Anti-Candida activity of *Thymus pulegioides* (*Lamiaceae*) essential oils depends on the plant chemotype

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**SUMMARY**

The essential oils isolated from linalool (L), geranial/geraniol/neral (G/G/N) and thymol (T) chemotypes of *Thymus pulegioides* L. were investigated for antifungal properties using pathogenic fungi of *Candida* species. Tested chemotypes showed different fungicidal activities. The *Candida* genus was most significantly affected by essential oils of T chemotype and most weakly affected by essential oil of L chemotype. The G/G/N chemotype showed the strongest effect on *C. albicans* CA1 and the weakest on *C. parapsilosis* CP1. Assessment of viability of yeast cells showed that cell viability after 60 min. of incubation with different chemotypes decreased 5 times in comparison with control.

**Key words:** *Thymus pulegioides* L., chemotypes, essential oils, *Candida*, antifungal activity

**INTRODUCTION**

The essential oils possess antibacterial [1-4], antifungal [5], antiviral [6-8], antioxidant [9] and wide spectrum of pharmacological activities [10, 11]. These properties of essential oils are used in pharmacy [12, 13] and food industry [14, 15]. The essential oils became officinal drugs in many countries what has
been documented in their pharmacopoeias [11]. The essential oils have found the widest use in the treatment of infectious pathologies of the respiratory and gastrointestinal systems, urinary tract as well as at various skin diseases [13].

Various species of the *Thymus* genus were reported to be strongly antibacterial [16-21], antifungal [22-25] and antioxidant [26, 27] activities. These properties depend on the essential oil composition. Thyme oils are listed in Pharmacopoeias of Europe, Germany and United Kingdom [11] and used as natural preservatives in the food industry [28, 29]. The volatile components are important in determining the biological activity of *Thymus* species [30-33]. However, chemosystematic investigations revealed the presence of infra-specific variability of essential oil chemical composition in different *Thymus* species [34]. The chemical polymorphism is also characteristic to *Thymus pulegioides* L. growing wild in Lithuania. Six chemotypes were defined for this species according to the main essential oil constituents, namely thymol, carvacrol/γ-terpinene/p-cymene, thymol/carvacrol/γ-terpinene/p-cymene, linalool, geranial/geraniol/neral and α–terpenyl acetate [35-38]. It can be expected that antimicrobial activities of essential oils from different chemotypes of *T. pulegioides* may vary in a wide range.

*Candida* is a heterogeneous genus containing about a quarter of all yeast species. It includes not only species of uncertain affiliation but also unrelated strains whose phylogenetic relationships have not been resolved. Fungi of *Candida* species are mainly associated with plants, rotting vegetation as well as insects feeding on plants or with food. Probably up to 10% of *Candida* species may be of medical importance [39]. Candidiasis is mainly caused by *Candida albicans*. Although, other species, such as *C. tropicalis* and *C. glabrata*, are emerging as pathogens [40, 41]. The species are commonly present as a saprophyte in human mucosa. It may become invasive as a result of local or systemic decrease of defense mechanisms of the host. Deficiency in T-cell number or function, as occurs in AIDS, leads to (muco)cutaneous candidiasis, whereas neutropenia is an important factor predisposing to systemic candidiasis [41]. *Candida albicans* is the cause of such children diseases as pneumonia, oral candidasis, and nappy rush and can localize in bloodstream, respiratory tract, skin or mouth [11].

In Lithuania the number of patients suffering from candidiasis has been growing constantly; from 4.34% of all isolated and identified cultures from pathogenic material of patients suffering from dermatomycoses and urogenital system diseases in 1981-1985 to, 7.64% in 1986–1990, 20.0% in 1996-2000 and 24.2% in 2001–2005 [40]. Various drugs are used for mycosis treatment. Antifungal pharmaceuticals are categorized with dependence on their site of action, mechanism of action or chemical nature [42, 43]. On the basis of action site antifungal medicines are systemic; they are used to treat deep and topical infections, meaning that the infection is superficial and occurs on the skin. Considering the mechanism action, antifungal drugs belong to two groups. Agents of the first group disturb the cell wall and membrane, and the drugs of the second group act on the intracellular processes [44, 43].
Natural products of botanical origin are associated with alternative therapies. The fungicidal activity of tea tree (Melaleuca alternifolia) oil against Candida albicans was analysed [45]. Thirteen of 35 studied medicinal plants containing 1,8-cineole, geranial, germacrene D, limonene, linalool and menthol in their essential oils possessed the anti-Candida activity [46]. The lowest minimum inhibitory concentration of thyme oil against Candida albicans was 0.03% (v/v) [5]. Anise fluid extract showed antimycotic activity against Candida albicans, C. parapsilosis, C. tropicalis, C. pseudotropicalis and C. krusei with MIC values between 17 and 20% (v/v). No activity was observed against C. glabrata [47]. The essential oil of leaf from Juniperus oxycedrus ssp. oxycedrus showed remarkable activity against Candida, particularly, C. krusei, C. glabrata and C. albicans D5 [48].

