**IN VITRO ANTICANDIDAL ACTIVITY OF ESSENTIAL OIL OF THYMUS VULGARIS**

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**ABSTRACT**

The objective of the present study is to evaluate the anticandidal activity of thyme essential oil against clinical isolates of *Candida* species from genital fungal infections and oral thrush. Thyme oil is popularly used as antifungal treatments in aromatherapy and potent essential oil in the traditional Indian medicinal systems. Anticandidal activity of 73 clinical isolates to thyme oil and diluted thyme oil with DMSO (25%, 50% and 75%) was determined by agar disc diffusion method. Undiluted thyme oil was efficiently inhibited growth of all isolates of *Candida* species with growth inhibition zones ranging from 42 to 62 mm, whereas diluted thyme oil invulnerability to *Candida* species decreased with increased dilution rate. Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) were evaluated by broth microdilution and broth macrodilution method. The broth microdilution assay gave MIC and MFC values ranging from 0.02–1.25 μl/ml. The broth macrodilution assay shows MIC values ranging from 0.02–0.62 μl/ml and MFC ranging from 0.04–0.62 μl/ml. The results showed that the *C. krusei* exhibited higher sensitivity to thyme oil than other isolates with lowest MIC of 0.02 μl/ml (v/v). The result of this study suggests that thyme oil contain potential active anticandidal components.
KEYWORDS
Anticandidal activity, Candida species, Thymus vulgaris and thyme oil

INTRODUCTION

The anticandidal properties of plant origin essential oils, and their constituents from a variety of plants have been studied\(^1,2,3\) and reviewed\(^4,5\). It is known that most of the essential oils have potential uses in medical procedures and demonstrated against a wide range of microorganisms, including bacteria, protozoa, virus, fungi, and antioxidant properties. In addition, essential oils have also been used in various cancer treatments\(^6\), food preservation\(^7,8\), aromatherapy\(^9\), pharmaceutical\(^10\), insecticides\(^11\), fragrance\(^12,13\) and cosmetic industries.\(^14,15\). Biological activity of essential oils depends on their chemical constituents and exhibited in-vitro cytotoxicity to human breast tumour \(^16\).

There are a limited number of antifungals available, most just providing fungistic but not fungicidal effects. Continuous exposure to the antibiotics and synthetic chemical drugs results in the development of resistance in the organisms. Fluconazole is one of the most widely used antifungal agent, both for prophylaxis and therapy of Candida infections. Several mechanisms of azole resistance in Candida albicans have already been described \(^17\). The increasing ineffectiveness of these drugs and unavailability of alternative antimicrobials in developing countries caused to spread major infectious diseases\(^18\). So there is a need to develop new effective therapeutic agents.

Thymus vulgaris belongs to the Lamiaceae family, and is an important medicinal plant, which has been used in traditional medicine in the treatment of bronchitis, asthma and other respiratory diseases. Thyme oil (essential oil extracted from Thymus vulgaris) and its components are widespread as naturally occurring antimicrobial and antioxidant agents. The antimicrobial activity of thyme oil is associated with thymol, linalool, carvacrol, α-pinene, 1, 8-cineole, camphor, 4-terpineol and are major volatile components\(^19,20\). It also plays an important role to serve as a protective agent to the damaged tissues by decreasing the nitric oxide level in burn cases\(^21\). Thyme oil inhibits the cell growth of human head and neck squamous cell carcinoma \(^22\).

It is widely perceived that the incidence of invasive Candida spp. infections is rising. Significant increases in the number of Candida spp. infections are the fourth commonest cause of bloodstream infections\(^23,24\). Candida albicans has accounted for virtually all mucosal candidiasis. However, other species such as C. glabrata, C.parapsilosis, C.tropicalis and C. krusei also causes serious oropharyngeal candidiasis and occasionally esophageal candidiasis\(^25\). Candida species is also the most common cause of vaginal candidiasis or thrush and approximately 80-90% of thrush cases are caused by Candida species \(^26\).

The objective of this study is to evaluate the in-vitro activity of thyme oil using broth microdilution and macrodilution assay which is suitable for the assessment of anticandidal activity of thyme oil of T. vulgaris against clinical isolates of Candida species. This study shows for the first time that in vitro anticandidal activity of essential oil of T. vulgaris determined by using broth microdilution and macrodilution methods for clinical isolates of Candida.

