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Evaluating the efficacy of an essential oil extract of thyme (*Thymus vulgaris*) against methicillin-sensitive and methicillin-resistant strains of Staphylococci

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Abstract

An essential oil extract of thyme (*Thymus vulgaris*) was evaluated against MSSA and MRSA strains of Staphylococci. Minimum Inhibitory Concentrations (MICs) were determined using cation-adjusted Mueller-Hinton broth with 2% sodium chloride and modified with 0.2 % agar for suspension of Thyme oil. Oxacillin and vancomycin were used as positive controls. MICs were determined by visual inspection and optical density (OD). *S. aureus* ATCC 29213 showed MICs of 0.125 µg/ml in media with agar and 0.25 µg/ml in media without agar by visual inspection for oxacillin, but an MIC of 0.25 µg/ml was determined by OD for oxacillin in both media. Vancomycin MICs were 1 µg/ml for *S. aureus* ATCC 29213 by visual inspection but 2 µg/ml using OD in both media with and without agar. *S. aureus* ATCC BAA-1720 showed MIC values of 1 µg/ml in media without agar but 2 µg/ml in media with agar both by visual inspection and OD. MICs determined by optical density (OD) allowed detection of small amounts of growth not apparent by visual inspection, allowing better detection of heteroresistant strains against the standard antibiotics oxacillin and vancomycin. The MIC for thymeoil was 0.3125 µl/ml against both strains of *S. aureus* by visual inspection as well as OD. Essential oils provide an important new mode of action against these strains.

Keywords: Tube macrodilution method, 0.2% agar, antimicrobial activity, thyme essential oil, MRSA

1. Introduction

Staphylococcus aureus is a major cause of nosocomial infections, but antimicrobials commonly used to treat these infections tend to promote the emergence of resistant strains [1]. Staphylococci became resistant to many penicillins due to production of β-lactamase [2], penicillin-binding proteins [3], or changes in peptidoglycan biosynthesis [3,4].

In an effort to slow down development of resistance, current trends include limiting use of these antimicrobials and finding new agents with novel mechanisms of action [2]. An alternate approach in control of these and other infections might be use of essential oils [5, 6]. Due to their complex chemical composition, these oils possess several modes of action, including disruption of the outer and inner membranes by terpenoids such as thymol [7]. Thyme (*Thymus vulgaris* L.) essential oil contains thymol as a major component and has been evaluated in a number of studies for its efficacy against bacterial and other infections [8, 9, 10, 11]. However, standard guidelines for evaluating antimicrobial activity of essential oils have not been developed, and there is some question as to the accuracy of methods used for determining antibacterial activity of these oils [12]. Major difficulties in testing essential oils are their low solubility in standard media used for growing bacteria and their volatility. Several studies [12, 13] suggest that a modified broth dilution method can provide accurate results in quantifying antibacterial activity of essential oils. These methods are modified by using emulsifying agents or other chemicals to increase solubility of the oils, such as tween 80 [12, 14], ethanol [14], dimethylsulfoxide [15], acetone [10], and agar [13, 14]. Remmal *et al.* compared tween 80, ethanol and agar as dispersing agents and found that addition of 0.2% agar to test media allowed the formation of stable dispersions of essential oils without interfering with the antimicrobial activity of the essential oils [14]. However, in agar dilution MIC tests with standard antibiotics, trailing endpoints are not uncommon making it difficult to determine endpoints [3]. In addition, essential oils can produce discoloration of the medium at higher concentrations that interfere with reading of the MIC values [13].

Guidelines have been developed for evaluating standard antimicrobials against MRSA strains since, among other things, differences in basal medium, sodium chloride concentration and

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temperature of incubation can influence expression of resistance [3]. For dilution testing methods, these guidelines include the use of cation-adjusted Mueller-Hinton broth, addition of sodium chloride for assessing oxacillin sensitivity, and incubation for a full 24 hours at a temperature not above 35°C, since temperatures above this may not detect MRSA [3, 16]. Addition of sodium chloride is not required for determining vancomycin MICs; however, it could be essential for detecting vancomycin heteroresistant strains of *Staphylococci* [17, 18].

