Food Chemistry 119 (2010) 731–737

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Chemical composition and antimicrobial properties of essential oils of three Australian *Eucalyptus* species

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ARTICLE INFO

Article history:
Received 5 February 2009
Received in revised form 12 June 2009
Accepted 13 July 2009

Keywords:
Eucalyptus
Essential oil
Chemical composition
Antimicrobial properties

ABSTRACT

The chemical composition and antimicrobial properties of the essential oils of three common Australian *Eucalyptus* species, namely *E. olida*, *E. staigeriana* and *E. dives* were determined by gas chromatography/mass spectrometry and the agar disc diffusion method, respectively. A total of 24 compounds were identified from the essential oil of *E. dives*, with the dominant compounds being piperitone (40.5%), α-phellandrene (17.4%), *p*-cymene (8.5%) and terpin-4-ol (4.7%). For *E. staigeriana*, 29 compounds were identified with 1,8-cineole (34.8%), neral (10.8%), geranial (10.8%), α-phellandrene (8.8%) and methyl geranate (5.2%) being the dominant ones. In contrast, a single compound, (*E*)-methyl cinnamate, accounted for 99.4% of the essential oils of *E. oilda*, although 20 compounds were identified. The essential oils displayed a variable degree of antimicrobial activity with *E. staigeriana* oil showing the highest activity. In general, Gram-positive bacteria were found to be more sensitive to the essential oils than Gram-negative bacteria. *Staphylococcus aureus* was the most sensitive strain while *Pseudomonas aeruginosa* was the most resistant.

1. Introduction

Eucalyptus is a large genus of the Myrtaceae family that includes some 900 species and subspecies (Brooker & Kleinig, 2004). Although *Eucalyptus* is widely grown in many countries all over the world, Australia is probably the only one where such a single group of plants dominate most of the landscape. The Aborigines, believed to have settled in Australia over 60,000 years ago, developed a sophisticated empirical understanding of indigenous plants such as *Eucalyptus*. They traditionally used its leaves to heal wounds and fungal infections. While much of this knowledge has vanished with its keepers, there is currently a renewed interest in native herbal traditions (Chevallier, 2001).

Leaf extracts of *Eucalyptus* have been approved as food additives, and the extracts are also currently used in cosmetic formulations. Recently, attention has been focused on the functional properties of these extracts. Research has shown that the extracts exhibit various biological effects, such as antibacterial, antihyperglycemic and antioxidant activities (Takahashi, Kokubo, & Sakaino, 2004), with essential oils playing a central role in these biological functions. Essential oils are the odorous, volatile products of the secondary metabolism of an aromatic plant, which are often concentrated in a particular organ of the plant such as leaves, stems, bark or fruit (Conner, 1993). An estimated 3000 essential oils are known, of which about 300 are commercially important, destined chiefly for the flavours and fragrances market (Burt, 2004).

Most essential oils have some degree of antimicrobial activity attributable to the presence of a number of terpenoid and phenolic compounds, which, in pure form, have been shown to exhibit antimicrobial activity. These properties are partly associated with their lipophilic character, leading to accumulation in membranes and to subsequent membrane-associated events such as energy depletion (Conner, 1993). Phenolic components of essential oils sensitise the phospholipids bi-layer of the cell membrane, causing an increase of permeability and leakage of vital intracellular constituents or impairment of microbial enzyme systems (Moreira, Ponce, del Valle, & Roura, 2005). The chemical composition and biological effects of essential oils, including antimicrobial properties and potential applications in food products, have been reviewed (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Burt, 2004).

A number of studies have demonstrated the antimicrobial properties of *Eucalyptus* essential oils against a wide range of microorganisms. These studies, however, are focused on a few *Eucalyptus* species, especially *E. citriodora* (lemon-scented *Eucalyptus*) oil, which has been shown to have a wide spectrum of antifungal activity. Other studies have also mostly focused on the antifungal properties of *Eucalyptus* essential oils (Dhaliwal, Thind, & Chander,

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doi:10.1016/j.foodchem.2009.07.021

2004; Fiori et al., 2000; Lee et al., 2007; Ramezani, Singh, Batish, & Kohli, 2002; Somda, Leth, & Sereme, 2007; Tripathi, Dubey, & Shukla, 2008), while only a few studies investigated their activity against pathogenic and food spoilage bacteria and yeasts (Chaibi, Ababouch, Belasri, Boucetta, & Busta, 1997; Delaquis, Stanich, Girard, & Mazza, 2002; Moreira et al., 2005; Sartorelli, Marquioreto, Amaral-Baroli, Lima, & Moreno, 2007).

