SYNERGISTIC INTERACTION BETWEEN LAMIACEAE ESSENTIAL OILS AND ANTIFUNGAL DRUGS AGAINST CANDIDA ALBICANS ATCC 10231

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Abstract

Several Candida species are part of the normal human microbiota. One of the main characteristics of Candida is its ability to form highly drug tolerant biofilms in the human host. The antifungal therapeutic options are still limited to only a few drug classes, none of which fulfills all desired expectations. Systemic Candida infections demonstrate the advantages of combined therapies carried out with combinations of at least two different antifungal drugs, or/and combinations with natural antifungal agents such as essential oils (EOs), peptide molecules etc. In this work we evaluated the antifungal effect of thyme and oregano essential oils against C. albicans ATCC10231 and their potential synergistic interactions with frequently used antifungal agents – nystatin and fluconazole. Synergic interactions between nystatin and fluconazole and EOs were determined by the checkerboard microtiter assay. The results of the study showed that fluconazole has a synergistic effect with Thymus vulgaris EO in combination with 9.37 μg/mL antifungal and 2 μg/mL essential oil, respectively (FIC index values 0.49). In combination with other concentrations of antimicrobial agents, the effect is additive or interfering. Fluconazole did not show a synergistic interaction with oregano EO at any of the combinations in different concentrations of antimicrobial agents (FIC index values 0.75). The MIC of fluconazole was again reported as 37.5 μg/mL and that of nystatin and fluconazole 0.75. The MIC of fluconazole was again reported as 37.5 μg/mL and that of nystatin, thyme EO and fluconazole and Thymus vulgaris EO.

Rezumat

Infectiile sistemiche cu Candida beneficiază de terapii combinate cu cel puțin două medicamente antifungice diferite și/sau combinații cu agenți antifungici naturali, cum ar fi uleiurile esențiale (UE), moleculele peptidice etc. În acest studiu am evaluat efectul antifungal al uleiurilor esențiale de cimbr și oregano împotriva C. albicans ATCC 10231 și potențialele lor interacțiuni sinergice dintre nistatina și fluconazol. Interacțiunile sinergice dintre nistatina și fluconazol și UE au fost determinate prin testul de microtitrat. Rezultatele studiului au arătat că fluconazolul are un efect sinergic cu Thymus vulgaris UE, în combinația 9,37 μg/mL antifungal și, respectiv, 2 μg/mL ulei esențial (valori ale indicelui FIC 0,49). Pentru alte concentrații de agenți antimicrobiieni, efectul este aditiv sau de interferență. Fluconazolul nu a prezentat interacțiune sinergică cu uleiul esențial de oregano la niște din combinațiile testate (valori ale indicelui FIC 0,75). Rezultatele noastre au arătat existența unei interacțiuni sinergice între și oregano EU și nistatin, cimbr și nistatin și între fluconazol și Thymus vulgaris UE.

Keywords: Lamiaceae essential oils, antifungal drugs, Candida albicans, synergism

Introduction

Several Candida species are part of the normal human microbiota. They are mostly localized in the gastrointestinal tract, respiratory and genitourinary systems, as well as on the skin of healthy patients [28]. Candida species have been proven to be a leading cause of the circulatory and cardiovascular systems infections. Respectively, they are responsible for 50% of the lethal outcome of infected patients [16]. Candida albicans most commonly causes infections in immunocompromised patients. Risk factors for invasive Candida albicans include: surgery interventions, long-term stay in Intensive Care Unit, inappropriate application of broad spectrum antibiotics immunosuppressive drugs [21]. Candida spp. are being isolated from urine in 1 - 9% of patients with hospital stay in Intensive Care Units, Paediatric Intensive Care Units and Combustion Units [8]. One of the main characteristics of Candida is its ability to form highly drug tolerance biofilms in the human host. Candida albicans is known to build mixed species
biofilms with other Candida species and with various other bacterial species in different host niches [14]. Prerequisite for the above include organ transplantation, haemodialysis, parenteral nutrition, catheterization, implementation of various medical devices and etc. [21].

