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Antimicrobial activity of limonene enantiomers and 1,8-cineole alone and in combination

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ABSTRACT: Two common essential oil constituents, 1,8-cineole and limonene, were assessed for antimicrobial activity, using the minimum inhibitory concentration (MIC) microtitre plate method against eight organisms. The limonene enantiomers, i.e. (+), (–) and the racemate, singularly and in combination (1:1) with 1,8-cineole, were investigated to establish possible interactions. The MIC values were in the ranges 3–27 mg/ml for (+)-limonene; 2–27 mg/ml for (–)-limonene and 23 mg/ml for 1,8-cineole, depending on the pathogen studied. The combinations, when investigated in a 1:1 ratio, mostly indicated reduced activity. Using various ratios of limonene and 1,8-cineole, the specific interaction was further investigated against *Staphylococcus aureus* (Gram-positive), *Pseudomonas aeruginosa* (Gram-negative) and a yeast, *Cryptococcus neoformans*. A figurative representation of the results using isobologram construction indicated that, depending on the ratio and specific enantiomer, an additive, synergistic or antagonistic interaction may be observed. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: antimicrobial activity; limonene; 1,8-cineole; enantiomer

Introduction

Essential oils are complex mixtures with a number of constituents that may impact on the biological activity, either independently or in combination. The biological activities of several oil constituents have been studied.[1,2] In these studies, 1,8-cineole featured predominantly as a major or minor constituent occurring in a number of popular biologically active aromatic plants and spice oils, such as *Coriandrum sativum* (coriander), *Origanum vulgare* (oregano), *Rosmarinus officinalis* (rosemary), *Thymus vulgaris* (thyme) and *Zingiber officinale* (ginger).[3] Limonene is a constituent of popular spice oils, such as *Thymus vulgaris* (thyme) and *Citrus medica* (citron),[4] and studies have shown pathogen-selective antimicrobial activity for limonene.[5–7]

In addition to single-component studies, some research has been conducted on essential oil combinations[8,9] and a synergistic interaction has previously been demonstrated for camphor and 1,8-cineole.[10] Few studies, however, have considered the specific stereochemistry of optically active molecules such as limonene or linalool. It is well known for several pharmaceutically active compounds that stereochemistry is often related to the structure–activity relationships of a molecule. For example, levofloxacin is the optically active L-isomer of ofloxacin. Not only is levofloxacin twice as active as ofloxacin, but it also has a broader spectrum of antimicrobial activity.[11] Studies on the enantiomers of limonene, and later pinene, have shown that biological activity is influenced by the enantiomeric configuration.[6,12] Another study[13] demonstrated a few instances where essential oil constituents show synergistic and antagonistic interactions. Even though it is known that biological activity is influenced by different enantiomeric configurations[14] and is of concern in the pharmacodynamic differences of chiral drug development, no investigation could be found on how the biological activity may be affected when specific enantiomers are combined with other molecules. In this study, the interactions (antagonistic, synergistic or additive) between 1,8-cineole and limonene enantiomers were investigated. The combination of limonene and 1,8-cineole was selected on the basis of their abundance in nature and frequent co-occurrence.[15–19]

Experimental

Chemicals

1,8-Cineole at 98.0% purity (Lot 1054365) was obtained from Sigma-Aldrich. *S*(–)-limonene at 99.0% purity (Lot 054076) was obtained from Fluka and (*R*)-(+)-limonene at 97.0% purity (Lot 301Tl-101) was obtained from Sigma-Aldrich.

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MIC determination

Essential oil constituents were investigated for antimicrobial activity, using the MIC microtitre plate method.[20] All bacterial cultures were subcultured from stock agar plates and grown in Tryptone Soya broth for 18 h. Investigations were undertaken on three Gram-positive (*Staphylococcus aureus, Bacillus cereus* and *Enterococcus faecalis*) and four Gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Moraxella catarrhalis*) bacteria. The yeast (*Cryptococcus neoformans*) was incubated for a further 24 h. All cultures have standard reference numbers (Table 1), with the exception of the clinical *M. catarrhalis* strain, which was obtained from The National Health Laboratory Services, Johannesburg, South Africa.

