

# Antimicrobial activity of four volatile essential oils

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# Antimikrobiella aktiviteten för fyra essentiella oljor

## Antimicrobial activity of four volatile essential oils

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### ABSTRACT

There has been an increasing interest in essential oils during recent years because of the need of new therapies against microbes. Bacterial resistance is spreading throughout the world primarily due to excessive use of antibiotics and poor infection control practices in hospitals, making it one of our times biggest issues. In this study, the volatile oils of *Syzygium aromaticum*, *Thymus serpyllum*, *Lavandula angustifolia* and *Lavandula x intermedia* were assessed for antimicrobial activity using the microatmosphere method. An assay was also performed to determine how different exposure times to essential oils effected the inhibition of microbes. The oils were tested against five organisms; methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Candida albicans*. The results showed considerable variability in the size of zone of inhibition depending which oil was used and no essential oil was observed to be the “best” against all organisms. *Pseudomonas aeruginosa* was the only bacterium not susceptible for any of the tested oils and *C. albicans* was the only microorganism that was susceptible to essential oils after an exposure time of 6 h. This study demonstrated that the volatiles from the tested essential oils have good antimicrobial effects.

### INTRODUCTION

Essential oils have been traditionally used for treatment of infections and diseases all over the world for centuries (Rios et al. 2005). Today the use of essential oils is a growing market and there are a considerable range of applications. The oils are used, for example, in the food and beverages industry and as fragrances in perfumes and cosmetics, but the oils also cover a broad spectrum of biological activity which has lead to an increased interest among researchers. In recent years there has been extensive research to explore and determine the antimicrobial activity of essential oils. All oils tested to date have displayed some antimicrobial activity and some have been shown to be more effective than others. Thymol, carvacrol, linalool and eugenol are main constituents of some plant essential oils

that have been shown to have a wide spectrum of activity against microbes (Kalemba et al 2003; Dorman et al. 2000). Members of this class are known to be either bactericidal or bacteriostatic, depending upon the concentration used. The mechanism of action is still unclear but some studies suggest that compounds penetrate the cell, where they interfere with cellular metabolism. Other studies suggest that phenols such as carvacrol and eugenol, disturb the cellular membrane and react with active sites of enzymes (Guynot et al 2003).

In the last decade there has also been an increased interest in essential oils and their antimicrobial activity due to the spread of antibiotic resistance. Since the discovery of penicillin by Alexander Fleming in 1929 many new classes of antibiotics have become available for treatment of bacterial infections, but due to excessive and often unnecessary use of antibiotics in humans and animals, bacterial resistance has now been reported against every currently available antibiotic (Gopal Rao 1999). Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE) and resistant strains of *Pseudomonas* are examples of multiresistant bacteria that are becoming an alarming problem within the healthcare system. MRSA is probably the most common antibiotic resistant bacterium found in hospitals throughout the world and it naturally colonises skin and infects wounds. Today the prevalence of MRSA is between 25-50 percent in parts of the world, including the USA, Australia, South America and central parts of Europe. Even in Scandinavian countries, where MRSA rates have been low, the frequency is beginning to rise (Grundmann et al 2006). VRE has also spread throughout the world since it was first discovered and isolated in the late 80's and can now be found in every continent. *Enterococci* can cause bacteremia, wound infection and urinary tract infection, but serious infections of VRE usually only occurs in patients with significantly compromised host defences (Linden 2002). *Candida* and *Pseudomonas* are other opportunistic pathogens that usually only lead to serious infections in immunocompromised individuals. Therapies for *Candida* have been difficult because of the limited number of antifungal agents, and for *Pseudomonas* even drug-susceptible strains have considerable defences against antibiotics (Livemore 2002; Rex et al 1995).

The factors responsible for the spread of resistant bacteria do not differ so much compared to the ordinary strains of bacteria and they are most frequently seen in hospitals. The most common route of spread is through indirect transmission from the healthcare staff to their patients. Staff may carry the resistant bacteria on their hands or clothing and even equipment in the hospital can become contaminated and a source of infection (Guynot et al 2003). New therapies are therefore necessary and of great value.

The aim of this study was to evaluate the antimicrobial activity of plant volatile oils and to determine how the inhibition was effected by different exposure times to the essential oil vapours. Four essential oils were tested against five different microorganisms: MRSA, VRE, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Candida albicans*.