There is a number of natural and synthetic antibacterial drugs for the treatment of bacterial infections. The antifungal therapeutic options are still limited to only a few drug classes, none of which fulfills all desired expectations.

Depending on their origin and chemical consistence, antifungal agents are divided into 2 groups: synthetic (azole derivatives, allylamines) and antifungal antibiotics (polymes, echinocandins). Polymes are toxic and exhibit low water solubility, and echinocandins can only be administered intravenously, while allylamines such as terbinafine lack anti-candidal activity. Currently, azoles are the main used class of antifungal drugs [31]. Among azoles, the most widely used in the pharmacotherapy remains fluconazole. The considerable number and intensity of side effects, mainly related to the usage of high drug concentrations and prolonged therapies, together with the constantly increasing number of drug-resistant isolates [12], still makes fungal infections a serious clinical problem [4, 25]. Systemic Candida infections demonstrate the advantages of combined therapies carried out with combinations of at least two different antifungal drugs [6], or and combinations with natural antifungal agents such as essential oils, peptide molecules and etc.

Plant derived products and essential oils are used against various ailments including infectious diseases since long in traditional system of medicine [29]. Among some of the most important biological actions noted for EOs are the antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, neuroprotectant and anti-diabetic activities [9]. The essential oil (EO) plants of Lamiaceae’s family – oregano, thyme, etc. – possessed significant antibacterial and antifungal activities against resistant microbial isolates and referent strains such as: G (+) – Acinetobacter baumannii, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa; G (+) – Staphylococcus aureus and fungus Trichophyton rubrum and Candida albicans [10, 15]. Their antifungal activity is related with affecting mainly the cell wall and the membranes of the yeast [4]. Thymus vulgaris and Oreganum vulgare essential oils and their active constituent, thymol and carvacrol have been used as an antioxidant, anti-inflammatory, local anaesthetic, antiseptic, antibacterial and antifungal agents [22]. Antiofilm activity of essential oils have been subject of recent investigation [7, 18, 30]. However, little work has been reported on T. vulgaris and O. vulgaris against drug resistant Candida species [17].

Therefore, in order to fill these gaps, we evaluated the antifungal effect of Thyme and Oregano essential oils against C. albicans ATCC 10231 and their potential synergistic interactions with frequently used antifungal agents – nystatin and fluconazole.

Materials and Methods

Microbial strains, antifungal drugs and essential oils

In this study we used a reference strain Candida albicans ATCC 10231. The strain susceptibility to fluconazole, nystatin (as the most common antifungal drugs prescribed in Bulgaria) and EOs from Lamiaceae family was first evaluated by disk diffusion method in Mueller-Hinton (MH) agar medium + 2% glucose + 0.5 μg/mL methylene blue and Serial dilution method in Mueller-Hinton (MH) broth – both techniques according to Clinical and Laboratory Standards Institute (CLSI, 2020). Disk tests are inexpensive and easy to set up, and provide an ideal screening test. Clinical Laboratory Standards Institute (CLSI) recommends the use of Mueller-Hinton agar supplemented with 2% glucose, providing a suitable growth for most yeasts, and 0.5 mg/L methylene blue dye medium (enhances the zone edge definition) minimizing the trailing effect [2]. Serial dilution technique is also suitable to testing antifungal activity of drug agents’ against Candida spp. [19].

In both techniques, we used different antifungal drugs concentrations – ranging from 2.34 to 75 μg/mL fluconazole and 0.5 to 16 μg/mL nystatin and EOs concentration – ranging from 1 to 32 μg/mL. Essential oils of Thymus vulgaris and Oreganum vulgare were purchased from community pharmacy, nystatin and fluconazole from Sigma-Aldrich. DMSO (1%) was used to dilute essential oils and antifungal drug powders. DMSO is an important polar aprotic solvent that dissolves both hydrophilic and lipophilic compounds and is miscible in a wide range of organic solvents as well as water [3]. Antimicrobial potential of DMSO solution was also tested as a control on agar medium. The results showed that it did not demonstrate antimicrobial activity against the tested microbial species. After that, we investigated the potential synergistic interaction between Lamiaceae EOs and antibiotics by checkerboard test in MH broth.

