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Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact

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The antibacterial activity of 14 essential oils and their major constituents in the gaseous state was evaluated against *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus*. For most essential oils examined, *H. influenzae* was most susceptible, followed by *S. pneumoniae* and *S. pyogenes*, and then *S. aureus*. Penicillin-susceptible and -resistant *S. pneumoniae* were comparable in susceptibility. *Escherichia coli*, which was used as a control, showed least susceptibility. A minimal inhibitory dose (MID) was introduced as a measure of the vapour activity. Among 14 essential oils, cinnamon bark, lemongrass and thyme oils showed the lowest MID, followed by essential oils containing terpene alcohols as major constituents. The essential oils containing terpene ketone, ether and, in particular, hydrocarbon had high MIDs. The vapour activity on short exposure was comparable to that following overnight exposure, and rapid evaporation was more effective than slow evaporation of essential oils. The vapour concentration and absorption into agar of essential oils reached a maximum 1 or 2 h after rapid evaporation. These results indicate that the antibacterial action of essential oils was most effective when at high vapour concentration for a short time.

Introduction

Essential oils produced by plants have been traditionally used for respiratory tract infections, and are used nowadays as ethical medicines for colds.[1,2] In the medicinal field, inhalation therapy of essential oils has been used to treat acute and chronic bronchitis and acute sinusitis. Inhalation of vapours of essential oils augmented the output of respiratory tract fluid,[3] maintained the ventilation and drainage of the sinuses,[4] had an anti-inflammatory effect on the trachea[5] and reduced asthma.[6]

Essential oils are known to possess antimicrobial activity, which has been evaluated mainly in liquid medium. Systematic evaluation of the vapour activity was first reported by Maruzzella *et al.*[7,8] and Kienholz[9] in 1959, using the inverted Petri dish technique. The technique, in which a volatile compound placed in a cup or a paper disc was exposed to the inverted agar medium plate inoculated with test strains at about 5 mm distance, was convenient and has been used by subsequent researchers.[10,11] Under these conditions, the air space was too small to measure the vapour concentration of essential oil. We employed an airtight box of 1 L air capacity for the measurement of vapour activity.[12,13] Although evaluation of cinnamon bark oil against respiratory tract mycoses has been reported,[14] there are no reports describing vapour activity of essential oils against major bacterial respiratory tract pathogens.

In contrast to antibiotics, essential oils are highly volatile at room temperature. We therefore investigated a potential role for these oils as inhalation therapy, and determined the antibacterial activity of a wide variety of them against five pathogens: *Haemophilus influenzae*, penicillin-susceptible and -resistant *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus*, by gaseous contact in an airtight box. *Escherichia coli* was used as a control.

Materials and methods

Essential oils and their major constituents

Essential oils used are listed in Table I, together with their respective major constituents and distributors. The same lots of each oil were used throughout the experiments.

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D-Limonene was obtained from Nippon Terpene Co. (Kobe, Japan), perillaldehyde from Kohken Koryo Co. (Yokohama, Japan), perillyl alcohol from Aldrich Chemical Corp. (Milwaukee, WI, USA), perillic acid from Alexis Corp. (San Diego, CA, USA), camphor, 1,8-cineole, cinnamic acid, linalool, menthol, menthone, nonanal, α-pinene and thymol from Wako Pure Chemical (Osaka, Japan), citral, cinnamaldehyde and octanal from Nacalai Tesque, Co. (Kyoto, Japan) and geraniol from Kanto Chemical Co. (Kyoto, Japan). Other compounds were purchased from Tokyo Kasei Kogyo, Co. (Tokyo, Japan). The high quality of these chemicals (purity >95%) was confirmed by gas chromatographic (GC) analysis.

Bacterial strains

H. influenzae ATCC 33391 and *S. pyogenes* ATCC 12344 were obtained from the American Type Culture Collection. Penicillin-susceptible *S. pneumoniae* IP-692, penicillin-resistant *S. pneumoniae* PRC-53, *S. aureus* FDA 209P JC-1 and *E. coli* NIHJ JC-2 were stock cultures from the Pharmaceutical Research Centre, Meiji Seika Kaisha, Ltd (Yokohama, Japan).

Media and inocula

Mueller–Hinton agar (MHA) was used as a basal medium and employed for *S. aureus* and *E. coli*. MHA containing 5% defibrinated horse blood (chocolate agar) and MHA containing 5% defibrinated sheep blood (blood agar) were used for *H. influenzae* and the streptococci, respectively.

