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Synergistic properties of the terpenoids aromadendrene and 1,8-cineole from the essential oil of *Eucalyptus globulus* against antibiotic-susceptible and antibiotic-resistant pathogens

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ABSTRACT

The aim of the present study was to investigate the chemical composition of the essential oil of the fruits of *Eucalyptus globulus* and to examine the potential application of the fruit oil against multidrug-resistant bacteria. GLC/MS analysis in the fruit oil showed that aromadendrene was the main compound followed by 1,8-cineole and globulol. The three most abundant components of the fruit oil were also tested individually against microorganisms. In addition, the synergistic effects of combinations of the major constituents (aromadendrene and 1,8-cineole) of the fruit oil were also investigated. All Gram-positive bacteria were susceptible to the fruit oil with different degrees of susceptibility as determined by microdilution method. The oil exerted a marked inhibition against multidrug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) *Enterococcus faecalis*. The results indicated that aromadendrene might be responsible for the antimicrobial properties, whereas 1,8-cineole and globulol exhibited low activities. The checkerboard assay demonstrated that combinations of 1,8-cineole and aromadendrene reduce the MIC in most cases in an additive way, whereas the time-kill assay indicates a synergistic effect.

Introduction

Members of the genus *Eucalyptus* (Myrtaceae) originate from Australia but have been naturalized on most continents. *Eucalyptus* has been used in folk medicine throughout the world and the medicinal properties of these plants have been investigated. This family is an important source of essential oils with a wide range of biological activities such as antibacterial, antifungal, analgesic and anti-inflammatory properties (Ramezani et al. 2002; Sartorelli et al. 2007; Silva et al. 2003). Essential oils from *Eucalyptus* species are widely used in modern pharmaceutical, food and cosmetic industries (Lis-Balchin et al. 1998).

The essential oil of leaves of *Eucalyptus globulus* Labill. has been used all over the world as an antiseptic and for relieving symptoms of cough, cold, sore throat and other infections (Kumar et al. 2007; Van Wyk and Wink 2004). On the other hand, the essential oil of fruits of *E. globulus* has not been much explored yet. The chemical composition of the fruit oil has been studied (Cimanga et al. 2002; Ghalem and Mohamed 2008; Pereira et al. 2005), but antimicrobial properties have not been examined. Additionally, the contribution of the major components of the fruit oil to the antimicrobial activity has not been investigated. A previous study reported that the main compound of the fruit oil was 1,8-cineole (Basias and Saxena 1984), but also a different composition has been reported with aromadendrene as the main constituent (Pereira et al. 2005). In the present study we have reinvestigated the chemical composition of the fruit oil. Antibiotic-susceptible and antibiotic-resistant microorganisms were tested towards the fruit oil and the three major components (aromadendrene, 1,8-cineole, and globulol). Moreover, we have studied whether aromadendrene and 1,8-cineole produce additive or synergistic antibacterial effects, when applied in combination (as is the case of the oils).

Materials and methods

Plant material

The fruits of *E. globulus* were kindly provided by Prof. Thomas Efferth. The identity of the plant has been authenticated by Dr. Wahyono from Department of Pharmacognosy, Gadjah Mada University, Indonesia and the voucher specimen (P6868) was deposited at the Department of Biology, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany.

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Essential oils and monosubstances

The dried powder of fruits of *E. globulus* was subjected to hydrodistillation for 6 h using a Clevenger-type apparatus. After separation, the essential oil was kept in separate sealed vials at 4 °C for further analysis. (+)-Aromadendrene (≥97% purity) was purchased from Fluka, Switzerland, (−)-globulol (≥98.5% purity) and 1,8-cineole (99% purity) from Sigma–Aldrich, USA. The purity of the isolated compounds was confirmed by GLC/FID and GLC–MS.

GLC/FID

The quantitative analysis was carried out by high-resolution GLC using a Varian 3400 gas chromatograph equipped with flame ionization detector (FID) and OV-1 column (30 m × 0.25 mm × 0.25 μm) (Ohio Valley, OH, USA). The operating conditions were as follows: carrier gas was helium with a flow rate of 2 ml/min, split ratio 1:20. The oven temperature was programmed with an initial temperature of 40 °C, 2 min isothermal, 300 °C, 4 °C/min, then 10 min isothermal. Injector and detector temperatures were set at 250 and 300 °C, respectively. The PeakSimple® 2000 chromatography data system (SRI Instruments, California, USA) was used for recording and integrating the chromatograms.

