

Characterisation and screening of antimicrobial essential oil components against clinically important antibiotic-resistant bacteria using thin layer chromatography-direct bioautography hyphenated with GC-MS, LC-MS and NMR.

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Abstract

Introduction: The antimicrobial activity of many Essential Oils (EOs) is well established, indicating that EOs may be a source of compounds for antimicrobial drug development. Thin Layer Chromatography-Direct Bioautography (TLC-DB) can quickly identify antimicrobial components in complex mixtures and can be applied to the screening of EOs for lead compounds. **Objectives:** This study aimed to identify antimicrobial components of oregano, rosewood and cumin EOs against antibiotic-sensitive and -resistant bacteria using TLC-DB and a multi-faceted approach of GC-MS, LC-MS and NMR techniques to characterise bioactive compounds. The study also aimed to quantify the antimicrobial activity of bioactive compounds in order to evaluate their potential for the development of therapies against antibiotic-resistant bacteria. **Materials and Methods:** EOs were eluted on TLC plates and sprayed with a suspension of *Staphylococcus aureus*, *Enterococcus faecium*, *Escherichia coli* or *Pseudomonas aeruginosa* (antibiotic-sensitive and -resistant isolates). Zones of inhibition, visualised with iodinitrotetrazolium chloride, were subject to GC-MS, LC-MS and NMR to characterise the bioactive compounds. **Results:** Seven compounds were identified from the three EOs using GC-MS, while LC-MS and NMR failed to detect the presence of any further non-volatile or heat labile compounds. Carvacrol was most antimicrobial compound identified, with minimum inhibitory concentrations ranging 0.99-31.62 mM. **Conclusion:** The identified antimicrobial compounds present in oregano, rosewood and cumin EOs including carvacrol may be candidates for the development of novel antimicrobial therapies against antibiotic-resistant bacteria.

Short Abstract

In this study, components of oregano, rosewood and cumin essential oils with antimicrobial activity against antibiotic-sensitive and -resistant bacteria were identified using TLC-DB

coupled with GC-MS, LC-MS and NMR as a multi-faceted approach for the characterisation of antimicrobial essential oil fractions. Carvacrol was the most antimicrobial compound identified, with minimum inhibitory concentrations ranging from 0.99-31.62 mM, and may be a candidate for the development of therapies against antibiotic-resistant bacteria.

INTRODUCTION

Over recent decades, Antimicrobial Resistance (AMR) has become widely disseminated amongst many hospital and community-associated pathogens. In some European countries, over 25% of bacterial clinical isolates are now resistant to at least one antibiotic drug.¹ New antimicrobials are needed to continue treating AMR infections, yet few are in the pipeline and only around 30 antibiotics have been approved for use since 2000.²

Natural products are an important source of novel and chemically diverse molecules for drug discovery.³ Indeed, 69% of currently employed antibiotics are derived from natural products.⁴ As such, in response to the increase in AMR, extensive screening of natural products for novel antimicrobial compounds has been conducted. Many reports in the published literature have indicated that Essential Oils (EOs) possess broad spectrum antimicrobial activity, for example, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Propionibacterium acnes*, *Brevibacterium* spp. and *Escherichia coli* were strongly inhibited by 59 EOs, with Minimum Inhibitory Concentration (MICs) below 2 mg/mL.⁵ This suggests that EOs could be a source of lead compounds for antimicrobial development. Several EO constituents have been identified as potent antimicrobials, such as carvacrol, cinnamaldehyde, eugenol and terpinen-4-ol which possess MICs ranging 0.006-1.6% (v/v) against *S. aureus*, *S. epidermidis* and *Enterococcus faecalis*.⁶

The activity of EOs and their components appears to be generally independent of antibiotic resistance profiles, indicating a lack of cross-resistance between antibiotics and EO

compounds. MICs of aromendrene and eucalyptus (*Eucalyptus globulus*) EO against Methicillin-Resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. were equivalent to that of antibiotic susceptible isolates.⁷ Similarly, Kifer et al.⁸ reported that MRSA was as susceptible as Methicillin-Sensitive *S. aureus* to the monoterpenes thymol (MICs = 0.250-0.375 mg/mL), menthol (MICs = 1 mg/mL) and 1,8-cineole (MICs = 4-8 mg/mL). Oregano (*Origanum* spp.) EO was strongly antimicrobial against extended spectrum β -lactamase-producing *E. coli* (MICs = 0.596 mg/mL), carbapenem-resistant *E. coli* (MICs = 0.596 mg/mL) and carbapenem-resistant *Acinetobacter baumannii* (MICs = 0.298 mg/mL). This activity was equivalent to that of sensitive isolates.⁹ The activity of EOs and their components against antibiotic-resistant isolates supports their investigation as a source of antimicrobial lead compounds for drug development.

EOs are typically extracted by steam or hydro-distillation of plant material.¹⁰ The resulting EO is a mixture of secondary metabolites, with around 20 to 100 different constituents. Most components are volatile, low molecular weight organic compounds¹¹ including terpenes and terpenoids, the most abundant class of compound present in EOs.¹² The complex composition of EOs means that isolation of each EO and determination of their bioactivity using fractional techniques can be time consuming. Thin Layer Chromatography-Bioautography (TLC-B) is an analytical technique used to identify antimicrobial compounds separated using Thin Layer Chromatography (TLC); Zones of Inhibition (ZoIs) of bacterial growth corresponding to spots on eluted TLC plates are indicative of antimicrobial compounds. This method affords several advantages over techniques relying on fractionation to screen for antimicrobial EO components. The lack of sample preparation or separation needed makes this a rapid, low cost method for identifying bioactive compounds from complex mixtures, prevents the loss/destruction of compounds and means only small quantities of the crude extract are needed.¹³ In addition, minor components can be overlooked and not fractionated using

techniques such as high-performance liquid chromatography¹⁴ as many EO components are present in trace quantities and some are not UV active.¹⁵ The application of Two-Dimensional (2D)-TLC to TLC-B assays, first described by Wedge and Nagle¹⁶ can also be used to improve resolution, which is especially useful for natural products containing lipophilic compounds, including EOs. However, TLC-B is a qualitative method and so the activity of lead compounds must be quantified by supplementary assays, usually determination of MICs.¹⁷

There are several variations in TLC-B method. Contact bioautography involves overlaying agar seeded with bacteria onto eluted TLC plates before incubation and assessment for zones of inhibition or placing TLC plates onto an inoculated agar surface for a defined amount of time, after which the TLC plate is removed and the agar layer is incubated. A major disadvantage of contact bioautography is that diffusion of lipophilic components into the aqueous agar medium is limited,¹³ making this method unsuitable for EOs. Other disadvantages of contact bioautography include adherence of the TLC adsorbent (e.g. silica) onto the agar layer, and difficulties in obtaining complete contact between the plate and agar layer.¹⁸

TLC-DB overcomes these limitations by applying a bacterial suspension directly onto the TLC plate.¹⁹ ZoIs of bacterial growth are visualised using metabolic stains, typically tetrazolium salts such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (INT).²⁰⁻²⁴ These salts are reduced by dehydrogenases in viable cells to produce purple formazan, thus ZoIs are visualised as white spots on a purple background.^{20,25} Lead compounds are then extracted and characterised.

Mass Spectrometry (MS) is a useful technique in natural product research for compound identification and dereplication due to its sensitivity and ability to provide

accurate mass measurements.²⁶ Gas Chromatography coupled with MS (GC-MS) is the most commonly used method to characterise EOs, as many EO components are low boiling point volatile liquids.²⁷ However, a major disadvantage of GC is that the high temperatures necessary can result in decomposition or structural alteration of heat-labile or non-volatile components.²⁸ For example, the content of sesquiterpenes possessing an (*E,E*)-1,5-cyclodecadiene structure in *Vepris unifoliolata* EO was underestimated by GC due to thermal rearrangement.²⁹ Both Liquid Chromatography coupled with MS (LC-MS) and Proton (¹H) Nuclear Magnetic Resonance (NMR) spectroscopy are conducted at ambient temperature, potentially improving detection of heat-labile and non-volatile components^{26,28}. However, these techniques are less commonly employed than GC-MS in EO analysis. A multi-faceted approach to the characterisation of antimicrobial EO fractions from TLC-DB could therefore be warranted to ensure that heat-labile and non-volatile compounds are not overlooked.