The objectives of the present study are to determine the chemical composition of the essential oils of three common Australian *Eucalyptus* species, namely *E. olida*, *E. staigeriana* and *E. dives* and to investigate their antimicrobial properties against some of the common pathogens and food spoilage bacteria and yeasts. We further examined two samples of *E. olida* grown in different geographical regions to determine the influence of growth conditions on the chemical composition and antimicrobial properties of the essential oils.

2. Materials and methods

2.1. Plant material and chemicals

Fresh leaves of *E. dives* and a specimen of *E. olida* (designated as VIC) were provided by Tarnuk Bush Food and Flower, Korumburra, Victoria, Australia. These specimens were grown in the Southern Victorian region and were hand harvested in October 2006. *E. staigeriana* and another specimen of *E. olida* (designated as NSW) were provided by Australian Rainforest Products Pty Ltd., Blue Knob, New South Wales (NSW), Australia. These specimens were grown in the Northern River district of NSW and were hand harvested at approximately the same time (October 2006) as the VIC specimen. Immediately after harvest, all the specimens were packed with dry ice and shipped to our laboratory by air. All chemicals used were of analytical grade and purchased from Sigma–Aldrich (Castle Hill, NSW, Australia), unless otherwise stated.

2.2. Extraction of essential oils

Samples (100 g each) of *E. dives* and *E. staigeriana* frozen leaves were cut into 5 mm strips and subjected to steam distillation for 3 h using an indirect steam distillation apparatus with 600 ml of de-ionised water for each sample. The essential oil floating on the top of the condensed water was collected into a separation funnel and then into a scintillation vial before being dried overnight in a desiccator and stored at −18 °C.

Because the essential oil from *E. olida* was found to be heavier than water in preliminary experiments, a Clevenger-type apparatus was used for its extraction. Samples (100 g) of frozen leaves were cut into 5 mm strips and steam-distilled for 3 h with 600 ml of de-ionised water. The essential oil appeared as a white opaque suspension at the bottom of the condensation and was collected into a scintillation vial and freeze dried (Christ Alpha 2-4 LDplus, Martin Christ, Osterode am Harz, Germany) under 0.011 mbar at −85 °C for 15 h before being stored at −18 °C.

2.3. Gas chromatography/mass spectrograph (GC/MS) analysis of essential oils

The essential oils, extracted as described above, were analysed by GC/MS to identify their components. GC/MS was performed using a Varian 3800 gas chromatograph directly coupled to a Varian Saturn 2000 Ion Trap (ITD) mass spectrometer with the whole system controlled by the Saturn GC/MS workstation (v5.2). The column used was a J and W DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm). GC/MS operating conditions followed those described by Adams (1995): injector temperature, 220 °C; transfer line, 240 °C; oven temperature, from 60 to 240 °C at 3 °C/min; carrier gas, He at 0.8 ml/min at 220 °C; sample injection volume, 0.2 μl; split ratio, 1:20. MS acquisition parameters were as follows: full scan with scan range 41–300 amu; scan time, 1.0 s; threshold, 1 count; AGC mode: on; microscans: 5; filament delay: 120 s; manifold – 60 °C. Column head pressure was adjusted to 9.4 psi with *Lavandula angustifolia* essential oil, which was used as an internal standard to set the flow rate at 0.8 ml/min, at which the retention times matched those reported by Adams (1995).

A 10% (v/v) hexane solution as well as a 1% (v/v) hexane solution of each essential oil was injected to allow a better identification of compounds. This was necessary to increase the sensitivity for minor compounds, especially for the essential oils of the two *E. olida* specimens which were similar in their major compounds. The chromatograms obtained with the 10% (v/v) solution allowed the identification of some minor compounds while those obtained with the 1% (v/v) solution allowed a more accurate quantification of the major compounds, which were saturated at 10%. The analysis was repeated three times for each sample.

Compounds were identified by comparing their GC retention times and mass spectra with Adams (1995, 2001), aided with authentic compounds and NIST mass spectra library. Camphor, borneol and terpin-4-ol were used as reference compounds. Quantification of essential oil components (expressed as percentage of total peak area of chromatogram) was carried out by peak area normalisation measurements.

2.4. Microbial strains

Essential oils of each *Eucalyptus* species were tested against a panel of food-related microorganisms, including the Gram-positive *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 19433, Gram-negative *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and the yeast *Candida albicans* ATCC 10231. The cultures were purchased from the Australian Cultural Collection at the School of Microbiology, University of Queensland, Queensland, Australia and were maintained on slants of Nutrient Agar (Oxoid, CM0003) for bacteria and Sabouraud Dextrose Agar (SDA, Oxoid, CM0041) for the yeast at 4 °C.