Taking these guidelines and other studies for evaluating oxacillin and vancomycin by broth dilution against *Staphylococci* into account, along with suggestions from studies evaluating essential oils, we decided to use cation-adjusted Mueller-Hinton broth with 2% sodium chloride and incubation for a full 24 hours at a temperature not above 35°C. The essential oil we used was a commercially available extract of thyme (*Thymus vulgaris*), and was tested using the same conditions except with the addition of 0.2% agar to the medium. To determine if the 0.2% agar influenced MIC values, both oxacillin and vancomycin were also tested in medium modified with 0.2% agar. *Staphylococcus aureus* ATCC 29213 and *Staphylococcus aureus* ATCC BAA-1720 were used as methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* strains, respectively.

The aim of our study was to evaluate the ability of this method of testing in determining a minimum inhibitory concentration for a commercially available thyme oil product against methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus* that can be used for comparison to standard antibiotics.

2. Materials and methods

2.1 Microorganisms and culture maintenance

Test organisms included *Staphylococcus aureus* ATCC 29213 as a strain sensitive to methicillin and *Staphylococcus aureus* ATCC BAA-1720 as a methicillin-resistant strain (American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110). *S. aureus* ATCC 29213 is recommended for dilution testing as a control strain since it is a weak, β -lactamase-producing strain [16]. Mueller-Hinton Agar (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103) was used for maintenance of both strains. Typically, on a weekly basis, each strain was maintained by inoculation onto an MHA plate and incubated at 35°C for 18-24 hours. Stock plates were then stored at 4°C. Prior to testing, strains were inoculated on to fresh MHA plates and incubated at 35°C for up to 24 hours.

2.2 Determination of minimum inhibitory concentration (MIC) endpoints

With broth dilution testing, minimum inhibitory concentration (MIC) is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the tubes (button or definite turbidity) as detected by the unaided eye [16]. Cation-adjusted Mueller-Hinton broth supplemented with 2% sodium chloride was used for testing both oxacillin and vancomycin [3, 16]. Testing vancomycin with 2% sodium chloride should increase detection of vancomycin heteroresistant strains [17]. This same medium, modified with the addition of 0.2% agar, was used for testing the essential oil thyme extract and to determine any influence of the agar on reading of endpoints of standard antibiotics. Following recording of visual readings, all tubes were vortexed and optical density (OD) was measured at 625 nm using a Genesys 10 S UV-VIS spectrophotometer (Thermo Scientific). Uninoculated tubes of the test medium

were used as blanks for oxacillin and vancomycin assays. However, since essential oils themselves can cause variation in optical density due to increased turbidity as well as increased discoloration of the media as their concentration increases, a series of uninoculated tubes for each dilution were incubated under the same conditions as the inoculated tubes and each concentration of inoculated tube was blanked with its equivalent concentration of uninoculated tube.

2.3 Inoculum

Enough colonies from a MHA plate were transferred to 4 ml of MHB to achieve an absorbance at 625 nm between 0.08 to 0.13 OD for comparison to the 0.5 McFarland standard. Suspensions were vortexed for uniformity prior to measuring OD. A reading between 0.08 to 0.13 OD yields approximately 1.5×10^8 cfu/ml. From this tube, 0.1 ml was removed and added to 9.9 ml of MHB and vortexed to yield approximately 1×10^6 cfu/ml. This will give a final inoculum of 5×10^5 cfu/ml when mixed with equal volumes of antimicrobial solution [16].

2.4 Preparation of serial dilutions of oxacillin and vancomycin

Stock solutions of antimicrobials were prepared to account for potency of the powder as per CSLI guidelines [16]. These were prepared at twice the final desired concentrations and serially diluted to provide a range from 256 to 0.0312 $\mu\text{g/ml}$. To these tubes were added equal volumes of inoculated media, prepared as above, to yield final concentrations of antimicrobial agent from 128 to 0.0156 $\mu\text{g/ml}$. Negative control tubes contained no antibiotics. All tubes were vortexed and incubated at 35°C for 24 hours. Following incubation, tubes were inspected visually to determine MIC values and then optical density readings were taken to document turbidity.

