Antifungal potential of mangrove extracts against *Aspergillus flavus* and *Penicillium italicum*

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ABSTRACT

The interest in plants antimicrobial properties has been revived because of the current problems associated with the use of antibiotics. Nowadays, the fact that microorganisms tend to develop drug resistance, besides the side effects of certain antibiotics has offered considerable potentials for the development of new effective antifungal and antioxidant agents; medicinal plants are prolific sources. *Avicennia marina*, commonly known as grey mangrove, is a species of mangrove tree classified in the plant family Acanthaceae. The aim of this experimental study is determination of antifungal effect of the aqueous and ethanolic extracts of *Avicennia marina* on *Aspergillus flavus* and *Penicillium italicum "in vitro"*. In this study, quantitative bioassay was done using disc diffusion method, Minimum Inhibition Concentration (MIC) and Minimum Fungicidal Concentration (MFC) was carried out using broth dilution methods. The results showed that the ethanol extract of *Avicennia marina* leaves with "antimicrobial activity method" in 2000 µg/ml, were inhibited the growth of *Aspergillus flavus* and *Penicillium italicum*. In "disk agar diffusion test" the mentioned extract were shown inhibiti on effect on pathogenic molds growth ($p<0.05$). The result showed that MIC of ethanolic extract of *Avicenna marina* leaves for *Aspergillus flavus* and *Penicillium italicum* was 16 and 8 mg/ml respectively. The results showed that MFC of ethanolic extract of *Avicenna marina* for *Aspergillus flavus* was 32 mg/ml, and for *Penicillium italicum* was 16 mg/ml. Ethanolic extract compared to the aqueous extract was more effective and has a greater inhibition effect. This study showed that the extract of *Avicennia marina* had antifungal effects that justify their traditional use as medicinal plants. Finally, the data suggested that Avicennia marina extracts could have notable antifungal effects.

Keywords: Antifungal potential; *Avicennia marina*; *Aspergillus flavus*; *Penicillium italicum*.

INTRODUCTION

Human fungal infections pose serious medical issues. Up to now, more than a hundred thousand fungal species are considered as natural contaminants. During the last decade, the incidence of superficial and deep mycotic infections has continued to increase explosively. There is a general consensus among researchers, clinicians and pharmaceutical companies that new, potent, effective and safe antifungal drugs are needed [1]. The increasing prevalence of antibiotic resistance is a major health concern, worldwide. The World Health Organization (WHO) and the European Commission (EC) have recognized the importance of studies on the emergence and determinants of antimicrobial resistance and the need for strategies to control drug resistance [2]. Avicennia marina, commonly known as grey mangrove or white mangrove, is a species of mangrove tree classified in the plant family Acanthaceae (formerly in the Verbenaceae or Avicenniaceae) [3]. As with other mangroves, it occurs in the intertidal zones of estuarine areas. Grey mangroves grow as a shrub or tree to a height of three to ten meters, or up to 14 meters in tropical regions [4].The habit is a gnarled arrangement of multiple branches. It has smooth light-grey bark made up of thin, stiff, brittle flakes. This may be whitish, a characteristic described in the common name. The leaves are thick, five to eight centimeter long, a bright, glossy green on the upper

surface, and silvery-white, or grey, with very small matted hairs on the surface below [5]. As with other Avicennia species, it has aerial roots (pneumatophores); these grow to a height of about 20 centimeters, and a diameter of one centimeter. The grey mangrove can experience stunted growth in water conditions that are too saline, but thrive to their full height in waters where both salt and fresh water are present [6]. The species can tolerate high salinity by excreting salts through its leaves. The chemical component extracted Iranian mangroves are used mainly in folkloric medicine (*e.g.* bush medicine), as insecticides and pesticides [7].

At the beginning of the 20th century, bacterial epidemics were a global and important cause of mortality. In contrast, fungal infections were almost not taken into account. Since the late 1960s when antibiotic therapies were developed, a drastic rise in fungal infections was observed, and they currently represent a global health threat. This increasing incidence of infection is influenced by the growing number of immunodeficient cases related to AIDS, cancer, old age, diabetes, cystic fibrosis, and organ transplants and other invasive surgical procedures [8].

