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ORIGINAL ARTICLE

Essential oil combinations against *Clostridium perfringens* and *Clostridium septicum* - the causative agents of gas gangrene

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Abstract

Aims: The inhibitory and bactericidal effect of a wide range of essential oils, and their selected combinations against two pathogens (*Clostridium perfringens* and *Clostridium septicum)*, the causative pathogens of gas gangrenous infections were investigated. Fractional inhibitory indices were also calculated to determine the interactions.

Methods and Results: The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays were used to determine the efficacy of the essential oils. *Santalum austrocaledonicum* demonstrated the highest activity inhibiting both Clostridial pathogens at the lowest concentration of 0·02 mg ml^{-1}. *Santalum austrocaledonicum* combined with *Cymbopogon martinii* had the strongest inhibition against *C. perfringens* (MIC 0·02 mg ml^{-1}) and *C. septicum* (MIC 0·01 mg ml^{-1}). Selected combinations demonstrated synergy ($\Sigma FIC \leq 0{\cdot}50$) in combination against both pathogens tested. Antagonism was also observed in many combinations.

Conclusions: Selected essential oils, when studied either individually or in combination, have high inhibitory and bactericidal effects against both Clostridial strains. Nine combinations have proven to be synergistic with 23 combinations additive; 96 indifferent and 77 having an antagonistic effect against the pathogenic strains. Some combinations demonstrated extreme antagonism and as such, careful consideration needs to be given to essential oil selection against these pathogens.

Significant Impact of the Study: Very few essential oils have been antimicrobially screened (MIC and MBC) against Clostridial strains and furthermore, the efficacies in combination are not known.

Introduction

Natural products, in particular essential oils have piqued the interest of researchers due to their medicinal properties, and more specifically hold promise with respect to antimicrobial properties. Previous studies have identified and reviewed the potential of these natural products in terms of food preservation and various types of microbial infections (Raeisi *et al.* 2016; van Vuuren and Holl 2017; Chouhan *et al.* 2017; Aminzare *et al.* 2018). The pique in interest of these natural antimicrobials is also attributed to the decrease in effective antimicrobial treatments, as well as the lack of new classes of clinically useful antimicrobials (Swamy *et al.* 2016). The extensive use of antibiotics over the years have now resulted in emerging resistance, with a significant number of microbial strains becoming multidrug resistant (Chouhan *et al.* 2017).

Essential oils are widely known and have been reviewed for their antibacterial activity against many pathogens. (Nazzaro *et al.* 2013; Orchard *et al.* 2017; Orchard and van Vuuren 2017). The variant chemical properties and the hydrophobic nature the essential oils allows them to penetrate lipids in bacterial cell membranes resulting in

an increased cell permeability causing the deactivation of enzymes (Chouhan *et al*. 2017; Serra *et al*. 2020).

There are many studies which prove efficacy of different essential oils in combination against multiple pathogens relative to skin diseases (de Rapper *et al*. 2013; Honorio *et al*. 2015; Adrar *et al*. 2016; Orchard and van Vuuren 2017). Various studies and reviews have also been conducted with the focus on essential oils in combination with either compounds, other essential oils or antimicrobials (Langeveld *et al*. 2014; Freires *et al*. 2015; Stefanello *et al*. 2020). Even with the extensive research which has already been conducted on essential oils, there is very little data available on essential oils against Clostridial strains. A recent study was conducted on the activity of essential oils in combination specifically focusing on wound pathogens (Orchard *et al*. 2018), and in total, 247 combinations were tested against five pathogens. The study, however, did not focus on Clostridial strains and the authors recommended that further investigations be conducted on these neglected pathogens.

Clostridium perfringens and *C. septicum* are anaerobic, Gram-positive rod-shaped bacilli which have previously been isolated and identified in various diseases such as infections of the gut of humans and ruminants, foodborne illnesses, necrotic enteritis among other infections (Choi *et al*. 2020; Hamad *et al*. 2020; Harry *et al*. 2020; Lu *et al*. 2020). The pathogens have also been isolated for gangrenous infections, aortitis, and pneumorachis (Junior *et al*. 2020; Ranchal *et al*. 2020; Wongboonsin *et al*. 2020).

Clostridial strains have shown poor susceptibility against multiple antibiotics as far back as the 1980's (Marrie *et al*. 1981), and resistance is still evident (Srivastava *et al*. 2017). Treatment has since progressed to intravenous therapy (Srivastava *et al*. 2017) due to failed oral therapy. Furthermore, antibiotic resistance amongst Clostridial strains is an ever-increasing concern. Essential oils have become a natural alternative. Some essential oils have been said to heal wounds related to gas gangrene and *C. perfringens* (Sellar 1992; Buckle 2003). An online layman skin blog, documents the overall healing effects of lavender. The author of the blog refers to a previous publication (Wannissorn *et al*. 2005) which states that lavender essential oil should be used for gangrenous infections. However, there is no scientific, quantitative evidence to substantiate these claims. From a clinical perspective, *Melaleuca alternifolia* Cheel (tea tree) has been used on a patient with a Clostridial gas gangrenous wound and results indicated an improvement in the wound healing (Cooney and Cooney 2011). However, this study focused on a single patient, making the results somewhat inconclusive. This does, however, provide some promise for other essential oil possibilities as possible treatment regimens for gangrenous wounds.

Given the in-depth analysis of the antibacterial activity of essential oils tested against the broad-spectrum of pathogens, there is very little data in comparison, of the antibacterial effects of essential oils against Clostridial pathogens. Aminzare *et al*. (2018) conducted a study on the antibacterial effect of *Cinnamomum zeylanicum* Blume (cinnamon) and grape seed extract individually and in combination against *C. perfringens* for food preservation. *Cinnamomum zeylanicum* proved to drastically decreased the colony forming unit (CFU) count of the *C. perfringens* culture, which was injected into sausages, proving its antibacterial efficacy.

A few essential oils, *Pimpinella anisum* L. (aniseed), *Thymus vulgaris* Sm. (thyme), *Mentha piperita* L. (peppermint), *Origanum basilicum* L. (basil), *Rosmarinus officinalis* L. (rosemary) and *Origanum majorana* L. (majoram), of Brazilian origin, were tested against *C. perfringens* (Radaelli *et al*. 2016). *Achillea millefolium* L. (yarrow) (Candan *et al*. 2003) as well as *C. zeylanicum* (Unlu *et al*. 2010) have been tested against *C. perfringens*. A few more studies have been conducted regarding essential oil constituents against *C. perfringens* (Kačániová *et al*. 2014; Alanazi *et al*. 2018).

This study aimed to investigate the antimicrobial efficacy of a wide range of essential oils and selected essential oil combinations, against two main Clostridial pathogens that cause gas gangrene. This will be achieved by employing the minimum inhibitory and minimum bactericidal concentration assays for the essential oils individually, and in combination. Furthermore, The fractional inhibitory index (ΣFIC) will be calculated to determine which combinations are either synergistic, additive, indifferent or antagonistic.

Materials and methods

Essential oil selection

The 56 essential oils have been selected based on their efficacy against other dermatological pathogens (Orchard *et al*. 2017). The selection was further narrowed down by selecting oils which were proven to be effective against pathogens specific to wounds (Orchard *et al*. 2018). Additionally, a range of aromatherapy books were consulted which documented the use of essential oils for skin conditions and infections (Sellar 1992; Lawless 1995; Curtis 1996; Harding 2002; Buckle 2003; Farrer-Halls 2005; Harding 2008). The oils selected in this study were procured from Prana Monde (Leuven, Germany) Scatters oils (Johannesburg, South Africa), Burgess & Finch (Cape Town, South Africa), Natural oils and Essentia,

(Johannesburg, South Africa) and once procured, stored in amber bottles at −20°C. The major chemical components of each essential oil are recorded in Table 1.

Selection and culture of pathogens

The two pathogens (*Clostridium perfringens*, ATCC 13124 and *Clostridium septicum*, ATCC 12464) selected for this study are the two strains which are most often the causative pathogens in gas gangrene (Kuroda *et al*. 2005; Srivastava *et al*. 2017). These strains were obtained from Davies Diagnostics (Johannesburg, South Africa). Both were cultured in Thioglycolate broth (TGB) (Thermofisher Scientific Oxoid, Basingstoke, UK) and incubated at 37°C for 24 h along with Anaerogen sachets and an anaerobic indicator (Thermofisher Scientific Oxoid) which were placed in an anaerobic vacuum sealed container (Thermofisher Scientific Oxoid) which is designed to maintain an anaerobic environment. These cultures were then streaked out onto Tryptone Soya agar (TSA) plates (Thermofisher Scientific Oxoid) supplemented with 5% sheep's blood (South African vaccine producers, Johannesburg, South Africa) to ensure the viability and purity of the cultures. These cultures were also streaked out onto TSA plates and incubated aerobically at 37 °C for 24 h to ensure that the cultures were not contaminants.