GLC/MS analysis

GLC/MS was carried out on a Hewlett-Packard gas chromatograph (GC 5890 II) equipped with a DB-5 column. Samples (2 μl) were injected with a split mode (split ratio, 1:15) with the carrier gas helium at a flow rate of 2 ml/min. The capillary column was coupled to a quadrupole mass spectrometer (SSQ 7000, Thermo-Finnigan, Bremen, Germany). The injector temperature was 250 °C. All mass spectra were recorded in the following conditions: electron energy, 70 eV; ion source, 175 °C. The oil components were identified by their retention indices relative to C8–C28 *n*-alkanes, computer matching with the Wiley Registry of Mass Spectral Data, 8th edition, NIST Mass Spectral Library (December 2005) and by comparison of their mass spectra with data already available in the literature and in our own date base (Adam 2004; Ashour et al. 2009).

Microbial strains

Multidrug-resistant bacteria were clinical isolates from patients including nine methicillin-resistant *Staphylococcus aureus* (MRSA) strains, three vancomycin-resistant enterococci (VRE) *Enterococcus faecium* strains and one VRE *E. faecalis* strain. Also, the two strains MRSA NCTC 10442 and VRE *E. faecalis* ATCC 51299 were used for comparison.

The essential oils were also tested against 11 different bacteria and two yeast species. Gram-positive bacteria: *Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus saprophyticus* ATCC 15305, *Streptococcus pyogenes* ATCC 12344, *Streptococcus agalactiae* ATCC 27956, *Enterococcus faecalis* ATCC 29212. Gram-negative bacteria: *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Acinetobacter baumanii* ATCC BAA 747. Yeasts: *Candida albicans* ATCC 90028 and *Candida glabrata* ATCC MYA 2950. All microorganisms were obtained from the Department of Hygiene and Medical Microbiology, Institute of Hygiene, Heidelberg University, Germany. The strains were subcultured on appropriate agar plates 24 h prior to any antimicrobial test.

Culture media

Prior to testing, the bacteria were cultivated on Columbia Agar supplemented with 5% sheep blood (Becton Dickinson, Germany) and the yeasts were cultivated on CHROMagar Candida medium (Becton, Dickinson, Germany) at 36 °C. Mueller Hinton (Fluka, Switzerland) was used for antibacterial activity test, except for streptococci in Brain Heart Infusion (BHI) (Merck, Germany). Determination of antifungal activity was performed in Sabouraud Dextrose medium (Merck, Germany).

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

Broth microdilution assays were performed according to CLSI (Clinical and Laboratories Standards Institute 2006). Briefly, the samples were pipetted into 96-well microtiter plates in an appropriate medium followed by a two-fold serial dilution. Fifty microliters of microbial suspension was added to give a final concentration of 5×10^5 cfu/ml. After incubation at 36 °C for 24 h, MIC were determined as the lowest concentration at which no growth occurred (no color change in MTT assay). The MBC was determined by subculturing 3 μl from each well without apparent microbial growth on an appropriate medium and incubated at 36 °C for 24 h. The lowest concentration without apparent microbial growth was taken as the MBC. Vancomycin (Applichem, Germany) and nystatin (Cellpharm, Germany) were also included as positive control.

The checkerboard method

The checkerboard method was used to determine potential synergistic, additive or even antagonistic effects of combinations of individual compounds at different concentrations. In this method, aromadendrene and 1,8-cineole were added to a medium in 96-well microtiter plates to give two-fold dilutions in the vertical and horizontal direction, respectively as described elsewhere (Iten et al. 2009). Also, aromadendrene and 1,8-cineole as single compounds have also been tested on the same plate for comparison. The concentration of aromadendrene and 1,8-cineole were tested ranging from about 4 × MIC to 1/64 × MIC. Each well of the microtiter plate was started with inoculum at a concentration of 5×10^5 cfu/ml. Plates were incubated at 36 °C for 24 h. Fractional inhibitory concentration indexes (FICIs) were calculated as follows: FICI = (MIC of substance A in combination/MIC of substance A alone) + (MIC of substance B in combination/MIC of substance B alone). The combination of two compounds was considered to be synergistic when the FICI value was ≤0.5, additive when it was 0.5 to ≤1, indifferent when it was 1–4.0, and antagonistic when it was >4 (Matsumura et al. 1999; Pillai et al. 2005).

