-way ANOVA analysis was used to measure the diameter of the fungal growth inhibition halo at different concentrations of garlic essential oil. A larger growth inhibition halo is indicative of a higher fungal susceptibility to the antimicrobial agent; therefore, MIC and MFC would be lower, which denotes that the essential oil has a higher antimicrobial effect in a lower concentration and that microbes disappear at lower concentrations (Figure 1).

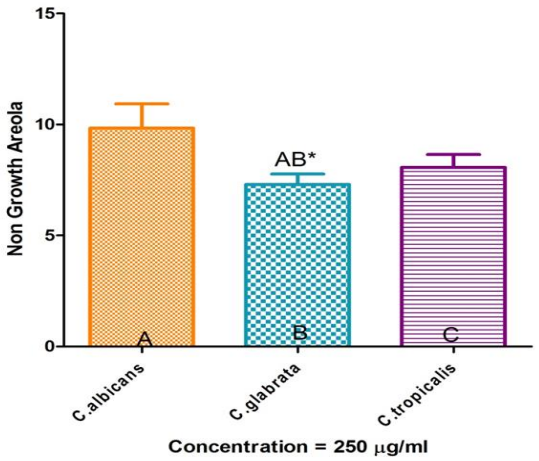


Figure 1. Comparison of the growth inhibition zones of garlic essential oil at a concentration of 250 µg/mL against *Candida albicans*, *C. glabrata* and *C. tropicalis*

The results of One-Way ANOVA revealed that at a concentration of 250 µg/ml, garlic essential oil had a larger inhibition zone against *C. albicans* than against *C. tropicalis*. However, their statistical comparison showed that this difference in halo size was not statistically significant between the two species ($p>0.05$). Furthermore, the inhibition zone of garlic essential oil (250 µg/ml) was larger against *C. tropicalis*, compared to that against *C. glabrata*. Nonetheless, this difference was not statistically significant between the two *Candida species* ($p>0.05$). Additionally, this essential oil (250 µg/ml) displayed a significantly larger zone of inhibition against *C. albicans* than against *C. glabrata* ($p=0.0386$).

As displayed in Figure 2, at a concentration of 500 µg/ml, garlic essential oil produced a higher inhibition zone against *C. tropicalis* than against *C. glabrata*. Nevertheless, this difference was not statistically significant between these species ($p>0.05$). Furthermore, the zone of growth

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inhibition of garlic essential oil (500 µg/ml) was significantly larger against *C. albicans*, compared to those against *C. glabrata* and *C. tropicalis* (p=0.0273and p=0.0488, respectively).

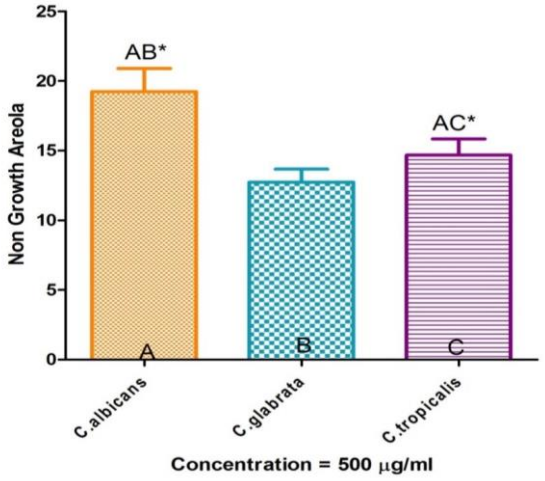


Figure 2. Comparison of the growth inhibition zone of garlic essential oil at a concentration of 500 µg/ml against *Candida albicans*, *C. glabrata*, and *C. tropicalis*

According to the results of the one-way ANOVA, garlic essential oil at a concentration of 1000 µg/ml showed a larger zone of growth inhibition against *C. albicans* than against *C. glabrata* and *C. tropicalis* (P=0.004 and P=0.0425, respectively) (Figure 3). These differences were revealed to be statistically significant. Furthermore, the growth inhibition zone of this plant essential oil (1000 µg/ml) against *C. tropicalis* was larger than that against *C. glabrata*. The statistical comparison of the two species revealed that they were significantly different in this regard (P=0.0383).