Minimum inhibitory concentration assay

Each essential oil used in this assay was diluted in acetone to form a stock concentration of 32 mg ml^{-1} and thereafter stored at 2–8 °C. The micro-dilution method adapted from de Rapper *et al*. (2013), was carried out to determine the inhibitory activity of the selected essential oils. Micro-titre plates (AEC Amersham, Midrand, South Africa), were prepared by adding 100 μl of TGB into each of the wells of the plate under sterile conditions. The oils were then added to the first row of the micro-titre plate at a volume of 100 μl and then were serially double diluted, resulting in first row of essential oils having a concentration of 8·00 mg ml^{-1}. The selected pathogens were inoculated into TGB at a 1:100 dilution (0·5 McFarlands standard equivalent) in order to achieve an approximate concentration of 1×10^6 CFU per ml and this was then added to all the wells at a volume of 100 μl. This results in an approximate concentration of 5×10^4 CFU the bacterial strain and a final volume of 200 μl per well.

Antibiotics (ciprofloxacin, clindamycin and penicillin from Sigma-Aldrich, Steinheim, Germany) were used as positive controls at concentrations of 0·01 mg ml^{-1}. All antibiotics obtained are HPLC grade with ≥98% purity. Water diluted in acetone (32 mg ml^{-1}) was the negative control and TGB with culture acted as the culture control. The micro-titre plates were incubated at 37 °C for 24 h. Once incubation was complete, a volume of 40 μl of *p*-iodonitrotetrazolium violet solution (INT) at a concentration of 0·04 mg ml^{-1} was added into each well of the micro-titre plate. The INT solution interacts with the pathogen which has not been inhibited, therefore a colour change (clear to a pink) is visible in the well where there has been no inhibition, whereas the well colour will remain clear where the pathogen has been inhibited. The lowest dilution with a clear well was considered the MIC value for that oil.

Noteworthy inhibitory activity of an essential oil was considered for values ≤1·00 mg ml^{-1} (Orchard and van Vuuren 2017). Essential oils which are regarded to have noteworthy activity can be further classified according to various degrees of noteworthy inhibition. Moderate activity is seen where the inhibitory value of an essential oil is between 0·50 and 1·00 mg ml^{-1} (van Vuuren and Holl 2017). Strong activity is seen where the MIC value is 0·10–0·50 mg ml^{-1} (Freires *et al*. 2015) and very strong activity is seen with an MIC value of <0·10 mg ml^{-1} (Freires *et al*. 2015).

All essential oils which demonstrated noteworthy activity were then combined in a 1 : 1 ratio. A few additional combinations were tested due to their inhibitory efficacy against wound pathogens (Orchard *et al*. 2018). The individual and combined MIC values were recorded and used to calculate the ΣFIC (van Vuuren and Viljoen 2011). The ΣFIC was calculated according to Equations 1–3.

$$\text{Division factor} = \frac{(\text{MIC of EOs in combination})}{2}, \quad (1)$$

$$\text{FIC A} = \frac{\text{Division factor}}{\text{MIC of EO 1}} \qquad \text{FIC B} = \frac{\text{Division factor}}{\text{MIC OF EO 2}}, \quad (2)$$

$$\text{FIC Index} = \text{FIC A} + \text{FIC B}. \quad (3)$$

The ΣFIC for each essential oil combination was interpreted as follows; ≤0·5 is synergistic activity; >0·5–1·0 is additive; >1·0–≤4·0 is an indifferent reaction and >4·0 indicates antagonistic activity (van Vuuren and Viljoen 2011). All assays were carried out in at least triplicate on consecutive days for each sample.

Minimum bactericidal concentration assay (MBC)

The lowest concentration of the essential oil that is able to cause 99·9% bacterial death is reported as the MBC. Once the MIC assays were conducted, an MBC assay was carried out by taking a loop full of sample mixed with culture out of the wells (MIC assay) where inhibition was observed and streaking this onto a subdivided TSA agar plate supplemented with 5% sheep's blood. This assay

Table 1 Selected essential oils with chemical composition and supplier

Essential oil	Essential oil composition (major compounds)*	Supplier
Abies balsamea Mill. (balsam fir)	β-Pinene (30·1%); δ-3-Carene (16·8%); Bornyl acetate (11·4%); α-Pinene (9·7%); Limonene (7·8%); 1,8-Cineole (6·9%); Camphene (5·8%)	Prana Monde†
Achillea millefolium L. (yarrow)	β-Pinene (11·7%); Sabinene (16·7%); β-Caryophyllene (17·0%); δ-Cadinene (16·3%)	Prana Monde
Allium sativum L. (garlic)	Diallyl sulfide (57·1%); Diallyl trisulfide (19·5%)	Essentia
Artemisia dracunculus L. (tarragon)	Estragole (82·2 %)	Prana Monde
Boswellia carteri Birdw. (frankincense)	α-Pinene (47·9%); Limonene (12·8%)	Prana Monde
Cananga odorata Hook.f.&Thomson (ylang ylang)	Benzyl acetate (30·5%); Linalyl acetate (27·7%); Methyl benzoate (10·3%); Methylanisol (10·9%)	Burgess & Finch
Canarium luzonicum Miq. (elemi)	Limonene (47·5%); Elemol (18·4%); α-Phellandrene (9·2%)	Prana Monde
Carum carvi L. (caraway)	Limonene (34·0%); Carvone (64·1%)	Prana Monde
Cinnamomum camphora J. Presl (camphor)	a-Pinene (9·7%); Limonene (27·5%); 1,8-Cineole (41·1%); *p*-Cymene (7·3%)	Prana Monde
Cinnamomum zeylanicum Blume (cinnamon)	Eugenol (84·7%); β-Caryophyllene (6·8%)	Prana Monde
Citrus aurantifolia (Christm.) Swingle (lime)	α-Pinene (9·1%); γ-Terpinene (9·7%); *p*-Cymene (9·3%); Terpinolene (11·2%); α-Terpineol (16·4%)	Burgess & Finch
Citrus aurantium var. Amara flower L. (neroli)	β-Pinene (10·1%); Limonene (15·3%); Linalyl acetate (41·8%)	Prana Monde
Citrus bergamia Risso & Poit. (bergamot)	Limonene (39·1%); Linalyl acetate (9·3%); Linalyl formate (33·0%)	Prana Monde
Citrus limon (lemon)	β-Pinene (7·2%)	Prana Monde
Citrus paradisi Macfad (grapefruit)	Limonene (95·1%)	Prana Monde
Citrus sinensis Pers. (orange)	Limonene (93·5%)	Prana Monde
Commiphora myrrha Engl. (myrrh)	Furanoeusdema-1,3-diene (52·9%); Lindestrene (15·8%)	Prana Monde
Coriandrum sativum L. (coriander)	Linalool (72·4%)	Prana Monde
Cupressus sempervirens L. (cypress)	α-Pinene (50·9%); δ-3-Carene (22·5%)	Prana Monde
Cymbopogon citratus Stapf (lemongrass)	Limonene (11·7%); Neral (28·9%); Geranial (42·7%)	Prana Monde
Cymbopogon martinii Stapf (palmarosa)	Geraniol (80·7%)	Prana Monde
Daucus carota L. (carrot seed)	Carotol (42·1%)	Scatters oils†
Eucalyptus globulus Labill. (eucalyptus)	1,8-Cineole (63·6%); α-Terpineol (10·5%)	Prana Monde
Ferula galbaniflua Boiss & Buhse (galbanum)	α-Pinene (8·3%); β-Pinene (54·3%); δ-3-Carene (7·8%)	Essentia
Helichrysum italicum G. Dhon (immortelle)	β-Pinene (7·6%);1,8-Cineole (19·4%) β-Caryophyllene (12·4%); α-Humulene (15·0%)	Prana Monde
Hyssopus officinalis L. (hyssop)	Isopinocamphone (47·9%); Pinocamphone (15·1%); β-Pinene (10·1%)	Prana Monde
Juniperus virginiana Endl. (cedarwood)	α-Pinene (22·4%); Myrcene (11·5%); Benzyl benzoate (15·4%)	Prana Monde
Kunzea ericoides Joy Thomps (kanuka)	α-Pinene (52·9%); *p*-Cymene (11·9%)	BM Oils‡
Laurus nobilis Cav. (bay)	Eugenol (54·4%); Myrcene (18·5%); Chavicol (11·5%)	Prana Monde
Lavandula angustifolia Moench (lavender)	Linalyl acetate (35·6%); Linalool (32·8%); β-Caryophyllene (10·2%)	Prana Monde
Lavandula spica Loisel. (lavender spike)	Linalool (43·1%); 1,8-Cineole (26·0%); Camphor (10·9%)	Prana Monde
Leptospermum scoparium J.R. forst (manuka)	Neral (23·0%); Geranial (35·0%);	BM Oils

