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The effect of ethanolic extract of walnut thin shell on the growth of *Aspergillus spp.*

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ABSTRACT

Aspergillus spp. are the most important fungi for production of Aflatoxin. They are heat resistant and can be easily adapted to the environment. Toxin of these fungi can enter the bodies through food chain and causes major health problems. It can also make serious troubles in many fields such as food industry, animal husbandry and economic. Some studies have investigated the effects of herbal and plant extracts to reduce the growth of Aflatoxin resulting inhibition of toxin production. Several benefits have been addressed for different parts of walnut. Thin shell around the walnut is full of phenolic and antioxidant compounds that may have effect on *Aspergillus flavus* and *Aspergillus fumigates*. This study explored the effects of ethanol extract obtained from walnut thin shells on standard and isolated species of *Aspergillus flavus* and *Aspergillus fumigatus*. The extract dilutions of 500 to 3.75 mg per ml were exposured to the desired fungi using the broth dilution method. minimum inhibitory concentrations (MIC) of fungi were determined and compared with the effects of Nystatin and fluconazole. The minimum inhibitory concentration for *Aspergillus fumigatus* and *Aspergillus flavus* were 15 mg/ml and 61.5 mg/ml respectively. walnut Thin shells has antifungal activity and could inhibit the growth of *Aspergillus flavus* and *Aspergillus fumigatus*.

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1. Introduction

Nowadays, antioxidant compounds found in fruits and vegetables have been extensively investigated. They play a vital role in maintaining health and preventing diseases. The pressure imposed by the consumers to avoid using chemical antimicrobial compounds as well as increased resistance of the body against synthetic antibiotics has led to a growing interest in the use of natural ingredients in food industry (1). In order to reduce chemical additives consumption in foods, plenty of researches have been carried out to replace chemicals with natural alternatives. In this regard, some efforts have been made to find antimicrobial and antioxidant compounds from herbal sources. Due to producing some secondary metabolites which are a rich source of antimicrobial agents, medicinal herbs can be used as antimicrobial compounds (2,3). In numerous studies, the antimicrobial effects of native herbs of Iran, including walnut, on *Salmonella, Shigella, Listeria, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Bacillus cereus* have been investigated (4-6). Several benefits have been addressed for different parts of Iranian walnut. For instance, the walnuts reduce blood triglycerides and increase HDL (7, 8). Some studies have shown the anti-inflammatory and antioxidant effects of walnut shell extract (9). Moreover, antimicrobial effect of volatile compounds, phenols and tannins in walnut green shells has been proved too (10). The role of methanol extract of green walnut shells in preventing the growth of different fungi such as dermatophytosis was examined in order to be used as

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an alternative to synthetic drugs. Walnut hulls had an inhibitory effect on four selected species of fungi and prohibited the fungal growth by 60% (11). Furthermore, the polysaccharides antibacterial effect of thin walnut shells on *Bacillus subtilis* and *Salmonella typhimurium* was specifically examined in another study (12).

Fungi are one of the microorganisms involved in food spoilage, especially stored foods, reduce the nutritional value of food sources and cause economic losses and health problems. Major fungi that grow predominantly on food products are *Aspergillus, Fusarium* and *Penicillium*. *Aspergillus spp.* is widely spread in various environments such as in soil, vegetables, decaying organic materials, food debris, medicines and buffers. All species of *Aspergillus* are heat resistant and can be easily adapted to the environment; hence, they induce a variety of diseases. Mycotoxins, which are secondary metabolites of fungi, cause toxicity for the consumers of productive fungi or contaminated products. These toxins are often produced by *As. flavus* and *A.parasiticus* and can result in carcinogenic, mutagenic and toxigenic effects in animals and humans. The optimum condition for the growth and production of mycotoxins among these fungi is provided with room temperature (25°C) and humidity above 85% (1,13). Another health problem caused by fungi is the emergence of different species resistant to conventional antifungal compounds, such as certain types of fungi *Candida, Dermatophytes and Cryptococcus neoformans*. These made researchers to develop new methods to control the fungi (14). The results of studies have shown that some plant extracts can reduce or completely inhibit the growth of fungi. The antifungal and antibacterial effects of shallot, garlic and onion extracts were evaluated and it was found that, compared to bacteria, the fungal species are more sensitive to shallot extract. The amount of MIC obtained for shallot dried extract in three species (namely *Aspergillus fumigatus, Aspergillus flavus* and *Aspergillus Niger*) was 20 mg per ml (15). In another study, the antifungal effects of seven species of Allium genus on three *Aspergillus* species was described (16).