The aim of this study was to identify compounds within cumin, oregano and rosewood EOs with antimicrobial activity against antibiotic-sensitive and -resistant bacteria (clinical and type strains) using TLC-DB and a multi-faceted approach employing GC-MS, LC-MS and ¹H NMR techniques. Moreover, this study aimed to quantify the antimicrobial activity of bioactive compounds in order to evaluate their potential for the development of therapies against antibiotic-resistant bacteria.

EXPERIMENTAL

EOs

Cumin (*Cuminum cyminum*), oregano (*Origanum compactum*) and rosewood (*Aniba rosaeodora*) EOs were obtained from Penny Price Aromatherapy Ltd. (Hinckley, UK).

Authentic terpene standards (-)- β -pinene (99%), carvacrol (98%), cuminaldehyde (98%), linalool (97%), *p*-cymene (99%), thymol ($\geq 98.5\%$), β -caryophyllene ($\geq 80\%$) and γ -terpinene (97%) were obtained from Sigma Aldrich (Gillingham, UK).

Microorganisms

Methicillin-Sensitive *S. aureus* NCTC 1298 (MSSA), Methicillin-Resistant *S. aureus* NCTC 12497 (MRSA), *E. coli* NCTC 8003, Ciprofloxacin-Resistant (CR) *E. coli* (clinical isolate), *P. aeruginosa* NCTC 6749 and CR *P. aeruginosa* (clinical isolate) were cultured using nutrient broth and agar (Oxoid, Basingstoke, UK) and grown under aerobic conditions at 37°C. Vancomycin-Sensitive *Enterococcus faecium* (VSE; clinical isolate) and Vancomycin-Resistant *E. faecium* NCTC 12202 (VRE) were cultured using Brain-Heart Infusion (BHI) broth and agar (Sigma Aldrich) and grown under aerobic conditions at 37°C. All clinical isolates were obtained from Leicester Royal Infirmary (Leicester, UK).

Screening of EOs for Antimicrobial Activity

Oregano, cumin and rosewood EOs were screened for antimicrobial activity using a Kirby-Bauer disc diffusion method.³⁰ Agar plates were spread with 100 μ L of a suspension (10^8 Colony Forming Units (CFU)/mL) of test organism before paper discs (20 mm) were placed in the centre and impregnated with 50 μ L EO. Plates were incubated for 24 hr. at 37°C before ZoI (mm) was measured. Experiments were conducted in triplicate and replicated twice (n=6).

GC-MS of EOs

Oregano, rosewood and cumin EOs were diluted 1:100 in GC-grade hexane (Fisher Scientific, Loughborough, UK). GC-MS was conducted using a Bruker (Billerica, USA) 450-RC GC equipped with a Zebron (Phenomenex, Torrance, USA) ZB-5MSi column (30 m x 0.25 mm; 0.25 μ m film thickness) coupled with a Bruker 300-MS SQ electron impact MS. Sample volumes of 1 μ L were injected into the GC with an injection temperature of 280°C

and a split ratio 1:100 using a Bruker (Billerica, USA) CP8400 autosampler. Helium was used as a carrier gas at a flow rate of 1.5 mL/min. Column temperature was held at 60°C for 5 minutes before a 4°C min⁻¹ ramp to 220°C and then a 11°C min⁻¹ ramp to 280°C, where the temperature was held for 10 minutes. MS was conducted in positive mode with a source temperature of 150°C, CID gas pressure of 1.5 mTorr, collision energy of 5 eV, and detector voltage of 1000 V. Mass spectra were acquired over an *m/z* range of 50 to 300. Identification of compounds was based on comparison with retention time of standard reference samples of EO components (Sigma Aldrich). Further assignments were made by NIST (Gaithersburg, USA) MS Search Program version 2.0 database matching and comparison of mass values to the published literature.

TLC

Oregano, rosewood and cumin EOs and EO compound reference standards were subject to two-dimensional (2D) TLC to separate components. EOs dissolved in ethyl acetate (Fisher Scientific) to 45 mg/mL and an aliquot sufficient to deliver a 5 mm circular spot to the TLC plate was manually spotted onto aluminium-backed silica 60 TLC plates with F₂₅₄ (VWR Chemicals, Lutterworth, UK) using a capillary pipette. TLC plates were then eluted in a mixture of petroleum ether (boiling point 60-80°C) and ethyl acetate (Fisher Scientific) in a glass TLC developing tank which was saturated with the solvent system prior to elution. Solvent systems were optimised to increase resolution of each EO, with final concentrations of 1:19 (v/v) ethyl acetate:petroleum ether for cumin EO, 1:9 (v/v) ethyl acetate:petroleum ether for oregano EO and 1:3 (v/v) ethyl acetate:petroleum ether for rosewood EO. To achieve 2D resolution, following the elution of a TLC plate, the mobile phase was allowed to fully evaporate before rotating the plate by 90° and eluting for second time along the orthogonal axis.

Following elution, separated compounds were visualised using UV light (254 nm) and iodine vapour derivatization, whereby dried, eluted plates were suspended in a glass chamber containing iodine crystals for 5 minutes. The Retention Factor (Rf) for each spot was calculated as the ratio of the distance (mm) that a spot was eluted on a plate relative to the total distance (mm) that the solvent front travelled. Images of derivatized TLC plates were taken using a digital smartphone camera.

TLC-DB

Overnight cultures of test microorganism (10 mL) were centrifuged at 5000 rpm for 15 minutes and the supernatant discarded. Cells were resuspended in 10 mL broth and diluted to approximately 10^6 CFU/mL. The bacterial suspension was then transferred to a spray-equipped plastic bottle and approximately 1.5 mL was sprayed onto pre-eluted 2D TLC plates before incubation in a humid chamber for 24 hours at 37°C. Microbial growth was visualised by spraying incubated plates with approximately 1.5 mL of 20 mg/mL 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (INT; Sigma Aldrich) suspended in deionised water, which were left to develop for 4 hours at 37°C. Following this, ZoIs were observed. Images of the TLC-DB plates were taken using a digital smartphone camera.

Characterisation of Antimicrobial Fractions

¹H NMR Spectroscopy. Silica was extracted from eluted TLC plates in areas relating to the antimicrobial ZoIs by scraping with a plastic spatula and transferred into 1 mL deuterated chloroform (Cambridge Isotope Laboratories Inc, Tewksbury, USA), mixed, and left overnight at 4°C for extraction. Extracts were filtered with grade 1 qualitative filter papers (Whatman, Maidstone, UK) to remove silica, diluted to a final volume of 600 µL in deuterated chloroform, and transferred to 5 mm NMR tubes. NMR analysis was conducted on

a Bruker Avance 500 MHz spectrometer with a probe temperature of 25°C. Chemical shift values of resonances were referenced to the residual solvent peak.

GC-MS. Silica extracts recovered from the TLC plate by scraping with a plastic spatula were transferred into 1 mL GC grade hexane (Fisher Scientific) and filtered using 0.45 µm PTFE syringe filters (Merck Millipore) before analysis. Samples were injected into a Bruker 450-RC GC coupled with a Bruker 300-MS SQ MS (Bruker) and analysed using the same method as the EOs.

LC-MS. Samples for LC-MS were prepared as for GC-MS analysis except acetonitrile (Fisher Scientific) was used as a solvent. LC-MS analysis was performed using an Agilent 1100 series HPLC (Agilent, Santa Clara, USA) with a UV-Vis detector coupled to a Bruker MicroTOF electrospray ionisation spectrometer (Bruker) operating in positive mode. Samples were injected using a 5 µL injection loop and chromatographed using a Phenomenex analytical reversed phase C-18 column (250 mm x 4.6 mm; 5 µm particle size). An isocratic mobile phase consisting of 10% water (v/v) in acetonitrile with 0.1% (v/v) formic acid (Fisher Scientific) was used. UV-detection was conducted at 230 nm prior to mass analysis in positive mode over a range of m/z 100 to 2500.

