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Research Article

Comparison of Anti-Fungal Effect of thyme and Pennyroyal Essential Oils with Nystatin against Standard and Clinical Species of Oral *Candida albicans*

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ABSTRACT

Backgroundandaims: Candidiasis is one of the most common opportunistic fungal infections caused by Candida albicans. In addition to antifungal chemical drugs, recently traditional medicine approach using essential oils has been considered. Other studies have shown that Thymus vulgaris (thyme) and Menthapulegium (pennyroyal) essential oils can inhibit the growth of Candida albicans.The aim of this studywas comparing anti-Candida albicans activity of Thymus vulgaris and Menthapulegium essential oils with that ofnystatin.

Materials andmethods: Total antioxidant activity and total phenolic content of thyme and pennyroyal were assayed by FRAP assay and Folin□Ciocalteu (FCR) reagent, respectively. Flavonoid content was assayed using aluminum chloride and photometry. Minimum inhibitory concentration (MIC)against *Candida albicans* was determined, in vitro. clinical species *Candida albicans* were isolated from the dorsum of the tongue in 15 healthy children and one of their healthy parents.

Results: Total antioxidant concentration of thyme (1.76 molar) was more than that of pennyroyal (0.146×10^{-3} molar). the concentration of polyphenols in thyme (79.5 mg/mL) was more than that of in pennyroyal (0.132 mg/mL). MIC of thyme (73.83-2362.5 µg/mL) was significantly less than that of pennyroyal (578.75-9260 µg/mL) against *Candida albicans* ($P<0.001$). MIC of nystatin was 2–8 µg/mL. MIC of these solutions was not significantly different between children and parents.

Conclusion: Considering the limitations of this study, thyme essential oil can be used in the production of anti-candida agents in certain situations for better results as complementary synthetic drugs but after more experiments.

Key words: Essential oil, Thyme, Pennyroyal, nystatin, candida albicans

INTRODUCTION

Oral candidiasis is one of the most common opportunistic infection that affects the oral cavity. It is common among the elderly and infants (1). The management of candidiasis is problematic because of the toxicity of commonly used antifungal drugs against host cells (2). Nystatin is

the most commonly used antifungal agent for the treatment of candidiasis (3). Nystatin has various forms, such as oral suspension, topical cream and oral pastille. It is also used in the prophylaxis of oral and systemic candidiasis in newborns, infants and immunocompromised patients.It has low incidence of drug interactions and acceptable cost. Some studies have shown that poor taste and gastrointestinal adverse reactions were the most common side effects of nystatin (1). Because of resistance and side effects of antifungal drugs, many studies have been carried out on the use of herbal antifungal essential oils (4).Previous studies have shown anti-candidal activity of herbal medicines (4, 5, 6).Research has shown that herbal medicinesare rich in compounds with biological activities, preventing the productionof oxidants (7). Flavonoids and other phenolic compounds are secondary metabolites that exist in plants. Phenol compounds have biologic effects like anti-allergic, antimicrobial and anti-inflammatory effects. The beneficial effects of phenol components are related to their antioxidant activities (8). Of course essential oils have side effects (9, 10) but in general they are safer than synthetic drugs (4). Over 80% of the world population relies on traditional medicine for their health needs (11). Iranian traditional medicine is rich in various plants which have been employed as drugs to treat different diseases and disorders since the ancient times (12). Natural active products against *Candida* species have increased in recent years and include nearly 258 plant species (13). In this study we compared the antifungal effects of thyme and pennyroyal and nystatin against *Candida albicans*in vitro. In addition, as the secondary aim of this study we compared the antifungal effect of nystatin and thyme and pennyroyal between children and adults by considering the drug resistance issue in adults. We assayed total antioxidant activity, total phenolic and flavonoid contents of thyme and pennyroyal essential oils. Therefore,given the high prevalence of candidiasis in different populations, if these plants have anti-candidal effect, they might be used in the production of anti-*Candida*agents.

MATERIALS AND METHODS

Ethical considerations

The protocol of this study was approved by the Ethics Committee of Babol University of Medical Sciences under the code MUBABOL.REC.1395.139 andinformed consent was taken from all the volunteers.

Study population

Standard strain of *Candida albicans*was purchased from Institute of Industrial and Scientific Research Organization of Iran (PTCC 5027).In addition, 30 clinical samples were isolated from the dorsum of the tongue of 15 healthy children and from one of their healthy parents (n=30). These subjects had not received antifungals, antibiotics and antiseptics in the last month. In addition, they were healthy clinically without any metabolic and systemic conditions.

