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Antifungal Effects of Oregano Essential Oil on Dermatophyte and Non-Dermatophyte Fungi Isolates from Sport Mats: A Report from the Golestan Province, Iran

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ABSTRACT

Fungal infections are a major health problem worldwide due to their high prevalence. Proper disinfection is the most effective way to control such infections. The aim of this study was to determine the frequency of fungi isolates from sport mats and to investigate antifungal properties of oregano essential oil against the isolates. In this cross-sectional study, 48 samples were collected from six sports clubs between spring and summer 2020. The samples were analyzed using the sterile carpet method (4×4). After microscopic examination and mycological culture, the antifungal effect of oregano essential oil was evaluated using the dilution-neutralization method according to the protocols of Iran's national standards 2824 and 9899.Overall, fungal isolates were found in 23 samples (47.9%), of which 56.6% were dermatophytes and 43.4% were non-dermatophyte species. *Trichophyton tonsurans* (34.8%) and *Aspergillus niger* (21.8%) were the most common dermatophyte and non-dermatophyte isolates, respectively. The oregano essential oil at concentration of 400 mg/ml, inhibited growth of 65% of non-dermatophyte isolates ($P<0.05$). This effect was more profound against *Candida albicans*. However, the essential oil had no significant effect on dermatophytes ($P>0.05$). Given the satisfactory antifungal effect of oregano essential oil in high concentration, it is suggested to use this essential oil as a natural disinfectant to control growth of non-dermatophyte fungi in sports clubs.

1. Introduction

Infectious diseases caused by fungi are common major public health problems worldwide (Chowdhry and Gupta, 2013). Such infections have been reported among athletes and wrestlers. Sport mats can spread infectious microorganisms, including fungi, to athletes. Among fungal infections, superficial mycosis caused by dermatophytes has recived more of the attention since it is highly contagious. Dermatophytes are a group of widely-spread fungi that are mainly transmitted through direct contact with infected human, animals and soil (Oliveira et al., 2006, Lange et al., 2004). Although these infections are rarely life-threatening, they can negatively affect mood, daily activities and ultimately the economic status of the affected person (Adams,2002). The prevalence of dermatophytes in athletes has been reported to be between 20 to 77%. Although dermatophytes, including *Trichophyton*, are the main cause of mycosis, non-dermatophyte molds and yeasts can sometimes cause skin infections.

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Given the high importance of such infections, tracking their reservoirs seems crucial. Sports clubs have been regarded as one the reservoirs for these fungal infections. In this regard, moisture caused by sweating is a key factor that provides a suitable environment for the growth and multiplication of various pathogenic microorganisms. Contact with contaminated surfaces such as exercise mats increases the risk of transmission of various infections, particularly in highly humid regions (Teklebirhan, 2015, Frisk, 1996), including the Golestan Province in northern Iran. Therefore, identifying effective disinfectants for sterilization of these places and sports equipment, especially exercise mats can be effective in reducing the frequency of fungi and subsequently the incidence of fungal skin infections. Medicinal plants are among natural compounds that are effective in sterilization. Oregano is a native plant in Iran and cultivated in the northern and western provinces of the country. This plant has good antimicrobial and antioxidant properties due to its essential oil components such as alpha-thujene, alpha-pinene, octanone and thymol (Namvar et al., 2016, Moradi et al.,2014). The aim of this study was to investigate the frequency of fungi isolates from exercise mats and the anti-fungal properties of oregano essential oil against the isolates.

2. Materials and Methods

2.1. Fungi Isolation

In this cross-sectional study, sampling was done from six sports clubs in the Golestan Province (Gorgan and Kordkuy) between March and August 2020. Ambient temperature of the clubs varied between 22 and 35 °C depending on the season. In each club, four samples were taken from two exercise mats (eight samples per club) using sterile swabs. The samples were examined using the sterile carpet method (4×4). A part of the samples was cultured in sabouraud dextrose agar containing chloramphenicol without cyclohexamide (Merck, Germany) and the other part in mycobiotic agar (Merck, Germany). After four weeks of incubation at 25 ^{o}C, macroscopic characteristics of the grown fungi such as growth, colony color as well as microscopic characteristics such as size and shape of the reproductive organ were examined with lactophenol cotton blue. *Trichophyton* species were identified by urease test. *Candida* species were identified using chromogenic medium (Himedia, India). *Malassezia* yeasts were detected by growth in Dixon agar, catalase test and Tween assimilation test.

