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Antifungal effects of six herbal extracts against *Aspergillus* sp. and compared to amphotericin B and nystatin

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ABSTRACT

Aspergillus is a filamentous fungus of wide distribution having widespread species diversity. Among pathogenic species of Aspergillus, *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus flavus* are regarded as the most important pathogenic agents. The aim of this research is to study the antifungal effects of *Artemisia dracunculus L, Achillea wilhelmsii C. Koch, Bunium persicum, Cuminum cyminum L, Zataria multiflora Boiss,* and *Satureja hortensis* extracts against *Aspergillus* Sp. The herbal extracts were prepared using maceration method. 50gr of ground plant was introduced into a 1-litre flask and macerated with 400ml of 70% ethanol for 24h and then was shaken on shaker. Afterwards, different concentrations of the extract were prepared in dimethyl sulfoxide solvent (DMSO). The extracts were filtered and sterilized. Suspension of each fungus was separately prepared with 1×10^6 CFU/ml concentration. The antifungal effects of the extracts were measured through NCCLS protocols and Broth dilution method. The control and comparison were done between the antifungal effects of the mentioned herbal extracts and Amphotericin B and Nystatin. The results indicated that the range of MIC and MFC values of these drugs was significantly higher ($p\leq0.05$) compared to the prepared herbal extracts of different dilutions and control group of drug solvent, too. Results showed that the extracts of all herbal extracts have antifungal properties against Aspergillus sp.

1. Introduction

The prevalence of drug resistance among infectious agents is considered as a common problem that affects the individuals' health around the world. The incidence and spread of drug resistance have been observed in various agents including bacteria, fungi, protozoa, and viruses (Doust et al., 2013). Recently, with an increase in the infections resistant to antibiotics, conducting research on drugs with new efficacy against infections is an absolute necessity (Kalemba and Kunicka, 2003). The essences are a mixture of the secondary volatile metabolites from plants which are obtained in several ways. They are highly complex natural compounds which may compose of approximately 20 to 60 components in completely different concentrations (Bakkali et al., 2008). Aspergillus mold fungus is a large genus consisted of over 200 species to which humans are constantly exposed. Only few of these species are pathogenic among which more than 95% of the infections caused by three species of Aspergillus including *A. fumigatus, A. flavus, A. niger* (Anaissie et al., 2009). *Aspergillus* sp. is among pathogenic fungi causing infection through spores entering human body and its infection is invasive and very serious in individuals with deficient immune systems. Even in healthy people, Aspergillus may cause local infections in lungs, sinuses, and other organs of the body (Teles and Seixas, 2015). Among the diseases resulting from Aspergillus infections are acute invasive pulmonary aspergillosis, bronchial obstruction and bronchiolitis aspergillosis, cerebral aspergillosis, cutaneous aspergillosis, pulmonary aspergillosis, and chronic necrotizing pulmonary aspergillosis. The selective drugs for treatment of Aspergillus infections are Amphotericin B,