(Continued)

Table 1 (Continued)

Essential oil	Essential oil composition (major compounds)*	Supplier
	Citronellol (15·4%)	
Melaleuca alternifolia Cheel (tea tree)	c-Terpinene (16·6%); *p*-Cymene (9·6%); Terpinen-4-ol (44·6%)	BM Oils
Melaleuca cajuputi Roxb. (cajeput)	Limonene (14·2%); 1,8-Cineole (56·7%); Benzyl alcohol (10·6%)	Prana Monde
Melissa officinalis L. (melissa)	Citronellal (35·6%); Neral (7·1%); Terpinyl acetate (14·2%); α-Terpineol (7·3%); Geranial (8·4%); *cis*-Geraniol (7·2%)	Burgess & Finch
Mentha piperita L. (peppermint)	Menthol (47·0%); Menthone (18·8%)	Prana Monde
Myrtus communis Blanco (myrtle)	Myrtenyl acetate (22·1%); Limonene (10·0%) 1,8-Cineole (27·4%); α-Pinene (16·7%)	Prana Monde
Ocimum basilicum L. (basil)	Linalool (55·2%); Eugenol (10·4%)	Prana Monde
Origanum majorana L. (majoram)	1,8-Cineole (51·9%); Linalyl formate (22·4%)	Prana Monde
Origanum vulgare L. (origanum)	Carvacrol (51·7%); Thymol (22·5%); γ-Terpinene (10·2%); *p*-Cymene (7·2%)	Prana Monde
Pelargonium odoratissimum L. L'Hér. (geranium)	Citronellol (26·7%); Eudesmol (12·5%); Citronellyl formate (11·5%); Neryl formate (5·4%) Menthone (7·8%); Linalool (7·2%)	Essentia
Pimpinella anisum L. (aniseed)	*trans* Anethole (86·9%); α-Terpineol (1·7%); *p*-Anisaldehyde (5·0%); Estragol (3·1%); Linalyl formate (1·0%); Anisketone (1·0%)	Scatters Oils
Pinus sylvestris Baumg (pine)	Bornyl acetate (34·8%); Camphene (22·3%) α-Pinene (11·1%); δ-3-Carene (12·6%)	Prana Monde
Piper nigrum Wall. (black pepper)	β-Caryophyllene (28·0%) Limonene (18·1%); β-Pinene (10·7%)	Prana Monde
Pistacia lentiscus L. (matic)	α-Pinene (18·7%); Myrcene (20·2%); Limonene (14·5%)	Prana Monde
Pogostemon cablin Benth. (patchouli)	α-Himachalene (13·1%); β-Patchoulene (38·3%); α-Bulnesene (13·0%); α-Guaiene (11·9%)	Prana Monde
Rosa damascena Mill. (rose)	Citronellol (29·9%); α-Pinene (14·7%) Phenelyl alcohol (22·4%); Geraniol (20·8%); Nerol (10·2%)	Prana Monde
Rosmarinus officinalis L. (rosemary)	Camphene (5·0%); 1,8-Cineole (6·9%); Camphor (19·1%); Bornyl acetate (12·2%); Verbenone (12·3%)	Prana Monde
Salvia sclarea L. (clary sage)	Linalyl acetate (57·9%); Linalool (12·4%)	Prana Monde
Santalum austrocaledonicum Vieill. (sandalwood)	α-Santalol (52·7%); β-Santalol (15·7%); Bergamotol (7·1%); Cis-Santalol (3·8%)	Prana Monde
Styrax benzoin Dryand. (benzoin)	Benzyl acetate (7·2%); Ethyl cinnamate (5·7%); Dimethyl phthalate (72·2%)	Natural Oils
Syzygium aromaticum Merr. & L.M. Perry (clove)	Eugenol (81·9%); Isoeugenol (13·1%)	Scatters Oils
Tagetes minuta L. (tagetes)	(*E*)-β-Ocimene (47·8%) (*E*)-Tagetone (10·9%); Verbenone (9·2%)	Essentia
Thymus vulgaris Sm. (thyme)	Thymol (18·9%); γ-Terpinene (7·2%); *p*-Cymene (41·0%)	Prana Monde
Vetiveria zizanoides Stapf (vetiver)	β-Vetirenene (8·8%); Zizanol (12·8%)	Prana Monde
Zingiber officinale Roscoe (ginger)	α-Curcumeme (37·6%); *Ar*-Curcumene (8·62%); (*E*,*E*),-a-Farnescene (5·3%) Himachalene (14·4%)	Prana Monde

*Most essential oil stocks have been previously analysed by GC-MS (Orchard *et al*. 2017).
†Selected Essential oil GC-MS data provided by the supplier.
‡Distilled directly from organic supplier (BM Oils, Polokwane, Limpopo).

was carried out for all assays on the individual oils as well as the oils in combination. The streaked-out blood agar plates were then incubated anaerobically at 37 °C for 24 h. If the endospores and the culture were not killed, Clostridial growth was observed after anaerobic incubation. Essential oils with a bactericidal efficacy $\leq$1·00 mg ml^{-1} was considered as noteworthy.

Results

From the total of 56 essential oils which were tested against each strain, 18 and 26 essential oils inhibited *C. perfringens* and *C. septicum* at a noteworthy value respectively (Table 2). *Santalum austrocaledonicum* proved to have the strongest inhibition against both *C. perfringens* and *C. septicum* at a concentration of 0·02 mg ml^{-1} each. Other oils demonstrating noteworthy inhibition against both strains were *C. sativum* and *O. vulgare* both inhibiting the two Clostridial strains at 0·06 mg ml^{-1} each. Other oils inhibiting both pathogens include *C. martinii*, *C. zeylanicum. C. citratus, D. carota*, *C. martini, J. virginiana, L. scoparium, M. alternifolia, M. officinalis, M. piperita, P. odoratissimum, P. cablin, R. damascena, S. aromaticum, T. vulgaris* and *V. zizanoides*.

Five essential oils (*C. martinii*, *M. piperita*, *M. officinalis, P. cablin* and *T. vulgaris*) proved to be bactericidal at a noteworthy value ($\leq$1·00 mg ml^{-1}) against *C. perfringens*; and against *C. septicum* (*C. martinii*, *M. piperita*, *D. carota*, *M. officinalis*, and *O. vulgare*). The average MIC results can be seen in Table 2 along with the respective average MBC results.

Essential oil activity of combinations against *C. perfringens*

From the 119 combinations tested against *C. perfringens*, 105 of these combinations have inhibitory activity at a noteworthy concentration ($\leq$1·00 mg ml^{-1}) (Table 3). It is interesting to note that *S. austrocaledonicum* which has proved to have the strongest inhibition against *C. perfringens*, also demonstrates the strongest inhibition when combined with other essential oils. *Santalum austrocledonicum* in a 1 : 1 combination with *C. zeylanicum*, *C. sativum*, *C. citratus, J. virgiana*, *M. officinalis*, *O. vulgare*, *P. anisum* and *S. aromaticum* each, have all shown an MIC value of 0·06 mg ml^{-1}. *Thymus vulgaris* and *C. martinii* have each shown an inhibition of *C. perfringens* at 0·02 mg ml^{-1} when combined with *S. austrocaledonicum*.

When the interactive profiles were calculated using the FIC index, five combinations proved to have synergistic activity against *C. perfringens*, namely, *C. citratus* with *R. damascena* (ΣFIC 0·12), *T. vulgaris* with *M. officinalis* (ΣFIC 0·26), *R. damascena* with *P. cablin* (ΣFIC 0·42), *R. damascena* with *S. aromaticum* (ΣFIC 0·43) and *P. odoratissimum* with *M. officinalis* (ΣFIC 0·50). Antagonism was, however, observed in a selection (23) of the combinations. The most antagonistic essential oil combinations against *C. perfringens* were *J. virginiana* with *C. zeylanicum* (ΣFIC 79·00), *C. myrrha* with *P. odoratissimum* (ΣFIC 37·33) and *C. myrrha* with *S. austrocaledonicum* (ΣFIC 16·67). Figure 1 represents a summary of the interactions of the essential oil combinations against *C. perfringens*.

Fifteen combinations demonstrated bactericidal activity against *C. perfringens* with two oil combinations (*S. austrocaledonicum* with *C. zeylanicum* and *T. vulgaris* with *V. zizanoides*) demonstrating noteworthy (1.00 mg ml^{-1}) cidal effects.

