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On Guard®! Antimicrobial activity of a proprietary essential oil blend against skin microbes

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SUMMARY There is ample research highlighting the antimicrobial activity of the essential oils (EOs) like cinnamon, clove, orange, rosemary, and eucalyptus found in dōTERRA©'s proprietary On Guard® blend. This suggests that the essential oil blend (EOB) may have its own antimicrobial properties; however, currently there is no published literature to confirm this. The purpose of this study was to investigate the antimicrobial activity of dōTERRA© On Guard® products against *Staphylococcus epidermidis*, *Bacillus subtilis, Staphylococcus aureus*, and *Pseudomonas aeruginosa*. To address this, the EOB was tested using a Kirby-Bauer disc diffusion assay in order to determine the antimicrobial activity of the EOB against skin microbes. Based on previously published research, it was proposed that the On Guard® EOB would have antimicrobial activity towards some or all of the selected microbes since its EO components have been shown to have antimicrobial properties. It was found that the EOB showed statistically significant antimicrobial activity against all four species of skin bacteria. Interestingly, the cinnamon EO showed the highest antimicrobial activity, and even outperformed the chloramphenicol positive control in some trials. The EOB also seemed to more selectively inhibit *S. aureus* over *S. epidermidis*. These findings strongly suggest that the On Guard® EOB does have antimicrobial activity but further research is needed in order to confirm the statistical significance of the selectivity of the EOB, to solidify its potential as a treatment for bacterial skin conditions.

INTRODUCTION

Mainstream antimicrobial products, such as topical antibiotics, alcohol-based disinfectants, and surface cleansers such as Lysol® and Clorox®, can be effective at eliminating pathogens; however, they also have high cytotoxic activity against human cells (1, 2). A more natural, plant-based product that is gentle on the skin but also has antimicrobial properties may be less harmful than current antimicrobial products. By discovering powerful natural antimicrobial agents, effective sanitization may be achieved with minimal concern about the negative effects of common commercial cleansers.

Unfortunately, many natural products often make claims that are not fully explored in the literature. For example, the proprietary On Guard® essential oil blend (EOB) produced by dōTERRA©, used in their hand sanitizer, hand soap, toothpaste, and Cleaner Concentrate, is labelled as an immune boosting, non-toxic cleanser that can be used internally, topically, and aromatically (3). While this EOB has been shown to be anti-inflammatory, effective in modulating immune responses in skin disease models (4), and able to attenuate influenza virus PR8, what is missing in the literature is the antibacterial properties of this proprietary EOB (5).

There is ample evidence to support the diverse antimicrobial properties of the pure essential oils (EOs) found in the EOB, including cinnamon, clove, eucalyptus, rosemary, and

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wild orange, suggesting that the On Guard® EOB could also have similar antimicrobial properties (6-26).

Human skin is host to a multitude of microorganisms, including Gram-positive and Gram-negative bacteria as well as fungi (27). The ratio between resident skin microbes versus those with more pathogenic potential is important in maintaining homeostasis, and disruption to these ratios can lead to skin conditions and diseases, such as atopic dermatitis, acne, eczema, and psoriasis (27-29). Of the many microbes found on human skin, several have been shown to be susceptible to various natural products, including the individual EOs present in the On Guard® EOB (6, 9, 10, 13, 20, 24). Examples of these microbes include *Staphylococcus epidermidis* the most abundant resident skin microbe, and *Bacillus subtilis*, which is a resident skin microbe that can produce antimicrobial substances that are competitive to pathogenic skin bacteria and fungi (28). Additionally, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are common skin bacteria that contribute to various skin conditions, including furuncles, impetigo, folliculitis, ecthyma gangrenosum, and acute external otitis (30, 31). These microbes were chosen based on previous literature showing the antimicrobial effects of EOs on these species; moreover, their presence on human skin was an important factor in order to determine the effect of the EOB on common skin microbes of different species and with different structural properties (6, 9, 10, 13, 16, 20, 32). Using Gram-negative and Gram-positive bacteria may show important differences between the susceptibility of the different types of microbes to the EOB, since many Gram-positive and Gram-negative bacteria react differently to antimicrobial substances due to differences in their cellular structure (22).