2.5 Preparation of serial dilutions of Thyme oil extract

Thyme (*Thymus vulgaris*) essential oil supplement was obtained from dōTERRA, Pleasant Grove, UT 84062, USA. Preliminary tests performed in our lab indicated that the highest final concentration of oil we could test was 5 $\mu\text{l/ml}$. Above this concentration the oil produced significant cloudiness and color changes in the medium following incubation at 35°C to interfere with both visual inspection and OD measurements of tubes for growth. Starting with 5 $\mu\text{l/ml}$, the oil was serially diluted using a 1:2 dilution to give a range of oil from 5 to 0.078 $\mu\text{l/ml}$. A negative control contained no essential oil. For each replicate these dilutions were repeated a second time. One set was inoculated with the test organism and the second set was left uninoculated. All tubes were incubated at 35°C for 24 hours. Each tube was visually inspected for growth as indicated by cloudiness and then vortexed and OD was measured. Each tube was blanked with an un-inoculated tube of the same concentration of oil to adjust for normal variation in absorption caused by the essential oil following 24 hours of incubation. The MIC is defined as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism as detected by the unaided eye [16]. Optical Density measurements were used for purposes of comparison.

3. Results

3.1 MIC determinations of oxacillin and vancomycin

Dilution tests for oxacillin against *S. aureus* ATCC 29213 showed an MIC of 0.25 $\mu\text{g/ml}$ in media without agar both by OD readings (Fig. 1A) and visual inspection (Table 1). All tubes examined at OD 0.125 $\mu\text{g/ml}$ had a visible cloudiness even though the OD was considerably less than that of the negative

control tube. In media with agar, the MIC of oxacillin for *S. aureus* ATCC 29213 was 0.125 µg/ml by visual inspection (Table 1); however, OD readings (Fig. 1A) were slightly higher in these tubes than most tubes deemed clear. In addition, OD readings in both types of media indicate a trailing effectiveness of the antibiotic at concentrations lower than the MIC.

Dilution tests for vancomycin (Fig. 1B) against *S. aureus* ATCC 29213 showed greater variation in endpoints from trial to trial compared to oxacillin, both in OD readings and by visual inspection. The average MIC endpoint in both broth and agar supplemented media for vancomycin was 1 µg/ml by visual inspection (Table 1) but 2 µg/ml using OD (Fig. 1B).

S. aureus ATCC BAA-1720 showed no sensitivity to oxacillin (Fig. 2A, Table 1) growing in all concentrations tested up to 128 µg/ml. Both OD readings and visual inspection for vancomycin against *S. aureus* ATCC BAA-1720 (Fig. 2B, Table 1) were an average of 1 µg/ml in media without agar and 2 µg/ml in media with agar.

3.2 MIC determination of Thyme oil

The MIC for thyme oil was 0.3125 µl/ml against both strains of

S. aureus as determined by OD (Fig. 3) and visual inspection (Table 1). Media tended to become increasingly hazy beginning at 1.25 µl/ml of oil and a purple discoloration of media was visible at 5 µl/ml.

Table 1: MIC (range) values by visual inspection for oxacillin (µg/ml), vancomycin (µg/ml) and thymeoil (µl/ml) in Mueller-Hinton broth (MHB) and Mueller-Hinton broth with 0.2% agar.

Strain	Agent	MHB	MHB+0.2% agar
<i>S. aureus</i> ATCC 29213	Oxacillin	0.25 (0.25-0.25)	0.125* (0.125-0.125)
	Vancomycin	1* (0.5-2)	1* (0.5-2)
	Thyme oil		0.3125 (0.3125-0.3125)
<i>S. aureus</i> ATCC BAA-1720	Oxacillin	>128	>128
	Vancomycin	1 (0.5-1)	2 (0.5-2)
	Thyme oil		0.3125 (0.156-0.3125)

*Denotes different from OD determination.