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Voriconazole, and Itraconazole (Shadzi, 2007; Sullivan et al., 2004). Aspergillus resistance too many antifungals used in clinic presupposes an alarming prognosis for the people invaded by Aspergillus (Curtis et al., 2005). Today, research on antifungal substances and compounds found in plants are being supported and encouraged by medical centers and communities; in this regard, multiple drugs have been tested so far (Ghasemi Dehkordi, 2002). *Artemisia dracunculus* L, is a small perennial herb belonging to Asteraceae family. It contains insecticidal and radical's elimination activities. It has antifungal antitumor effects. Its antibacterial effects have been less studied. *A. dracunculus* is used for treatment of epilepsy in traditional medicine in Iran (Meepagala et al., 2002; Sayyah et al., 2004). *Cuminum cyminum* L, is a small herbaceous from Apiaceae family. Its fruit is very fragrant. It is commonly used as a condiment in Iran and many other countries. There are some reports on its antibacterial and antifungal activities in medical science (Boyraz and Özcan, 2005). For many years, it has been used in traditional and experimental medicine as a medicinal plant especially in relieving flatulence and stomachache. *C. cyminum* essence is among carminative herbal medicinal products available in the world pharmaceutical market (Sağdıç and Özcan, 2003). *Bunium persicum*, is an economically important plant belonging to apiaceae family that grows wild in the areas of dry climate. Its seeds are rich in extracts and widely used as a spice. In native medicine, these seeds are used as stimulant and carminative medicines and are useful in treating diarrhea and dyspepsia (Baser et al., 1997). *Zataria multiflora Boiss* is a medicinal plant whose antibacterial properties have been proven. The plant extract consists of two phenolic isomers named thymol and carvacrol that intense antimicrobial property of the plant is being attributed to the presence of these two substances (Salgueiro et al., 2003; Daferera et al., 2003). *Satureja hortensis*, is one of the most important 12 species of available savory in Iran which is cultivated in various areas of the country. All dried parts of the plant especially its aerial parts are widely used as condiment in food. In traditional medicine, it has ant flatulence, orexigenic, anti-diarrhea, and diuretic properties. In some areas, it is being used as a treatment for pain and inflammatory diseases (Hajhashemi et al., 2002). *Achillea Wilhelmsii C. Koch* includes 85 species from which 7 species are exclusive to Iran and have a relatively wide dispersion in different provinces. The alcoholic extract of the aerial parts of this flowering plant is antihypertensive and anti-hyperlipidemic. Aqueous-ethanol extract of *Achillea wilhelmsii* has an inhibitory effect on gastric acid basal secretion through inhibition of stomach vagus nerve (Asgary et al., 2000). Knowing the fact that the side effects of antifungal drugs have always been one of the problems of patients treated with these drugs and since *A. dracunculus, Z. multiflora Boiss, S. hortensis, B.persicum, C. cyminum,* and *A. wilhelmsii* grow in vast areas of Iran and are native, and also antifungal effects of the extracts of these plants have not been studied on different species of aspergillus until now, the aim of study antifungal effects of six herbal extracts against aspergillus sp. and compared to amphotericin b and nystatin.

2. Methodology

This study was conducted in cooperation with Mycology Department of Kurdestan University of Medical Sciences and Islamic Azad University of Sanandaj from Aban 1390 to Mehr 1391, over one year. *A. niger, A. flavus,* and *A. fumigatus* species isolated from patients' clinical samples were used. Three clinical samples were used from each species, and each species was confirmed based on morphological features of the colony grown on Sabourand dextrose agar culture medium (Merck, Germany) and also in terms of microscopic features.

2.1. Extraction

Artemisia dracunculus, A. wilhelmsii, Bunium persicum, Cuminum cyminum, Zataria multiflora Boiss, and Satureja hortensis were prepared and confirmed by the researchers of Agricultural Research Center of Kurdestan. The mentioned plants were washed with distilled water to separate their waste materials, and they were completely dried and totally ground using the grinder, and then coarse particles were removed by passing through a sieve and the obtained powder of mentioned plants was used for extraction. The herbal extracts were prepared through maceration. For this purpose, 50gr of ground plants was introduced into a 1-liter flask, macerated with 400ml of 70% ethanol for 24 h, and then was shaken on shaker for 1hr. Using Buchner funnel (Minisart, Sartorius Stedim Biotech GmbH, Germany), flask contents were percolated and the residue was extracted again for two more times. In the last stage, the filtered products were added together and dried in a vacuum distillation unit. Finally, using dried extractions, 5, 12, 25, 50, 100, 200, 500 mg/ml concentrations of extract were prepared in Dimethyl sulfoxide solvent (Merck, Germany). The resulting solution of extracts was filtered using a 0.2µ filter and sterilized (Shanmugam et al., 2010).

2.2. Antimicrobial susceptibility testing of herbal extracts using Broth dilution method

Microbial suspension of each one of the fungi was prepared separately with 1×10^6 CFU/ml concentration. Based on what was conducted in the prior study, fungi were grown on Sabour and dextrose agar culture medium from the mentioned suspensions in sterile conditions (Rashidi et al., 2012). The study of the antifungal effect of fungi was conducted using Broth dilution method based on NCCLS protocols. 1ml of each dilution of the

mentioned plant extracts was added to each tube and every 1ml of fungal suspension was added to the tubes of each dilution series. The tubes were placed in the incubator at 22-28°C for 24-48 h. The first tube found with no growth was reported as the minimum inhibitory concentration (MIC) and the first tube without making any growth in the solid medium was reported as the minimum fungicidal concentration (MFC). In this experiment, the control tube containing DMSO was used to study the effect of extracts' solvent on the considered fungi. All experiments were repeated three times for each fungal species and the average of all three times was considered for each species (Mahmoudabadi et al., 2007).

