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# Enhancing the in vitro activity of *Thymus* essential oils against *Staphylococcus aureus* by blending oils from specific cultivars

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**Astract**

The in vitro activity of a blend of essential oils derived from four UK grown cultivars of thyme (*Thymus zygis* L.) against two strains of methicillin-sensitive *Staphylococcus aureus* (MSSA) and two strains of epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA) was examined, and the results compared to those obtained for an unblended linalool chemotype of thyme oil. The principal components of the thyme oil blend, designated 'Oil B' were thymol, linalool, terpinen-4-ol, and $\alpha$-terpinene. The oil blend exhibited significant inhibitory and bactericidal effects, as determined by disc diffusion assay, modified broth micro-dilution and time-kill studies. Oil B had mean MIC and MBC values of 0.3% and 0.6% (v/v) against the staphylococcal isolates compared to 0.4% and 0.8% (v/v) for the linalool chemotype thyme oil. Time-kill studies exhibited a decrease in viability of more than 3 $\log_{10}$ cfu/ml within two hours using a final Oil B concentration of 5% (v/v). The results for the Oil B compare favourably to those for single linalool chemotype thyme oils, and the blend shows good in vitro potential for the treatment of MRSA colonisation.

**Keywords:** thyme oils, cultivars, components, blending, MRSA

## Introduction

The antimicrobial properties of essential oils from certain plants, trees, herbs and spices have been recognised for hundreds of years, but scientific evidence of their efficacy has been described only since the 1940s and 1950s [1]. The antimicrobial activity of essential oils is dependent on their chemical constituents, which is determined by the genotype of the plant species and the growth, environmental and harvesting conditions [2-4]. Consequently different cultivars, batches and blends of essential oil may vary in their composition and hence vary in their apparent antimicrobial effects.

Oils extracted from species and cultivars of the genus Thymus (family Lamiaceae) are widely used by the food, beverage, confectionery and cosmetics industries [4]. There are several species and varieties in the genus *Thymus* and the essential oil composition of some of them have been studied. The main chemical components noted are $\alpha$-thujone, $\alpha$-pinene, camphene, $\beta$-pinene, p-cymene, $\alpha$-terpinene, linalool, borneol, $\beta$-caryophyllene, thymol and carvacrol [5]. Piccaglia [6] and colleagues recorded six chemotypes of French thyme (geraniol, linalool, $\gamma$-terpineol, carvacrol, thymol and terpinen-4-ol). Studies by Sáez [7] on *Thymus zygis* subsp. *gracilis* growing wild in southeastern Spain, showed that the most common chemotype was thymol, although a pure linalool chemotype was also recorded. Sáez [8] also reported that the most abundant chemotypes of the essential oil from wild *T. hyemalis* from the same region were thymol, carvacrol, borneol and linalool.

The aim of the present study was to investigate the antibacterial properties of Oil B, a proprietary blend of essential oils derived from four cultivars of UK grown *Thymus zygis* (Loefl).L. against two strains of methicillin-sensitive *Staphylococcus aureus* and two strains of epidemic methicillin-resistant *Staphylococcus aureus* (MRSA). The

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results for Oil B were compared to those for a single linalool-containing chemotype of thyme oil that was assayed in parallel. The essential oils were steam distilled from the aerial parts of the plant and the blend of thyme oils used in this study was formulated by a distillation chemist especially for Benchmark Oils Ltd. It was designed to contain a high concentration of linalool (23.6%) and thymol (31.1%), and relatively high concentrations of α-terpinene (13.2%) and terpinen-4-ol (11.7%), which are usually found in significant amounts in tea tree oil (*Melaleuca alternifolia*) [9] but in smaller amounts in thyme oils (0.72% and 0.69% respectively in *Thymus vulgaris* [5]).

Following initial disc diffusion studies, the bacteriostatic and bactericidal concentrations of Oil B and the single chemotype of thyme oil were established for the staphylococcal test strains, and the efficacy of the oils were determined by time-kill assays by measuring the reduction of staphylococcal numbers exposed to the oil over time.

