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RESEARCH ARTICLE

α-Cyclodextrin encapsulation enhances antimicrobial activity of cineole-rich essential oils from Australian species of *Prostanthera* (Lamiaceae)

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Abstract

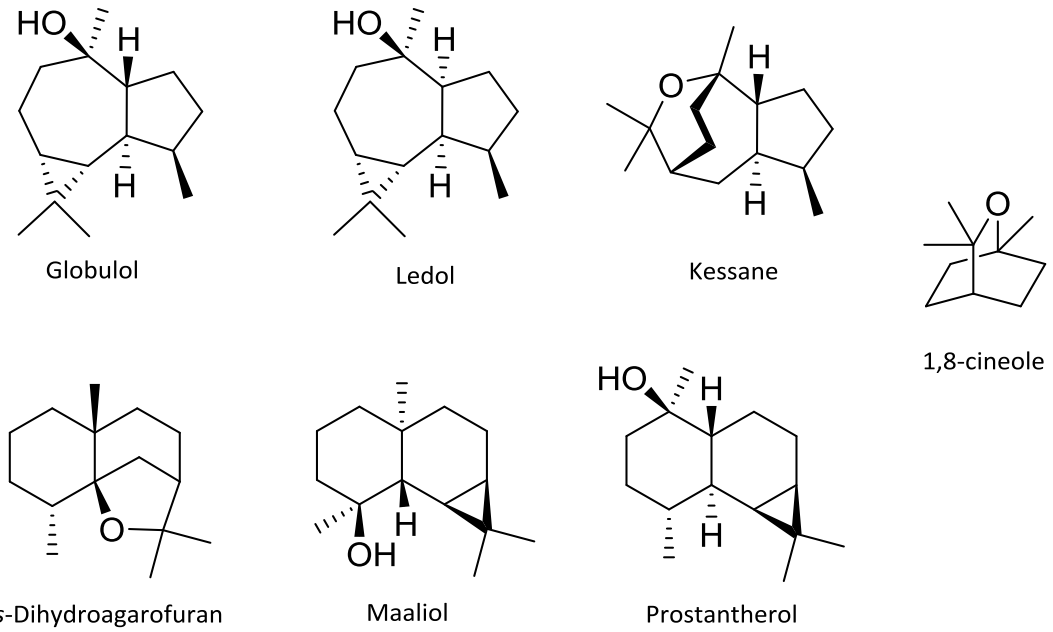
Highly chemically variable cineole-rich essential oils were produced from cultivated specimens of the genus *Prostanthera* (Lamiaceae), currently taxonomically assigned to *P. ovalifolia*, *P. rotundifolia*, *P. incisa* and *P. lasianthos*. Essential oils were chemically characterised using GC-MS and NMR. The mean inhibitory concentrations against Gram-positive and Gram-negative bacterial species were measured using a microtitre plate broth dilution assay. A selection of these oils were further assayed for antimicrobial activity after being encapsulated at a 1:1 molar ratio using α-cyclodextrin. Cineole-rich essential oils are chemically differentiated by the character of the sesquiterpene oxides cis-dihydroagarofuran and kessane; and the sesquiterpene alcohols globulol, prostantherol and ledol. Within the wider context of common essential oils, this selection of essential oils from *Prostanthera* demonstrated relatively low inhibitory concentrations (high antimicrobial activity), particularly against Gram-positive organisms. When some of these oils were encapsulated in α-cyclodextrin the antimicrobial activity was generally enhanced by two to four-folds. This enhancement may be a result of encapsulation with reduced evaporation during the assay and emulsion formation which may facilitate delivery to bacterial species. The use of cyclodextrins as a feed and formulation additive should be considered within the context of the antimicrobial activity of cineole-rich essential oils from *Prostanthera*.

