Inhibitory effect of hydroalcoholic extract of Olive leaf (*olea Olea europaea*) on growth of *Candida albicans*

Abstract

Introduction and aim: *Candida albicans* is the most common and pathogenic species of *Candida* genus, known to be the fourth most common cause of blood infections. The present study investigates the inhibitory effect of hydroalcoholic extract of Olive leaf on growth of this yeast in vitro.

Materials and Methods: In this experimental study, fresh Olive leaf were collected from its natural habitat in Gotwand city and after washing the leaves were dried in a sterile environment. After verifying the plant species and receiving the herbarium code, the leaf of the plant was completely ground and the 80% hydroalcoholic extract of the plant was prepared by maceration method. Serial dilutions of extract were then prepared in RPMI 1640 medium from 256 to 1 mg/**ml**mL. Afterwards, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of this extract for fungus was achieved after 48 hours exposure of the extract to the yeast Suspension using micro broth dilution method and re-culturing on Sabouraud dextrose agar (SDA) medium and colony counting.

Results: After counting the yeast colonies grown on Sabouraud dextrose agar and performing one-way ANOVA statistical analysis at the significance level less than 0.05, Although all concentrations of equal to or greater than 4 mg / **ml** mL of extract had a significant difference from positive control colonies count, according to the concepts of MIC and MFC, concentrations of 8 and 32 mg / **ml** mL were considered as them, respectively.

Conclusion: Olive seems to be a proper complementary drug in the treatment of *Candida albicans*. Needless to say, this requires extensive pharmacological studies and the evaluation of the possible toxicity of the derivatives and compounds of this plant.

Keywords: olives, *Candida albicans*, medicinal plants, fungicides, microbial sensitivity tests.

Abbreviations

MIC (mg/**ml**mL): Minimum inhibitory concentration

MFC (mg/mL**l**): Minimum fungicidal concentration

SDA: Sabouraud dextrose agar

ATP: Adenosine triphosphate

Introduction

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Candidiasis has been rising in the world in recent decades and is one of the major causes of mortality among special patients [1]. Among the various *Candida* species, ***Candida C. albicans*** is one of the most common commensal species of humans and also the most pathogenic one [2,3]. It is one of the major causes of oral and systemic candidiasis [2]. ***Candida C. albicans*** have been identified as the fourth cause of blood infections in clinical settings among the causes of acquired hospital infections [4]. It also causes more than 400,000 cases of invasive diseases annually, the mortality rate of which is high [5,6]. ***Candida C. albicans*** is normal flora of mucosa, and exists as an opportunistic pathogen in the gastrointestinal mucosa of healthy people [2,4]. But this fungus causes infections in patients and individuals with underlying conditions such as diabetics, the elderly, pregnant women, patient with immunodeficiency, and patients who use anticancer drugs [2,4,7,8]. Being infected with Candidiasis (as an important factor in hospital infections) can cause an infection in the circulatory system of patients. This led to an increase in the length of hospital stay and a significant increase in the cost of treatment for the individual, which in some cases also leads to death [9]. ***Candida C. albicans*** is also able to stick to various medical devices and form biofilms of the fungus in implants, urinary catheters, central vascular catheters, pacemakers, artificial heart valves, joint prostheses and contact lenses [4]. One of the most important natural resources suggested by researchers and pharmacists is herbal medicine, and if they are used at a specific dose, they will show the highest levels of compatibility to the immune system and these plants are able to reduce the side effects of chemical drugs [10,11]. Today, traditional medicines are widely used, and plants are still considered as an important source of antioxidants that can play a major role in the development of new drugs [**11**12-14]. Olive leaf extract is widely used in medicine against microbial diseases, which is due to the presence of polyphenols including Oleuropein, Hydroxytriazole and their derivatives [**12,13**15,16]. Among phenolic compounds, Oleuropein is the most widely used compound in Olive leaves [**14**17]. These compounds are antioxidant, anti-inflammatory, anti-diabetes, and have antimicrobial activity against bacteria, fungi, mycoplasma and viruses, especially in the digestive and respiratory tract [**13**16, **15-17**18-20]. In recent years, due to drug resistance and side effects associated with the use of chemical drugs, progress has been made in the field of medicinal plants, which might also prove useful as antifungal compounds [**18**21]. Therefore, in view of the increasing trend of candidiasis in different clinical forms and their increased resistance to common chemical drugs and their impact on community health, in this study the inhibitory effect of hydroalcoholic extract of Olive leaf on growth of ***Candida C. albicans*** has been investigated.