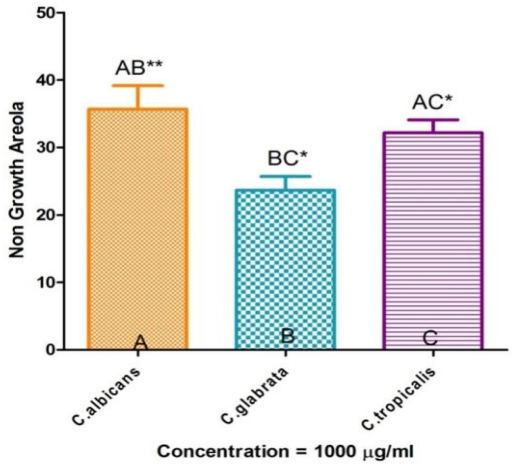


Figure 3. Comparison of the inhibition zone of garlic essential oil at a concentration of 1000 µg/ml against *Candida albicans*, *C. glabrata* and *C. tropicalis*

Minimum Inhibitory Concentration and Minimum Fungicidal Concentration

The MIC and MFC of the essential oil were determined using the macrodilution broth method (Figures 4 and 5, Table 1). Comparison of the MIC of garlic essential oil showed that *C. albicans* had the lowest MIC value (0.4 µg/ml); therefore, this Candida species were concluded to have the highest susceptibility to garlic essential oil among the other investigated species (i.e., *C. tropicalis* and *C. glabrata*). In other words, the lowest concentration of garlic essential oil was effective in the inhibition of fungal growth. Furthermore, *C. tropicalis* had a lower MIC value (0.5 µg/ml) than *C. glabrata* (0.6 µg/ml); accordingly, it was more susceptible to garlic essential oil, compared to *C. glabrata.*

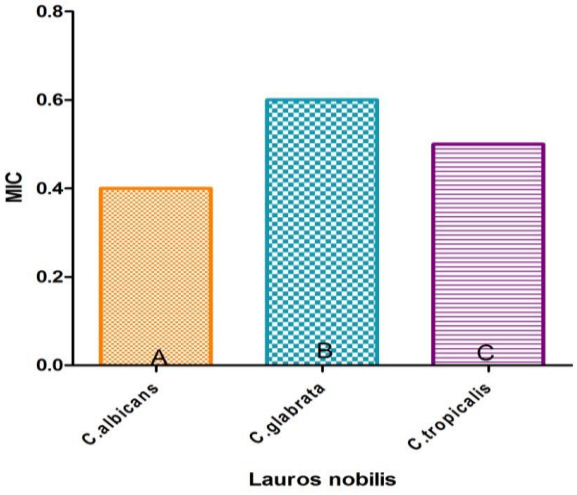


Figure 4. Comparison of minimum inhibitory concentration of garlic essential oil against *Candida albicans*, *C. glabrata* and *C. tropicalis*

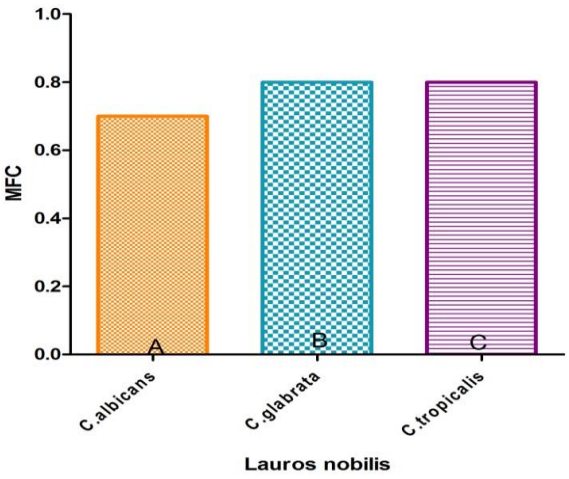


Figure 5. Comparison of minimum fungicidal concentration of garlic essential oil against *Candida albicans*, *C. glabrata* and *C. tropicalis*

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Table 1. Effective concentrations of garlic essential oil against the studied species by disk diffusion and macrodilution (µg/ml)

	Inhibition zone (mm) at 250µg/ml	Inhibition zone (mm) at 500µg/ml	Inhibition zone (mm) at 1000µg/ml	MIC (µg/ml)	MFC (µg/ml)
C.albicans	12	22.5	42	0.4	0.7
(mm)	8.5	17	30		
	9	18.2	35		
C.glabrata	6.4	11	20	0.6	0.8
(mm)	8	14.2	27		
	7.5	13	24		
C.tropicalis	7	12.5	28.5	0.5	0.8
(mm)	8.3	15	33		
	9	16.5	35		

MIC: minimum inhibitory concentration,
MFC: minimum fungicidal concentration

Comparison of the MFC of garlic essential oil demonstrated that *C. albicans* had the lowest MFC (0.7 µg/ml) among the other species. Therefore, *C. albicans* was the most susceptible species to garlic essential oil. In other words, the lowest concentration of garlic essential oil was effective in the inhibition of this fungus.