Mycotoxicosis is the term used for poisoning associated with exposures to mycotoxins. In humans, *Aspergillus flavus* aflatoxin production can lead to acute hepatitis, immunosuppression, hepatocellular carcinoma, and neutropenia [9]. It is highly possible for *Aspergillus flavus* to invade arteries of the lung or brain and cause infarction. The absence of any regulation of screening for the fungus in countries that also have a high prevalence of viral hepatitis highly increases the risk of hepatocellular carcinoma [10]. After Aspergillus fumigatus, *Aspergillus flavus* is the second leading cause of aspergillosis. Primary infection is caused by the inhalation of spores; bigger spores have a better chance of settling in the upper respiratory tract. The deposition of certain spore sizes could be a leading factor of why *Aspergillus flavus* is a common etiological cause of fungal sinusitis and cutaneous infections and non invasive fungal pneumonia. Two allergens have been characterized in *Aspergillus flavus*: Asp fl 13 and Asp fl 18. In tropical and warm climates, *Aspergillus flavus* has been shown to cause keratitis in approximately 80 percent of infections. *Aspergillus flavus* infection is typically treated with antifungal drugs like amphotericin B, itraconazole, voriconazole, posaconazole, and caspofungin; however, some antifungal resistance has been shown in amphotericin B, itraconazole, and voriconazole [11]. The aim of this study was to determine antifungal effect of the aqueous and ethanolic extracts of *Avicennia marina* on *Aspergillus flavus* and *Penicillium italicum" in vitro".*

MATERIALS AND METHODS

Plant material

This experimental study was conducted at Department of Food Science and Technology, Ferdowsi University of Mashhad in 2013.The leaves of *Avicennia Marina* were collected from the mangrove forests of Qeshm Iran, which extends from 26°50′N and 56°0′E.

Extract preparation

The amount of 25 gram of *Avicennia marina* leaves powder was added to 125 ml ethanol 96% or distilled water. The ethanolic and aqueous extracts mixture was preserved at laboratory temperature 25 ˚C for 48 hours and was stirred every few hours with a glass rod. The collecting supernatant was centrifuged by 9000 rpm for 5 min. The supernatant was removed and reached to the original volume with ethanol or distilled water, then the samples packed in dark containers and stored at refrigerator temperature after filtered by 0.45 μ Whatman filter paper [12].

Antimicrobial activity

Dried plant extracts were dissolved in the same solvent (methanol or water) to a final concentration of 30 mg/mL and sterilized by filtration through 0.45 μg Millipore filters. Antimicrobial tests were then carried out by the disk diffusion method using 100 μg of suspension containing 1.5×10^8 CFU/ml of fungi spread on sabouraud dextrose agar medium. Disks (7 mm in diameter) were impregnated with 20, 40, 60 and 80 mg/ml were placed on the inoculated agar. Negative controls were prepared using the same solvents employed to dissolve the plant extracts. The inoculated plates were incubated at 27 °C 72 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms in comparison to a control of negative and reference standards. The experiment was done three times and the mean values are presented [13, 14].

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of the pure compound that inhibits visible growth of the microorganism. MIC was determined according to agar dilution method. Various concentrations (2, 4, 8, 16, 32, 64, 128, 256 mg/ml) of extract was prepared in 10 cm experimental tubes containing SD broth. Each tube contains 9 ml of SDA and was sterilized in autoclave. After cooling, 1 ml of each extract concentration were added to each tube, to make the final concentrations of 2, 4, 8, 16, 32, 64, 128, 256 mg/ml. The mixture of SDA and extract was poured into plates aseptically in a laminar flow cabinet. After solidification of the agar medium, 2 µl of adjusted spore suspension were added to each plate by micropipette and incubated at 27°C. The SDA without any herbal extract served as control. The MIC was regarded as the lowest concentration of the extract that did not show any visible growth after 12 days of incubation compared with control [15].

Determination of Minimum Fungicidal Concentration (MFC)

MFC was determined according to agar dilution method with slight modifications. The MFC was determined by incorporating various concentrations of extracts (2-256 mg/ml) in SD broth in tubes. One milliliter adjusted spore suspension was added to each tube and incubated at 27°C for 3 days. The SD broth without incorporation of dried herbal extract and 1 ml of adjusted spore suspension served as positive control and SD broth alone served as negative control. Those tubes which showed no visible growth after 3 days incubation were subculture on extract free SDA plates and incubated at 27°C for 7 days. The MFC was regarded as the lowest concentration of the extract that prevented the growth of any molds colony on the solid medium [16, 17].

Statistical analysis

Antifungal disk diffusion, MFC and MIC determination based on tube dilution method were performed in triplicate. Inhibition zones diameters (mm) of various Myrtle leaf extracts were measured and expressed as mean ± SD. The data were analyzed using one way analysis of variance (ANOVA) using SPSS version 17.

RESULTS

Results of antifungal effect's mangrove leaf ethanolic extract in screening antimicrobial activity method were showed that mangrove leaf extracted in screening antimicrobial activity method in 2000 µg/ml, inhibit *Aspergillus flavus* and *Penicillium italicum* growth.