## **MATERIALS AND METHODS**

### **Essential oils**

The essential oils used in this study were: clove (*Syzygium aromaticum*), wild thyme (*Thymus serpyllum*) and two varieties of lavender (*Lavandula angustifolia* and *Lavandula x intermedia*). The clove oil was purchased from Pharma Core, wild thyme oil from Australian Botanicals and the lavender oils from Norfolk respectively Bunyip Nursery.

### **Microorganisms and media**

The essential oils were assayed against following microorganisms: MRSA, VRE, *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus pyogenes* and *Candida albicans* (ATCC 10231). MRSA, VRE and *Strep.pyogenes* were clinical strains obtained from Wagga Wagga Base Hospital.

The bacteria were grown in nutrient broth (10 g tryptone, 5 g yeast extract, 10 g sodium chloride, water to 1 L) and Mueller-Hinton agar (beef- dehydrated infusion from 300 g, 17.5 g casein hydrolysate, 1.5 g starch, 17 g agar, water to 1 L). Sabouraud dextrose agar (10 g mycological peptone, 40 g glucose, 15 g agar, water to 1 L) was used as growing medium for *C.albicans*. All media was purchased from Oxoid, UK.

### **Statistical analysis**

Differences between size of zones of inhibition were tested using Students' *t* test or ANOVA with differences being deemed significant where  $p < 0.05$ .

### **Experimental method**

First a primary screening was performed to ensure that there were no differences in the bacterial growth between agar plates made out of glass and plastic due to the vapour from the essential oils. Positive controls were also used to ensure that MRSA and VRE were resistant against methicillin and vancomycin respectively. The methods and volumes used for these assays were the same as described below except for the antibiotic susceptibility test, where the activity was determined using the disc diffusion assay. Broth cultures (250  $\mu$ L) of MRSA and VRE were spread on to different agar plates and antibiotic discs were then placed on to the surface of the agar. All discs used in this study, antibiotic- as well as blank discs, were purchased from Oxoid.

The antimicrobial activity was determined using the microatmosphere method. Agar plates were made at least one day before use and broth culture of bacteria was prepared fresh for each assay. The concentration of the broth culture was measured using a spectrophotometer (standard curves were obtained from Charles Sturt University, Wagga Wagga) and a standard plate count of viable microorganism was also performed to insure that a sufficient cell concentration was used (bacteria:  $10^6$  CFU/ml, candida:  $10^4$  CFU/ml). The plates were dried (37  $^{\circ}$ C, 20 min) before 250  $\mu$ L of broth culture

was spread on to the surface. Cultures were vortexed for 15 s before being pipetted on to the agar. A coverslip was fixed with a droplet of water on to each lid of the agar plates and blank discs (sets of; 2, 4 and 6) were then transferred on to the cover slips. The cover slips were placed in the centre of the lids and the discs were placed as close together as possible. 10 µL of essential oils were then pipetted on to each disc giving a volume range of 20, 40 and 60 µL between the agar plates. For control plates 10 µL sterile water was pipetted on to the discs. The agar plates were then sealed with parafilm and separated into plastic bags, with only one kind of essential oil in each bag. After 18 h of incubation at 37°C, the results were recorded by measuring the clear inhibition zone with a slide calliper. For oils that had a good antimicrobial effect, an additional assay was performed to determine how the inhibition was effected by different exposure times to the essential oils. The microorganisms were exposed for 1 h, 3 h and 6 h respectively to 60 µL of essential oil. The lids were changed after the exposure and then the plates were reincubated overnight. The assays were completed in duplicate and repeated independently three times.

An assay was also performed to determine whether the antimicrobial effects were bacteriostatic or bacteriocidal. After incubation, a sterile loop was used to transfer bacteria from the agar plates exposed to 60 µL of essential oils on to fresh plates. If a total reduction in growth density was seen, colonies were taken randomly and spread on to the new plates. Otherwise the bacteria were taken from the inhibition zone. The fresh agar plates as well as the old were then incubated (37 °C, 18 h). On the backside of the old plates a circle was drawn before incubation, marking the initial inhibition zone.

## RESULTS

There were no differences in bacterial growth between the use of plastic and glass petri dishes and the antibiotic susceptibility test confirmed that VRE and MRSA were resistant against vancomycin and methicillin respectively. The results of the microatmosphere assay of four essential oils against five microorganisms are shown in Table 1. *Pseudomonas aeruginosa* was the only bacterium not susceptible to any of the oils and was therefore excluded from further assays. The antimicrobial effects of the essential oils were bacteriocidal against all organisms except for VRE, where *T. serpyllum* was the only oil with a cidal effect.

