Antimicrobial effect against different bacterial strains and bacterial adaptation to essential oils used as feed additives

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Abstract

The aim of this study was to evaluate the antimicrobial activity and determine the minimum bactericidal concentration (MBC) of the essential oils derived from *Origanum vulgare* (oregano), *Melaleuca alternifolia* (tea tree), *Cinnamomum cassia* (cassia), and *Thymus vulgaris* (white thyme) against *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*. The study also investigated the ability of these different bacterial strains to develop adaptation after repetitive exposure to sub-lethal concentrations of these essential oils. The MBC of the essential oils studied was determined by disc diffusion and broth dilution methods. All essential oils showed antimicrobial effect against all bacterial strains. In general, the development of adaptation varied according to the bacterial strain and the essential oil (tea tree > white thyme > oregano). Therefore, it is important to use essential oils at efficient bactericidal doses in animal feed, food, and sanitizers, since bacteria can rapidly develop adaptation when exposed to sub-lethal concentrations of these oils.

Résumé

*La présente étude avait pour but d'évaluer l'activité antimicrobienne et de déterminer la concentration bactéricide minimale (CBM) des huiles essentielles dérivées d'*Origanum vulgare *(origan), de* Melaleuca alternifolia *(l'arbre à thé), de* Cinnamomum cassia *(cassia), et de* Thymus vulgaris *(thym blanc) contre* Salmonella *Typhimurium,* Salmonella *Enteritidis,* Escherichia coli, Staphylococcus aureus, *et* Enterococcus faecalis. *L'étude visait également à examiner la capacité de ces différentes souches bactériennes à développer une capacité d'adaptation après une exposition répétée à des concentrations sub-létales de ces huiles essentielles. La CBM des huiles essentielles étudiées a été déterminée par des méthodes de diffusion en disque et de dilution en bouillon. Toutes les huiles essentielles ont démontré un effet antimicrobien contre toutes les souches bactériennes. En général, le développement de la capacité d'adaptation variait selon la souche bactérienne et l'huile essentielle (arbre à thé > thym blanc > origan). Il est donc important que les huiles essentielles soient utilisées à des doses bactéricides efficaces dans la nourriture animale, les aliments, et les assainisseurs, étant donné que les bactéries peuvent rapidement développer une adaptation lorsqu'exposées à des concentrations sub-létales de ces huiles.*

(Traduit par Docteur Serge Messier)

Introduction

The use of antibiotics as growth promoters has attracted significant attention and scrutiny due to its potential risk as a source of bacterial resistance, which is a current public health concern. In fact, many countries have already banned the use of antibiotics in animal production (1). The use of essential oils (EOs) as a feed additive has increasingly gained interest due to their potential use as alternatives to the antibiotics commonly applied in the livestock industry to promote animal growth.

In recent years, several studies have shown that bacteria are susceptible to different essential oils, including those derived from *Origanum vulgare* (oregano), *Melaleuca alternifolia* (tea tree), *Cinnamomum cassia* (cassia), and *Thymus vulgaris* (white thyme). The general compounds found in these essential oils are shown in Table I (2–9). Although essential oils are viewed as natural, their current use in the animal and food industries leads us to an important question. Does the use of essential oils at sub-lethal concentrations promote bacterial adaptation/resistance, since the development of microbial adaptation can dramatically reduce the effectiveness of the essential oils and cannot guarantee that the animal will not pose a health risk to humans (10)?

Another potential issue in the study of the antimicrobial activity of essential oils concerns the differences among the methods applied. Broth dilution and disc diffusion methods are often used to measure the antimicrobial activity of these compounds (11,12), but results may differ when different tests are used.

The present study was conducted to evaluate the antimicrobial activity and determine the minimum bactericidal concentration (MBC) of essential oils against Gram-positive and Gram-negative

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Received March 27, 2015. Accepted May 8, 2015.