Materials and methods

Preparation of Olive leaf extract

At first, Olive was collected from its habitat in Gotvand, in Khuzestan province. For confirmation of the accuracy of the herbal sample, it was sent to the Pharmacology Department of the Faculty of Pharmacy, Ahwaz University of Medical Sciences. *Olea europaea* L. of the *Oleaceae* family was identified as an Olive plant and was registered with JPS016101 in the herbarium. Maceration method was used to prepare the hydroalcoholic extract of Olive leaves. First, the plant was dried in a sterile environment and then completely milled and 100 g of plant

powder were mixed in one liter of 80% ethanol and divided into two 500 **ml** mL sterile Erlenmeyer flask. The flasks were placed in a shaker incubator (Arian Andish Co., Iran) for 3 days at 25 ° C. After being filtered through sterilized gauze pads several times, the remaining plant particles were removed from the mixture using the Whatman paper(No.1), and a clear pure solution was obtained. This solution was placed in incubator at 30 ° C for condensation and solvent removal and final extraction. Finally, the hydroalcoholic extract of the Olive plant weighing five grams, equal to five percent of the initial weight of the plant, was obtained [**18**21].

Preparation of Yeast Suspension

Candida albicans yeast strain ATCC 10231 was prepared from the Department of Medical Mycology, Tarbiat Modares University and cultured in the Sabouraud dextrose agar (SDA) medium and incubated at 35 ° C for 24 hours. In order to obtain standard cell suspension for use in the test for determining the susceptibility of the fungus to the Olive extract, fresh colonies were harvested at 24 hours and were washed with the sterile phosphate buffered saline by centrifugation for 10 minutes at a speed of 1,500 rpm for three times. Then, using standard RPMI 1640 without red phenol and containing 0.2% glucose, standard yeast suspension was prepared and the concentration of these suspensions was determined using cell counting with Neobar slides, being about 3×10^3 cells per ml of liquid culture medium, which was twice as high as the optimal final concentration. In the next steps, due to the addition of other compounds to the test tube, this concentration was reduced to 1.5×10^3 cells per ml [**18**21].

Preparing serial dilutions of Olive leaf extract and exposing yeast suspension to the dilutions

At this stage, approximately 2048 mg of hydroalcoholic extract of Olive leaf was dissolved in 2 ml of RPMI 1640 without phenol, containing 0.2% glucose that was produced final concentration of 1024 mg per ml. Next, 100 **µl** µL of RPMI 1640 medium was added to 11 wells of a 96-well U-shaped microplate each. Then 100 **µl** µL of prepared concentration of extract was added to the first well, which decreased the extract concentration to 512 mg per **ml**mL. After mixing, 100 **µl** µL of the solution in the first well was transferred to the second well, and the technique was continued down to the ninth well and 100 **µl** µL was discarded out of the ninth well. 100 **µl** µL of yeast suspension was then added all the 9 wells. Thus, the same concentration of yeast cells (1.5×10^3 cells per **ml**mL) with serial dilutions of Olive leaf extract with final concentrations of 256, 128, 64, 32, 16, 8, 4, 2 and 1 mg per **ml** mL was achieved. The 10th well was merely containing yeast suspension and culture medium, used as a positive control. The 11th well contained the highest concentration of Olive leaf extract and culture medium and lacked yeast cells, were used as negative control. The above mentioned steps were performed on three separate well series (triplicate) [**18**21].

