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ORIGINAL ARTICLE

The additive and synergistic antimicrobial effects of select frankincense and myrrh oils – a combination from the pharaonic pharmacopoeia

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Abstract

Aims: The *in vitro* antimicrobial activity of three essential oil samples of frankincense (*Boswellia rivae*, *Boswellia neglecta* and *Boswellia papyrifera*) and two essential oil samples of myrrh and sweet myrrh (*Commiphora guidotti* and *Commiphora myrrha*), collected from different regions of Ethiopia, was investigated independently and in combination to determine their anti-infective properties.

Methods and Results: The microdilution minimum inhibitory concentration (MIC) assay was performed, whereby it was noted that generally *Cryptococcus neoformans* (MIC values in the range of 0·8–1·4 mg ml^{-1}) and *Pseudomonas aeruginosa* (MIC values in the range of 0·5–1·3 mg ml^{-1}) often appeared to be the most susceptible micro-organisms against oils of both *Boswellia* and *Commiphora* spp. When assayed in various combinations, the frankincense and myrrh oils displayed synergistic, additive and noninteractive properties, with no antagonism noted. When investigating different ratio combinations against *Bacillus cereus*, the most favourable combination was between *B. papyrifera* and *C. myrrha*. The composition of the oils was determined by gas chromatography coupled to mass spectrometry (GC–MS) to document the specific chemotypes used in the study, and the chemical profiles were found to be congruent with previously reported data.

Conclusions: The majority of interactions identified synergistic and additive effects, with strong synergism noted between *B. papyrifera* and *C. myrrha*.

Significance and Impact of the Study: Frankincense and myrrh essential oils have been used in combination since 1500 BC; however, no antimicrobial investigations have been undertaken to confirm their effect in combination. This study validates the enhanced efficacy when used in combination against a selection of pathogens.

Introduction

Frankincense (*Boswellia* spp.) and myrrh (*Commiphora* spp.) are members of the Burseraceae, a family of resinous and aromatic plants that occur in the southern Arabian Peninsula, north-east Africa, Somalia, Kenya, Ethiopia and the Sudan (Vollesen *et al.* 1989; Dharmananda 2003).

The historical use of frankincense and myrrh essential oils date back to biblical times, where frankincense has been cited extensively in the bible (Tucker 1986). The antimicrobial use of frankincense can be traced back to the 11th century when the Persian physician and philosopher Avicenna used frankincense to treat inflammation and infections of the urinary tract (Michie and Cooper 1991). Furthermore, frankincense oils have been associated with the treatment of wounds and inflammation, firming of aged skin, cystitis, treatment of rheumatic joints as well as the treatment of irregular menstrual and

nose bleeds. Internally, the oils have been used for the treatment of throat and larynx infections, respiratory infections, stomach and liver ailments as well as constipation.

The earliest antimicrobial use of myrrh dates back to 1100 BC, where Sumerians used myrrh to treat infected teeth and intestinal worms (Michie and Cooper 1991). In ancient times, myrrh was used by the Egyptians for embalming. Myrrh oil has also long been used for the treatment of skin wounds and fungal infections caused by *Candida albicans* and *Tinea pedis* (Stevenson 1998). *The British Herbal Pharmacopoeia* (BHMA 1996) indicates myrrh tincture as a mouthwash for gingivitis and ulcers. The European Commission (Blumenthal *et al.* 2000) approved myrrh for topical treatment of mild inflammation of the oral and pharyngeal mucosa. Myrrh is also an important drug in Chinese Traditional Medicine where it has been used for the treatment of syphilis, leprosy and rheumatism (Yen 1992; Nomicos 2007). In Somalia and Ethiopia, a decoction of myrrh resin is used traditionally to treat stomach ache.