Table I. Five major compounds commonly found in *Thymus vulgaris* (White thyme), *Origanum vulgare* (Oregano), *Melaleuca alternifolia* (Tea tree), and *Cinnamomum cassia* (Cassia) essential oils

Number	Compound	%	References
White thyme			
1	Thymol	48.9–57.7	(2,3)
2	p-cymene	16.0–19.0	(3,2)
3	γ-terpinene	4.1–8.4	(2,3)
4	Carvacrol	2.8–3.5	(3,2)
5	β-caryophyllene	0–3.5	(2,3)
Oregano			
1	Carvacrol	14.5–65.0	(5,4)
2	Thymol	0.3–12.6	(4,5)
3	γ-terpinene	4.3–11.6	(4,5)
4	p-cymene	0–9.0	(4,5)
5	α-terpinene	0.1–3.7	(4,5)
Tea tree			
1	Terpinen-4-ol	39.8–40.4	(6,7)
2	γ-terpinene	17.8–19.5	(6,7)
3	α-terpinene	7.7–8.3	(7,6)
4	1,8-cineole	4.5–5.2	(6,7)
5	p-cymene	2.3–4.7	(6,7)
Cassia			
1	Cinnamaldehyde	30.4–85.0	(8,9)
2	Methoxy-1,2-propanediol	0–29.3	(9,8)
3	δ-Methoxy-cinnamaldehyde	8.8–25.4	(9,8)
4	Coumarin	0–6.4	(9,8)
5	Glycerin	0–3.0	(9,8)

bacteria using two different methods, as well as to investigate the ability of different bacterial strains to develop adaptation after repetitive exposures to essential oils at sub-lethal concentrations.

Materials and methods

Essential oils

Commercially available essential oils from *Origanum vulgare* (oregano), *Melaleuca alternifolia* (tea tree), *Cinnamomum cassia* (cassia), and *Thymus vulgaris* (white thyme) were obtained from NOW Foods (Bloomingdale, Illinois, USA).

Bacterial strains

The essential oils were tested against *Salmonella* Typhimurium (*S.* Typhimurium, ATCC 13311), *Salmonella* Enteritidis (*S.* Enteritidis, ATCC 13076), *Escherichia coli* (*E. coli*, ATCC 25922), *Staphylococcus aureus* (*S. aureus*, ATCC 29213), and *Enterococcus faecalis* (*E. faecalis*, ATCC 29212). These bacterial strains were acquired from the American Type Culture Collection (ATCC, Rockville, Maryland, USA).

Determination of antimicrobial activity and minimum bactericidal concentration

Each bacterial species was individually grown in test tubes containing 10 mL of Müller-Hinton broth (MHB; Difco Laboratories, Franklin Lakes, New Jersey, USA) for 24 h at 37°C. A 100-μL aliquot was then transferred to a test tube containing 9.9 mL of fresh MHB, resulting in a final population of 7 to 8 log CFU/mL. The antimicrobial activity of the essential oils was determined for each bacterial species by the disc diffusion method. Aliquots were drawn from each bacterial solution using a sterile cotton swab, which was then used to inoculate the surface of plates containing Müller-Hinton agar (MHA; Difco). Five sterile paper discs, 6 mm in diameter (VWR, Foster City, California, USA), were placed on the agar surface using sterile tweezers. Four paper discs were embedded with 20 μL of 1 essential oil (pure) each and 20 μL of sterile water was added to the fifth disc as a control. The plates were then incubated at 37°C for 24 h. After incubation, the diameter of each inhibition zone formed under the paper disc was measured using a caliper (Precision Plastic Vernier Calipers; VWR) and the sensitivity of the bacteria to each essential oil was classified according to the size of this zone [non-sensitive for a diameter smaller than 8 mm, sensitive for a diameter of 9 to 14 mm, very sensitive for a diameter of 15 to 19 mm, and extremely sensitive for a diameter larger than 20 mm (13)].