Keywords: prostantherol, encapsulation, α cyclodextrin, antimicrobial, cineole

Introduction

The genus *Prostanthera* comprises approx. 100 species (CHAH Australian Plant Census) in the family Lamiaceae. The number of species is expected to increase dramatically as heterogeneous species aggregates are revised and new species are subsequently described. By the end of the last century nearly half of these species had been examined for essential oil character (Baker & Smith, 1912; Dellar, Cole, Gray, Gibbons, & Waterman, 1994; Lassak, 1980; Southwell & Tucker, 1993). In these previous studies it was demonstrated that a significant number of species yielded essential oils dominated by 1,8-cineole and a sesquiterpene alcohol or oxide that gives each chemotype its defining character. Examples of such characteristic sesquiterpenes include the decahydronapthalenic prostantherol, maaliol or cis-dihydroagarofuran and the decahydroazulenic kessane, ledol or globulol (Figure 1).

Figure 1. Major components in essential oils from species of *Prostanthera*



The greatest extent of intraspecific variability of essential oils occurs within the *P. lasianthos*, *P. ovalifolia* and *P. rotundifolia* heterogeneous species aggregates. This variability correlates with morphological features (I.RH. Telford, 2014 pers. comm.). In light of this, chemotaxonomy has therefore become a complement to the ongoing taxonomic revision of these three species.

In general, *Prostanthera* species yield appreciable amounts of essential oil upon hydrodistillation (1-3% w/w wet leaves). The species *P. incisa, P. ovalifolia* or *P. rotundifolia* are generally easily cultivated, and due to the abundance of 1,8-cineole they could be used in antimicrobial applications. As far as we can tell, only one previous study has examined antimicrobial activities of compounds from *Prostanthera*, and focused on using three isolated sesquiterpenes against phytopathogens (Dellar et al., 1994). In this previous study, no concentrations higher than 100 µg ml^{-1} were used, which is relatively low in the context of essential oils. At these lower concentrations the sesquiterpene alcohol prostantherol inhibited just one of the phytopathogens, *Streptomyces scabies*, with a mean inhibitory concentration of 60 µg ml^{-1}.

One of the challenges faced in antimicrobial studies that employ lipophilic compounds such as essential oils, is in the slowing of evaporation of active volatiles and the formation of a stable solutions or emulsions. Currently the most widely employed method for emulsifying essential oils in antimicrobial assays is to thicken the broth with a small amount of agar (Mann & Markham, 1998). It is also possible to emulsify solutions using common formulations including a mineral oil and surfactants such as polysorbate 80 (Viyoch et al., 2006). This has substantially enhanced the reproducibility of antimicrobial assays using essential oils, however in using only agar, complete dispersion is not achieved. This is particularly evident with sesquiterpene-rich essential oils that can sometimes form aggregates in the agar. In addition, antimicrobial activities of essential oils in agar emulsions do not necessarily translate to the same activity in a lotion that uses borax, bees wax or chitosan.

There is increasing interest in the use of both lipophilic polymers and cyclodextrins to encapsulate essential oils to facilitate even dispersal and stability of emulsions. The advantage is that cyclodextrins can be used in both the antimicrobial assays and the end product, whether it be a cream or feed additive (Karlsen, 2010). Cyclodextrins are biocompatible, non-toxic and are able to form inclusion complexes that stabilize oil in water emulsions (Mathapa & Paunov, 2013) and slow the rate of oil evaporation. There is increasing interest in the use of cyclodextrins to encapsulate essential oils to disperse the oils, which will aid in the uniformity of emulsions.

In the current study, several cultivated specimens of *Prostanthera* were sampled for essential oil analysis, with subsequent antimicrobial studies against Gram-positive and Gram-negative human pathogens, and the yeast *Candida albicans*. With the view of subsequent formulation work, essential oils were encapsulated in α-cyclodextrin as a preliminary attempt to examine for either positive or negative effects on antimicrobial activity.

Materials and Methods

Leaf Collection, Essential Oil Production and Characterization

Field specimens of selected *Prostanthera* specimens were collected and locations are indicated in Table 1. Cultivated specimens were taken from local gardens with provenances also included in Table 1. Vouchers from the wild and from garden specimens were lodged with the N.C.W Beadle Herbarium at the University of New England, Armidale, NSW Australia, labelled numerically with the prefix NJSadgrove. For example, NJSadgrove239 represents *P. sp. aff. ovalifolia*, which is cited numerically in the current study as 239.