The test strains were suspended in skimmed milk, and kept at –45°C until use. *H. influenzae* ATCC 33391 was grown on chocolate agar and incubated overnight at 37°C, and then colonies were suspended in Mueller–Hinton broth (MHB), which was diluted with fresh MHB to give 10^6 cfu/mL. *S. pneumoniae*, *S. pyogenes*, *S. aureus* and *E. coli* strains were inoculated on to blood agar plates or MHA, and incubated at 37°C overnight, and the inoculum prepared as for *H. influenzae*.

Determination of minimal inhibitory dose (MID) of essential oil by gaseous contact

Petri dishes containing chocolate agar, blood agar or MHA (10 mL each) were inoculated with the respective test strains (5 μL of 10^6 cfu/ml) using a microplanter (Sakuma, Tokyo, Japan), and placed in 1.3 L airtight boxes (Jallee Co., Tokyo, Japan). The inside of the boxes was covered with aluminum foil to protect the wall of the container from direct contamination by essential oil, which is difficult to remove by washing.

A two-fold dilution series of an essential oil or its major constituent dissolved in ethyl acetate was prepared: 4000, 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 mg/mL. Part of the solution (260 μL) was soaked homogeneously on filter paper (9 cm in diameter), which was dried for 1 min to remove ethyl acetate, and placed in the airtight box apart from the Petri dish. A filter paper impregnated with ethyl acetate alone and dried at room temperature was inserted in the control box to confirm no solvent effect on the bioactivity. In the second experiment, an absolute essential oil in a glass vessel (3 cm in diameter) was placed in the airtight

Table I. Essential oils used and their major constituents

Essential oil	Major constituent (content)	Distributor
Cinnamon bark oil	cinnamaldehyde (63.1%)	La Florina (Germany)
Lemongrass oil	neral (33.2%), geranial (37.8%)	Sanoflore (France)
Perilla oil	limonene (18.9%), perillaldehyde (60.8%)	Kohken Koryo (Japan)
Thyme (wild) oil	carvacrol (80.0%)	Neal's Yard Far East (Japan)
Thyme (red) oil	limonene (25.8%), γ-terpinene (19.4%), thymol (25.5%)	La Florina (Germany)
Thyme (geraniol) oil	geraniol (32.7%), geranyl acetate (55.6%)	Sanoflore (France)
Peppermint oil	*p*-menthone (19.5%), menthol (63.5%)	Sanoflore (France)
Tea tree oil	γ-terpinene (17.7%), terpinen-4-ol (42.5%)	Thursday Plantation (Australia)
Coriander oil	linalool (73.2%)	Sanoflore (France)
Lavender (spike) oil	1,8-cineole (23.7%), linalool (46.3%), camphor (17.1%)	Sanoflore (France)
Lavender (true) oil	linalool (30.1%), linalyl acetate (36.6%)	Sanoflore (France)
Rosemary oil	α-pinene (24.1%), 1,8-cineole (23.5%), camphor (19.7%)	Sanoflore (France)
Eucalptus (radiata) oil	1,8-cineole (74.3%), α-terpineol (10.3%)	Sanoflore (France)
Citron oil	limonene (83.1%)	Kohken Koryo (Japan)

Content of each major constituent was determined from a peak area relative to the total peak area in GC analysis.
Major constituents of >15% content are listed here, with the exception of eucalyptus oil. Multiple constituents are arranged in the order of retention time in GC.

box to give concentrations of 800, 400, 200, 100 and 50 mg/L space. Ethyl acetate was selected as a solvent because it is less toxic than solvents containing halogen or benzene and is volatile enough to be removed rapidly by drying at room temperature. Ethyl acetate is also useful for the extraction of essential oil from the agar medium. Diethyl ether, which was frequently used in the past, was too volatile to maintain a constant volume of solution.

The boxes were incubated at 37°C overnight, and the MID of essential oil that did not allow bacterial growth was recorded. The MID values were expressed as weight per unit volume (mg/L air). In the third experiment, culture was terminated after 2 h, and the Petri dish was taken from the box and incubated overnight under open conditions. MID was determined simultaneously in parallel with the overnight culture.