The purpose of this study was to investigate the antimicrobial activity of the dōTERRA© On Guard® EOB against skin microbes. Currently, there is no published information about how the On Guard® EOB affects skin microbes. To address this, the EOB was tested using a Kirby-Bauer disc diffusion assay in order to determine the antimicrobial activity of the EOB against skin microbes. Based on previously published research, it was proposed that the On Guard® EOB would have antimicrobial activity towards some or all of the selected microbes, as each of its individual EO components have been shown to have antimicrobial properties.

METHODS AND MATERIALS

Essential Oils and Antibiotic Discs. The On Guard® EOB was the oil of interest in this study; however, the individual pure EOs present in the EOB were tested against each microbe as well, in order to replicate literature findings and to confirm that the model performed as expected. The EOs used in this study were dōTERRA© brand: On Guard® EOB, cinnamon bark, clove, eucalyptus, rosemary, and wild orange. Mineral oil was used as a negative control in order to confirm that the active molecules in the EOs were contributing to the observed antimicrobial effects, rather than the hydrophobicity of the EOs themselves (33). Autoclaved 6mm punches of Whatman No. 3 filter paper were impregnated with 10 µL of each EO and the mineral oil and left to absorb (34). The antibiotic discs used as positive controls were Chloramphenicol BD BBL™ Sensi-Disc™ 30µg (product number 230733), and Gentamicin BBL™ Sensi-Disc™ 10µg (product number 231227). Chloramphenicol was chosen as it is often used as a positive control in experiments involving the antimicrobial activity of EOs and has susceptibility information available for many microbes in the *M100-S23 Performance Standards for Antimicrobial Susceptibility Testing* (9, 35).

Bacterial Strains. The following bacterial strains were used: *S. epidermidis* (ATCC cat# 35983), *S. aureus* (ATCC cat# 6538), *B. subtilis* Ward's® Live Cultures # 470176-524, and *P. aeruginosa* Ward's® Live Cultures # 470179-204.

Determination of Antimicrobial Activity. Antimicrobial activity of the EOs was investigated using the Kirby-Bauer Disc Diffusion method as outlined in Clinical and Laboratory Standards Institute *Performance standards for antimicrobial disk susceptibility tests* (36). Each bacterial species was inoculated into 4mL of tryptic soy broth (TSB) and placed in a shaking incubator for 4-5 hours at 35°C ± 2°C and 150 rpm. The turbidity of the

inoculated TSB was measured using a spectrophotometer set to 625 nm and adjusted (using sterile TSB) to an optical density of 0.08 - 0.10, with control TSB used as a blank. This was based on the 0.5 McFarland standard, which approximates the CFUs of the culture as 1-2 x 10^8 CFU/mL. Two 21 mL 1.7% Mueller-Hinton (MH) agar plates (100 mm x 15 mm) received 200 µL each of the adjusted suspension using the spread plate method (37). The lids were left slightly ajar for three to five minutes to dry. The EO impregnated discs were then applied to the plates, along with one mineral oil negative control disc and one Chloramphenicol positive control disc per plate. Gentamicin was also used in later trials as a secondary positive control. These plates were incubated at 35°C ± 2°C, within 15 minutes after the discs were applied, for 18-24 hours. A standard plate count with CFU assays, and sterilized water used as a diluent were also conducted to confirm that an OD reading of 0.08 - 0.1 produced consistent bacterial lawn densities and that the zones of inhibition (ZIs) were repeatable amongst the trials within each bacterial species (37, 38).

The results of the disc diffusion assays were obtained by measuring each ZI using a ruler, to the nearest millimeter (39). These counts were then divided by the ZI diameter of the Chloramphenicol on that plate in order to normalize the measurements and allow for comparison between plates and trials.

Statistical analysis. The normalized counts were used to compare the antimicrobial activity of each of the EOs and the EOB using one-way ANOVA and Tukey tests. The normalized ZI values were used in ANOVA and Tukey tests to determine the statistical significance of the antimicrobial activity of the EOs and the EOB compared to the negative control. The Tukey test was carried out at a 95% confidence interval. The tests were carried out in StatCrunch Online. Figures 1-2, 4-6 were also made in StatCrunch Online and edited in PowerPoint.