Microtitre plates were aseptically prepared and 100 µl distilled, sterile water was added to each well. Limonene and 1,8-cineole, independently and in various combinations, were transferred into the first rows of a microtitre plate at starting stock concentrations of 128 mg/ml and serial dilutions were performed. Microbial cultures were diluted in fresh Tryptone Soya broth at a 1:100 ratio, yielding an approximate inoculum size of 1×10^6 colony forming units (CFU)/ml and 100 µl was added to all wells. Optimal incubation conditions (37 °C for 24 h for bacteria and 48 h for *C. neoformans*) followed. Positive bacterial controls (i.e. ciprofloxacin or amphotericin B) at starting stock concentrations of 0.01 mg/ml were included in each assay to confirm the antimicrobial susceptibility of the test organism. A 0.4 mg/ml *p*-iodonitrotetrazolium violet solution (INT) was prepared and 40 µl transferred to all inoculated wells. The microtitre plates were examined after 6 h to determine a colour change in relation to concentration of microbial growth. The yeast *C. neoformans* was examined after 24 h. Assays were undertaken in triplicate and the means documented in Table 1.

Synergy studies

Once the independent MIC was determined, the fractional inhibitory concentration (FIC) was calculated for the 1:1 combinations. The FIC is expressed as the interaction of two agents, where the concentration of each test agent in combination is expressed as a fraction of the concentration that would produce the same effect when used singularly.[21] The FIC index is determined as the correlation between the two combined test substances and may be classified as either synergistic (≤0.5), additive (>0.5–1), indifferent (>1–<4) or antagonistic (≥4).[22]

The optically active monoterpene limonene was used to determine whether microbes respond differently when exposed to the (+) or (–) forms and/or racemic mixture of limonene in combination with 1,8-cineole. The isobologram ratio method[21,23] was used and nine ratios (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80 and 10:90) of each enantiomeric form and the racemate were thoroughly mixed with 1,8-cineole.

The MIC values (mg/ml) were determined for each concentration independently and for the nine ratios. The MIC values of all relevant ratios (within the range 0–1.25) were plotted as points on an isobologram relative to the MIC of limonene and

Table 1. The mean MIC (mg/ml) for the major constituents, limonene and 1,8-cineole, independently and in combination with FIC (in parentheses), determined for 1:1 combinations

Constituent	*Staphylococcus aureus* ATCC 12600	*Bacillus cereus* ATCC 11778	*Enterococcus faecalis* ATCC 29212	*Escherichia coli* ATCC 11775	*Pseudomonas aeruginosa* ATCC 9027	*Klebsiella pneumoniae* ATCC 13883	*Moraxella catarrhalis* (clinical strain)	*Cryptococcus neoformans* ATCC 90112
(+)-Limonene	13	3	27	11	4	12	8	3
(–)-Limonene	4	3	27	8	4	6	4	2
1,8-Cineole	8	2	23	8	4	8	16	2
1:1 (+) and (–)-Limonene	8 (1.1)	4 (2.6)	8 (0.6)	8 (1.8)	4 (1.0)	8 (2.0)	1 (0.4)	4 (2.3)
1:1 (+)-Limonene and 1,8-cineole	16 (3.2)	8 (6.6)	16 (1.3)	32 (6.9)	8 (4.0)	16 (3.3)	16 (3.0)	3 (2.5)
1:1 (–)-Limonene and 1,8-cineole	8 (3.0)	4 (3.3)	8 (0.6)	27 (6.8)	8 (4.0)	16 (4.6)	16 (5.0)	2 (2.0)
1:1 (±)-Limonene and 1,8-cineole	8 (2.0)	4 (1.5)	8 (1.4)	16 (2.5)	8 (4.0)	16 (4.0)	16 (16)	2 (1.5)
Control*	0.30×10^{-3}	0.30×10^{-3}	1.25×10^{-3}	0.40×10^{-4}	0.30×10^{-3}	0.60×10^{-3}	0.30×10^{-3}	1.60×10^{-3}

* Antimicrobial controls are ciprofloxacin for bacteria and amphotericin B for the yeast *Cryptococcus neoformans*.

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1,8-cineole independently (shown as a straight line), allowing for a figurative representation of the interaction of the various combinations. The isobologram can be interpreted by examining the position of the ratio points and extrapolating synergy (below the line), antagonism (above the line) and additive in the vicinity closest to or on the line.[21] Investigations were undertaken in duplicate on the following reference test organisms: *S. aureus* ATCC 12600 (Gram-positive); *P. aeruginosa* ATCC 9027 (Gram-negative); and a yeast, *C. neoformans* ATCC 90112. Conventional antimicrobials, as used in the MIC assay (not shown on the isobologram) were included in all repetitions to ensure susceptibility of the test organism.