Head space and agar layer analyses

A 5 mm diameter hole was bored in the side wall of the airtight box 4 cm up from the bottom, and sealed with a silicone plug, which was fixed with plastic cement. A Petri dish containing 10 mL of MHA was placed in the box, and pre-warmed at 37°C for 1 h. Filter paper impregnated with the MID of each essential oil against *S. pneumoniae* was introduced, the box maintained at 37°C, and an air sample (1 mL each) was taken out 0.5, 1, 2, 4 and 16 h later using an airtight syringe (Hamilton, Reno, NV, USA). The sample was introduced immediately through bubbling into an airtight vial (Target DP vial; Hewlett Packard, Palo Alto, CA, USA) containing 0.5 mL of ethyl acetate. The vial was shaken vigorously for 3 min to dissolve the vapour of the essential oil into the solvent layer. Four microlitres of the ethyl acetate extract was subjected to GC analysis. Preliminary work indicated that the injection of organic extract was superior to the direct injection of the air sample, in respect of the reproducibility of GC peaks and protection of the GC column, which is very sensitive to oxygen.

Absorption of vapour into the agar layer was determined after extraction with ethyl acetate (30 mL) for 3 h at room temperature, followed by drying over anhydrous sodium sulphate and GC analysis. A preliminary experiment revealed that a major constituent could be extracted from the agar layer at a yield of 75–92%, and that homogenization of the agar layer before extraction did not raise the yield. The amount absorbed on the agar layer is expressed based on 100% recovery.

GC analysis

GC analysis was carried out using a GC apparatus (Model 353B; GL Sciences, Tokyo, Japan) equipped with a DB-5 column (0.5 mm × 30 m; J & W Scientific, Folsom, LA, USA) and a hydrogen ionization detector. Helium was used as a carrier gas with a flow rate of 3.8 mL/min. The column was raised from 60 to 160°C at a rate of 5°C/min. Terpene carboxylic acids that could not be analysed under these conditions were converted to trimethylsilylesters by treating the ethyl acetate extract with 10% trimethylsilyldiazomethane in methanol at room temperature for 30 min before analysis.

Gas chromatography/mass spectrometry (GC/MS) analysis was carried out using a GC apparatus (Model HP5890, Hewlett Packard) coupled with an MS apparatus (model HP5989, Hewlett Packard). The TC-5 column (0.25 mm × 30 m; GL Sciences) was raised from 60 to 200°C at a rate of 5°C/min. GC peaks were identified by comparing MS fragmentation pattern and relative retention time with those of the reference compounds. Quantitative determination of a constituent was made using the calibration curve of the dose-peak area of a pure compound.

Results

Antibacterial activity of essential oils and their major constituents by gaseous contact

Table II shows MID values obtained under closed conditions. All of the essential oils examined exhibited antibacterial activity, which generally increased in the following order: *E. coli* < *S. aureus* < streptococci < *H. influenzae*. No cross-resistance was observed between penicillin-resistant and -susceptible *S. pneumoniae* and no significant difference in susceptibility between *S. pneumoniae* and *S. pyogenes* was identified. Among the 14 essential oils, cinnamon bark, lemongrass and thyme (wild and red) oils showed the highest activity, inhibiting six strains at <12.5 mg/L air, with the exception of lemongrass oil, which exhibited weak activity against *E. coli* (MID, 100 mg/L air). Perilla, thyme (geraniol), peppermint, tea tree, coriander and lavender (spike and true) oils showed moderate activity against all isolates except *E. coli*. However, tea tree and coriander oils showed activity against *E. coli* comparable to that against other strains. Eucalyptus (radiata) oil, and especially citron oil, were weakest in activity.

Table III summarizes the MID values of major constituents and related compounds. The MIDs of major constituents were close to those of the parent essential oils. Among aldehyde constituents, cinnamaldehyde of cinnamon bark oil showed the highest activity, with a MID of <6.25 mg/L air against six strains. This was followed by citral, and then perillaldehyde, octanal and nonanal. Thymol, a phenolic constituent of thyme oil, showed activity comparable to that of cinnamaldehyde. Among terpene alcohols, the highest activity was demonstrated by geraniol. Menthol, terpinen-4-ol and linalool showed moderate activity and were comparable to each other. α-Terpineol was tested only against *S. aureus,* showing a MID of 25 mg/L in air. The activity of terpene ketones, menthone and camphor

as well as terpene ether (1,8-cineole) was weaker, and terpene hydrocarbons such as D-limonene and α-pinene were lowest in activity. The activity of ester constituents geranyl acetate and linalyl acetate exhibited very weak activity against *S. aureus*, showing an MID of 800 mg/L in air.