The minimum bactericidal concentration (MBC) of the essential oils was determined by disc diffusion and broth dilution methods applied according to the Clinical Laboratory Standards Institute (14). To determine the MBC with the agar disc diffusion test, the essential oils were diluted in ultrapure water until the minimum concentration able to inhibit the bacterial growth was visually observed by

Table II. Antimicrobial activity of 20 μL of pure essential oils determined by disc diffusion method

	Inhibition zone (mm)			
Bacteria	Oregano	White thyme	Tea tree	Cassia
S. Typhimurium	$50.9 \pm 1.32^{a,b}$	62.4 ± 11.0^{a}	$50.8 \pm 4.67^{a,b}$	39.5 ± 4.9^{b}
S. Enteritidis	$37.2 \pm 2.71^{a,b}$	60.27 ± 1.62^{a}	$37.4 \pm 4.37^{a,b}$	35.3 ± 3.15^{b}
E. coli	$38.7 \pm 0.99^{a,b}$	64.4 ± 1.44^{a}	$35.03 \pm 1.78^{b,c}$	30.47 ± 1.40^{c}
S. aureus	$39.6 \pm 1.65^{b,c}$	64.8 ± 1.06^{a}	29.03 ± 2.89^{c}	$40.43 \pm 2.61^{a,b}$
E. faecalis	31.8 ± 1.01^{b}	50.67 ± 2.52^{a}	23.43 ± 4.3^{c}	33.2 ± 1.84^{b}

^{a,b,c} Different letters in the same line represent statistical difference ($P < 0.05$) in the size of inhibition zones formed under the paper disc by each essential oil.

Table III. Minimum bactericidal concentration (MBC) of essential oils determined by disc diffusion (DD) and broth dilution (BD) methods

	MBC of each essential oil (μL/mL)							
	Oregano		White thyme		Tea tree		Cassia	
Bacteria	DD	BD	DD	BD	DD	BD	DD	BD
S. Typhimurium	$0.25^{C,a}$	$0.06^{B,b}$	$0.12^{D,a}$	$0.03^{C,b}$	$4^{A,a}$	$0.25^{A,b}$	$2^{B,a}$	$0.03^{C,b}$
S. Enteritidis	$0.12^{C,a}$	$0.03^{C,b}$	$0.12^{C,a}$	$0.015^{A,b}$	$4^{A,a}$	$0.12^{B,b}$	$2^{B,a}$	$0.03^{C,b}$
E. coli	$0.12^{C,a}$	$0.03^{B,b}$	$0.12^{C,a}$	$0.03^{B,b}$	$4^{A,a}$	$0.12^{A,b}$	$2^{B,a}$	$0.12^{A,b}$
S. aureus	$0.25^{C,a}$	$0.06^{B,b}$	$0.12^{D,a}$	$0.03^{C,b}$	$8^{A,a}$	$0.25^{A,b}$	$4^{B,a}$	$0.25^{A,b}$
E. faecalis	$0.25^{B,a}$	$0.06^{B,b}$	$0.12^{C,a}$	$0.06^{B,b}$	$4^{A,a}$	$0.25^{B,b}$	$4^{A,a}$	$0.25^{B,b}$

^{A,B,C} Same letters in the same line represent statistical difference ($P < 0.05$) between the method used to determine the MBCs.

^{a,b,c} Different letters in the same line represent statistical difference ($P < 0.05$) between the MBCs of each essential oil.

the absence of bacterial growth in the plates. Overnight, bacterial cultures (7 to 8 log CFUs/mL) were used as an inoculum as previously described. The MBC for each bacterial strain was determined in parallel by the broth microdilution method, whereby the essential oils were serially diluted (2-fold) in 9.9 mL of MHB and added to 0.1 mL of bacterial inoculum. The tubes were screw-capped and incubated overnight at 37°C on a shaker (150 rpm) to minimize the separation of the essential oils from the aqueous phase. After incubation, 100 μL from each tube was inoculated in MHA and incubated overnight at 37°C. The lowest concentration that was able to completely inhibit bacterial growth in the MHA plates was considered to be the minimum bactericidal concentration (MBC). For the disc diffusion method, different concentrations of each essential oil were added to 6 mm paper discs and placed in inoculated petri dishes as described previously. The lowest concentration that formed an inhibition halo observed visually was considered as the MBC. A total of 3 independent replicates was tested for each bacterial strain, essential oil, and method.