Preparation of essential oils and drugs

Two pure herbal essential oils of thyme and pennyroyal were purchased from the Zardband Pharmaceutical Company (Tehran, Iran). These essential oils were prepared by hydrodistillation method. Nystatin was purchased from AppliChem, Germany. Then, 9 concentrations of essential oils were prepared, including thyme (73.83-18900 mg/L), pennyroyal (72.34-18520 mg/L) and nystatin (0.125-32 mg/L).

Medium preparation

Roswell Park Memorial Institute (RPMI 1640) medium (10.4 g) with glutamine and phenol red, without bicarbonate(gibco, UK), wasdissolved in 900 mLof distilled water; then 34.53 g of MOPS (3-[N-morpholino] propanesulfonic acid) buffer (Merck, Germany) was added. The pH was adjustedat 7.0 at 25°Cand additional water was added to achieve a final volume of 1 L.The medium was sterilized by with the use of a filter,

0.22 µm in pore size (Carriglwohill, Ireland) and store at 4°C until used.

Preparation of fungal suspension

The samples were cultured on*Candida* CHROMagar (Dr. Rambach, France) Petridish spontaneously and incubated at 35°C for 48 hours.All the standard and *Candida*isolates from previous cultures were re-cultured on Sabouraud Dextrose Agar supplemented with Chloramphenicol (HiMedia Labratories, India). Candida albicans spieces according to green color created in the environment, creation a germ tube in human serum and creation vesicles in cornmeal agar with 1% tween 80 (Micro Master Labratories, India), is confirmed. After preparation of a suspension of these colonies, the concentration of yeast cell was adjustedat 0.5 McFarland standard. To this end, fungal suspensions were adjusted at 1×10^6 – 5×10^6 cells/mL using a spectrophotometer. Then, yeast cellswere adjusted at a final concentration of 2.5 – 5×10^3/mL after two dilutions by RPMI 1640 (Roswell Park Memorial Institute).

MIC

Micro-dilutiontest was used to determine minimum inhibitory concentration (MIC) of essential oils and nystatin by using M27-A3 standard presented by Clinical Laboratory Standard Institution (CLSI). To this end, essential oils and nystatin accompanying medium were added to the sterile round-bottom ELISA plates. These essential oils and nystatin dilutions were used as duplicates. The plates were incubatedfor 48 hours at 35°C.

The growth of fungiin wells with essential oils and nystatin were compared to the growth of positive and negativecontrol wellsby using a mirror. The MIC was defined as the lowest concentration of drug or essential oils that prevented any fungal growth (14).

Total antioxidant activity assay

Total antioxidant activity was estimated by a FRAP assay. The FRAP reagent contained 2.5 mL of 10-mmol/LTPTZ (2, 4, 6-tripyridyl-s-triazine; Sigma) solution in 40-mmol/LHCl plus 2.5 mL of 20-mmol/L $FeCl_3$ and 25 mL of 0.3-mol/L acetate buffer (pH=3.6). The reagent was freshly prepared and warmed at 37°C. The working FRAP reagent (1.5 mL) was mixed with 50 µLof the sample or standard in a test tube. After 10 minutes at 37°C, the absorbance was determined at 593 nm. $FeSO_4$, at a concentration of 1 mmol/L, was used as the standard solution. The final result was expressed as the concentration of antioxidant with a ferric reducing ability equivalent to that of 1-mmol/L $FeSO_4$ (15).

Total phenolic content

Folin-Ciocalteu reagent use was adapted from McDonald to determine total phenolic content. The diluted extract (0.5mL of 1:10, v/v) and phenolic standard were mixed with Folin-Ciocalteu reagent (5mL, 1:10 diluted) and aqueous Na_2CO_3 (4mL, 1M). The solutions were heated for 15 minutes in a water bath at 45°C and the total phenols were determined spectrophotometrically at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 and 500 mg/L solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values were expressed as gallic acid equivalents (mg/g dry weight) (16).

Total flavonoid content

Total flavonoid content was determined using aluminum chloride and photometry.The plant extract (0.5 mL of 1:10 g/mL) in methanol was mixed with 1.5 mLof methanol and 0.1 mL of aluminum chloride (1%), 0.1 mLof potassium acetate (1 M) and 2.8 mLof distilled water. After 30 minutes of incubation at room temperature, sample absorbance was read at 415 nm. Quercetin solution calibration curve was prepared in the range of 0□50 mg/mL in methanol. The results were reported as quercetin equivalent, mg/g dry weight (17).

