In vitro and in vivo activity of tea tree oil against azole-susceptible and -resistant human pathogenic yeasts

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A tea tree oil (TTO) preparation of defined chemical composition was studied, using a microbroth method, for its in vitro activity against 115 isolates of Candida albicans, other Candida species and Cryptococcus neoformans. The fungal strains were from HIV-seropositive subjects, or from an established type collection, including reference and quality control strains. Fourteen strains of C. albicans resistant to fluconazole and/or itraconazole were also assessed. The same preparation was also tested in an experimental vaginal infection using fluconazole–itraconazole-susceptible or -resistant strains of C. albicans. TTO was shown to be active in vitro against all tested strains, with MICs ranging from 0.03% (for C. neoformans) to 0.25% (for some strains of C. albicans and other Candida species). Fluconazole- and/or itraconazole-resistant C. albicans isolates had TTO MIC50s and MIC90s of 0.25% and 0.5%, respectively. TTO was highly efficacious in accelerating C. albicans clearance from experimentally infected rat vagina. Three post-challenge doses of TTO (5%) brought about resolution of infection regardless of whether the infecting C. albicans strain was susceptible or resistant to fluconazole. Overall, the use of a reliable animal model of infection has confirmed and extended our data on the therapeutic effectiveness of TTO against fungi, in particular against C. albicans.

Keywords: antifungal activity, tea tree oil, animal model, Candida spp.

Introduction

In recent years, interest has grown in natural medicinal products, essential oils and other botanicals, in response to the ever increasing incidence of adverse side effects associated with conventional drugs, and the emergence of resistance to antibiotics, synthetic disinfectants and germicides. There has been particular resurgence of interest in Australian tea tree oil (TTO), which has been employed for its germicidal activity since 1925.1 The leaves of Melaleuca alternifolia (tea tree) have long been used in aboriginal traditional medicine of New South Wales (Australia) as remedies for wounds and cutaneous infections, and the essential oil obtained by steam distillation from their leaves was used early in the last century to treat many pathological conditions such as empyema, ringworm, paronychia, tonsillitis, stomatitis and vaginal infections.2,3

In view of the anecdotal nature of most of the reports on the effects of TTO, more controlled investigations have been started recently, particularly dealing with extensive in vitro testing of antimicrobial activity using established standard methodology.4–6 However, there is a substantial lack of preclinical testing using suitable animal models of infection, a necessary prerequisite to large-scale clinical testing. Thus, the aim of this work is to report on further investigations of the in vitro antifungal activity of TTO against clinical isolates of pathogenic yeasts and, particularly, to assess TTO activity against azole-resistant and -susceptible strains of Candida albicans in a well-established experimental model of rat vaginal candidiasis.7

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Materials and methods

Melaleuca alternifolia (tea tree oil)

Australian TTO Pharmaceutical Grade MAIN CAMP, marketed by Ballina Ltd NSW, Australia, was kindly supplied by Variati (Milan, Italy). Although complying with the International Standard ISO 4730, as described previously, the oil was further analysed for exact determination of single constituents, as shown below. Terpinen-4-ol and 1,8-cineole [purchased from Fluka (Buchs, Switzerland) and Sigma-Aldrich (St Louis, MO, USA), respectively] were used as positive markers.

Gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS)

Gas chromatography appliances used included a Perkin Elmer AutoSystem equipped with two fused-silica SPB columns (60 m × 0.25 mm i.d.; film thickness 0.25 μm), mounted in parallel in the same oven, with two detectors: FID and Q-Mass 910 (electron ionization 70 eV electron energy, transfer line 220°C). Carrier gas was oxygen and moisture-free helium obtained from a SUPELCO High Capacity Heated Carrier Gas Purifier (Sigma–Aldrich, Milan), provided with an OMI-2 indicating tube, at an average flow rate of 1 mL/min. The oven temperature programme was 60°C for 4 min, then 2°C/min until 180°C had been reached, then 3°C/min until 250°C. The detector temperature was 280°C, and the injector temperature 280°C. The volume of injected essential oil or pure substance was 0.1 μL, and the split ratio was 1:50. Two distinct data systems were connected to the GC-FID or GC-MS: Turbochrom and Q-mass Analytical Workstation Software (Perkin–Elmer, Milan) with a NIST/EPA/MSDC Mass Spectral database, respectively.