2.5. Preparation of microbial cultures for antimicrobial tests

Cultures for antimicrobial activity tests were prepared by transferring a loop of cells from the agar slant to a test tube containing 10 ml of Mueller Hinton Broth (MHB, Oxoid, CM0405) for bacteria and Sabouraud Liquid Medium (SLM, Oxoid, CM0147) for the yeast, respectively. They were then incubated overnight (16 h) at 37 °C for bacteria and 30 °C for the yeast. Cultural purity was checked by streaking each culture on plates of Mueller Hinton Agar (MHA, Oxoid, CM0337) for bacteria and SDA for the yeast.

In order to use an appropriate inoculum concentration in the agar disc diffusion tests, a standard growth curve for each microorganism was prepared. Briefly, the overnight culture of each microorganism, obtained as described above, was diluted 100 times in 10 ml MHB for bacteria and SLM for the yeast. The test tubes containing the diluted cell suspension were incubated at 37 °C for bacteria and 30 °C for the yeast. Optical density was measured at 420 nm every hour for *E. coli*, *S. aureus* and *P. aeruginosa* and every 2 h for *C. albicans*. The corresponding microbial growth (CFU/ml) was determined by plating 100 μl of a serial dilution of each suspension on Plate Count Agar (Oxoid, CM0003) for bacteria and on SDA for the yeast. Each dilution was plated in duplicate and the plates were incubated for 24 h at 37 °C for bacteria and 48 h at 30 °C for the yeast.

2.6. Assay of antimicrobial activity

Antimicrobial activity of the essential oils was tested using the agar disc diffusion method (The Oxoid Manual, 1995; National Committee for Clinical Laboratory Standard, 2000, 2002). MHA and SDA were sterilised in an autoclave and cooled to 45–50 °C before being poured into 90 mm Petri dishes. To help in differentiating bacterial colonies and clarifying the zone of inhibition, 0.01% (w/w) of 2,3,5-triphenly tetrazolium chloride (TTC) was added to MHA after sterilisation (Elgayyar, Draughon, Golden, & Mount, 2001; Mourrey & Canillac, 2002). The agar plates were stored at 4 °C before being used.

Fresh bacterial and yeast inoculums were prepared from overnight cultures to obtain microbial inoculates in the logarithmic growth phase. The overnight culture of each microorganism was diluted to 1/100 in 10 ml MHB for bacteria or 10 ml SLM for the yeast, and incubated for 4 to 6 h at 37 °C for bacteria and 30 °C for the yeast. Optical density was measured at 420 nm and appropriate dilutions were carried out following the standard growth curve to achieve a cell concentration of 10^5–10^6 CFU/ml as suggested by the Oxoid Manual (1995). Aliquots (100 μl) of the fresh inoculums were spread over the surface of MHA for bacteria and SDA for the yeast. Agar plates were incubated at 30 °C for approximately 15 min until the microbial overlay had dried. Six susceptibility test discs (Oxoid, CT0998B, Ø = 6 mm) were placed onto the agar plates using a six cartridges disc dispenser (Oxoid, ST6090). Only one or two discs were used when essential oils showed great inhibition zones. Discs were then individually impregnated with 10 μl of essential oils or standard antibiotics (positive controls). Because of its high content of methyl cinnamate, *E. olida* essential oils needed to be re-solubilised by heating before application. Three standard antibiotics were used as positive controls: chloramphenicol (30 μg/disc, Oxoid CT0013B) for *E. coli*, *E. faecalis* and *S. aureus*; gentamicin (30 μg/disc, Oxoid CT0072B) for *P. aeruginos*; and nystatin (3 mg/ml) for the yeast, while Milli-Q water (Millipore) was used as a negative control. The agar plates were sealed with Parafilm (PM-996, Pechiney Plastic Packaging, Sydney, Australia) and kept at 4 °C for 2 h in order to lower the detection limit (Rios, Recio, & Villar, 1988). They were then incubated for 24 h at 37 °C for *E. coli*, *P. aeruginosa* and *S. aureus* and 48 h at 30 °C for *C. albicans*. The diameters of the inhibition zones were measured with a digital calliper (Absolute Digimatic, Melboune, Australia). Three agar disc diffusion tests were carried out on each microorganism. Each test was performed in duplicate.

2.7. Statistical analysis

Value dispersion was analysed by relative standard deviation (standard deviation divided by the mean value) for the quantification of the compounds indentified by GC/MS, and by standard deviation for the measurements of inhibition zones in the antimicrobial activity tests. Student's two-tailed *t*-test was used to compare means of two populations, which were deemed to be significantly different when the calculated *p*-value was smaller than the 5% significance level.

