



**A Multifactorial Comparison of Ternary Combinations of Essential Oils in Topical Preparations to Current Antibiotic Prescription Therapies for the Control of Acne Vulgaris-Associated Bacteria.**

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6 A Multifactorial Comparison of Ternary Combinations of Essential Oils in Topical  
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8 Preparations to Current Antibiotic Prescription Therapies for the Control of Acne Vulgaris-  
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10 Associated Bacteria.  
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42 *epidermidis*; antimicrobial synergy.  
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**ABSTRACT**

Acne vulgaris, a chronic condition associated with overgrowth of *Propionibacterium acnes* and *Staphylococcus epidermidis*, is commonly treated with antibiotics. However, the emergence of antibiotic resistance has resulted in a need for alternative therapies. The aim of this study is to develop a topical preparation incorporating Essential Oils (EOs) for use against acne-associated bacteria and assess its efficacy against prescription therapies Dalacin T and Stiemycin. Antimicrobial screening of rosewood, clove bud and litsea EOs against was conducted before interactions between binary and ternary combinations were determined against *P. acnes* and *S. epidermidis* (type and clinical isolates) using Minimum Inhibitory Concentrations (MICs) and Fractional Inhibitory Concentrations (FICs). The EOs were characterised by both GC-MS and NMR. A combination of 0.53 mg/ml litsea, 0.11 mg/ml rosewood and 0.11 mg/ml clove bud was formulated into herbal distillates and compared to Dalacin T and Stiemycin against antibiotic sensitive and resistant isolates (erythromycin). The distillate with EO had synergistic activity against *P. acnes* (7log<sub>10</sub> reduction) and indifferent activity against *S. epidermidis* (6log<sub>10</sub> reduction), antimicrobial activity was either significantly ( $p \leq 0.05$ ) more antimicrobial or equivalent to that of Dalacin T and Stiemycin. This formulation may serve as a valuable alternative for the control of acne vulgaris-associated bacteria.

## INTRODUCTION

Acne vulgaris, a chronic inflammatory disease of the pilosebaceous unit (Williams et al., 2012), affects approximately 80% of all 11 to 30 year olds (Bergler-Czop, 2014). The disease manifests as seborrhoea, non-inflammatory lesions (comedones), inflammatory lesions (papules, pustules, nodules and cysts), and scarring on the face, neck, chest and back (Williams et al., 2012), and is associated with substantial physical and psychological morbidity (Koo et al., 2014).

The pathogenesis of acne vulgaris is not fully understood, however, increased sebum production and follicular hyperkeratosis provides an anaerobic, lipid-rich environment in which the anaerobic commensal *Propionibacterium acnes* proliferates and stimulates inflammation (Contastot and French, 2014). In addition, *Staphylococcus epidermidis*, an aerobic skin commensal, may be a secondary coloniser that potentiates inflammation (Bek-Thomsen et al., 2008).

Topical and oral antibiotics, such as tetracyclines or erythromycin, are commonly prescribed for acne as they inhibit the proliferation of *P. acnes* and exert anti-inflammatory activity (Dawson and Dellavalle, 2013). However, resistance to these antibiotics has been observed in *P. acnes*; resistance to at least one antibiotic was found in 51-94% of isolates in 6 European countries and was strongly correlated with prescribing patterns (Ross et al., 2003). Antibiotic resistance in *S. epidermidis* and other coagulase-negative staphylococci also increases in prevalence with the duration of therapy (Humphrey, 2012). This provides a rationale for seeking alternative antimicrobial treatments for acne vulgaris.

Essential oils (EOs) are volatile mixtures of plant secondary metabolites obtained by steam distillation of plant material (Solorzano-Santos and Miranda-Novales, 2012). Many EOs possess broad-spectrum antimicrobial activity, including against antibiotic resistant strains, *in*

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3 *vitro*. For example, lemongrass (*Cymbopogon flexuosus*) EO inhibited methicillin-sensitive  
4 *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) at 0.06% (Adukwu et al.,  
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2012). While long term use of synthetic antibiotics is linked to resistance, EOs are complex  
mixtures and are believed to have multiple non-specific mechanisms of action, suggesting  
resistance may be less likely to develop (Alexopoulos et al., 2011). To date, no significant  
antimicrobial resistance to EOs has been reported (Bakkali et al., 2008). Furthermore, EOs  
often possess other pharmacological activities, such as anti-inflammatory activity, which may  
enhance their therapeutic benefits as acne treatments (Tsai et al., 2011).

This study aims to identify a synergistic antimicrobial combination of EOs against *P. acnes*  
and *S. epidermidis*, and analyse its *in vitro* efficacy in natural product-based topical  
formulations. The efficacy of the most active topical formulation was then compared to that  
of the topical antibiotic solutions Dalacin T and Stiemycin and their respective active  
ingredients, clindamycin phosphate and erythromycin.

## MATERIALS AND METHODS

**Essential Oils.** Clove bud (*Syzygium aromaticum*), litsea (*Litsea cubeba*) and rosewood  
(*Aniba rosaeodora*) EOs were obtained from Penny Price Aromatherapy Ltd. (Hinckley,  
UK).