It was also shown that aqueous extract of garlic and onion can have a deterring effect on keratinases fungus growth and performance (17). Maskouki et al., proved that the growth of *A.parasiticus* is prevented by natural extracts (18). Two common fungal infections include *Aspergillus fumigatus* and *Aspergillus flavus*. *Aspergillus fumigatus* is considered as one of the important pathogenic fungi and *Aspergillus flavus* contaminate a wide range of food products and causes health hazards by aflatoxin production. The fungus has caused stored food spoilage, resulting in waste and economic losses. (19, 20) Additionally, the present study offers the opportunity to reduce the growth of these fungi and to remove them from food products and cultures. The results could also be beneficial in the treatment of fungal diseases.

2. Materials and methods

2.1. Materials & devices

All devices, chemicals and solvents were of analytical grade and included as follows; Electrical grinding machine (Molyneux), Laminar Hood Class II (Witeg, Germany), Rotary evaporator (IKA , Germany), Ethanol %80, Dimethyl sulfoxide solvent, Syringes and filters with a diameter of 0.22 micron, Nystatin powder (Royan Darou Co., Iran) and fluconazole (Pars Darou, Iran), Growth medium of fungi Sabouraud Dextrose Agar (Merck, Germany), Growth medium Sabouraud Dextrose Broth for MIC (Merck,Germany), Microdilution plates (48U-shaped wells, Nunc, Roskilde, Denmark).

In this study, two fungi (namely *A. fumigatus* (ATCC No. 42202) and *Aspergillus flavus* (ATCC No. 5004) were used. The standard species of both fungi were prepared from fungus bank located in Tehran University of Medical Sciences, School of Public Health.

2.2. Preparing ethanolic extract of walnut thin shell

In order to prepare the extract, Iranian walnuts with no traces of mold were purchased from different stores in Tehran. After the removal of thin walnut shells, they were dried at room temperature. Then, they were powdered by electrical grinding machine. Prior to taking the extract, the powder became totally sterile using a laminar hood and under the influence of UV ray. Following this step to produce ethanol extract, 20 grams of dried powder was mixed with 100 cc of 80% ethanol and kept for 24 hours at room temperature (about 22 °C). Then, the extract was filtered by filter papers and poured into the rotary device (to remove ethanol). The obtained alcoholic extract was dried at 40 °C. After that, one gram of dried alcoholic extract was added to 5 cc of dimethyl sulfoxide solvent, and filtered and sterilized by syringes having filters with a diameter of 0.22 micron (6).

2.3. Preparing fungi suspension

The fungi were cultured in the Sabouraud Dextrose Agar (SDA) in the form of transplanting and were held for three days at 25 °C. After three days, the plates were examined macroscopically. Slide cultures were also prepared to determine the microscopic form of fungi. New fresh culture *Aspergillus* spores were solved in distilled water and its concentration was set equal to the McFarland standard of 0.5 (9.95 mL of sulfuric acid and 1% and 0.05 ml of Barium chloride). Standard Nystatin powder and fluconazole were prepared. Dimethyl sulfoxide (DMSO) and distilled water were used to solve Nystatin (insoluble in water) and Fluconazole (soluble in water) respectively then walnut extract, Nystatin, and fluconazole were diluted in a 22 series for the precise comparison MIC and MFC between walnut extract and the other ones.

2.4. Minimum inhibitory concentration (MIC) of fungus growth Determination

Antifungal susceptibility test was carried out by using the broth microdilution method described in the Clinical and Laboratory Standards Institute (CLSI). In this part for determining of inhibitory concentration on fungus, 22 dilution series of thin shell walnut methanol extract from 350mg/ml to 5 liter were prepared then these series were added to wells also 22 dilution series for Nystatin and Fluconazole were prepared separately. For determining of MIC, 200 micro liter Sabouraud Dextrose Broth were added to 24 wells of microdilution plate after that 200 micro liter of walnut extract, Nystatin and Fluconazole were injected to 22 dilution series. Of 24 wells, Two wells were considered as a growth controls; one of them as a positive control and the other as a negative one. Negative control contained media and positive control consisted media and microbial suspension. Subsequently 50 micro liter of fungus suspension according to McFarland standard were added to all wells except the negative one. These steps were repeated for Nystatin and Fluconazole. All tests were carried out three times. At last, microdilution plates were incubated at 25 °C for 48-72 hours then they were observed for the presence or absence of visible growth. The growth in each well and turbidity were comparison with McFarland standard. No turbidity regard as no growth and success of extract. (21).