A number of studies have been undertaken in support of the traditional use of frankincense (both *Boswellia* and *Commiphora* spp.) to treat infectious diseases and other medical conditions (Michie and Cooper 1991; El-Ashry *et al.* 2003; Nomicos 2007; Paraskeva *et al.* 2008; El-Sherbini *et al.* 2009; Van Vuuren *et al.* 2010; Walsh *et al.* 2010; Wanner *et al.* 2010; Jarić *et al.* 2011). Furthermore, over many centuries, ranging from 1500 BC until present day, these oils have been used in combination. The earliest evidence of their use in combination for pharmaceutical purposes is found in the 'Papyrus Ebers', an ancient Egyptian formulary of prescriptions dating to around 1500 BC, wherein they were prescribed for the treatment of wounds and skin sores (Michie and Cooper 1991). The use of frankincense and myrrh has also reportedly been combined with other medicinal substances, such as opium and red ochre to treat ailments such as pruritus and infections of burn wounds (Scarborough 1983). There is further evidence of the pharmacological activities of frankincense and its use in combination therapy with other medicinal plants (Scarborough 1983; Vollesen *et al.* 1989; Michie and Cooper 1991; Dharmananda 2003; Moussaieff *et al.* 2005; Shen and Lou 2008; Yigit *et al.* 2008). Similarly for myrrh, there are many publications supporting the pharmacological use in combination with other medicinal plants (Tucker 1986; Michie and Cooper 1991; Jarić *et al.* 2011). In spite of the historical and traditional use of a combination of frankincense with myrrh, no supporting evidence could be found to validate their use in combination to treat infectious diseases. With this in mind, an *in vitro* study was designed to evaluate the antimicrobial interactive efficacy of three frankincense essential oils (*Boswellia rivae*, *Boswellia neglecta* and *Boswellia papyrifera*) with two myrrh oils (*Commiphora guidotti* and *Commiphora myrrha*). In addition to the antimicrobial evaluation of these oils, both independently and in combination, the chemical composition (major compounds) is also reported to document the specific chemotypes studied.

Materials and methods

Plant material and preparation of essential oils

Resins of *B. rivae* Engl., *C. guidotti* Chiov. and *C. myrrha* (Nees) Engl. were collected from the Ogaden region in south eastern Ethiopia; *B. neglecta* S. Moore from Borena region in the south and *B. papyrifera* (Del.) Hochst T. Nees. from north Ethiopia. Voucher specimens for all species have been deposited at the National Herbarium, Addis Ababa University, and species identification was made by Kaj Vollesen of Kew Botanic Garden, UK. The respective essential oils were obtained from distillation (for *c.* 3 h) in a Clevenger apparatus and thereafter stored in amber bottles.

Chemical composition

The composition of the oils was determined using gas chromatography coupled to a mass spectrometer and flame ionization detector (GC–MS–FID). The GC–MS methodology was adapted from Van Vuuren *et al.* (2010). The percentage composition of the individual components was quantified by integration measurements using flame ionization detection (FID, 250°C). Component identifications were made by comparing mass spectra from the total ion chromatogram and retention indices using NIST® and Mass Finder® GC–MS libraries (Van Vuuren *et al.* 2010). Major compounds were noted if % area was ≥10% and compared with previous studies where major and minor compounds for the species have been reported.

Micro-organism selection and preparation

The micro-organisms selected for this study were *Staphylococcus aureus* (ATCC 12600), *Bacillus cereus* (ATCC 11778), *Pseudomonas aeruginosa* (ATCC 27858), *Escherichia coli* (ATCC 8739), *C. albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 90112). Selection was based on the traditional use of these essential oils for the treatment of dermatological and gastrointestinal infections. The Clinical Laboratory Standards Institute (CLSI), formerly known as the National Committee for Clinical Laboratory Standards (NCCLS) (CLSI 2003), guidelines

were used to ensure that accurate microbiological assay and transfer techniques were followed.

Minimum inhibitory concentration (MIC) determination

The microdilution MIC method was undertaken to quantify inhibitory activity of the essential oils (Carson *et al.* 1995; Eloff 1998; CLSI 2003). The method, as indicated in Van Vuuren *et al.* (2010), was followed. Briefly, essential oils were diluted to yield a concentration of 64 mg ml^{-1} using acetone as the diluent and 100 μl (where tested individually) and 50 : 50 μl (where tested in combination) added to 100 μl of sterile water. The positive control was 0·01 mg ml^{-1} ciprofloxacin for bacteria, and 0·01 mg ml^{-1} amphotericin B for the yeasts. The negative control was a water/acetone solution at a concentration of 64 mg ml^{-1}, to determine antimicrobial efficacy of the solvent. Media (sterile media incubated) and culture (culture inoculated into microtitre plate without inhibitor) controls were included to confirm sterility and viability, respectively. The micro-organisms were added at an approximate concentration of 1×10^6 colony-forming units (CFU) per ml followed by optimal incubation conditions. Visualization of the end point MIC was undertaken using *p*-iodonitrotetrazolium violet. Assays were undertaken in triplicate, and further repetitions conducted where necessary.