Test for bacterial resistance/adaptation to essential oils

The broth dilution method was used to determine the possibility of bacterial adaptation after exposure to sub-lethal concentrations of the essential oils. Concentrations representing half of the MBC from oregano, tea tree, and white thyme essential oils (only the 3 strongest essential oils were selected for this phase of the study) were added to 10 mL of MHB inoculated with 7 to 8 log CFUs/mL of each bacterial species and incubated at 37°C for 24 h. Then, 100 μL of the EO-exposed cultures was added to fresh MHB containing half the minimum bactericidal concentration (MBC) of each essential oil again and incubated at 37°C for 24 h. This step was repeated for a third time, when the bacteria were exposed to the previously determined MBC of the essential oils. After 24 h of incubation at 37°C, 20 μL of the bacterial suspensions was cultured in MHA. After a 24-h incubation period at 37°C, each plate was examined for bacterial growth and classified as positive (+) or negative (−), which indicated the presence or absence of growth, respectively. As 3 independent replicates were carried out, each combination of bacterial strain and essential oil received a total of 3 scores (+ and/or −). The method carried out for this study was adapted from Becerril et al (10).

Statistical analysis

The data obtained from the 3 independent replicates tested to determine the antimicrobial activity and minimum bactericidal concentration were analyzed by applying analysis of variance (ANOVA) and the differences between the means and standard deviation were tested by Tukey test, considering $P < 0.05$.

Results

The antimicrobial activity of the essential oils of white thyme, oregano, cassia, and tea tree against *S.* Typhimurium (ATCC 13311), *S.* Enteritidis (ATCC 13076), *E. coli* (ATCC 25922), *S. aureus* (ATCC 29213), and *E. faecalis* (ATCC 29212) was determined by the disc diffusion method. It was found that all bacterial strains used were sensitive to all essential oils tested (based on large and clear growth-inhibition zones), which suggests a broad spectrum against both

Table IV. Bacterial growth or inhibition after repetitive exposure to sub-lethal doses of different essential oils

	Growth (+) or Inhibition (−)			
Bacteria	Control	Oregano	White thyme	Tea tree
S. Typhimurium	+ + +	+ + −	+ + +	+ + +
S. Enteritidis	+ + +	+ + +	+ + +	+ + +
E. coli	+ + +	+ − +	+ + +	+ + +
S. aureus	+ + +	+ − −	+ + +	+ + +
E. faecalis	+ + +	− − −	− + −	+ + +

Each symbol (+ or −) represents 1 replicate for a total of 3 independent replicates.

Gram-positive and Gram-negative bacteria (Table II). In most cases, however, MBCs for Gram-negative bacteria were lower in comparison to MBCs for Gram-positive bacteria, which suggests higher sensitivity to the oils tested. Overall, the white thyme oil had the strongest ($P < 0.05$) MBCs by the broth dilution method (0.015% to 0.06%), followed by the oregano (0.03% to 0.06%), cassia (0.03% to 0.25%), and tea tree (0.12% to 0.25%) oils. The MBCs determined by disc diffusion presented higher values, with white thyme still having the strongest effect (0.12% to 0.12%), followed by oregano (0.12% to 0.25%), cassia (2% to 4%), and tea tree (4% to 8%) (Table III). The MBCs determined by broth dilution were significantly lower ($P < 0.05$) than MBCs determined by disc diffusion (Table III).

In general, it was observed that the bacterial strains could grow or not grow, according to bacterial strain and essential oil (tea tree > white thyme > oregano) after the exposure to sub-lethal concentrations of EOs (Table IV). *Enterococcus faecalis* did not develop adaptation to oregano oil when re-exposed to the previously determined MBC (Table IV).

Discussion

A broad spectrum of antimicrobial activity was observed for all essential oils tested as reported in previous studies (3,15). However, the varied potency among essential oils (Table II) can be explained by the differences among the chemical compounds, as well as the different concentrations of compounds found in each essential oil (3). Furthermore, essential oils can have a varied degree of penetration on the agar and volatilization, which can alter the size of the inhibition zone and consequently reduce the antimicrobial effect (16). The difference observed in bacterial sensitivity to the essential oils tested may also be due to the solubility of these oils. In addition, the different chemical composition of the same essential oil can be attributed to a number of factors, including the part of the plant used to extract the oil, drying stress, the development stage of the plant when harvested, and growth conditions, such as the type of soil, temperature, and fertilizers used, as well as the extraction procedure itself.