MATERIALS AND METHODS

Essential oils:
Thyme oil used in the present study was procured from Rakesh Products, Kanpur (India). The sterility of the oils was checked by inoculating oil on Sabouraud dextrose agar (SDA) and nutrient agar (Hi-Media, Mumbai)
slants, and subsequently assessed the growth. The essential oil was stored in the dark at 25°C before experiments. Different concentrations of thyme oil with DMSO (25%, 50% and 75%) were prepared for experiments.

**Organisms:**
73 clinical *candida* species were isolated from genital fungal infection and oral thrush including *Candida albicans* (n=28), *C. krusei* (n=5), *C. glabrata* (n=9), *C. parapsilosis* (n=7), *C. tropicalis* (n=12), *C. pseudotropicalis* (n=6), *C. guilliermondii* (n=3) and *C. stellatoidea* (n=3). Each isolate was originated from a different patient with clinical manifestations. All isolated strains were maintained on SDA at 4°C in refrigerator until ready for the study. Prior to testing, each isolate was checked for purity and viability.

**Inoculum preparation:**
Overnight broth culture of the test *Candida* species isolates was inoculated on SDA slant and subsequently transferred into 5 ml of 0.85% sterile saline. The resulting inoculum density was adjusted according to National Committee for Clinical Laboratory Standards\(^2\) compared their turbidity to MacFarland’s standard of 0.5 to yield a suspension of 1x10\(^4\) cfu/ml.

**Anticandidal sensitivity testing:**

(i) **Disc Diffusion Assay:**
In brief, 90 mm-diameter plates containing Sabouraud dextrose agar at a depth of 4 mm were used. The agar surface was seeded with inoculums of *Candida species* containing 1x10\(^4\) spore/ml by using a sterile swab. Sterile disc (SD 067, Hi-Media, Mumbai) of 6 mm diameter was impregnated with 20 μl of undiluted oil (100%) and diluted (25%, 50%, 75% in DMSO) placed at center of seeded agar surfce. The plates were then left undisturbed for 30 min to allow diffusion of the essential oil into the agar and incubated at 35°C for 24 hrs. Studies were performed in triplicate and anticandidal activity was evaluated by measuring the developed clear zone of inhibition around the well measured in mm. The scale of zone size interpretation as followed by Elgayyar *et al.*,\(^2\) was used (disk diameter included). Inhibition zone of isolates with ≥ 28 mm were classified as strongly sensitive, with a zone diameter of 16 to < 28 mm as moderately sensitive, with a zone diameter of 12 to < 16 mm as weakly sensitive and zone diameter of < 12 mm as resistant.

(ii) **Broth Microdilution Assay:**
The minimal inhibition concentration (MIC) values were also studied for the anticandidal activity, which were determined as sensitive to the thyme oil in disc diffusion assay. MIC values of thyme oil against *Candida* species isolates were determined based on a micro-well dilution method\(^2\) The inocula of *Candida* species were prepared from 18 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The inocula were diluted to the appropriate cell densities in Sabouraud dextrose broth containing 0.15% (w/v) agar (Hi-Media, Mumbai) as a stabilizer of the oil water mixture.\(^3\) Serial two-fold dilutions of thyme oil were made in a concentration from 0.02-10 μl/ml (v/v) with a final thyme oil concentration range 0.01- 5.0 μl/ml in 96-well microtitre plates. 100 μl *Candida* suspensions were added to each well. Well containing only the Sabouraud dextrose broth with 0.15% agar without microorganism was used as sterility control. The lowest concentration of oil that inhibited visible growth after incubation at 35°C for 48 ± 2 hrs without shaking was taken as minimum inhibitory concentration. Minimum concentration of oil that inhibited 50%, 70% and 90% of the isolates tested were defined as MIC\(_{50}\), MIC\(_{70}\) and MIC\(_{90}\), respectively. To determine minimum fungicidal concentration (MFC), a loopful of broth was removed from each well and spot inoculated onto SDA plate and after incubation at 35°C for 48 hours, the lowest concentration of oil that inhibited the complete growth of clinical isolates of *Candida* species was considered as MFC. The concentration of thyme oil fungicidal for 90% of the clinical isolates tested was defined as MFC\(_{90}\). Control tubes with 0.15% agar without
thyme oil were essayed simultaneously. All tests were carried out for three sample replications and the results were averaged.