2.5. Minimum fungicidal concentration (MFC) of fungus growth Determination

For determining MFC, samples were picked up from transparent tubes with a sterile loop and transferred to culture media contained Sabouraud Dextrose Agar (SDA) and cultivated through streak culture method then they were incubated at 25 0C for 48-72 hours. After that plates were checked on behalf of growing fungus. Ones with minimum extract concentration and without any fungus colonies, considered as MFC (22).

3. Results

Inhibitory effect of thin shell walnut on two kinds of *Aspergillus* was investigated in Mycology laboratory of School of Health, Tehran University of Medical Sciences, Tehran, Iran in about 6 months. The results were shown in tables 1 and 2. Findings indicated that ethanolic shell walnut extract had inhibitory effect on *Aspergillus spp.,* prominently but fungicidal effect was not notable. As shown in tables, MIC of thin shell walnut for *A.fumigates* were 15 mg/ml whereas this figure was 30 mg/ml and 10 mg/ml for Nystatin and fluconazole respectively (table 1).

MIC of the extract on *A.flavus* was 50 mg/ml which is less than Nystatin MIC with 60 mg/ml and more than fluconazol MIC with 30 mg/ml (table 2).

Table 1. Minimum Inhibitory Concentration (MIC) for *Aspergillus flavus* (mg/ml)

Concentration	Extract	Nystatin	fluconazole
5 mg/ml	+	+	+
10 mg/ml	+	+	-
15 mg/ml	-	+	-
20 mg/ml	-	+	-
30 mg/ml	-	-	-*
40 mg/ml	-	-	-
50 mg/ml	-	-	-
60 mg/ml	-	-*	-
70 mg/ml	-	-	-
80 mg/ml	-	-	-
90 mg/ml	-	-	-
100 mg/ml	-	-	-
125 mg/ml	-	-	-
150 mg/ml	-	-	-
175 mg/ml	-	-	-
200 mg/ml	-	-	-
225 mg/ml	-	-	-
250 mg/ml	-*	-	-
275 mg/ml	-	-	-
300 mg/ml	-	-	-
325 mg/ml	-*	-	-
350 mg/ml	-	-	-
Control +	+	+	+
Control -	-	-	-

* Minimum fungicidal concentration (MFC)

Also MFC for *Aspergillus Fumigant* in walnut extract was 250mg/ml and for Nystatin and Fluconazole were 60 and 30 mg/ml respectively. MFC for *Apergillus flavu*s with walnut extract was 325 mg/ml and for Nystatin and Fluconazole were 90 and 50 mg/ml in that order. It means that MFC of thin walnut methanol extract for both fungus was more than Nystatin and Fluconazole.

Table 2. Minimum Inhibitory Concentration (MIC) for *Aspergillus flavus* (mg/ml)

Concentration	Extract	Nystatin	fluconazole
5 mg/ml	+	+	+
10 mg/ml	+	+	+
15 mg/ml	+	+	+
20 mg/ml	+	+	+
30 mg/ml	+	+	-
40 mg/ml	+	+	-
50 mg/ml	-	+	-*
60 mg/ml	-	-	-
70 mg/ml	-	-	-
80 mg/ml	-	-	-
90 mg/ml	-	-*	-
100 mg/ml	-	-	-
125 mg/ml	-	-	-
150 mg/ml	-	-	-
175 mg/ml	-	-	-
200 mg/ml	-	-	-
225 mg/ml	-	-	-
250 mg/ml	-	-	-
275 mg/ml	-	-	-
300 mg/ml	-	-	-
325 mg/ml	-*	-	-
350 mg/ml	-	-	-
Control +	+	+	+
Control -	-	-	-

* Minimum fungicidal concentration (MFC)

4. Discussion

According to the results, thin shell walnut had notable inhibitory and acceptable fungicide effects on *Aspergillus spp.* The prepared walnut extract can play a key role in controlling health hazards and to be used as a safe and effective additive. Although a large number of different chemical compounds exist in herbal extracts and have similar antimicrobial function, they do not have a specific mechanism. They have several targets in cells. Three main sections for the interaction of antimicrobial substances are the cell wall, cytoplasmic membrane and Cytoplasm (23, 24). Antifungal effect of common herbal antioxidants such as tocopherols and flavonoids and relevant compounds such as coumarins, derivatives of cinnamic acid and phenolic acids which are produced by plants as secondary products could damage mitochondrial DNA and cell walls and result in microorganism death (25, 26) several studies have investigated the role of herbal extracts in preventing the growth of fungal and bacterial pathogens (27).