RESULTS

Staphylococcus epidermidis. For *S. epidermidis*, the EOB, eucalyptus, and wild orange showed statistically significant antimicrobial effects when compared to the mineral oil negative control (Fig 1). Cinnamon and clove showed even higher antimicrobial activity when compared to the negative control (Fig 1). Rosemary did not show significant antimicrobial activity (Fig 1). Interestingly, the cinnamon performed almost as well as the chloramphenicol in most of the trials (Fig 1). Chloramphenicol ZIs were consistent amongst all trials with a mean ± SEM of 25 ± 0.4mm.

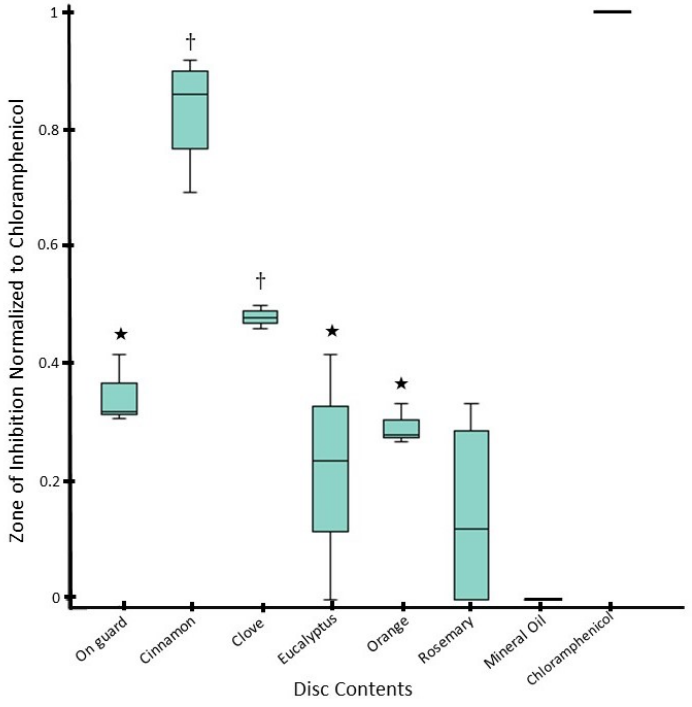

FIG. 1 On Guard® EOB exhibits antimicrobial activity against *S. epidermidis*. A Kirby-Bauer disc diffusion assay was performed, the ZIs were normalized against chloramphenicol, and plotted as a boxplot data summary. The normalized counts were compared using one-way ANOVA and Tukey tests comparing the EOs and the EOB to the negative control. The Tukey test was carried out at a 95% confidence interval. Raw data is shown in Supplemental Table S1.

n = 4; ★ denotes $p < 0.05$; † denotes $p < 0.0001$.

Bacillus subtilis. For *B. subtilis*, the EOB, cinnamon, clove, and wild orange all showed statistically significant antimicrobial effects when compared to the negative control (Fig 2). Cinnamon and clove showed higher antimicrobial activity when compared to the negative control (Fig 2). The eucalyptus and rosemary did not show significant antimicrobial activity (Fig 2). Chloramphenicol ZIs were consistent amongst all trials with a mean ± SEM of 17 ± 0mm.