**Gas Chromatographic Analysis.** Major components of EOs were determined using a  
Thermo Focus gas chromatograph (GC) equipped with a flame ionization detector and  
Zebron ZB-5MS column (30m x 0.25 mm, 0.25 µm film thickness). EO samples were diluted  
1:100 in GC-grade hexane (Fisher Scientific, Loughborough, UK) and 0.30 ml volumes  
injected into the GC. Injection temperature was 280°C and the split ratio 1:34. The column  
temperature was retained at 60°C for 5 minutes, before increasing to 220°C at a rate of  
4°C/min, and to 280°C at a rate of 11°C/min. Identification of major components was based

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3 on comparisons to standard reference samples (Sigma Aldrich, Gillingham, UK) where  
4 possible. GC coupled with mass spectrometry (GC-MS) was performed using a Bruker 450-  
5 GC gas chromatograph coupled with a Bruker 300-MS SQ mass spectrometer (Bruker,  
6 Billerica, USA) in order to identify remaining major components by NIST library database  
7 matching.  
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14 **<sup>1</sup>H NMR Measurements.** Proton (<sup>1</sup>H) NMR measurements of EOs were conducted on a  
15 Bruker Avance 400 MHz spectrometer operating at a frequency of 399.94 MHz and a probe  
16 temperature of 298 K. A 0.20 ml aliquot of each EO sample was diluted to a final volume of  
17 0.60 ml with deuterated chloroform (C<sup>2</sup>HCl<sub>3</sub>) containing 3.67 x 10<sup>-3</sup> mol.dm<sup>-3</sup>  
18 tetramethylsilane (TMS), the former providing a field frequency lock, the latter as an internal  
19 chemical shift reference (δ = 0.00 ppm) and quantitative <sup>1</sup>H NMR standard; these solutions  
20 were then placed in 5 mm diameter NMR tubes. Typical pulsing conditions were: 128 free  
21 induction decays (FIDs) using 65,536 data points and a 4.5 s pulse repetition rate, the latter to  
22 allow full spin-lattice (T<sub>1</sub>) - relaxation of protons in the samples investigated. Chemical shift  
23 values of resonances were referenced to TMS (δ = 0.000 ppm, internal) and/or residual  
24 chloroform (δ = 7.262 ppm). Resonances present in each spectrum were routinely assigned  
25 by a consideration of chemical shift values, coupling patterns and coupling constants.  
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41 **Microorganisms and Culture Methods.** Test species were *P. acnes* (NCTC 737) and *S.*  
42 *epidermidis* (NCTC 7291). Clinical isolates obtained from Leicester Royal Infirmary  
43 (Leicester, UK) were *S. epidermidis* PL02202 (erythromycin-sensitive), PL021862  
44 (erythromycin-resistant) and PL022186 (erythromycin-resistant), and *P. acnes* PL017109  
45 (erythromycin-sensitive). All microorganisms were grown in brain heart infusion (BHI) broth  
46 and cultured on solid media using BHI agar (Sigma Aldrich). *P. acnes* NCTC 737 was grown  
47 in a CO<sub>2</sub> incubator (Innova CO-170, New Brunswick Scientific, Edison, USA) at 37°C, and  
48 *P. acnes* PL017109 in an anaerobic cabinet (MiniMACS Anaerobic Workstation, Don  
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3 Whitley Scientific, Shipley, UK). *S. epidermidis* was grown under aerobic conditions at  
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5 37°C.  
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7 **Screening EOs for Antimicrobial Activity.** A Kirby-Bauer disc diffusion method was  
8  
9 used. Agar plates were spread with 0.10 ml test bacterial culture before 2 cm filter paper discs  
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11 spotted with 0.05 ml EO were placed on the agar surface. Plates were incubated for 24 hr. at  
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13 37°C and zone of inhibition (ZOI) measured for each EO tested.  
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15 **Determination of Minimum Inhibitory Concentrations (MIC) and Minimum**  
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17 **Bactericidal Concentrations (MBC) of EOs.** Serial two-fold dilutions of EOs (litsea: 0.53-  
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19 35.6 mg/ml; rosewood and clove bud: 0.66-44 mg/ml) were prepared in BHI broth with 10%  
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21 (v/v) dimethyl sulfoxide (Fisher Scientific) to a final volume of 9.90 ml. A 0.10 ml aliquot of  
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23 either *P. acnes* ( $10^6$  colony forming units (CFU)/ml<sup>-1</sup>) or *S. epidermidis* ( $10^5$  CFU/ml<sup>-1</sup>) was  
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25 added before incubation at 37°C for 24 hr. Controls were BHI broth with 10% (v/v) dimethyl  
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27 sulfoxide and 0.10 ml organism only. MICs were determined as the lowest concentration of  
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29 EO where no bacterial growth was observed. MBCs were determined by sub-culturing 0.10  
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31 ml aliquots of the test solutions after incubation onto BHI agar plates, which were then  
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33 incubated for 24 hr. and any growth observed.  
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38 **Assessment of Synergistic Antimicrobial Interactions.** The checkerboard method  
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40 (Mulyaningsih et al., 2010) was used to investigate potential synergistic, indifferent or  
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42 antagonistic relationships between binary and ternary combinations of EOs by determination  
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44 of Fractional Inhibitory Concentrations (FIC) and FICIs. Serial two-fold dilutions of EOs to  
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46 yield final concentrations between the MIC and 0.89 mg/ml for litsea and 0.11 mg/ml for  
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48 rosewood and clove bud EOs were prepared in BHI broth with 10% (v/v) dimethyl sulfoxide  
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50 and inoculated with either *P. acnes* or *S. epidermidis*. Binary and ternary combinations of  
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52 these variable concentrations were added to 96-well plates. The optical densities (595 nm) of  
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54 the samples were obtained using a Spectramax Plus 384 microplate reader and Softmax Pro  
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version 6.4 software (Molecular Devices, Sunnyvale, USA) at 0 hr., and again after 24 hr. incubation at 37°C.