Table 1. An antimicrobial effect of ethanolic and aqueous *Avicenna marina* extracts concentrations on *Aspergillus flavus* and *Penicillium italicum*

Microorganism	**Antimicrobial effect of *Avicenna marina* leaves extracts**
Aqueous *Aspergillus flavus*	R
Aqueous *Penicillium italicum*	S
Ethanolic *Aspergillus flavus*	S
Ethanolic *Penicillium italicum*	S

R: Resistant S: Sensitive

Table 2. Average diameter (mm) of microbial free zone area of ethanolic and aqueous *Avicenna marina* leaves extract on *Aspergillus flavus* and *Penicillium italicum* (disk agar diffusion method)

	concentration (mg/ml)			
Microorganism	**20**	**40**	**60**	**80**
Aqueous *Aspergillus flavus*	9.4±0.28[a]	11.5±0.58[b]	13.6±0.54[c]	15.2±0.50[d]
Aqueous *Penicillium italicum*	10±0.28[a]	12±0.54[b]	14±0.28[c]	17±0.50[d]
Ethanolic *Aspergillus flavus*	7.8±0.28[a]	9.6±0.54[b]	11.5±0.58[c]	13.6±0.54[d]
Ethanolic *Penicillium italicum*	8.6±0.25[a]	11.7±0.58[b]	12.6±0.29[c]	15.8±0.50[d]

[a]Values are means ± standard deviations, n=3.

Table 3. Minimum Inhibitory Concentration (MIC) of ethanolic and aqueous *Avicenna marina* leaves extract on *Aspergillus flavus* and *Penicillium italicum*

	concentration (mg/ml)								
Microorganism	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**control**
Aqueous *Aspergillus flavus*	+	+	-	-	-	-	-	-	-
Aqueous *Penicillium italicum*	+	-	-	-	-	-	-	-	-
Ethanolic *Aspergillus flavus*	+	+	+	-	-	-	-	-	-
Ethanolic *Penicillium italicum*	+	+	-	-	-	-	-	-	-

+: Grow –: Not grow, N=3.

Table 4: Minimum Fungicidal Concentration (MFC) of ethanolic and aqueous *Avicenna marina* leaves extract on *Aspergillus flavus* and *Penicillium italicum*

	concentration (mg/ml)								
Microorganism	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**control**
Aqueous *Aspergillus flavus*	+	+	+	-	-	-	-	-	-
Aqueous *Penicillium italicum*	+	+	-	-	-	-	-	-	-
Ethanolic *Aspergillus flavus*	+	+	+	+	+	-	-	-	-
Ethanolic *Penicillium italicum*	+	+	+	-	-	-	-	-	-

+: Grow –: Not grow, N=3.

Results of antifungal activity of mangrove leaf aqueous extract in screening antimicrobial activity method were showed that the mangrove leaf extract in 2000 µg/ml, inhibit *Penicillium italicum* growth. However, 2000 µg/ml concentration aqueous extracts, had no significant antibacterial effect on *Aspergillus flavus* and it is not able to prevent the growth of fungi on culture (Table 1).

Results show that the mangrove leaf ethanolic extracts had inhibition effect on both *Aspergillus flavus* and *Penicillium italicum* (in 20, 40, 60 and 80 mg/ml) but the mangrove leaf aqueous extract had inhibition effect on *Penicillium italicum* (in 20, 40, 60 and 80 mg/ml) and *Aspergillus flavus* (in 40, 60 and 80 mg/ml) (Table 2).

Results show that MIC of *Avicenna marina* leaves ethanolic extract for *Aspergillus flavus* and *Penicillium italicum* was 8 and 4 mg/ml respectively. The results shows that MIC of aqueous extract of *Avicenna marina* leaves for *Aspergillus flavus* was 16 mg/ml, and for *Penicillium italicum* was 8 mg/ml (Table 3).

Also MFC of aqueous extract of *Avicenna marina* leaves for *Aspergillus flavus* was 64 mg/ml, and for *Penicillium italicum* was 16 mg/ml. The results shows that MFC of ethanolic extract of *Avicenna marina* leaves for *Aspergillus flavus* was 16 mg/ml, and for *Penicillium italicum* was 8 mg/ml (Table 4).