Determining MIC and MFC using micro-dilution

Microplates were incubated at 35 ° C for 48 hours and then, 20 **µl** µL of the wells were cultured on three series (triplicates) on the Sabouraud dextrose agar to determine the MIC and MFC, and after 24 hours, the colonies count were compared. The cultures were kept for up to 48 hours and were re-examined for colonies count [**18**21].

Statistical analysis

In order to study the significance level of the difference in the count of grown colonies of ***Candida*** *C. albicans* on the Sabouraud dextrose agar from samples exposed to different concentrations of Olive leaf hydroalcoholic extract, one-way ANOVA test was performed on the obtained data.

Results

Determining MIC and MFC results

The Determination of the effects of different dilutions of the hydroalcoholic extract of Olive leaf on the *Candida albicans* yeast suspension were not possible by spectrophotometric assay due to the color darkness of the hydroalcoholic extract of the plant. Therefore, the results were analyzed by re-culture on the SDA for colony counting (Table 1). MIC and MFC were determined based on the count of colonies grown on that medium for each dilution of the hydroalcoholic extract of Olive leaf and compared with the positive control sample. The first dilution of the extract in which the count of yeast colonies grown in the medium reached half that of the positive control, was considered as the MIC, and the first dilution of the extract in which no yeast colony was grown on the medium, was considered as MFC (Table 2).

Table 1. The count of grown colonies on the SDA from wells with different concentrations of Olive leaf extract

Concentrations of Olive leaf extract (mg/mlmL)	**256**	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**Positive control**
Count of yeast colonies	0±0	0±0	0±0	0±0.5	3±1.2	12±1.4	20±1.2	28±2.7	31±2.9	31±3.1

Table 2. MIC and MFC results of the exposure of hydroalcoholic extract of Olive leaf to ***Candida*** *C. albicans*

MIC/ MFC	**MIC**	**MFC**
Concentration of Olive leaf extract (mg/**ml**mL)	8±0	32±0

After performing one-way ANOVA and considering the significance level less than 0.05, all concentrations of equal to or greater than 4 mg / **ml** mL of extract had a significant difference from positive control regarding the count of grown colonies.

Discussion

Evaluation of the sensitivity or resistance of the pathogen to the drug requires performing in vitro sensitivity test. However, MIC is only able to predict the clinical outcome of a medication in some cases. There are various variables affecting the antifungal efficacy of the medication at the clinic. The advent of drug resistance to common synthetic drugs, particularly in the ***Candida*** *C.*

genus, as well as adverse side effects that have always been taken into consideration, has led researchers more than ever to research in the field of medicinal herbs [18,21].

The Olive tree and its products have had positive health effects over the past years. There are compounds such as Oleuropein in Olive leaves that prevent growth of molds and yeasts [13,16]. The results of this study showed that with increasing concentrations of Olive leaf extract, the growth rate of the fungus decreases. Comparison of wells containing the Olive leaf extract with wells lacking the extract, demonstrated its ability to inhibit growth of the fungus. As the results show, the first well indicating a significant difference than the positive control in the count of yeast colonies, had a concentration of 4 mg / **ml**mL. However, the reason for rejecting it as MIC is that the count of colonies obtained from this well is more than half the count of colonies derived from positive control which contradicts the concept of "minimal inhibitory concentration ". Therefore, the concentration of 8 mg / **ml** mL of the hydroalcoholic extract, whose count of grown colonies was less than half that of the positive control, is considered as a MIC. The minimum fungicidal concentration of the fungus (MFC) was equivalent to 32 mg / **ml** mL for this extract, at which no yeast colony had grown.

So far, several other studies have been carried out to find the antifungal effects of this plant against *Candida* yeast, among which Nasrallahi et al. [16] **(2009)** studied the inhibitory effect of aqueous extract of Olive leaves on growth of a fluconazole resistant strain of ***Candida*** *C. albicans* and reported MIC and MFC for the aqueous extract of this plant to be 24 and 48 µg/**ml**mL, respectively [13,16]. However, in the present study, considerably higher amounts of hydroalcoholic extract of this plant were concluded as MIC and MFC.