MICs and FICIs of the combinations of the EOs were then calculated:

$$FICI = FIC_{EO1} + FIC_{EO2} = \frac{MIC_{EO1} \text{ in combination}}{MIC_{EO1} \text{ alone}} + \frac{MIC_{EO2} \text{ in combination}}{MIC_{EO2} \text{ alone}}$$

Synergistic, indifferent or antagonistic interactions were concluded from the following limits: Synergy:  $FICI \leq 0.5$ , indifferent:  $0.5 > FICI < 4$ , antagonism:  $FICI > 4$  (Odds, 2003). FIC data for each EO in the ternary combination was then employed to generate 3D isobolograms (Yoon et al., 2004) using Minitab version 17.2.1 (Minitab Inc., State College, USA).

**Efficacy of EO Blend within Topical Formulations.** A ternary combination of 0.53 mg/ml litsea, 0.11 mg/ml clove bud and 0.11 mg/ml rosewood EOs found to inhibit both *P. acnes* and *S. epidermidis* was tested for antimicrobial activity in two topical cosmetic toner preparations comprising of either neroli (*Citrus aurantium* var *amara*) or Roman chamomile (*Chamaemelum nobile*) herbal distillate (hydrolat) with 0.5% (v/v) ethanol (Fisher Scientific) added as an EO solvent. A method adapted from the British Standard preservative efficacy test (British Standards Institute, 2012) was used. Formulations were prepared and inoculated with either *P. acnes* ( $10^7$  CFU/ml<sup>-1</sup>) or *S. epidermidis* ( $10^6$  CFU/ml<sup>-1</sup>) before being incubated for 24 hr. at either 25°C (skin surface temperature) or 37°C (pilosebaceous unit temperature), spread plated onto BHI agar and enumerated. This was repeated with the most efficacious topical preparation against *P. acnes* and *S. epidermidis* clinical isolates. Controls were hydrolat alone and hydrolat with 0.5% (v/v) ethanol.

**Comparison of Efficacy of Neroli Toner to Current Prescription Therapies.** The antimicrobial efficacy of the neroli toner was compared to the prescription only topical antibiotic preparations Dalacin T and Stiemycin by determining log<sub>10</sub> CFU/ml reductions of *S. epidermidis* and *P. acnes* type strains together with clinical isolates *S. epidermidis*



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3 PL021862, PL022186 and PL022202 and *P. acnes* PL017109 after 24 hr. incubation at 25°C  
4 and 37°C according to the adapted British Standard preservative efficacy test method. In  
5 addition, the ternary combination of 0.53 mg/ml litsea, 0.11 mg/ml clove bud and 0.11 mg/ml  
6 rosewood EOs was compared to that of the Dalacin T and Stiemycin active ingredients, 10  
7 mg/ml clindamycin phosphate and 20 mg/ml erythromycin (Sigma Aldrich), respectively.  
8 Active ingredients were suspended in BHI broth and 0.5% (v/v) ethanol was used as a solvent  
9 for the EO combination. Controls were BHI broth with 0.5% (v/v) ethanol, and BHI broth  
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20 All investigations were carried out in triplicate on at least two separate occasions.

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23 **Statistical Analysis.** Two-way analysis of variance with Tukey's comparisons was  
24 conducted using Minitab Statistical Software version 16 (Minitab Inc., State College, USA)  
25 in order to determine the significance of any differences ( $p \leq 0.05$ ) in mean  $\log_{10}$  reductions  
26 between topical preparations and their controls.  
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## 34 RESULTS AND DISCUSSION