Results and Discussion

The results outlined in Table 1 show that in general (–)-limonene is the more active isomer, with higher sensitivities for five of the eight pathogens studied. A disc diffusion study[8] on the antimicrobial activity of different volatile oil components indicated moderate to no inhibition for (+)-limonene, depending on the pathogen studied, with the highest sensitivity noted for *E. coli* (11.2 mm diameter zone of inhibition). Although the disc diffusion results may not be comparable to the MIC data presented here, the general antimicrobial trend for (+)-limonene, indicating moderate activity, was also noted in this study.[24,25] The highest sensitivities for (+)-limonene were noted for *Bacillus cereus* and *C. neoformans*, both having MIC values of 3 mg/ml. In another study,[25] antimicrobial activity was also found to be greater for the (–)-enantiomer. Contrary to results reported from other studies[8,25] and those reported here, were studies which indicated higher antimicrobial activity for (+)-15 limonene when using the disc diffusion method.[6,26,27] A more recent study[26] indicated sensitivities only for (+)-limonene, with no values given for (–)-limonene. Furthermore, it has been shown that the influence of stereochemistry on antimicrobial activity is pathogen-specific.[28]

One common finding in all the above-mentioned studies is that antimicrobial activity is greatly influenced by stereochemistry. The greatest variation in this study was noted for *S. aureus*, where there was at least a three-fold difference between the enantiomeric forms of limonene (Table 1). The lowest antimicrobial activity for the racemate in comparison to the two isomeric forms was noted for *M. catarrhalis*, where the racemic form had an MIC value of 1 mg/ml, and the (+) and (–)-limonene had MIC values of 8 and 4 mg/ml, respectively. This pattern was also noted for *E. faecalis*, with MIC values for the racemate at 8 mg/ml and both the (+) and (–)-limonene having MIC values of 27 mg/ml.

1,8-Cineole, known for its biological activity,[29] demonstrated the highest activity (2 mg/ml) against *B. cereus* and *C. neoformans*. The 1:1 combinations of 1,8-cineole with (+), (–) and the racemate of limonene mostly showed an indifferent interaction. The 1:1 combinations of 1,8-cineole with (+), (–) and the racemate of limonene mostly showed an indifferent interaction, with FIC's of 1 to <4 for 13 samples.

Although the MIC data given in Table 1 present the antimicrobial activity of the enantiomers of limonene and 1,8-cineole independently and in a 1:1 combination with FIC values, it is important to consider the specific ratio of compounds when tested in combination, as plants seldom accumulate phytoconstituents in a 1:1 ratio. Using the isobologram method, limonene and 1,8-cineole combinations in various ratios were investigated against three pathogens (Figures 1–3).

For *S. aureus*, the various isomeric forms of limonene together with 1,8-cineole showed antagonism for each isomer (Figure 1). Where (+)-limonene and 1,8-cineole were combined, the antimicrobial activity was dependent on the concentration of the ratios assessed. Antagonistic profiles were observed for all combinations where (+)-limonene was at a higher ratio. Where 1,8-cineole and (+)-limonene were in a 1:1 combination, a synergistic