Statistic analysis

Data were analyzed with SPSS 22. On the basis of Kolmogorov–Simonov test result, the normality assumption was rejected (P<0.001). Therefore for analysis of the study data Mann-Whitney nonparametric test was used. P-values <0.05 were considered significant.

RESULTS

Total antioxidant concentration of thyme (1.76 molar) was more than pennyroyal (0.146×10^{-3} molar). In addition, the amount of polyphenols in thyme (79.5 mg/mL) was more than that in pennyroyal (0.132 mg/mL).

Flavonoid test for thyme and pennyroyal did not exhibit absorption due to formation of a biphasic medium and oil-seeds or creation of colorless solutions, in addition, its absorption could not be verified with a spectrophotometer because of the light. The anti-candida activity of herbal essential oils showed that thyme essential oils had more inhibitory effect on *Candida albicans* than pennyroyal (P<0.001).

All the isolates of candida according to green color produced in the environment (figure 1), creation a germ tube in human serum and creation vesicles in cornmeal agar with 1% tween 80, were confirmed as candida albicans. MIC of thyme was variable from 73.83 to 2362.5 µg/mL. MIC of pennyroyal was variable from 578.75 to 9260 µg/mL. MIC of nystatin was variable from 2 to 8 µg/mL (table 1). MIC of these solutions did not exhibit significant differences between children and parents. MIC of thyme against Candida albicans was significantly less than that of pennyroyal in two groups of children and parents.MIC of essential oils and nystatin did not have significant difference between children and parent (table 2).

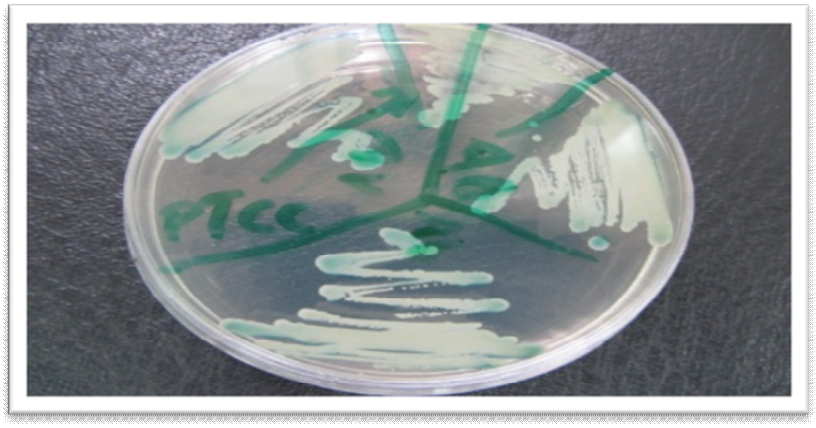

Figure 1. candida albicans isolates cultured on chrome agar and green color after 48 hours at 35° c.

Table 1. The minimum inhibitory concentration (MIC) of study solutions against standard and clinical species of oral candida albicans.

Sample code	MICof nystatin µg/m	MIC of pennyroyal µg/m	MIC of thyme µg/m
1	8	2315	1181.25
2	8	4630	147.66
3	4	2315	295.31
4	8	2315	1181.25
5	4	2315	590.62
6	4	4630	295.31
7	8	4630	2362.5
8	8	2315	590.62
9	8	9260	1181.25
10	8	4630	1181.25
11	4	9260	1181.25
12	4	4630	295.31
13	8	9260	590.62
14	8	4630	73.83
15	2	2315	295.31

16	8	4630	590.62
17	4	2315	147.66
18	8	2315	1181.25
19	4	4630	590.62
20	8	4630	295.31
21	4	2315	295.31
22	4	2315	147.66
23	4	4630	590.62
24	4	2315	590.62
25	4	578.75	295.31
26	8	2315	590.62
27	4	1157.5	590.62
28	8	2315	590.62
29	8	1157.5	590.62
30	8	2315	295.31
31	4	2315	73.83
32	8	4630	1181.25

Table 2. Means±SD (Median) of MIC of study solutions

groups	Parent	Childeren	p-value
Thymus vulgaris	664.45 ± 597.83 (590.62)	558.32 ± 360.64 (590.62)	0.922
Menthapulegium	4398.5 ± 2773.86 (4630)	2857.58 ± 1327.66 (2315)	0.163
Nistatin	6.15 ± 2.07 (8)	5.89 ± 2.22 (6)	0.767

DISCUSSION

The results of this study showed that thyme essential oil had more inhibitory effects on *Candida albicans* than pennyroyal (P<0.001).