3. Results and discussion

3.1. Extraction and yield of essential oils

Indirect steam distillation worked well for *E. dives* and *E. staigeriana*, but very poorly for *E. olida*. With this method, essential oils extracted from *E. olida* were found to form crystals within the condenser, which sank to the bottom of the separation funnel. It was thus concluded that the essential oils of *E. olida* were heavier than water and crystallised in the presence of a small amount of water, probably due to the high concentration of the amphipathic methyl cinnamate (mp: 34–37 °C) present. Curtis et al. (in Smale, Nelson, Porter, & Hay, 2000) reported that essential oils from fresh leaves of *E. olida* contained 94–99% (*E*)-methyl cinnamate and our results (reported in following section) confirmed this. Therefore, in later experiments, *E. olida* essential oils were extracted with a Clevenger-type apparatus, which is adapted for collecting essential oils heavier than water.

The yield of essential oils ranged from 2.13% to 3.12% (w/w), on a fresh weight basis, for the different *Eucalyptus* species. The highest yield was obtained from *E. olida*^{VIC} (3.12%), followed by *E. dives* (2.97%), while *E. staigerina* gave the lowest yield at 2.13%. Curtis et al. (in Smale et al., 2000) reported that fresh leaves of *E. olida* gave 2–6% yield in essential oils. The extraction yield of 3.12% obtained for *E. olida*^{VIC} fit into this range while the yield of 1.12% obtained for *E. olida*^{NSW} was lower than expected. As reported by McGimpsey and Douglas (1994) and Salgueiro et al. (1997), climate, genotype, growth location, rainfall and harvesting regime all can affect the total essential oil content of plants.

3.2. Composition of the essential oils

Tables 1–4 show the chemical composition of the essential oils extracted from *E. dives*, *E. staigeriana* and *E. olida* (VIC and NSW), respectively, together with the retention times of the compounds. A total of 24 compounds were identified from the essential oils of *E. dives*, which represented 95.9% of the oils extracted. The dominant compounds were piperitone at 40.5%, α-phellandrene (17.4%), *para*-cymene (8.5%) and terpin-4-ol (4.7%) (Table 1). For *E. staigeriana*, 29 compounds, representing 97.7% of the essential oils, were identified, with 1,8-cineole (34.8%), neral (10.8%), geranial (10.8%), α-phellandrene (8.8%) and methyl geranate (5.2%) being the dominant ones (Table 2). In contrast, a single compound,

Table 1
Chemical composition of *E. dives* essential oil.

No.	Retention time (min)	Compound	Content average (%[a])	RSD[b] (%)
1	4.76	Tricyclene	0.9	22.3
2	4.88	α-Thujene	3.1	24.9
3	5.06	α-Pinene	0.4	32.2
4	6.04	Sabinene	0.1	31.5
5	6.53	Myrcene	1.1	44.0
6	6.88	*para*-Mentha-1(7),8-diene	3.3	64.7
7	**6.98**	**α-Phellandrene**	**17.4**	**4.5**
8	7.32	α-Terpinene	1.2	2.6
9	**7.56**	***para*-cymene**	**8.5**	**6.9**
10	7.72	β-Phellandrene	2.8	15.5
11	7.79	1,8-Cineole (eucalyptol)	0.7	81.5
12	8.35	(*E*)-β-Ocimene	0.3	8.6
13	8.76	γ-Terpinene	0.8	8.9
14	9.84	Terpinolene	2.4	10.0
15	10.21	Linalool	0.9	2.4
16	11.06	*cis*-*para*-Menth-2-en-1-ol	1.3	2.9
17	11.79	*trans*-*para*-Menth-2-en-1-ol	0.9	4.3
18	**13.41**	**Terpin-4-ol**	**4.7**	**3.5**
19	13.94	α-Terpineol	1.0	6.9
20	14.67	*trans*-Piperitol	0.5	21.5
21	**16.90**	**Piperitone**	**40.5**	**7.3**
22	20.87	α-Terpinyl acetate	0.3	11.6
23	22.22	(*E*)-Methyl cinnamate	2.0	12.5
24	27.18	Bicyclogermacrene	0.8	21.4

The dominant compounds are indicated in bold.
[a] Relative proportions as percent of the total peak area.
[b] Relative standard deviation (standard deviation divided by the mean).

Table 2
Chemical composition of *E. staigeriana* essential oil.