There was considerable variability in size of zone of inhibition depending which oil was used and statistically significant differences in antimicrobial activity was also observed between the use of different volumes. Greater zones of inhibition were observed when 60 µL of essential oil was used rather than 40 µL for MRSA against *T. serpyllum* ( $p = 0.0002$ ), *L. intermedia* ( $p < 0.0001$ ), *S. aromaticum* ( $p < 0.0001$ ) and for *Strep. pyogenes* against *L. intermedia* ( $p = 0.02$ ). If not mentioned, further comparison indicates volumes of 60 µL.

No essential oil was observed to be the “best” against all organisms, with *C. albicans* having constantly larger zones than the other organisms. *T. serpyllum* produced significantly

**Table 1** Activity of essential oils against bacteria and the yeast, *C. albicans*.

	MRSA	VRE	C.albicans	Strep.pyogenes
<i>Clove (Syzygium aromaticum)</i>				
6x10 $\mu$ l	20.1 $\pm$ 1.9	q	30.9 $\pm$ 1.6	q
4x10 $\mu$ l	13.25 $\pm$ 1.5	q	27.6 $\pm$ 1.9	q
2x10 $\mu$ l	q	q	19.6 $\pm$ 1.9	q
<i>Wild thyme (Thymus serpyllum)</i>				
6x10 $\mu$ l	29.8 $\pm$ 1.1	18.7 $\pm$ 1.2	1+, 1+	24.7 $\pm$ 3.6
4x10 $\mu$ l	21.6 $\pm$ 2.2	7.7 $\pm$ 3.3	32 $\pm$ 3.2 / 2+, 1+	19.2 $\pm$ 3.8
2x10 $\mu$ l	12.1 $\pm$ 1.3	4+	27.3 $\pm$ 4/ 2+, 1+	q
<i>Lavender (Lavandula angustifolia)</i>				
6x10 $\mu$ l	14.2 $\pm$ 1.5	q	1+	22.6 $\pm$ 4
4x10 $\mu$ l	q	4+	1+/ 2+	q
2x10 $\mu$ l	4+	4+	2+	q
<i>Lavender (Lavandula x intermedia)</i>				
6x10 $\mu$ l	27.9 $\pm$ 1.6	q	0	25.4 $\pm$ 3.5
4x10 $\mu$ l	20.7 $\pm$ 1.3	4+	1+	q
2x10 $\mu$ l	q	4+	1+/ 2+	q
Control (sterile water) <i>No inhibition was recorded</i>				

Results are presented as mean ( $\pm$ standard deviation) size of inhibition (in mm). Growth density, market cursive, is presented in a scale from 0 to 4+. With zero indicating no growth and 4+ indicating the growth of the control. q = Not a clear inhibition zone for one or several of the samples.

larger zones of inhibition against MRSA than VRE ( $p < 0.0001$ ) and *Strep. pyogenes* ( $p = 0.008$ ). For the same oil *Strep. pyogenes* was observed to have a larger zone compared to VRE ( $p = 0.0036$ ). Statistically significant differences in antibacterial activity were also observed between MRSA and *Strep. pyogenes* against *L. angustifolia* ( $p = 0.0007$ ). *L. intermedia* and *L. angustifolia* had the best effect against *C. albicans* with a total reduction in growth, while a clear zone of inhibition was obtained against *T. serpyllum* (30.9  $\pm$  1.6 mm). The most effective oils against MRSA were *T. serpyllum* and *L. intermedia*, with no significant difference in the size of inhibition between the two oils. *S. aromaticum* produced larger zones of inhibition than *L. angustifolia* against MRSA ( $p < 0.001$ ).

No inhibition was observed for VRE after exposure the bacteria to *T. serpyllum* for 6 h. The same result was observed for MRSA against all essential oils except for *S. aromaticum* and *T. serpyllum*,

where inhibition zones (although not clear) could be noticed. After 6 h exposure, clear inhibition zones were observed for *C. albicans* against *S. aromaticum* ( $18.1 \pm 2.9$  mm) and *T. serpyllum* ( $17 \pm 1.6$  mm). *L. angustifolia* and *L. intermedia* gave a total reduction in growth, but the reduction was not as great as after 18 h of exposure. *C. albicans* was also observed to be susceptible to *L. angustifolia* and *L. intermedia* after a 3 h exposure, giving inhibition zones of  $15.5 \pm 1.3$  mm respectively  $22.8 \pm 3.3$  mm. No results were obtained for *Strep. pyogenes* because of growth problems following subculture. The most likely explanation is that the bacteria on the stock-plate were dead when they were transferred to the nutrient broth.