Isobologram

The isobologram illustrates the result of the checkerboard assay and the FICI values. The axis of the isobologram represents the dose of substance A and the ordinate represents the dose of B. The straight line connecting the intercept points represents zero interaction (Williamson 2001). Below this line we find the area of synergistic (FICI ≤ 0.5) and additive (0.5 < FICI < 1) interactions. Values above of the straight line represent antagonistic interactions (FICI > 4) (Iten et al. 2009).

Time-kill method

Time-kill experiments were performed with selected antibacterial combinations according to the results of the checkerboard assay. Aromadendrene and 1,8-cineole were tested alone and in

combination at sub-MIC level (below original MIC values). The mixtures were inoculated with an overnight culture of the test strain adjusted to give a final concentration of approximately 5×10^5 cfu/ml. After 0, 2, 4, 6, 8, and 24 h of incubation at 36 °C, aliquots were withdrawn and diluted with physiological saline solution. The dilutions were spread onto 5% Columbia blood agar and the colonies were counted after incubation at 36 °C for 24 h. The number of colonies were expressed as colony forming units per milliliter (cfu/ml) (Suschke et al. 2007). Reduction in viable cell count $\geq 2 \log_{10}$ after 24 h incubation in comparison with the cell count of the most active single substance was interpreted as synergy (Matsumura et al. 1999).

Data analysis

All experiments were performed in duplicate and repeated at least twice. The data were analyzed with Student's *t*-test or one-way ANOVA followed by Bonferroni test (GraphPad Prism 5.01; GraphPad Software, Inc., San Diego, USA). The criterion for statistical significance was taken as $P < 0.05$.

Results

Chemical composition of the essential oils

The essential oil of fruits from *E. globulus* (EGF) yielded 0.71% (w/w) with a pale yellow color and a pleasant odor. The chemical composition of EGF was determined by GLC/MS analysis (Table 1). The sesquiterpene aromadendrene (31.17%) was the most abundant component followed by 1,8-cineole (14.55%), globulol (10.69%), and ledene (7.13%).

Antimicrobial activity of the essential oil, aromadendrene, 1,8-cineole, and globulol

MICs of EGF and the main constituents (aromadendrene, 1,8-cineole, and globulol) tested against 13 microorganisms are shown in Table 2. The EGF exerted a good inhibitory activity against all Gram-positive bacteria with MIC values between 0.06 and 1 mg/ml. *Streptococcus pyogenes* was the most sensitive strain of bacteria to EGF (MIC 0.06 mg/ml) and to aromadendrene (MIC 0.12 mg/ml). Of the tested Gram-negative bacteria, EGF did not show a substantial inhibition against *P. aeruginosa*, *K. pneumonia*, and *E. coli*, except *A. baumanii* (MIC = 1 mg/ml). However, EGF exhibited a moderate activity against yeasts with MIC values of 1–4 mg/ml.

To study the potential application of the oils to treat of multidrug-resistant bacteria, the fruit oil was tested against MRSA and VRE (reference strains and clinical isolates). The results are given in Table 3. All antibiotic-resistant bacteria were susceptible to EGF with MIC values between 0.25 and 1 mg/ml. The antimicrobial activity of aromadendrene was slightly lower than EGF except against *S. saprophyticus*, *S. epidermidis*, VRE *E. faecalis*, and yeasts. All of the tested microorganisms were less affected by 1,8-cineole, except yeasts which were inhibited at 8 mg/ml. In our investigation, globulol inhibited only streptococci, *E. faecalis* and *A. baumanii* with MIC values ranging from 1 to 4 mg/ml.

Effect of combinations of aromadendrene and 1,8-cineole

Most antimicrobial activities of aromadendrene and 1,8-cineole as single entities were lower than those of EGF. In order to study whether aromadendrene and 1,8-cineole in combination produce a higher inhibition via an additive or synergistic interaction, checkerboard and time-kill experiments were performed.