Antifungal agents

A stock solution of fluconazole (5000 mg/L; Pfizer Inc., NY, USA) was prepared in sterile distilled water, and a stock solution of itraconazole (1000 mg/L; Janssen Pharmaceutica, Beerse, Belgium) was prepared in polyethylene glycol 400, by heating at 75°C for 45 min.

Yeast isolates

A total of 101 clinical isolates of Candida species and 14 isolates of Cryptococcus neoformans were used for the study. The clinical isolates comprised C. albicans (n = 61), Candida krusei (n = 12), Candida glabrata (n = 13), Candida tropicalis (n = 4) and Candida parapsilosis (n = 11). All isolates of C. albicans were from oropharyngeal swabs from HIV-seropositive subjects. All other Candida strains and C. neoformans were from the established type collection of the Istituto Superiore di Sanità, Rome, Italy. Among C. albicans isolates, seven were resistant to fluconazole, 13 to itraconazole and six to both drugs. All clinical isolates were identified according to morphology on corn meal agar, followed by germ tube formation and assimilation–fermentation profiles in the API 20 system (bioMérieux, Marcy l’Étoile, France), as reported elsewhere.

Reference isolates were C. albicans ATCC 24433, C. tropicalis ATCC 750, C. parapsilosis ATCC 22019, C. krusei ATCC 6258, C. glabrata ATCC 90030, C. neoformans ATCC 90113. C. parapsilosis ATCC 22019 and C. krusei ATCC 6258 were also quality control isolates.

Determination of minimum inhibitory and fungicidal concentration

Susceptibility testing of C. albicans and other yeasts to TTO, fluconazole and itraconazole was performed according to the NCCLS method for broth dilution antifungal susceptibility testing of yeasts, slightly modified in the MIC definition (see below). Each antifungal was diluted using RPMI 1640 medium with L-glutamine, without sodium bicarbonate (Sigma Chemical Co., St Louis, MO, USA) and buffered to pH 7.0 with 0.165 M MOPS buffer (Sigma). Aliquots of 50 μL of two-fold dilutions of drug solutions were dispensed into each well of 96-well microtitre plates. The final concentration of the antifungal agents ranged from 0.0078 to 4 mg/L for itraconazole, 0.125 to 64 mg/L for fluconazole and 0.0078% to 2% for TTO. Tween-80 (final concentration 0.001% v/v) was included to facilitate oil solubility. At this concentration, no inhibitory effect on yeast growth was shown by the detergent.

The cell density of the suspensions was estimated by direct cell count using a Thoma camera, and adjusted to a cell density ranging from 0.5 × 10⁵ to 2.5 × 10⁶ cfu/mL (twice the final inoculum size); 50 μL was added to each well of the microdilution plate, followed by incubation at 35°C for 48 h.

Minimum inhibitory and fungicidal concentrations (MIC and MFC, respectively) were determined. The MIC was defined as the lowest concentration that produced a 50% reduction of growth compared with growth of the drug-free, growth control, and the MFC as the lowest drug concentration resulting in the death of 99.9% or more of the initial inoculum. To determine MFCs, 10 μL of broth was taken from the well without microbial growth, inoculated onto Sabouraud’s dextrose agar (SDA) and incubated at 35°C. After 48 h the cfu were counted to assess viability.

The minimum concentration of drug that inhibited 90% and 50% of the isolates tested was defined as MIC₉₀ and MIC₅₀, respectively. The criteria for definition of susceptibility/resistance to fluconazole/itraconazole were those established by the NCCLS.
Time–kill studies

The fungal inoculum was prepared by growing C. albicans isolates on SDA for 48 h at 35°C. The inoculum suspension was prepared by picking five colonies of at least 1 mm in diameter and suspending them in sterile distilled water. The cell density of the suspension was adjusted to 2 × 10⁶ cells per mL.¹¹

A further dilution was made by inoculating 200 μL of the cell suspension in glass tubes containing RPMI 1.8 mL with Tween-80 and TTO, pH 7, and in other tubes containing Sabouraud broth 1.8 mL with Tween-80 and TTO, pH 5. The final cell density was ~2.5 × 10⁶ cfu/mL, whereas the final concentrations of TTO and Tween-80 were 1% (v/v) and 0.001% (v/v), respectively. Glass tubes containing only RPMI with Tween-80 pH 7 and Sabouraud broth with Tween-80 pH 5 were the negative control.