The results obtained by Akbari et al. showed that thin shell around the walnut is full of phenolic and antioxidant compounds and, as a defective layer, protects fatty acids, especially polyunsaturated fatty acids, against free radicals. (28) The extract tested in the current research had an acceptable effect on *A.flavus* and *A.fumigates* because of existence of phenoicl and antioxidant compounds in thin shell walnut.

Furthermore, Molyneux et al. indicated that hydrolyzable tannins which are in a physical and chemical defensive tissue surrounding the edible portion of walnuts have role in eliminating formation of aflatoxins. These tannins can be hydrolyzed by a fungal tannase present in *A. flavus*, yielding gallic acid and ellagic acid, testing of which showed that only gallic acid had potent inhibitory activity (29).

In an experimental study, the inhibitory effect of nettle and Mentha piperita on the growth of *A.flavus* and consequently reducing level of aflatoxin B1 was studied. Essential oil of M.piperita and nettle at the 1000 mg/l concentration decreased the dried mycelia weight 79.40% and 53.30% and decreased AFB1 production 88.25% and 57.3%, respectively. The ethanol extract of M. piperita at 6000mg/l decreased mycelial growth 95.25% and FB1 production 89.58%. Aqueous extracts of nettle and M. piperita at the 4000 mg/l concentration decreased AFB1 content 78.15% and 56.1%, respectively. It seems that the extract of thin walnut shell can prohibit the growth of *A.flavus* in less concentration (30). Another study revealed that the production of fungal mycelium decreased with increasing concentrations of licorice extract. The highest inhibitory concentration was 500 mg/ml of the licorice extract also 10 mg/ml concentration of licorice extract inhibited toxin production by 99.9% which is less than of 50 mg/ml that obtain from the extract of thin walnut shell (31).

An experimental study investigated that the effects of thyme (Thymus vulgaris L.), milk thistle (Silybum marianum L.) and cape aloe (Aloe vera) essences on the growth of *A. flavus* and AFB 1production. Findings showed that 300 and 400 ppm of thyme essences had the highest MIC and MFC on fungal colonies growth and AFB1production. This MIC is less than the extract of thin walnut shell may be more phenolic compound – –*Thymus vulgaris* L. caused better inhibition than the other essential oils (32).

Another research conducted effect of the leaf extract of Aloe vera on growth of *A.flavus*. Results showed the maximum antifungal activity was observed in acetone extract and in concentration of 2000µL. the inhibition of Aflatoxin production in 2000µL and 2µL was 40.94% and 18.14% respectively. This MIC is less than the extract of thin walnut shell (33). The other study showed the effect of ethanol leaf extract of pinus eldarica on the inhibiting growth of *A.flavus* and *A.niger* in corn biomass in vitro conditions. Findings revealed that 1 and 2% of pine leaf extract effectively reduced the number of fungi colonies at days 10 and 20 of incubation and the growth of fungi were completely inhibited on days 30 and 40 of incubation (33). About inhibitory effects of different essence, existence and amount of phenolic compounds and antioxidant, method of extract and concentration of extracted essence are very remarkable (34), regarding these thin shell walnut extract have better inhibitory effect rather than Nystatin and some other essence such as nettle but in comparison to Fluconazole and other extract like licorice and mustard extract should be used more.

The mechanism of the extract effect on the standard fungi *A.flavus* and *A.fumigatus* maybe due to affecting free radicals and creating apoptosis. Furthermore, it seems that the methanol extract of thin walnut shell prevents the pathogenicity of *A.fumigatus* and *A. flavus* because of its high antioxidant effect on phospholipase Group B and aflatoxin secreted from fungi.

5. Conclusion

The results of the current study showed that walnut shell extract has significant antifungal effects. Given the effectiveness of the extract for the growth of *A.flavus* and *A.fumigatus*, further research may confirm it as a safe compound with antifungal function to prevent fungal contamination in laboratory environments, treat fungal infections and increase the food product lifetime.

It also should be noted that more research is required to examine the use of extract as a treatment of fungal infections. Moreover, if it is proved that the extract has no effect on the bacteria growth, it may be used in the microbiological culture media. Additionally, due to the inhibitory effect of walnut shell extract on the growth of *A.flavus* which is the most important producer of aflatoxins in food products and regarding the increasing insist of the community health authorities and consumer demand for natural and safe additives and concerns about overuse and resistance of antibiotics further more better effect on inhibiting fungus rather than Fluconazole this extract can be used as an natural and effective fungicide in food products.

Conflict of interest

The authors have no conflict of interest.

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