No.	Retention time (min)	Compound	Content average (%)[a]	RSD[b] (%)
1	4.88	α-Thujene	1.8	11.2
2	5.06	α-Pinene	0.5	9.4
3	6.06	Sabinene	0.7	6.3
4	6.52	Myrcene	1.5	4.9
5	**6.97**	**α-Phellandrene**	**8.8**	**2.4**
6	7.34	α-Terpinene	0.9	1.5
7	7.57	*para*-Cymene	1.1	15.8
8	**7.83**	**1,8-Cineole (eucalyptol)**	**34.8**	**3.0**
9	8.38	(*E*)-β-Ocimene	0.5	3.4
10	8.77	γ-Terpinene	1.3	2.6
11	9.85	Terpinolene	1.5	2.8
12	10.25	Linalool	0.9	0.8
13	11.10	*cis-para*-Menth-2-en-1-ol	0.5	0.8
14	11.81	*trans-para*-Menth-2-en-1-ol	0.3	2.1
15	12.05	(*E*)-Tagetone	0.2	2.9
16	12.86	lavandulol	0.6	7.6
17	12.92	*para*-Mentha-1,5-dien-8-ol	0.2	4.3
18	13.41	Terpin-4-ol	2.3	1.6
19	13.63	Thuj-3-en-10-al	1.0	3.2
20	13.95	α-Terpineol	0.7	1.6
21	14.69	*trans*-Piperitol	0.3	1.0
22	15.61	Nerol	1.4	2.3
23	**16.20**	**Neral**	**10.8**	**3.4**
24	16.80	Geraniol	3.2	3.0
25	**17.54**	**Geranial**	**10.8**	**4.0**
26	19.84	Methyl geranate	5.2	3.8
27	21.61	Neryl acetate	2.1	4.4
28	22.24	(*E*)-Methyl cinnamate	0.6	2.7
29	22.46	Geranyl acetate	3.1	5.7

The dominant compounds are indicated in bold.
[a] Relative proportions as percent of the total peak area.
[b] Relative standard deviation (standard deviation divided by the mean).

Table 3
Chemical composition of *E. olida*^{NSW} essential oil.

No.	Retention time (min)	Compound	Content average (%)[a]	RSD[b] (%)
1	5.61	Benzaldehyde	<0.1	44.4
2	8.35	(*E*)-β-Ocimene	<0.1	39.6
3	10.24	Linalool	<0.1	26.8
4	12.39	Isoborneol	<0.1	32.8
5	13.96	α-Terpineol	<0.1*	62.9*
6	15.62	Citronellol	0.1	16.8
7	16.12	Neral	<0.1	31.9
8	16.75	Geraniol	0.1	24.0
9	17.05	Methyl citronellate	<0.1	61.0
10	17.42	Geranial	<0.1	23.8
11	17.60	Nopol	<0.1	36.8
12	18.90	(*Z*)-Methyl cinnamate	0.1	17.5
13	19.82	Methyl geranate	<0.1	43.9
14	**22.59**	**(*E*)-Methyl cinnamate**	**99.4**	**0.5**
15	23.97	(*E*)-Caryophyllene	<0.1*	41.3*
16	25.72	Seychellene	<0.1*	55.0*
17	27.18	Viridiflorene	<0.1*	36.7*
18	28.33	δ-Cadinene	<0.1*	41.1*
19	30.46	Spathulenol	<0.1*	47.1*

The dominant compounds are indicated in bold.
[a] Relative proportions as percent of the total peak area.
[b] Relative standard deviation (standard deviation divided by the mean).
* Average and relative standard deviation calculated from two values instead of three.

Table 4
Chemical composition of *E. olida*^{VIC} essential oil.

No.	Retention time (min)	Compound	Content average (%)[a]	RSD[b] (%)
1	5.61	Benzaldehyde	<0.1*	58.2*
2	8.00	(*Z*)-β-ocimene	<0.1*	90.0*
3	8.35	(*E*)-β-ocimene	<0.1	42.1
4	10.23	Linalool	<0.1*	104.5*
5	16.61	Piperitone	<0.1*	28.3*
6	16.83	Linalool acetate	<0.1*	110.0*
7	17.58	Nopol	<0.1*	28.3*
8	18.86	(*Z*)-Methyl cinnamate	0.1	12.3
9	**22.29**	**(*E*)-Methyl cinnamate**	**98.8**	**1.0**
10	25.71	(*E*)-Ethyl cinnamate	0.1	13.2
11	27.23	Bicyclogermacrene	<0.1	10.2
12	30.46	Spathulenol	<0.1	25.6
13	30.69	Globulol	<0.1	10.4
14	31.03	Viridiflorol	<0.1	31.5
15	31.13	Khusimone	<0.1	24.2
16	31.46	Guaiol	<0.1	10.6
17	32.26	10-Epi-γ-eudesmol	<0.1	17.3
18	32.61	γ-Eudesmol	<0.1	19.3
19	33.29	α-Eudesmol	<0.1	24.1
20	33.43	7-Epi-α-eudesmol	0.1	4.0

The dominant compounds are indicated in bold.
[a] Relative proportions as percent of the total peak area.
[b] Relative standard deviation (standard deviation divided by the mean).
* Average and relative standard deviation calculated from two values instead of three.