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37 **Antimicrobial Activity of EOs.** Initial screening showed that clove bud, litsea and  
38 rosewood had mean ZOI of 3.9 mm  $\pm$  0.03, 9.0 mm  $\pm$  0.00 and 9.0 mm  $\pm$  0.00 for *P. acnes*,  
39 and 2.8 mm  $\pm$  0.03, 1.7 mm  $\pm$  0.06 and 3.2 mm  $\pm$  0.03 against *S. epidermidis*.  
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45 MICs of clove bud and rosewood EOs were 1.38 mg/ml against both *S. epidermidis* and *P.*  
46 *acnes*. Litsea had an MIC of 1.11 mg/ml against *S. epidermidis*; however, it exerted less  
47 antimicrobial activity against *P. acnes* (MIC = 2.23 mg/ml). EOs were bactericidal against *S.*  
48 *epidermidis*, with MBCs of 2.23 mg/ml for litsea, and 2.75 mg/ml for rosewood and clove  
49 bud EOs, however, only the clove bud EO was bactericidal against *P. acnes* (MBC = 5.5  
50 mg/ml). Previous investigations have reported MICs of 0.25% (v/v) for clove bud EO against  
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3 *S. epidermidis* (Fu et al., 2007) in accordance with the current findings. However, the MIC of  
4 clove bud EO against *P. acnes* was greater than the previously reported MIC of 0.31 mg/ml  
5 Fu et al., 2009) which may be the result of variation in isolate susceptibility or EO  
6 composition between studies (Verma et al., 2015). Antimicrobial activity of litsea and  
7 rosewood EOs has been reported against species such as *S. aureus* (MICs = 0.2 and 0.5  
8 mg/ml, respectively; Wang and Liu, 2010; Rosato et al., 2010)