Essential oils from wild and cultivated specimens were produced using hydrodistillation. Approximately 600 g of leaf was removed from the twig then chopped into 0.5 mm fragments and placed into a 5 L round bottomed flask with 2.5 L of deionised distilled water (ddH_20). Leaves were heated for 4 hr by a 6 L mantle and the steam/oil mix was condensed and collected in a 500 ml separating funnel then separated from the hydrosol. Oils were stored away from light at 4˚C until used.

Prior to GC-MS analysis oils were treated with anhydrous sodium sulphate ($NaSO_4$) powder (0.5g in 10ml oil) for more than 24 hr to remove hydrosol emulsions. Afterwards, oils were dissolved in dichloromethane (DCM) at a ratio of 1:1000. Analyses were performed using an Agilent Technologies 7890A GC-System coupled with an Agilent 5975C mass selective detector (insert MSD with triple-Axis detector). An autosampler unit (Agilent Technologies 7693 – 100 positions) was used to perform the 1 µL injections. Separation was accomplished with a HP-5MS Agilent column (30m X 250µm X 0.25µm). Operating conditions were as follows: Injector: split ratio 25:1; Temperature: 250˚C; carrier gas: helium, 1.0 mL/min, constant flow; column temperature, 60˚C (no hold), 5˚C per minute then @ 250 hold 15 minutes. MS was acquired at -70 eV using a mass scan range of 30 – 400 m/z.

Primary identifications were performed by comparison of mass spectra with an electronic library database (NIST08) and subsequently confirmed using comparison of temperature programmed retention indices (IUPAC, 1997) with published values. Most discrepancies in identification were resolved by comparison of mass spectra with a second and third library (Joulain & Koenig, 1989; Adams, 2007; NIST, 2011). Quantification was achieved using GC-MS operating software to calculate area under the curve, using data with a minimum peak area of 0.1%.

Secondary identifications were performed using NMR spectra, by ^{1}H (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$), and comparing to published values. Sesquiterpenes which had structures confirmed using NMR spectra were ledol (Bombarda et al., 2001), globulol (Toyota, Tanaka, & Asakawa, 1999), cis-dihydroagarofuran/kessane (Southwell & Tucker, 1993) and prostantherol (Dellar et al., 1994).

Antibacterial and Anti-Candida activity

Working stocks of all species were maintained on Nutrient Agar (NA) with the exception of *Candida albicans* which required Yeast Extract Peptone Agar (YEPA). All growth media were purchased from Oxoid (Thebarton, South Australia) and prepared as per the manufacturer's instructions.

Minimum inhibitory concentrations (MIC) of the oils were determined using a micro-titre plate two-fold broth dilution method (CLSI, 2009; Wiegand, Hilpert, & Hancock, 2008) with the following modifications. Oil emulsions were prepared by vortexing a measured combination of oil and the appropriate broth with 0.15% w/w agar (Mann & Markham, 1998). Where encapsulation of α-cyclodextrin was used, oils were mixed at a 1:1 molar ratio with the concentration of α-cyclodextrin not exceeding 50 mg ml^{-1} and the molar weight of the oil averaged for the major components. α-Cyclodextrin and the essential oils were dispensed directly into the sterile broth and irradiated with UV for two hours until encapsulation/complexation had finished, indicated by a plateau in the development of a translucent emulsion. Most species were assayed in Tryptone Soya Broth (TSB) containing 0.15% w/w agar, with the exception of *C. albicans* which required YEP broth. Broth dilutions were executed using 96-well plates.

Inoculation was achieved by collecting colonies from fresh working stocks and dispensing into 0.9% w/v NaCl and diluting to match a 0.5McFarland $BaSO_4$ Turbidity Standard (McFarland, 1907) using a spectrophotometer at 600 nm (or 530nm for *C. albicans*). To achieve a final inoculation concentration of 5×10^5 the adjusted saline suspension was diluted into 40 volumes of the appropriate medium and 20µL was used to inoculate 80µL of media bringing the total volume to 100µL and reducing the essential oil concentration to the appropriate starting concentration. Following inoculation the 96-well plates were sealed using parafilm and placed into an incubator at 37˚C for approximately 20 hr before dispensing 40µL of sterile 0.2 mg/mL p-iodonitrotetrazolium dye and examining for colour changes, which indicated organism growth. Positive inhibition controls included tetracycline for bacterial growth and nystatin for *C. albicans*. Experiments were repeated three or more times and the results are reported as a range.