Essential oil activity in combination against *C. septicum*

Clostridium septicum was inhibited by 114 of the 119 essential oil combinations tested, with 100 of these combinations proving to have noteworthy inhibitory activity (Table 3). Furthermore, from these 100 combinations, 86 combinations have strong inhibition against *C. septicum* (MIC value 0·10–0·50 mg ml^{-1}). *Pogostemon cablin* in combination with *R. damascena,* and *C. martiniii* in combination with *S. austrocaledonicum* proved to have the strongest inhibitory activity against *C. septicum* with an MIC value of 0·01 mg ml^{-1}. *Santalum austrocaledonicum* in a 1 : 1 combination, with *C. zeylanicum*, *C. sativum*, *C. citratus, J. virgiana*, *M. officinalis*, *O. vulgare*, *P. anisum, P. cablin*, *S. benzoin* and *S. aromaticum*, each showed an inhibitory value of 0·06 mg ml^{-1} against *C. septicum*. *Thymus vulgaris* showed an inhibition of 0·03 mg ml^{-1} when combined with *S. austrocaledonicum*.

Four interactions displayed synergistic activity against *C. septicum*. These combinations are *R. damascena* with *P. cablin* (ΣFIC 0·26), *S. austrocaledonicum* with *T. vulgaris* (ΣFIC 0·29), *S. austrocaledonicum* with *C. martinii* (ΣFIC 0·33) and *M. officinalis* with *P. odoratissimum* (ΣFIC 0·50). Antagonism was observed in 54 combinations. The most antagonistic combinations against *C. septicum* were *P. odoratissimum* with *R. damascena* (ΣFIC 55·26), and *P. odoratissimum* with *M. officinalis* (ΣFIC 54·00). Figure 2 represents an overview of the essential oil interactions against *C. septicum*.

Twenty-two combinations demonstrated bactericidal activity against *C. septicum* with a selection of combinations (*V. zizanoides* with *D. carota*, *V. zizanoides* with *C. zeylanicum*, *P. odoratissimum* with *C. zeylanicum, S. austrocaledonicum* with *V. zizanoides, T vulgaris* with *M. officinalis, M. officinalis* with *S. austrocaledonicum* and *T vulgaris* with *S. austrocaledonicum*) demonstrating noteworthy ($\leq$1.00 mg ml^{-1}) cidal effects.

Table 2 Antibacterial activity of individual essential oils against both Clostridial pathogens

	Activity of oil (mg ml^{-1})			
	C. perfringens ATCC 13124		*C. septicum* ATCC 13124	
Essential oil tested	MIC average	MBC average	MIC average	MBC average
Abies balsamea	2·00 ± 0·00	>8·00	2·00 ± 0·00	>8·00
Achillea millefolium	>8·00	>8·00	>8·00	>8·00
Allium sativum	8·00 ± 0·00	>8·00	**0·63** ± 0·22	>8·00
Artemisia dracunculus	8·00 ± 0·00	>8·00	>8·00	>8·00
Boswellia carteri	>8·00	>8·00	>8·00	>8·00
Cananga odorata	>8·00	>8·00	8·00 ± 0·00	>8·00
Canarium luzonicum	>8·00	>8·00	>8·00	>8·00
Carum carvi	>8·00	>8·00	>8·00	>8·00
Cinnamomum camphora	>8·00	>8·00	>8·00	>8·00
Cinnamomum zeylanicum	**0·38** ± 0·13	>8·00	**0·16** ± 0·09	>8·00
Citrus aurantifolia	>8·00	>8·00	>8·00	>8·00
Citrus aurantium var. Amara flower	8·00 ± 0·00	>8·00	>8·00	>8·00
Citrus bergamia	>8·00	>8·00	2·00 ± 0·00	>8·00
Citrus limon	>8·00	>8·00	6·00 ± 2·00	7·00
Citrus paradisi	>8·00	>8·00	>8·00	>8·00
Citrus sinensis	>8·00	>8·00	>8·00	>8·00
Commiphora myrrha	>8·00	>8·00	>8·00	>8·00
Coriandrum sativum	**0·06** ± 0·00	>8·00	**0·06** ± 0·00	>8·00
Cupressus sempervirens	>8·00	>8·00	8·00 ± 0·00	>8·00
Cymbopogon citratus	**0·38** ± 0·13	>8·00	**0·05** ± 0·02	>8·00
Cymbopogon martinii	**0·25** ± 0·00	**1·00**	**0·06** ± 0·00	**0·06**
Daucus carota	**0·50** ± 0·00	5·00	**0·06** ± 0·00	**0·06**
Eucalyptus globulus	>8·00	>8·00	1·25 ± 0·43	1·50
Ferula galbaniflua	>8·00	>8·00	**0·63** ± 0·22	>8·00
Helichrysum italicum	>8·00	>8·00	**0·32** ± 019	>8·00
Hyssopus officinalis	>8·00	>8·00	**1·00** ± 0·00	6·00
Juniperus virginiana	**0·27** ± 0·12	>8·00	**0·04** ± 0·01	>8·00
Kunzea ericoides	1·50 ± 0·50	>8·00	**0·25** ± 0·00	>8·00
Laurus nobilis	4·00 ± 1·00	6·00	**0·06** ± 0·00	>8·00
Lavandula angustifolia	4·00 ± 0·00	>8·00	**0·88** ± 0·22	2·00
Lavandula spica	2·00 ± 3·00	8·00	2·00 ± 0·00	8·00
Leptospermum scoparium	**1·00** ± 0·00	>8·00	**0·25** ± 0·00	8·00
Melaleuca alternifolia	**1·00** ± 2·00	5·00	**0·33** ± 0·12	8·00
Melaleuca cajuputi	2·00 ± 2·50	7·00	2·00 ± 0·00	3·00
Melissa officinalis	**0·50** ± 0·19	**0·88**	**0·06** ± 0·00	**0·75**
Mentha piperita	**1·00** ± 0·00	**1·00**	**0·31** ± 0·11	**0·38**
Myrtus communis	3·00 ± 1·00	8·00	2·00 ± 0·00	2·00
Ocimum basilicum	8·00 ± 0·00	>8·00	4·00 ± 0·00	>8·00
Origanum majorana	4·00 ± 0·00	>8·00	2·00 ± 0·00	>8·00
Origanum vulgare	**0·06** ± 0·00	2·50	**0·06** ± 0·00	**0·25**
Pelargonium odoratissimum	**0·50** ± 0·00	1·50	**0·50** ± 0·00	>8·00
Pimpinella anisum	>8·00	>8·00	>8·00	>8·00
Pinus sylvestris	4·00 ± 0·00	>8·00	4·00 ± 0·00	>8·00
Piper nigrum	>8·00	>8·00	6·00 ± 2·00	>8·00
Pistacia lentiscus	4·00 ± 0·00	8·00	4·00 ± 0·00	6·00
Pogostemon cablin	**0·08** ± 0·03	**0·67**	**0·02** ± 0·01	4·00
Rosa damascena	**0·69** ± 0·32	1·50	**0·38** ± 0·13	>8·00
Rosmarinus officinalis	6·00 ± 2·00	6·00	1·50 ± 0·50	6·00
Salvea sclarea	6·00 ± 2·00	>8·00	2·00 ± 0·00	8·00
Santalum austrocaledonicum	**0·02** ± 0·00	6·00	**0·02** ± 0·00	8·00

(Continued)

Table 2 (Continued)

	Activity of oil (mg ml^{-1})			
	C. perfringens ATCC 13124		*C. septicum* ATCC 13124	
Essential oil tested	MIC average	MBC average	MIC average	MBC average
Styrax benzoin	8·00 ± 0·00	>8·00	4·00 ± 0·00	4·00
Syzigium aromaticum	**0·50** ± 0·00	>8·00	**0·50** ± 0·00	>8·00
Tagetes minuta	8·00 ± 0·00	>8·00	4·00 ± 0·00	>8·00
Thymus vulgaris	**0·15** ± 0·05	**0·50**	**0·13** ± 0·00	2·00
Vetiveria zizanoides	**0·03** ± 0·00	8·00	**0·06** ± 0·00	>8·00
Zingiber officinale (ginger)	6·00 ± 2·00	>8·00	**1·00** ± 0·00	>8·00
Negative control (water in acetone)	>8·00	>8·00	>8·00	>8·00
Culture control	>8·00	>8·00	>8·00	>8·00
Positive control Clindamycin	0·08 ± 0·00	>8·00	0·08 ± 0·00	>8·00
Positive control Penicillin	0·63 ± 0·00	0·63	0·63 ± 0·00	0·63
Positive control Clindamycin: Penicillin (1:1).	0·63 ± 0·00	>8·00	0·63 ± 0·00	>8·00
Positive control Ciprofloxacin	0·08 ± 0·00	0·63	0·08 ± 0·00	0·63

Values in bold indicate noteworthy activity; shaded area are antibiotic controls in μg ml^{-1}.

Discussion

The effectiveness of the antibacterial or antifungal activity of the essential oil is dependent on the chemical composition of the oil (Nazzaro *et al.* 2013). The major compounds of each essential oil has thus been included in Table 1.