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17 **Assessment of Synergistic Antimicrobial Interactions Between EOs.** Synergistic  
18 interactions were observed between all binary combinations of litsea, clove bud and  
19 rosewood EOs against *S. epidermidis*. MICs of these EOs in combination were 0.11 mg/ml  
20 rosewood with 0.33 mg/ml clove bud (FICI = 0.32), and 0.09 mg/ml litsea with 0.11 mg/ml  
21 rosewood (FICI = 0.16) or 0.33 mg/ml clove bud EO (FICI = 0.32). A combination of litsea  
22 (0.53 mg/ml) and rosewood EOs (0.33 mg/ml) was synergistic against *P. acnes* (FICI = 0.48),  
23 however, combinations containing clove bud EO were indifferent (FICIs = 0.58 - 1.08).  
24 Synergistic binary combinations of bergamot (*C. bergamia*) and basil (*Ocimum basilicum*)  
25 EOs reportedly engendered a greater leakage of cellular contents of *B. subtilis*, *E. coli* and *S.*  
26 *aureus* compared to EOs alone, suggesting enhanced cell membrane disintegration (Lv et al.,  
27 2011). Furthermore, disintegration of the membrane by one component may increase the  
28 cellular uptake of other antimicrobial components (Santiesteban-Lopez et al., 2007).  
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45 A combination of 0.53 mg/ml litsea, 0.11 mg/ml rosewood and 0.11 mg/ml clove bud EOs  
46 was synergistic against *P. acnes* (FICI = 0.40), whilst a combination of 0.09 mg/ml litsea,  
47 0.11 mg/ml clove bud and 0.11 – 0.33 mg/ml rosewood EOs was synergistic against *S.*  
48 *epidermidis* (FICI = 0.24), as illustrated by concave areas of 3D isobolograms (Figures 1a  
49 and 1b). The lowest combination of EOs required to inhibit both test species was 0.53 mg/ml  
50 litsea, 0.11 mg/ml rosewood and 0.11 mg/ml clove bud EOs. As such, the quantity of EOs  
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3 required to inhibit these microorganisms was further reduced, which may decrease the  
4 likelihood of adverse effects with topical application (Casetti et al., 2012).  
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8 **Chemical Composition of EOs.** GC analysis indicated that citral was the most abundant  
9 compound present in litsea EO (49.64%), while eugenol was most abundant in clove bud EO  
10 (60.90%), and linalool represented the predominant agent present in rosewood EO (86.73%),  
11 in accordance with previous research (Fu et al., 2007; Rosato et al., 2010; Wang and Liu,  
12 2010). <sup>1</sup>H NMR spectroscopy data acquired is in agreement with that from GC analysis  
13 (Figure 2).  
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22 **Antimicrobial Efficacy of the Ternary EO Combination in Cosmetic Toner Topical**  
23 **Formulations.** The ternary combination of EOs was tested for antimicrobial activity in two  
24 toner formulations based on neroli or Roman chamomile hydrolats. Hydrolats are water-  
25 based by-products of steam distillation of plant material, and contain some water-soluble  
26 components of EOs (Lin et al., 2011). Both hydrolats were antimicrobial, resulting in  
27 reductions of between 0.67 and 4.76 log<sub>10</sub> (Table 1). Few studies have investigated the  
28 antimicrobial activity of hydrolats; however, Chinese olive (*Canarium album*) hydrolat was  
29 reportedly antimicrobial against *P. acnes* and *S. epidermidis* (ZOIs = 15.2 mm and 21.0 mm,  
30 respectively; Lin et al., 2011). Temperature did not significantly ( $p \geq 0.05$ ) affect the activity  
31 of Roman chamomile hydrolat, however, activity of neroli hydrolat was significantly ( $p \leq$   
32 0.05) increased at 37°C.  
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48 Ethanol (0.5% v/v) was added to the hydrolat as an EO solvent. This concentration of  
49 ethanol was not antimicrobial (data not shown), however significantly ( $p \leq 0.05$ ) increased  
50 the activity of neroli hydrolat against *S. epidermidis* and *P. acnes*, resulting in complete  
51 inhibition of *P. acnes* at 37°C and *S. epidermidis* at both temperatures. Furthermore, Roman  
52 chamomile hydrolat was significantly ( $p \leq 0.05$ ) more antimicrobial against *S. epidermidis*  
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3 with the addition of ethanol, with 5.5 and 5.2 log<sub>10</sub> reductions being observed at 25°C and  
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5 37°C, respectively. Although antimicrobial interactions between ethanol and hydrolats have  
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7 not previously been studied, a non-lethal concentration of ethanol was synergistic with the  
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9 lipid sapienic acid against MRSA. Ethanol is a penetration enhancer, and in this manner may  
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11 increase partitioning of antimicrobial components into the cell membrane or enhance entry  
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13 into the cytoplasm (Drake et al., 2008).  
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17 The complete toner formulations (hydrolat, ethanol and EOs) completely destroyed *P.*  
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19 *acnes* at 25°C. This was significantly ( $p \leq 0.05$ ) greater than reductions achieved by hydrolat  
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21 with ethanol, suggesting that EOs significantly contribute to the antimicrobial activity of the  
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23 formulation. *S. epidermidis* was also completely destroyed by neroli and Roman chamomile  
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25 toners; this activity was equivalent ( $p \geq 0.05$ ) to that of a combination of hydrolat with  
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27 ethanol, indicating that the EOs do not provide any additional benefit to the topical  
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29 formulation for the control of *S. epidermidis*.  
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34 Additionally, the neroli toner with EOs completely inhibited all three clinical isolates of *S.*  
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36 *epidermidis*, and one of *P. acnes*, including the antibiotic resistant strains *S. epidermidis*  
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38 PL021862 and PL022186. Considering the variation in antimicrobial susceptibility of isolates  
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40 from individual acne patients, and between patients (Humphrey, 2012) this data demonstrates  
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42 the potential use of this formulation as a novel product for the control of *P. acnes* and *S.*  
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44 *epidermidis*.  
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47 **Comparison of Neroli Toner Efficacy to that of Dalacin T and Stiemycin.** The topical  
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49 antibiotic solutions Dalacin T and Stiemycin resulted in complete inhibition of all test  
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51 species. The neroli toner formulation possesses an equivalent antimicrobial activity (Table 1),  
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53 further supporting its use for the control of acne vulgaris.  
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3 **Comparison of the Neroli Toner, Dalacin T and Stiemycin Active Ingredients.** Overall,  
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5 there was a high level of variability in antimicrobial activity between the active ingredients,  
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7 which was dependent on strain/isolate and incubation temperature (Table 2). Erythromycin  
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9 produced significantly ( $p \leq 0.05$ ) greater reductions than clindamycin phosphate and EOs  
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11 against *S. epidermidis* PL021862 at 37°C, and *S. epidermidis* PL02202 and *P. acnes* type  
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13 strain, at both temperatures (Table 2). Interestingly, erythromycin completely inhibited the  
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15 erythromycin-resistant *S. epidermidis* isolate PL021862 at 37°C. However, the concentration  
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17 of 20 mg/ml found in Stiemycin is significantly greater than the MIC breakpoint for  
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19 resistance to erythromycin of 2 mg/ml in staphylococci (BSAC, 2015). EOs were  
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21 significantly ( $p \leq 0.05$ ) less antimicrobial than clindamycin phosphate against *S. epidermidis*  
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23 PL021862 and *P. acnes* at 37°C. However, EOs were not significantly ( $p \geq 0.05$ ) different in  
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25 activity to clindamycin phosphate against *S. epidermidis* isolate PL021862 at 25°C, and  
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27 PL022186 and PL02202 at both temperatures. Furthermore, EOs were significantly ( $p \leq$   
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29 0.05) more antimicrobial than clindamycin phosphate against *P. acnes* at 25°C, and both  
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31 antibiotics against the *P. acnes* clinical isolate PL017109 and *S. epidermidis* type strain,  
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33 indicating that the EOs could be useful as novel antibiotic alternatives.  
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40 Log<sub>10</sub> CFU/ml reductions achieved by all three active ingredients were significantly ( $p \leq$   
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42 0.05) lower than the complete inhibition achieved by their complete formulations. Dalacin T  
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44 contains 50% (v/v) isopropyl alcohol, and Stiemycin contains absolute ethanol (unspecified  
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46 quantity), which are both bactericidal agents and may increase the antimicrobial activity of  
47  
48 the complete formulations over that of their active ingredients. The combination of neroli  
49  
50 hydrolat and ethanol were demonstrated to be significantly antimicrobial and hence increase  
51  
52 the antimicrobial activity of the toner when incorporated (Table 1). This indicates that the  
53  
54 complete formulation is essential to the inhibition of bacteria contributing to acne vulgaris.  
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3 Novel antibiotic alternatives for the control of acne vulgaris-associated bacteria are required  
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5 to combat antibiotic resistance. In this study, a neroli toner formulation was equivalent in  
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7 activity to two current topical antibiotic solutions, including against erythromycin-resistant  
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9 clinical isolates of *S. epidermidis*. The active ingredients of the neroli toner, a ternary  
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11 combination of litsea, clove bud and rosewood EOs, had equivalent or even greater  
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13 antimicrobial activity than that of clindamycin phosphate, erythromycin, or both against all  
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15 but the *P. acnes* type strain at 37°C, and one clinical isolate of *S. epidermidis*. Therefore, the  
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17 toner formulation may be a useful novel alternative for the control of acne vulgaris-associated  
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19 bacteria.  
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## 26 **ACKNOWLEDGEMENTS**