(*E*)-methyl cinnamate, accounted for about 99% the essential oils of *E. olida*, although 19 compounds were identified in *E. olida*^{NSW} and 20 in *E. olida*^{VIC} oils (Tables 3 and 4).

Results of the GC/MS analyses showed that *E. staigeriana* and *E. dives* essential oils contained mostly volatile compounds (Fig. 1), with the majority appearing in the first 20 min. In contrast, essential oils of both *E. olida* specimens comprised of heavier compounds, with most of those coming out after 18 min. (*E*)-Methyl cinnamate, which appeared after 22.29 min, represented more than 98.8% of the total essential oil content of both *E. olida* specimens. This agreed with the finding of Curtis et al. (in Smale et al., 2000) that (*E*)-methyl cinnamate accounted for 94–99% of the essential oils extracted from fresh leaves of *E. olida*.

The major compounds present in essential oils of the three *Eucalyptus* species showed expected similarities since they are of the same genus. Essential oils of *E. dives* and *E. staigeriana*, in particular, had 18 common compounds. Surprisingly, although the two *E. olida* specimens were from the same species, they had only seven compounds in common, compared with the 18 common compounds present in *E. dives* and *E. staigeriana*. According to Smale et al. (2000), the chemical composition of *E. olida* essential oils shows significant interspecies variability which appears to depend on both the genetic characteristics of the plants as well as the conditions under which they are grown.

Very little information is available in the literature on the chemical composition of *Eucalyptus* essential oils. Delaquis et al. (2002) reported that the heavy end of an *E. dives* essential oil from South Africa was rich in piperitone while the light end of the distillate contained high levels of α-phellandrene, α-thujene and α-terpinolene combined with several minor volatiles. Oussalah, Caillet, Saucier, and Lacroix (2006) reported that piperitone (42.9%) and α-phellandrene (30.0%) were the dominant compounds of *E. dives* essential oil extracted from Australian leaves of the plant. The result for piperitone agreed closely with ours (40.5%) while the figure for α-phellandrene (30.0%) was much higher than our result (17.4%).

