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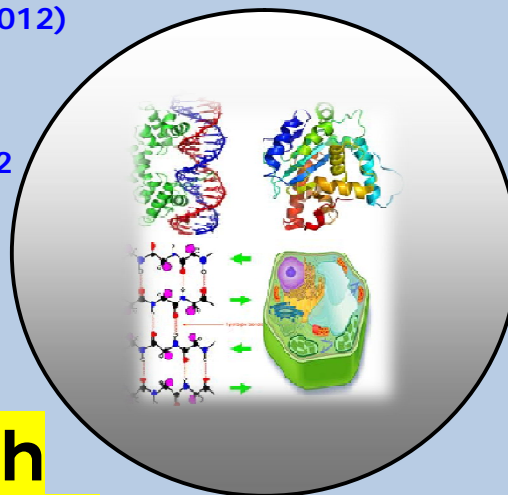
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RESEARCH PAPER

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In Vitro Inhibitory Effect of the Hydro-Alcoholic Extract from the *Avicennia marina* (Hara) on *Candida albicans*

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ABSTRACT

Candida albicans is one the most common opportunistic fungal diseases in humans. For a long time and even now the leaf of the *Avicennia marina* (hara) herb has been used to treat many infections in southern parts of Iran. The purpose of this study is to determine the inhibitory effects of the hydro alcoholic extract of the *Avicennia marina* leaf on *Candida albicans* under in vitro conditions.

The *Avicennia marina* leaf was collected from the southern shores of Iran and after being washed and dried the extraction of the hydro alcoholic extract was carried out. The effect of the extract was applied using the disk diffusion method and diffusion in agar by creating soak away pits (well) in different concentrations. The amount of MIC and MFC was determined by Macrobroth dilution method. The data analysis was done using the SPSS (version 18) software and the repeat measure test.

In the disk diffusion method in 80 µg/disk concentration the inhibition zone was equal to 18.7mm and in the agar well diffusion method in the 80 mg/ml concentration the inhibition zone was equal to 20mm. The values for MIC and MFC for the clinical samples were 55 and 70 µg/disk consecutively.

Hydro alcoholic extract of *Avicennia marina* has significant effect on *Candida albicans* in vitro, thus, Recommended that further studies be done about this extract under In vivo conditions.

Key words: Hydro-Alcoholic Extract, *Avicennia Marina*, *Candida Albicans*, MIC and MFC.

INTRODUCTION

The spread of opportunistic fungal infections in susceptible patients on one hand and the increase in drug resistance and its side effects on the other has caused the study of anti fungal herbs to become of importance (Havasian et al. 2013, Nasrolahi et al. 2009). In fact traditional treatments are cheaper and more effective when compared to modern methods and in communities who use herb medications the chance of an infection with a resistant pathogen occurring are lower (Panahi et al. 2013). This perspective that herb have a therapeutic potential goes back years (Behdani et al. 2009). The *Avicennia marina* species is from the Verbenaceae or Avicenniaceae family. This species is one of the dominant species in the Mangrove eco system and is more resistant to weather temperatures and salt water compared to other species (Taghizade et al. 2009). Some of the identified substances in this herb are Saponins, Steroids, Tanins, Triterpenes, Flavonoids and Alkaloids (Bandaranayake et al. 1995, Abeysinghe et al. 2006, Homhual et al. 2004). Considering the traditional use of this herb's leaf and fruit extract in curing skins diseases, tooth ache, leprosy, abortions... it seems that this extract also has antifungal properties (Zargari et al. 1997, Robert et al. 2000, Bandaranayake et al. 2002). The *Candida albicans* yeast is the body's normal flora and rarely causes disease in healthy individuals, but as an opportunistic infection it can be found in susceptible people such as those with AIDS, wide spectrum antibiotic users and transplant patients. The importance of the diseases caused by this yeast, besides causing infections in susceptible people, is related to AIDS patients and the main index in them is candidiasis in the oesophagus (Menon et al. 2001). Moreover, the *Candida* species are the fourth cause of blood infections in hospitalized patients and causes the death of 40% of these patients in America (Panáček et al. 2009). Considering the ever increasing drugs resistance in fungi and dosage of common drugs and their subsequent side effects, cures with a natural base such as herbs have been receiving attention (Kanafani et al. 2008, Pfaller et al. 2007). The objective of this study is determining the inhibitory effect of the hydro alcoholic extract of the *Avicennia marina* leaf on *Candida albicans* obtained from fungal vaginitis under in vitro conditions based on finding the Minimum Inhibitory Concentration, MIC 90 and the Minimum Fungicidal Concentration, MFC.