MICs

MICs of EOs and antimicrobial terpenes identified using TLC-DB were determined using a microdilution method.³¹ Serial two-fold dilution of the EO or terpene in nutrient or BHI broth supplemented with 10% (v/v) dimethyl sulfoxide (Fisher Scientific) was carried out in polystyrene 96-well plates to give a total well volume of 75 µL. Each well was inoculated with an equal volume of bacterial suspension to yield a final well concentration of 5×10^5 CFU/mL. A control of broth with 10% dimethyl sulfoxide and microorganism only was included. Bacterial growth was determined by measuring optical density (595 nm) of samples using a Spectramax Plus 384 microplate reader and Softmax Pro version 6.4 software

(Molecular Devices, Sunnyvale, USA) at 0 hr. and again after 24 hr. incubation at 37°C.

Minimum Bactericidal Concentrations (MBCs) were determined following 24 hr. incubation by observing bacterial growth of samples from wells corresponding to the MIC and above on fresh agar plates. All experiments were conducted in triplicate and replicated twice (n=6).

RESULTS AND DISCUSSION

Antimicrobial Activity of Whole EOs

Oregano, cumin and rosewood EOs exhibited ZOI against all test species in the Kirby-Bauer disc diffusion method (Table 1), indicating the presence of antimicrobial compounds, however the antimicrobial activity of the EOs varied significantly, ranging from 0.29 to 37.20 mg/mL (Table 2). Oregano EO was strongly antimicrobial against *S. aureus*, *E. faecium* and *E. coli* isolates, with MICs ranging from 0.29-1.15 mg/mL (Table 2). Similarly, Castilho et al.³² reported MICs of 0.1 mg/mL against *S. aureus*, 0.05-0.1 mg/mL against *E. faecium* and 0.2 mg/mL against *E. coli*. Cumin EO was also strongly antimicrobial against antibiotic-sensitive and -resistant *S. aureus* (MICs = 0.58-2.33 mg/mL) and *E. coli* (MICs = 0.29 mg/mL) isolates. Bag and Chattopadhyay³³ reported similar MICs of 0.13 mg/mL against *S. aureus* and 0.30 mg/mL against *E. coli*, and moderate activity against a range of bacteria including *Bacillus cereus*, *Micrococcus luteus*, *Listeria monocytogenes* and *Salmonella enterica* subsp. *enterica* ser. Typhimurium. Conversely, oregano was less antimicrobial against *P. aeruginosa* (MICs = 4.60-9.20 mg/mL) and cumin EO was weakly antimicrobial against both *E. faecium* (MICs = 18.60-37.20 mg/mL) and *P. aeruginosa* (MICs = 4.65-18.60 mg/mL). It is difficult to ascertain the differences in susceptibility to EOs of different species, as their mechanism of action is poorly understood. It has been hypothesised that *Pseudomonas* spp. are more resistant to EOs and their components due to multiple efflux

systems and a particularly impermeable outer membrane.³⁴ Conversely, Dussault et al.³⁵ observed a considerably lower MIC (2083 ppm) of oregano EO against *P. aeruginosa*. Differences in bacterial susceptibility or chemical composition between EOs may account for variation in observed MICs between studies. EO composition, and therefore concentration of antimicrobial principles, can vary significantly as a result of differing growth conditions such as harvest season,³⁶ geographical location³⁷ and water availability³⁸ or EO storage conditions.³⁹ Rosewood EO was relatively inactive, with MICs ranging 8.80-35.20 mg/mL against *S. aureus*, *E. coli*, *E. faecium* and *P. aeruginosa*. Rosewood EO had lower MICs of 1.38 and 2.23 mg/mL against *S. epidermidis* and *Propionibacterium acnes*, respectively, in previous work.³⁰

Chemical Composition of EOs

GC-MS analysis identified 99.95% of cuminaldehyde, 99.94% of oregano EO and 95.40% of rosewood EO (Table 3). Oregano, rosewood and cuminaldehyde EOs were complex mixtures, containing upwards of 47 compounds in cuminaldehyde EO, 22 in oregano and 14 in rosewood EO. The major components of cuminaldehyde EO were β -pinene (15.64%), cuminaldehyde (17.66%), terpinen-7-al (14.61%) and γ -terpinene (18.18%), which are within the International Standards Office (ISO) standard chromatographic ranges for GC.⁴⁰ The major identified components in rosewood EO were linalool (54.75%), 1,8-cineole (11.04%), *p*-cymene (10.98%) and α -terpineol (10.49%) This does not conform to British Standards Institute guidelines for rosewood EO,⁴¹ as an excess of 1,8-cineole and α -terpineol was observed compared to the maximum permitted 3% and 7%, respectively. Moreover, a low concentration of linalool was present compared to the ISO range of 70-90%. No ISO standard has been issued for oregano (*O. compactum*) EO, however the major composition was identified here as 66.61% carvacrol, 12.32% *p*-cymene, 4.80% β -caryophyllene. El Babili et

al.⁴² and Santamarina et al.⁴³ reported a similar carvacrol content of 66.2% and 64.9%, respectively, and the *p*-cymene content was concordant with Santamarina et al.⁴³ who reported a content of 13.95%. In both studies, the β -caryophyllene content was lower (0.78 and 1.83%) and the thymol content greater (29.74 and 21.64%). Discrepancies in chemical composition of these EOs from the published literature and ISO standard ranges are unsurprising due to the influence of differing growth conditions on composition.

TLC-DB

Although activity varied, oregano, rosewood and cumin EOs were inhibitory against all test species, thus TLC-DB was used to ascertain the presence of antimicrobial compounds within each EO. Coupled with a suitable analytical technique, compounds with antimicrobial activity can be characterised providing a rapid and effective method to identify candidates for antimicrobial development from a complex mixture.

TLC-DB analysis revealed the presence of two antimicrobial fractions in cumin EO that inhibited all test species except for CR *P. aeruginosa* (Figure 1a; Table 4). Oregano EO possessed one fraction at $R_f = 0.32$ that inhibited all test species and another fraction at $R_f = 0.67$ that inhibited VSE only (Figure 1b; Table 4). Rosewood EO inhibited all test species at $R_f = 0.43$, and all species except CR *P. aeruginosa* at $R_f = 0.25$ (Figure 1c; Table 4). A further antimicrobial fraction on the baseline ($R_f = 0.00$) only inhibited *S. aureus* and *E. coli*. Characterisation of antimicrobial compounds extracted from TLC-DB plates was accomplished using GC-MS in addition to LC-MS and NMR to potentially enhance detection of heat-labile and non-volatile compounds.^{26,28} GC-MS analysis revealed that the antimicrobial fractions were mixtures of several compounds. Cuminaldehyde was present in both antimicrobial fraction of cumin EO, while minor components γ -terpinene, terpinen-7-al and linalool were also identified in these fractions. Linalool was identified in all three antimicrobial fractions of rosewood EO, and *p*-cymene was present as a minor component of

fractions at Rf = 0.00 and Rf = 0.25. Carvacrol was a major component of the oregano EO fraction at Rf = 0.32, alongside *p*-cymene and β -caryophyllene, while the fraction at Rf = 0.67 contained only *p*-cymene and β -caryophyllene (Table 4). Rf values of the antimicrobial spots were concordant with standard reference samples of the compounds identified by GC-MS (Table 5). GC-MS has been previously employed to identify antimicrobial EO fractions from TLC-DB. For example, thyme (*Thymus vulgaris*) EOs were screened against *Bacillus subtilis* and *Pseudomonas syringae*, with thymol, carvacrol, linalool and α -terpineol being identified as the antimicrobial components.⁴⁴ Falcao et al.⁴⁵ investigated the activity of lemongrass (*Cymbopogon citratus*) and detected EO components with activity against *S. aureus*, *P. aeruginosa* and *Salmonella enterica*, concluding that citral and myrcene were antimicrobial against *S. aureus*. Naveed et al.⁴⁶ attributed ZoIs of *Salmonella enterica* subsp. *enterica* ser. Typhi, *Salmonella enterica* subsp. *enterica* ser. Paratyphi, *Bacillus licheniformis*, *E. coli* and *Pseudomonas fluorescens* on bioautographs of cumin (*C. cyminum*), cinnamon (*Cinnamomum verum*), cardamom (*Amomum subulatum*) and clove (*Syzygium aromaticum*) EOs to the major components cuminaldehyde, cinnamaldehyde, 1,8-cineole and eugenol, respectively.