In the MBC evaluation, the broth dilution method provided a more reliable comparison, since the oils are more freely dispersed when homogenized in broth, whereas there is a great variability of penetration and diffusion of these compounds when they are added to agar. Moreover, this method is conducted in a tightly closed test tube that prevents volatilization of the oil, while the Petri dish can lose some of the oil content. Thus, the method used to determine the MBC of different compounds should take into account the physicochemical properties of the substances tested (17).

According to the test used to investigate the bacterial adaptation after repetitive exposures to sublethal doses of the essential oils, if at least 1 repetition shows bacterial growth, bacterial adaptation to essential oils can occur. It should be noted, however, that the process of the development of bacterial adaptation varied according to bacterial strain and the essential oil tested (tea tree > white thyme > oregano). Curiously, *E. faecalis* (ATTCC 29212) was the only strain that did not develop adaptation to oregano oil in all repetitions. According to Cordeiro et al (18), *E. coli* O157:H7 strain 02-0304 was able to adapt after repetitive exposures to sub-MBC of allyl isothiocyanate (mustard essential oil). The bacterium did not develop resistance, however, since its sensitivity to allyl isothiocyanate returned after the growth of *E. coli* O157:H7 strain 02-0304 in broth without the essential oil. The authors showed that the BaeSR two-component system was associated with the adaptation against allyl isothiocyanate (18). The mechanisms in which the BaeSR system promotes bacterial resistance against extrinsic stressors are not fully understood, but it was shown to regulate the expression of *spy* (19). This gene is involved with the formation of the cell envelope and membrane protein folding. Moreover, the BaeSR system also regulates the *mdtABCD* operon (19), and these genes codify a multi-drug efflux pump that confer bacterial resistance against several antibiotics.

Similar to our study, *Bacillus cereus* (IFR-NL94-25) became less sensitive to carvacrol after exposure to sub-lethal concentrations of this compound (0.4 mM) (20). Lower membrane fluidity was observed in the adapted *B. cereus* cells due to alterations in the fatty acid composition, which presented increased levels of *iso*-$C_{13:0}$, $C_{14:0}$ and lower concentrations of *iso*-$C_{16:1}$ and $C_{18:0}$. In addition, *Morganella morganii*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*, which were isolated from clinical human samples with varied susceptibility profile to antibiotics, showed higher MBCs after exposure to sub-lethal concentrations of oregano essential oil. Some bacterial strains of *P. aeruginosa* were also less responsive to tea tree essential oil and its monoterpene components, terpinen-4-ol, 1,8-cineole, and alpha-terpineol, which was attributed to the role of MexAB-OprM and its interplay with MexCD-OprJ in mediating efflux pumps (21). The same strains and *Serratia marcescens* did not show changes in MBCs after exposure to *Cinnamomum zeylanicun* essential oil (10). The complex composition of essential oils (Table I) result in a multi-targeted mechanism of antimicrobial activity. This complexity may result in the need of different mechanisms to develop microbial resistance against EOs, and therefore, it is thought that the resistance to EOs rarely happens.

In conclusion, this study demonstrates the potential use of EOs against Gram-positive and Gram-negative bacteria. However, it also shows that results need to be cautiously interpreted and the analytical method used should be taken into consideration. Broth dilution method was more sensitive and reliable for determining the MBC of essential oils than the disc diffusion method. This study also reports the tendency of bacteria to adapt to essential oils when exposed to sub-lethal concentrations *in vitro*. Although essential oils are perceived as natural alternatives to antibiotics,

their misuse could have unintended consequences as bacteria can rapidly develop adaptation when exposed to sub-lethal concentrations of these oils. Further studies are needed to better understand the occurrence of this adaptation under *in vivo* conditions, particularly when essential oils are administered to animals as feed additives.

Acknowledgments

Antonio Diego Brandão Melo thanks the Science Without Borders Program (CNPq) from the Brazilian government for financing his internship at Purdue University. The authors also would like to thank Purdue University, the Pontifícia Universidade Católica do Paraná (PUCPR) and the Universidade Estadual de Santa Cruz (UESC) for funding this work.

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