From an antimicrobial perspective, essential oil studies against *C. perfringens* (Radaelli *et al.* 2016), demonstrated that six essential oils, namely, *O. basilicum*, *R. officinalis*, *O. majorana*, *M. piperita*, *T. vulgaris* and *P. anisum* have shown inhibition at concentrations ≥1·00 mg ml^{-1} which is not regarded as noteworthy activity. This is congruent with the inhibitory results seen in this study for five of the essential oils, namely, *O. basilicum*, *R. officinalis*, *O. majorana*, *M. piperita*, and *P. anisum*. However, *T. vulgaris* inhibited *C. perfringens* at a concentration of 0·15 mg ml^{-1}, whereas the previous study (Radaelli *et al.* 2016) reports that *T. vulgaris* did not show noteworthy inhibition. The variation in the results could be due to the difference in chemical constituents. *Thymus vulgaris* which was used in the previous study (Radaelli *et al.* 2016) contains 40·07% less *p*-Cymene, 13·59% less Thymol and 33·80% more γ-Terpinene in comparison to the *T. vulgaris* tested in this study. The oils tested by Radaelli *et al.* (2016), have also shown a bactericidal effect at concentrations ≥5·00 mg ml^{-1} with the exception of *T. vulgaris* having an MBC concentration of 1·25 mg ml^{-1}. This study also demonstrated *T. vulgaris* to be bactericidal but at a much lower concentration (0·50 mg ml^{-1}). In another study, a handful of essential oils were tested against *C. perfringens* (Dias *et al.* 2015). The authors of that study found that *C. limon*, *C. paradisi*, *O. basilicum*, *R. officinalis* and *Z. officinale* showed no antibacterial activity (MIC) or cidal activity (MBC). This is in agreement with the current study regarding *C. limon* and *C. paradise,* as no antibacterial activity was noted either. However, *O. basilicum*, *R. officinalis* and *Z. officinale* have shown inhibitory activity in the current study. The same strain of *C. perfringens* was used (ATCC 13124) in both studies and therefore the difference in antibacterial activity could be attributed to a difference in chemotype of the essential oils. The chemotype data was not provided in the previous study for comparison.

In another study where *C. zeylanicum* and *R. officinalis* were tested against four pathogens (Raeisi *et al.* 2016), it was reported that *R. officinalis* proved to have a stronger inhibitory and bactericidal activity in comparison to *C. zeylanicum* with MIC values of 312·00 and 625·00 parts per million (ppm) and MBC values of 312·00 and 625·00 ppm against *Listeria monocytogenes* for *R. officinalis* and *C. zeylanicum* respectively. This translates to the MIC values being 0·31 mg ml^{-1} and 0·63 mg ml^{-1} and the MBC values being 0·31 mg ml^{-1} and 0·63 mg ml^{-1} for *R. officinalis* and *C. zeylanicum* respectively, displaying noteworthy inhibitory and bactericidal activity. These noteworthy results are indicative of good antibacterial activity. However, the noteworthy antibacterial activity was not observed in *R. officinalis* in this study. This study reports MIC and MBC values of 6·00 mg ml^{-1} against *C. perfringens* and an MIC and MBC value of 1·50 mg ml^{-1} 6·00 mg ml^{-1} against *C. septicum* respectively. The variance in antibacterial activity

Table 3 Antibacterial activity with FIC indices of essential oil combinations against both Clostridial strains

Essential oil combinations tested		Activity of essential oils in combination (mg ml^{-1})					
		Clostridium perfringens (13124)			*Clostridium septicum* (12464)		
		MIC average	ΣFIC	MBC average	MIC average	ΣFIC	MBC average
Boswellia carteri	*Ocimum basilicum*	>8·00	N/A	>8·00	>8·00	N/A	>8·00
Cinnamomum zeylanicum	*Rosa damascena*	0·50 ± 0·00	1·02	1·50	0·50 ± 0·00	2·22	5·00
	Juniperus virginiana	0·25 ± 0·00	*79·00**	6·00	0·38 ± 0·13	*5·86*	8·00
	Coriandrum sativum	0·19 ± 0·06	1·81	6·00	0·06 ± 0·00	0·69	6·00
	Cymbopogon citratus	0·38 ± 0·13	0·99	8·00	0·38 ± 0·13	*4·99*	6·00
	Cymbopogon martinii	0·38 ± 0·13	1·24	6·00	0·38 ± 0·13	*4·35*	2·00
	Daucus carota	1·00 ± 0·00	2·32	>8·00	0·50 ± 0·00	*5·73*	4·00
	Melissa officinalis	0·50 ± 0·00	1·16	>8·00	0·50 ± 0·00	*5·73*	1·50
	Pelargonium odoratissimum	0·50 ± 0·00	1·16	8·00	0·50 ± 0·00	2·06	**1·00**
	Pogostemon cablin	0·13 ± 0·00	0·95	3·00	0·25 ± 0·00	*7·03*	6·00
Coriandrum sativum	*Rosa damascena*	0·25 ± 0·00	2·26	>8·00	0·38 ± 0·13	3·67	>8·00
	Cymbopogon citratus	0·13 ± 0·00	1·21	>8·00	0·25 ± 0·00	*4·58*	>8·00
	Cymbopogon martinii	0·13 ± 0·00	1·29	>8·00	0·25 ± 0·00	*4·17*	>8·00
	Daucus carota	0·13 ± 0·00	1·17	>8·00	0·25 ± 0·00	*4·17*	>8·00
	Juniperus virginiana	0·13 ± 0·00	1·27	>8·00	0·38 ± 0·13	*7·92*	>8·00
	Melissa officinalis	0·19 ± 0·06	1·75	>8·00	0·38 ± 0·13	*6·33*	>8·00
	Pogostemon cablin	0·13 ± 0·00	1·82	>8·00	0·25 ± 0·00	*8·33*	>8·00
Cymbopogon citratus	*Rosa damascena*	0·06 ± 0·03	**0·12**†	>8·00	0·50 ± 0·00	0·71	>8·00
	Pelargonium odoratissimum	0·50 ± 0·00	1·16	>8·00	0·75 ± 0·25	*8·25*	>8·00
	Cymbopogon martinii	0·50 ± 0·25	1·66	>8·00	1·00 ± 0·00	*18·33*	>8·00
	Daucus carota	1·00 ± 0·00	2·32	>8·00	0·75 ± 0·25	*13·75*	>8·00
	Juniperus virginiana	1·00 ± 0·00	3·17	>8·00	0·50 ± 0·00	*11·25*	>8·00
	Melissa officinalis	0·50 ± 0·00	1·16	>8·00	0·75 ± 0·25	*13·75*	>8·00
	Pogostemon cablin	0·25 ± 0·00	1·89	>8·00	0·50 ± 0·00	*17·50*	>8·00
Cymbopogon martinii	*Pelargonium odoratissimum*	1·00 ± 0·00	3·00	>8·00	1·00 ± 0·00	*9·33*	>8·00
	Pogostemon cablin	0·28 ± 0·00	2·06	>8·00	0·50 ± 0·00	*16·67*	>8·00
	Rosa damascena	0·75 ± 0·25	2·04	>8·00	0·75 ± 0·25	*7·24*	>8·00
	Daucus carota	0·75 ± 0·25	2·25	>8·00	1·50 ± 0·50	*25·00*	>8·00
	Juniperus virginiana	0·50 ± 0·00	1·93	>8·00	0·50 ± 0·00	*10·42*	>8·00
	Melissa officinalis	0·75 ± 0·25	2·25	>8·00	1·00 ± 0·00	*16·67*	>8·00
Daucus carota	*Rosa damascena*	1·50 ± 0·50	2·59	>8·00	2·00 ± 0·00	*19·30*	6·00
	Juniperus virginiana	>8·00	N/A‡	>8·00	2·00 ± 0·00	*41·67*	>8·00
	Melissa officinalis	1·50 ± 0·50	3·00	>8·00	1·00 ± 0·00	*16·67*	>8·00
	Pelargonium odoratissimum	2·00 ± 0·00	*4·00*	>8·00	1·50 ± 0·50	*14·00*	>8·00
	Pogostemon cablin	0·50 ± 0·00	3·63	>8·00	1·00 ± 0·00	*33·33*	>8·00
Juniperus virginiana	*Melissa officinalis*	1·00 ± 0·00	2·85	1·50	2·00 ± 0·00	*41·67*	>8·00
	Pelargonium odoratissimum	1·00 ± 0·00	2·85	2·00	4·00 ± 0·00	*54·00*	>8·00
	Pogostemon cablin	0·50 ± 0·00	*4·05*	2·00	>8·00	N/A	>8·00
	Rosa damascena	2·00 ± 0·00	*5·15*	>8·00	4·00 ± 0·00	*55·26*	>8·00
Melissa officinalis	*Pelargonium odoratissimum*	0·25 ± 0·00	**0·50**	>8·00	0·25 ± 0·00	**0·50**	>8·00
	Pogostemon cablin	0·11 ± 0·03	0·80	>8·00	0·08 ± 0·03	2·02	>8·00
	Rosa damascena	0·31 ± 0·11	0·53	>8·00	0·31 ± 0·11	0·72	>8·00
Mentha piperita	*Rosmarinus officinalis*	>8·00	N/A	>8·00	>8·00	N/A	>8·00
Origanum vulgare	*Cinnamomum zeylanicum*	0·50 ± 0·00	*4·82*	>8·00	0·75 ± 0·25	*8·59*	>8·00
	Juniperus virginiana	0·25 ± 0·00	2·55	>8·00	0·50 ± 0·00	*10·42*	>8·00
	Coriandrum sativum	0·25 ± 0·00	*4·17*	>8·00	0·10 ± 0·03	1·58	>8·00
	Cymbopogon citratus	0·25 ± 0·00	2·41	>8·00	0·06 ± 0·00	1·10	>8·00
	Cymbopogon martinii	0·13 ± 0·00	1·34	>8·00	0·25 ± 0·00	*4·17*	>8·00
	Daucus carota	0·50 ± 0·00	*4·67*	>8·00	0·50 ± 0·00	*8·33*	>8·00
	Pogostemon cablin	0·06 ± 0·03	0·88	>8·00	0·38 ± 0·13	*12·67*	>8·00
	Rosa damascena	0·25 ± 0·00	2·26	>8·00	0·38 ± 0·13	3·67	>8·00