DISCUSSION

Microorganisms especially fungi and bacteria are the major pathogenic organisms having potential to cause human diseases and diseases of aquatic organisms. The interest in plants antimicrobial properties has been revived because of the current problems associated with the use of antibiotics. Nowadays, the fact that microorganisms tend to develop drug resistance, besides the side effects of certain antibiotics has offered considerable potentials for the development of new effective antimicrobial and antioxidant agents; medicinal plants are prolific sources [18]. The effectiveness of the active compounds present in plant extracts cause the production of growth inhibition zones that appear as clear areas surrounding the disk. Antifungal activity may be due to active components which are present in plant extracts. However, some plant extracts were unable to exhibit antimicrobial activity against tested fungi strains. These fungi strains may have some kind of resistance mechanisms e.g. enzymatic inactivation, target sites modification and decrease intracellular drug accumulation [19]. In this study, by increasing the amount of ethanolic extract on disc diffusion method, inhibition zone around the disc was increased. The maximum size of inhibition zone for *Avicenna marina* leaves ethanol extract in the disc method was 17

mm (80 mg/ml) and *Avicenna marina* leaves aqueous extract was 15.8 mm (40 mg/ml), respectively. The results show that ethanolic and aqueous extract of *Avicenna marina* in all concentrations (20, 40, 60 and 80 mg/ml) had the inhibitory effect on *Aspergillus flavus* and *Penicillium italicum. Avicenna marina* leaves are rich in a wide variety of phytochemicals like alkaloids, terpenoids, tannins, flavonoids, antimicrobial peptides, etc., that have been found to have antimicrobial activities (4, 5 and 6). In almost all tests, crude ethanolic extract showed better inhibition against all tested fungi strains, indicating that active ingredients in plant materials could be extracted into ethanol.

The results indicate that the ethanolic and aqueous extract of *Avicenna marina* leaves mostly had been effective on *Penicillium italicum* and has the least impact on *Aspergillus flavus*. Ogbe Raphael et al. (2012) reported that most of the antimicrobial active compounds were soluble in polar solvent such as ethanolic instead of water [20]. This result is comparable to the study by Alo et al. (2012) using ethanol extract of *Mangifera indica* that showed effective antibacterial activity on Escherichia coli, Salmonella typhi and Klebsiella pneumoniae [21].Malini et al (2013) showed that ethanol fraction of *Aloe vera* and *Coleus aromaticus* exhibited more promising results in suppressing the fungal growth rather than aqueous extract [22].

The result shows that MIC of *Avicenna marina* leaves ethanolic extract for *Aspergillus flavus* and *Penicillium italicum* was 8 and 4 mg/ml respectively. The results shows that MIC of aqueous extract of *Avicenna marina* leaves for *Aspergillus flavus* was 16 mg/ml, and for *Penicillium italicum* was 8 mg/ml (Table 3). The results indicated that ethanolic and aqueous extract of *Avicenna marina* leaves mostly had been effective on *Penicillium italicum* and has the least impact on *Aspergillus flavus*. Different levels of mangrove extract have been used to consider its antimicrobial effect.

The results shows that MFC of aqueous extract of *Avicenna marina* leaves for *Aspergillus flavus* was 64 mg/ml, and for *Penicillium italicum* was 16 mg/ml. The results shows that MFC of ethanolic extract of *Avicenna marina* leaves for *Aspergillus flavus* was 16 mg/ml, and for *Penicillium italicum* was 8 mg/ml (Table 4). Fatty acids are widely occurring compounds in natural fats and dietary oils, and they are known to have antibacterial and antifungal properties [23]. The results of this study showed that the extract of *Avicennia marina* has antifungal properties that justify their traditional use as medicinal plants. Finally, the data suggest that Avicennia marinat extracts may be a useful source of antifungal use.

Advances in medicine have led to more patients living longer. Commensurate with the growth in patients at risk, the number of patients with severe fungal infections has dramatically increased. Concern regarding the development of resistance to any of the few antifungal drugs available has developed. Although we are able to define certain mechanisms of drug resistance, continued efforts for a deeper understanding of the cellular and molecular mechanisms as well as the clinical components of antifungal resistance will be important [24]. In addition, new diagnostic tools for rapid, sensitive, and specific detection of fungi in clinical material, such as PCR-based techniques, are mandatory.

The result of this work indicated that ethanol is better solvent than water for the extraction of the active ingredients of these Avicenna marina leaves. The results indicated that ethanolic and aqueous extract of *Avicenna marina* leaves mostly had been effective on *Penicillium italicum* and has the least impact on *Aspergillus flavus*. Finally, our knowledge of drug-resistance mechanisms should maximize the utility of current drugs and assist in the development of new antifungal drugs and new treatment strategies.

ACKNOWLEDGMENT

The authors wish to express their profound gratitude and thank sincerely to Research's Deputy of Ferdowsi University of Mashhad for providing the cost of this project and help to implementation of this project with code 3/24158 which adopted of the Research Council of Agricultural faculty.

The authors wish to express their profound gratitude to Ms. Afsharian who helps about experiments and Ms. Khademi pour helped us to prepare samples.

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