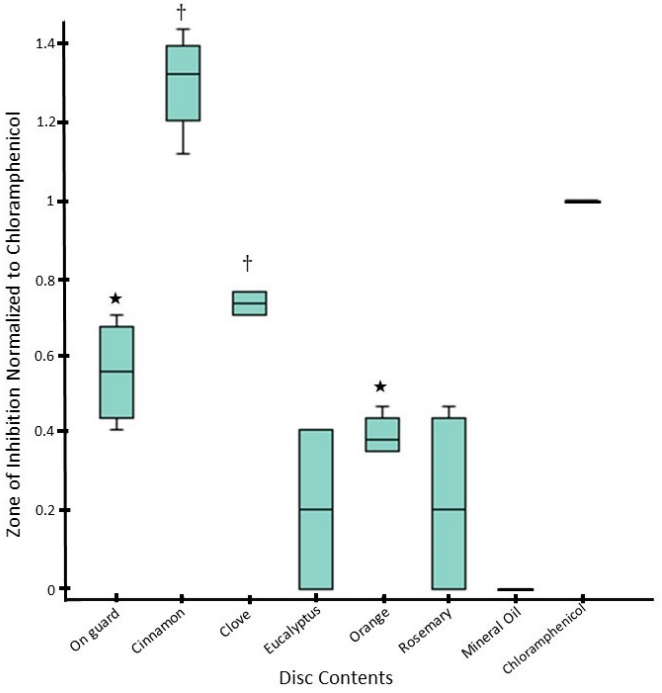

FIG. 2 On Guard® EOB exhibits antimicrobial activity against *B. subtilis*. A Kirby-Bauer disc diffusion assay was performed, the ZIs were normalized against chloramphenicol, and plotted as a boxplot data summary. The normalized counts were compared using one-way ANOVA and Tukey tests comparing the EOs and the EOB to the negative control. The Tukey test was carried out at a 95% confidence interval. Raw data is shown in Supplemental Table S2.

n = 4; ★ denotes $p < 0.05$; † denotes $p < 0.0001$.

Pseudomonas aeruginosa. The *P. aeruginosa* cultures exhibited a striking aqua/turquoise colour on the MH plates, which was "muted" in areas where the bacteria were inhibited (Fig 3). The ZIs of the chloramphenicol were not clearly defined as there was streaking noted (Fig 3). Gentamicin was included in subsequent trials to confirm that the bacteria could be inhibited, and some streaking was also noted within the ZIs against gentamicin. The EOB, cinnamon, and clove showed statistically significant antimicrobial effects when compared to the mineral oil negative control, while the eucalyptus, wild orange, and rosemary did not (Fig 4). Chloramphenicol ZIs were consistent amongst all trials with a mean ± SEM of 12.75 ± 0.1mm.

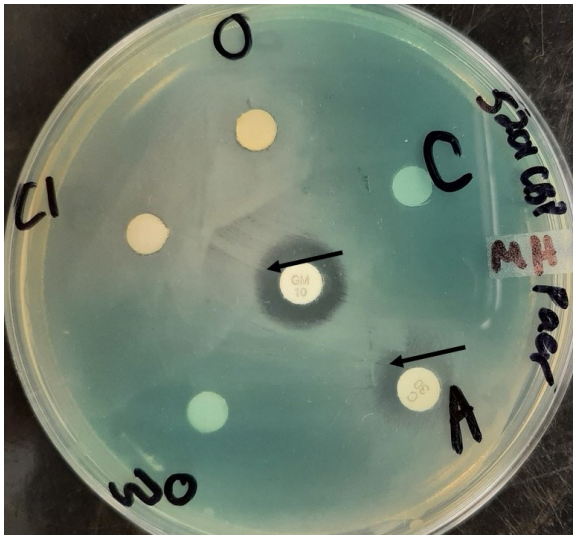

FIG. 3 *P. aeruginosa* Mueller Hinton Plate with turquoise shading and swarm-like streaks. The *P. aeruginosa* exhibited a striking aqua/turquoise colour on the MH plates, which was muted in areas where the bacteria were inhibited such as around the clove (Cl) and On Guard® (O) discs, as well as the gentamicin (GM) and Chloramphenicol (A) antibiotics. Swarm-like streaks indicated by bold arrows.