Checkerboard assays and isobolograms of all four tested bacteria gave additive or synergistic profiles when aromadendrene was combined with 1,8-cineole at sub-inhibitory concentrations (Table 4 and Fig. 1). Synergistic effects were observed with at least one dose pair of combination against MRSA, *B. subtilis*, *S. aureus*, and *S. pyogenes*. Synergy was noted at 0.12 mg/ml aromadendrene plus 16 mg/ml 1,8-cineole for MRSA, 0.12 mg/ml aromadendrene plus 16 mg/ml 1,8-cineole for *S. aureus*, and 0.03 mg/ml aromadendrene plus 4 mg/ml 1,8-cineole for *S. pyogenes*. A predominant synergy was also observed against *B. subtilis* when 0.25 mg/ml aromadendrene was combined with either 4 or 2 mg/ml 1,8-cineole. All other dose pair combinations resulted in additive effects. Neither indifferent nor antagonistic effects were found in the combinations.

Time-kill experiments were performed with combinations of aromadendrene and 1,8-cineole against *S. pyogenes* and MRSA (Figs. 2 and 3), of which *S. pyogenes* exhibits the highest susceptibility. Fig. 2 illustrates the synergistic kinetics of combinations of aromadendrene and 1,8-cineole in *S. pyogenes* in the time-kill assay. Combinations of 0.03 mg/ml aromadendrene plus 4 mg/ml 1,8-cineole, and of 0.06 mg/ml aromadendrene plus 4 mg/ml 1,8-cineole revealed a $2 \log_{10}$ decrease of colony counts after 24 h

Table 1
Composition of the essential oil of the fruits of *Eucalyptus globulus* determined by GLC–MS.

Constituents	RI (DB5)	Relative abundance (%) EGF
α-Pinene	925	1.53
α-Phellandrene	1002	2.61
p-Cymene	1024	0.49
1,8-Cineole	1030	**14.55**
γ-Terpinene	1057	0.18
τ-Terpinene	1087	0.18
Isoterpinolene	1098	0.27
Linalool	1111	0.12
Carvenone	1164	0.07
Borneol	1166	0.41
Terpinen-4-ol	1176	1.87
α-Terpineol	1189	0.85
Sabinol	1199	1.14
p-Ment-1(7)-en-2-one	1231	0.62
Piperiton	1249	0.31
Geraniol	1252	Trace
Thymol	1302	Trace
Exo-2-hydroxycineole acetate	1338	0.14
α-Terpinyl acetate	1348	1.27
Geranyl acetate	1373	0.20
Isoledene	1378	0.81
3,3,7,11-Tetramethyl-tricyclo(6.3.0.0(2.4))undec-8-ene	1396	0.18
α-Gurjunene	1412	5.10
Aromadendrene	1446	**31.17**
allo-Aromadendrene	1466	3.68
γ-Gurjunen	1476	0.70
α-Selinene	1490	0.84
Longifolene	1493	1.75
Ledene	1504	7.13
γ-Cadinene	1518	0.24
Dehydroaromadendrene	1526	0.75
δ-Cadinene	1543	0.64
α-Calacorene	1555	0.16
Epiglobulol	1566	5.17
Palustrol	1581	0.22
Viridiflorol	1593	0.24
Globulol	**1595**	**10.69**
τ-Eudesmol	1600	1.24
Guaiol	1607	0.79
β-Eudesmol	1611	0.31
Cubenol	1616	0.11
Sesquiterpene alcohol	1627	0.55
τ-Cadinol	1631	0.17
α-Eudesmol	1657	0.18

Trace: trace amount, less than 0.05%.
EGF: *E. globulus* fruits.

Table 2
The MIC values of essential oil of *Eucalyptus globulus* fruits, aromadendrene, 1,8-cineole, and globulol determined with microdilution method.