(Continued)

Table 3 (Continued)

Essential oil combinations tested		Activity of essential oils in combination (mg ml^{-1})					
		Clostridium perfringens (13124)			*Clostridium septicum* (12464)		
		MIC average	ΣFIC	MBC average	MIC average	ΣFIC	MBC average
	Pelargonium odoratissimum	0·50 ± 0 00	*4·67*	>8·00	0·38 ± 0·13	3·55	>8·00
	Melissa officinalis	0·13 ± 0·00	1·21	>8·00	0·25 ± 0·00	*4·17*	>8·00
	Pimpinella anisum	0·25 ± 0·00	N/A	>8·00	0·50 ± 0·00	N/A	>8·00
	Vetiveria zizanoides	0·50 ± 0·00	*12·50*	**1·00**	0·50 ± 0·00	*12·50*	**1·00**
Pelargonium odoratissimum	*Commiphora myrrha*	4·00 ± 0·00	*37·33*	>8·00	4·00 ± 0·00	N/A	6·00
Pimpinella anisum	*Rosa damascena*	1·00 ± 0·00	N/A	>8·00	1·50 ± 0·50	N/A	>8·00
	Pelargonium odoratissimum	2·00 ± 0·00	N/A	>8·00	2·00 ± 0·00	N/A	>8·00
	Coriandrum sativum	0·75 ± 0·25	N/A	>8·00	0·25 ± 0·00	N/A	>8·00
	Cymbopogon citratus	1·00 ± 0·00	N/A	>8·00	0·50 ± 0·00	N/A	>8·00
	Cymbopogon martinii	1·00 ± 0·00	N/A	>8·00	0·75 ± 0·25	N/A	>8·00
	Daucus carota	>8·00	N/A	>8·00	>8·00	N/A	>8·00
	Juniperus virginiana	0·75 ± 0·25	N/A	>8·00	1·00 ± 0·00	N/A	>8·00
	Melissa officinalis	2·00 ± 0·00	N/A	>8·00	2·00 ± 0·00	N/A	>8·00
	Pogostemon cablin	1·00 ± 0·00	N/A	>8·00	1·50 ± 0·50	N/A	>8·00
	Cinnamomum zeylanicum	0·50 ± 0·00	N/A	>8·00	0·50 ± 0·00	N/A	>8·00
Pogostemon cablin	*Rosa damascena*	0·06 ± 0·00	**0·42**	>8·00	0·01 ± 0·00	**0·26**	>8·00
Rosa damascena	*Citrus aurantium var. Amara* flower	2·00 ± 0·00	1·57	6·00	1·50 ± 0·50	N/A	>8·00
	Boswellia carteri	2·00 ± 0·00	N/A	3·00	3·00 ± 1·00	N/A	6·00
Santalum austrocaledonicum	*Cinnamomum zeylanicum*	0·06 ± 0·00	1·64	**1·00**	0·06 ± 0·00	1·69	>8·00
	Commiphora myrrha	0·50 ± 0·00	*16·67*	>8·00	0·13 ± 0·00	N/A	>8·00
	Coriandrum sativum	0·06 ± 0·00	2·08	>8·00	0·06 ± 0·00	2·00	>8·00
	Cymbopogon citratus	0·06 ± 0·00	1·58	>8·00	0·06 ± 0·00	2·10	>8·00
	Cymbopogon martinii	0·02 ± 0·00	0·54	>8·00	0·01 ± 0·00	**0·33**	>8·00
	Daucus carota	0·13 ± 0·00	3·25	>8·00	0·25 ± 0·00	*8·33*	**0·75**
	Juniperus virginiana	0·06 ± 0·00	1·68	>8·00	0·06 ± 0·00	2·25	>8·00
	Melissa officinalis	0·06 ± 0·00	1·56	>8·00	0·06 ± 0·00	1·56	**0·06**
	Origanum vulgare	0·06 ± 0·00	2·00	>8·00	0·06 ± 0·00	2·00	>8·00
	Pimpinella anisum	0·06 ± 0·00	N/A	>8·00	0·06 ± 0·00	N/A	>8·00
	Pogostemon cablin	0·25 ± 0·00	*7·81*	>8·00	0·06 ± 0·00	3·00	6·00
	Styrax benzoin	0·13 ± 0·00	3·13	>8·00	0·06 ± 0·00	1·51	**0·06**
	Syzygium aromaticum	0·06 ± 0·00	1·63	>8·00	0·06 ± 0·00	1·56	>8·00
	Thymus vulgaris	0·02 ± 0·01	0·57	>8·00	0·03 ± 0·00	0·87	**0·25**
	Vetiveria zizanoides	0·25 ± 0·00	*10·42*	>8·00	0·25 ± 0·00	*8·33*	>8·00
Syzygium aromaticum	*Cinnamomum zeylanicum*	0·25 ± 0·00	*4·42*	>8·00	0·50 ± 0·00	2·06	>8·00
	Rosa damascena	0·25 ± 0·00	**0·43**	>8·00	0·25 ± 0·00	0·58	>8·00
	Pelargonium odoratissimum	0·50 ± 0·00	1·00	>8·00	0·50 ± 0·00	1·00	>8·00
	Coriandrum sativum	0·19 ± 0·06	1·75	>8·00	0·13 ± 0·00	1·21	>8·00
	Cymbopogon citratus	0·25 ± 0·00	0·58	>8·00	0·25 ± 0·00	2·75	>8·00
	Cymbopogon martinii	0·25 ± 0·00	0·75	>8·00	0·25 ± 0·00	2·33	>8·00
	Daucus carota	0·50 ± 0·00	1·00	>8·00	0·50 ± 0·00	*4·67*	>8·00
	Juniperus virginiana	0·25 ± 0·00	0·71	>8·00	0·25 ± 0·00	3·38	>8·00
	Melissa officinalis	0·50 ± 0·00	1·00	>8·00	0·50 ± 0·00	*4·67*	>8·00
	Pogostemon cablin	0·25 ± 0·00	1·81	>8·00	0·25 ± 0·00	*6·50*	>8·00
	Pimpinella anisum	1·00 ± 0·0 0	N/A	>8·00	1·00 ± 0·00	N/A	>8·00
	Origanum vulgare	0·13 ± 0·0 0	1·21	>8·00	0·25 ± 0·00	2·33	>8·00
Thymus vulgaris	*Cinnamomum zeylanicum*	0·50 ± 0·00	2·32	2·50	0·38 ± 0·13	2·61	1·50
	Coriandrum sativum	0·38 ± 0·13	*4·38*	>8·00	0·50 ± 0·00	*6·09*	>8·00
	Cymbopogon citratus	0·38 ± 0·13	*4·38*	>8·00	0·50 ± 0·00	*6·09*	>8·00
	Cymbopogon martinii	0·25 ± 0·00	1·33	>8·00	0·38 ± 0·13	*4·63*	>8·00
	Daucus carota	0·50 ± 0·00	2·17	>8·00	0·50 ± 0·00	*6·09*	>8·00
	Juniperus virginiana	0·50 ± 0·00	2·59	>8·00	1·00 ± 0·00	*16·35*	>8·00

(Continued)

Table 3 (Continued)