27  
28 This work was supported by the European Regional Development Fund (ERDF) Partnerships  
29  
30 in Knowledge Transfer Scheme.  
31  
32

## 33 **Conflict of Interest**

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35  
36 P. Price is an employee of Penny Price Aromatherapy which in part funded the ERDF  
37  
38 Partnerships in Knowledge Transfer Scheme. The experimental aspect of the research was  
39  
40 conducted by academics and was fully objective.  
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**Table 1.** Log<sub>10</sub> reductions of *P. acnes* and *S. epidermidis* in cosmetic toner formulations after 24 hr. incubation at 25°C and 37°C (n=6, mean ± SEM).

Formulation	<i>P. acnes</i>				<i>S. epidermidis</i>			
	Roman chamomile		Neroli		Roman chamomile		Neroli	
	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C
Hydrolat	0.75 ± 0.19	2.51 ± 0.55	0.67 ± 0.10	4.76 ± 1.40	1.37 ± 0.00	2.65 ± 0.12	0.90 ± 0.01	3.65 ± 0.06
Hydrolat, 0.5% ethanol	1.69 ± 0.12	4.57 ± 0.25	1.14 ± 0.10	7.19 ± 0.00 <sup>a</sup>	5.55 ± 0.20	5.19 ± 0.22	6.05 ± 0.00 <sup>a</sup>	6.05 ± 0.00 <sup>a</sup>
Hydrolat, 0.5% ethanol, EOs (toner)	7.19 ± 0.00 <sup>§</sup>	4.63 ± 0.22	7.19 ± 0.00 <sup>a</sup>	7.19 ± 0.00 <sup>a</sup>	6.05 ± 0.00 <sup>a</sup>	6.05 ± 0.00 <sup>a</sup>	6.05 ± 0.00 <sup>a</sup>	6.05 ± 0.00 <sup>a</sup>

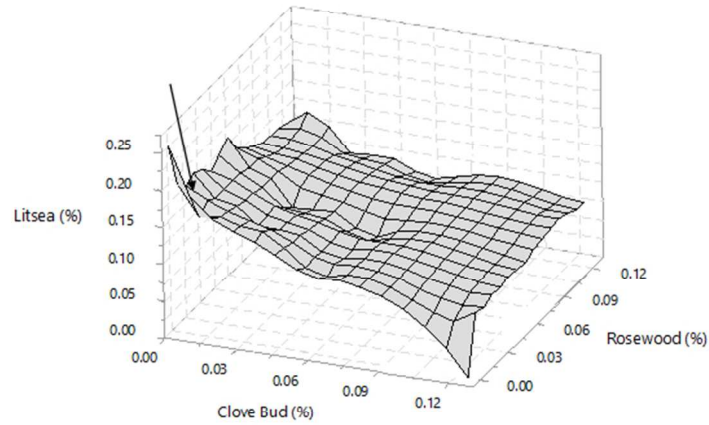
<sup>a</sup> Complete inhibition.

**Table 2.** Log<sub>10</sub> reductions of *P. acnes* and *S. epidermidis* by active ingredients of the neroli toner, Dalacin T and Stiemyacin at 25°C and 37°C (n=6, mean ± SEM).

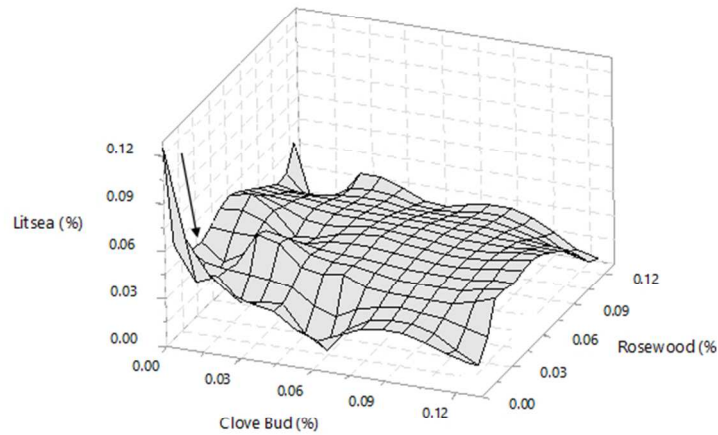
Organism	EOs		Clindamycin Phosphate		Erythromycin	
	25°C	37°C	25°C	37°C	25°C	37°C
<i>P. acnes</i>	1.95 ± 0.09	-0.62 ± 0.15	0.36 ± 0.02	1.81 ± 0.01	7.19 ± 0.00 <sup>a</sup>	6.39 ± 0.46
<i>P. acnes</i> PL017109	4.15 ± 0.11	6.41 ± 0.16	0.17 ± 0.02	0.17 ± 0.03	2.50 ± 0.21	2.87 ± 0.01
<i>S. epidermidis</i>	2.51 ± 0.15	3.41 ± 0.25	-2.00 ± 0.00	-2.00 ± 0.00	0.34 ± 0.01	0.22 ± 0.06
<i>S. epidermidis</i> PL021862 (erythromycin -resistant)	-0.53 ± 0.21	-0.71 ± 0.23	2.00 ± 0.00	2.00 ± 0.00	0.81 ± 0.05	6.97 ± 0.00 <sup>§</sup>
<i>S. epidermidis</i> PL022186 (erythromycin -resistant)	1.56 ± 0.14	1.04 ± 0.21	-0.49 ± 0.73	-1.76 ± 0.00	-0.01 ± 0.14	0.50 ± 0.05
<i>S. epidermidis</i> PL02202	-0.04 ± 0.04	-2.27 ± 0.06	0.52 ± 0.02	-2.00 ± 0.00	3.13 ± 0.00	5.68 ± 0.39

