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Synergistic Antifungal Effects of Quince Leaf's Extracts and Silver Nanoparticles on *Aspergillus niger*

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Abstract

Invasive fungal disease represents a major threat to life in immunocompromised patients and is now one of the most common causes of infection in this group. There is a limited range of antifungal agents available to treat disease caused by *Aspergillus* including the polyenes, flucytosine, azoles and more recently the echinocandins. Therefore it is necessary to find new ways of treatment for *Aspergillus niger*. The aim of this study was to investigate the synergistic antifungal effects of Quince leaf's extracts and silver nanoparticles on *A.niger in vitro*. The ethanolic and acetonic extracts of Quince (*Cydonia oblonga)* leaf's and silver nanoparticles prepared. Antifungal effects of extracts and silver nanoparticles against *A. niger* investigated by agar dilution method. Then minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) determined by the broth macro dilution method.Then synergistic effect was studied between the most effective plant extract with silver nanoparticles. The results showed that the *C. oblonga* extracts and silver nanoparticles can inhibit the growth of *A. niger*. Ethanolic extract was more effective extract that has antifungal activity against *A. niger* and synergistic activity observed between ethanolic extract of Quince's leaf and silver nanoparticles against *A. niger*. Ethanolic extract of Quince's leaf and silver nanoparticles has synergistic antifungal effect against *A. niger* and can be effective in controlling of *A. niger*.

Key words: Antifungal Effect, synergism, *Aspergillus niger*, *Cydonia oblonga*, silver nanoparticles.

INTRODUCTION

Fungal infections have been increasing in recent years due to a growing number of high-risk patients, particularly immunocompromised hosts [1]. Aspergillosis causes patient afflictions that are classically defined as invasive, saprophytic, or allergic. Invasive diseases caused by Aspergillus species include infections of the lower respiratory tract, sinuses, and skin as portals of entry. The CNS, cardiovascular system, and other tissues may be infected as a result of hematogenous dissemination or direct extension from contiguous foci of infection. Saprophytic involvement includes Aspergillus otomycosis and pulmonary aspergilloma. Allergic conditions encompass allergic Aspergillus sinusitis and allergic bronchopulmonary aspergillosis [2, 3].The mortality rate due to invasive aspergillosis increased by 357% between 1980 and 1997 in the USA [4]. In spite of the introduction of new antifungal drugs, they are limited in number. The increase of fungal resistance to classical drugs, the treatment costs, and the fact that most available antifungal drugs have only fungistatic activity, justify the search for new strategies [5]. According to our preliminary results [1, 6], some essential oils show an important antifungal activity against yeasts, dermatophyte fungi and *Aspergillus* strains, which could predict therapeutic benefits, mainly for diseases with mucosal, cutaneous and respiratory tract involvement [1]. Medicinal plants remain a rich source of novel therapeutic agents. Many plant species are still unevaluated chemically or biologically. Several studies regarding the action of plant extracts against some phytopathogenic fungi have been performed. The quality and quantity of the biologically active compounds from the plant extracts significantly depend on the species, the plant organ and harvest time [7].

Quince (*Cydonia oblonga*) belongs to the group of the oldest cultural plants. They originate from Central Asia and were gradually penetrated also to other parts of the world [8].Chlorogenic acid is a polyphenolic natural compound which is commonly present in plant materials such as apples, coffee beans, grapes, pulp, peel, Quince and tea leaves. Structurally, it is an ester of caffeic acid with the 3-hydroxyl group of a quinic acid. It has been reported to possess many health benefits including antibacterial, antifungal, antiviral, antiphlogistic, antioxidant, chemopreventive, and other biological activities [9].

Recently, nanotechnology has amplified the effectiveness of silver particles as antimicrobial agents. So that, different studies showed that the antimicrobial effect of silver nanoparticles against bacteria and fungi [10,11].The aim of this study was to investigate the antifungal effects of *Cydonia oblonga* and silver nanoparticles on *Aspergillus niger in vitro*.