Microorganism		*E. globulus* fruits*		Aromadendrene*		1,8-Cineole*		Globulol		Standard drug (μg/ml)	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
G+	*B. subtilis*	0.25	0.5	1	1	32	NT	>4	NT	0.2	0.8
G+	*S. saprophyticus*	1	4	1	8	>8	NT	>4	NT	1.6	3.1
G+	*S. aureus*	0.25	1	0.5	2	64	NT	>4	NT	0.4	0.8
G+	*S. epidermidis*	0.5	4	0.5	1	>8	NT	4	>4	0.8	1.6
G+	*S. agalactiae*	0.25	0.5	0.5	1	>8	NT	1	>4	0.4	0.4
G+	*S. pyogenes*	0.06	0.12	0.12	0.12	16	NT	2	>4	0.1	0.2
G+	*E. faecalis*	1	2	2	2	>8	NT	4	>4	1.6	3.1
G−	*E. coli*	8	NA	>8	NT	>8	NT	>4	NT	NI	NT
G−	*P. aeruginosa*	>8	NT	>8	NT	>8	NT	>4	NT	NI	NT
G−	*K. pneumoniae*	>8	NT	>8	NT	>8	NT	>4	NT	NI	NT
G−	*A. baumanii*	1	1	2–4	2	8	8	4	>4	NI	NT
Yeast	*C. albicans*	4	4	4	4	8	8	>4	NT	1.6	1.6
Yeast	*C. glabrata*	2	4	2	4	8	8	>4	NT	1.6	1.6

Concentrations are given in mg/ml.
NT: not tested; NA: not active at the tested concentration.
Vancomycin was used as standard drug for bacteria, whereas nystatin for yeasts.
* Significant difference between MIC of the essential oil and of 1,8-cineole; and between aromadendrene and 1,8-cineole ($P < 0.05$ in both cases).

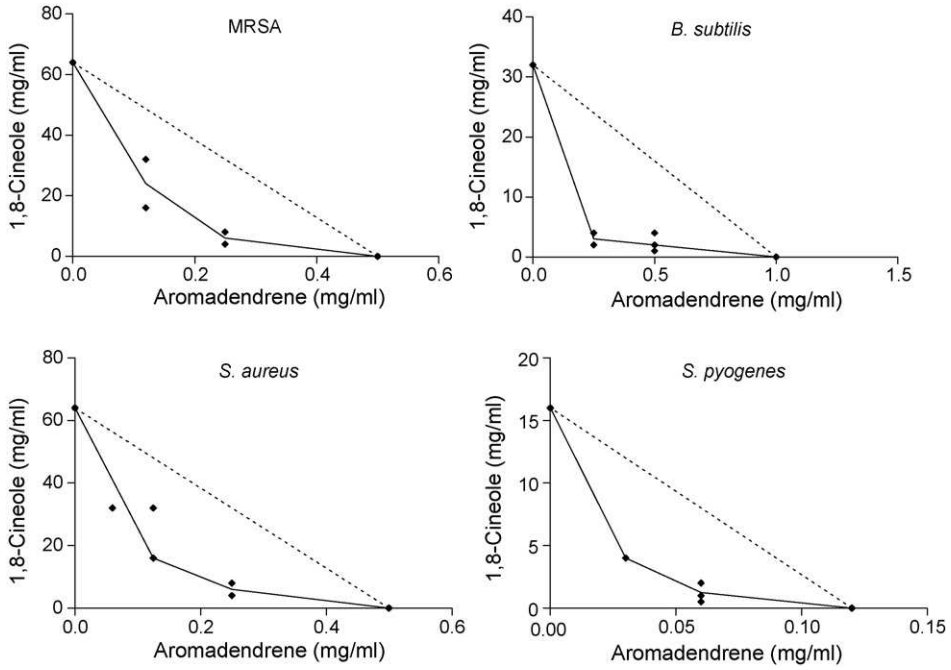

Fig. 1. Isobologram depicting the effect of aromadendrene and 1,8-cineole against MRSA, *S. aureus*, *B. subtilis*, and *S. pyogenes*.

compared to the single substance (aromadendrene). Particularly, the combination of 0.06 mg/ml aromadendrene and 8 mg/ml 1,8-cineole reduced the viable cell number of *S. pyogenes* approximately 5 $\log_{10}$ after 24 h.

Similarly, synergism was observed in MRSA when 0.25 mg/ml aromadendrene was combined with 32 mg/ml 1,8-cineole as presented in Fig. 3. The combinations significantly reduced the number of MRSA colonies (4 $\log_{10}$) compared to aromadendrene.

Table 3
Antibacterial activity of the essential oil of fruits of *Eucalyptus globulus*, aromadendrene, 1,8-cineole, and globulol against MRSA and VRE strains.