**(iii) Broth Macrodilution Assay:**
A range of doubling dilutions of thyme oil from 0.02–10 µl/ml (v/v) with a final oil concentration range 0.01–5 µl/ml (v/v) was prepared in Sabouraud dextrose broth in round bottom sterile glass tubes (12x75mm). Bacteriological agar was included at a concentration of 0.15% (w/v) to enhance oil solubility. A working inoculum suspension of 1x10^4 cfu/ml was added to each tube except sterility control. Sabouraud dextrose broth containing 0.15% agar without essential oil served as growth control. The tubes were then incubated at 35°C for 48 ± 2 hrs without agitation and observed for the presence or absence of visible growth. The MIC was defined as the lowest concentration of oil inhibiting visible growth.

**RESULTS AND DISCUSSION**

**Anticandidal Activity**
The preliminary screening of in vitro anticandidal activity of thyme oil at different concentrations was studied against isolates Candida species by disc diffusion and the activity was determined by measuring the zone of inhibition. The results showed the variation in anticandidal activity of thyme oil (Table 1). Clinical isolates of Candida species were sensitive to 25%, 50%, 75% and 100% thyme oil concentrations. The activity exhibited zone inhibition range between 42-62 mm with average inhibition zone of 52 mm. According to this result, the thyme oil was most effective against C. krusei followed by C. pseudotropicalis but comparatively oil was least effective against the isolates of C. parapsilosis.

**Table 1**
_Inhibition zones obtained by disc diffusion method of the thyme oil assayed against eight different Candida species._

<table>
<thead>
<tr>
<th>Species (No. of isolates)</th>
<th>Inhibition Zones (in mm)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>4:0 (100% oil)</td>
</tr>
<tr>
<td></td>
<td>MIZ</td>
</tr>
<tr>
<td><em>C. albicans</em> (n = 28)</td>
<td>51</td>
</tr>
<tr>
<td><em>C. glabrata</em> (n = 9)</td>
<td>52</td>
</tr>
<tr>
<td><em>C. tropicalis</em> (n = 12)</td>
<td>52</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> (n = 7)</td>
<td>47</td>
</tr>
<tr>
<td><em>C. pseudotropicalis</em> (n = 6)</td>
<td>56</td>
</tr>
<tr>
<td><em>C. krusei</em> (n = 5)</td>
<td>57</td>
</tr>
<tr>
<td><em>C. guilliermondii</em> (n = 3)</td>
<td>50</td>
</tr>
<tr>
<td><em>C. stellatoidea</em> (n = 3)</td>
<td>51</td>
</tr>
<tr>
<td><strong>Total (n = 73)</strong></td>
<td>52</td>
</tr>
</tbody>
</table>

MIZ, Mean Inhibition Zone; IZR, Inhibition Zone Range; 4:0 represents undiluted oil; 3:1 represents 3 parts oil and 1 part DMSO solvent; 2:2 represents 2 parts oil and 2 parts DMSO solvent; 1:3 represents 1 part oil and 3 parts DMSO solvent. Data are means of triplicates determinations.
Evaluation of antcandidal activity by broth microdilution assay

In our study, various concentrations of thyme oils have been used to know the MIC₉₀ and MFC₉₀ by broth micro-dilution assay. The results exhibited a broad spectrum inhibitory activity against C. krusei at minimum concentrations (0.02 μl/ml) of thyme oil followed by C. pseudotropicalis (Table 2). On the contrary, high concentrations (1.25 μl/ml) of thyme oil was not vulnerable to C. parapsilosis. However, thyme oil showed optimum inhibitory activity at 0.15 μl/ml concentration against C. albicans, C. glabrata, C. tropicalis, C. guilliermondii and C. stellatoidea. Present results indicated that C. krusei was most susceptible among the Candida species, and showed its inhibition with MIC₅₀, MIC₇₀, MIC₉₀ and MFC₉₀ at lowest concentration of 0.02 μl /ml by broth microdilution.

