In vitro Anti-Candidal effects of Aqueous and Methanolic Extracts of Walnut (Juglans regia) Tree Fruit Peel in Comparison with Fluconazole

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ABSTRACT

The appearance of fungal species resistant to antifungal drugs among the Candida genus and also their numerous side effects, convince the researchers to work on new therapeutic methods with minimal side effects for humans fungal infections. The aim of this study was to assess of antifungal activities of aqueous and methanolic extracts of walnut Fruit Peel on common Candida species. In this study, the antifungal effects of aqueous and methanolic extracts of fruit peel of walnut (Juglans regia) and fluconazole against four Candida species were evaluated using broth Microdilution method, based on the Clinical and Laboratory Standards Institute (CLSI) M38-A3 guideline. And then the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of these extracts and antifungal drug were obtained for tested Candida species. Both aqueous and methanolic extracts of fruit peel of walnut showed antifungal effects against Candida species. The MIC of fluconazole, aqueous and methanolic extracts of walnut fruit peel for different Candida species were 0.001-0.032, 6.25-50, 3.125-25 mg/ml, respectively. The MFC of fluconazole, and methanolic extracts of fruit peel of walnut were 0.001-0.032 and 6.25-25 mg/ml, respectively. The aqueous extracts of walnut fruit peel were without fungicidal effects. The most important result of this study is to show that fruit peel extracts of walnut are effective against named fungal species and it looks promising that in future, we can obtain some effective antifungal agents with minimal side effects from shallot extract.

Keywords: Candida, walnut (Juglans regia), aqueous and methanolic extracts, broth microdilution method

INTRODUCTION

The incidence and prevalence of invasive fungal infections have been increasing since the 1980s, especially in immunocompromised patients and hospitalized patients with serious underlying diseases [1, 2]. Candida species have isolated from healthy individual’s mucosal oral cavity, gastrointestinal tract and vagina as normal flora [3] and are responsible for various clinical manifestations e.g. mucocutaneous infections, bloodstream infections [4]. More than 17 different Candida species are isolated from human infections; however, approximately 90% of invasive infections are caused by 5 species including; Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis and Candida krusei[5].The factors that contribute to the increase of these infections are immunocompromised patients with intravenous catheters for long times, increasing use of broad-spectrum antibiotics, cytotoxic chemotherapies and transplantation [6].
Currently, an increase in the number of resistant yeasts to antifungal drugs is identified worldwide [7]. Therefore, new antimicrobial agents are needed for treatment of humans and animals infections by drug resistant pathogens [8]. In the recent decades, there has been much interest in natural products as new antimicrobial agents. Antimicrobial effects of different extracts from traditional medicinal plants have been tested and proved [9]. Main factors for development of new antimicrobial agents with natural origin are including: the increasing resistance to available drugs, the problems caused by antimicrobial agents added in food and public's pressure on the food industry to produce products without chemical preservatives [10, 11].

The Juglans genus (family Juglandaceae) includes several species and is distributed worldwide [12]. Walnut (Juglans regia L.) Fruit Peel has several properties such as anti-inflammatory, anticancer, blood purify, diuretic, depurative, and laxative activities [13]. Some studies have proved the antimicrobial effects of walnut products, particularly of Fruit Peel [14, 15]. The extract of walnut Fruit Peel inhibited the growth of several Gram-positive bacteria and Gram-negative bacteria [16].

The aim of this study is evaluation of anti-candidal activities of aqueous and methanolic extracts of walnut Fruit Peel on common Candida species including: Candida albicans, Candida glabrata, Candida tropicalis and Candida krusei.

**MATERIALS AND METHODS**

The Fruit Peel of walnut tree (Juglans regia) was collected from Bardaskan city, Khorasan Razavi province, Iran.

**Extraction**

The Fruit Peel of walnuts were dried and ground into a powder by a blender. The powder was extracted by Maceration method for 48 hours. The extraction was performed using two solvents; water and methanol 70%. The methanolic and aqueous extracts were filtered and evaporated to produce pure extracts by a vacuum rotary evaporator. Afterwards, the extracts were dried and stored at -20°C.

**Microorganisms and Inoculum preparation**

The Candida species, including Candida albicans (PTCC 5027), Candida glabrata (PTCC 5297), Candida tropicalis (PTCC 5028) and Candida krusei (PTCC5295) were obtained from the Iranian Research Organization for Sciences and Technology, Tehran, Iran.

Stock inoculum suspensions of the Candida species were obtained from 24h cultures on Sabouraud Dextrose Agar (SDA) (Conda, Spanish) at 35°C. A few colonies were suspended in sterile normal saline. The cell densities of the Candida suspensions were measured spectrophotometrically at a wavelength of 530 nm, and the transmission was adjusted to 75-77%. The suspensions were diluted to yield final inoculums concentration ranged from 0.5-2.5×10^3 CFU/ml.

**Antifungal susceptibility testing**

Antifungal susceptibility test was performed according to the Clinical and Laboratory Standards Institute (CLSI) using micro dilution method as describe previously with minor modification [17, 18] and RPMI 1640 medium (Sigam, USA) supplemented with L-glutamine, without sodium bicarbonate buffered at PH 7.0 with 0.165 M morpholinepropaesulfonic acid (MOPS) (Sigma, Aldrich Chemie).