Examination of factors that affected the MID values

Table IV shows the effect of the evaporation rate of essential oil on MID against *S. aureus* and *E. coli*. It was evident that rapid evaporation via filter paper resulted in lower

Table II. Antibacterial activity of essential oils by gaseous contact

	MID (mg/L air)					
Essential oil	*H. influenzae* ATCC 33391	*S. pyogenes* ATCC 12344[a]	*S. pneumoniae* IP-692[a]	*S. pneumoniae* PRC-53[a,b]	*S. aureus* 209P[a]	*E. coli* NIHJ[a]
Cinnamon bark oil	3.13	6.25	3.13	1.56	6.25	12.5
Lemongrass oil	1.56	6.25	6.25	6.25	12.5	100
Perilla oil	12.5	12.5	12.5	25	50	>800
Thyme (wild) oil	3.13	6.25	3.13	3.13	12.5	12.5
Thyme (red) oil	3.13	3.13	6.25	6.25	6.25	12.5
Thyme (geraniol) oil	12.5	12.5	6.25	6.25	50	>1600
Peppermint oil	12.5	25	25	25	25	>1600
Tea tree oil	25	50	50	50	50	50
Coriander oil	12.5	25	25	25	50	50
Lavender (spike) oil	12.5	25	25	25	50	>1600
Lavender (true) oil	25	50	50	50	100	>1600
Rosemary oil	50	50	50	50	100	>1600
Eucalyptus (radiata) oil	25	50	50	100	200	>1600
Citron oil	200	200	400	400	800	>1600

MID was determined in airtight boxes using chocolate agar at 37°C for 18 h incubation.
[a]Blood agar.
[b]Penicillin-resistant strain.

Table III. Antibacterial activity of major constituents of essential oils and related compounds

	MID (mg/L air)					
Constituent	*H. influenzae* ATCC 33391	*S. pyogenes* ATCC 12344[a]	*S. pneumoniae* IP-692[a]	*S. pneumoniae* PRC-53[a,b]	*S. aureus* 209P[a]	*E. coli* NIHJ[a]
Cinnamaldehyde	1.56	3.13	3.13	3.13	6.25	6.25
Citral	3.13	3.13	6.25	6.25	12.5	>12.5
Perillaldehyde	12.5	12.5	12.5	12.5	50	>50
Octanal	12.5	25	25	25	50	25
Nonanal	12.5	12.5	12.5	12.5	25	>50
Thymol	3.13	3.13	3.13	3.13	3.13	>3.13
Geraniol	6.25	12.5	6.25	6.25	>25	>25
Menthol	6.25	25	25	25	25	>50
Terpinen-4-ol	12.5	25	25	25	25	25
Linalool	12.5	25	25	25	50	50
Camphor	50	100	100	100	400	>400
Menthone	50	200	200	200	200	>400
1,8-Cineole	25	200	200	200	>200	>200
D-Limonene	200	400	200	400	800	>800
α-Pinene	>800	800	400	400	800	>800

Experimental conditions are described in Table II.

Table IV. Effect of evaporation rate on MID values

Essential oil	MID (mg/L air)			
	S. aureus 209P		*E. coli* NIHJ	
	rapid evaporation[a]	slow evaporation[b]	rapid evaporation[a]	slow evaporation[b]
Cinnamon bark oil	6.25	>100	12.5	>100
Lemongrass oil	12.5	>100	100	>100
Perilla oil	50	>100	>100	>100
Thyme (wild) oil	12.5	>100	12.5	>100
Tea tree oil	50	800	50	>800
Lavender (true) oil	100	>800	>800	>800

MID was obtained using MHA after incubation at 37°C for 18 h in an airtight box.
[a]Rapid evaporation through a filter paper impregnated with a solution of essential oil in ethyl acetate.
[b]Slow evaporation of net essential oil from a glass vessel.