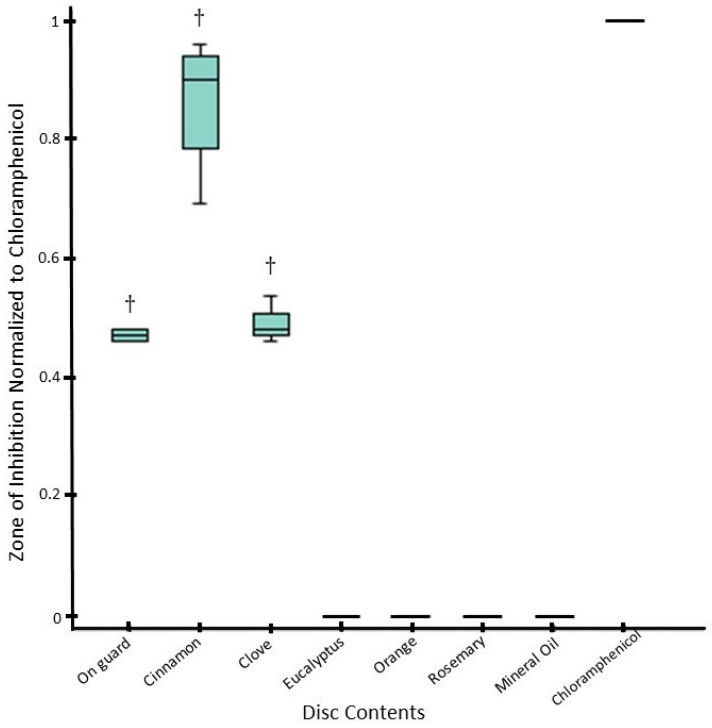

FIG. 4 On Guard® EOB exhibits antimicrobial activity against *P. aeruginosa*. A Kirby-Bauer disc diffusion assay was performed, the ZIs were normalized against chloramphenicol, and plotted as a boxplot data summary. The normalized counts were compared using one-way ANOVA and Tukey tests comparing the EOs and the EOB to the negative control. The Tukey test was carried out at a 95% confidence interval. Raw data is shown in Supplemental Table S3.

n = 4; ★ denotes $p < 0.05$; † denotes $p < 0.0001$.

Staphylococcus aureus. For *S. aureus*, the EOB, cinnamon, clove, eucalyptus, wild orange, and rosemary all showed statistically significant antimicrobial effects when compared to the negative control (Fig 5). The EOB, cinnamon, and clove showed the highest antimicrobial activity (Fig 5). Interestingly, the cinnamon performed similarly to the chloramphenicol in most of the trials (Fig 5). Chloramphenicol ZIs were consistent amongst all trials with a mean ± SEM of 24.5 ± 0.8mm.

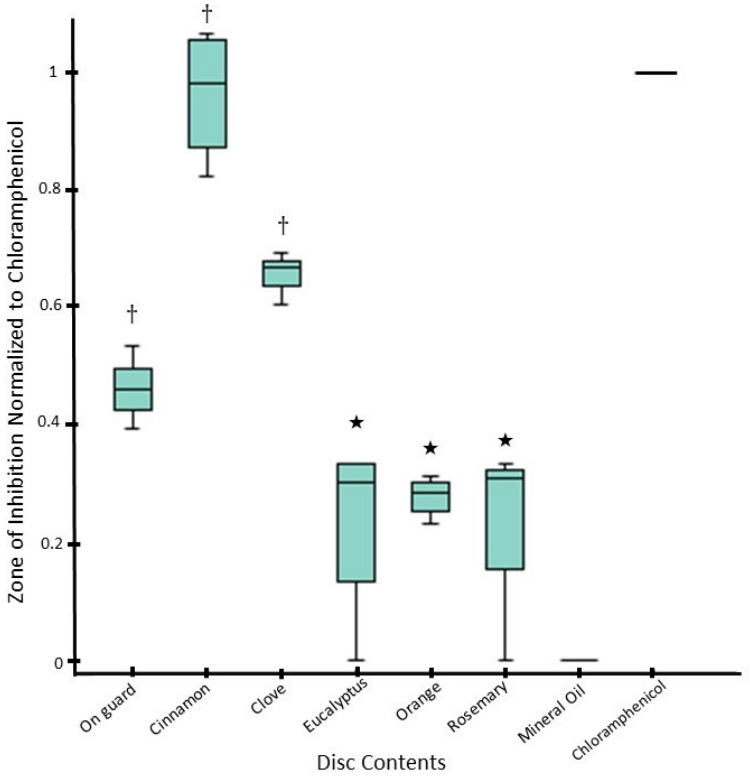

FIG. 5 On Guard® EOB exhibits antimicrobial activity against *S. aureus*. A Kirby-Bauer disc diffusion assay was performed, the ZIs were normalized against chloramphenicol, and plotted as a boxplot data summary. The normalized counts were compared using one-way ANOVA and Tukey tests comparing the EOs and the EOB to the negative control. The Tukey test was carried out at a 95% confidence interval. Raw data is shown in Supplemental Table S4.