NMR spectroscopy is rarely coupled with TLC-DB assays of EOs, and is mainly used in combination with other techniques, for example Faria et al.⁴⁷ and Dhingra et al.⁴⁸ employed NMR spectroscopy in combination with GC-MS to identify antifungal fractions of basil (*Ocimum gratissimum*) EO and turmeric (*Curcuma longa*) EOs, respectively. However, NMR spectroscopy is a non-destructive technique conducted at ambient temperatures, and so may be of value in combination with GC-MS to enhance detection of any thermolabile and non-volatile EO constituents.^{26,28} That said, NMR spectroscopy was only able to identify the major components present in antimicrobial fractions of cumin EO at Rf = 0.27 as cuminaldehyde, oregano EO at Rf = 0.32 as carvacrol and rosewood EO at Rf = 0.40 as

linalool (Table 4). Other antimicrobial fractions could not be identified using NMR spectroscopy as no resonances were observed. NMR spectroscopy has lower sensitivity than MS techniques, and thus trace compounds present within the antimicrobial fractions were below the limits of detection. Therefore, this technique was unable to determine if minor heat-labile and non-volatile EO compounds are present within these antimicrobial fractions.

The use of LC-MS for the identification of TLC-DB fractions of EOs does not appear to have been reported in the published literature, however this technique has been employed frequently for the analysis of other plant extracts. For example, HPLC-ESI-MS identified the antimicrobial compounds of St. John's Wort (*Hypericum perforatum*) ethanol extract determined with TLC-DB as 3,8'-biapigenin, quercetin, apigenin, kaempferol, and linolenic acid⁴⁹ while apigenin, kaempferide and kaempferol glycosides were concluded to be antimicrobial components of the *Cistus incanus* flavonoid fraction by TLC-DB and HPLC-Diode Array Detector (DAD)-ESI-MS.⁵⁰ In this study, LC-MS spectra, using positive-mode Electrospray Ionisation (ESI) MS, showed the presence of common phthalate plasticiser contaminant peaks ($m/z= 391.3, 413.3$ and 803.6) but no peaks relating to the EO constituents could be detected. NMR analysis of antimicrobial fractions and dioctyl phthalate (Sigma Aldrich, UK) ruled out plasticiser contamination of the EOs and indicates that these peaks arise from internal sources of contamination in the LC-MS equipment. Simple terpenes are semi-inert to ESI⁵¹ which may be attributed to the lack of observed peaks for compounds identified by GC-MS and NMR, however no further heat-labile or non-volatile compounds were found. Turek and Stitzing^{28,39} argued that the atmospheric pressure chemical ionisation (APCI-MS) technique is preferable to GC-MS (the most common method for EO analysis), as it can detect non-volatile or thermolabile constituents in addition to volatile molecules. APCI may therefore be a more suitable MS ionisation method than ESI.

Antimicrobial Activity of Bioactive Compounds

The presence of multiple compounds within each antimicrobial EO fraction is due to similar polarity and distribution of major components over the TLC plate. While this is a disadvantage of TLC-DB, the bioactive compounds in these fractions were identified by subjecting isolated compounds to the Kirby-Bauer disc diffusion method. Carvacrol and *p*-cymene inhibited all microorganisms, while cuminaldehyde and linalool inhibited all species except for *P. aeruginosa*, suggesting that these compounds were at least in part responsible for the activity of the antimicrobial TLC fractions. γ -Terpinene inhibited four isolates, indicating limited potential for antimicrobial activity. β -Caryophyllene only inhibited VSE, indicating that this compound may have had an antimicrobial effect at the ZoI at $R_f = 0.67$ (Table 6).

β -Caryophyllene was excluded as an antimicrobial compound from oregano EO against all test species except VSE, where moderate activity was observed (MIC = 5.50 mM; Table 7); this compound at $R_f = 0.67$ may have inhibited VSE where the remaining test species were not inhibited. β -Caryophyllene has previously shown weak antimicrobial activity against *S. aureus*, *S. Typhimurium*, *E. coli* and *E. faecalis*, with MICs ranging 0.5-1.0 mg/mL.⁵² The activity of β -caryophyllene against *E. faecium* does not appear to have been reported in recent published literature. The moderate activity of this compound against *E. faecium* indicates that further investigation might be developed in order to evaluate if this compound inhibits VSE compared to the other test species regarding the development of antimicrobial formulations.

Linalool and γ -terpinene were weakly antimicrobial against all test species, with MICs ranging 57.05-912.80 mM (Table 7), suggesting that these compounds are unlikely to be useful in the development on new antimicrobial formulations. In agreement, Zengin and Baysal⁵³ reported MICs of linalool ranging from 1-2% against three Gram-positive and four

Gram-negative pathogenic and food spoilage bacteria. Hoferl et al.⁵⁴ reported that *p*-cymene was not antimicrobial against *E. coli*, *P. aeruginosa*, *Klebsiella pneumoniae* and *Proteus vulgaris*, but had some activity (MIC = 600 ppm), against *S. aureus* and *E. faecalis*. Cuminaldehyde was also weakly antimicrobial, however moderately inhibited *E. coli* with an MIC of 2.10 mM.

Carvacrol was the most antimicrobial compound identified, presenting the lowest MICs against all test species (0.99-15.81 mM; Table 7), and was bactericidal against all test species except CR *E. coli* and *P. aeruginosa*. Carvacrol's antimicrobial activity is well established; Espina et al.⁵⁵ reported similar MICs of 0.02% against MSSA and MRSA. MICs of carvacrol against *E. coli*, VSE and VRE were greater than those reported in the literature; Gutierrez-Larrainzar et al.⁵⁶ and Castilho et al.³² reported MICs of 0.18 mg/mL against *E. coli* and 0.2 mg/mL against *E. faecium*, compared to 1.98 and 3.95 mM (equivalent to 0.31 and 0.63 mg/mL), respectively (Table 7). Phenolic monoterpenes such as carvacrol are known as the most antimicrobial EO components. Comparative analysis of the antimicrobial activity of carvacrol and structurally related monoterpenes with substituted functional groups indicated that the presence of phenolic structures and free hydroxyl groups enhance antimicrobial activity.⁵⁷ Carvacrol is inserted between acyl chains in the phospholipids of the bacterial cytoplasmic membrane, increasing membrane fluidity and permeability.⁵⁸ It was hypothesised that the delocalised electrons present in phenolic structure enhance proton exchange through the hydroxyl group, which results in collapse of proton motive force across the cell membrane.^{59,60}

The whole, unfractionated, rosewood and cumin EO were generally more antimicrobial than their individual bioactive components (Tables 2 and 6). Bioactive EO components have been known to act synergistically, producing a greater antimicrobial effect in the crude extract than when administered alone. *Filipendula vulgaris* EO was more

antimicrobial than its major salicylaldehyde component, while a combination of salicylaldehyde and linalool was more antimicrobial than the whole EO.⁶¹ Moreover, inactive compounds may influence antimicrobial activity; *p*-cymene, which is not antimicrobial against *B. cereus*, causes expansion of the cell membrane and so increases carvacrol's interaction with the membrane.⁶² In this study, carvacrol was more antimicrobial than oregano EO against all test species except MSSA, CR *E. coli* and CR *P. aeruginosa*, suggesting that the greater concentration of carvacrol compared to the whole EO, where it is present at 67.04%, results in a lower MIC. This contrasts with the study by Ultee et al.⁶³ that reported increased activity of carvacrol in the presence of *p*-cymene, which would suggest a greater activity of the whole EO than carvacrol alone. This could be species specific to *B. subtilis*.

The activity of the EOs were generally comparable between antibiotic-sensitive and -resistant isolates, suggesting no cross-resistance between EOs and antibiotics. The mechanism of action of EOs and their isolated components is primarily attributed to disruption of bacterial cytoplasmic membrane structure and function,⁵⁸ so they may therefore bypass antibiotic resistance mechanisms.