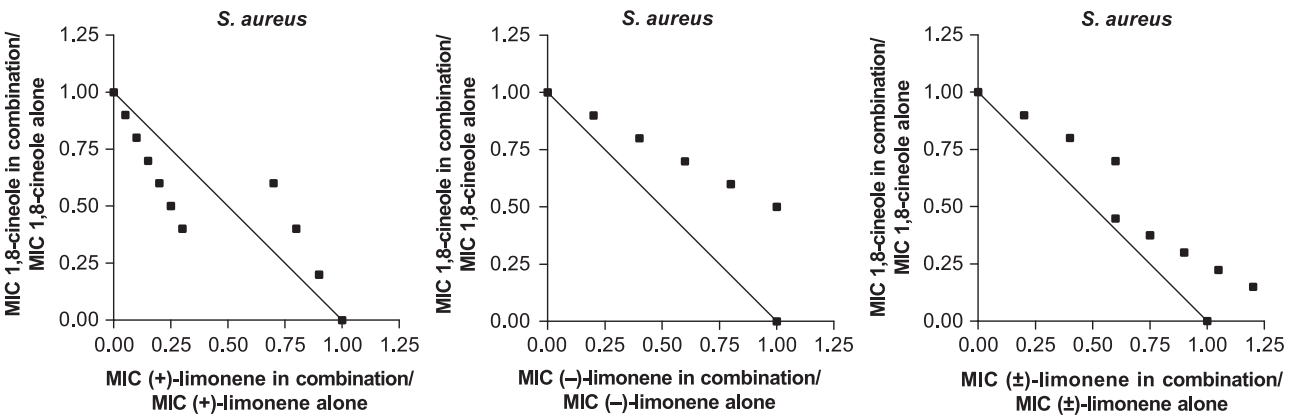

Figure 1. Isobologram plots for *S. aureus* (ATCC 12600) when exposed to the combinations of (+)-limonene with 1,8-cineole, (–)-limonene with 1,8-cineole and a racemic mixture of limonene with 1,8-cineole

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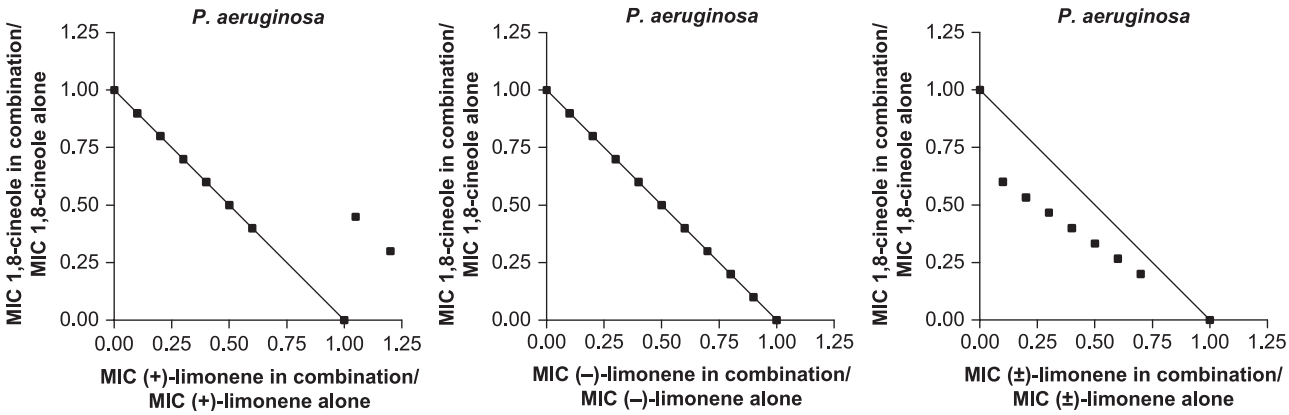

Figure 2. Isobologram plots for *P. aeruginosa* (ATCC 9027) when exposed to the combinations of (+)-limonene with 1,8-cineole, (–)-limonene with 1,8-cineole and a racemic mixture of limonene with 1,8-cineole