n = 4; ★ denotes $p < 0.05$; † denotes $p < 0.0001$.

Figure 6 shows the side-by-side comparison of the antimicrobial effect of the On Guard® EOB on each bacterial species tested. It appeared that the pathogenic microbe *S. aureus* may be more selectively inhibited than the resident skin microbe *S. epidermidis* since the ZIs for *S. aureus* were larger than those of *S. epidermidis,* while the ZIs against chloramphenicol were similar between the two species. However, further trials are necessary to confirm statistical significance.

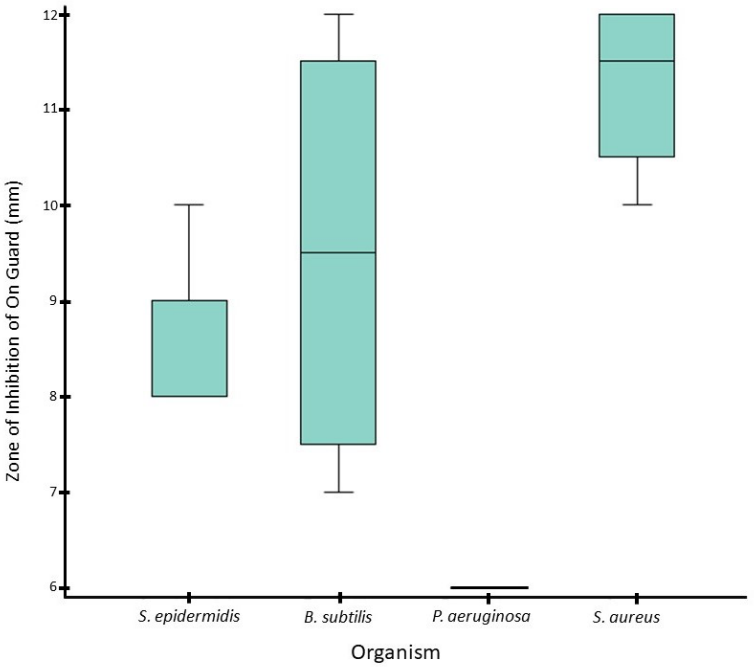

FIG. 6 Comparison of antimicrobial activity of On Guard® against *S. epidermidis*, *B. subtilis*, *P. aeruginosa*, and *S. aureus*. A Kirby-Bauer disc diffusion assay was performed, the ZIs in mm were plotted as a boxplot data summary in order to visualize the comparative antimicrobial activity of the On Guard® EOB between species. Raw data is shown in Supplemental Table S5.

DISCUSSION

The purpose of this study was to investigate the antimicrobial activity of dōTERRA© On Guard® products against skin microbes, as there is currently no published information about the extent to which the On Guard® EOB affects skin microbes. To address this, the EOB was used in a Kirby-Bauer disc diffusion assay in order to determine the antimicrobial activity of the EOB against the following skin microbes: *S. epidermidis*, *B. subtilis, S. aureus*, and *P. aeruginosa*. The Kirby-Bauer disc diffusion susceptibility protocol is a valid, reliable, and standardized method that has been used for decades as an accurate technique for determining the susceptibility of certain microbes to different antimicrobial substances (32). This method is commonly used in the literature for determining the antimicrobial properties of EOs (9, 10, 12, 16, 20, 21, 24, 40).