Cuminaldehyde was less active against antibiotic-resistant isolates of *S. aureus*, *E. faecium* and *P. aeruginosa* than antibiotic-sensitive isolates, while linalool was less active against antibiotic-resistant Gram-negatives and carvacrol was less active against antibiotic-resistant *P. aeruginosa* (Table 7). This was no more than a two-fold difference in most cases, which suggests that antibiotic cross-resistance is unlikely to have resulted in these variations in susceptibility. The inherent variability in the microdilution method which puts the MIC within a three-dilution range⁶⁴ or inherent susceptibility differences between isolates independent of antibiotic resistance mechanisms may account for these results. In exception, linalool was more antimicrobial against antibiotic-sensitive *P. aeruginosa* (MIC = 228.20

mM) compared to CR *P. aeruginosa* (MIC = 912.90 mM). Moreover, cuminaldehyde was more antimicrobial against antibiotic-sensitive *E. coli* (MIC = 2.10 mM) compared to CR *E. coli* (MIC = 8.40 mM) and VSE (MIC = 134.41 mM) than VRE (MIC = 537.65 mM). Further studies would be needed to determine if this was the result of cross-resistance with antibiotics, for instance through extrusion by efflux pumps. The multidrug efflux pump MexAB-OprM appears to confer tolerance to EOs, as MexAB-OprM deficient *P. aeruginosa* were more susceptible than wild type strains to tea tree (*Melaleuca alternifolia*) EO, respectively,⁶⁵ suggesting that further studies are needed to determine if the current findings are the result of tolerance or inherent susceptibility differences.

Cumin, oregano and rosewood EOs were screened for the presence of antimicrobial compounds as potential candidates for the further development of antimicrobial formulations against antibiotic-resistant bacteria using TLC-DB coupled with GC-MS, LC-MS and NMR. Comparison of LC-MS, NMR and GC-MS techniques revealed that GC-MS is the most suitable technique for the characterisation of antimicrobial EO fractions from TLC-DB. A total of seven bioactive compounds were identified using GC-MS, while LC-MS and NMR failed to identify the presence of any further heat-labile or non-volatile compounds. Carvacrol, the major component of oregano EO, was the most potent antimicrobial compound isolated with TLC-DB. Carvacrol did not appear to confer cross-resistance with antibiotics, suggesting that, in agreement with previous studies,^{32,55,56} it may be a suitable candidate for development of new antimicrobial formulations against antibiotic-resistant bacteria.

References

1. Prestinaci F, Pezzotti P, Pantosti, A. Antimicrobial resistance: a global multifaceted phenomenon. *Pathog Global Health* 2015;109:309-318.
2. Butler MS, Blaskovich MAT, Cooper MA. Antibiotics in the clinical pipeline at the end of 2015. *J Antibiot* 2017;70:3-24.
3. Pye CR, Bertin MJ, Lokey RS, Gerwick WH, Linington RG. Retrospective analysis of natural products provides insights for future discovery trends. *Proc Natl Acad Sci USA* 2017;114:5601-5606.
4. Patridge E, Gareiss P, Kinch MS, Hoyer D. An analysis of FDA-approved drugs: natural products and their derivatives. *Drug Discovery Today* 2016;21:204-207.
5. Orchard A, Sandasi M, Kamatou G, Viljoen A, van Vuuren S. The *in vitro* antimicrobial activity and chemometric modelling of 59 commercial essential oils against pathogens of dermatological relevance. *Chem Biodiversity* 2017;14:e1600218. DOI: 10.1002/cbdv.201600218.
6. Hammer KA, Heel KA. Use of multiparameter flow cytometry to determine the effects of monoterpenoids and phenylpropanoids on membrane polarity and permeability in staphylococci and enterococci. *Int J Antimicrob Agents* 2012;40:239-245.
7. Mulyaningsih S, Sporer F, Zimmermann S, Reichling J, Wink M. Synergistic properties of the terpenoids aromadendrene and 1,8-cineole from the essential oil of *Eucalyptus globulus* against antibiotic-susceptible and antibiotic-resistant pathogens. *Phytomedicine* 2010;17, 1061–1066.

8. Kifer D, Muzinic V, Klaric MS. Antimicrobial potency of single and combined mupirocin and monoterpenes, thymol, menthol and 1,8-cineole against *Staphylococcus aureus* planktonic and biofilm growth. *J Antibiot* 2016;69:689-696.
9. Scandorieiro S, de Camargo LC, Lancheros CA, Yamada-Ogatta SF, Nakamura CV, de Oliveira AG, Andrade CG, Duran N, Nakazato G, Kobayashi RK. Synergistic and additive effect of oregano essential oil and biological silver nanoparticles against multidrug-resistant bacterial strains. *Front Microbiol* 2016;7:760. DOI: 10.3389/fmicb.2016.00760.
10. El Asbahani, A, Miladi K, Badri W, Sala M, Addi EA, Casabianca H, El Mousadik A, Hartmann D, Jilale A, Renaud FNR, Elaissari A. Essential oils: from extraction to encapsulation. *Internat J Pharm* 2015;483:220-243.
11. Raut J, Karuppayil SM. A status review on the medicinal properties of essential oils. *Ind Crops Prod* 2014;62:250–264.
12. Rehman R, Hanif MA, Mushtaq Z, Al-Sadi AM. Biosynthesis of essential oils in aromatic plants: a review. *Food Rev Int* 2016;32:117–160.
13. Moricz AM, Habe TT, Boszormenyi A, Ott PG, Morlock GE. Tracking and identification of antibacterial components in the essential oil of *Tanacetum vulgare* L. by the combination of high-performance thin-layer chromatography with direct bioautography and mass spectrometry. *J Chromatog A* 2015;1422:310-317.
14. Weller MG. A unifying review of bioassay-guided fractionation, effect-directed analysis and related techniques. *Sensors* 2012;12:9181-9209.
15. El-Alfy A, Wilson L, ElSohly MA, Abourashed EA. Towards a better understanding of the psychopharmacology of nutmeg: activities in the mouse tetrad assay. *J Ethnopharmacol* 2009;126:280-286.
16. Wedge DE, Nagle DG. A new 2D-TLC bioautography method for the discovery of novel antifungal agents to control plant pathogens. *J Nat Prod* 2000;62:1050-1054.

17. Marston A. Thin-layer chromatography with biological detection in phytochemistry. *J Chromatog A* 2011;1218:2676-2683.
18. Dewanjee S, Gangopadhyay M, Bhattacharya N, Khanra R, Dua TK. Bioautography and its scope in the field of natural product chemistry. *J Pharm Anal* 2015;5:75-84.
19. Jesionek W, Grzelak E, Majer-Dziedzic B, Choma I. Thin-layer chromatography – direct bioautography for the screening of antimicrobial properties of plant extracts. *J Planar Chromatog - Mod TLC* 2013;26:109-113.
20. Ngezahayo, J, Pottier L, Ribeiro SO, Delporte C, Fontaine V, Hari L, Stevigny C, Duez P. *Plastotoma rotundifolium* aerial tissue extract has antibacterial activities. *Ind Crops Prod* 2016;86:301–10.
21. Jamshidi-Aidji M, Morlock GE. Bioprofiling of unknown antibiotics in herbal extracts: development of a streamlined direct bioautography using *Bacillus subtilis* linked to mass spectrometry. *J Chromatog A* 2015;1420:110–118.
22. Kasote D, Ahmad A, Chen W, Combrinck S, Viljoen A. HPTLC-MS as an efficient hyphenated technique for the rapid identification of antimicrobial compounds from propolis. *Phytochem Lett* 2015;11:326-331.
23. Taha N, Krawinkel MB, Morlick GE. High-performance thin-layer chromatography linked with (bio)assays and mass spectrometry – a suited method for discovery and quantification of bioactive components? Exemplarily shown for turmeric and milk thistle extracts. *J Chromatog A* 2015;1394:137–147.
24. Valagas C, de Souza SM, Smania EFA, Smania Jr A. Screening methods to determine antibacterial activity of natural products. *Braz J Microbiol* 2007;38:369-380.
25. Zhang W, Zhu M, Wang F, Cao D, Ruan JJ, Su W, Ruan BH. Mono-sulfonated tetrazolium salt based NAD(P)H detection reagents suitable for dehydrogenase and real-time cell viability assays. *Anal. Biochem.* 2015;509:33-40.