MATERIAL AND METHODS

The Hara herb leaf was collected from the southern shores of Iran, washed and dried in 25° shade and away from direct sunlight. The Maceration method was used to obtain the extract (Eidi et al. 2009). In order to collect the extract, each gram of leaf powder was mixed with 5ml of solvent (composed of 30% water and 70% 96 degree ethanol). The solvents were kept under 15 – 20 degrees centigrade temperature for 72 hours. The collected solutions were filtered using the Whatman No 1: 5mm (diameter) filtering paper and the resultant extracts were made more viscous in vacuum using a distiller in laboratory temperature. The Agar well diffusion assay and Filter paper disk embedding methods were used to determine the antifungal activity against *Candida albicans* (Duarte et al. 2005).

Micro organism and culture mediums

30 samples were collected from patients with vaginitis, and the *Candida albicans* ATCC 2091 was used as the standard strains.

Agar well diffusion assay

The agar diffusion was carried out with minor modifications (Bagiu et al. 2012, Jainkittivong et al. 2009). For these purpose 100 micro litres of *Candida albicans* McFarland 0.5 suspension was mixed or 0.85% sterile distilled water (SDW) and using a sterile loop it was spread across the surface of the plate containing SDW. Using sterile pipettes Pasteur, four pits (5mm in diameter) were made in each of the plates. In the first well 35µl of the extract solvent was placed as the negative control and in the second well 35µl of different concentrations or the extract was added. The cultured plates were incubated at 37°C and the results were recorded after 24 hours. The halos around each well were recorded in millimeters which showed the antifungal activity of the extract (Panahi et al. 2013, Hosainzadegan et al. 2012).

Filter paper disk embedding

Filter paper disks (Whatman No 1: 5mm diameter) were smeared with different concentrations of the extract (200µg/disk-6.4mg/disk) and in order to dry them they were incubated at 37°C for an hour (Khalaji et al. 2007). Then the disks smeared with herb extracts and standard antifungal drugs (20µg/disk Fluconazole, Ketoconazole) were placed on SDW culture media containing 100 micro liters of McFarland 0.5 *Candida albicans* suspension for 24 hours at 37°C. The disks were of three kinds, one containing pure solvent, one with herb extract and another with standard antifungal drugs. The index for activity on average for the inhibition zone was calculated by dividing the average inhibition zone by the standard drugs (Hosainzadegan et al. 2012, Iwalokun 2002).

MIC and MFC

To determine MIC and MFC the Macrobroth dilution method and BHI broth culture media was used (NCCLS 1999, Dangi et al. 2013).

Statistical Analysis

The results acquired were analyzed using the SPSS (version 18) software and the repeated measure statistic test.

RESULTS

Results of the inhibitory effect using the disk diffusion method

In this method the least and most inhibitory effects were recorded at 200 and 1600 µg/disk consecutively and the inhibition zone for them were 10.5 and 18.7 mm. the Fluconazole and Ketoconazole disks showed no inhibitory effect on the clinical samples and showed effect on the standard strains ATCC 2091 (P<0.01)(Table 1).

Table 1. Inhibitory effects using the disk diffusion method.

35 µl of Concentration mg/ml	Inhibition zone (10mm hole size)	Filter disk impregnated	Inhibition zone (10mm hole size)
10 mg/ml	8 mm	200 µg/disk	10.5 mm
20 mg/ml	13mm	400 µg/disk	12.3 mm
40 mg/ml	16 mm	800 µg/disk	15mm
80 mg/ml	20 mm	1600 µg/disk	18.7 mm
Candida albicans ATCC 2091 , 80 mg/ml	21 mm	1600 µg/disk	19 mm

Results of the inhibitory effects using the well diffusion method

The results showed the least and most effective inhibitory effect on *Candida albicans* at 10 and 80 mg/ml consecutively and the no growth halos were 8 and 20 mm ($P < 0.01$) (table 1).