MID values than slow evaporation in a glass vessel. Thus, cinnamon bark oil exhibited 6.25–12.5 mg/L in air by rapid evaporation, but no inhibition was observed at 100 mg/L in air by slow evaporation. With tea tree oil, rapid evaporation gave a MID 16 times lower than slow evaporation. The effect of exposure time on MID was examined using two essential oils against *H. influenzae*. The MID values of tea tree and lemongrass oils after 2 h exposure were <50 and 3.13 mg/L, respectively, which were equivalent to, or double those, by overnight exposure. To elucidate the efficacy following rapid evaporation and short exposure, the time course of vapour concentration and absorption on an agar layer were examined using four representative constituents, and the results are shown in Figure 1. A single constituent was selected in place of a complex mixture of essential oil for ease of analysis of the results obtained.

Figure 1(a) shows the time course of citral, a major constituent of lemongrass oil. When the MID (6.25 mg/L in air) for *S. pneumoniae* was added to a closed box, the vapour level reached a maximum of 0.3 mg/L air around 1 h later, and then decreased rapidly. Similarly, absorption into the agar layer reached a maximum of 215 mg/L at 1–2 h, and then decreased in parallel with a decrease in vapour concentration. Figure 1(b) shows the time course of thymol of thyme oil at MID. Similar to citral, the maximum (0.1 mg/L air) in the head space was reached at 1 h, but then decreased gradually, differing from citral. The absorption maximum in the agar layer was at 2–4 h. The time course of terpinen-4-ol of tea tree oil (Figure 1c) was similar to that of thymol, showing a vapour maximum of 0.86 mg/L air, and agar maximum of 1100 mg/L at around 1–2 h. D-Limonene of citron oil (Figure 1d) showed a maximal vapour level (7.4 mg/L air) at 1 h, but, differing from other major constituents, it had accumulated very little in the agar layer, showing a maximum of 4.1 mg/L. Though not shown in the figure, perillaldehyde at MID (12.5 mg/L air) and octanal (12.5 mg/L air) showed a similar time course of vapour and agar levels to those of citral, while the behaviour of 1,8-cineole at MID (200 mg/L air) and α-pinene (400 mg/L air) was similar to that of terpinen-4-ol and D-limonene, respectively.

Alteration of essential oil constituents absorbed in agar layer during incubation

Table V shows the compositional analysis of five essential oils absorbed on blood or chocolate agar after overnight incubation at the MID. The chemical structures of major derivatives are shown in Figure 2. In the case of lemongrass oil, new degradation products, nerol, geraniol and a furan derivative, were formed, in addition to the original constituents of neral and geranial. Moreover, some formation of geranic acids b and a was indicated by the analysis of trimethylsilylester. In the case of perilla oil, reduction (perillyl alcohol) and oxidation (perillic acid) products and the *p*-menthane derivative were newly formed. In octanal and nonanal, no reduction product was observed, but oxidation products, octanoic acid and nonanoic acid, were predominantly formed. In D-limonene and α-pinene, a variety of oxygenated products were newly formed as shown in Table V and Figure 2.

Discussion

Since there has been no recognized measure to express the antimicrobial activity by gaseous contact, a new parameter, MID, was introduced in this investigation to compare the relative vapour activity of essential oils. The MID value was defined as the minimal inhibitory dose per unit space required to suppress the growth of microorganism in a closed system. The MID value expressed as mg/L air did not necessarily represent the actual vapour concentration, which changed constantly during incubation, as shown in

Figure 1. The maximal vapour concentration was far less than that calculated from the MID value. For example, the highest vapour levels for citral, thymol, terpinen-4-ol and limonene were 1/17 to 1/58 of the calculated levels. The lower vapour level was caused by loss of vapour such as by absorption into the medium and chamber, leakage outside and occasionally by spontaneous decomposition. However, the MID value was reproducible when the assay conditions were identical, and allowed comparison of the bioactivity of a series of volatile compounds.