2.3. The effect of amphotericin B and nystatin on fungal species of *Aspergillus*

Control and comparison of the antifungal effect of the mentioned plant extracts were done compared with Amphotericin B and Nystatin (Sigma, America). For this purpose, 12.8mg/ml of drug was dissolved separately in 1ml of Dimethyl sulfoxide and left at laboratory temperature for 30min, and then was sterilized using a syringe filter. Thereafter, 1ml volumes of pharmaceutical stocks were prepared in sterile vials and stored at -70°C for future uses. To determine the MIC and MFC values on fungal species tested, Amphotericin B and Nystatin were divided into different dilutions from 0.08 to 12.8mg/ml in sterile conditions and their effects were studied.

2.4. Data analysis

The resulting data were entered into software SPSS 23 and t-test was used to compare the mean of quantitative variables in two test groups. The significant level $p \leq 0.05$ was considered in all tests.

3. Findings

The obtained mean of MIC/ MFC values was in the range of 1.8-5mg/ml for Amphotericin B and 2-8mg/ml for Nystatin. The obtained results of the range of MIC and MFC values of these drugs were significantly higher compared to the prepared herbal extracts in various dilutions and control group of drug solvent, too ($p \leq 0.05$). MIC and MFC values in all data obtained from extracts were normally distributed. MIC and MFC values of each extract on fungal *A. niger, A. fumigatus*, and *A. flavus* species separately did not have any significant difference compared to each other ($p>0.05$). MIC and MFC values of extracts were obtained in total of three Aspergillus sp. (Table 1 and 2). The extracts' effect of a total of three species of Aspergillus showed that although the MIC mean of *A. dracunculus* was higher than *S. hortensis*, this increase was not significance ($p=0.054$). Furthermore, the MIC mean of *Z. multiflora Boiss* extract was significantly lower than *C. cyminum* extract ($p=0.022$) and likewise, their MFC mean were significant ($p=0.001$). The MFC mean of *Z. multiflora Boiss* extract was significantly lower than *A. dracunculus* ($p=0.004$) and *B. persicum* ($p=0.011$). The MIC mean of *S. hortensis* extract was significantly lower than *B. persicum* ($p=0.049$) and *C. cyminum* ($p=0.009$) extracts. Additionally, the MFC mean of *S. hortensis* extract was significantly lower compared with *A. dracunculus* $p=0.022$) and *B. persicum* ($p=0.044$). The MIC mean of *A. wilhelmsii* extract was significantly lower than *C. cyminum* ($p=0.011$) and its MFC mean was lower than *A. dracunculus* ($p=0.004$), *B. persicum* ($p=0.009$), and *C. cyminum* ($p=0.001$). Also, the MIC mean of *B. persicum* was higher than *A. wilhelmsii* but this increase was not significant ($p=0.062$). In addition, the MIC mean of *B. persicum* was higher than *Z. multiflora Boiss* but the increase was not significant ($p=0.095$) and the MFC mean of *S. hortensis* was higher than *A. wilhelmsii* but the difference was not significant as well ($p=0.055$). Meanwhile, *A. flavus* was the most susceptibile and *A. fumigatus* the most resistant of fungal species against antifungal effects of extracts and the mentioned drug ($p \leq 0.05$) (Table 3). The mean of minimum inhibitory concentration (MIC) of *A. dracunculus* extract was obtained within the range of 25-250mg/ml in total of three fungal species studied in liquid Sabourand dextrose agar medium, within the range of 25-62.5mg/ml for *A. wilhelmsii*, 25-250mg/ml for *B. persicum* extract, 50-250mg/ml for *C. cyminum* extract, 12.5-125mg/ml for *Z. multiflora Boiss* extract, and 12.5-62.5mg/ml for *S. hortensis* extract. The mean of the minimum fungicidal concentration (MFC) of *A. dracunculus* extract in total of three Aspergillus sp. on Sabourand dextrose agar medium was obtained within the range of 50-250mg/ml, 31.5-62.5mg/ml for *A. wilhelmsii* extract, 25-250mg/ml for *B. persicum* extract, 50-250mg/ml for *C. cyminum* extract, 12.5-125mg/ml for *Z. multiflora Boiss* extract, and 50-125mg/ml for *S. hortensis* extract.