Free radical (DPPH) Scavenging activity

The method used to assess free radical scavenging activity was the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by Brand-Williams et al. (1995) with the following modifications. Assay volumes were 2mL and kept in 4mL cuvettes, which were sealed with parafilm to slow the evaporation of methanol. Assay concentrations of DPPH were aimed at 12-15mg/L, however actual concentrations were later determined using the calculations proposed by Szabo et al. (2007). Spectrophotometer readings were taken at 517nm after 12 hr.

Results are taken as the quantity of essential oil required to quench 50% of DPPH molecules. Data are firstly presented as µg/ml of essential oil or control, and secondly presented as a molar ratio. The number of moles of essential oil was estimated by averaging the molecular weight of the essential oil components. The molar ratio was produced by dividing number of moles of essential oil or control treatment required to quench 50% of DPPH, by the number of moles of DPPH quenched. This figure represents the molar quantity of essential oil or control required to quench 1 mol of DPPH.

Three replicates were prepared for each treatment. Statistical analyses were performed using linear regression analysis of averaged replicates per treatment. Quantity of essential oil, or positive control, required to quench 50% of dissolved DPPH was determined from a data range that produced r^2 values above 90% in linear regression analysis. Flanking data was trimmed to include only data with DPPH concentrations within the approx. range of 80-20%.

Results and Discussion

Due to the inherent complexity in determination of *Prostanthera* to species level the affiliated taxonomic classification of specimens included in the current study may be subsequently described. However at present these species are not up to date. Thus, wherever possible the provenances of cultivated specimens have been included in Table 1. However, where data related to provenance is lacking, voucher specimens have been lodged according to collection number with the NE Herbarium, Armidale NSW 2351 Australia. In conjunction with phytochemical data provided here, the specimens in the current study can be traced to their most up to date taxon in subsequent investigation. As a general guide, figures depicting some of the morphological features of the leaves have been provided to scale for select specimens of *P. sp. aff. ovalifolia* (Figure 2) and *P. sp. aff. rotundifolia* (Figure 3) examined in the current study.

The chemical character of specimens chosen for this study demonstrated a high degree of variability in the composition of the major sesquiterpene components (Table 2). This variability was evident between species affiliates and also within these apparent species, which demonstrated some degree of correlation with morphological variants. This corroborates the chemotaxonomic approach as a potential tool to facilitate taxanomic revision of these heterogeneous species aggregates of *Prostanthera*.

Table 1. Collection numbers (Coll. no.) of cultivated specimens (except 321). The *sp. aff.* refers to species most affiliated with. Currently there are no concerns related to the delimitation of *P. incisa*.

Coll. no.	Species	Lat	Long	Location
NJSadgrove321	*P. sp. aff. ovalifolia*	28°29'09.6''	153°21'16.9''	Hell's Hole, Mt Jerusalum NP, Mullumbimby NSW
NJSadgrove393	*P. sp. aff. ovalifolia*	-	-	Cultivated ex. Blue Mts (Katoomba NSW)
NJSadgrove394	*P. sp. aff. rotundifolia*	-	-	*Cultivated ex. Unknown.*
NJSadgrove398	*P. sp. aff. ovalifolia*	-	-	Cultivated ex. Brundah (near Cowra NSW)
NJSadgrove399	*P. sp. aff lasianthos*	-	-	Cultivated ex. Edgar's lookout (Wollomombi Falls Armidale NSW)
NJSadgrove400	*P. sp. aff. rotundifolia*	-	-	*Cultivated ex. Unknown.*
NJSadgrove401	*P. sp. aff. rotundifolia*	-	-	*Cultivated ex. Unknown.*
NJSadgrove402	*P. incisa*	-	-	*Cultivated ex. Unknown.*
NJSadgrove404	*P. sp. aff. rotundifolia*	-	-	*Cultivated ex. Unknown.* (Resembles Piliga Type; *P. cotinifolia* Benth)
NJSadgrove405	*P. sp. aff. ovalifolia*	-	-	Cultivated ex. Ellenborough Falls, Comboyne NSW

Figure 2 - Morphological variability of *P. sp. aff. ovalifolia* specimens sampled in the current study. Numbers refer to collection no. of vouchers lodged at the herbarium.