Table 2
Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) obtained by broth microdilution of the thyme oil assayed against eight different Candida species.

<table>
<thead>
<tr>
<th>Species (No. of isolates)</th>
<th>MIC (μl /ml)</th>
<th>MFC (μl /ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC Range</td>
<td>MIC₅₀</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------</td>
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</tr>
<tr>
<td>C. albicans (n = 28)</td>
<td>0.02 – 0.15</td>
<td>0.04</td>
</tr>
<tr>
<td>C. glabrata (n = 9)</td>
<td>0.02 – 0.31</td>
<td>0.08</td>
</tr>
<tr>
<td>C. tropicalis (n = 12)</td>
<td>0.02 – 0.31</td>
<td>0.08</td>
</tr>
<tr>
<td>C. parapsilosis (n = 7)</td>
<td>0.04 – 1.25</td>
<td>0.15</td>
</tr>
<tr>
<td>C. pseudotropicalis (n = 6)</td>
<td>0.04 – 0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>C. krusei (n = 5)</td>
<td>0.02 – 0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>C. guilliermondii (n = 3)</td>
<td>0.08 – 0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>C. stellatoidea (n = 3)</td>
<td>0.08 – 0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>Total (n = 73)</td>
<td>0.02 – 1.25</td>
<td>0.08</td>
</tr>
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</table>

MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration (MIC and MFC values are expressed in μl /ml). Data are means of triplicates determinations.

Evaluation of antcandidal activity by broth macrodilution assay

Table 3 illustrated the MIC and MFC in vitro susceptibility of antcandidal activity by thyme oil assessed by broth macrodilution assayed at different concentrations. The broth macrodilution study also showed that the C. krusei was most susceptible to thyme oil at minimum concentration of 0.04 μl /ml, whereas C. parapsilosis was least susceptible to thyme oil. In the aftermath, a wide MIC range was found in C. parapsilosis by broth microdilution assay between 0.04 – 1.25 μl /ml, and 0.02 – 0.62 μl /ml by broth macrodilution assay. In contrast, the present results revealed the most susceptible species C. krusei showed narrow MIC range between 0.02 – 0.02 μl /ml by broth microdilution assay and 0.02 – 0.04 μl /ml by broth macrodilution assay.
Table 3
Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) obtained by broth macrodilution of the thyme oil assayed against Candida species.

<table>
<thead>
<tr>
<th>Species (No. of isolates)</th>
<th>MIC (µl/ml)</th>
<th>MFC (µl/ml)</th>
</tr>
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<tr>
<td></td>
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<td>0.04 – 0.15</td>
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</tr>
<tr>
<td>C. krusei (n = 5)</td>
<td>0.02 – 0.04</td>
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</tr>
<tr>
<td>C. guilliermondii (n = 3)</td>
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<td>0.08 – 0.15</td>
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<td>Total (n = 73)</td>
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MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration (MIC and MFC values are expressed in µl/ml). Data are means of triplicates determinations.

In the present study, essential oil showed good diffusibility in the medium using paper disc diffusion method, however partitioning of essential oil components through the agar according to their affinity with water was reported earlier<sup>31</sup>. Previous reports also demonstrated that addition of agar, DMSO, Tween-20 and Tween-80 enhanced the oil solubility in the test medium<sup>25,30,32,33,34</sup>. In the present study, the addition of 0.15 % (w/v) bacteriological agar before sterilization of test medium was found to give better solubility and homogeneous diffusibility commonly encountered while determining the antimicrobial effects of essential oils. The outstanding feature of thyme oil was the effective inhibition of all the Candida species isolated from vaginal and oral thrush with 42-62 mm inhibition zones and 0.02 – 2.50 µl/ml MIC range. Therefore, it is possible that the presence of active components in thyme oil as reported earlier<sup>36</sup> are likely to be responsible for antifungal activity.

