

An in vitro study to determine the minimum inhibitory concentration of *Melaleuca alternifolia* against the dermatophyte *Trichophyton rubrum*

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Abstract

This in vitro study assessed the antifungal activity of a well-known and widely used essential oil, *Melaleuca alternifolia* (Australian tea tree oil), against the ubiquitous dermatophyte *Trichophyton rubrum*. The literature has reported the antifungal properties of *M. alternifolia* citing the minimum inhibitory concentration needed to secure this effect.

Following a study which determined that the oil was a potent antifungal and that the inverse relationship between essential oil concentration and fungal growth was not influenced by random variability ($P = 0.05$); the minimum inhibitory concentration (MIC) was then determined at 0.1% (v/v). This MIC is approximately 10% lower than results from other similar studies, and could have important therapeutic significance. © 2003 Elsevier Ltd. All rights reserved.

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1. Introduction

Many studies have welcomed the latest antifungals to the armamentarium of therapies available to the practitioner for treating onychomycosis [1–6].

The latest conventional antifungals may be very effective in treating onychomycosis and tinea pedis and have reputedly reduced side effects. However, it has been reported they can still produce a number of adverse reactions. Current research suggests that tea tree oil (TTO) may be a safe, economically viable alternative topical therapy with relatively few side effects.

Trials involving terbinafine (allylamine) and itraconazole (triazole) have reported very high success rates in treating tinea unguium. Roberts [7] in a comparative study of antifungals in the treatment of onychomycosis, found terbinafine to be the most effective treatment, providing a cure rate of 70–80%. Furthermore, it was also shown to have an excellent tolerability profile. The question arising from such work is what happens to the patients who inevitably fail treatment [8]. This position raises a future challenge in the treatment of onychomycosis, especially with high failure rates of 20%. Roberts suggests, in many of these cases surgery may need

to precede drug therapy to maximise the prospects of cure. In addition to surgical intervention, the possibility of combining oral and topical therapy warrants further investigation. Buck et al. [9] compared two topical preparations for the treatment of onychomycosis; TTO and clotrimazole. After 6 months of treatment, the two groups were comparable based on culture cure and clinical assessment documenting partial or full resolution. Three months later, approximately 50% of each group reported continued improvement or resolution. They concluded, all current therapies have high recurrence rates. Work by Concha et al. [10] in an in vitro study, demonstrated TTO to have an inhibitory activity against a selection of clinical isolates, including *Trichophyton rubrum*. The only exception to the isolates not to show an inhibitory effect was one strain of *Epidermophyton floccosum*.

Dermatophytes have survived several generations of therapeutic regimens; there is certainly no guarantee that they would not become resistant to the latest antifungals. There have been to date, no reported cases of resistance to TTO. Scientists have genetically modified *Melaleuca alternifolia* (tea tree) to produce a more chemically defined agent. However, it is unknown whether a more refined form of TTO, instead of its naturally occurring complex composition will allow microbes to develop resistance. Williams [11] reports on the cloned production of TTO, high in terpinen-4-ol, which will soon provide commercial quantities of a par-

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ticularly active oil of consistent quality for therapeutic use.

Given this level of interest and academic research it is surprising that only limited studies have been undertaken which have attempted to test the MIC and the toxicity of TTO. Initial work by Altman [12] suggested the MIC for TTO to be in the region of 1% (v/v). Similar work by Bassett et al. [13] managed to obtain MIC values of between 0.5 and 1.0% (v/v). With this variation in concentrations, the question this research set out to answer was to secure a definite MIC for TTO using modern microbiological laboratory techniques.

2. Methodology

The TTO was incorporated into the medium by using a “Polytron” ultrasonicator, as used by Garg et al. [14] under aseptic conditions. The speed of the Polytron and the duration of the sonification period were standardised to ensure that the oil became evenly mixed as fine micelles throughout the media. The oil was sterilised by passage through a 0.22 µm pore size Millipore filter. The speed of the Polytron gave a maximum RPM of 20,000 and the duration of application was between 2 and 3 s.

A standard sized inoculum of *T. rubrum* (25 µl), derived from a spore suspension, was applied to Sabouraud’s dextrose agar (SDA). Preparation of the spore suspension was standardised by applying 3 ml of autoclaved distilled water to a 2-week-old *T. rubrum* culture, which was then gently rubbed over the surface with a sterile spreader. The water was then drawn off with a pipette and stored in a sterile universal bottle, ready for inoculation. The spore suspension gave an absorbency reading of 0.14. Data from our standard calibration curves for fungal spores show that an absorbance of 1.0 was equivalent to 5×10^8 colony forming units/ml (cfu/ml). The calibration curve is linear up to this point so a suspension with an absorbance of 0.14 would contain $0.14 \times 5 \times 10^8$ cfu/ml = 7×10^7 cfu/ml.