MATERIALS AND METHODS

Material and Media

Aspergillus niger PTCC: 5012 were purchased from Iranian Research Organization for Science and Technology (IROST). Silver nanoparticles were purchased from NANOCID company, Iran in the size range of 3-18 nm and concentration of 4000 ppm. All the microbial media and chemical solvents were obtained from Merck, Germany.

Preparation of extracts

Quince leaf's collected in September from Hashtrood, East Azarbayjan, Iran. Then the leaf's dried at room temperature in the shadow and ground into fine powder using a mechanical grinder. 50 g of the powdered sample was soaked in 500 ml of solvent (in this study water, ethanol and acetone). The solution shakes 24 hours at shaker. The solution is then refrigerated centrifuge (HETTICH ROTIX DA 50) to remove larger particles with 2500rpm for 20 min at 4°C. Supernatant obtained by distillation in vacuum concentrator and filtered by microbial filter 0.45 µ. Prepared extracts stored in sterile microtube at - 80°C for later usage [8].

Antifungal susceptibility assay

Agar dilution method

The serial concentrations of the extracts of Quince's leaf (25, 50, 100, 200, 400 and 800 mg/ml) and silver nanoparticles (5, 10, 20, 40, 80 and 160 ppm) were prepared in Potato Dextrose Agar (PDA) medium.One plate was used as a control without any treatment.Fungal suspension was added to each plate. The fungal suspension with turbidity of 2.5×10^3 CFU/ml in normal saline was prepared. Then 1 ml of the prepared suspension was added to each plate and spread on agar by sterile platinum wire loop. The prepared plates incubated at 35°C for 48h. All experiments were repeated three times [8, 12].

Broth macrodilution assay

The activity of Quince's leaf extracts and silver nanoparticles against *A. Niger* was also determined by the broth macrodilution method. Dilutions of extracts (25, 50, 100, 200, 400 and 800 mg/ml) and dilution of silver nanoparticles (5, 10, 20, 40, 80 and 160 ppm) were prepared in RPMI 1640 Medium in 0·5 ml volumes in glass test tube. 0.5 ml *A. niger* with turbidity of 2.5×10^3 CFU/ml was added to each test tube. After 48h incubation, MIC and MFC were determined. MIC was determined as the lowest concentration of agent resulting in the maintenance or reduction of the inoculum and MFC were determined as the lowest concentration of agent resulting in no growth [12, 13].

Synergistic assay

To investigation of synergistic activity of Quince's leaf extract with silver nanoparticles was operate like the broth macro dilution method. So that, dilutions of ethanolic extract of Quince's leafincluded that 25, 50, 100, 200, 400 and 800 mg/ml combined with dilution of silver nanoparticlesincluded that 5, 10, 20, 40, 80 and 160 ppm, respectively in RPMI 1640 Medium in 0·5 ml volumes in glass test tube. 0.5 ml*A. Niger* with turbidity of 2.5×10^3 CFU/ml was added to each test tube. After 48h incubation, MIC and MFC were determined as described above [13, 14].

Statistical analysis

The results were analyzed by one way ANOVA test by SPSS 18 software. $P \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

Medical centers worldwide have reported that invasive aspergillosis (IA) has become a leading infection-related cause of death in immunocompromised patients [15]. On the other hand acute invasive aspergillosis is resistant to multiple antifungal drugs developed within the past few decades. Some scientists believe that the major determinant of treatment response appears to be the underlying condition rather than the antifungal drug used (16). However, since the drug therapy is the best way to deal with fungal infections.But the side effects of chemical drugs and the high cost of treatment is causing a lot of problems. For this reason, efforts are continuing to find ways to therapeutic without side effects and high costs. Today, treatment with herbal products because of fewer side effects and lower costs are highly regarded [17]. On the other hand, silver nanoparticles have been of interest to scientists because of their antimicrobial properties (10). The availability of new antifungal agents with novel mechanisms of action has stimulated renewed interest in combination antifungal therapies. With the recent publication of the first large randomized trial of antifungal combination therapy to be conducted in two decadesand the rapid proliferation of new in vitro and in vivo data on antifungal combinations (19). Combinations of antifungal agents, in comparison with single agents, may confer the benefit of increasing efficacy, sparing toxicity, or both [18].