Therefore, the search of means of natural origin against the Candida genus pathogenic yeasts is purposeful. The main aim of this study was to examine the anti-Candida properties of the essential oils of three different T. pulegioides chemotypes.

MATERIAL AND METHODS

The individual parent plants of T. pulegioides were moved from different natural habitats of Lithuania into the experimental field collection of the Institute of Botany, Vilnius, Lithuania, in 1997, where they were re-planted vegetative and cultivated for nine years. According to the main components of their essential oil, the plants were previously attributed to chemotypes [49]. Voucher specimens were deposited in the Herbarium of the Institute of Botany (BILAS, Vilnius, Lithuania); labeled 60878, 60879 and 60881 for the chemotypes G/G/N, T and L, respectively. The aerial parts of plants were collected at the flowering stage in the year 2006, air-dried at room temperature and ground before analysis.

The essential oils were isolated by hydrodistillation in European Pharmacopoeia apparatus for two hours. They were diluted in diethyl ether (20 µl in 1 ml) and analysed with Fisons 8261 gas chromatograph with flame ionisation detector (FID) on a fused silica capillary column DB-5, 25 m, i.d. 0.32 mm, film thickness 0.5 µm. Helium was used as a carrier gas with a flow rate of 1.6 ml/min; detector temperature was 260°C, oven temperature was programmed from 40°C to 250°C at the rate of 4°C/min. Split injector was heated at 250°C, split ratio was 15:1. The data was processed on a DP 800 integrator. For identification essential oils were also analysed on a HP 5890 (II) instrument equipped with a 5971 series mass selective detector in the electron impact ionisation mode at 70eV, and the following GC parameters: split inlet 1:10; helium as carrier gas at a flow rate of 2 ml/min; fused silica HP5 MS column (Hewlett Packard, crosslinked 5% phenyl methyl silicone) 30 m length, 0.25 mm id, 0.25 µm film thickness, temperature program from 40 to 250°C increasing at 4°C/min. Identification was based mainly on the comparison of retention indices (Ri) [50, 51] and mass spectra (NIST/EPA/NIH Mass Spectral Database NBS75K).
The following *Candida* genus clinical strains were isolated from the patients with the diagnosis of dermatomycosis and onychomycosis in Vilnius University Hospital Santariškių Klinikos Diagnostic Centre: *Candida albicans* (CA1) and *C. albicans* (CA2) (Robin) Berkhout, *C. glabrata* (H. W. Anderson) S. A. Meyer et Yarrow, *C. parapsilosis* (CP1) and *C. parapsilosis* (CP1.1) (Ashford) Langeron et Talice, *C. tropicalis* (CT1) and *C. tropicalis* (CT2) (Castellani) Berkhout. The yeasts were tested for sensitivity to L, G/G/N and T chemotypes of *T. pulegioides* L. The yeasts were screened on Sabouraud Agar (Oxoid, England) and Corn Meal Agar (Oxoid, England). The identification of yeast strains was performed applying methods and diagnostics systems “Candifast” and “Fungichrom” (International Microbio, France) and using previously published recommendations [52, 41].

The essential oils from L, G/G/N and T chemotypes of *T. pulegioides* L. were tested for their anti-*Candida* activities. Susceptibility of the test organisms was determined by employing the standard disk diffusion technique [20]. In addition, fluconazole (Liofilchem, Italy) and nystatin (Liofilchem, Italy) were used as reference antifungal agents.

The studied yeasts were incubated on Sabouraud Dextrose Agar (SDA) (LAB M, England) for 24 h. Sabouraud Dextrose Agar, sterilized in a flask and cooled to 45–50°C, was distributed to sterilized 90 mm Petri dishes after injecting with 1 ml of yeast cultures (10⁵ cells per ml) and distribution onto the medium in Petri dishes homogenously. The disks (6 mm) were placed on the agar plates and then 10 µl of essential oil of each chemotype was put on the disks. The dishes were incubated at 25°C for 48 h. At the end of the period, inhibition zones formed on the SDA were evaluated in millimeters.