<sup>a</sup> Complete inhibition.

a)

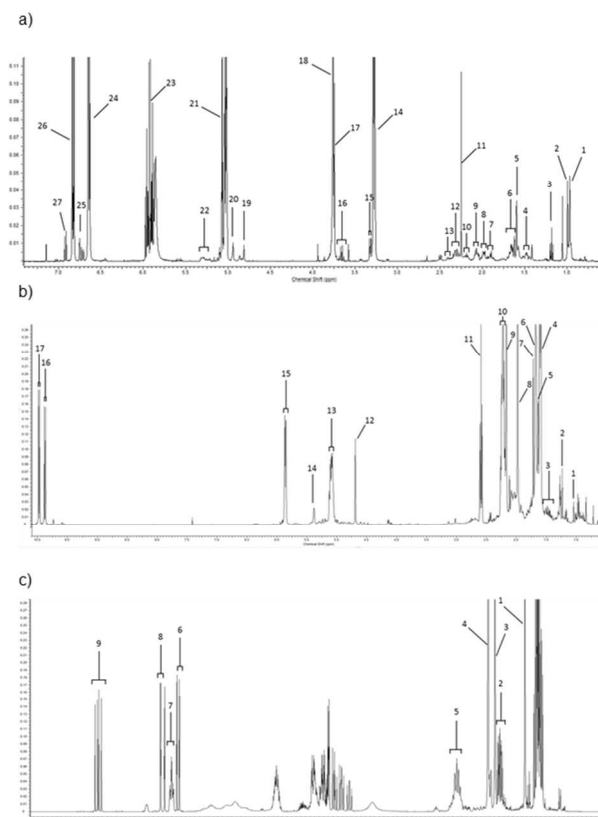


b)



Lucy Owen. Figure 1: 3D plot of fractional inhibitory concentrations of litsea, rosewood and clove bud EOs against a) *P. acnes* and b) *S. epidermidis*.

Figure 1: 3D plot of fractional inhibitory concentrations of litsea, rosewood and clove bud EOs against a) *P. acnes* and b) *S. epidermidis*.  
(Figures 1a and 1b).  
153x245mm (96 x 96 DPI)



Lucy Owen. Figure 2: 400 MHz  $^1\text{H}$  NMR spectra of a) clove, b) litsea and c) rosewood EOs.

Assignments:

a) 1,  $\beta\text{C-C14-CH}_3$ ; 2,  $\beta\text{C-C15-CH}_3$ ; 3, Ethanol- $\text{CH}_3$ ; 4,  $\beta\text{C-C2-CH}_{2a}$ ; 5,  $\beta\text{C-C13-CH}_3$ ; 6,  $\beta\text{C-C2-CH}_{2b}/\text{C10-CH}_2$ ; 7,  $\beta\text{C-C3-CH}_{2a}$ ; 8,  $\beta\text{C-C6-CH}_{2a}$ ; 9,  $\beta\text{C-C3-CH}_{2b}$ ; 10,  $\beta\text{C-C7-CH}_{2a}$ ; 11, AcEug-CO- $\text{CH}_3$ ; 12,  $\beta\text{C-C7-CH}_{2b}/\text{C6-CH}_2$ ; 13,  $\beta\text{C-C9-CH}$ ; 14, Eug-Ar- $\text{CH}_2$ ; 15, AcEug-Ar- $\text{CH}_2$ ; 16, Ethanol- $\text{CH}_2\text{OH}$ ; 17, AcEug-O- $\text{CH}_3$ ; 18, Eug-O- $\text{CH}_3$ ; 19,  $\beta\text{C-C12-CH}_{2a}$ ; 20,  $\beta\text{C-C12-CH}_{2b}$ ; 21, Eug/Ac-Eug- $\text{CH}_2=\text{CH}$ ; 22,  $\beta\text{C-C5-CH}$ ; 23, Eug/Ac-Eug- $\text{CH}_2=\text{CH}$ ; 24, Eug-Ar<sub>1</sub>; 25, AcEug-Ar<sub>1</sub>; 26, Eug-Ar<sub>2</sub>; 27, AcEug-Ar<sub>2</sub>.