Figure 3 - Morphological variability of *P. sp. aff. rotundifolia* specimens sampled in the current study. Numbers refer to collection no. of vouchers lodged at the herbarium.

The abundance of cis-dihydroagarofuran and kessane is limited to species currently assigned to *P. ovalifolia*. However, Prostantherol yields from species currently assigned to *P. ovalifolia* and *P. rotundifolia* but not the others. All specimens collected for the current study yielded appreciable amounts of 1,8-cineole and a moderate amount of *p*-cymene.

The antimicrobial activity of the essential oils was generally higher if essential oils were dominated by approximately equal concentrations of 1,8-cineole and sesquiterpene alcohols, particularly prostantherol.

Table 2. Chemical character of essential oils from species of *Prostanthera*

	Species 'sp. aff.'			***P. oval***	***P. oval***	***P. rotu[a]***	***P. oval***	***P. lasi***	***P. rotu[a]***	***P. rotu[a]***	***P. inci***	***P. rotu[b]***	***P. oval[c]***
	Voucher			**321**	**393**	**394**	**398**	**399**	**400**	**401**	**402**	**404**	**405**
	Yield % g/g			0.5	1.1	1.0	3.1	0.3	1.3	1.1	1.7	1.3	1.1
Compound Name	**AI**		**Pub. AI**										
α-Pinene		934	932	-	0.8	1.1	0.5	11.6	-	-	1.6	12.2	-
Camphene		950	946	-	-	0.7	6.7	0.6	-	-	0.5	1.9	-
Sabinene		973	969	-	0.9	-	0.9	1.4	-	-	1.7	0.5	-
β-Pinene		979	974	-	0.8	-	1.0	17.7	-	-	1.4	1.3	-
α-Phellandrene		1005	1002	-	2.0	-	3.8	0.5	1.0	0.8	3.9	-	-
p-Cymene		1025	1020	2.8	9.2	1.9	8.8	2.6	6.3	4.6	10.8	3.3	4.5
Limonene		1029	1024	-	-	-	-	7.2	-	0.9	-	1.5	0.7
1,8-Cineole		1034	1031	13.7	36.1	64.8	15.6	32.6	58.5	49.7	21.4	23.9	7.9
Linalool		1096	1095	-	-	-	0.5	-	-	-	1.9	-	-
Camphor		1149	1144	-	-	5.1	-	-	-	-	-	0.8	-
Borneol		1169	1165	-	-	4.2	-	0.6	-	-	0.5	2.9	-
Terpinen-4-ol		1180	1174	-	0.5	-	0.5	2.0	-	-	0.5	-	-
α-Terpineol		1192	1186	-	-	-	0.9	2.0	-	-	1.2	-	-
Myrtenol		1199	1194	-	-	-	0.5	0.5	-	-	0.5	-	-
Myrtenal		1201	1195	-	-	-	0.5	0.6	-	-	0.5	0.5	-
Linalool acetate		1252	1254	-	-	-	0.6	-	-	-	4.6	0.5	-
Bornyl acetate		1287	1288	-	-	0.8	3.1	0.5	-	-	-	2.3	-
Isodihydro carveol acetate		1335	1326	-	2.2	-	-	-	-	-	-	-	-
α-Terpineol acetate		1349	1346	-	-	-	0.5	-	-	-	3.4	-	-
Unknown A		1352	-	-	-	-	-	-	1.2	3.7	-	-	-
Aromandendrene		1443	1439	-	-	-	-	0.7	-	-	0.5	-	-
Alloaromadendrene		1462	1458	-	0.5	-	2.2	0.5	-	-	2.1	1.0	1.2
Rotundene		1465	1457	-	-	0.8	-	-	-	-	-	-	-
4,5-di-epi-Aristolochene		1470	1471	-	-	-	-	-	-	-	-	4.8	-
Eremophilene		1487	1486	-	-	-	-	-	-	-	-	0.9	-
β-Selinene		1494	1489	-	-	-	-	1.8	-	-	1.2	-	0.6