The all extracts of walnut Fruit Peel were dissolved in dimethylsulphoxide(DMSO), Andfluconazole (Daroupakhsh, Iran) were dissolved in sterile distilled water. Serial dilutions of the extracts and drug were performed in 96-well microplates trays using RPMI 1640. The concentrations of extracts were prepared at a range of 0.02441-50 mg/ml. MIC was determined after incubations of the 96-well microplates at 35°C for 24-48 hours. The growth in each well was compared with control wells. MIC, were visually determined and defined as the lowest concentration inhibiting growth of Candida species. MFC was determined by culturing 20 μl of the mixed broth culture from the wells with no turbidity on SDA at 35°C for 24 hours on the MIC assay. The MFC was defined as the lowest concentration completely inhibiting the growth of the agents.

73
RESULTS

The MIC of methanolic and aqueous extracts of walnut Fruit Peel and fluconazole are presented in Table 1. The MIC values of methanolic and aqueous extracts of walnut Fruit Peel ranged from 3.125 to 25 mg/ml and 6.25 to 50 mg/ml, respectively. The least sensitive species to methanolic extract were Candida albicans and Candida glabrata and the more sensitive species were Candida tropicalis and Candida krusei. The later species were also more sensitive to aqueous extract. The MFC values of methanolic extracts and fluconazole ranged from 6.25 to 25 mg/ml and 0.001 to 0.032 mg/ml, respectively and the aqueous extracts of walnut fruit peel were without fungicidal effects [Table 2].

<table>
<thead>
<tr>
<th>Candida species</th>
<th>MIC (mg/ml)</th>
<th>Methanolic extract</th>
<th>Aqueous extract</th>
<th>Fluconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>25</td>
<td>25</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>25</td>
<td>50</td>
<td>0.032</td>
<td></td>
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<tr>
<td>Candida tropicalis</td>
<td>3.125</td>
<td>6.25</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>Candida krusei</td>
<td>3.125</td>
<td>12.5</td>
<td>0.002</td>
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</tr>
</tbody>
</table>

**DISCUSSION**

The emergence of drug-resistant strain of various fungal species such as Candida, Dermatophytes and Cryptococcus neoformans has prompted into developing new strategies for treatment of fungal infections which may be less toxic to human [19]. Some studies have demonstrated the antibacterial and antifungal effects of walnut extract. Noumi et al. evaluated anticandidal effect of three type extracts (methanol, acetone and ethyl acetate) of Salvadorapersica and Juglansregia on different Candida species at 2009. In this study, it is indicated that ethyl acetate extract of walnut Fruit Peel was more effective than other extracts. Candida albicans was the most sensitive species to ethyl acetate extract of juglansregia Fruit Peel and Candida glabrata was the most resistant species to this extract[12]. In other study, Citoglu et al. investigated antibacterial and antifungal effects of methanolic extract of walnut leaves. The results of this study showed that Candida albicans in comparison with Candida glabrata and Candida krusei was more sensitive to walnut extract[19]. In a study by Yigt et al., antifungal and antibacterial activities of aqueous and methanolic extracts of leaves and husk of walnut were proved. The results showed that the MIC values of aqueous extracts of leaves and husk on three Candida species were 2.5 mg/ml and the MIC of alcoholic extracts on all Candida species were 1.5 mg/ml[20]. In a study at 2010, Coban et al. assessed antibacterial and antifungal properties of hydro-alcoholic extract of walnut leaves. In this study, the most sensitive species was Candida utilis and Candida tropicalis was the most resistant species in comparison with other tested Candida species[21].

The results of present study are in agreement with the results of some researchers, but differ from the other studies [20, 21]. It seems that the main effective factors in differences between the results of these studies might be include extraction method, fungal species differences, soil ingredients, climatic conditions of the regions, age of plants, and time of collection of plant materials [22, 23].

The results indicated that anticandidal activity of methanolic extract of walnut Fruit Peel on tested Candida species is more than aqueous extract. It shows that effective ingredients of walnut Fruit Peel with antifungal effects dissolve in methanol better than water, therefore; it is recommended that alcoholic extracts are used for prospective complementary researches.

Comparison of the MIC of methanolic and aqueous extracts of walnut with the MIC values of fluconazole indicated that the effective concentrations of extracts on tested Candida species are higher than fluconazole. According to the
fact that the methanolic and aqueous extracts of walnut were crude extracts and contain ineffective ingredients, it seems that the differences between the MIC of extracts and fluconazole are reasonable.

Interestingly, the MFC results show that the aqueous extract of walnuts has no fungicidal effects and only stops Candida species growth, whereas the alcoholic extract kill the Candida cells and even inhibit growth of fungi by killing the Candida albicans and Candida glabrata cells.

CONCLUSION

The most important result of this study is to demonstrate the effectiveness of walnut extract against Candida species and it looks promising that in future, we can achieve an appropriate antifungal drug with minimal side effects from walnut extract for treatment of candidiasis, but in vivo studies are required for evaluation of pharmacokinetic effects of walnut.

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REFERENCES