Investigation of viability of yeast cells was performed with the following yeast species: *C. albicans* CA1, *C. parapsilosis* CP1 and *C. tropicalis* CT1. The yeasts were grown on Sabouraud Dextrose Agar (LAB M, England) and the inoculum for the assays was prepared by diluting scraped cell mass in 9% NaCl solution. The yeasts were isolated by centrifugation at 8000 rpm. Ten µl of essential oil of each chemotype were added into a suspension. The solutions of the yeast cells and essential oils were incubated at 26 °C for 1, 5, 10, 15, 20, 25, 30 and 60 min in an incubator shaker. The samples were withdrawn after the time intervals and diluted. Serial dilutions were performed to obtained the concentration amounting for approximately 10⁵ cells per ml and cultured on SDA for 48 h at 25±2 °C. The tests were repeated in triplicate. Yeasts colonies were counted after the incubation period and the total number of viable cells per ml was calculated.

The obtained data were handled using Microsoft Excel XP (mean, standard deviation). The number of viable cells was expressed as a percentage.

**RESULTS AND DISCUSSION**

The composition of essential oils of T, G/G/N and L chemotypes of *T. pulegioides* is presented in table 1. In total, 43 compounds were identified by capillary GC and
GC/MS. Thymol was the main component of the T chemotype essential oil (37.5%); the content of its methyl ether was also high (9.8%). The high amount of thymol in *T. pulegioides* was established in southern Italy (39.1%) [53]. Thymol possesses strong antibacterial [3] and antioxidant properties [27, 33]. The studied chemotype was also rich in γ-terpinene (24.7%) and *p*-cymene (5.3%), which are chemical precursors of thymol and carvacrol.

**Table 1.**

The composition of essential oils of different *Thymus pulegioides* L. chemotypes on GC area (%) (T – thymol, G/G/N – geranial/geraniol/neral, L – linalool chemotypes)

<table>
<thead>
<tr>
<th>component</th>
<th>Thymus pulegioides L. chemotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
</tr>
<tr>
<td>α-thujene</td>
<td>1.0</td>
</tr>
<tr>
<td>β-pinene</td>
<td>1.0</td>
</tr>
<tr>
<td>1-octen-3-ol</td>
<td>0.3</td>
</tr>
<tr>
<td>myrcene</td>
<td>2.3</td>
</tr>
<tr>
<td>α-phellandrene</td>
<td>0.4</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>2.2</td>
</tr>
<tr>
<td><em>p</em>-cymene</td>
<td>5.3</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>0.5</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>24.7</td>
</tr>
<tr>
<td>cis-sabinene hydrate</td>
<td>2.4</td>
</tr>
<tr>
<td>terpinolene</td>
<td>0.1</td>
</tr>
<tr>
<td>linalool</td>
<td>-</td>
</tr>
<tr>
<td>camphor</td>
<td>-</td>
</tr>
<tr>
<td>borneol</td>
<td>-</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>0.3</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>-</td>
</tr>
<tr>
<td>nerol</td>
<td>-</td>
</tr>
<tr>
<td>thymol methyl ether</td>
<td>9.8</td>
</tr>
<tr>
<td>nerol</td>
<td>-</td>
</tr>
<tr>
<td><em>trans</em>-sabinene hydrate acetate</td>
<td>-</td>
</tr>
<tr>
<td>geraniol</td>
<td>-</td>
</tr>
<tr>
<td>geranial</td>
<td>-</td>
</tr>
<tr>
<td>thymol</td>
<td>37.5</td>
</tr>
<tr>
<td>carvacrol ethyl ether</td>
<td>-</td>
</tr>
<tr>
<td>carvacrol</td>
<td>tr</td>
</tr>
<tr>
<td>α-elemene</td>
<td>tr</td>
</tr>
<tr>
<td>α-cubebene</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Geraniol (40.6%), geranial (16.4%) and neral (12.8%) were major components in the G/G/N chemotype. The biggest content of geraniol (41.1%) in *T. pulegioides* was determined in the plants originating from Croatia [54]; its antibacterial activity was previously reported [32].

The percentage of linalool (84.1%) in L chemotype was exceptionally high: to our best knowledge the biggest content of linalool (60.2%) was previously reported in *T. pulegioides* from Slovakia [54], while similar chemotype of *T. zygis* with 91.4% of linalool was found in Spain [55]. Linalool was examined for its antifungal [30] and antibacterial properties, particularly against *Citrobacter freundii* and *Clostridium sporogenes* [3].