Bicyclogermacrene	1504	1500	-	2.1	0.9	1.0	1.5	1.5	-	0.5	6.3	0.7
cis-Dihydroagarofuran	1525	1519	58.7	-	-	2.6	-	-	-	-	15.4	50.0
Kessane	1532	1529	17.2	-	-	14.2	-	-	0.9	7.5	-	-
Unknown B	1583	-	-	4.1	-	-	-	-	-	-	4.7	2.3
Spathulenol	1587	1577	-	-	-	2.2	2.3	-	1.3	0.7	-	-
Caryophyllene oxide	1590	1582	-	-	-	-	-	-	3.3	-	-	-
Globulol	1590	1590	-	-	-	3.9	-	-	-	9.0	-	-
Viridiflorol	1601	1592	-	-	-	-	-	-	-	-	-	1.3
Prostantherol	1602	-	1.3	37.4	1.8	-	-	30.0	32.6	-	3.0	12.3
Ledol	1610	1602	5.5	0.6	0.8	11.5	5.0	1.5	1.2	13.7	-	18.0

Prostanthera species were *P. incisa, P. ovalifolia, P. lasianthos and P. rotundifolia,* [a] *Large round leaves,* [b] *Deep,* [c] *Medium incised leaves purple flowers small incised leaves*

The antimicrobial activity of cis-dihydroagarofuran and kessane rich oils was generally lower when compared to the other oils tested (Tables 2 and 4). It is unusual that oils were able to inhibit *Pseudomonas aeruginosa*, as this is not common. The radical scavenging activity of a selection of these oils was very low (Table 3).

All of the oils that were selected for encapsulation with α-cyclodextrin inhibited bacterial species at lower concentrations relative to concentrations without encapsulation (Table 5). Concentrations depicted in Table 5 are in mg ml^{-1}. Oils were encapsulated with α-cyclodextrin at a 1:1 molar ratio, not exceeding the solubility of α-cyclodextrin, at approx. 50 mg ml^{-1}. Encapsulation of most oils at this concentration resulted in a white turbid emulsion. It has been hypothesised that a Pickering emulsion is formed which is stabilized by a-cyclodextrin micrcrystal precipitation at the oil-water interface (Mathapa & Paunov, 2013).

Table 3. DPPH scavenging ability of *Prostanthera* essential oils in µg DPPH per one mg of essential oil or positive control. Positive controls in this experiment were ascorbic acid (Vit. C) and Trolox.

Species	***P. incisa***	***P. sp. aff. ovalifolia***	***P. sp. aff. lasianthos***		
Voucher	**402**	**398**	**399**	**Vit C.**	**Trolox**
DPPH quenched (µg DPPH per mg^{-1} of essential oil)	3.5	3.8	0.8	2188	4484
Averaged molar ratio w/w (essential oil/DPPH)	683	618	2955	0.5	0.25

Table 4. The mean inhibitory concentrations from essential oils hydrodistilled from cultivated *Prostanthera* specimens, presented as % v/v of essential oil in agar. Results are presented as a range in some cases. > indicates inhibition not observed.