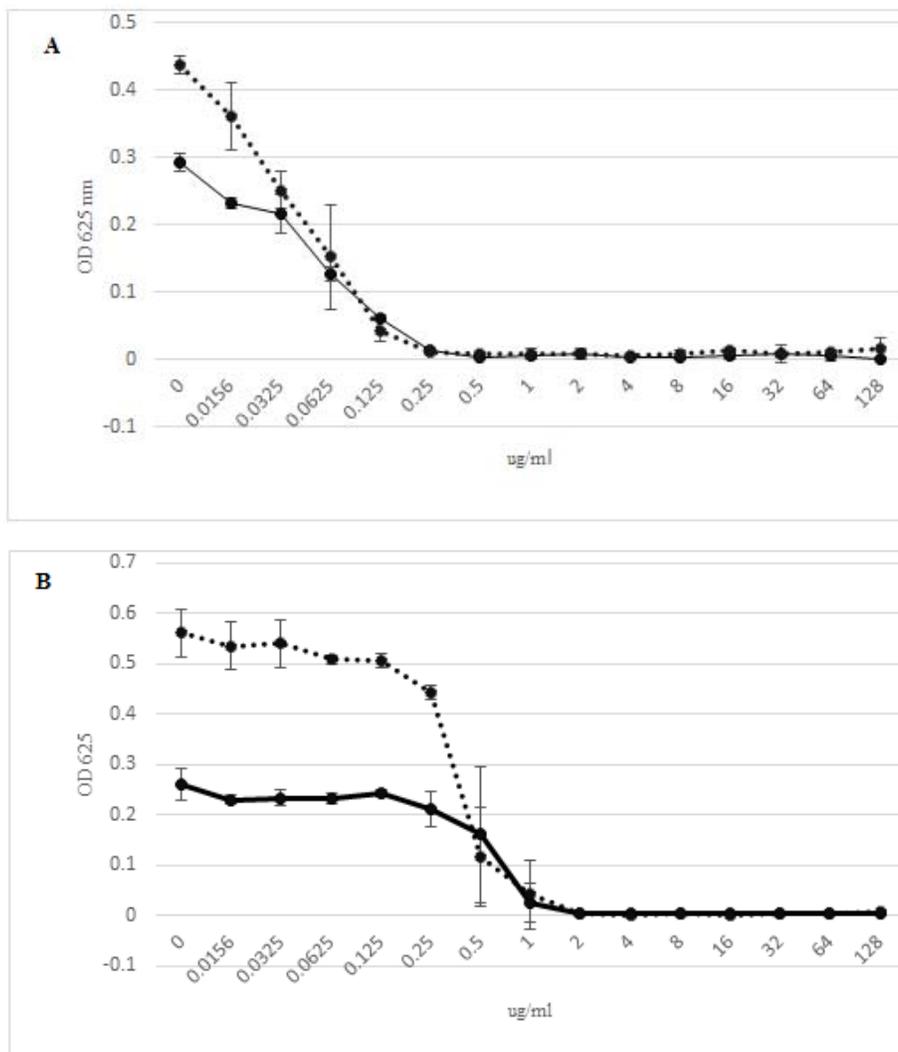


Fig 1: A) Minimum inhibitory concentration (µg/ml) against *S. aureus* ATCC 29213 as determined by optical density for oxacillin in cation-adjusted Mueller-Hinton Broth supplemented with 2% sodium chloride without 0.2% agar (solid line) and in cation-adjusted Mueller-Hinton Broth supplemented with 2% sodium chloride and 0.2% agar (dashed line). B) Minimum inhibitory concentration (µg/ml) of vancomycin in cation-adjusted Mueller-Hinton Broth supplemented with 2% sodium chloride without 0.2% agar (solid line), and in cation-adjusted Mueller-Hinton Broth supplemented with 2% sodium chloride and with 0.2% agar (dashed line) against *S. aureus* ATCC 29213, as determined by optical density (OD) at 625 nm. Error bars represent standard deviation.