Antifungal activity of fluconazole, nystatin and EOs tested by serial dilution method - determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBCs)

According to Cid-Chevechich et al. [11], different concentrations of fluconazole (2.34 to 75 μg/mL), nystatin (0.5 to 16 μg/mL) and two EOs (1 to 32 μg/mL) were prepared in four rows of tubes. After serial dilution of the antimicrobial agents, 0.1 mL of standardized (0.5 MF) Candida albicans microbial suspension was added to all tubes. We also set up controls on antimicrobial agents and the fungi strain. The tubes were incubated for 48 h at 35°C. All samples were performed in triplicate. MIC is defined as the lowest concentration...
of the antimicrobial agent that inhibits the microbial growth after incubation.
After determining the MICs of the tested antimicrobial agents, from all tubes, in which no visual turbidity was reported, bacterial seeds were made on Sabouraud agar. The lowest concentration at which bacterial growth is inhibited to 99.9% is reported as the minimum bactericidal concentration (MBC).

**Antifungal activity of fluconazole, nystatin and EOs tested by disc diffusion test**

The disk diffusion method is used to evaluate antimicrobial activity of fluconazole, nystatin, and oregano and thyme EOs. The antifungal drugs and EOs were dissolved in DMSO, loaded over sterile filter paper discs (8 mm in diameter) to obtain final concentration of 75 μg/mL fluconazole/disc, 16 μg/mL nystatin/disc and 32 μg/mL of both of the EOs. Twenty millilitres of Mueller-Hilton agar medium + 2% glucose + 0.5 μg/mL methylene blue was poured into sterile Petri dishes, and after complete solidification of the agar media, standardized bacterial suspension (0.5 MF) was spread on the surface of the agar by dense seeding. Sterile filter paper discs loaded with different concentration of antibiotics or EOs were placed on the top of MH agar plates. All the samples were incubated at 35°C for 48 hours. The presence of inhibition zones were measured, recorded and considered as indication for antibacterial activity. The antifungal susceptibility of the isolates was interpreted as sensitive (S), dose dependent-susceptible (DDS) and resistant (R). The results were interpreted as per the CLSI (Clinical and Laboratory Standards Institute) guidelines [13].

**Synergistic interaction between EOs and antifungal drugs**

Synergistic interactions between nystatin and fluconazole and EOs were determined by the checkerboard microtiter assay. Serial dilutions of two antimicrobial agents were mixed together so that each row and column contained a fixed amount of the first agent and increasing amounts of the second one. Six serial two-fold dilutions of each antibiotic and five serial two-fold dilutions of each EO were prepared. The dilutions were prepared beforehand using 1 mL BHI broth in each well. After that, 0.1 mL of fresh yeast suspension (0.5 MF) were added to each well and cultured for 48 hours at 35°C.

To quantify the interactions between the antimicrobial agents being tested (the FIC index), the following equation is used:

\[ \frac{A}{\text{MIC}_A} + \frac{B}{\text{MIC}_B} = \text{FIC}_A + \text{FIC}_B = \text{FIC Index} \]

where, A and B are the MIC of each antimicrobial agents in combination (in a single well), and MIC\_A and MIC\_B are the MIC of each drug individually. The FIC (fractional inhibitory concentration) index value is then used to categorize the interaction of the two antibiotics tested. Total synergism (FIC value < 0.5), additive or indifference (FIC value = 0.5 - 4) or antagonism (FIC value > 4) between two compounds were deduced from the values of the FIC index numbers.