26. Prichystal J, Schug KA, Lemr K, Novak J, Havlicek V. Structural analysis of natural products. *Anal. Chem.* 2016;88:10338–10346.
27. Tranchida PQ, Zoccali M, Bonaccorsi I, Dugo P, Mondello L, Dugo G. The off-line combination of high performance liquid chromatography and comprehensive two-dimensional gas chromatography–mass spectrometry: a powerful approach for highly detailed essential oil analysis. *J Chromatog A* 2013;1305:276–284.
28. Turek C, Stintzing FC. Stability of essential oils: a review. *Compr Rev Food Sci Food Saf* 2-13;12:40–53.
29. Garcia G, Charmillon JM, Roux E, Sutour S, Rakotozafy JB, Desire O, Paoli M, Tomi F and Rabehaja DJR. Chemical composition of leaf and bark essential oil of *Vepris unifoliolata* from Madagascar. *J Essent Oil Res* 2017;29:214-220.
30. Owen L, Grootveld M, Arroo R, Ruiz-Rodado V, Price P, Laird K. 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. *Phytother Res* 2017;31:410-417.
31. Owen L, Laird K, Wilson PB. Structure-activity modelling of essential oils, their components, and key molecular parameters and descriptors. *Mol Cell Probes* 2018;38:25-30.
32. Castilho, P.C., Savluchinske-Feio, S., Weinhold, T. S., and Gouveia, S. C. (2012) Evaluation of the antimicrobial and antioxidant activities of essential oils, extracts and their main components from oregano from Madeira Island, Portugal. *Food Control* 23, 552-558.
33. Bag A, Chattopadhyay RR. Evaluation of synergistic antibacterial and antioxidant efficacy of essential oils of spices and herbs in combination. *PLoS One* 2015;10: e0131321. DOI: 10.1371/journal.pone.0131321.

34. Mota AS, Martins MR, Arantes S, Lopes VR, Bettencourt E, Pombal S, Gomes AC, Silva LA. Antimicrobial activity and chemical composition of the essential oils of Portuguese *Foeniculum vulgare* Fruits. *Nat Prod Commun* 2015;10:547-690.
35. Dussault D, Vu KD, Lacroix M. *In vitro* evaluation of antimicrobial activities of various commercial essential oils, oleoresin and pure compounds against food pathogens and application in ham. *Meat Sci* 2014;96:514-520.
36. Riahi L, Chograni H, Elferchichi M, Zaouali Y, Zoghlami N, and Mlik A. Variations in Tunisian wormwood essential oil profiles and phenolic contents between leaves and flowers and their effects on antioxidant activities. *Ind Crops Prod* 2013;46:290–296.
37. Satyal P, Crouch RA, Monzote L, Cos P, Awadh Ali NA, Alhaj MA, and Setzer WN. The chemical diversity of *Lantana camara*: analyses of essential oil samples from Cuba, Nepal, and Yemen. *Chem Biodivers* 2016;13:336-342.
38. Maatallah S, Nasri N, Hajlaoui H, Albouchi A, Elaissi A. Evaluation changing of essential oil of laurel (*Laurus nobilis* L.) under water deficit stress conditions. *Ind Crops Prod* 2016;91:170-178.
39. Turek C, Stintzing, FC. Impact of different storage conditions on the quality of selected essential oils. *Food Res. Int.* 2012;46:341–353.
40. International Standards Office. *ISO 9301:2003 (R09) Ed 1: Oil of cumin fruit (Cuminum cyminum Linnaeus)*. Geneva: International Standards Office; 2003.
41. British Standards Institute. *BS ISO 3761:2005: Oil of rosewood, Brazilian type [Aniba rosaeodora Ducke var. amazonica Ducke or Aniba parviflora (Meissner) Mez]*. London: British Standards Institute; 2005.
42. El Babili F, Bouajila J, Souchard JP, Bertrand C, Bellvert F, Fouraste I, Moulis C, Valentin A. Oregano: chemical analysis and evaluation of its antimalarial, antioxidant, and

cytotoxic activities. *J Food Sci* 2011;76: C512-C518. DOI: 10.1111/j.1750-3841.2011.02109.x.

43. Santamarina MP, Rosello J, Sempere F, Gimenez S, Blazquez MA. Commercial *Origanum compactum* Benth. and *Cinnamomum zeylanicum* Blume essential oils against natural mycoflora in Valencia rice. *Nat Prod Res* 2015;29:2215-2218.

44. Moricz, A.M., Ott, P.G., Boszormenyi, A., Lemberkovics, E., Mincsovics, E., Tyihak, E. Bioassay-guided isolation and identification of antimicrobial compounds from thyme essential oil by means of overpressured layer chromatography, bioautography and GC-MS. *Chromatographia* 2012;75:991-999.

45. Falcao MA, Fianco AL, Lucas AM, Pereira MA, Torres FC, Vargas RM, Cassel E. Determination of antibacterial activity of vacuum distillation fractions of lemongrass essential oil. *Phytochem Rev* 2012;11:405-412.

46. Naveed R, Hussain I, Tawab, A, Tariq M, Rahman M, Hameed S, Mahmood MS, Siddique AB, Iqbal M. Antimicrobial activity of the bioactive components of essential oils from Pakistani spices against *Salmonella* and other multi-drug resistant bacteria. *BMC Complementary Altern Med* 2013;13:265. DOI: 10.1186/1472-6882-13-265.

47. Faria, TDJ, Ferreira RS, Yassumoto L, Souza JRPD, Ishikawa NK and Barbosa ADM. Antifungal activity of essential oil isolated from *Ocimum gratissimum* L. (eugenol chemotype) against phytopathogenic fungi. *Braz Arch Biol Technol* 2006;49:867-871.

48. Dhingra OD, Jham GN, Barcelos RC, Mendonca FA and Ghiviriga I. Isolation and identification of the principle fungitoxic component of turmeric essential oil. *J Essent Oil Res* 2007;19:387-391.

49. Jesionek W, Moricz A, Alberti A, Ott, PG, Kocsis B, Horvath G and Choma IM. TLC-direct bioautography as a bioassay guided method for investigation of antibacterial compounds in *Hypericum perforatum* L. *J AOAC Int* 2015;98:1013-1020.

50. Moricz AM, Szeremeta D, Knas M, Dlugosz E, Ott PG, Kowalaska T and Sajewicz M. Antibacterial potential of the *Cistus incanus* L. phenolics as studied with use of thin-layer chromatography combined with direct bioautography and *in situ* hydrolysis. *J Chromatog A* 2018;1534:170-178.
51. Weckwerth W. Metabolomics in systems biology. *Annu. Rev. Plant Biol.* 2003;54:669-689.
52. Neta MCS, Vittorazzi C, Guimaraes AC, Martins JD, Fronza M, Endringer DC, Scherer, R. Effects of β -caryophyllene and *Murraya paniculata* essential oil in the murine hepatoma cells and in the bacteria and fungi 24-h time-kill curve studies. *Pharm Biol* 2016;S5:190-197.
53. Zengin H, Baysal AH. Antibacterial and antioxidant activity of essential oil terpenes against pathogenic and spoilage-forming bacteria and cell structure-activity relationships evaluated by SEM microscopy. *Molecules* 2014;19:17773-17798. DOI: 10.3390/molecules191117773.
54. Hoferl M, Buchbauer G, Jirovetz L, Schmidt E, Stoyanova A, Denkova Z, Slavchev A, Geissler M. Correlation of antimicrobial activities of various essential oils and their main aromatic volatile constituents. *J. Essent Oil Res* 2009;21:459-463.
55. Espina L, Pagan R, Lopez D, Garcia-Gonzalo D. Individual constituents from essential oils inhibit biofilm mass production by multi-drug resistant *Staphylococcus aureus*. *Molecules* 2015;20:11357-11372.
56. Gutierrez-Larraínzar M, Rua J, Caro I, de Castro C, de Arriaga D, Garcia-Armesto MR, del Valle P. Evaluation of antimicrobial and antioxidant activities of natural phenolic compounds against foodborne pathogens and spoilage bacteria. *Food Control* 2012;26:555-563.