The On Guard® EOB showed statistically significant antimicrobial activity against all the four bacterial species *S. epidermidis*, *B. subtilis*, *P. aeruginosa*, and *S. aureus* (Fig 1-2, 4-5) when compared to the negative control. When the activity of the EOB against all four organisms is compared between species, there appears to be some selectivity (Fig 6). The ZIs against chloramphenicol were consistent between *S. epidermidis* and *S. aureus* species, which makes the different effects of the On Guard® EOB between these species more apparent. Since the ZIs against chloramphenicol differed between species, further studies could use an alternate antibiotic, such as gentamicin, as its positive control.

The EOB may show selective properties towards more pathogenic skin bacteria. After determining how the EOB performed between species, it was noted that the EOB showed a consistently higher activity against *S. aureus* than *S. epidermidis*. Since the variation in the *B. subtilis* trials was high, it was difficult to compare the data with that of the other species. This may suggest that the EOB can more selectively inhibit pathogenic skin bacteria, which would prove useful in treating bacterial skin conditions. A similar selectivity phenomenon was also noted when the effects of EOs were tested by Ambrosio *et al*., 2017 (41). The

mechanism of this selectivity remains unclear, especially when considering two species from the same family, such as *S. epidermidis* and *S. aureus*, which are both Gram-positive and share similar structural and functional components. How could a product be able to tell the difference between 'good' and 'bad' when considering these two bacterial species? Further research is needed in order to clarify this phenomenon.

Cinnamon and clove showed the highest antimicrobial activity against all bacterial species tested, with the cinnamon having outperformed or performed similarly to the chloramphenicol in each trial (Fig 7). These results are mostly consistent with findings reported in previously published research (6, 10). Interestingly, the cinnamon outperformed the chloramphenicol in every trial against *B. subtilis*, suggesting that it may be even more effective than chloramphenicol at inhibiting the growth of *B. subtilis* (Fig 2, Fig 7). The results for the antimicrobial activity of clove were higher for *S. epidermidis* than what was previously published by Abers *et al*., 2021 (6), but lower than what Fu *et al*. 2007 noted (10). However, the antimicrobial effect of clove against both *B. subtilis* and *S. aureus* was higher than what was published by Abers *et al*. (6) and Fu *et al*. (10). Rosemary underperformed in all tests in this study when compared to findings published by Abers *et al*. (6), and Fu *et al*. (10). This could be due to different manufacturing processes, sourcing, or various other factors.

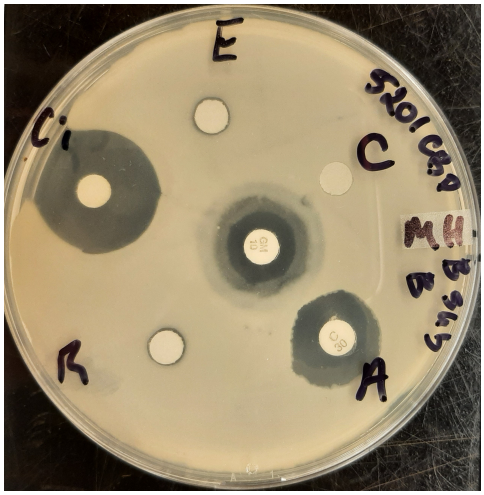

FIG. 7 *B. subtilis* Trial Sample Mueller Hinton Plate. Cinnamon EO (Ci) showed a larger ZI when compared to the chloramphenicol (A) and gentamicin (GM) antibiotic ZIs. Also seen are the negative control (C), eucalyptus (E), and rosemary (R).

As per the 2021 EUCAST *disk diffusion method for antimicrobial susceptibility testing reading guide*, reading of the ZI edges is the point where no growth is seen from the back of the plate on a black surface. In the event that the ZI edges are cloudy, fuzzy, or otherwise, the innermost area without growth is measured (39). Sometimes these results can indicate swarming, or other forms of motility; however, the edge can still be measured in this case (39). Growth within the ZI was observed in the *P. aeruginosa* trials, where there was streaking into the ZIs of chloramphenicol (Fig 3) which could be mistaken for swarming; however, *P. aeruginosa* typically only exhibits this in up to 0.7% agar thickness (42). Because of this, another positive control, gentamicin, was added to confirm that the microbes can be inhibited and some streaking was noted with the gentamicin as well (Fig 3). Interestingly, the cinnamon ZI was clear with a sharp edge, suggesting that the cinnamon more effectively inhibited motility in *P. aeruginosa* than chloramphenicol. Also of note, the *P. aeruginosa* cultures exhibited a striking aqua pigmentation on the MH plates (Fig 3). This may be due to the production of pyocyanin and other pigments through the metabolic activity of *P. aeruginosa* (43) or quorum sensing which is influenced by bacterial density (44). Muting of this coloration suggests that the metabolic activity or bacterial density of *P. aeruginosa* is being affected by gentamicin, chloramphenicol, clove and On Guard® as seen in Figure 3.