## Materials and methods

*Essential oils*

The blend of oils, designated Oil B, from four UK grown cultivars of thyme (*Thymus zygis*), a single linalool-chemotype thyme oil (*Thymus zygis*), and jojoba oil (*Simmondsia chinensis*) were obtained from Benchmark Oils Ltd., Crawley, West Sussex, UK. Jojoba oil was used as inactive diluent and was used as the control oil. The quantitative compositions of the oils were derived from gas liquid chromatography and mass spectrometry analyses provided by Benchmark Oils Ltd. The blend of essential oils (Oil B) from the four UK grown cultivars of *Thymus zygis* used in this study were composed of 31.1% thymol, 23.6% linalool, 13.2% α-terpinene, and 11.7% terpinen-4-ol. GC/MS conditions: 30m DB5 column, 60-250°C, 4°C/min, start isothermal 2.5 min, quadrapole sensing scan from mass 40, NIST and Adams libraries (personal communication, Benchmark Oils Ltd).

*Bacterial strains*

Two strains of methicillin-sensitive *Staphylococcus aureus* (NCTC 8511, NCTC 10788) and two strains of epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA15/ NCTC 13142, EMRSA16/ NCTC 13143) were used in all experiments and were obtained from the culture collection of the School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, UK.

Prior to initial use, the identity of the test strains were confirmed by Gram's staining and positive reactions in the catalase and the coagulase tests. Methicillin resistance was confirmed by disc diffusion assay against 1 µg oxacillin (data not shown).

*Disc diffusion assay*

Preliminary screening of the antimicrobial activity of Oil B blend and the single chemotype thyme oil was performed using the disc diffusion assay. A modification of a method described by the British Society for Antimicrobial Chemotherapy was used [10]. Each staphylococcal test strain was inoculated into Tryptone Soya Broth (Oxoid Ltd; Basingstoke, UK) which was incubated overnight at 37°C with shaking. The overnight cultures were diluted with fresh broth to give final inocula of approximately $10^5$ colony forming units per ml (cfu/ml). Diagnostic Sensitivity Test Agar plates (Oxoid) were inoculated with each bacterium to form semi-confluent growth. Sterile 6 mm diameter filter-paper discs were impregnated with 20 µl of each undiluted essential oil and applied to the surface of the inoculated agar plates. As a control, filter-paper discs were impregnated with 20 µl of the inactive jojoba 'carrier' oil. The agar plates were inverted and incubated at 37°C for 18 hours and zones of growth inhibition around the discs were measured to 0.1 millimetre accuracy using a vernier caliper. The discs had a diameter of 6 mm, and a recorded value of 6 mm or less represents no activity.

*Broth microdilution assay*

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the oils were determined for the staphylococcal strains using a broth microdilution method [11]. All the tests were performed in Diagnostic Sensitivity Test Broth containing Tween 80 at a final concentration of 0.5% to assist the emulsification of the oils. Serial doubling dilutions from 6% to 0.05% (v/v) of each essential oil were prepared by vortexing the oil in 0.15% (w/v) Diagnostic Sensitivity Test Agar (Oxoid).

Bacterial inocula were prepared as for the disc diffusion assay and adjusted so that the final concentration of the test bacteria in the wells was ~$10^5$ cfu/ml. Well contents were mixed using a micropipettor and the trays incubated at 37°C for 18 hours. The highest oil dilution not exhibiting bacterial growth was taken as the MIC. After the MICs were read, 100 µl aliquots from each well were plated onto Tryptone Soya Agar and incubated at 37°C for 18 hours. Following incubation, the highest dilution not exhibiting bacterial growth was recorded as the MBC.

*Time-kill assay*

A method based on that described by May et al. [12] was employed. Overnight cultures of the test bacteria grown in Tryptone Soya Broth incubated at 37°C, were diluted to approximately $10^7$ cfu/ml. 200 µl of each bacterial suspension were added to 20 ml Diagnostic Sensitivity Test Broth (Oxoid) in a flask to give approximately $10^5$ cfu/ml. The broth cultures were incubated in a shaking water-bath at 150 rpm, for 90 minutes at 37°C to get the cells into the logarithmic growth phase. 100 µl aliquots were aseptically removed from each flask to determine the initial inocula. The aliquots were plated onto Tryptone Soya Agar plates, which were incubated at 37°C for 18 hours and the number of colonies per ml counted.