All tubes were incubated by shaking at 35°C to ensure uniform oil dispersion; the incubation was stopped with cold acetate buffer (pH 3.5) after 2, 5, 10, 15, 30 and 60 min, and 100 μL aliquots were removed for cfu enumeration. The cfu were determined by making appropriate dilutions in acetate buffer, plating 100 μL of each dilution on SDA with chloramphenicol (50 mg/L, Sigma) and incubating for 48 h at 35°C. In control experiments, acetate buffer alone had no inhibitory effect on the growth of C. albicans in vitro.

Each experiment was performed in triplicate, independently. A fungicidal effect was defined as a 3 log decrease in the cfu/mL, or a >99.9% kill over a specified time.¹¹,¹²

Viability assays of fluconazole- and itraconazole-resistant strains

The inoculum was prepared as in time–kill studies by growing an azole-resistant C. albicans isolate (AIDS 68) on SDA. Serial two-fold dilutions (from 2% to 0.0078%) of TTO were then prepared using RPMI 1640 (pH 7) supplemented with TTO at the following concentrations: 0.125, 0.25, 0.5, 1, 2, 4, 8 (% v/v) were prepared. A 10 μL aliquot of the suspension was added to the lowest TTO concentration, corresponding to the MIC for that isolate, and incubated for 48 h at 35°C.

Culture aliquots (100 μL) were removed and subcultured for 48 h at 35°C to check viability on SDA plates. If growth was present, a subculture to the next incremental tube was attempted. This cycle was repeated until visible growth no longer occurred. Isolates from the highest incremental tube were checked and retained for MIC determination.

Experimental vaginal infection

A rat vaginal model was used, as previously described, for the experimental vaginal infection.⁷ Experiments were carried out with fluconazole-susceptible and -resistant strains of C. albicans. Two independent experiments with each fungal strain were conducted and in each experiment groups of five rats were used.

In brief, oophorectomized female Wistar rats (80–100 g; Charles River Calco, Italy) were injected subcutaneously with oestradiol benzoate 0.5 mg (Estradiolo, Amia Farmaceutici srl, Rome, Italy). Six days after the first oestradiol dose, all animals were inoculated intravaginally with 10⁷ yeast cells of each C. albicans strain tested in 0.1 mL of saline. The strains used for the challenge were C. albicans SA40, which was susceptible to both fluconazole and itraconazole, and AIDS 68, which was resistant to both these drugs. The inoculum was dispensed into the vaginal cavity through a syringe equipped with a multipurpose calibrated tip (Combitip; PBI, Milan, Italy). The yeast cells had been grown previously in YPD broth (yeast extract 1%, peptone 2%, dextrose 2%) at 28°C on a gyrator shaker (200 rpm), harvested by centrifugation (1500g), washed, counted in a haemocytometer, and suspended to the required number in saline solution. The number of cells in the vaginal fluid was counted by culturing 1 μL samples (using a calibrated plastic loop, Disponio; PBI) taken from each animal, on SDA containing chloramphenicol (50 mg/L) as previously described. The kinetics of Candida vaginal infection were monitored by the number of cfu/mL of vaginal lavage fluid.

TTO was administered intravaginally (0.1 mL at 1%, 2.5% and 5%, in 0.001% Tween-80), at 1, 24 and 48 h after intravaginal C. albicans challenge. Rats receiving fluconazole (three doses of 100 μg intravaginally) or Tween-80 served as positive or negative controls, respectively. The infection was monitored for at least 21 days after the challenge, with vaginal fluid sampling usually being made at 1, 24 and 48 h, then on days 5, 7, 14 and 21.

The animal experimentation referred to in this paper was approved by the ad hoc committee of the Istituto Superiore di Sanità, Rome, Italy.
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Statistical analysis

The significance of cfu differences in the vaginal infection was assessed by Student’s t-test and set at \( P < 0.05 \) (two-tailed).