Microorganism	*E. globulus* fruit*		Aromadendrene*		1,8-Cineole*		Globulol		Standard drug (μg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MRSA NCTC 10442	0.25	0.25	0.5	1	64	>64	>4	NT	0.8	1.6
MRSA (clinical isolates)	0.12–1	0.25–2	0.25–1	0.5–4	NA	NT	4 to >4	NT	0.8–50	1.6 to >50
VRE *E. faecalis* ATCC 51299	1	2	1	2	NA	NT	4	NT	50	50
VRE *E. faecalis* (clinical isolates)	1	2	1	2	NA	NT	>4	NT	50 to >50	>50
VRE *E. faecium* (clinical isolates)	0.5–1	0.5–2	1	2–4	NA	NT	>4	NT	>50	>50

Concentrations are given in mg/ml.
NT: not tested, NA: not active at the tested concentration.
Vancomycin was used as standard drug.
* Significant difference between MIC of the essential oil and of 1,8-cineole; and between aromadendrene and 1,8-cineole ($P < 0.05$ in both cases).

Table 4
Result of the checkerboard assay: ratio of aromadendrene and 1,8-cineole (Aro/Cin), concentration of aromadendrene (Aro) and 1,8-cineole (Cin) in mg/ml, and FICI values. The interaction as reflected by FICI values is considered to be synergistic at ≤0.5, additive at >0.5–1, indifferent at >1–4.0, and antagonistic at >4.0.

Aro/Cin	(Aro)	(Cin)	FICI	Interpretation
MRSA				
0.5/0	0.5	0	–	–
1/16	0.25	4	0.6	Additive
1/8	0.25	2	0.56	Additive
1/4	0.25	1	0.52	Additive
1/266	0.12	32	0.75	Additive
1/133	0.12	16	0.49	Synergistic
0/32	0	64	–	–
B. subtilis				
1/0	1	0	–	–
1/4	0.5	2	0.56	Additive
1/2	0.5	1	0.53	Additive
1/1	0.5	0.5	0.52	Additive
1/0.5	0.5	0.25	0.51	Additive
1/16	0.25	4	0.38	Synergistic
1/8	0.25	2	0.31	Synergistic
0/32	0	32	–	–
S. aureus				
1/0	0.5	0	–	–
1/8	0.25	16	0.75	Additive
1/4	0.25	8	0.63	Additive
1/2	0.12	32	0.75	Additive
1/1	0.12	16	0.49	Synergistic
1/533	0.06	32	0.56	Additive
0/64	0	64	–	–
S. pyogenes				
0.12/0	0.12	0	–	–
1/32	0.06	2	0.63	Additive
1/16	0.06	1	0.56	Additive
1/8	0.06	0.5	0.53	Additive
1/133	0.03	4	0.50	Synergistic
0/16	0	16	–	–

Discussion

Resistance toward antibiotics is increasingly observed in some pathogenic microorganisms including Gram-positive (MRSA and VRE) and Gram-negative bacteria like *P. aeruginosa* and have become a tremendous problem on a global scale. These bacteria are the major causes of nosocomial infections (Coates et al. 2002; Taubes 2008). To overcome resistance, many antimicrobial agents have been investigated and essential oils were also included as alternative agents.

Eucalyptus oil has been integrated in medical systems all around the world since they possess potent antibacterial properties. Eucalyptus oils commonly found in health care systems are derived from leaves. To our knowledge, this investigation for the first time reports the antimicrobial effect of the fruit oil of *E. globulus*, particularly against multidrug-resistant bacteria. The results revealed that the essential oil of EGF (MIC values of 0.25–1 mg/ml) may serve as a potential drug in this context (Aqil et al. 2006). Furthermore, low MIC against MRSA and VRE might be advantageous in certain therapeutic applications such as inhalations or topical applications with regard to toxicity and stability of formulations (Guba 2001).