Table 1: The mean of MIC/MFC values (mg/ml) for the extracts, and amphotricin B & nystatin against of *Aspergillus* sp. in liquid Sabourand dextrose medium

Extract	MIC (Mean±SD) (mg/ml)	MFC (Mean±SD) (mg/ml)
Artemisia dracunculus	90.97±73.76	154.16±93.95
Achillea Wilhelmsii	42.36±16.76	48.61±15.55
Bunium persicum	104.16±98.57	147.22±98.57
Cuminum cyminum	120.83±91.85l	175±91.85
Zataria multiflora Boiss	46.55±34.81	46.52±34.81
Satureja hortensis	38.19±30.47	72.22±30.47
Amphotericin B	2.82±1.21	3.5±1.32
Nystatin	4.85±2.13	5.52±2.68

Table 2: The mean of MIC/MFC of the extracts and amphotricin B & nystatin in terms of the effect on fungal sp. separately in liquid Sabourand dextrose medium

Fungal species	*Aspergillus niger*		*Aspergillus fumigatus*		*Aspergillus flavus*	
Drug and selected antifungal extracts	MFC(mg/ml) Mean±SD	MIC(mg/ml) Mean±SD	MFC(mg/ml) Mean±SD	MIC(mg/ml) Mean±SD	MFC(mg/ml) Mean±SD	MIC(mg/ml) Mean±SD
A.dracunculus	137.5±99.21	60.41±56.01	183.33±115.47	91.66±57.33	141.67±101.03	120.84±112.03
A.wilhelmsii	47.91±15.72	47.91±15.72	58.33±7.21	39.58±20.09	39.58±20.09	39.58±20.09
B. persicum	200±86.60	100±43.30	120.83±112.03	102.08±128.13	120.83±112.03	110.41±121.24
C. cyminum	183.33±115.47	141.66±101.03	200±86.60	100±43.30	141.66±101.03	120.83±112.03
Z.multiflora Boiss	35.5±25.23	35.5±25.23	56.25±60.27	56.25±60.27	47.91±15.72	47.91±15.72
S.hortensis	79.16±40.18	39.58±20.09	79.16±40.18	35.41±25.25	58.33±7.21	39.58±20.09
Amphotricin B	3±1.25	2.5±1.03	5±2.48	4.17±2.12	2.5±1.03	1.8±1.01
Nystatin	4.86±2.22	3.3±2.11	8±4.95	6.8±3.46	3.3±2.11	2.2±1.18

Table 3: Statistical analysis results of the comparison of MIC/MFC (p≤0.05)

Mean Comparison of MIC/MFC of extracts	P value
MFC mean of *Achillea Wilhelmsii* compared to *Cuminum cyminum*	P=0.001
MFC mean of *Achillea Wilhelmsii* compared to *Bunium persicum*	P=0.009
MFC mean of *Achillea Wilhelmsii* compared to *Artemisia dracunculus*	P=0.004
MIC mean of *Achillea Wilhelmsii* compared to *Cuminum cyminum*	P=0.011
MFC mean of *Satureja hortensis* compared to *Bunium persicum*	P=0.044
MFC mean of *Satureja hortensis* compared to *Artemisia dracunculus*	P=0.02
MIC mean of *Satureja hortensis* compared to *Cuminum cyminum*	P=0.009
MIC mean of *Satureja hortensis* compared to *Bunium persicum*	P=0.049
MFC mean of *Zataria multiflora Boiss* compared to *Cuminum cyminum*	P=0.001
MFC mean of *Zataria multiflora Boiss* compared to *Bunium persicum*	P=0.011
MIC mean of *Zataria multiflora Boiss* compared to *Cuminum cyminum*	P=0.022