Results

Chemical identification and quantitative estimations

TTO composition was determined by comparing GC retention times, the Kovat’s Indices (Adams, 1995) and GC/MS spectra with those of the co-injected reference substances. In the absence of reference substances, the structure of the components was tentatively assigned by the Official NIST/EPA/MSDL Spectral Library. Quantitative data were based on peak area normalization without using a correction factor. By this approach, the oil was shown to contain: 42.35% terpinen-4-ol; 11.9% \( \alpha \)-terpinene; 9.76% \( \alpha \)-terpineol; 3.71% terpinolene; 3.57% 1,8-cineole; 2.82% \( p \)-cimene; 2.42% \( \alpha \)-pinene; 1.75% limonene; 1.05 \( \delta \)-cadinene; 0.94% \( \alpha \)-thujene; 0.94% aromadendrene; 0.87% myrcene; 0.73% \( \beta \)-pinene; 0.4% sabine; 0.34% \( \alpha \)-phellandrene. The oil was therefore a terpinen-4-ol type according to the International Standard ISO 4730:1996.8

Antifungal activity

The results of TTO antifungal activity are shown in Table 1. TTO inhibited the growth of all isolates tested inclusive of those resistant to fluconazole and itraconazole. The MIC ranged from 0.015% to 0.5%. MIC\(_{90}\)s were 0.25% and 0.5% forazole-susceptible and -resistant C. albicans strains, respectively, 0.125% for C. krusei and C. glabrata, and 0.06% for C. neoformans and C. parapsilosis. Four isolates of C. tropicalis tested also had low MICs, ranging from 0.06 to 0.125%.

The interpretation of susceptibility was easy because a distinct endpoint of growth inhibition was produced without trailing growth.14 At the MIC, TTO was generally also fungicidal, as determined by MFC. The MIC and MFC coincided for each isolate. As expected from previous reports,15 non-albicans species of Candida, in particular C. krusei and C. glabrata, were less susceptible to fluconazole with their MIC\(_{90}\) falling in the range 8–32 mg/L. As shown in Table 1, TTO was also active against fluconazole- and/or itraconazole-resistant strains, with MIC\(_{90}\)s of 0.25% and 0.5%, respectively, for both drugs.

All azole-resistant isolates of C. albicans were killed within 30 min by 1% TTO and within 60 min by 0.25% TTO, at pH 7. The pH of the medium slightly influenced the killing activity of TTO. At pH 5, the decrease in viable count was less rapid; nonetheless, a 100% killing within 30 min by TTO 1% was achieved (data not shown).

The two clinical yeast isolates tested for induction of resistance to TTO did not produce visible growth in broth containing 2% TTO, after five to seven serial subcultures in increasing TTO concentrations. Thus, no induction of resistance to TTO was achieved under our experimental conditions.

Experimental vaginal infection

After establishing activity in vitro, we examined the activity of TTO in vivo. For this purpose, we selected an experimental

Table 1. \textit{In vitro} antifungal activity of tea tree oil (TTO) compared with fluconazole (FCZ) and itraconazole (ITR)\(^a\)

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of isolates</th>
<th>MIC(_{50}) FCZ (mg/L)</th>
<th>MIC(_{90}) FCZ (mg/L)</th>
<th>TTO (%)</th>
<th>MIC(_{50}) ITR (mg/L)</th>
<th>MIC(_{90}) ITR (mg/L)</th>
<th>TTO (%)</th>
<th>MIC(_{50}) TTO (mg/L)</th>
<th>MIC(_{90}) TTO (mg/L)</th>
<th>TTO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans(^b)</td>
<td>47</td>
<td>0.125</td>
<td>0.03</td>
<td>0.125</td>
<td>0.25</td>
<td>0.03</td>
<td>0.25</td>
<td>0.125</td>
<td>0.0078</td>
<td>0.5</td>
</tr>
<tr>
<td>C. albicans(^c)</td>
<td>14</td>
<td>64 (7)</td>
<td>4 (13)</td>
<td>0.25</td>
<td>64 (7)</td>
<td>4 (13)</td>
<td>0.5</td>
<td>32–64</td>
<td>4</td>
<td>0.25–0.5</td>
</tr>
<tr>
<td>C. krusei</td>
<td>12</td>
<td>32</td>
<td>0.5</td>
<td>0.125</td>
<td>32</td>
<td>0.5</td>
<td>0.125</td>
<td>8–64</td>
<td>0.03–1</td>
<td>0.06–0.2</td>
</tr>
<tr>
<td>C. neoformans</td>
<td>14</td>
<td>2</td>
<td>0.03</td>
<td>0.03</td>
<td>4</td>
<td>0.06</td>
<td>0.06</td>
<td>1–4</td>
<td>0.0078–0.125</td>
<td>0.015–0.0</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>13</td>
<td>4</td>
<td>0.25</td>
<td>0.06</td>
<td>8</td>
<td>0.5</td>
<td>0.125</td>
<td>0.25–32</td>
<td>0.06–1</td>
<td>0.03–0.125</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>11</td>
<td>0.5</td>
<td>0.125</td>
<td>0.06</td>
<td>1</td>
<td>0.25</td>
<td>0.06</td>
<td>0.125</td>
<td>0.06–0.25</td>
<td>0.03–0.125</td>
</tr>
</tbody>
</table>