b) 1, Euc-C11- $\text{CH}_3$ ; 2, Euc-C9/10- $\text{CH}_3$ 's; 3, Euc-C3- $\text{CH}$ ; 4, G-C8- $\text{CH}_3$  / N-C8- $\text{CH}_3$ ; 5, L-C7- $\text{CH}_3$ ; 6, G-C8'- $\text{CH}_3$  / N-C8'- $\text{CH}_3$ ; 7, L-C10- $\text{CH}_3$ ; 8, N-C3'- $\text{CH}_3$ ; 9, G-C3'- $\text{CH}_3$ ; 10, N-C4/5- $\text{CH}_2$ 's / G-C5- $\text{CH}_2$ ; 11, G-C4- $\text{CH}_2$ ; 12, L-C7- $\text{CH}_2$ ; 13, N-C6- $\text{CH}$  / G-C6- $\text{CH}$ ; 14, L-C2- $\text{CH}$ ; 15, N-C2- $\text{CH}$  / G-C2- $\text{CH}$ ; 16, N-C1- $\text{CHO}$ ; 17, 16, G-C1- $\text{CHO}$ .

c) 1, Lin-C3- $\text{CH}_3$ ; 2, Lin-C4- $\text{CH}_2$ ; 3, Lin-C7- $\text{CH}_3$ ; 4, Lin-C8- $\text{CH}_3$ ; 5, Lin-C5- $\text{CH}_2$ ; 6, Lin-

Figure 2: 400 MHz  $^1\text{H}$  NMR spectra of a) clove, b) litsea and c) rosewood EOs respectively. %"Assignments: a) 1,  $\beta\text{C-C14-CH}_3$ ; 2,  $\beta\text{C-C15-CH}_3$ ; 3, Ethanol- $\text{CH}_3$ ; 4,  $\beta\text{C-C2-CH}_{2a}$ ; 5,  $\beta\text{C-C13-CH}_3$ ; 6,  $\beta\text{C-C2-CH}_{2b}/\text{C10-CH}_2$ ; 7,  $\beta\text{C-C3-CH}_{2a}$ ; 8,  $\beta\text{C-C6-CH}_{2a}$ ; 9,  $\beta\text{C-C3-CH}_{2b}$ ; 10,  $\beta\text{C-C7-CH}_{2a}$ ; 11, AcEug-CO- $\text{CH}_3$ ; 12,  $\beta\text{C-C7-CH}_{2b}/\text{C6-CH}_2$ ; 13,  $\beta\text{C-C9-CH}$ ; 14, Eug-Ar- $\text{CH}_2$ ; 15, AcEug-Ar- $\text{CH}_2$ ; 16, Ethanol- $\text{CH}_2\text{OH}$ ; 17, AcEug-O- $\text{CH}_3$ ; 18, Eug-O- $\text{CH}_3$ ; 19,  $\beta\text{C-C12-CH}_{2a}$ ; 20,  $\beta\text{C-C12-CH}_{2b}$ ; 21, Eug/Ac-Eug- $\text{CH}_2=\text{CH}$ ; 22,  $\beta\text{C-C5-CH}$ ; 23, Eug/AcEug- $\text{CH}_2=\text{CH}$ ; 24, Eug-Ar<sub>1</sub>; 25, AcEug-Ar<sub>1</sub>; 26, Eug-Ar<sub>2</sub>; 27, AcEug-Ar<sub>2</sub>. %"b) 1, Euc-C11- $\text{CH}_3$ ; 2, Euc-C9/10- $\text{CH}_3$ 's; 3, Euc-C3- $\text{CH}$ ; 4, G-C8- $\text{CH}_3$  / N-C8- $\text{CH}_3$ ; 5, L-C7- $\text{CH}_3$ ; 6, G-C8'- $\text{CH}_3$  / N-C8'- $\text{CH}_3$ ; 7, L-C10- $\text{CH}_3$ ; 8, N-C3'- $\text{CH}_3$ ; 9, G-C3'- $\text{CH}_3$ ; 10, N-C4/5- $\text{CH}_2$ 's / G-C5- $\text{CH}_2$ ; 11, G-C4- $\text{CH}_2$ ; 12, L-C7- $\text{CH}_2$ ; 13, N-C6- $\text{CH}$  / G-C6- $\text{CH}$ ; 14, L-C2- $\text{CH}$ ; 15, N-C2- $\text{CH}$  / G-C2- $\text{CH}$ ; 16, N-C1- $\text{CHO}$ ; 17, 16, G-C1- $\text{CHO}$ . %"c) 1, Lin-C3- $\text{CH}_3$ ; 2, Lin-C4- $\text{CH}_2$ ; 3, Lin-C7- $\text{CH}_3$ ; 4, Lin-C8- $\text{CH}_3$ ; 5, Lin-C5- $\text{CH}_2$ ; 6, Lin-C1- $\text{CH}_{2a}$ ; 7, Lin-C6- $\text{CH}$ ; 8, Lin-C1- $\text{CH}_{2b}$ ; 9, Lin-C2- $\text{CH}$ . Abbreviations:  $\beta\text{C}$ ,  $\beta$ -Caryophyllene; Eug, Eugenol; AcEug, Eugenyl acetate; Ar, Aromatic-H; Euc, Eucalyptol; G, Geranial; N, Neral; L, Limonene; Lin, Linalool %" (Figure 2).

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For Peer Review