2.2. Molecular identification of fungal isolates

DNA was extracted from phenotypically identified isolates by the phenol-chloroform method. PCR was carried out in a 25 µl reaction solution containing 1.5 µl of forward and reverse primers, 0.5 µl of $MgCl_2$, 1 µl of sample DNA, 8 µl deionized water, 12.5 µl PCR Mastermix and 1.5 µl PCR buffer. The PCR process was done using universal fungal primers and specific primers for *Trichophyton rubrum* (Table 1).

PCR reaction was performed according to the following conditions: denaturation at 94 °C for 5 minutes, annealing at 94 °C for 45 seconds, 35 cycles of elongation at 72 °C for one minute and one cycle of final expansion at 72 °C for one minute. PCR products were mixed with a sampling buffer (5:1 v/v) and subjected to electrophoresis on 2% agarose gel. Sequencing was done using the Blast software and sequence similarity was examined in the GeneBank. Isolates were identified by detecting sequence similarity of 99-100% in the NCBI database.

2.3. Preparation of oregano essential oil

After collecting the oregano from plains in Gilan Province, the oregano seeds were separated and mixed with 70% ethanol in a ratio of 1 to 10 on a shaker. After 24 hours, the resulting mixture was filtered and placed in a rotary evaporator. The oregano essential oil was obtained by the maceration method. After filtration with Whatman® Grade 1 filter paper, the essential oil was cooled at room temperature and then stored at 4 °C.

2.4. Determination of antifungal properties of oregano essential oil

To determine the effective concentration of oregano essential oil, the dilution-neutralization method was applied according to Iran's national standards 2842 and 9899. First, a fungal suspension was prepared from *Aspergillus* and *Trichophyton* conidia (3×10^7 colonies/ml) and yeast cells (1×10^6 colonies/ml). Then, 1 ml of interfering substance (30 g/l skimmed milk) was

added to 1 ml of the fungal suspensions. Next, 5 ml of different concentrations (50, 100, 200, 400 mg/ml) of the oregano essential oil were added to tubes containing 1 ml of fungal suspensions. After 5 minutes, 4 ml of neutralizer (containing lecithin and thiosulfate) were added to the tubes to inhibit the possible effect of the essential oil. After culturing each sample and counting the colonies, a concentration of disinfectant that reduced the number of fungal colonies by at least 5 to 10 times was regarded as the effective concentration of disinfectant. In the case of the essential oil, logarithmic reduction was evaluated using the following formula: Log R = LogN0-LogNA.

2.5. Statistical analysis

Normal distribution of data was verified using the Kolmogorov-Smirnov test. Quantitative data were analyzed using independent t-test and qualitative data were analyzed using the Chi-square test. All statistical analyses were carried out at 95% confidence level.

Table 1. Primers sequences used in this study (Garg et al., 2007).

Primer Sequence	Sequence (5'-3')	Product (bp)
ITS1-Forward primer	5/-TCC GTAGGTGAACCTGCGG-3/	700
ITSu -Reverse primer	5/-TCC TCC GCT TAT TGA TAT GC-3/	
T-rub -Forward primer	5/-GCC TGT TGT TCC GCT CAT TCT T-3/	500
T-rub -Reverse primer	5/-CGG CTA GGA GGG CGT GGT AGA-3/	