Oil B was mixed with Tween 80 to give final concentrations of 5% and 0.5% respectively. 1.1 ml of the Oil B-Tween 80 mixture was added to each flask. Control flasks had only Tween 80 added. The broth cultures were incubated in a shaking water-bath at 150 rpm at 37°C, and 100 µl aliquots were aseptically removed from each flask every 30 minutes for up to six hours and after 24 hours incubation. The aliquots were used to inoculate Tryptone Soya Agar plates, which were incubated at 37°C for 18 hours and the number of colonies per ml counted. The logarithm of the viable count (cfu/ml) of each bacterium for the oil was plotted against exposure time, to produce a kill-curve.

## Results

Disc diffusion tests were carried out to investigate the anti-staphylococcal activity of Oil B compared to thyme essential oil derived from a single linalool chemotype (Figure 1). Oil B showed greater inhibitory activity than the single-chemotype thyme oil ($p$ <0.05), with a mean zone of inhibition size of 34.8 mm (range 25.0-48.7 mm), whereas the single-chemotype oil (linalool cultivar) produced mean zone sizes of 20.7 mm (18.2-26.0 mm). No difference was noted between the size of the zones of inhibition of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* strains.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Each oil/ bacterium combination was tested in duplicate on two separate occasions, and the mean MIC/MBC value recorded. Oil B had a mean MIC/MBC of 0.3/0.6% (v/v), whereas the linalool chemotype oil had mean MIC/MBC of 0.4/0.8% (v/v). No differences were observed between the action of the different oils on the methicillin-sensitive or methicillin-resistant staphylococci.

Time-kill curves to assess the bactericidal activity of Oil B and the single chemotype thyme oil. The time taken to reduce bacterial numbers by the essential oils was investigated. A bactericidal effect is defined as a 3 $\log_{10}$ decrease in the number of cells per millilitre or a 99.9% kill over a specified time [12]. The logarithm of the viable count (recorded as cfu/ml) for each of the staphylococci examined was plotted against the exposure time to the essential oil. The growth controls not exposed to the essential oils showed a $10^2$-$10^3$ increase in bacterial numbers by six hours (data not shown). All the thyme oils caused a 2-3 $\log_{10}$ reduction in all the staphylococcal strains within two to three hours. Single chemotype thyme oil took two and half hours to reduce the numbers to zero (Figure 2), whereas Oil B caused the same reduction in numbers by two hours (Figure 3). In all cases, a 3 $\log_{10}$ decrease in bacterial numbers was observed, indicating a strong bactericidal effect (Figure 4).

## Discussion

There is a large body of anecdotal evidence regarding

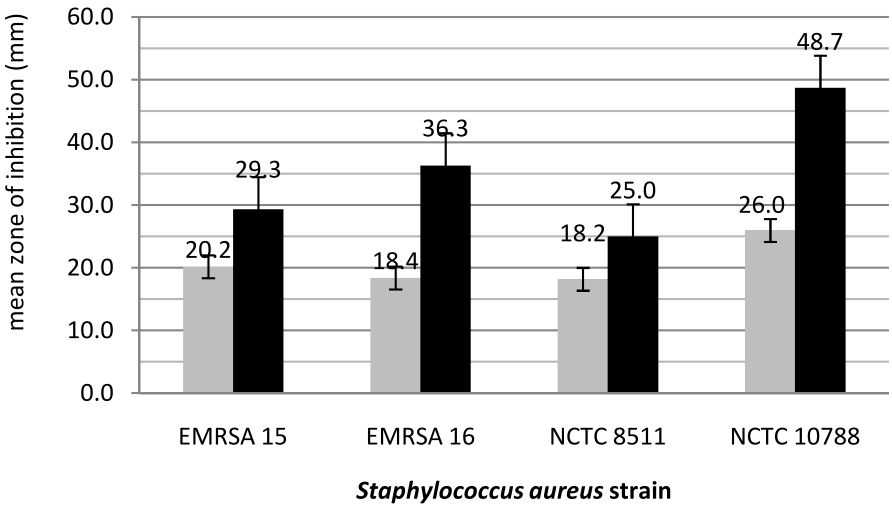


**Figure 1. Comparison of the anti-staphylococcal activities of a single chemotype thyme oil and Oil B by disc diffusion assay.**
(Key: grey fill = thyme oil; black fill = Oil B blend).