The SDA plates were prepared containing a predetermined concentration of the essential oil and were allowed to dry for 24 h before being inoculated. SDA plates were then incubated at 25 °C for 7 days.

All procedures were carried out aseptically and inside a laminar flow cabinet, thus reducing any incidences of contamination by bacteria or other fungi.

2.1. Procedure 1

Using the Polytron mixer, varying amounts of the TTO were added to molten SDA. The concentrations were as follows—5, 1 and 0.1% (v/v). When the agar set (after 24 h) the application of spore suspension from the 2-week-old *T. rubrum* was added. Data were recorded for fungal growth.

2.2. Procedure 2

Following this, a Polytron mixer was again used to prepare SDA plates containing the following oil concentrations, 0.1, 0.05, 0.01, 0.0083, 0.0066, 0.005, 0.0033 and 0.0016% (v/v). The spore suspension was added in single droplets, and data were recorded for fungal growth after the standardised incubation period of 7 days.

2.3. Procedure 3

The previous procedure was repeated to test experimental reliability. On this occasion one set of plates had the spore suspension applied in a single droplet; the other set had the spore suspension applied and then spread over the surface with a sterile spreader.

Using replicates of each plate allowed for eight colonies to be produced for each concentration, each colony had their diameter measured, the average diameter was then calculated. Pearson’s product moment correlation coefficient was used to assess the strength of the linear relationship between the two variables, i.e. oil concentration and colony size.

2.4. Procedure 4—recovery

Any plates from the previous procedure that had failed to produce growth after 7 days, had 3 ml of sterilised water added to the plate. The water was gently swilled around the plate or gently rubbed over the agar surface. It was then drawn off after 5 min and used to inoculate a SDA plate, using 100 µl of the inoculum, processed. This was incubated for 7 days at 25 °C. Data of fungal growth were recorded. This indicated if the concentrations of oil used in this experiment were either fungicidal or just fungistatic.

3. Results

3.1. Procedure 1

The results of procedure 1 are given in Table 1. All concentration of TTO inhibited growth whilst the control, grape seed oil, had good even growth across the plates.

Table 1
Determining the MICs (NG: no growth G: growth), Procedure 1

Concentration of oil (% , v/v)	Growth/no growth after 1 week	Growth/no growth after 2 weeks
Control SDA only	Signs of good even growth	Very good growth
Control 10% (grape seed oil)	Signs of good even growth	Very good growth
5% TTO	NG	NG
1% TTO	NG	NG
0.1% TTO	NG	NG

Table 2
Determining the MICs, Procedure 2

Oil concentration (% v/v)	TTO plate 1	TTO plate 2	TTO plate 3
0.1	NG 100%	NG 100%	NG 100%
0.05	G 100%	G 100%	G 100%
0.01	G 100%	G 100%	G 100%
0.0083	G 100%	G 100%	G 100%
0.0066	G 100%	G 100%	G 100%
0.005	G 100%	G 100%	G 100%
0.003	G 100%	G 100%	G 100%
0.0016	G 100%	G 100%	G 100%

Table 3
Determining the MICs, Procedure 3, readings after 7 days

Oil concentration (% v/v)	Plate 1 TTO (spore suspension in single droplet)	Plate 2 TTO (spore suspension spread over surface)
0.1	NG	NG
0.09	G	G
0.08	G	G
0.07	G	G
0.06	G	G
0.05	G	G
0.04	G	G
0.03	G	G
0.02	G	G
0.01	G	G

3.2. Procedure 2

Table 2 illustrates growth on all plates with TTO concentrations below 0.1% (v/v).

3.3. Procedure 3

Table 3 illustrates a repeat of procedure 2 with different methods of application of the spore suspension. Furthermore, Table 4 gives the association between TTO concentration and colony size (Pearson's product moment correlation coefficient, $r = 0.9$). Fig. 1 illustrates a selection of 10 plates with the differing concentrations of TTO and the amount of growth associated with each (high to low concentration).

Table 4
Oil concentration and the equivalent colony size in mm

Subject	Oil concentration (% v/v) TTO	Colony diameter (mm)
1	0.1	0
2	0.09	9
3	0.08	9.62
4	0.07	11.75
5	0.06	18.31
6	0.05	17.37
7	0.04	22.69
8	0.03	26.94
9	0.02	25.44
10	0.01	24.94

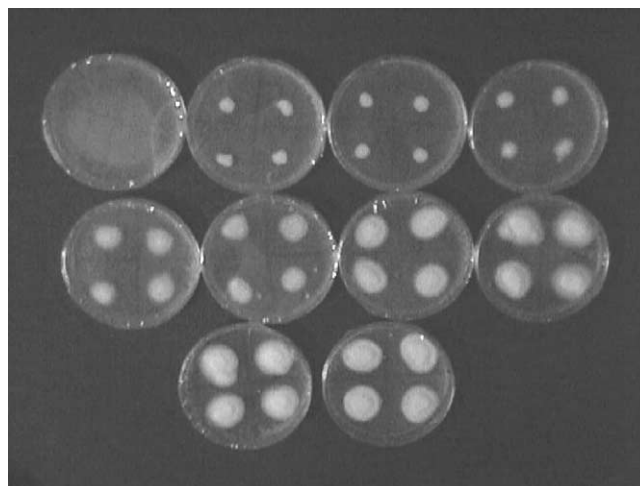


Fig. 1. Ten plates of differing TTO concentrations.