57. Veldhuizen EJ, Tjerdema-van Bokhoven JLM, Zweijtzer C, Burt SA, Haagsman HP. Structural requirements for the antimicrobial activity of carvacrol. *J Agric Food Chem* 2006;54:1874-1879.
58. Radulovic NS, Blagojevic PD, Stojanovic-Radic ZZ, Stojanovic NM. Antimicrobial plant metabolites: structural diversity and mechanism of action. *Curr Med Chem* 2013;20:932-952.
59. Andrade-Ochoa S, Nevarez-Moorillon GV, Sanchez-Torres LE, Villanueva-Garcia M, Sanchez-Ramirez BE, Rodriguez-Valdez LM, Rivera-Chavira BE. Quantitative structure-activity relationship of molecules constituent of different essential oils with antimycobacterial activity against *Mycobacterium tuberculosis* and *Mycobacterium bovis*. *BMC Complementary Altern Med* 2015;15:332. DOI: 10.1186/s12906-015-0858-2.
60. Ben Arfa A, Combes S, Preziosi- Belloy L, Gontard N, Chalier P. Antimicrobial activity of carvacrol related to its chemical structure. *Lett Appl Microbiol* 2006;43:149-154.
61. Radulovic N, Misic M, Aleksic J, Dokovic D, Palic R, Stojanovic G. Antimicrobial synergism and antagonism of salicylaldehyde in *Filipendula vulgaris* essential oil. *Fitoterapia* 2007;78:565-570.
62. Ultee A, Bennik MHJ, Moezelaar R. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl Env Microbiol* 2002;68:1561-1568.
63. Ultee A, Slump RA, Steging G, Smid EJ. Antimicrobial activity of carvacrol on rice. *J Food Prot* 2000;63:620-624.
64. Odds FC. Synergy, antagonism and what the checkerboard puts between them. *J Antimicro. Chemother* 2003;52:1.
65. Papadopoulos CJ, Carson CF, Chang BJ, Riley TV. Role of the MexAB-OprM efflux pump of *Pseudomonas aeruginosa* in tolerance to tea tree (*Melaleuca alternifolia*) oil and its

monoterpene components terpinen-4-ol, 1,8-cineole, and α -terpineol. *Appl Env Microbiol* 2008;74:1932-1935.

Tables

Table 1: ZoI (mm) of cumin, oregano and rosewood EOs according to the Kirby-Bauer disc diffusion method (n=6 ± standard error of the mean [SEM])

Microorganism	ZoI (mm)		
	Oregano	Cumin	Rosewood
MSSA	90.0 ± 0.0	58.2 ± 3.1	90.0 ± 0.0
MRSA	66.8 ± 1.4	44.7 ± 3.1	40.3 ± 1.8
VSE	66.2 ± 1.8	36.3 ± 1.0	64.3 ± 3.4
VRE	73.8 ± 5.4	43.2 ± 3.0	87.0 ± 3.0
<i>E. coli</i>	68.0 ± 2.9	49.5 ± 1.0	90.0 ± 0.0
CR <i>E. coli</i>	58.5 ± 5.8	33.0 ± 1.4	49.5 ± 2.7
<i>P. aeruginosa</i>	28.0 ± 0.4	22.5 ± 1.2	24.0 ± 0.4
CR <i>P. aeruginosa</i>	26.2 ± 0.8	23.5 ± 0.6	18.8 ± 2.89

Table 2: MICs and MBCs (mg/mL) of cumin, oregano and rosewood EOs according to the broth microdilution method (n=6).

Microorganism	MIC (MBC; mg/mL)			
	10% DMSO Control	Oregano	Cumin	Rosewood
MSSA	No inhibition	0.29 (0.29)	2.33 (18.60)	8.80 (17.60)
MRSA	No inhibition	0.58 (0.58)	0.58 (18.60)	8.80 (17.60)
VSE	No inhibition	1.15 (4.60)	18.60 (37.20)	17.60 (35.20)
VRE	No inhibition	0.58 (0.58)	37.20 (74.40)	17.60 (35.20)
<i>E. coli</i>	No inhibition	1.15 (1.15)	0.29 (18.60)	17.60 (35.20)
CR <i>E. coli</i>	No inhibition	0.29 (2.30)	0.29 (37.20)	17.60 (35.20)
<i>P. aeruginosa</i>	No inhibition	9.20 (9.20)	18.60 (37.20)	35.20 (70.40)
CR <i>P. aeruginosa</i>	No inhibition	4.60 (9.20)	4.65 (37.20)	8.80 (35.20)

Table 3: Chemical composition (%) of oregano, cumin and rosewood EOs.

Compound	Composition (%)		
	Cumin	Oregano	Rosewood
1,8-Cineole	0.261	0.455	11.04
1,8-Terpin	0.031	-	-
Acoradiene	0.799	-	-
α -Phellaledrene	2.368	0.208	-
α -Phennadrene	-	-	-
α -Pinene	1.368	1.392	0.214
α -Terpinene	0.463	1.145	-
α -Terpineol	-	-	10.49
β -Caryophyllene	0.173	4.796	-
β -Cedrine	0.090	-	-
Borneol	-	0.305	-
β -Phelladrene	-	0.363	-
β -Pinene	15.642	-	-
Camphene	0.049	-	-
Carene	-	0.207	-
Carotol	0.745	-	-
Carvacrol	0.054	66.608	-
Caryophyllene oxide	0.161	-	-
Cedryl acetate	-	-	0.209
Citronellyl formate	-	-	2.456

Cuminaldehyde	17.662	-	-
Cymen-9-ol	0.102	-	-
D-limonene	1.232	3.330	-
Dihydrocarveol	3.002	-	2.301
Dihydrocarveone	-	-	-
Dodecane	-	0.259	-
Elemol	-	-	0.178
Estragol	0.178	-	-
Eudesmol	0.076	-	-
Farnesol	0.091	-	-
Farnesyl acetate	0.083	0.103	-
γ -Cadinene	0.022	-	-
Geraniol	-	0.076	0.55
Geranyl n-			
butyrate	-	0.094	-
Germacrene	0.074	-	-
Isoborneol	-	-	0.463
Isobornyl acetate	0.037	-	-
Isocedranol	0.081	-	-
Linalool	0.802	1.521	54.75
Linalyl acetate	0.091	-	-
Longipinene	0.043	-	-
Menth-2-en-1-ol	0.056	-	-
Methyl gamma			
ionone	0.043	-	-

Methyl			
isothyonate	-	-	0.501
Muurolol		0.082	-
Myrcene	-	1.801	-
Myrtenol	0.114	-	-
Nerolidol	0.136	-	-
Nerolidyl acetate	-	-	-
<i>P</i> -Cymene	10.638	12.394	10.98
Perrilaldehyde	1.607	-	-
Pinocarveol	0.104	-	-
Pinocarveone	0.042	-	-
Pulegol	-	0.112	-
Sabinene hydrate	0.057	-	-
Sabinyl acetate	0.795	-	-
Santilene	0.074	-	-
Terpinen-4-ol	0.62	-	0.199
Terpinen-7-al	14.906	-	-
Terpineol	-	0.610	0.497
Terpinolene	0.162	3.197	-
Terpinyl acetate	-	0.123	-
Thymol	-	0.599	-
Trans linalool			
oxide	0.033	-	-
Trans carveol	0.684	-	-
Trans- β -terpineol	-	-	0.571

Undecane	-	0.275	-
Valencene	0.093	-	-
γ -Terpinene	18.183	-	-
Total identified	99.947	99.941	84.419

Table 4: Antimicrobial components identified using TLC-DB followed by GC-MS. *Compounds detected by NMR in addition to GC-MS.

EO	Mean Rf Value	Antimicrobial Compound	Inhibited Microorganism								
			MSSA	MRSA	VSE	VRE	<i>E. coli</i>	CR <i>E. coli</i>	<i>P. aeruginosa</i>	CR <i>P. aeruginosa</i>	
Cumin	0.00	Cuminaldehyde, γ -terpinene, terpinen-7-al	✓	✓	✓	✓	✓	✓		✓	
	0.27	Cuminaldehyde*, linalool, terpinen7-al	✓	✓	✓	✓	✓	✓		✓	
Oregano	0.32	Carvacrol*, β -caryophyllene, <i>p</i> -cymene	✓	✓	✓	✓	✓	✓		✓	✓
	0.67	β -caryophyllene, <i>p</i> -cymene			✓						
Rosewood	0.00	Linalool, <i>p</i> -cymene	✓	✓			✓	✓			
	0.25	Linalool, <i>p</i> -cymene	✓	✓	✓	✓	✓	✓		✓	
	0.40	Linalool*	✓	✓	✓	✓	✓	✓		✓	✓

Table 5: Comparison of Rf values for EO compound reference standards and TLC-DB antimicrobial spots from which the compounds were recovered.