The standard plate count was conducted in order to confirm that an OD reading of 0.08-0.1 produced consistent bacterial lawn densities and that the ZIs were repeatable between trials (37, 38). If the bacterial lawn was too thick, the diffusion of the antimicrobial substances may have decreased and would negatively affect the results. By performing the standard plate count it was possible to determine that the bacterial lawns were consistent with the 0.5

McFarland standard which approximates for a lawn consisting of 1.5 x 10^8 CFU/mL. This growth is ideal for disc diffusion assays. Any lower or higher densities outside of an OD range of 0.08-0.10 would result in larger or smaller ZIs, respectively, due to differences in diffusion ability, bacterial density, and thickness of agar (45).

Limitations Using a relatively simple and standard method of determining antimicrobial activity, this study was able to highlight the antimicrobial effects of the EOB and the pure EOs in it. However, some limitations should be noted. The limited trials of each experiment decreased the certainty of the experimental results. This study was not able to compare the concentrations of the EOB and EOs, since determining the concentration of the active components in the EOB was outside of the scope of these experiments. The mechanisms of action cannot be determined without further understanding of the active components. Antibiotic resistance is common in bacterial species such as *P. aeruginosa* and *B. subtilis* which may have an effect on how well the positive controls inhibit growth. Using different positive controls would be useful in this type of experiment. Furthermore, each of the microbes were tested separately from each other; however, most skin microbes that participate in the skin microbiome will respond differently to perturbations in commensals and at different ratios than they will in pure cultures (28). In order to address these limitations, more trials are needed to increase the certainty of the results, more research is required to determine the minimum inhibitory concentration of the EOB with each of the chosen microbes, and the active components need to be tested against the microbes in order to determine the mechanism of action of the EOB. Additionally, further research should be conducted with these microbes in biofilms to model the microbiome, as well as with the skin microbiome itself in order to better understand the action of the EOB against microbes that participate in commensals in the microbiome.

Conclusions The purpose of this study was to investigate the antimicrobial activity of dōTERRA© On Guard® products against *S. epidermidis*, *B.s subtilis*, *S. aureus*, and *P. aeruginosa*. This was achieved using a Kirby-Bauer disc diffusion assay. It was proposed that the On Guard® EOB would have antimicrobial activity towards some or all of the selected microbes, since its EO components have previously been shown to have antimicrobial properties. It was found that the EOB showed statistically significant antimicrobial activity against all four species of skin bacteria. Interestingly, the cinnamon EO outperformed the chloramphenicol positive control in some trials (Fig 7). The EOB also seemed to more selectively inhibit the pathogenic microbe *S. aureus* over the resident microbe *S. epidermidis*. These findings strongly suggest that the On Guard® EOB does have antimicrobial activity; however, further research is required to confirm the statistical significance of the selectivity of the EOB and to solidify its potential as a treatment for bacterial skin conditions.

Future Directions There is ample literature outlining the antimicrobial activity of different pure essential oils, but next to nothing on EOBs. EOBs should be included more often in this kind of research, as well as in vivo studies, to determine their potential in treating bacterial conditions, or for use in sanitization. Using more natural, plant-based products can help to limit one's exposure to cytotoxic ingredients, and having effective EOB-based treatments and cleaners may benefit those that are allergic or do not respond well to traditional pharmaceutical treatments or cytotoxic (and foul smelling) disinfectants and sanitizers.

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