Table 5
Recovery experiment

Plate A	Growth/no growth	Plate B	Growth/no growth
0.2% (v/v) TTO—rubbed	Growth	0.1% (v/v) TTO—rubbed	Growth
0.2% (v/v) TTO—swilled	No growth	0.1% (v/v) TTO—swilled	No growth

3.4. Procedure 4

Table 5 illustrates the concentrations at which growth or no growth was recorded for the recovery experiment.

4. Discussion

Tea tree oil demonstrated antifungal properties. The minimum inhibitory concentration (MIC) for TTO against the fungus *T. rubrum*, was 0.1% (v/v) after 7 days, incubated at 25 °C. The MIC was taken as the lowest oil concentration that inhibited growth. At oil concentration, 0.09% (v/v) of TTO, fungal growth was severely restricted and difficult to measure.

Altman [12] indicated the MIC to be about 1% (v/v) for most commonly occurring pathogenic bacteria and fungi. Although he suggested that this MIC information may not accurately assess the true potential of TTO as an antimicrobial. The reason being that MIC tests use an aqueous medium, and the antimicrobial activity of a sparingly soluble oil would be expected to reflect only the "activity of the saturation concentration calculated on the amount of oil added to the test medium". The use of the Polytron ultrasonicator in this study may have reduced this solubility problem, hence the lower MIC values in this current project. The importance of this in relation to the project (and indeed clinically) is that no emulsifying agents were used to disperse

the TTO in the SDA, which are known to reduce the potency of the TTO as an antifungal.

Bassett et al. [13] demonstrated the inhibition of a wide range of pathogenic micro-organisms with concentrations of TTO varying between 0.5 and 1.0% (v/v). Previous work by Beylier [15] and Walsh and Longstaff [16] yielded a similar range of MIC values against pathogenic organisms. But variations in the results do occur for certain bacterial species, this variation is likely to be due to methodological variations.

Hammer et al. [17] gave a range of MICs for TTO of 0.25–3.0% (v/v), organisms tested were all bacteria, many of them common commensals. Interestingly they suggested that their results did not compare with other studies possibly because of methodological differences.

Remmal et al. [18] found that MIC values varied depending on the emulsifying agent used. When no solvent or detergent was used, MIC values were reduced. This may suggest that the solvents/detergents used in antimicrobial studies may lead to decreasing the antimicrobial activity of the essential oils. Our study utilised the Polytron ultrasonicator, which we feel led to yielding lower and more accurate MIC values.

In terms of clinical implications, the authors would suggest that it is of no clinical advantage to carry out similar investigations using the same strain of *T. rubrum*. Other in vitro studies should not only vary the strain of the *T. rubrum* but also the fungal species. It is important to determine the MICs for all dermatophyte and non-dermatophyte species, which are more likely to manifest in the clinical situation.

Similarly, it is of no advantage to carry out further investigations using the same batch number of essential oil. It is important to know the “average” MIC for TTO and other essential oils. The source of any oil is important to any future research and therefore it is essential that researchers obtain the purest available oil for experimental purposes. Ideally a tea tree bottle should have *M. alternifolia* written on it, indicating the Australian variety, and not a substandard or chemical form.

The “recovery” experiment was carried out, to ascertain if the oils were having a fungistatic or fungicidal effect. There was no growth evident from the fungal ‘plugs’ (obtained from preliminary experiments) when placed on 0.1% (v/v) TTO after 14 days. When these ‘plugs’ were removed and placed on fresh SDA plates, after 7 days incubated at 25 °C, growth became evident, suggesting 0.1% (v/v) TTO was fungistatic rather than fungicidal. The autoclaved distilled water used in procedure 4, the recovery experiment, was used to inoculate fresh SDA plates, which were incubated for 7 days. The water was either swilled around the agar plate, or gently rubbed over the agar surface using a sterile spreader. On the plates where the water was just swilled around, no growth was apparent. However where the water had been rubbed over the SDA surface, growth was now apparent, suggesting one of two things:

- Simply swilling the water around the plate was not sufficient to dislodge any viable spores/mycelium, leading to the assumption that the MIC had been fungicidal.
- Rubbing the surface did dislodge spores/mycelium, which were still capable of growing, now indicating that the MIC of 0.1% (v/v) for TTO had actually only been fungistatic.