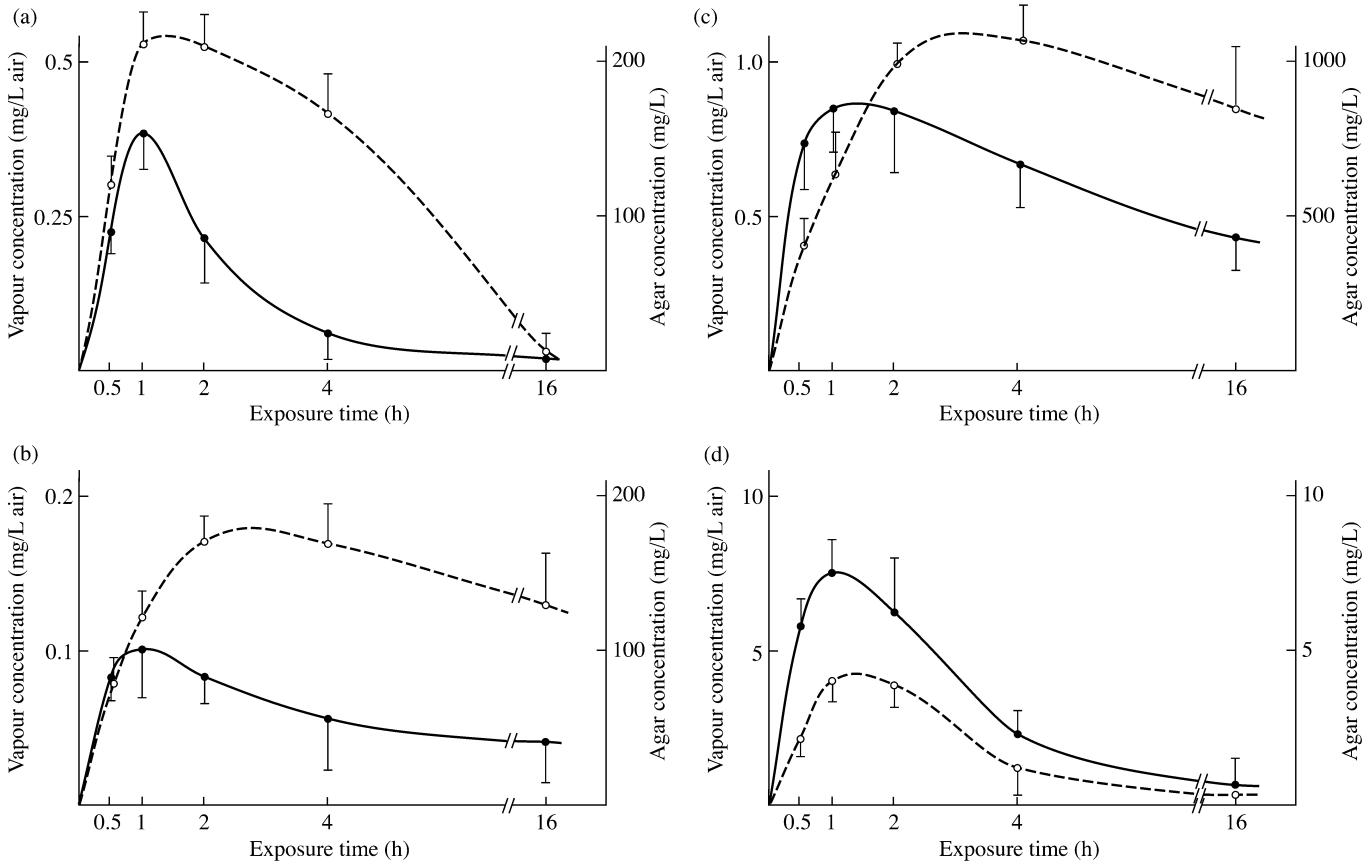

Figure 1. Time courses of vapour and MHA concentrations of essential oils at MID against *S. pnuemoniae* at 37°C in an airtight box. Concentration was expressed by average ± S.D. (n = 3). A solid line and a dotted line indicate vapour and agar concentrations, respectively. (a) Citral at 6.25 μg/mL air; (b) thymol at 3.13 μg/mL air; (c) terpinen-4-ol at 50 μg/mL air; (d) D-limonene at 200 μg/mL air.

Table V. Air-oxidation products of major constituents of essential oils during overnight incubation at 37°C

Essential oil/ constituent	Initial dose[a]	Compounds detected in agar (concentration, mg/L)
Lemongrass oil (blood agar)	6.25 mg/L air	linalool (1.8), furan derivative (2.1), nerol (4.8), neral (10.5), geraniol (8.4) geranial (15.9), geranic acid b (1.2), geranic acid a (2.1)
Perilla oil (blood agar)	12.5	*p*-menthane derivative (8.3), perillaldehyde (39.3), perillyl alcohol (9.0), perillic acid (4.2)
Octanal (chocolate agar)	12.5	octanal (4.8), octanoic acid (88.8)
Nonanal (blood agar)	12.5	nonanal (11.7), nonanoic acid (73.8)
D-Limonene (blood agar)	200	limonene (1.5), oxygenated limonene (M+, 134) (6.3), *p*-menthane derivative (8.1) *trans*-carveol (7.2), *cis*-carveol (3.3), unidentified (3.3)
α-pinene (blood agar)	400	α-pinene (1.4), dehydro-α-pinene (M+, 134) (2.2), oxygenated α-pinene (M+, 152) (7.8), verbenone (8.4), hydroxy-ether derivative (4.2), *t*-sobrerol (3.3)