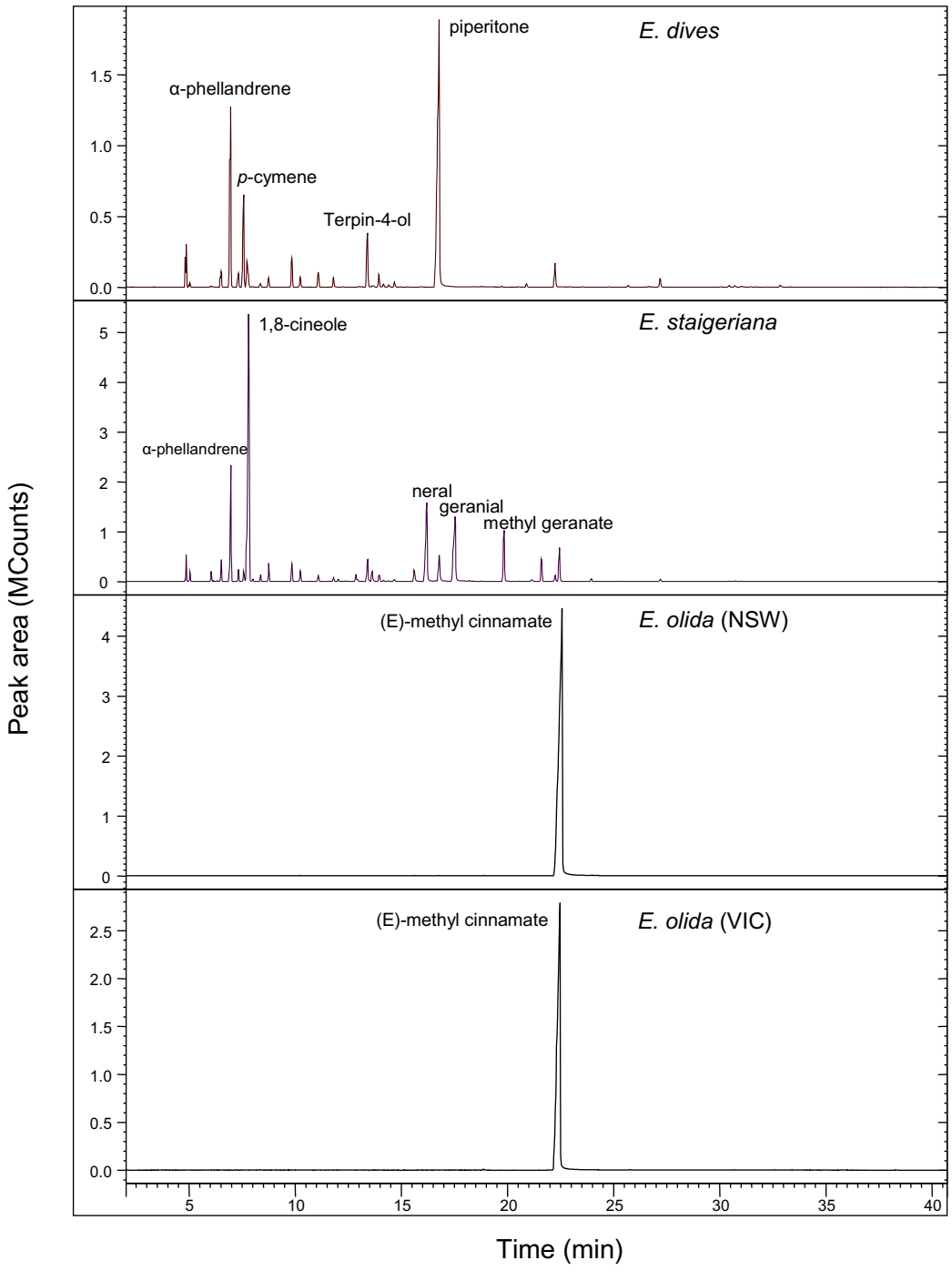

Fig. 1. Typical GC/MS chromatograms showing the chemical profiles of the essential oils of *E. dives*, *E. staigeriana*, *E. olida*^{NSW} and *E. olida*^{VIC}. The sample injected into the GC/MS system were a 1% solution of each of the essential oils.

3.3. Antimicrobial activity of the essential oils

Results from the agar disc diffusion tests for antimicrobial activity of the essential oils are presented in Table 5. The essential oils displayed a variable degree of antimicrobial activity against the different strains tested. The Gram-positive pathogen, *S. aureus*, was the most sensitive microorganism to the essential oils, followed by the yeast *C. albicans*, while the Gram-negative spoilage bacterium, *P. aeruginosa*, was the most resistant.

The antimicrobial activity of the essential oils displayed considerable variations among the different *Eucalyptus* species. The essential oil of *E. staigeriana* showed the highest activity against all the microorganisms tested, especially against the pathogen *S. aureus* with an inhibition zone greater than 90 mm (the growth of the organism was inhibited over the entire Petri dish). *E. dives* exhibited a considerably stronger activity than that of *E. olida* when tested against *S. aureus*, but the two *Eucalyptus* species showed similar activity against the Gram-positive *E. faecalis* and the yeast *C. albicans*. Essential oils from *E. oilda* showed no inhibitory activity against the Gram-negative bacterium *P. aeruginosa*.

The antimicrobial activity of the essential oils, especially those of *E. staigeriana* and *E. dives*, compared very favourably with that of the standard antibiotics tested. The essential oil of *E. staigeriana*, in particularly, had an inhibition zone diameter more than four

Table 5
Antimicrobial activity of investigated essential oils and standard antibiotics against a microorganisms inoculum concentration of about 10^5–10^6 CFU/ml.

Microorganisms	Inoculum conc.[a] (CFU/ml)	Inhibition zone diameter[b] (mm)						
		Essential oils				Antibiotics		
		E. dives	*E. olida*^{NSW}	*E. olida*^{VIC}	*E. staigeriana*	CH	GE	NY
Gram$^+$								
E. faecalis	3.88E+06	11.1 ± 0.4	11.6 ± 1.1	9.1 ± 0.2	20.3 ± 1.2	22.5 ± 0.7	–	–
S. aureus	2.75E+06	52.3 ± 5.6	25.4 ± 0.5	25.0 ± 2.7	>90	21.9 ± 1.1	–	–
Gram$^-$								
E. coli	6.43E+05	14.8 ± 0.4	10.8 ± 0.7	10.4 ± 0.6	15.8 ± 1.1	21.9 ± 0.9	–	–
P. aeruginosa	3.05E+06	9.1 ± 0.3	nd	nd	7.7 ± 0.4	–	22.8 ± 0.5	–
Yeast								
C. albicans	3.72E+06	15.4 ± 0.8	15.6 ± 1.0	12.6 ± 1.2	26.7 ± 0.7	–	–	11.0 ± 1.0

–: not tested; nd: not detected; >90: inhibition zone diameter exceeded Petri dishes diameter.
CH: chloramphenicol 30 μg/disc; GE: gentamicin 30 μg/disc; NY: nystatin 30 μg/disc.
[a] Results are presented as mean ($N = 3$).
[b] Results are presented as mean ± standard deviation ($N = 6$). The diameter of the discs (Ø = 6 mm) was included.

times greater than the antibiotic chloramphenicol (CH) against the pathogen *S. aureus*. The diameter of the inhibition zone against the yeast was more than twice greater than that from the antibiotic nystatin. The essential oil of *E. dives* was also effective against *S. aureus*, having an inhibition zone of 52.3 mm in diameter. Its activity against the yeast was slightly higher than the antibiotic nystatin, but the activity against the other bacteria was lower than the antibiotics tested. The essential oil of *E. olida* had a lower inhibitory activity against all the microorganisms tested, compared with the antibiotics.