The results of *in vitro* antifungal activity of organic(ethanolic and acetonic) and aquaticextracts of Quince'sleaf were presented in table 1. The results were showed that the ethanolic extract of leaf of Quince has superlative antifungal effect against *A.niger*.In this study ethanolic extract was effective extract against tested fungus. The aquatic extract was weakand tested fungus was resisting to it.The results of this study showed that the ethanolic and acetonic extracts of Quince's leaf and silver nanoparticles have antifungal activity against *Aspergillus niger in vitro*. The aquatic extract of Quince's leaf doesn't show any antifungal effect. These findings are perfectly matching with the study of Alizadeh et al in 2013 [8, 11].

The results of antifungal activity of silver nanoparticles in agar dilution method showed that the growth of *Aspergillus niger* is inhibited at concentrations higher than 80 ppm (Table 2).

The results of determination of MIC and MFC of Quince's leaf extracts and silver nanoparticles in macro dilution broth method is shown in Table 3.

Table 1. Growth of *Aspergillus niger* in agar dilution method in the presence of extracts

Extracts	Concentration of extracts (mg/ml)						
	25	50	100	200	400	800	Control
Ethanolic	+	+	+	-	-	-	+
Acetonic	+	+	+	+	-	+	+
Aquatic	+	+	+	+	+	+	+

(+): Growth, (-): No Growth

Table 2. Growth of*Aspergillus niger* in agar dilution method in the presence of extracts

	Concentration of silver nanoparticles (ppm)						
	5	10	20	40	80	160	Control
Growth	+	+	+	+	-	-	+

(+): Growth, (-): No Growth

Table 3. MIC & MFC of Quince's leaf extracts and silver nanoparticles on *Aspergillus niger*

	MIC	MFC
Ethanolic extract (mg/ml)	100	200
Acetonic extract (mg/ml)	200	400
Aquatic extract (mg/ml)	-	-
Silver nanoparticles (ppm)	40	80

The results showed synergistic activity against *Aspergillus niger* between ethanolic extract of Quince's leaf and silver nanoparticles. So that, the MIC and MFC concentration of ethanolic extract of Quince's leaf reduced to 50 and 100 mg/ml, respectively and the MIC and MFC concentration of silver nanoparticles reduced to 20 and 40 respectivley. Combination antifungal therapy has been of interest because of the poor outcomes associated with treatment with either azole antifungals or amphotericin B formulations alone, especially in Hematopoietic stem cell transplantation (HSCT) recipients [19]. The ethanolic extract of Quince's leaf and silver nanoparticles showed synergistic antifungal activity against *Aspergillus niger in vitro*. The synergistic antifungal effects of various drugs on Aspergillus in previous studies also confirmed that it corresponded to this study. Comparison of the most objective parameter of therapeutic success—survival—showed that the combination of voriconazole and caspofungin was associated with better outcomes, compared with voriconazole administered alone, for patients receiving salvage therapy to treat aspergillosis. These data support the use of the combination regimen in treating patients who require salvage therapy, but given the retrospective nature of this study, a randomized trial will be necessary to determine whether combination regimens will be effective for primary therapy for aspergillosis [15, 16, 19].This study have been carried out only in vitro, but has considering that acceptable results, can be used in the future it be performed in animal models and it can be used as a new therapy for Aspergillus infections.

Overall it can be concluded from this study that the Quince's leaf extracts and silver nanoparticles have synergistic antifungal activity and can be used in controlling of *Aspergillus niger*.

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