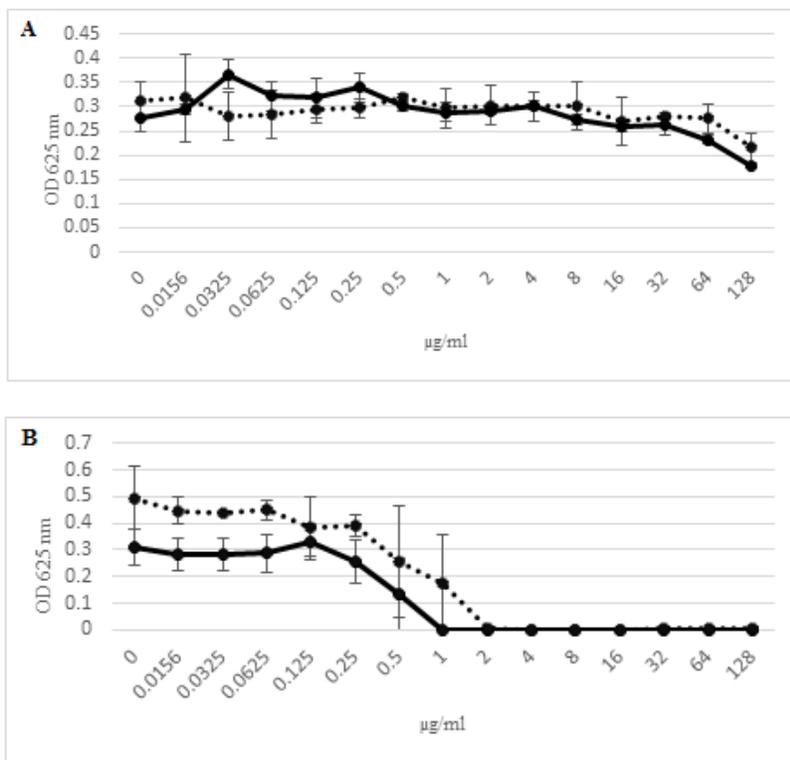


Fig 2: A) Minimum inhibitory concentration (µg/ml) of oxacillin in cation-adjusted Mueller-Hinton Broth supplemented with 2% sodium chloride without 0.2% agar (solid line) and in cation-adjusted Mueller-Hinton Broth supplemented with 2% sodium chloride and with 0.2% agar (dashed line). B) Minimum inhibitory concentration (µg/ml) of vancomycin in cation-adjusted Mueller-Hinton Broth supplemented with 2% sodium chloride without 0.2% agar (solid line) and in cation-adjusted Mueller-Hinton Broth supplemented with 2% sodium chloride and with 0.2% agar (dashed line) against *S. aureus* ATCC BAA-1720, as determined by optical density (OD) at 625 nm. Error bars represent standard deviation.

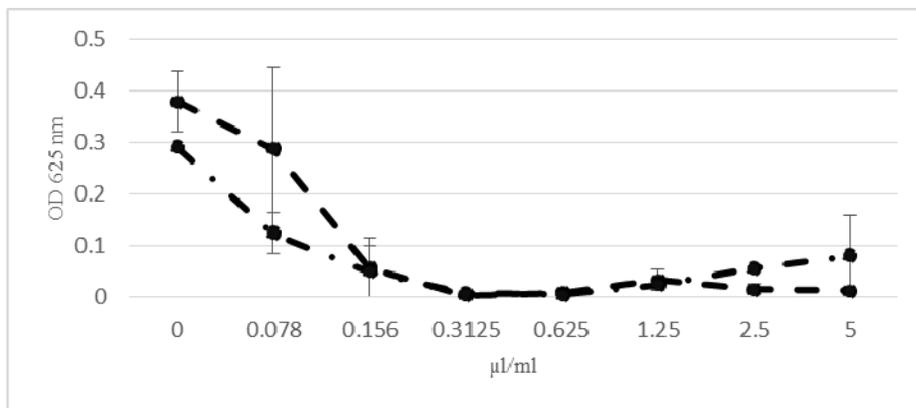


Fig 3: Minimum inhibitory concentration (µl/ml) of thyme oil against *S. aureus* ATCC 29213 (dashes) and *S. aureus* ATCC BAA-1720 (dots and dashes) in cation-adjusted Mueller-Hinton Broth supplemented with 2% sodium chloride and 0.2% agar as determined by optical density (OD) at 625 nm. Error bars represent standard deviation.

4. Discussion

Our goal was to determine if a standard macrodilution method for determining MICs of oxacillin and vancomycin against MSSA and MRSA strains could be modified to evaluate efficacy of essential oils against these strains. Breakpoints for oxacillin using tube macrodilution MIC tests for *S. aureus* are ≤ 2 µg/ml for susceptible and ≥ 4 µg/ml for resistant [16]. Using visual inspection, the MIC for oxacillin against *S. aureus* ATCC 29213 was 0.25 µg/ml in media without agar but 0.125 µg/ml in media with agar (Table 1). OD readings (Fig. 1A) however, indicate an MIC value of 0.25 µg/ml both in media with and without agar. Slight growth occurring as a button on top of media containing agar was more difficult to detect by the