Volatile oil composition of different *T. pulegioides* chemotypes was monitored in 1998–2002 and it was observed that the contents of thymol, geraniol and linalool are genetically stable in the studied T, G/G/N and L chemotypes, respectively [49]. Some variation of the mentioned secondary metabolites may depend on various factors such as the differences in the climatic conditions in different years, e.g. air temperature, the rainfall, the amount of hours of sunlight [56, 57], exact time of flowering phase at which the plants were harvested [53].

The results obtained clearly show that the tested chemotypes of *T. pulegioides* manifested different fungicidal effects on different pathogenic yeasts of the genus *Candida* (tab. 2, fig. 1-3).
The fungicidal effect of the essential oil of different chemotypes of Thymus pulegioides L. on pathogenic yeasts of the Candida genus (L – linalool, T – thymol, G/G/N – geranial/geraniol/neral chemotypes)

<table>
<thead>
<tr>
<th>strain</th>
<th>Chemotype of Thymus pulegioides L.</th>
<th>inhibition zone [mm, includes disc diameter (6mm)]</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L 10µl T 10µl G/G/N 10µl fluconazole 100 µg nystatin 100 IU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida albicans CA1</td>
<td>25±1 28±2.64 42.3±4.72 26.7±2.16 23.3±0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida albicans CA2</td>
<td>21.3±16.1 27.3±1.52 21.3±2.3 20.8±1.16 21.2±0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>11.3±2.3 33.6±0.57 14.3±0.57 12.3±4.76 22.1±2.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida parapsilosis CP1</td>
<td>15.6±5.13 22.6±0.57 11.6±0.57 26.5±0.88 23.3±2.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida parapsilosis CP1.1</td>
<td>10±0 22.6±1.15 17±1 22.5±2.35 20.2±1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida tropicalis CT1</td>
<td>6.6±0.57 9±0 22±1.73 27.2±4.02 20.7±1.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida tropicalis CT2</td>
<td>10.3±0.57 9.6±0.57 19±1 15.4±1.01 22±0.86</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.

The fungicidal effect of the essential oil of Thymus pulegioides L. on pathogenic yeasts of the Candida genus (L – linalool, T – thymol, G/G/N – geranial/geraniol/neral chemotypes)

Figure 1. Fungicidal effect of the essential oil of the thymol chemotype of Thymus pulegioides L.: A – Candida albicans CA1; B – Candida glabrata; C – Candida parapsilosis CP1.1; D – Candida tropicalis CT1 yeasts

The strongest fungicidal effect on Candida yeasts was measured for the T chemotype essential oil (fig. 1). C. glabrata was the species most vulnerable to the T che-
motype oil. The size of fungicidal zones was 33.6±0.57 mm, as in antifungal agent fluconazole fungicidal zone was 12.3±4.76 mm. *C. tropicalis* CT1 and *C. tropicalis* CT2 were resistant to the essential oil of this chemotype – in this case fungicidal zones were from 9±0 to 9.6±0.57 mm. Fungicidal effect of the essential oil of the thymol chemotype of *T. vulgaris* on *C. albicans* as assessed by the MIC 80%=0.016 µl/ml was reported previously [58]. Fungicidal properties of the essential oil are associated with such phenolic compounds as carvacrol, eugenol and thymol [3]; the latter compound was abundant in the T chemotype *T. pulegioides* used in the present study.

The essential oil of the G/G/N chemotype possessed weaker fungicidal effect on the pathogenic *Candida* yeasts than the T chemotype essential oil (fig. 2). *C. albicans* CA1 were most sensitive to the G/G/N chemotype; the size of fungicidal zones was 42.3±4.72 mm, whereas antifungal agents fluconazole fungicidal zones were 26.7±2.16 and nystatin – 23.3±0.5 mm. The weakest effect was observed in *C. parapsilosis* CP1; the size of fungicidal zones was 11.6±0.57 mm (tab. 2). Most likely geraniol present at the level of 40.6% was the strongest antifungal compound in this chemotype. Its antimicrobial properties were previously reported [32].