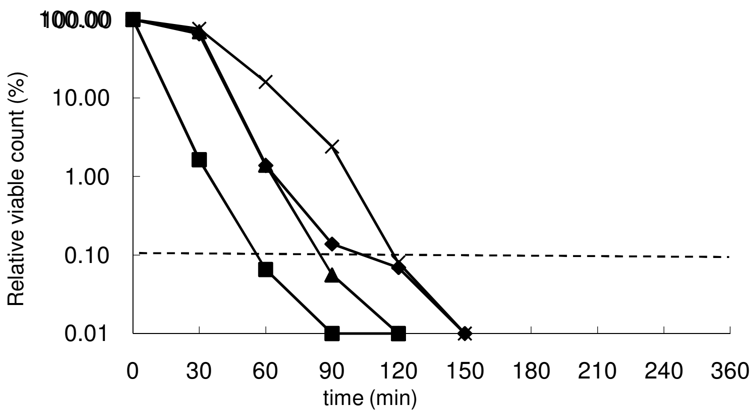


**Figure 2. Time-kill curves for staphylococci exposed to a single chemotype thyme oil.**
(Key: diamond = EMRSA 15; square = EMSA 16; triangle = NCTC 8511; cross = NCTC 10788).

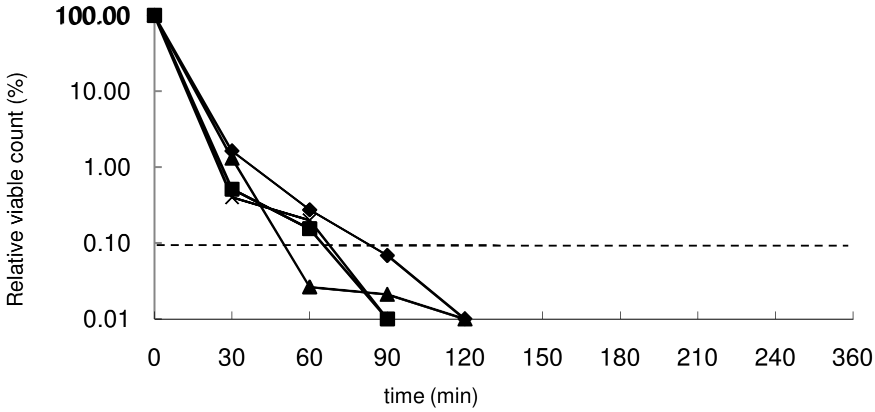


**Figure 3. Time-kill curves for staphylococci exposed to thyme oil blend Oil B.**
(Key: diamond = EMRSA 15; square = EMSA 16; triangle = NCTC 8511; cross = NCTC 10788).

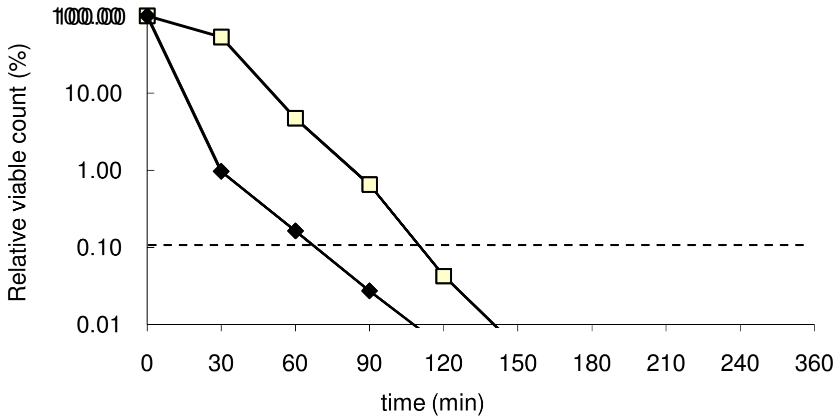


**Figure 4. Time-kill curves for all staphylococci exposed to thyme oil and Oil B blend.**
(Key: square = thyme oil; diamond = Oil B blend).

the use of plant essential oils in the treatment of certain medical conditions although there is little published, scientifically validated data. The search for novel antimicrobial compounds and the rise in interest in alternative and 'natural' therapies means that quantitative data on essential oils are needed. Thyme oil has a history of use in perfumes and as a preservative in the food industry but has rarely been used as an antimicrobial in a clinical setting. The antifungal and antibacterial activity exhibited by *Thymus* genus essential oils has been demonstrated by several researchers [5, 13-15], but the variability between species may result in differing degrees of in-vitro and in-vivo activities.