\(^a\)For the four strains of C. tropicalis, the MIC ranges were: 0.25–8 and 0.06–0.25 (mg/L) for fluconazole and itraconazole, respectively. For the same strains, TTO MICs ranged from 0.06 to 0.125%. For other details see the text. The MICs for the QC strain of C. krusei were 32 and 0.25 (mg/L) for fluconazole and itraconazole, respectively, and 0.25 (\%/v/v) for TTO.

\(^b\)Fluconazole- and itraconazole-susceptible isolates. The MICs for the QC strain of C. parapsilosis were 2.0 and 0.06 (mg/L) for fluconazole and itraconazole, respectively, and 0.125 (\%/v/v) for TTO.

\(^c\)Fluconazole- and/or itraconazole-resistant isolates; six isolates were cross-resistant.
mucosal infection (oestrogen-dependent rat vaginitis) in which the animals were challenged with either a fluconazole–itraconazole-susceptible (SA-40) or a fluconazole-resistant (AIDS 68) C. albicans strain. Two experiments were performed with each strain, and these produced substantially overlapping results. Figures 1 and 2 show the details of one of the two experiments conducted with the fluconazole-susceptible and -resistant strains, respectively.

As shown in Figure 1, which shows the results with fluconazole-susceptible C. albicans, TTO exerted a marked acceleration of clearance of the yeast, as demonstrated by a statistically significant decrease in cfu counts in the first 2 weeks after the vaginal challenge, compared with the control (TTO-untreated animals, given the Tween-80 diluent). The parallel curves of clearance with different TTO concentrations suggest a substantial TTO dose dependence of fungus clearance, although the difference was not statistically significant. As with all dose regimens, the infection was cleared in 3 weeks, whereas the untreated control rats remained infected (∼2.5 × 10^4 C. albicans cfu/mL of vaginal fluid). Fluconazole treatment, used as a positive control, showed a pattern of clearance comparable to that induced by TTO. No effect on the rate of fungal clearance was observed in rats treated with TTO diluent Tween-80.

As shown in Figure 2, TTO (5%) also caused a rapid clearance of the fluconazole-resistant strain from the vagina of experimentally infected rats. There was a highly statistically significant difference at all time-points considered between control (or fluconazole-treated rats) and those treated with TTO. Here again, the infection was resolved in 3 weeks by TTO, whereas all other animals, either untreated or fluconazole-treated, were still infected.

**Discussion**

Interest in the therapeutic use of non-conventional, non-prescription, or so-called natural medicinals in the field of infectious diseases has increased remarkably in recent years, mostly driven by the well-known side effects of conventional drugs as well as by the spread of antimicrobial resistance to otherwise efficacious and well-tolerated drugs. One of the most popular of the above medicinals is the essential oil of *M. alternifolia*, TTO,11,16 for which extensive work has been published on antimicrobial activity in vitro.4,5,17–19 There are also several anecdotal or empirical reports on the clinical benefits of TTO use, not firmly based, however, on evaluation of in vivo activity in experimental animal models. A small, single-centre, pilot study of oropharyngeal candidiasis in AIDS patients treated with an oral solution of TTO has shown favourable clinical response in most of the few patients treated,20 despite some compliance problems. In another instance, the TTO, although unsuccessful alone, was shown to have additive effects with butanefine in curing toenail onychomycosis.21 Overall, there is a need for more extensive and appropriate evaluation of both pre-clinical and clinical investigations in experimental animal models and in patient groups. In this paper, we have confirmed and extended the data on in vitro activity of TTO against an elevated number of clinical isolates of *C. albicans*, other Candida species and *C. neoformans*. The TTO mixture used throughout our investigation was predominantly composed of terpinen-4-ol, considered to be the active antimicrobial compound,22 whereas the percentage of 1,8-cineole, generally considered to affect

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**Figure 1.** Outcome of vaginal infection by a fluconazole-susceptible strain of *C. albicans* in oophorectomized, oestradiol-treated rats inoculated intravaginally with TTO 5% v/v (white squares), 2.5% v/v (white triangles), 1% v/v (white circles), fluconazole 100 µg (black circles), Tween-80 (0.001% v/v) (control; black squares) at 1, 24 and 48 h after intravaginal *C. albicans* challenge. Each curve represents the mean (±S.E.) of cfu of five rats. The data are from one of two independent experiments with similar results.