**Results and Discussion**

**MICs and MBCs determination of EOs and antifungal drugs**

In our study, we initially determined the minimum inhibitory concentrations of the two antibiotics nystatin and fluconazole, as well as the essential oils of oregano and thyme, in a liquid culture medium. For this purpose, we used serial two-fold dilution method in MH broth. A number of studies have shown that the MICs of nystatin vary within range 3.7 to 7.4 IU/mL (0.625 to 1.25 μg/mL) for many Candida species [1, 23]. According to other authors, the values reach 100 IU [26]. Inhibitory concentrations of fluconazole can also vary widely - ranged from 8 - 1024 μg/mL against C. albicans and C. tropicalis strains [17]. Most reports, however, show MICs within limits 8 - 32 μg/mL [20, 24]. Based on these data, we used antibiotic concentrations – ranging from 2.34 to 75 μg/mL fluconazole and 0.5 to 16 μg/mL nystatin. Our studies have largely confirmed the MICs values described above from other studies - MIC of fluconazole against Candida albicans ATCC 10231 was 37.5 μg/mL, MIC of nystatin – 16 μg/mL. Regarding MICs of Lamiaceae EOs, according to [4], Candida albicans is more sensitive to different essential oils compared to the main used drugs. According to various studies, MICs of Thyme and Oregano EOs is possible to vary between 0.3 and 25 μg/mL. In the study of [17], MICs of T. vulgaris EO were 25, 3.12 and 1.56 μg/mL against the C. albicans (CAJ-01), C. albicans CAJ-12 (KGMU028) and C. albicans MTCC-3017 respectively. Investigation study of [5] presented results of MICs varied from 0.3 to 0.15 μL/mL for yeast species. Our results showed that the MIC of Thymus vulgaris EO against C. albicans ATCC 10231 is 2 μg/mL. The Oregano oil also demonstrated antifungal activity against C. albicans and a MIC value was 4 μg/mL. After MICs were determined, volume equal to one bacterial loop was taken from each of the test tubes without visible turbidity, and was transferred on the surface of Sabouraud agar for MBCs determination.

The observed MBCs against C. albicans ATCC 10231 for Thyme and Oregano EOs were 4 μg/mL. This results of MICs and MBCs determination of Thyme and Oregano EOs showed that C. albicans demonstrate high sensitivity and they have the potential to be used in therapeutic practice, and some of them probably in combination with other antimicrobial agents. Tests with nystatin and fluconazole showed MBCs 16 and 75 μg/mL, respectively. The MICs and MBCs of EOs and antifungal drugs are presented in Table I.
Table I

<table>
<thead>
<tr>
<th>MIC</th>
<th>Nystatin (μg/mL)</th>
<th>Fluconazole (μg/mL)</th>
<th>Thyme EO (μg/mL)</th>
<th>Oregano EO (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC</td>
<td>16</td>
<td>37.5</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>MBC</td>
<td>16</td>
<td>75</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration

Antimicrobial activity of antifungal drugs and EOs tested by disc diffusion test

The Kirby-Bauer disk diffusion susceptibility test is used to evaluate antimicrobial activity of fluconazole, nystatin and Oregano and Thyme EOs against C. albicans. The antibiotics and EOs were loaded over 8 mm sterile filter paper discs to obtain final concentration of 25 μg/mL fluconazole/disc, 16 μg/mL nystatin/disc and 32 μg/mL of both of the EOs.

Our results showed that Candida albicans ATCC 10231 is sensitive of all of the tested antifungal agents, so that the inhibitory zones for nystatin were 20 ± 2 mm, for fluconazole were 21 ± 0.89 mm and for EOs reached 40 ± 3 mm. Interpretation of antimicrobial susceptibility of the yeast strain to antifungal drugs (susceptible S, susceptible dose dependent [SDD], and resistant R) was done according to CLSI standards – for 17.4 μg/mL nystatin (≥ 15 – S; 10 - 14 – SDD; ≤ 10 – R) and for fluconazole (≥ 19 – S; 15 - 18 – SDD; ≤ 14 – R).