3. Results

Out of 48 samples collected from six sports clubs, 23 samples (47.9%) were positive for fungal contamination in phenotype and genotype tests. *Trichophyton tonsurans* (n=8, 34.8%) and *Aspergillus* (n=5, 21.8%) were the most common dermatophyte and non-dermatophyte isolates, respectively. The highest dermatophyte frequency (17.4%) was observed in sports club 1, while the lowest frequency of dermatophytes (4.3%) was seen in club 4. In clubs 3 and 5, 13% of dermatophyte strains were *T. tonsurans* and *Trichophyton rubrum*. In the same clubs, 8.7% and 13% of non-dermatophyte strains were *Aspergillus niger* and *Candida albicans*, respectively. Thus, non-dermatophyte strains (*A. niger*) were prominent in club 5. Both *T. tonsurans* and *T. rubrum* with a frequency of 8.7% and *A. niger* (4.3%) were isolated from club 6. In clubs 5 and 6, only one case of *Malassezia.spp* and *Alternaria.spp* (each one 4.3%) were isolated, which were not included in the next experiments due to their low frequency. No dermatophyte was isolated from club 2 (Table 2). According to the results of the Chi-square test, there was no significant difference between the frequency of fungal strains (P=0.076).

3.1. Antifungal effects of oregano essential oil

After examining the effect of oregano essential oil, it was found that 65.2% of non-dermatophyte fungal strains including *A. niger* and *C. albicans* were eliminated at a concentration of 400 mg/ml (Figure 1). According to the national standard 2842 and the dilution-neutralization method, the oregano essential oil significantly decreased the number of fungal colonies ($P < 0.05$) but had no effect on *T. tonsurans* and *T. rubrum* isolates. The number of *C. albicans* isolates decreased significantly at concentration of 200 mg/ml of essential oil. Nevertheless, no antifungal effect was observed at lower concentrations of the essential oil (Table 3).

Table 2. Fungal isolates from the sport mats of the tested sports clubs

Clubs	Sample 1	Sample 2	Sample 3	Sample 4
Club1-1	*T. tonsurans*	-	-	*T. rubrum*
Club2-1	-	*T. tonsurans*	-	-
Club1-2	-	-	-	-
Club2-2	-	-	-	-
Club1-3	*A. niger*	-	-	*C. albicans*
Club2-3	-	*T. rubrum*	-	*T. tonsurans*
Club1-4	-	*A. niger*	-	*C. albicans*
Club2-4	-	-	*T. tonsurans*	-
Club1-5	*T. rubrum*	*A. niger*	-	-
Club2-5	*C. albicans*	*T. rubrum*	*T. tonsurans*	*Malassezia* spp.
Club1-6	*T. tonsurans*	*T. rubrum*	*A. niger*	
Club2-6	-	*Alternaria* spp.	-	-

Club1-1: Sport mat 1 from club 1
Club2-1: Sport mat 2 from club 1

Table 3. Antifungal effect of oregano essential oil against fungal isolates from Sport mats

Fungi isolates Log (number of Isolates CFU/ml)	Oregano Essential oil Concentrations(mg/ml)				P-value
	50	100	200	400	
T. tonsurans	$2.5 X10^7$	$1.8 X10^7$	$4.5 X10^6$	$1.5 X10^6$	0.076
T. rubrum	$2 X10^7$	$2 X10^7$	$1.8 X10^7$	$1.2 X10^6$	0.053
A. niger	$4 X10^6$	$3.4 X10^5$	$2 X10^3$	0	0.041*
C. albicans	$1.9 X10^3$	$1.5 X10^3$	$1 X10^2$	10	0.03*