Fractional inhibitory concentration ($\sum$FIC) interpretations

For the 1 : 1 combinations, the fractional inhibitory index ($\sum$FIC) was calculated to determine the interaction of the oils. The $\sum$FICs were calculated by dividing the MIC value of the combined essential oils (taking into account half the concentration is attributed to 1 : 1 mixes) by the MIC value of each essential oil placed in the combination. The $\sum$FIC is then calculated by adding these two FIC values. Previously, the $\sum$FIC for each combination was interpreted as synergistic where the $\sum$FIC was <1·00, and antagonistic with a $\sum$FIC of >1·00; however, a more conservative approach has been recommended (Odds 2003) and adapted, with the $\sum$FIC for each combination interpreted as synergistic where the $\sum$FIC is ≤0·50. For additive properties, the $\sum$FIC is interpreted as >0·50–1·00. For indifference, $\sum$FIC values are >1·00–≤4·00 and antagonism occurs when an $\sum$FIC is >4·00 (Van Vuuren and Viljoen 2011).

Variable ratio analysis

To determine what antimicrobial interactions could be apparent if variable concentrations of the two test essential oils were mixed, a selection of *Boswellia* and *Commiphora* oils were combined in nine ratios, i.e., 9 : 1; 8 : 2; 7 : 3; 6 : 4; 5 : 5; 4 : 6; 3 : 7; 2 : 8 and 1 : 9. MIC values were determined for all nine ratios as well as for the essential oils independently against the pathogen *B. cereus*. All tests were undertaken at least in duplicate, and where results differed by more than one dilution factor, a third replicate was undertaken. Isobolograms were constructed using GraphPad Prism version 5® to present the mean MIC values of the combinations as ratios (Suliman *et al.* 2010). The isobolograms were interpreted by examining the data points for each ratio in relation to the MICs for the oils independently. All points below or on the 0·5 : 0·5 line on the isobologram were interpreted as synergistic. Points between the 0·5 : 0·5 and 1·0 : 1·0 line were interpreted as additive, and points above the 1·0 : 1·0 line were observed as noninteractive. Antagonism (not observed in this study) would be noted for points above 4·0 (Van Vuuren and Viljoen 2011). Ciprofloxacin was included as the positive control in all repetitions to confirm antimicrobial susceptibility. Negative controls were included to confirm sterility and effect of solvents.

Results

Chemical composition

The two *Commiphora* species included in the study have a very different oil composition. *Commiphora myrrha* possessed major constituents such as furanogermacrene (15·9%), and furanoeudesma-1,3-diene (44·3%) present only as minor components in *C. guidotti*. Major constituents present in *C. guidotti* include (*E*)-β-ocimene (52·6%), α-santalene (11·1%) and (*E*)-α-bisabolene (16·0%).

Antimicrobial analysis – independent samples

When testing the essential oils individually, it was noted that *C. neoformans* and *Ps. aeruginosa* were the most susceptible micro-organisms with MIC values ranging between 0·80 and 1·40 mg ml^{-1}, and 0·50 and 1·50 mg ml^{-1}, respectively (Table 1). Noteworthy antimicrobial activity was considered for MIC values ≤2·00 mg ml^{-1} (Van Vuuren 2008). Therefore, the majority (80·0%) of the oils investigated in this study had noteworthy antimicrobial activity. Antimicrobial activity against *Staph. aureus* varied greatly with MIC values ranging from 1·30 to 6·00 mg ml^{-1}. Similarly, the antimicrobial activity against *B. cereus* demonstrated MIC values ranging from 0·40 to 2·90 mg ml^{-1}. The oil sample showing the most broad-spectrum activity was *C. myrrha* with a mean MIC value of 1·00 mg ml^{-1}.