**Figure 2.** Outcome of vaginal infection by fluconazole–itraconazole-resistant strain of *C. albicans* (AIDS 68) in oophorectomized, oestradiol-treated rats inoculated intravaginally with TTO 5% v/v (white squares), fluconazole 100 µg (black circles), Tween-80 0.001% v/v (black squares) at 1, 24 and 48 h after *C. albicans* challenge (10^7 cells in 0.1 mL). Each curve represents the mean (±S.E.) of the fungal cfu of five rats.
the therapeutic performance of the oil mixture negatively, was well below the established standard. However, it is not clear whether terpinen-4-ol is the only antimicrobially active compound of the mixture, or whether other components, even in trace amounts, add synergically to the activity. Our TTO mixture was fully characterized by gas chromatography and mass spectrometry. It was shown to contain another 14 compounds in addition to terpinen-4-ol and cineole, constituting >50% of the whole composition, and it can be safely anticipated that the antimicrobial activity of terpinen-4-ol is somewhat modulated by their presence.

It is of some interest that the MIC\textsubscript{90} for \textit{C. albicans} strains determined in our study matched that (0.25%) reported by Hammer et al.\textsuperscript{5} against the same fungus using a TTO mixture with relatively similar proportions of terpinen-4-ol and cineole, thus indirectly attesting to the reproducibility of results in different laboratories. Importantly, the MIC\textsubscript{90} were still lower for other fungal strains (from 0.06% for \textit{C. parapsilosis} and \textit{C. neoformans} to 0.125% for \textit{C. krusei} and \textit{C. glabrata}), among which some natural refractoriness to important therapeutically active compounds, such as the triazoles—in particular fluconazole—is frequently observed. It is of interest that the TTO mixture employed had a fungicidal concentration equal to the MIC, thus demonstrating the rapid cytocidal activity of TTO. This was also demonstrated by the time–kill experiments, and confirms and extends earlier data.\textsuperscript{12} Neither fungistatic nor fungicidal activities were strongly influenced by lowering the pH of the incubation medium to pH 5, a fact in keeping with justifications of TTO usage for skin and mucosal infections.\textsuperscript{21,24}

We have examined specifically the therapeutic activity of TTO in a well-established experimental model of rat vaginal candidiasis, in which the effect of immunotherapy by passive transfer of antibodies, or active vaccination with whole \textit{Candida} cells or subunit antigens, has been assessed extensively.\textsuperscript{25–27} This model has also been shown to be a valuable tool in determining and predicting the antifungal activity of various drugs, including the HIV-protease inhibitors.\textsuperscript{28} This investigation was instigated following several claims and anecdotal reports on the therapeutic activity of TTO against several forms of vaginal infection, including vaginal candidiasis. There are also reports that TTO inhibits germ-tube formation in \textit{C. albicans}, a morphogenetic event of critical importance in the onset of vaginal and other infections by this fungus.\textsuperscript{6} A potential advantage of novel therapeutics is their capacity to inhibit microorganisms that are resistant to existing drugs; we therefore tested the \textit{in vivo} activity of TTO against a strain of \textit{C. albicans} resistant to fluconazole, one of the most popular and medically effective anti-\textit{Candida} drugs.

The results of our investigations demonstrate that TTO treatment is efficacious in resolving experimental \textit{Candida} infection, with both fluconazole-susceptible and -resistant isolates. In the case of the drug-susceptible organism, treatment with TTO was comparable to a standard treatment with fluconazole.

In all cases, the infection was resolved (using 5% TTO) by the third week of treatment. Importantly, TTO treatment was equally efficacious against a fluconazole–itraconazole-resistant organism.

Throughout this investigation, there was no evidence of suffering by the animals following TTO treatment, or any sign of allergic response to a treatment that was easily dispensed and non-chronic in nature (one intravaginal application a day, for the first 3 days only, after intravaginal challenge). Overall, our experimental data give substantial support to previous anecdotal or empirical evidence of the efficacy of treatment of vaginal candidiasis with TTO. These data also encourage adequately controlled and randomized clinical investigations, including studies on the mechanisms of antifungal activity, of this long-established medicinal plant extract.

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