The Gram-negative spoilage bacterium *P. aeruginosa* was found to be the most resistant to the essential oils tested. *E. olida* essential oils did not show any antimicrobial activity against *P. aeruginosa* while only weak inhibition (7–9 mm) was observed for *E. dives* and *E. staigeriana* oils. *P. aeruginosa* is known to have a high level of intrinsic resistance against many antimicrobials and antibiotics due to a very restrictive outer membrane barrier, being highly resistant even to synthetic drugs (Skočibušić, Bezić, & Dunkić, 2006).

Essential oils from the three *Eucalyptus* species showed high antimicrobial activity against the yeast *C. albicans* as they were all more effective than 30 μg of nystatin. The highest antimicrobial activity was observed for *E. staigeriana* oil with an inhibition zone diameter of 26.7 mm. The lowest activity against the yeast was recorded for the essential oil of *E. olida*^{NSW} at 12.6 mm while the essential oil of *E. dives* and *E oilda*^{VIC} had similar activities at 15.4 and 15.6 mm, respectively.

The relatively high antimicrobial activities of *E. staigeriana* and, to a lesser extent, *E. dives* essential oils are most likely due to the presence of compounds with antimicrobial properties in them. A number of compounds present in relatively high concentrations in the essential oil of *E. staigeriana* are known to have antimicrobial properties. Particularly worth noting is 1,8-cineole (eucalyptol), which accounted for approximately 35% of the essential oil of *E. staigeriana* and which has been found to have relatively strong antimicrobial properties against many important pathogens and spoilage organisms including *S. aureus* (Rosato, Vitali, De Laurentis, Armenise, & Milillo, 2007), *Fusarium solani* (Pitarokili, Tzakou, Loukis, & Harvala, 2003), *E. coli* and *Bacillus subtilis* (Sonboli, Babakhani, & Mehrabian, 2006). Geraniol, which represented 3.4% of the essential oil of *E. staigeriana* but has an antimicrobial activity against *S. aureus* 3–7 times higher than that of 1,8-cineole (Bakkali et al., 2008), could also be expected to make a significant contribution to the antimicrobial activity of the essential oil. Compounds such as linalool, γ-terpinene, *p*-cymene, α-pinene and α-terpineol also have relatively strong antimicrobial activities (Bakkali et al., 2008). Although individually their concentrations were low, collectively they made up more than 5% of the essential oil of *E. staigeriana*, and therefore may also have a moderate contribution to its antimicrobial activity.

Piperitone, which accounted for more than 40% of the essential oil of *E. dives*, may be partially responsible for its antimicrobial activity. The compound has been reported to have antimicrobial activity against *Mucora rouxii* (Bakkali et al., 2008). Furthermore, it is the main compound (38%) of the essential oil extracted from *Mentha pulegium* flowers (Mahboubi & Haghi, 2008) which was found to have an inhibitory zone diameter of 21 mm against *S. aureus*, a result similar to the data for *E. dives* essential oil recorded in the current study. Other compounds, including *p*-cymene linalool, γ-terpinene, α-pinene, α-terpineol, which were also present in *E. staigeriana* oil, may also collectively have a significant contribution to the antimicrobial activity of *E. dives* essential oil. With its predominance (99.7%) in the *E. olida* essential oil, (*E*)-methyl cinnamate would be expected to be mainly responsible for its antimicrobial properties, probably with lesser contributions from the minor constituents.

4. Conclusion

The three *Eucalyptus* species investigated in the present study showed a large variation in their chemical composition, even for the two *E. olida* samples grown in different regions. The essential oils were dominated by a few volatile compounds, and in the case of *E. olida*, just one compound, (*E*)-methyl cinnamate. The essential oils displayed a variable degree of antimicrobial activity with *E. staigeriana* oil having the highest activity. Gram-positive bacteria were found to be in general more sensitive to the essential oils than Gram-negative bacteria. *S. aureus* was the most sensitive strain while *P. aeruginosa* was the most resistant. The particularly high antimicrobial activity of *E. staigeriana* essential oil, especially against the Gram-positive pathogen S. *aureus*, suggests that it may have a promising prospect for applications in food and potentially, pharmaceutical products. Future studies could be directed at investigating the minimum inhibitory concentration (MIC) of the essential oils which have exhibited antimicrobial properties, extending the antimicrobial test to a wider range of microorganisms and applying the essential oils in food and possibly also pharmaceutical products.

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