Table 1 The mean MIC value (mg ml^{-1}) for frankincense and myrrh oil samples

	Test pathogen						
	Staphylococcus aureus	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Candida albicans*	*Cryptococcus neoformans*	
Species	(ATCC 12600)	(ATCC 11778)	(ATCC 27858)	(ATCC 8739)	(ATCC 10231)	(ATCC 90112)	Mean
Boswellia neglecta	6·00	2·70	**1·30**	3·00	**1·80**	**1·30**	2·68
Boswellia papyrifera	**1·50***	**1·60**	**1·50**	3·30	**1·40**	**1·40**	**1·78**
Boswellia rivae	2·50	2·90	**1·00**	3·00	4·00	**0·80**	2·36
Commiphora guidotti	**1·50**	**1·30**	**1·40**	4·00	**1·50**	**1·30**	**1·83**
Commiphora myrrha	**1·30**	**0·40**	**0·50**	**1·00**	**2·00**	**0·80**	**1·00**
B. neglecta + *C. guidotti*	**1·50**	3·20	**1·30**	2·40	2·80	**0·50**	**1·95**
B. neglecta + *C. myrrha*	**1·00**	**1·80**	**1·50**	3·00	**1·60**	**0·90**	**1·63**
B. papyrifera + *C. guidotti*	3·00	3·10	**1·60**	4·00	**1·20**	**0·80**	2·28
B. papyrifera + *C. myrrha*	**1·70**	2·70	**1·30**	3·30	**1·40**	5·30	2·62
B. rivae + *C. guidotti*	**1·00**	2·30	**1·00**	2·70	**2·00**	**0·50**	**1·58**
B. rivae + *C. myrrha*	**1·50**	2·70	**1·30**	3·00	**1·40**	**0·80**	**1·78**
Ciprofloxacin control	$0·3 \times 10^{-3}$	$0·2 \times 10^{-3}$	$0·6 \times 10^{-3}$	$0·6 \times 10^{-3}$	NA	NA	
Amphotericin control	NA†	NA	NA	NA	$0·6 \times 10^{-3}$	$0·6 \times 10^{-3}$	

MIC, minimum inhibitory concentration.
*Noteworthy activity is indicated with figures in bold.
†NA indicates the micro-organism to which the control was not used.

Table 2 The mean ∑FIC for frankincense and myrrh oil samples

	Test pathogen						
	Staphylococcus aureus	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Candida albicans*	*Cryptococcus neoformans*	
Species	(ATCC 12600)	(ATCC 1778)	(ATCC 27858)	(ATCC 8739)	(ATCC 10231)	(ATCC 10112)	Mean
Boswellia neglecta + *Commiphora guidotti*	2·50	0·63	1·04	1·46	0·59	2·60	2·63
B. neglecta + *Commiphora myrrha*	3·65	0·86	0·60	0·67	1·19	1·17	1·36
Boswellia papyrifera + *C. guidotti*	**0·50***	**0·47**	0·91	0·91	1·21	1·69	0·95
B. papyrifera + *C. myrrha*	0·82	**0·37**	0·77	0·65	1·21	**0·21**	0·67
Boswellia rivae + *C. guidotti*	2·00	0·91	1·20	1·30	1·38	2·10	1·48
B. rivae + *C. myrrha*	1·27	0·61	0·58	0·67	2·14	1·00	1·05

FIC, fractional inhibitory concentration.
*Synergistic activity is indicated with figures in bold.

Antimicrobial analysis – combined samples

In the 1 : 1 combinations, the oils predominantly showed noteworthy activity with the *B. rivae* and *C. guidotti* combination having the most prominent broad-spectrum activity (mean MIC value of 1·58 mg ml^{-1}) (Table 1). When FIC values were calculated for each 1 : 1 combination (Table 2), synergistic (11·11%), additive (41·67%) and noninteractive properties (45·95%) were observed. Synergistic effects were identified for *B. papyrifera* and *C. guidotti* against *Staph. aureus* (∑FIC = 0·50) and *B. cereus* (∑FIC = 0·47) and *B. papyrifera* in combination with *C. myrrha* against *B. cereus* (∑FIC = 0·37) and *C. neoformans* (∑FIC = 0·21). Of all the oil combinations tested, it was identified that *B. papyrifera* and *C. myrrha* possessed the best overall interaction with a mean ∑FIC value of 0·67. Interestingly, no antagonism was observed for any of the oils in any of the combinations.

As *B. cereus* demonstrated the most prominent synergistic profile, further in-depth analysis was undertaken to determine the antimicrobial effects of a selection of oils in nine different ratio concentrations. Isobolograms were plotted (Fig. 1) and from the data points observed, it is clear that the combination of *B. papyrifera* and *C. myrrha* demonstrates synergy not only when combined in equal proportions as noted in Table 2, but also in the majority of combinations, particularly where *C. myrrha* is in higher concentrations. A similar trend was also observed with