Results of MIC and MFC on *candida albicans*

The MIC and MFC of the extract on the clinical samples of *Candida albicans* were 55 and 70 $\mu\text{g/ml}$ consecutively (table 2).

Table 2. MIC and MFC results of the extract on *Candida albicans*.

$\mu\text{g/ml}$	Samples		
55	clinical samples	hydro alcoholic extract	MIC
50	standard strains		
70	clinical samples	hydro alcoholic extract	MFC
60	standard strains		

DISCUSSION

It has been proven that synthetic drugs with all their efficiency, have undesired side effects and it has also been noticed the few pure substance exist which do not have negative effects (Egorov et al. 1985), as a result the use of natural herbs has received attention recently. The drug resistance which has occurred in recent years has also been a cause in the increased use of herbal cures (Alizadeh-Behbahani et al. 2014). The antibacterial and antiviral effects of the *Avicennia marina* plant have been proven due to substances it contains such as Flavonoids, Brugierol and Carbamate (Taherzadeh et al. 2008, Bandaranayake et al. 2002, Kato et al. 1975, Abeyasinghe et al. 2006), also tanins in Mangrove plants act as an antibacterial agent and can defend against destructive factors (Jones et al. 1994, Bandaranayake et al. 1995). Punicalin and Punicalagin are two of the main tanins available in mangrove plants (Bandaranayake et al. 2002), which can have antibacterial effects (Asress et al. 2001). Due to the fact that the anti candida effects of the hydro alcoholic extract haven't been proven, this study analyzed the inhibitory effect of the extract on *Candida albicans* under in vitro conditions. As this herb was traditionally used in treating infections, it was expected for this plant to have strong antimicrobial effects which correlated with the results of this study. As the results showed the MIC and MFC for candida samples from clinical vaginitis were 55 and 70 $\mu\text{g/ml}$ consecutively and for the standard strains 50 and 60 $\mu\text{g/ml}$. a study by Alizadeh-Behbahani in 2013 which was carried out to analyze the antifungal effect of aqueous and ethanol extracts of mangrove plants showed the stronger effect of edible films containing mangrove extracts on *Penicillium digitatum* compared to *Alternaria citri* (Alizadeh-Behbahani et al. 2013). In the study carried out by Panahi et al. to analyze the anti candida effect of the alcoholic extract of inner stratum of oak fruit (jaft), this effect was proven but it was weaker than the anti candida effect of the hydro alcoholic extract of *Avicennia marina*. In Havasian et al.'s study to analyze the inhibitory effect of the hydro alcoholic extract of *Scrophularia striata* on candida, it was

shown that this extract has no effect on candida albicans. In the study done by Bahmani in 2011 to analyze the effect of the *Scrophularia deserti* plant compared to Amphotericin B on candida albicans, the MIC in first and second series of *Scrophularia deserti*'s alcoholic extract were 59 and 58 percent and for Amphotericin B it was 59 percent (Bahmani et al. 2011). In another study to analyze the inhibitory effect of the aqueous extract of the *Nectaroscordum Tripedale* herb on *Candida albicans*, even though the existence of saponins were proven in this extract there was no such effect (Panahi et al. 2013). On the studies carried out on the hematologic, biochemical and pathological effects of *Avicennia marina* on rats, the results showed no harmful or lethal effects (Ali et al. 1998). In an overall conclusion it can be stated that the hydro alcoholic extract of *Avicennia marina* under in vitro conditions showed noticeable anti Candida effects on different species of *Candida albicans*. Larger studies showed be carried out under in vivo conditions to determine the effective dosage of this extract against *Candida albicans* and also to find its side effects and the formulation needed to maximize its bioavailability and finally to introduce this extract as a new antifungal drug.

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