EO	Compound	Rf value	
		Reference Standard	TLC-DB
Cumin	Cuminaldehyde	0.00	0.00
		0.30	0.27
	γ -Terpinene	0.00	0.00
		0.74	-
	Linalool	0.09	0.00
		0.28	0.27
Oregano	Carvacrol	0.26	0.32
		0.68	0.67
	β -Caryophyllene	0.17	0.32
		0.72	0.67
	<i>p</i> -Cymene	0.14	0.32
		0.72	0.67
Rosewood	Linalool	0.00	0.00
		0.38	0.25
		0.48	0.40
	<i>p</i> -Cymene	0.31	0.25
		0.34	-

Table 6: ZoI (mm) of EO components present in antimicrobial fractions (n = 6 ± SEM).

Microorganism	ZoI (mm)					
	Carvacrol	Cuminaldehyde	Linalool	γ -Terpinene	<i>p</i> -Cymene	β -Caryophyllene
MSSA	70.7 ± 1.9	65.7 ± 1.4	90.0 ± 0.0	24.8 ± 1.1	49.7 ± 2.8	0.0 ± 0.0
MRSA	56.8 ± 0.9	56.8 ± 2.7	90.0 ± 0.0	19.5 ± 3.9	43.7 ± 0.6	0.0 ± 0.0
VSE	51.3 ± 0.5	25.2 ± 1.4	90.0 ± 0.0	7.7 ± 4.9	26.0 ± 1.9	15.2 ± 6.9
VRE	59.2 ± 3.2	32.7 ± 1.2	71.7 ± 4.1	0.0 ± 0.0	25.3 ± 1.5	0.0 ± 0.0
<i>E. coli</i>	63.2 ± 2.7	42.7 ± 4.6	90.0 ± 0.0	23.3 ± 0.5	37.0 ± 1.4	0.0 ± 0.0
CR <i>E. coli</i>	52.3 ± 1.0	24.2 ± 0.5	90.0 ± 0.0	0.0 ± 0.0	38.6 ± 0.9	0.0 ± 0.0
<i>P. aeruginosa</i>	26.7 ± 0.6	20.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	27.0 ± 0.8	0.0 ± 0.0
CR <i>P. aeruginosa</i>	22.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	29.7 ± 1.1	0.0 ± 0.0

Table 7: MICs and MBCs (mM) of bioactive components of cumin, oregano and rosewood EOs (n=6).

Microorganism	MIC (MBC; mM)						
	10% DMSO Control	β -caryophyllene	Carvacrol	Cuminaldehyde	Linalool	<i>p</i> -Cymene	γ -Terpinene
MSSA	No inhibition	-	1.98 (1.98)	33.60 (537.65)	114.10 (228.20)	8.01 (8.01)	62.61 (>500.92)
MRSA	No inhibition	-	0.99 (0.99)	67.21 (268.83)	57.05 (228.20)	16.02 (16.02)	125.23 (>500.92)
VSE	No inhibition	5.55 (>177.63)	3.95 (3.95)	134.41 (>268.83)	228.20 (228.20)	64.07 (256.30)	125.23 (>500.92)
VRE	No inhibition	-	3.95 (3.95)	537.65 (537.65)	57.05 (228.20)	64.07 (>256.30)	125.23 (>500.92)
<i>E. coli</i>	No inhibition	-	1.98 (1.98)	2.10 (134.41)	57.05 (228.20)	16.02 (128.14)	250.46 (>500.92)
CR <i>E. coli</i>	No inhibition	-	1.98 (15.81)	8.40 (268.83)	57.05 (228.20)	8.01 (32.04)	250.46 (>500.92)
<i>P. aeruginosa</i>	No inhibition	-	15.81 (31.62)	268.82 (>537.65)	228.20 (>456.40)	64.07 (128.14)	500.92 (>500.92)
CR <i>P. aeruginosa</i>	No inhibition	-	31.62 (126.48)	268.83 (537.65)	912.80 (>912.80)	64.07 (128.14)	250.46 (>500.92)

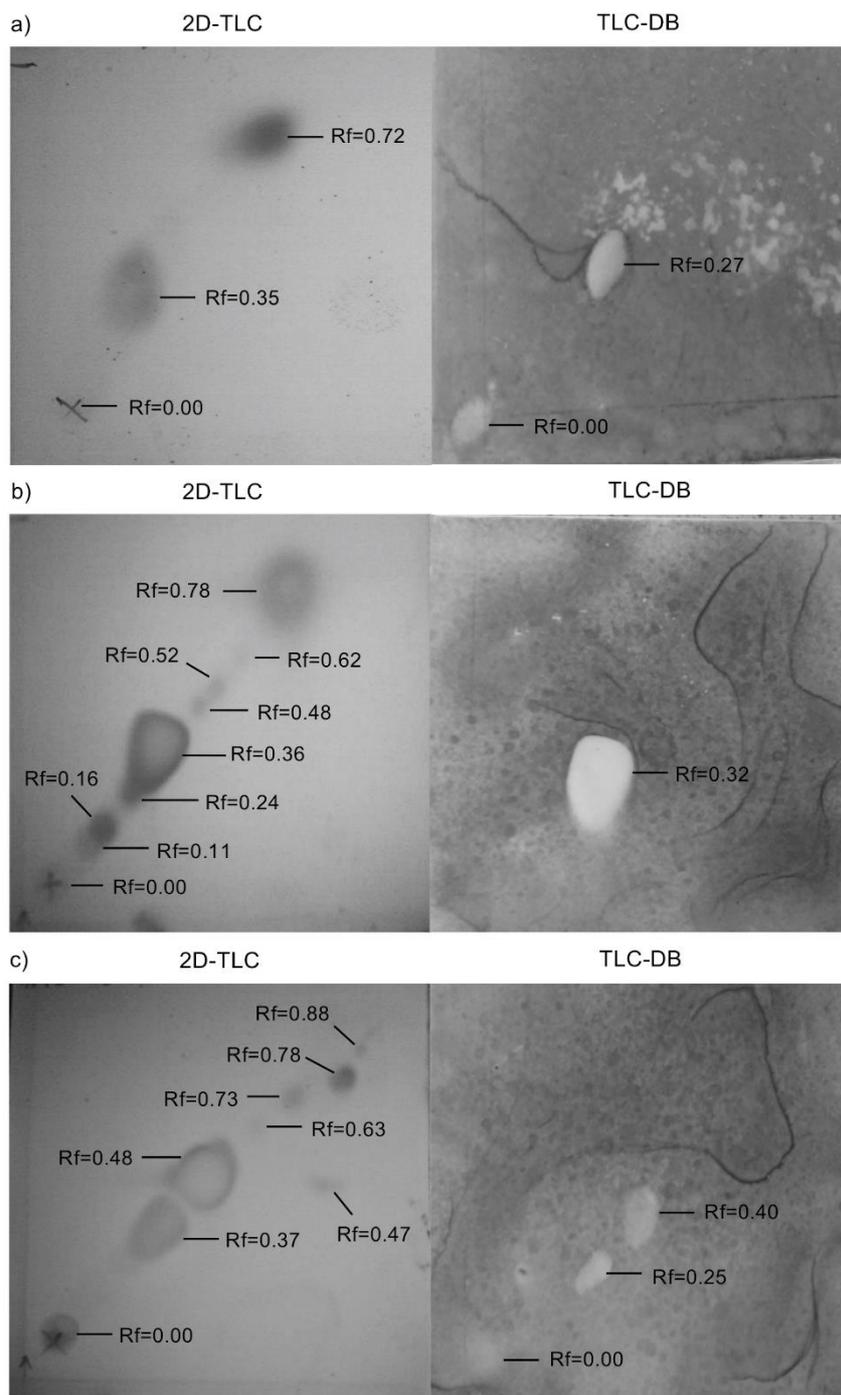


Figure 1: Side-by-side comparison of 2D-TLC and typical TLC-DB of a) cummin EO b) oregano EO and c) rosewood EO against *S. aureus*. Images were taken using a digital smartphone camera.