*statistically significant

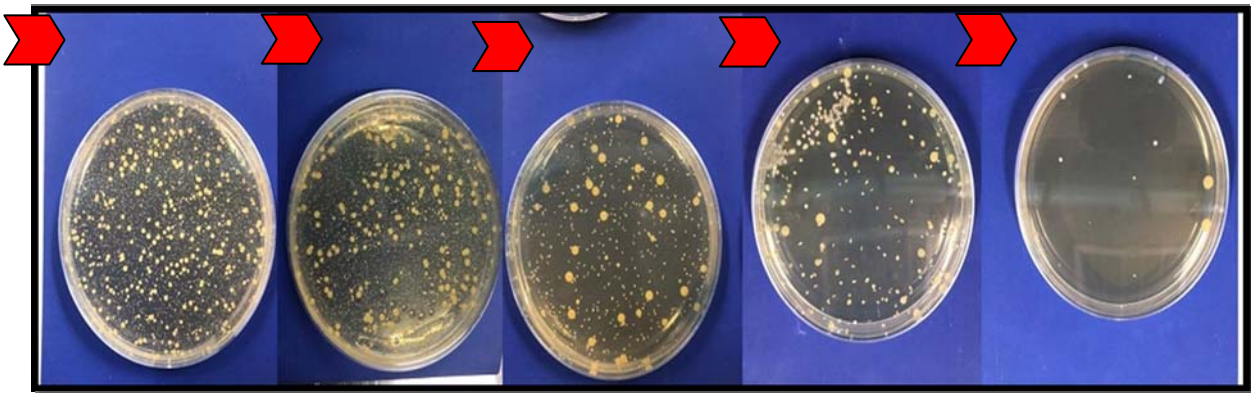

Figure1. Antifungal effect of Oregano essential oil on *C.albicans* isolate; from left to right: A: 48 h culture, B:, concentration of 50 mg/ml, C: concentration of 100, mg/ml, D:oncentration of 200 mg/ml, E: concentration of 400 mg/ml.

4. Discussion

This study is the first one to investigate the frequency of fungal strains in sports clubs of the Golestan Province, Iran. The results about the frequency of fungal strains may have been affected since gyms operated on a part-time basis because of the coronavirus pandemic. In general, the identification of fungal infections, especially skin infections, is of great importance since they affect all age groups and impose a substantial economic burden (Erbagci et al., 2005, Kohl et al., 2000). In the present study, the prevalence of dermatophytes from exercise mats was 56.6%. In a previous study, this frequency was reported to be 100% (Hedayati et al., 2007). In another study in Hamedan reported that 10% of exercise mats were contaminated with dermatophytes (Habibipour et al., 2012). This significant variation in the frequency of isolates could be due to the differences in the climate, sampling season and internal conditions of the sports clubs as non-air conditioned sports clubs were contaminated with a more diverse population of fungi. In the present study, the highest frequency of dermatophytes isolated from the sport mats was belonged to *T. tonsurans* (34.8%), which is similar to the reported rates in France (Poisson et al., 2005) and Sweden (Hradil et al., 1995). It seems that climate conditions, study season, humidity and lack of ventilation may affect on the growth of both dermatophytes and non-dermatophytes (Tan, 2005). As we know, different *Aspergillus* species are responsible for various infectious diseases, especially in immunocompromised individuals (Bongomin et al., 2018, Samson et al, 2014). In the present study, 21.8% of non-dermatophyte strains were *A. niger*. The frequency of non-dermatophyte strains, particular *Aspergillus* species isolated from superficial fungal infections was reported 14.5% and 46% in India and Ethiopia (Hazarika et al., 2019, Bitew et al., 2018), respectively. *Candida* species, particularly *C. albicans* is regarded as one of the most common opportunistic pathogens that cause skin problems (Bitew et al., 2018). Although there are many reports about the contamination of sport mats, there is still no comprehensive guideline for sterilization of these surfaces. Some researchers believe that spraying disinfectants directly onto sport mats can reduce microbial contamination (Young et al., 2017, Stehura and Jacobs, 2012). In our study, the oregano essential oil at concentration of 400 mg/ml prevented the growth of about 65% of non-dermatophytes. This inhibitory effect was more profound against yeast strains. The antimicrobial properties of oregano essential oil have been well established. These properties have been mainly attributed to carvacrol and thymol, as key components of oregano essential oil; however, these properties have been confirmed on bacteria (Nozohor et al., 2018, Dadalioglu et al., 2004). In this study the antifungal properties of oregano essential oil was reported for the first time. Given that novel antifungal disinfectants have been received attention of the many researchers (Young et al., 2017), it is suggested to evaluate the antifungal effect of oregano essential oil after direct application on exercise mats and sports equipment. The oregano essential oil in high concentrations was able to significantly reduce the frequency of non-dermatophyte isolates from sport mats. The results show that non-dermatophyte fungi play an important role in the contamination of sport mats as well. This highlights the need for more epidemiological studies to minimize the incidence of fungal skin infections in athletes. It is also recommended to evaluate the antifungal effects of oregano essential oil in a more comprehensive manner and on other pathogenic fungi to minimize their spread among athletes.

Refereces

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