[a]Initial dose was MID against *S. pneumoniae* IP-692 (blood agar) or *H. influenzae* ATCC 33981 (chocolate agar).

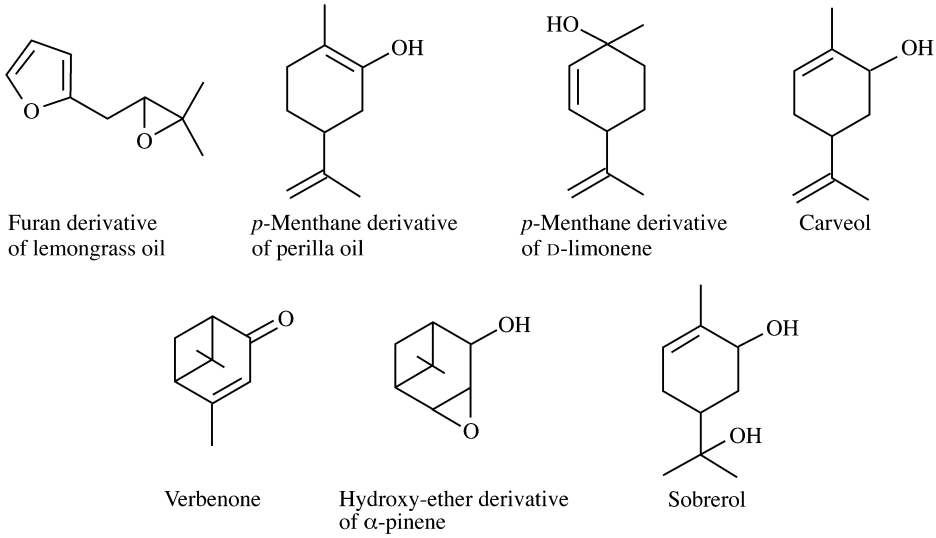

Figure 2. Chemical structures of air-oxidation products.

One of the most important findings in this investigation was that the MID values were dependent upon the rate of evaporation of essential oils; that is, rapid evaporation through impregnated filter paper showed <1/16 MID values than slow evaporation of absolute oils from a glass vessel. Under conditions of rapid evaporation, four major constituents showed maximal vapour and agar absorption within 2 h, and then gradually or rapidly decreased. Furthermore, vapour exposure for a short time (2 h) gave a comparable or two times greater MID than by overnight culture. The time course of vapour and agar concentrations under slow evaporation was not determined in this study, but was measured at 27°C during our study on the antifungal activity of essential oils (S. Inouye, K. Uchida and H. Yamaguchi, unpublished results). According to these results, the vapour of volatiles was maintained at a constant level during incubation, while accumulation into the agar layer was increased unilaterally in proportion to incubation time. These results could be rationalized if we assumed that the antibacterial activity by vapour contact was determined mainly by the maximal concentration of vapour and agar at the early stage of incubation, and that maintenance of vapour concentration might be not too important for the antibacterial activity.

As judged from MID values, the essential oils containing aldehyde or phenol as a major constituent were the most active, followed by those containing alcohol components. On the other hand, the essential oils containing ketone or ether, and especially hydrocarbon, as a major constituent were relatively weak. A 128-fold difference in MIDs was observed between the highly active cinnamon bark oil and the weakest citron oil. Furthermore, MID values of the major constituents were closely related to those of the respective essential oils, indicating that most of the activity of these oils was due to their major constituents, with the following exceptions.