unaided eye compared to seeing a slight cloudiness in media without agar. OD readings confirmed slight growth of organism at 0.125 µg/ml in media with agar where no button was visible. Although it is recommended that endpoints for MICs by tube macrodilution should be determined by the unaided eye (CLSI 2006), an MIC of 0.25 µg/ml for *S. aureus* ATCC 29213 is consistent with other studies using MH medium with 2% NaCl [19], indicating that OD readings are more accurate in tube dilutions when using media with 0.2% agar. Use of OD in determining MICs may overcome problems with trailing endpoints seen in agar dilution MIC tests [3]. Results for vancomycin (Fig. 1B) against *S. aureus* ATCC 29213 showed more variation in MIC endpoints from trial to

trial compared to oxacillin. MIC values ranged from 0.5 µg/ml to 2 µg/ml, both by OD readings and visual inspection. This was the same in media with and without agar. A consensus value by visual inspection suggests an MIC of 1 µg/ml; however, average OD readings suggest an MIC value of 2 µg/ml. Results from other studies for vancomycin against *S. aureus* ATCC 29213 report MIC values from 1 µg/ml using an agar dilution method^[19] to 2 µg/ml using a macrodilution method but with Todd-Hewitt broth^[20]. CLSI guidelines suggest abreakpoint of ≤ 2 µg/ml for susceptible for vancomycin using tube macrodilution MIC tests for *S. aureus*^[16]; however, there is concern in some cases that using vancomycin against strains of Staphylococci where the MIC for vancomycin between is between 1 and 2 µg/ml may not be effective^[21]. Together the information provided in testing both oxacillin and vancomycin in media containing sodium chloride improves detection of methicillin resistance and vancomycin heteroresistance^[3, 17]. Although additional testing is required, it appears that the 0.2% agar may aid in this effort also. This information may prove useful in treatment against specific strains of MSSA and MRSA Staphylococci that have MICs close to the susceptible value as well as strains of oxacillin-sensitive, methicillin-resistant *Staphylococcus aureus* (OS-MRSA)^[19, 21].

S. aureus ATCC BAA-1720 showed no sensitivity to oxacillin (Fig. 2A) growing in all concentrations tested up to 128 µg/ml. This compares with results obtained by Barbour *et al.* for this strain^[22]. Vancomycin MIC values as determined by OD and visual inspection (Fig. 2B; Table 1) ranged from 0.5 to 1 µg/ml in media without agar and from 0.5 to 2 µg/ml in media with agar, with an average MIC in media without agar of 1 µg/ml and in media with agar of 2 µg/ml. This strain would then be classified as heteroresistant^[4, 21].

In our study, thyme oil showed an MIC of 0.3125 µl/ml (Fig. 3) against both strains of Staphylococci as determined by OD and visual inspection. This broader spectrum of activity against both strains might be expected since thyme oil contains thymol, which disrupts the outer and inner membranes of bacteria^[7]. Donaldson *et al.* also demonstrated an MIC of 0.31 µl/ml for Thyme oil against *Staphylococcus aureus* using a tube macrodilution assay with Tryptic Soy Broth containing 0.2% agar and determined by OD assisted by *p*-iodonitrotetrazolium dye^[13]. Hammer *et al.* also found an MIC of 0.03% (v/v), or 3 µl/ml, of thyme oil against *Staphylococcus aureus* using a broth microdilution method and Mueller Hinton Broth^[8]. Not all studies have produced results consistent with these MIC values. For example, Bogavac *et al.* used a microdilution procedure with nutrient broth and found MICs for thyme oil against various strains of Staphylococci ranged from 11.4-45.4 µl/ml^[11]. In addition, Shukr and Metwally tested thyme oil by an agar well diffusion method and found an MIC of 4 µl/ml against their strain of MRSA^[23]. These differences in MIC values can result not only with type of medium but even using different batches or manufacturers of the same medium^[3]. There is also poor correlation between agar diffusion methods and tube dilution methods^[13]. The modified macrodilution method used in our study provided conditions suggested for oxacillin and vancomycin against MSSA and MRSA strains of Staphylococci. Under these conditions the MICs of essential oils should be directly comparable to the MICs of these antibiotics. The high level of activity shown by this essential oil against both strains of Staphylococci is an indication that they provide an important new mode of action against these strains.

5. Conflict of Interest

We acknowledge receipt of commissions by one of the authors for sales of essential oil extracts; however, the views expressed here are those of the authors and do not constitute endorsement by dōTERRA.

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