Synergistic or antagonistic effects between constituent compounds may affect the observed anti-staphylococcal activity of the oils. A synergistic action has been noted between thymol and carvacrol against some bacteria [16] and Oil B contains 31.1% (v/v) thymol and 1.1% (v/v) carvacrol. In an investigation of three different species of thyme (*Thymus vulgaris, Thymus zygis* and *Thymus hyemalis*), it was noted that most of the antimicrobial activity appeared to be associated with the thymol and carvacrol chemotypes [13]. However, *Thymus zygis* subsp. *gracilis* containing the alcohol linalool (82%) also had significant bactericidal and bacteriostatic effects. Pattnaik and colleagues [17] noted that linalool has a strong effect against a number of different bacteria and fungi, although they found that the presence of other compounds such as terpenic hydrocarbons ($\alpha$-terpinene, $\gamma$-terpinene, and limonene) and alcohols (terpinen-4-ol, $\alpha$-terpineol) increased the antimicrobial activity of the oil. The essential oil from *Thymus zygis* containing 39% linalool had no bacteriostatic or bactericidal activity against isolates of *Escherichia coli* unless thymol or carvacrol was also present [13].

In this study, Oil B exhibited substantial anti-staphylococcal activity in the disc diffusion assay, with a mean zone of inhibition size of 34.8mm, whereas the single-chemotype oil (linalool cultivar) produced a mean zone sizes of 20.7 mm. Other studies have produced zone sizes 25-45 mm for the thymol chemotypes of thyme [13]. The mean MIC values obtained for both the single-chemotype thyme oil and Oil B blend were comparable to values obtained by other workers using different strains of thyme oil and staphylococci. For example, an MIC of 0.25 (%v/v) against *S. aureus* NCTC 6591 was obtained using oil extracted from *T. vulgaris* [18]. Similar values were obtained in an analysis of Sardinian thyme oils from *T. capitus* and *T. herba-*

*barona* [5] and for Spanish thymes (*T. vulgaris*, *T. hyemalis* and *T. zygis*) against *S. aureus* CECT 239 [13]. No significant differences were observed between the action of the thyme oils on methicillin-sensitive and methicillin-resistant staphylococci. Measurements of the time of kill by the oils were made for each staphylococcal strain. The growth controls not exposed to the essential oils, showed at least a 2 log increase in bacterial numbers by six hours, whereas in all cases, a 3 $\log_{10}$ decrease in bacterial numbers was observed, indicating that the thyme oils had a significant bactericidal effect.

Oil B is a specially formulated blend of oils from *Thymus zygis*, with thymol and linalool as major components together with relatively high concentrations of $\alpha$-terpinene and terpinen-4-ol, compounds not usually found in thyme oil. Australian Standard tea tree oil contains a minimum of 30% terpinen-4-ol [19] and oils with increased concentrations of terpinen-4-ol are known to exhibit strong antimicrobial activity [20], possibly as a result of synergy with other components such as thymol and linalool.

Based on disc diffusion, MIC, MBC and time-kill studies, it may be concluded that Oil B has substantial inhibitory and bactericidal effects on the strains of staphylococci tested, and is more effective ($p < 0.05$) than a linalool chemotype thyme essential oil, against both methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. Additional investigation of its toxicity or irritant effects is also required if it is to be used as a topical medical agent. Hand disinfectants are an important means of reducing the spread of infectious microorganisms and the in vitro results from this study suggest that *Thymus zygis* Oil B blend might be a useful topical agent for reducing staphylococcal loads on skin and the decolonisation of nasal staphylococci.

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### Transparency declaration

Commissioned by Benchmark Oils Ltd. Crawley, West Sussex, UK.

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