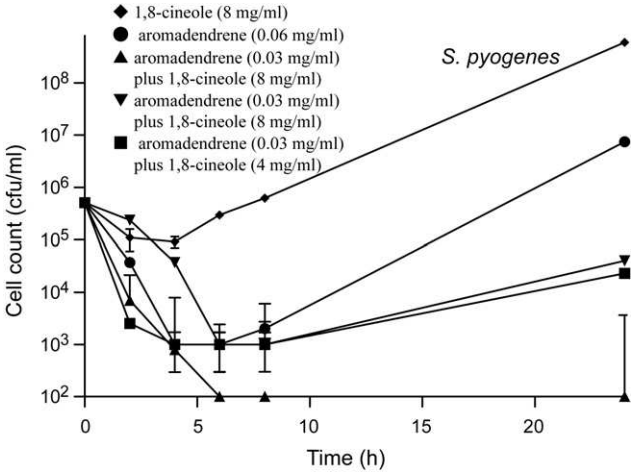

Fig. 2. Time-kill curve of aromadendrene and 1,8-cineole alone and in combination against *Streptococcus pyogenes*.

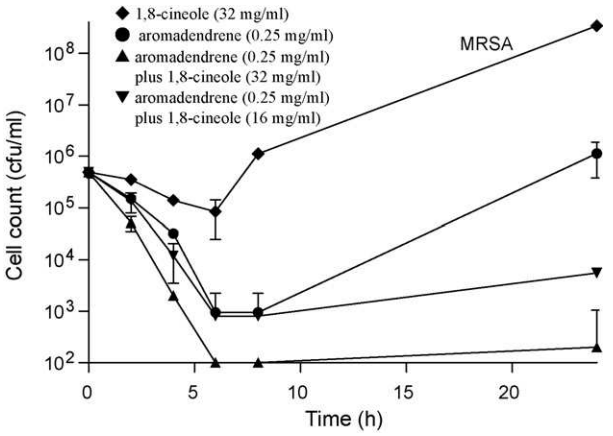

Fig. 3. Time-kill curve of aromadendrene and 1,8-cineole alone and in combination against MRSA.

Our GLC–MS study revealed aromadendrene to be the most abundant compound of EGF. A similar composition has been also reported from plants grown in Portugal (Pereira et al. 2005). It is most likely that aromadendrene was the main active principle of the EGF because aromadendrene showed higher antimicrobial properties than 1,8-cineole and globulol. The sesquiterpene aromadendrene bears a reactive exocyclic methylene group and a cyclopropane ring which can alkylate proteins and thereby disturb the conformation of protein. Additionally, since the compound is highly lipophilic, it causes disruption of cellular biomembranes (Sikkema et al. 1994; Wink 2007, 2008). It was surprising that globulol which had been reported as the active principle of the ethanol extract of EGF (Tan et al. 2008) showed low antimicrobial activities. On the other hand, 1,8-cineole (the second major constituent of EGF) exhibited low antimicrobial activities, in agreement with previous reports (Raman et al. 1995; Tzakou et al. 2001).

A previous study corroborated that minor components play a role in antibacterial activity, possibly by producing synergistic effects with other components (Burt 2004). Some studies had also shown synergistic effects of combinations such as limonene/1,8-cineole (van Vuuren and Viljoen 2007), cinnamaldehyde/eugenol, thymol/eugenol, carvacrol/eugenol, and thymol/carvacrol (Iten et al. 2009; Pei et al. 2009). In the present study, we could demonstrate that combinations of aromadendrene and 1,8-cineole apparently exhibited synergistic and additive antimicrobial properties. A clear synergistic effect of the aromadendrene and 1,8-cineole pair has been demonstrated in time-kill experiments. Synergistic effects of the combinations appeared only if 1,8-cineole (the weaker substance) was present in high doses. Most dose pair combinations produced additive effects in the checkerboard method. The differences occurred because the time-kill assay records a bactericidal effect, while the checkerboard method reveals inhibition of bacterial growth (Matsumura et al. 1999).

In conclusion, we have identified that aromadendrene apparently contributes significantly to the antimicrobial activity of EGF. Combinations of aromadendrene and 1,8-cineole showed additive effects in most cases, but also synergistic behavior in the time-kill assay. However, the possibility remains that other components of EGF contribute to the observed antimicrobial activity. Importantly, EGF exhibited a pronounced antimicrobial effect towards multidrug-resistant bacteria. Thus, EGF and its components, alone or in combination with other antibacterial agents, may provide a promising new scheme in phytotherapy.

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