Further investigations at concentrations >0.1% (v/v) are required to determine the fungicidal concentrations.

Interestingly, Kishore et al. [19] had tried a similar experiment. Fungal discs whose growth had been completely inhibited, were, after washing in distilled water, re-inoculated on fresh agar medium, and observed for revival of their growth. Although the fungus being used was *T. rubrum*, unfortunately the 16 essential oils being tested did not include TTO. Five of their oils were fungicidal at concentration of 1000 ppm, but the majority became fungistatic/fungicidal at 2000 ppm. They concluded that, compared with conventional drugs applied to control ringworm, essential oils are attractive for their availability, the indigenous origin and their renewable sources.

In relation to clinical practice, there certainly is an argument to move onto in vivo work. However, it is our opinion that there needs to be a lot more in vitro work before this therapeutic intervention is considered. For example, toxicity levels and the pharmaceutical mode of action of these essential oils are required. It is vital to know how safe these oils are to use in vivo and at what concentrations they should be applied. From this study the MIC values for TTO against *T. rubrum*, need to be compared to MICs for other dermatophytic and non-dermatophytic fungi.

Furthermore, it is also vital to distinguish between the fungicidal and fungistatic concentrations. One way of determining these properties is by employing the recovery experiment used in this study.

There are reported cases of sensitivity reactions and internal poisoning with TTO [20–27]. Prolonged use of the oil may have a cumulative effect, resulting in contact dermatitis-type reaction. In all cases, cessation of the oil application has resolved the reaction. Using essential oils (EOs) at their MICs, instead of concentrated topical applications, may reduce the occurrence of patients experiencing sensitivity reactions.

There also has to be research into the synergistic properties of essential oils. Research has shown that very low MIC for lemongrass (0.005%, v/v) is possible.

This project has attempted to incorporate certain standards to the experimental procedure and the MIC results obtained should be used as a starting point for future work. There is still much more preliminary work to consider before the final in vivo investigations are confidently carried out. Further work should distinguish between fungicidal and fungistatic values, using the recovery experiment. Investigations at concentrations >0.1% (v/v) are still required. Better clinical results occur when fungicidal preparations are employed rather than fungistatic [28].

Extending the range to other dermatophytes and non-dermatophytes (yeast) should be included. If the MIC values are found to be similar, this will reduce the need to carry out identification tests, before the oils are applied, as is occasionally done when prescribing antibiotics for bacterial infection. This identification of the fungus involved may be more important when considering which brand or batch number of EO to use. This idea for sensitivity testing is really for the safety of the patient. Irrelevant of the MIC value, sensitivity tests are essential before topical application of the oil, because regardless of the manufacturer or batch number, all oils should comply to the Australian Standard, meaning that the oil contains the principal ingredients of terpineol-4 and 1,8-cineole (cymene), the latter having the irritant properties. Therefore, cymene is not actually an active ingredient, i.e. antifungal. The idea of culture identification, would be important when considering which manufacturers' oil to use, because there is a vast difference in the antifungal effectiveness between differing sources of oil (as shown by recent work carried out at The University of Wolverhampton, personal communication, 2000). The importance of extending the range of microbes studied, is to ascertain an "average" MIC value, this would reduce the possible need for culture/identification testing.

Further work should also consider using other EOs with known antifungal properties such as garlic [29], castor oil, coconut oil and mustard seed oil [30]. Investigations using mixtures of these oils may be beneficial, although Lis-Balchin et al. [31] warned of the unpredictability of using mixtures of these complex, naturally occurring oils.

5. Conclusion

This paper presents a 'novel' method of identifying the MIC of TTO using techniques hitherto not applied to this question. It is acknowledged there may be variables within the methods adopted that proved to be difficult to quantify, for example, the standardisation of the 25 µl of fungal inoculum, however it has been demonstrated that TTO does exhibit antifungal characteristics. The minimum inhibitory concentration against *T. rubrum* proved to be 0.1% (v/v) (after an incubation time of 7 days and an incubation temperature of 25 °C). The inverse relationship between essential oil concentration and fungal growth, was not influenced by random variation ($P = 0.05$), as determined by using Pearson's product moment correlation coefficient for a two-tailed test.

Only the fungistatic value (0.1%, v/v) was determined for TTO, therefore further work is required to determine its fungicidal concentration.

The literature does give some theories as to how the oils exert their antimicrobial effects, however, little is known on the oil's effects on mammalian tissue. TTO is toxic if taken orally and sensitivity reactions are not uncommon when TTO is used topically. There is a pressing need to determine toxicity levels of TTO.

If this therapeutic agent and similar essential oils are to be used in the podiatric clinical environment, there is a clear, and indeed required body of knowledge to be investigated before they are utilised on the patient population.

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