Synergistic interaction between Lamiaceae EOs and antifungal drugs

Synergetic interactions between nystatin and fluconazole and EOs were determined by the checkerboard microtiter assay. The results are presented in Figure 1, Figure 2 and Table II. The results of the study showed that fluconazole has a synergistic effect with Thymus vulgaris EO in combination with 9.37 μg/mL antibiotic and 2 μg/mL essential oil, respectively (FIC index values 0.49). In combination with other concentrations of antimicrobial agents, the effect is additive or interfering.

The MIC of Thymus vulgaris EO against Candida albicans ATCC 10231 was 8 μg/mL and the MIC of fluconazole – 37.5 μg/mL. Fluconazole did not show a synergistic interaction with Oregano essential oil at any of the combinations in different concentrations of antimicrobial agents (FIC index values 0.75). The MIC of fluconazole was again reported as 37.5 μg/mL and that of Oregano EO – 16 μg/mL (Figure 1).

Figure 1.

Synergistic interaction between fluconazole and EOs (Oreganum vulgare EO and Thymus vulgaris EO) against Candida albicans, tested by checkerboard microtiter assay

In a study of the synergistic interaction between nystatin and Thyme essential oil against Candida albicans, we reported such an effect at a concentration of 2 μg/mL of nystatin and 2 μg/mL of Thymus vulgaris EO (FIC index values 0.22). At other concentrations the effect is additive. The MIC of Thymus vulgaris EO against Candida albicans ATCC 10231 is again 8 μg/mL and the MIC of nystatin – 16 μg/mL. In the combination of nystatin and Oregano EO synergism was also reported in 2 μg/mL antifungal drug and 2 μg/mL Oreganum vulgaris EO (FIC index values 0.29).

Therefore, the findings described here encourage the development of clinical trials to evaluate the efficacy of this combination treatment.

Natural products with intrinsic antimicrobial activity or products that promote the activity of commonly used antibiotic/antifungal agents may represent new ways to combat multifistant microorganisms and prevent the contact of these microorganisms with synthetic products, thus reducing the risk of selecting new or improved resistance mechanisms. Natural products may also be combined with traditional antimicrobials to enhance the antimicrobial activity of both [11, 19, 27].
Figure 2.
Synergistic interaction between nystatin and EOs (Oreganum vulgaris EO and Thymus vulgaris EO) against Candida albicans, tested by checkerboard microtiter assay.

Table II
The MIC of nystatin, fluconazole and EOs (Oreganum vulgaris EO and Thymus vulgaris EO), when used in association, and their combined FIC index values against C. albicans ATCC

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC of EOs</th>
<th>MIC of antifungal drug</th>
<th>FIC index</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>MIC of T. vulgaris EO</td>
<td>MIC of nystatin</td>
<td>0.22</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>MIC of T. vulgaris EO</td>
<td>MIC of fluconazole</td>
<td>0.49</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>37.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>MIC of O. vulgaris EO</td>
<td>MIC of nystatin</td>
<td>0.29</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>MIC of O. vulgaris EO</td>
<td>MIC of fluconazole</td>
<td>0.75</td>
<td>Additive</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>37.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conclusions
In conclusion, the essential oils of O. vulgaris and T. vulgaris appeared to be the effective antifungal agents, inhibiting Candida albicans evaluated in this study. The results of our investigation confirm synergistic interaction between Lamiaceae essential oils and antifungal drugs as nystatin and fluconazole against C. albicans. Our findings showed an experimental occurrence of a synergistic interaction between and Oregano EO and nystatin, Thyme EO and nystatin and between fluconazole and Thymus vulgaris EO. Their use is possible to be a promising approach for obtaining new, effective, and safer antifungal therapy.

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Conflict of interest
The authors declare no conflict of interest.

References