Species	***P. oval***	***P. oval***	***P. oval***	***P. oval***	***P. lasi***	***P. inci***	***P. rotu*[a]**	***P. oval*[b]**	**+Control**
Voucher	**-**	**321**	**393**	**398**	**399**	**402**	**404**	**405**	**-**
S. typhimurium	1	>	>	1-1.5	1.5	1.5-3	1	2	0.25
B. subtilis	0.13-0.25	0.13-0.25	0.02-0.03	0.13-0.15	0.75	0.06	0.13-0.15	0.06-0.25	0.13-0.15
K. aurogenes	-	-	-	0.06	0.375	0.3-0.5	-	-	0.25-0.7
S. epidermidis	2	>	5	0.13-3	0.13-3	0.13-3	1	2	0.5-0.75
S. aureus	0.13-0.5	0.5-1	0.06-0.13	0.2-0.7	0.2-0.3	0.05-0.5	0.13-0.25	0.06-0.25	0.13-0.25
S. pneumoniae	0.03	-	0.03	0.17	-	0.13	0.03	0.03	0.06
P. aeruginosa	2	>	3	0.5-1.5	0.5	0.5-1.5	1.5	>	0.75-1
C. albicans	0.13	1-3.5	0.06	0.06-0.13	0.5	0.06-0.13	0.13	1	1

[a] *Deep purple flowers small incised leaves,* [b] *Medium incised leaves,* Bacterial species were *Salmonella typhimurium, Bacillus subtili Klebsiella aurogenes, Staphylococcus epidermidis, S. aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa* and *Candic albicans*, *Prostanthera* species were *P. incisa, P. ovalifolia, P. lasianthos and P. rotundifolia*. +Control – positive (+) control tetracycline for bacterial species or nystatin for *C. albicans* in µg ml^{-1}.

Table 5. The antimicrobial activity of selected essential oils in mg ml^{-1} comparing free essential oil (EO) and oil encapsulated with α-cyclodextrin (α-CD).

Species aff.	***P. oval***		***P. oval***		***P. rot***		***P. rot***		***Nystatin, *Tetracycline***
Voucher	**321**		**393**		**401**		**405**		
Bacterial species	**EO**	**α-CD**	**EO**	**α-CD**	**EO**	**α-CD**	**EO**	**α-CD**	***+Control***
S. typhimurium	>8.3	5	8.3	2.5	8.3	2.5	>8.3	5	0.13
E. coli	>8.3	1.3	8.3	5	8.3	2.5	>8.3	5	0.25
B. subtilis	8.3	0.6	0.5	0.2	0.5	0.2	1	0.6	0.25
S. epidermidis	>8.3	5	8.3	2.5	8.3	2.5	8.3	5	0.06
S. aureus	2.1	0.6	0.3	0.2	0.3	0.2	1	0.2	0.06
C. albicans	8.3	2.5	2.1	1.3	2.1	1.3	2.1	1.3	1.3*

EO – essential oil, α-CD, α-cyclodextrin encapsulated, Bacterial species were *Salmonella typhimurium, Escherichia coli, Bacillus subtilis, Staphylococcus epidermidis, S. aureus,* and *Candida albicans*, *Prostanthera* species were *P. ovalifolia and P. rotundifolia*, Positive control - +Control.

In the study of Mathapa that used cyclodextrins to encapsulate n-alkanes or free fatty acids, cyclodextrin microcrystals were characteristically rod-shaped, formed by threading cyclodextrins over the carbon chains of the respective lipophilic compound. In the current study, essential oils were presumably formed into such microcrystals, but their appearances were not examined by the authors. Partial inclusion of oils into the small α-cyclodextrin core would create an amphiphilic complex around which crystal growth can occur. Formation of the emulsion took up to 2 hours consistent with the time required for crystal growth.

In the current study the observed Pickering emulsion visibly redissolved after two or three serial dilutions, which supports the previous proposal of α-cyclodextrin precipitation mechanism of stabilization. The enhanced antimicrobial activity did not correlate with this visible emulsion, as inhibition concentrations were measured lower than that required for an emulsion to be observed. The mechanism for the enhanced antimicrobial activity relative to non-encapsulated oils is not yet clear, but it most likely relates to an enhanced solubility of sesquiterpenes after formation of an inclusion complex. We did not observed any change in antimicrobial activity by encapsulation of monoterpenoid essential oils from various chemotypes of *Eremophila longifolia* using α- and γ-cyclodextrins (data not shown). The enhanced antimicrobial activity of purified sesquiterpene alcohols encapsulated in cyclodextrins will be published elsewhere.