Discussion

The purpose of the present study was to investigate The main objective of this study was to identify the effect of garlic essential oil on *C. albicans*, *C. glabrata*, and *C. tropicalis* and determine the susceptibility of these species to this plant essential oil and the related MIC and MFC values. Today, there are many reports regarding the failure in the treatment of patients with different clinical forms of candidiasis [12].

There are various antifungal drugs with different formulations for the treatment of this disease. However, in many cases, due to unresponsiveness to treatment, the disease becomes chronic and acute or sometimes recur. Nasir Wabe et al., (2011) studied the susceptibility pattern of *C. albicans* isolated from the oral cavity of HIV-infected patients in Ethiopia. To this end, they evaluated 42 isolates by microdilution broth and reported the resistance rates of 11.9%, 7.1%, 2.3%, and 4.7% to fluconazole, ketoconazole, amphotericin B, and nystatin, respectively [13]. The need for the prolonged use of antifungal drugs, which itself leads to side effects, has created limitations in the use of such compounds [14].

Epidemiological studies have shown that most of the major fungal infections are caused by the species that are resistant to antifungal medications. This issue has been confirmed, especially in terms of *C. albicans* and its resistance to fluconazole [15]. As a result, in recent years, researchers have focused on finding natural herbal compounds with fungal growth inhibitory properties. To this end, various types of plants, as well as plant extracts and essential oils, have been successfully used to inhibit fungal growth in laboratory conditions [17].

In the present study, using the disk diffusion method, the administration of 250, 500, and 1000 µg/ml of this essential oil on the culture medium produced an acceptable growth inhibitory zone against *C. albicans*, *C. glabrata*, and *C. tropicalis*. At a concentration of 250 µg/ml, no significant difference was observed among the three species in terms of inhibition zone. Nonetheless, the comparison of *C. albicans* with *C. glabrata* showed a significant difference between them in this regard; accordingly, *C. albicans* was more susceptible to garlic essential oil, compared to *C. glabrata*. At the concentrations of 500 and 1000 µg/ml, this difference between *C. albicans* results and those of *C. glabrata* and *C. tropicalis* was statistically significant.

Furthermore, there was a significant difference in the inhibition zone between *C. glabrata* and *C. tropicalis* at the concentration of 1000 µg/ml. The results of the MIC test showed that *C. albicans* had the lowest MIC value (0.4 µg/ml); therefore, it was the most susceptible species to garlic essential oil. In other words, the lowest concentration of garlic essential oil was effective in the growth inhibition of this species. In addition, *C. tropicalis* had a lower MIC value (0.5 µg/ml), compared to *C. glabrata* (0.6 µg/ml); consequently, this species had a higher susceptibility to garlic essential oil than *C. glabrata*. The results of MFC revealed that garlic essential oil at a concentration of 0.7 µg/ml had the greatest effect against *C. albicans*, compared to the other two species.

Iacobellis et al. investigated the antimicrobial activity of ajwain essential oil by means of agar diffusion method. They reported that the oil of ajwain showed a relatively high inhibitory effect against Rhodotorula, Erwinia, Xanthomonas, and Agrobacterium [8]. Likewise, Saksena et al. confirmed the antifungal activity of ajwain against dermatophytes [16]. Mahboubi et al. (2011) showed the antimicrobial and antifungal effects of ajwain and Thymus essential oils on a number of Gram-positive and Gram-negative bacteria (ATCC), as well as *C. albicans* (ATCC 10231), C. glabrata, and *Aspergillus* using broth microdilution. The results of the mentioned study revealed that ajwain essential oil had higher antimicrobial effects, compared to Thymus [11].

It has been well accepted that *Candida* is the most important pathogenic agent in oral

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candidiasis. Therefore, control of *C. albicans* and *C. glabrata* infections, along with the rapid diagnosis and prevention of candidiasis, is a matter of paramount importance. The implementation of accurate treatment requires the identification of *Candida* species and genotype analysis of clinical isolates, which also facilitate the evaluation and prevention of candidiasis, especially among inpatients.

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Conflicts of interest

None declared.

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