Essential oil combinations tested		Activity of essential oils in combination (mg ml^{-1})					
		Clostridium perfringens (13124)			*Clostridium septicum* (12464)		
		MIC average	ΣFIC	MBC average	MIC average	ΣFIC	MBC average
	Melissa officinalis	0·06 ± 0·00	**0·26**	>8·00	0·06 ± 0·00	**0·29**	**0·50**
	Origanum vulgare	0·13 ± 0·00	1·52	>8·00	0·13 ± 0·00	1·58	>8·00
	Pimpinella anisum	1·00 ± 0·00	N/A	>8·00	0·50 ± 0·00	N/A	>8·00
	Pogostemon cablin	0·13 ± 0·08	1·25	>8·00	0·06 ± 0·00	1·73	>8·00
	Syzygium aromaticum	0·25 ± 0·00	1·08	>8·00	0·25 ± 0·00	1·21	>8·00
	Vetiveria zizanoides	0·50 ±0·00	*10·00*	**1·00**	0·50 ± 0·00	*10·00*	>8·00
Vetiveria zizanoides	*Cinnamomum zeylanicum*	0·06 ± 0·00	1·12	6·00	0·06 ± 0·00	0·69	1·00
	Coriandrum sativum	0·08 ± 0·03	1·56	>8·00	0·16 ± 0·09	2·58	>8·00
	Cymbopogon citratus	0·25 ± 0·00	*4·50*	>8·00	0·38 ± 0·13	*6·97*	>8·00
	Cymbopogon martinii	0·25 ± 0·00	*4·67*	>8·00	0·19 ± 0·06	3·17	>8·00
	Daucus carota	0·50 ± 0·00	*8·83*	>8·00	0·50 ± 0·00	*8·33*	**0·75**
	Juniperus virginiana	0·50 ± 0·00	*9·26*	>8·00	>8·00	N/A	>8·00
	Melissa officinalis	0·06 ± 0·00	1·06	>8·00	0·06 ± 0·00	0·56	>8·00
	Pelargonium odoratissimum	0·06 ± 0·00	1·06	>8·00	0·50 ± 0·00	*4·67*	>8·00
	Pimpinella anisum	0·50 ± 0·00	N/A	>8·00	0·50 ± 0·00	N/A	>8·00
	Pogostemon cablin	0·50 ± 0·00	*11·46*	>8·00	0·19 ± 0·06	*6·25*	6·00
	Syzygium aromaticum	0·25 ± 0·00	*4·42*	>8·00	0·25 ± 0·00	2·33	>8·00

*Values in italics indicate antagonistic activity.

†Values in bold indicate synergistic activity or noteworthy activity.

‡N/A = Not applicable; no endpoint (value > 8·00 is evident for at least one of the samples, therefore ΣFIC cannot be calculated).

could be indicative of a higher resilience observed in the Clostridial strains in comparison to the studies undertaken on *L. monocytogenes*.

In this study, *C. zeylanicum* inhibited *C. perfringens* and C. *septicum* at noteworthy concentrations of 0·31 mg ml^{-1} and 0·16 mg ml^{-1} respectively. These results display a similar comparison to the MIC concentration of 0·31 mg ml^{-1} against *L. monocytogenes* (Raeisi *et al*. 2016). Bactericidal activity was not observed against either Clostridial strain in the current study. Cinnamon, black pepper, fennel, ginger and wormwood essential oils (Latin identification was not provided by the authors) have previously been tested for antibacterial activity against *C. perfringens* (Zhu *et al*. 2020). The authors reported the MIC data, however, none of the essential oils proved to have noteworthy inhibition. This emphasizes the potential resilience of Clostridial strains against antibacterial agents due to their spores which re-germinate under favourable conditions.

From a clinical perspective, *C. myrrha* and *L. angustifolia* has been reported to show a symptomatic improvement against Clostridial wounds (Sellar 1992). However, when these oils were tested against the two Clostridial pathogens *in vitro*, the MIC concentrations of *C. myrrha* were >8·00 mg ml^{-1} against *C. perfringens* and *C. septicum* demonstrating minimal *in vitro* antibacterial activity against both Clostridial strains. According to the current study, *L. angustifolia* inhibited *C. perfringens* at 4·00 mg ml^{-1} and *C. septicum* at 0·88 mg ml^{-1} with a bactericidal effect at 2·00 mg ml^{-1} against *C. septicum*. This oil proved to be only bacteriostatic against *C. perfringens* and inhibition was only noteworthy against *C. septicum*. *Origanum majorana* was reported to heal a Clostridial wound within 24 h (Buckle 2003). According to this *in vitro* study, *O. majorana* inhibited *C. perfringens* at a concentration of 4·00 mg ml^{-1} and *C. septicum* at 2·00 mg ml^{-1} and proved to have no bactericidal activity against either strain. This comparison shows that while layman literature and other internet sources available provide information on essential oils having antibacterial effects regarding Clostridial wounds, it is important that studies are conducted to scientifically quantify the efficacy. The chemotype of the essential oil is also important, due to the variations in the antibacterial results when different chemotypes are studied. The layman literature often lacks data on the chemotype of the essential oils being used.

When reviewing the antibiotic susceptibility against clostridial isolates it was found that of the fourteen antibiotics tested against *C. perfringens,* resistance was observed against eight antibiotics (Khan *et al*. 2019). Another study recommended that a combination of clindamycin and penicillin should be used to treat *C. septicum* (Stevens *et al*. 2005; Ranchal *et al*. 2020).

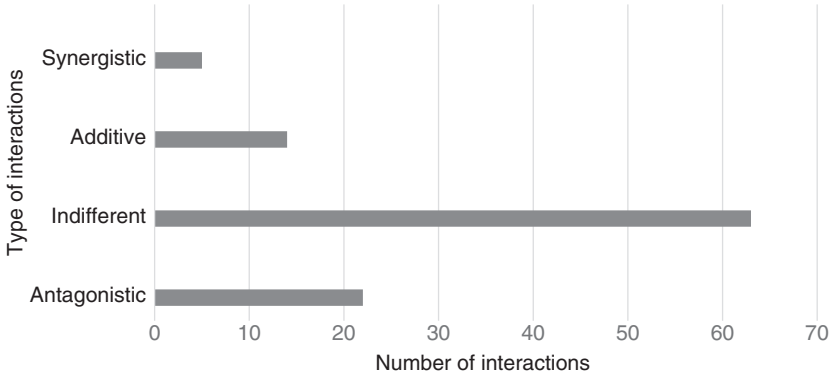

Figure 1 Overview of the interactions of essential oil combinations against *Clostridium perfringens*.

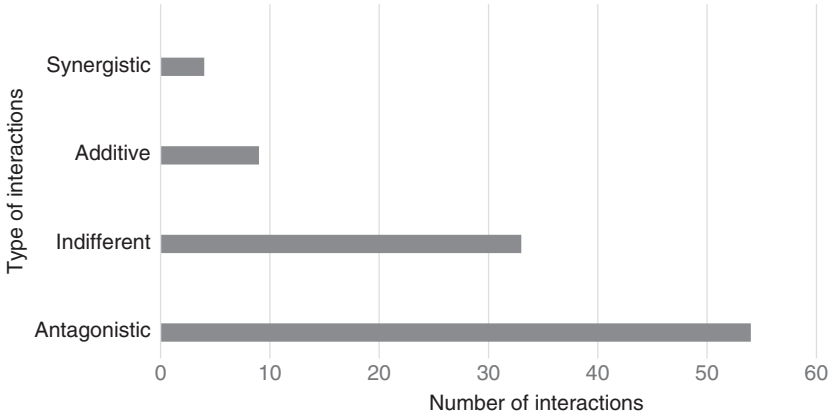

Figure 2 Overview of the interactions of essential oil combinations against *Clostridium septicum*.

A comparison of these antibiotics was carried out in this study regarding the antibacterial efficacy of these antibiotics against the Clostridial strains. Interestingly, the individual inhibitory activity of clindamycin proved to be more effective (MIC 0·08 μg ml^{-1}) in comparison to the inhibitory activity of the penicillin in combination clindamycin (0·63 μg ml^{-1}). A recent study (Ranchal *et al.* 2020), recommends that the combination should be used for the treatment of *C. septicum* which contradicts the *in vitro* activities observed herein. Ciprofloxacin has proven to be the most effective antibiotic with inhibitory (MIC 0·08 μg ml^{-1}) and bactericidal activity (MBC 0·63 μg ml^{-1}) observed against both Clostridial strains tested.

Nevas *et al.* (2004) conducted a study of a few essential oils against *C. perfringens*. Disc diffusion assays were was and therefore the results may be inaccurate, especially due to the inconsistencies associated with the volatility of essential oils. The current use of MIC and MBC assays are the preferred method used for essential oil studies (Kalemba and Kunicka 2003). However, an overview of the results of the previous study (Nevas *et al.* 2004) is in line with the current results, as the essential oils which have proven to have what is deemed as strong activity were *O. vulgare* and *T. vulgaris*. These two oils have also shown good antibacterial activity against *C. perfringens* when tested using the MIC and MBC assays studied herein.