Minimum inhibitory concentration (MIC) is the gold standard fordetermining the susceptibility of organisms to antimicrobials and is also used tojudge the performance of all the other methods of susceptibility testing. MIC is used indiagnostic laboratories to confirm unusual resistance, to give a definitive answer when aborderline result is obtained by other methods of testing, or when disc diffusion methodsare not appropriate. The range of drug concentrations used for determining MIC is universally accepted to be in doubling dilution steps up and down from 1 mg/L as required (18).

Previous studies have shown the anti-candidal activity of thyme and pennyroyal (4, 5, 6).Mahdavi evaluated the anti-candidal activities of thyme and pennyroyal against vaginal *Candida*. The results showed that thyme essential oil had more inhibitory effects on *Candida albicans* species than pennyroyal (4). Zia et al compared antifungal activity of thyme extract and nystatin against *Candida albicans*. The results showed that thyme extract can be used as an antifungal agent for the treatment of candidiasis compared to nystatin (13). We did not observed any study about comparison of pennyroyal and nystatin.

Nystatin and fluconazole are the most commonly used antifungal drugs for the treatment of candidiasis (3). Five controlled studies evaluated the safety profile of fluconazole compared with other antifungal drugs in immunocompromised children, with one beingnystatin. Children who received fluconazole exhibited more side effects, such as vomiting and diarrhea, than children who received nystatin (19). Fluconazole is the first choice for treatment, but the long-term use of this drug results in the development of fluconazole resistance(20).

In this study, the results showed that nystatin had more inhibitory effects on Candida albicans, but as synthetic drugs have limitations such as resistance, toxicity and allergy (4) maybe with more experiments,these plants could be used in certain situations for better results as complementary synthetic drugs.For example the incidence of oropharyngeal Candida infection has increased in AIDS patients. For the treatment, the patients use drugs like fluconazole and nystatin. Fluconazole has minor side effects, but long-term use of this drug results in the development of fluconazole resistance (20). Nystatin has some side effects like poor taste and gastrointestinal adverse reactions, including vomiting, nausea, anorexia and abdominal pain (1). Therefore, the authors recommend applying herbal medicine like thyme and pennyroyal in addition to these drugs. In cancer patients that are disposed to candidiasis offered these plants as a source of anti-candida, antioxidant and anticancer cells (6, 13, 21, 22, 23, 24). Of course essential oils have side effects (9, 10) but in general they are safer than synthetic drugs (4).In this context, we should be more careful when using essential oils in babies and children. Essential oils should be more diluted for consumption by infants as compared to adults. We should not administer essential oils to babies under 3 months of age because they cannot deal with the side effects of essential oils (25). Some studies have shown that thyme has side effects such as nausea and vomiting, but it does not have gastrointestinal complications unlike nystatin (10). Pennyroyal is highly suspected to be hepatotoxic. It has been responsible for fatal accidents. However, we should consider thefact that the correct dosage and good administration of essential oils might prevent such accidents (9). Thus, in general, herbal medicines are cheaper, safer and more nature-friendly.As a result, in some countries people use traditional medicines for treatment of diseases.Therefore it is necessary to compare antifungal drugs with herbal medicine (4). Total antioxidant capacity of thyme was more than that of pennyroyal;therefore, thyme essential oils are a more powerful source of antioxidants than pennyroyal. Tohidi evaluated the antioxidant activities of fourteen thymus species. The results showed that *Thymus vulgaris* had higher antioxidant activities than others. Therefore, the thymus species with high bioactive components might be used in food industry (24). Other studies showed that pennyroyal had potential antioxidant activity so that it can be used in food industry (22, 23). Ghazghazi evaluated biological activity of pennyroyal leaf extract. The results demonstrated that this plant can be used as a source of antioxidants to increase the shelf life of products (22). As synthetic drugs can lead to undesirable metabolic reactions and production of free radicals and peroxide, studies have shown that herbal medicinesare rich in compounds with biological activity in a way to prevent the production of oxidant compounds (7). In addition, because the presence of phenolic components in essential oils is one of the main reasons for their antimicrobial properties (3), maybe the lower amounts of phenolic components in pennyroyal versus thyme was the reason for the lower antifungal effect on *Candida albicans*. The anti-candidal effects of essential oils are different because of factors like environmental conditions, extraction methods and non-standardized processing; however,there are hopes for the futures of these plants. Essential

oils could be used as herbal medicines for some diseases but after more experiments with more species of *Candida* in animal models and human volunteers.

Recommendations

Methanol extractswere taken from different sources and different species of thyme and pennyroyal.This study had some limitations, including the absence of GCmass device for determination of flavonoid content.

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