Thyme oil demonstrates great potential as a novel antifungal compound with potent in-vitro antifungal activity against various isolates of Candida species, an opportunistic pathogen responsible for both superficial and systemic mycoses<sup>36</sup>. The clinical isolates of C. albicans exhibited the MIC values between 0.02 to 0.15 µl /ml by broth microdilution, whereas its upper limit of MIC range was two-fold greater than that of broth macrodilution method. Similarly, MIC<sub>90</sub> of C. glabrata, C. tropicalis, C. pseudotropicalis, C. guilliermondii and C. stellatoidea were found to be two-fold greater. In case of C. parapsilosis, MIC<sub>90</sub> was found to be remarkably 16-fold greater than MIC<sub>50</sub>. Nevertheless MIC<sub>90</sub> of C. albicans was found fourfold larger than MIC<sub>50</sub>. However MIC<sub>90</sub> of C. krusei was found to be same as MIC<sub>50</sub> by broth microdilution method. Comparing MIC<sub>90</sub> values obtained by broth microdilution and broth macrodilution resulted in variable susceptibility at different dilution concentrations. Thyme oil showed the least susceptibility to C. parapsilosis, with an MIC to 1.25 µl /ml by broth microdilution, and 0.62 µl /ml by broth macrodilution. Broth microdilution MIC
was two-fold higher in *C. parapsilosis* than broth macrodilution. In accordance with the results, broth macrodilution assay showed 54% test isolates inclined towards higher by 2 to 8-fold more than broth microdilution, while broth macrodilution MIC resulted at 22% isolates were 2 to 4-fold less than broth microdilution. These results indicated that broth micro and macrodilution methods have absolute agreement for 24% test isolates. However, in most cases (54% test isolates) it has been found that MICs by broth microdilution and broth macrodilution method agreed within a 2-fold range. Barchiesi et al. reported agreement within one doubling dilution of the macrodilution reference for fluconazole, itraconazole, flucytosine and amphotericin B higher than the MICs for microdilution test for yeast strains.

In the aftermath, the mean inhibitory data of all three methods revealed that isolated *C. krusei*, followed by *C. pseudotropicalis* were most sensitive to thyme oil, showed the lowest MIC and the highest inhibition diameter. In contrast, some of the other isolated *Candida* species such as *C. parapsilosis* that showed the lowest inhibition diameter and the highest requirement of MIC, reflecting its comparatively less sensitivity to thyme oil. In addition, *C. albicans*, *C. glabrata*, *C. guillermondii*, *C. tropicalis* and *C. stellatoidea* exhibited intermediate sensitivity to thyme oil. Comparison of the inhibition zone sizes produced by the thyme oil with the minimal inhibitory concentration showed that not always larger inhibitory zone corresponds to lower MIC. With few exceptions, the results for inhibition zones that were obtained by disc diffusion method showed an inverse correlation with those that were obtained with MICs by the broth macrodilution method.

MFC values for thyme oil were similar to MIC results. MFC<sub>90</sub> range for different *Candida* species was found to be 0.02 – 1.25 μl/ml by broth microdilution and 0.04 – 0.62 μl/ml by broth macrodilution method. MIC values reported in this study could be much significant as a concentration below the MIC values inhibits germ tube formation, an important virulence factor in the pathogenesis of *C. albicans*.

**CONCLUSION**

Over the past two decades, an increasing trend in the number of vaginal infections attributable to yeasts other than *C. albicans* has emerged. Of these non-albicans species, *C. tropicalis* and *C. glabrata* appear to be the more virulent. Many currently used drug therapies (e.g., imidazoles) for *C. albicans* vaginitis do not adequately eradicate non-albicans species. In the present investigation *C. albicans* and non-albicans species such as *C. tropicalis* and *C. glabrata* were inhibited strongly at a low concentration of thyme oil. Therefore, thyme oil could be promising and alternative to control pathogens and in designing the remedies for the treatment of vaginal candidiasis.

From the result, it can be concluded that the essential oil of *T. vulgaris* has got anticandidal activity which promoted its utilization in the vaginal candidiasis and *Candida* infections in immuno-compromised patients. Further research work will emphasize the isolation and characterization of active compounds from *T. vulgaris* responsible against virulent *Candida* species.

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REFERENCES

18. Cheesbrough M, Ed. Medical Laboratory Manual for Tropical Countries: Microbiology,