At present it is not clear if cyclodextrins are metabolised into a nutrient by bacteria, which could affect the delivery of antimicrobial compounds to bacterial cell walls, however one study observed increase of growth vigour of *Helicobacter pylori* in cultures supplemented with cyclodextrins (Marchini et al., 1995). Furthermore, the inclusion complexes of synergistic/antagonistic essential oil components may affect their biotransformation and availability. This may be of particular relevance to the interaction of 1,8-cineole and the other sesquiterpene alcohols. The small size of 1,8-cineole, which allows inclusion into the cage-like structures of the cyclodextrins, would counter its higher volatility and maintain its relative abundance during antimicrobial assays and in topical antimicrobial applications.

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REFERENCES

Baker, R. T., & Smith, H. G. (1912). On a new species of *Prostanthera* and its essential oil. *Journal and Proceedings of the Royal Society of NSW, 46*(1), 103-110.

Bombarda, I., Raharivelomanana, P., Ramanoelina, P. A. R., Faure, R., Bianchini, J.-P., & Gaydou, E. M. (2001). Spectrometric identifications of sesquiterpene alcohols from niaouli (*Melaleuca quinquenervia*) essential oil. *Analytica Chimica Acta, 447*, 113-123.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft und- Technologie, 28*, 25-30.

CLSI. (2009). Methods for dilution antimicrobial susceptibility testing for bacteria that grow aerobically; Approved Standard - Eight Edition. *M07-A8, 29*(2), 1-66.

Dellar, J. E., Cole, M. D., Gray, A. I., Gibbons, S., & Waterman, P. G. (1994). Antimicrobial sesquiterpenes from *Postanthera* aff. *melissifolia* and *P. rotundifolia*. *Phytochemistry, 36*(4), 957-960.

Karlsen, J. (2010). Chapter 17 - Encapsulation and other programmed release techniques for essential oils and volatile terpenes. In K. Hüsnü Can Baser & B. Gerhard. (Eds.), *Handbook of Essential Oils: Science, Technology and Applications*. Boca Raton: CRC Press - Taylor and Francis Group.

Lassak, E. V. (1980). *New essential oils from the Australian flora (October 1980) - perfumes and flavours symphony of nature*. Paper presented at the 8th International Congress of Essential Oils, Fedarom Grasse, France - Paper No. 120 pp. 409-415, Fedarom Grasse, France.

Mann, C. M., & Markham, J. L. (1998). A new method for determining the minimum inhibitory concentration of essential oils. *Journal of Applied Microbiology, 84*(4), 538-544.

Marchini, A., d'Apolito, M., Massari, P., Atzeni, M., Copass, M., & Olivieri, R. (1995). Cyclodextrins for growth of Helicobacter pylori and production of vacuolating cytotoxin. *Archives of Microbiology, 164*, 290-293.

Mathapa, B. G., & Paunov, V. N. (2013). Cyclodextrin stabilised emulsions and cyclodextrinosomes. *Physical chemistry chemical physics, 15*, 17903.

McFarland, J. (1907). Nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *Journal of the American Medical Association, 14*, 1176-1178.

Southwell, I. A., & Tucker, D. J. (1993). *cis*-Dihydroagarofuran from *Prostanthera* sp. aff. *ovalifolia*. *Phytochemistry, 22*(4), 857-862.

Szabo, M. B., Iditoiu, C., Chambre, D., & Lupea, A. X. (2007). Improved DPPH determination for antioxidant activity spectrophotometric assay. *Chemical Papers, 61*(3), 214-216.

Toyota, M., Tanaka, M., & Asakawa, Y. (1999). A revision of the ^{13}C NMR spectral assignment of globulol. *Spectroscopy, 14*, 61-66.

Viyoch, J., Pisutthanan, N., Faikreua, A., Nupangta, K., Wangtorpol, K., & Ngokkuen, J. (2006). Evaluation of *in vitro* antimicrobial activity of Thai basil oils and their micro-emulsion formulas against *Propionibacterium acnes*. *International Journal of Cosmetic Science, 28*(2), 125-133.

Wiegand, I., Hilpert, K., & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols, 3*(2), 163-175.