4. Discussion

Due to detecting new drugs and anti-fungal compounds on the one hand and observing the resistance of some fungal agents to several drugs including Fluorocytosine, Amphotericin B, and Nystatin, and also the increased incidence of fungal infections and different antifungal drugs use on the other hand, replacing effective and safe drugs against fungal agents seems necessary (O'Gorman and Hopfer, 1991). In the present study, the antifungal effect of the extracts of *A. dracunculus, A. wilhelmsii, B. persicum, C. cyminum, Z. multiflora Boiss,* and *S. hortensis* was examined on fungal species of Aspergillus. The obtained results suggested that all extracts had antifungal effect on the fungal species of Aspergillus. Concerning the obtained results from Broth dilution method, it was indicated that the extracts of *Z. multiflora Boiss* and *A. wilhelmsii* had a strong effect on Aspergillus species inhibition and even in higher concentrations had bactericidal effect. Regarding the effect of growth inhibition on fungal species of Aspergillus, the extracts of *Z. multiflora Boiss* and *A. wilhelmsii* showed a higher effect than the other extracts (p≤0.05). There have been done numerous studies on the effect of antimicrobial extracts of *C. cyminum* and *B. persicum*. In a study, the effect of the extracts of *A. nilotica, P. granatum*, *F. vulgare,* and *C. cyminum* was examined in vitro on *Candida albicans* fungus in which results showed that these extracts inhibited in-vitro *C. albicans* growth. In this research, mean of the antifungal effect of *C. cyminum* extract was reported 1.3±6.5µg/ml (Pai et al., 2010). In another study, antimicrobial effect of *C. cyminum* against *Escherichia coli* and *Salmonella typhimurium* bacteria has been studied and proven (Mekawey et al., 2009). While in the present study, MFC value for *B. persicum* and *C. cyminum* was obtained 98.57±147.22 and 91.85±175mg/ml, respectively. *Z. multiflora Boiss* is a medical plant whose antibacterial properties have been proven. The plant extract composes of two phenolic isomers named thymol and carvacrol that the intense antimicrobial property of this plant is attributed to the presence of these two substances (Salgueiro et al., 2003; Daferera et al., 2003). Marino and his coworkers showed that *Z. multiflora Boiss* essence has an extremely high bactericidal activity and the greatest effect on *Escherichia coli* (Marino et al., 1999). Okazaki and his coworkers stated that the obtained essence from *Z. multiflora Boiss* prevents the coagulation of platelets and has therapeutic aspects (Okazaki et al., 2002). Karaman confirmed the inhibitory effect of the obtained essence from aerial parts of *Z. multiflora Boiss* on the growth of *C. albicans*, *C. tropicalis*, and *S. cerevisiae* (Karaman et al., 2001). In Mahmoudabadi et al. (2007) antimicrobial effect of ethanol and methanol extracts of *Z. multiflora Boiss* were examined on *C. albicans* fungus. According to their findings, ethanol and methanol extracts of *Z. multiflora Boiss* have shown antifungal effect with MICs of 7, 70, and 127mg/ml, respectively (Mahmoudabadi et al., 2007). The results of this study demonstrated that the MIC mean of *Z. multiflora Boiss* extract was significantly lower than *C. cyminum* extract (p=0.022) and its MFC mean was similarly significant (p=0.001). The MFC mean of *Zataria multiflora Boiss* was significantly lower than the extracts of *A. dracunculus* (p=0.004) and *B. persicum* (p=0.011), and this also represented the fact that the results of the earlier studies corresponded to the present study results and confirmed the antibacterial effects of mentioned extracts. MIC and MFC values of *A. wilhelmsii* was 42.36±16.76 and 48.61±15.55mg/ml, respectively, and the most susceptible and resistance fungi to *A. wilhelmsii* effects were *A. fumigatus*, *A. flavus*, respectively, in the way that MIC mean of *A.*

wilhelmsii extract was significantly lower than *C. cyminum* (p=0.011) and its MFC mean was also lower than *A. dracunculus* (p=0.004), *B. persicum* (p=0.009), and *C. cyminum* (p=0.001).

5. Conclusion

The results of the study showed that the extracts of *A. dracunculus, A. wilhelmsii, B. persicum, C. cyminum, Z. multiflora Boiss,* and *S. hortensis* have antifungal properties against all studied Aspergillus sp. These three species of Aspergillus are clinically more important than the rest of the species. Regarding the effects of these extracts and the fact that the greatest anti-fungal effects of Aspergillus species was observed in the extracts of *A. wilhelmsii* and *Z. multiflora Boiss*, it will be possible to use the active substances of these plants to provide anti-aspergillus agents.

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