Rosemary oil contained numerous components of comparable percentage as shown in Table I, but camphor was assumed to be a major contributor to the bioactivity from the comparison of MID values. A major component of eucalyptus oil in percentage weight was 1,8-cineole, but a major contributor for the bioactivity was assumed to be α-terpineol, which showed eight-fold higher activity than 1,8-cineole against *S. aureus*. 1,8-Cineole has been reported not to be an active principle in other eucalyptus oils.[15] A similar situation was seen for thyme (geraniol) oil and lavender oil, where the respective major constituents, geranyl acetate and linalyl acetate, were not the active principles of the oils, and active contributors were geraniol and linalool, which co-existed in the respective parent oils. Likewise, limonene, one of the major components, might not contribute significantly to the antibacterial activity of thyme (red) oil.

It has already been shown that the antimicrobial activity of volatile compounds results from the combined effect of direct vapour absorption on microorganisms and indirect effect through the medium that absorbed the vapour.[16] A significant contribution of the volatile compounds through agar absorption was reported for *E. coli*.[17] Therefore, measurement of agar concentration was important to determine the exact contribution of the vapour effect. Absorption into aqueous media was determined by hydrophobicity, volatility and stability of a volatile compound. Thus, carvacrol and thymol, which are very stable, moderately soluble in water and of low volatility, were accumulated into the agar layer in greater amounts than citral, which is unstable and of moderate volatility. D-Limonene, which is highly volatile and extremely insoluble in water, was accumulated only slightly in the agar layer, and readily evaporated when the vapour concentration was reduced. An antibacterial contribution from constituents absorbed on agar therefore could not be expected for citron oil.

The amount absorbed in agar was expressed by concentration (mg/L), since high mobility and high diffusing ability of terpene components were proven (data not shown). In addition to the assessment of the activity contribution via agar medium, the agar concentration may be useful for the judgement of the antimicrobial activity of terpene components, which was reported to be closely related to their aqueous solubility.[18] Moreover, terpene hydrocarbons and esters, which show extremely low agar concentration, as shown in Figure 1(d), are expected to show only a small inhibitory zone on the agar diffusion assay, owing to poor diffusion in liquid media.

A phenomenon that occurred predominantly in the gaseous state but was not marked in the solution phase was the spontaneous degradation of unstable constituents by air. Thus, oxidation or oxido-reduction of aldehyde groups and unsaturated bonds, and rearrangement of linkage were observed in octanal, nonanal, citral, perillaldehyde, D-limonene and α-pinene. Oxidation of citral, D-limonene and α-pinene in air is already recognized.[19–22] As judged from GC analysis, the chemical change of citral and perillaldehyde was not significant within 2 h, indicating that the degraded products may not contribute to the activity. On the other hand, formation of oxygenated products of D-limonene and α-pinene occurred within 30 min. The antibacterial activity of the oxygenated products of limonene and pinene was not determined in this study, but, based on our findings and those of others,[23] these terpene alcohols may possess more activity than the parent hydrocarbons, implying that they contributed significantly to the bioactivity. Incidently, D-limonene was shown to be stable in aqueous solution.[24]

Gram-positive bacteria are known to be more susceptible to essential oils than Gram-negative bacteria.[25,26] The finding that *E. coli* was least susceptible to 14 essential oils in this investigation is in accord with previous studies. The weak antibacterial activity against Gram-negative bacteria was ascribed to the presence of an outer membrane,[27,28] which possessed hydrophilic polysaccharide chains as a barrier to hydrophobic essential oils. Accordingly, the high degree of susceptibility of *H. influenzae* was unexpected. One reason for this might be the hydrophobic nature of the outer membrane of *H. influenzae* forming rough colonies, in contrast to *E. coli* and *Pseudomonas aeruginosa*, which form smooth colonies.

Absence of cross-resistance between penicillin-susceptible and -resistant *S. pneumoniae* is due mainly to the different mechanism of action of essential oils compared with that of β-lactam antibiotics.[29,30]

Summarizing these results, we conclude that the antimicrobial action of essential oils by gaseous contact is most efficient when exposed at high vapour concentration for a short time. The results obtained in this investigation suggested that a maximal vapour level of 0.1–0.9 mg/L in air may suppress the growth of the bacterial pathogens of respiratory infection. This level is slightly higher than that required for the suppression of experimental cough in guinea pigs (30–500 ppb),[31] but could be tolerated by mice.[32] Accumulation of essential oil vapours on trachea of mice and rats was demonstrated by natural inhalation.[33,34] Before the vapour therapy of essential oils is applied in clinical practice, important factors such as the minimal exposure time for efficacy and the bactericidal vapour concentration should be determined, in addition to the safety assessment.

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