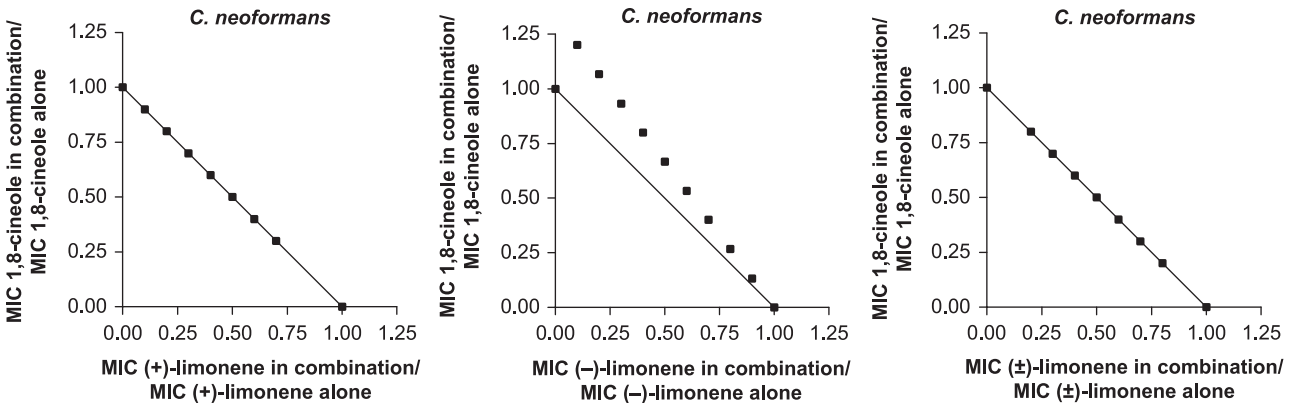

Figure 3. Isobologram plots for *C. neoformans* (ATCC 90112) when exposed to the combinations of (+)-limonene with 1,8-cineole, (–)-limonene with 1,8-cineole and a racemic mixture of limonene with 1,8-cineole

pattern was observed with less significant synergistic profiles for most of the ratios where 1,8-cineole is in a higher concentration than (+)-limonene. The (–)-limonene and 1,8-cineole combination showed antagonistic profiles for all ratios. Four antagonistic ratios were not plotted on the isobologram, as their values were greater than the 0–1.25 range. When a racemic mixture of limonene was combined with 1,8-cineole, ratios were predominantly antagonistic, with only one ratio (40 limonene:60 1,8-cineole) showing an additive or close to additive profile. The mixture of 1,8-cineole with (+)-limonene in a 1:1 combination showed the highest synergistic effect of all the combinations studied against *S. aureus*. Interestingly, with the single-compound study (Table 1), (–)-limonene showed the highest efficacy of the different enantiomers, yet it was when (+)-limonene was combined with 1,8-cineole that enhanced activity (synergy) became evident.

The isobologram for *P. aeruginosa* (Figure 2) mostly showed additive profiles when both (+) and (–) isomeric forms of limonene were combined with 1,8-cineole. Antagonistic activity was only noted for three ratios (90:10 ratio not noted on isobologram), where concentrations of (+)-limonene were much greater than those of 1,8-cineole. Synergy was observed for seven ratios where the racemate of limonene was combined with 1,8-cineole. For the 1:1 MIC study (Table 1), a reduced antimicrobial effect was noted for all combinations of limonene with 1,8-cineole.

In both isobolograms where (+)-limonene and the racemate were combined with 1,8-cineole and tested against *C. neoformans* (Figure 3), an additive profile was displayed for all ratios. For the (–)-limonene:1,8-cineole combination, an antagonistic profile was found for most ratios in which 1,8-cineole was in higher concentration. For the ratios in which (–)-limonene dominated, an additive or close to additive profile was noted. When observing the MIC data of compounds studied singularly against *C. neoformans*, (–)-limonene and 1,8-cineole indicated the highest activity (2 mg/ml) of all the pathogens studied; however, when investigated in various combinations, antagonism is observed for all ratios.

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Some discrepancies were displayed between the two methods (FIC and isobologram). Antimicrobial method variation has been noted many times in the literature, particularly when comparing the disc-diffusion and MIC methodologies. Variation may also be noted when looking at different methods to assess synergistic and antagonistic interactions. This has been highlighted previously,[30] where it was stated that reproducibility errors using MIC methodologies are common. There are a number of different methods that can be utilized to express synergy or antagonism. The FIC is a widely accepted means of measuring interaction and is used to interpret synergy from 1:1 MIC combination values (Table 1). The novel isobologram representation of synergy (Figures 1–3) has been poorly explored in spite of its wide acceptance in other interactive pharmacological studies, such as application in drug interactions, antiparasitic studies, antiviral combinations, toxicology interactions and numerous *in vivo* investigations. Researchers have been encouraged to seek more sophisticated approaches to the measurement of synergy, to overcome limitations imposed by the standard chequerboard methods.[30]

The antimicrobial efficacies of the various enantiomeric forms of limonene (Table 1) highlight the significance of stereochemistry in antimicrobial activity. The variation in results obtained for the (+), (−) and racemic forms of limonene when combined with 1,8-cineole, as well as their combination in different ratios, clearly impact on bioactivity. Variations depicting synergism, antagonism and an additive profile were noted for both *S. aureus* and *P. aeruginosa* when different enantiomers of limonene were combined with 1,8-cineole. These results suggest that the reductionist approach followed for the isolation of biologically active essential oil constituents is often short-sighted, as the activity may be due to interaction between the various components in a complex mixture.

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