Zorić et al., in a study indicated that MIC for Oleuropein as one of the derivatives of Olive against ***Candida*** *C. albicans* was found to be at least 12.5 mg / **ml**mL. They also revealed that exposure of yeast to this compound caused morphological changes in the fungal cell nucleus and cellular death process (apoptosis) of the fungus is observed when exposed to different concentrations of this compound. Yeast adhesion to epithelial surfaces has also been reduced, which has been shown to inhibit some important fungal virulence factors, including yeast hydrophobic power, as well as inhibition of secretory aspartate proteinases (SAPs) as another pathogenicity factor. Finally, it has been suggested in the study that exposure of yeast to Oleuropein decreases the amount of sterols in the cytoplasmic membrane of the fungus, and therefore it is likely that the antifungal property of this compound is also related to inhibiting the membrane sterol synthesis pathway [19,22]. It needs to be noted that the MIC value obtained from the study of Zorić et al. in inhibiting yeast growth by only one of the polyphenolic derivatives of the Olive tree, yielded higher values than MIC when exposed to total hydroalcoholic extract of this plant in this study. This might be attributed to a synergistic effect between the compounds in the total hydroalcoholic extract of the Olive plant.

Moreover, in the study by Korukluoglu et al. [23], Olive leaf extract was prepared using various solvents (water, ethanol, acetone and ethyl acetate) and the antimicrobial effects of these extracts were examined by sensitivity test. According to the results of this study, the yeast was susceptible to acetone and ethyl acetate extract and the MIC of ethyl acetate, acetone and ethyl alcohol of Olive leaf for ***Candida*** *C. oleophila* were obtained as 23, 12 and 28 µg / **ml**mL,

respectively [**20**23]. However, contrary to the results of Nasrallahi et al. [**13**16], no sensitivity was observed for the aqueous extract. The difference can be due to the difference in the performance of the researchers in various stages of the experiment or because of the use of two different methods (MIC and disk diffusion test) to evaluate the susceptibility of this fungus to the aqueous extract of Olive leaves. Nevertheless, the MIC of hydroalcoholic extract obtained by Korukluoglu et al. [23], has shown substantially lower values than the results of this study. **[20].**

In the study by Al-Waili, a mixture of honey and Olive oil was capable of inhibiting the growth of ***Candida*** *C. albicans* and ***Staphylococcus*** *S. aureus*. Olive oil alone reduces fungal growth, but combining it with a concentration of %66 of honey prevents fungal growth and creates a no-growth region of 3.5 mm. The results of a study also show that Olive oil has anti ***Candida*** *C. albicans* effects [**21**24]. Research demonstrates that the polyphenolic compounds in the plant and fruit of Olive do not only affect the growth of fungi, but also affect the growth of other microbial groups. In 2017, Amini et al. [25], did a broad study of the effects of some polyphenolic compounds in Olive, including Oleuropein, on inhibition of multi-strain growth from *Escherichia coli*. Not only did they prove the inhibitory effects of these compounds on the growth of the bacteria, but they also revealed their mechanism of action which is affecting the vital enzyme ATP synthase. **[22].**

Conclusion

According to the present study and the results of other studies, it is possible that the Olive plant may be considered as an appropriate drug supplement or even as a substitute for chemical drugs in the treatment of ***Candida*** *C. albicans* diseases. This, nonetheless, requires extensive pharmacological studies and evaluation of the possible toxicity of the derivatives and compounds of this plant. Therefore, it is recommended that detailed precautions be taken to fully identify the compounds in this plant and also to conduct in vivo tests to investigate the potential use of the drug. The limitations of this study were to investigate the effect of olive extract on a type of fungus. Therefore, it is suggested that more extensive pharmacological studies be carried out on other microbial and fungal species.

Conflict of interest

All authors declare no conflicts of interest in this paper.

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