## DISCUSSION

Previous studies carried out on the inhibitory effect of essential oil vapours are limited which makes it difficult to compare and confirm results. Most research that has been performed is based on the agar diffusion method and hasn't considered the possible antimicrobial effect by the vapour from the essential oils.

This study have shown that the vapours from *L. angustifolia*, *S. aromaticum*, *T. serpyllum* and *L. intermedia* have an antimicrobial effect and that no oil is superior to any other in this respect. The only organism that was not susceptible to any of the tested oils was *Ps. aeruginosa*. Many research works confirm that *Ps. aeruginosa* is more resistant against EOs (essential oils) because of the cell wall structure. Gram-negative bacteria have an outer lipopolysaccharide wall that can work as a barrier against toxic agents (Gaunt et al. 2005). *T. serpyllum* was proven to have good antimicrobial effect against MRSA, *Strep. pyogenes* and *C. albicans*. As with *S. aromaticum*, *T. serpyllum* consists of phenolic structures (eg. eugenol and carvacrol), which have been confirmed by many studies to have a good antimicrobial activity (Kalemba et al. 2003). Essential oils with predominant alcoholic compounds have in previous studies been shown to be slightly less active than compounds containing phenolic structures (Dorman et al 2000). This statement could not be confirmed by this current study. However the correlation between the composition and activities of essential oils have not been brought to satisfying conclusion yet and the good antimicrobial effect of lavender oils against MRSA, VRE and *Strep. pyogenes* have been investigated and confirmed by Moon et al (2006). *T. serpyllum* was the only oil that gave a clear zone of inhibition against VRE. Some essential oils, including the tested lavender oils, have been demonstrated to have *in vitro* activity against VRE using disk diffusion assays (Cavanagh 2002).

It has been noted that the inhibitory effect of EO can differ between the volatiles and the direct contact with microorganisms and the inhibitory effect of some essential oils on fungi have been reported to be greater when the oil volatile are used. *C. albicans* was susceptible to all of the tested essential oils and was the only organism where good antimicrobial activity was observed after been exposed to EO for 6 h. The antifungal activity of the volatile phase of essential oils has been

reviewed by Cavanagh (2007) and confirms that many oils possess strong activity against a wide range of fungi. It is unclear exactly how the volatiles are inhibiting fungal growth and why some essential oils have better activity against fungi than against bacteria. One explanation could be the fact that the antimicrobial activity of volatile compounds results from the combined effect of direct vapour absorption on microorganism and indirect effect through the medium that absorbed the vapour (Inouye et al 2000; Inouye et al 2001). Fungi grow mainly on the surface of the agar medium and might be more susceptible to direct vapour contact while the antimicrobial effect against bacteria might be more dependant on the vapour accumulation into the agar. This could explain why no clear zones of inhibition were observed after 6 h of incubation with essential oil against MRSA and VRE. Another explanation is that some fungi are more susceptible to essential oils than bacteria. The exact mechanism of action of essential oil volatile on fungi is unclear but the majority of reports agree that oil volatiles result in morphological changes to the hyphae (Cavanagh 2007).

The method used in this study for the estimation of essential oil activity is a well-tried method (Guynot et al. 2003). In this study a range of sterile discs were used to allow comparison of antimicrobial effects using different volumes of essential oils. It was proven to be difficult to place and keep the sterile discs close together, which could have increased the variance of the results. In future studies it may be better to use sterile discs of different sizes, rather than placing several discs next to each other.

This study has demonstrated that the volatile from the tested essential oils have good antimicrobial effect and that a longer exposure time than 6 h is necessary to obtain good results against bacteria. The good effect against MRSA for some of the oils should be further investigated. The result suggests that essential oils have the potential to be used as antimicrobial agents both for medical and for more commercial applications. Essential oil volatiles have the advantage that they can treat large areas and do not require direct contacts with liquid oils which can make it suitable for use as disinfectant of rooms and as a component in for example cleaning products. The oils might also be used as inhalation therapy against bacterial respiratory tract pathogens as *Strep. pyogenes* which can cause pharyngitis. Before the vapour therapy of essential oil is applied in clinical practise further studies are required to determine the range of the possibilities- important factors such as the minimal exposure time for efficacy, applicability and the possibility of toxicity needs to be further evaluated. It's also a challenge to determine which components in an essential oil that have the antimicrobial activity, and which may be the natural toxicant. Although extensive research have been done within this field, an essential oil may contain 40 or more different identifiable component chemicals which makes it difficult to determine the active components. Further research is still required.

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