Figure 2. Fungicidal effect of the essential oil of the geranial/geraniol/neral chemotype of *Thymus pulegioides* L.: A – *Candida albicans* CA1; B – *Candida glabrata*; C – *Candida parapsilosis* CP1.1; D – *Candida tropicalis* CT1 yeasts
Anti-Candida activity of *Thymus pulegioides* (*Lamiaceae*) essential oils depends on the plant chemotype

The essential oil of the L chemotype of *T. pulegioides* showed the weakest effect on pathogenic *Candida* yeasts (fig. 3). The strongest inhibition effect of this chemotype was observed on *C. albicans* CA1: the size of fungicidal zones size was $25 \pm 1$ mm, while *C. tropicalis* CT1 were the most resistant towards this essential oil, in this case the fungicidal zones were negligible, $6.6 \pm 0.57$ mm (tab. 2). It seems that linalool which was dominating terpene in this chemotype oil (84.1%) was the least effective antifungal compound as compared to thymol and geraniol. The contents of some other components in L chemotype oil were 2-4% (tab. 1). It is very unlikely that these compounds would possess any measurable anti-Candida activity after several dilutions.

![Figure 3. Fungicidal effect of the essential oil of the linalool chemotype of *Thymus pulegioides* L.: A – *Candida albicans* CA1; B – *Candida glabrata*; C – *Candida parapsilosis* CP1.1; D – *Candida tropicalis* CT1 yeasts.](image)

The viability of the tested pathogenic *Candida* yeasts after incubation lasting 60 min. with 10 µl of essential oils of the all tested chemotypes of *T. pulegioides* decreased 5 times in comparison with the control (fig. 4-6). The strongest fungicidal effect on cells of *C. albicans* CA1 was manifested by the essential oil of the G/G/N chemotype (fig. 4). After incubation during 25 min the viable cells of *C. albicans* CA1 were
absent. After the incubation of *C. albicans* CA1 cells with the essential oil of the L and the T chemotype essential oils for 60 min their viability varied from 7.1 to 19.19%.

![Figure 4. Effect of the essential oil of different *Thymus pulegioides* L. chemotypes on viability of *Candida albicans* CA1 cells](image)

Strong fungicidal activity against *C. parapsilosis* CP1 cells was exhibited by the essential oil of the T chemotype (fig. 5). No viable *C. parapsilosis* CP1 cells were found after their incubation for 15 min. The G/G/N chemotype needed 60 min. to eliminate viable cells in the same culture, while L chemotype oil was the weakest antifungal agent. The viability of *C. parapsilosis* CP1 cells after 60 min incubation with L chemotype oil was 19.62%.

![Figure 5. Effect of the essential oil of different *Thymus pulegioides* L. chemotypes on viability of *Candida parapsilosis* CP1 cells](image)
C. tropicalis CT1 cells were the most resistant to the essential oil of T. pulegioides chemotypes (fig. 6); after incubation for 60 min with the essential oils of different chemotypes of T. pulegioides the viability of yeast cells was from 3.17 to 17.07%.

The infraspecific chemical polymorphism is characteristic for the species of Thy-mus genus. Therefore, the investigations at chemotypes level are needed because the essential oils of different chemotypes have different chemical composition which may determine the different biological activity. The results of the present study suggest that the essential oils of T. pulegioides chemotypes characterize different fungicidal activities against pathogenic yeasts of the Candida genus. These results corroborate the importance of investigations of T. pulegioides chemotypes for possible phytotherapeutical use.

REFERENCES


Anti-*Candida* activity of *Thymus pulegioides* (*Lamiaceae*) essential oils depends on the plant chemotype


DZIAŁANIE OLEJKÓW ETERYCZNYCH \textit{THYMUS PULEGIOIDES} (LAMIACEAE) NA GRZYBY CANDIDA ZALEŻNE OD CHEMOTYPU ROŚLINY

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\textbf{Streszczenie}

Badano właściwości przeciwgrzybicze olejków eterycznych uzyskiwanych z następujących chemotypów \textit{Thymus pulegioides} L.: linaololu (L), geraniolu/geranialu/neralu (g/g/N) i tymolu (T) przy użyciu grzybów patogenicznych z gatunku \textit{Candida}. Badane chemotypy wykazały różne rodzaje działań przeciwgrzybiczych. Na grzyby z gatunku \textit{Candida} najsilniej działały olejki eteryczne chemotypu T a najsłabiej olejki chemotypu L. Chemotyp G/G/N działał najsilniej na \textit{C. albicans} a najsłabiej na \textit{C. parapsilosis} CP1. Z oceny wynika, że żywotność komórek drożdży po 60 min inkubacji z różnymi chemotypami była pięciokrotnie krótsza w porównaniu z kontrolą.

\textbf{Słowa kluczowe:} Thymus pulegioides L., chemotypy, olejki eteryczne, Candida, działanie przeciwgrzybicze