When reviewing the possibility of structure activity relationships against Clostridial pathogens, Thymol and Carvacrol were tested for their antibacterial efficacy against various pathogens (Du *et al.* 2015). Both compounds are also major components in various essential oils (*T. vulgaris* and *O. vulgare* respectively). These two compounds showed an MIC value of 0·38 mg ml^{-1} individually against *C. perfringens* with an MBC value of 0·75 mg ml^{-1} each.

Dias *et al.* (2015) also tested four essential oils (*C. citratus*, *O. vulgare*, *S. aromaticum* and *T. vulgaris*) against *C. perfringens* in various ratios of a combination of three essential oils. That study demonstrated that no synergy was found in any of the combinations tested which somewhat supports the data seen in this current study when two oils were combined. A previous study (Orchard *et al.* 2018), has shown that essential oil combinations prove to be efficacious in inhibiting wound pathogens such as *Pseudomonas aeruginosa, Escherichia coli* and various strains of *Staphylococcus aureus* at noteworthy MIC

values. This is in agreement with this study even though different wound pathogens were investigated. *Santalum austrocaledonium* and *V. zizanoides* proved to have noteworthy inhibition in all combinations against all three pathogens mentioned. *Origanum vulgare* proved to have noteworthy inhibitory activity in certain combinations, however, a wide range of combinations with this essential oil was not tested. This is possibly due to the efficacy of the individual oil not being noteworthy against these specific skin pathogens. The antibacterial results of the combined essential oils against the wound pathogens (Orchard *et al*. 2018) and other studies against multiple other pathogens (Becerril *et al*. 2012; De Rapper *et al*. 2012; Kon and Rai 2012; de Rapper *et al*. 2013) reported that few oils were synergistic. This can also be seen in combinations against the Clostridial strains. A total of five combinations proved to have noteworthy inhibitory efficacy and are synergistic combinations against *C. perfringens*. Four combinations proved to have noteworthy inhibition against *C. septicum* which are synergistic combinations. It is interesting to note that the combinations which have proven to be synergistic are the same combinations against both Clostridial strains which mainly include *M. officinalis* and *R. damascena*.

The ΣFIC of the combinations show that a total of 71 essential oil combinations indicate antagonistic activity. All combinations with *J. virginiana*, and *D. carota* were observed to be very antagonistic and should not be considered for combined anti-Clostridial activity. Every combination with *C. martinii* proved to be antagonistic when tested against *C. septicum*. However, there is an exception when *C. martinii* is combined with *S. austrocaledonicum* as this combination is very synergistic. This synergy shows a further increase in inhibitory efficacy against *C. septicum* as both essential oils have very good MIC values when tested independently. The ΣFIC results highlight that synergy should not be readily expected when combining two essential oils with noteworthy efficacy. Antagonism was found to be more evident than synergy even though the MIC values indicate noteworthy inhibition of the Clostridial strains.

According to Mackay *et al*. 2000, the criteria for fractional bactericidal concentration (ΣFBC) index of combinations is the same as those carried out to interpret ΣFIC values of combinations. The ΣFBC for all the combinations tested against *C. perfringens* and most of the combinations tested against *C. septicum* could not be calculated as there were no endpoints for the calculation.

The ΣFBC for the two combinations which were for *C. septicum*, namely *S. austrocaldeonicum* with *P. cablin* and *D. carota* respectively, were calculated and have proved to have no synergy. Three combinations against *C. septicum* proved to have a synergistic bactericidal effect. The ΣFBC for essential oils have not been reported in previous studies and therefore a comparison cannot be made. Is shows the evident gap of in-depth analysis of the potential synergistic benefits essential oil combinations or the noting of antagonistic essential oil combinations.

Santalum austrocaledonicum has proven to be a significant essential oil when dealing with Clostridial pathogens as it demonstrated the lowest inhibitory value against both tested strains when tested individually and in combination. Alone, *S. austrocaledonicum* may not have the strongest bactericidal effect, however, it clearly enhances the bactericidal effect of other essential oils as synergy in seen in two combinations against *C. septicum* with the MBC assay. *Melissa officinalis* and *O. vulgare* have noteworthy MIC and MBC values against both strains when used individually, however, these values are not the lowest concentration in the MIC and MBC assay (as seen with *S. austrocaledonicum* in the MIC assay). The combination of *S. austrocaldedonicum* with *M. officinalis* proves to be the most synergistic when the ΣFBC was calculated.

Although the toxicity of the essential oils and their combinations were not the major focus in this study, many of the essential oils which proved to have good bacterial efficacy have also proven to be toxic (Orchard *et al*. 2019). As reported in a previous study (Orchard *et al*. 2019), a Brine Shrimp Lethality assay was conducted to determine the biological toxicity of the essential oils and the combinations. Thereafter, a calculation was carried out to determine the % mortality of the brine shrimp over a stipulated period of 24 h. Any value calculated, which resulted in a 50% mortality of the brine shrimp was considered to be toxic and any value below 50% mortality was considered non-toxic. *Thymus vulgaris* and *C. citratus* demonstrated 100% mortality within 24 h. *Cymbopogon martinii*, *M. officinalis* and *C. zeylanicum* which have all shown over 87% mortality within 24 h in a Brine Shrimp Lethality assay. Although considered toxic, these properties maybe useful for various essential oil applications such as acting as pesticides or antitumor agents (Unlu *et al*. 2010). No matter what the oil or combination considered, it is important to establish the toxicity levels. Furthermore, clinical use of essential oils will require extensive testing and formulation prior to application.

Many essential oils, in particular a select few such as *S. austrocaledonicum*, *V. zizanoides*, *P. cablin*, *M. officinalis* and *C. martinii* have shown very strong antibacterial activity (MIC $< 0{\cdot}10$ mg ml^{-1}). These oils have great potential as antibacterial agents against the two specific *Clostridial* pathogens tested which are the major causes of infectious gas gangrene. Three combinations (*M. officinalis* with *S. austrocaledonicum* at ΣFBC 0·04, S. *austrocaledonicum* with *T. vulgaris* at ΣFBC 0·08 and *M. officinalis*

with *T. vulgaris* at ΣFBC 0·46) have demonstrated a synergistic bactericidal effect against *C. septicum* which is a definite indication of the strong antibacterial potential of these oils. Not only do they inhibit the pathogen, but they also are successful in killing the pathogenic spores which is often resistant and problematic.

This study has covered a broad range of essential oil antibacterial activity against two Clostridial strains which are commonly found in many infections. These two strains are also causative of most Clostridial gas gangrenous infections. The essential oils individually, and those in selected combination with noteworthy antibacterial activity were highlighted.

Eighteen and 26 essential oils have displayed noteworthy inhibition against *C. perfringens* and *C. septicum* respectively. Regarding the essential oil combination, a total of 119 combinations were tested against each pathogen, where 105 and 100 combinations inhibited *C. perfringens* and *C. septicum* at a noteworthy concentration respectively. Five different essential oils proved to be bactericidal against both Clostridial pathogens at a noteworthy value. *Thymus vulgaris* had the strongest bactericidal activity against *C. perfringens* (MBC 0·50 mg ml^{-1}). *Daucus carota* and *C. martinii* displayed the strongest bactericidal activity against *C. septicum* with an MBC value of 0·06 mg ml^{-1} each.

Santalum austrocaledonicum stood out as the essential oil with the strongest inhibition (MIC 0·02 mg ml^{-1}) against both Clostridial pathogens. This essential oil has also demonstrated the best inhibition when in combination with *C. martinii* against *C perfringens* and *C. septicum* with MIC values of 0·02 mg ml^{-1} and 0·01 mg ml^{-1} respectively.

Synergy is observed in various combinations which show that these combinations yield an even better inhibitory effect against the Clostridial pathogens. Five combinations proved to be synergistic against *C. perfringens* with four combinations displaying synergy against *C. septicum*. Three combinations in particular proved to be the most suitable essential oil combinations which are synergistic against both Clostridial strains. These combinations were *T. vulgaris* with *M. officinalis, R. damascena* with *P. cablin* and *M. officinalis* with *P. odoratissimum*.

However, while synergy is emphasized the role of antagonism must not be ignored as this may impact on essential oil selection. In fact, more combinations proved to have an antagonistic effect in comparison to those proving to be synergistic.

Anecdotal treatments should be scientifically tested in order to confirm the treatment efficacy or lack thereof, as many claims can be disproven once scientifically tested. For example, the combination of essential oils may help to potentially reduce the toxic effects of the individual essential oils; however, it must be noted that along with the reduction in toxicity, the antibacterial effect may also be reduced when the essential oils are combined as seen in this study. Further studies should be carried out in order to determine if toxicity of the essential oils is reduced when in combination. Further studies are also recommended for the effective oils to be considered in novel pharmaceutical formulations for the inhibition of Clostridial strains causing gas gangrene and therefore potentially aid in future developments in treating topical gas gangrenous wounds.

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Conflict of Interest

No conflict of interest is declared.

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