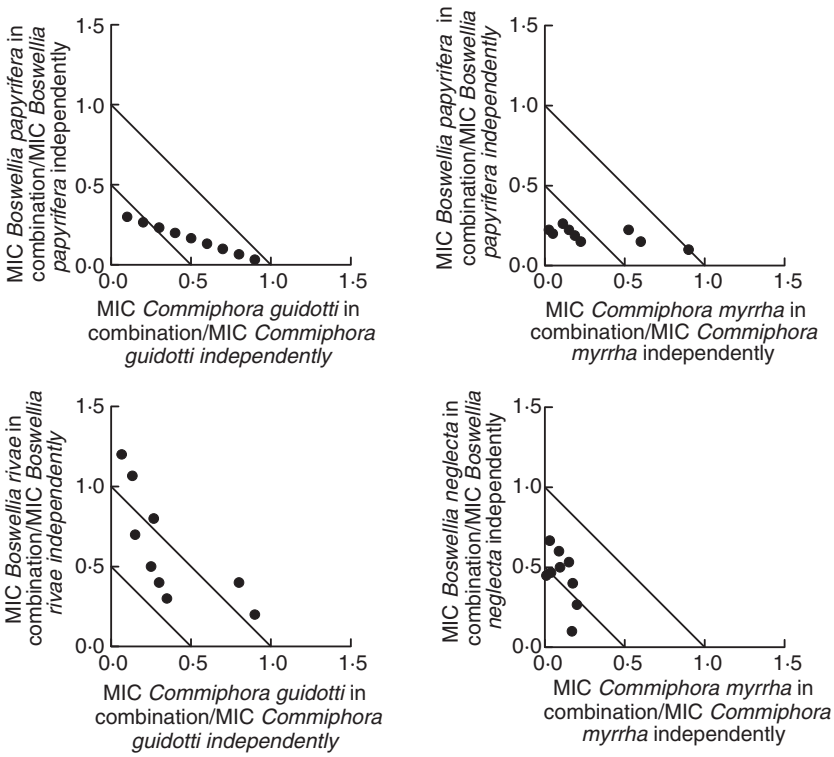

Figure 1 Isobologram representations of frankincense and myrrh oils in various ratios against *Bacillus cereus* (ATCC 11778).

the combination of *B. papyrifera* and *C. guidotti* where points closest to synergy are observed for concentrations where *C. guidotti* is present in higher ratios.

Discussion

Environmental conditions including climate, time of harvesting, storage conditions, etc. may play a role in the chemical composition of oils (Daferera *et al.* 2000). Thus, even though the essential oil composition of these oils has been previously reported, a detailed GC–MS analysis was undertaken to determine whether chemotypic variations are apparent. Major constituents of *C. myrrha* (furanodiene, 19·7%; furanoeudesma-1,3-diene, 34·0%; and lindestrene, 12·0%) have previously been reported from an Ethiopian species (Hanuš *et al.* 2005). An earlier study conducted by Craveiro *et al.* (1983) and later by Marongiu *et al.* (2005) also identified a similar composition to this study with furano-type compounds as major constituents. El-Ashry *et al.* (2003) reported the composition of *C. guidotti* oil (obtained from Egypt) to have high levels of α-santalene (22·0%), α-bisabolene (23·0%) and furanodiene (9·0%). Similarly, Hanuš *et al.* (2005) identified the major constituents of the Ethiopian *C. guidotti* to be (*E*)-*β*-ocimene (33·0%), α-santalene (15·8%) and (*E*)-α-bisabolene (22·2%). α-Pinene (36·1–67·7%) was present in high concentrations in both *B. rivae* and *B. neglecta.* Other major constituents from *B. rivae* include *δ*-3-carene (12·2%) and limonene (12·0%). In a separate study performed on the essential oil of *B. rivae*, Başer *et al.* (2003) demonstrated some variation in *B. rivae* oil obtained from Ethiopia with lower concentrations of α-pinene (5·3%), but with similar concentrations of *δ*-3-carene (9·6%) and limonene (14·8%). The only other major constituent from *B. neglecta* was terpinen-4-ol at 11·3%. For *B. papyrifera*, octyl acetate (64·8%) predominates as the major component. Our previous GC–MS analysis (Van Vuuren *et al.* 2010), as well as earlier reports (Başer *et al.* 2003; Camarda *et al.* 2007), demonstrated compositional congruency of these Ethiopian-derived oils.

Previous antimicrobial studies on *C. myrrha* oleo-gum resin using ethanol and ether solvents for extraction have been tested with poor activity (MIC values ranging from 10 to 40 mg ml^{-1}) (Omer *et al.* 2011). Usually, oils present lower activities than their respective extracts for the same plant species. The MIC results for the *C. myrrha* oil demonstrated in this study were more active than that found in the extracts previously. To find an essential oil exhibiting activities better than the extract of the same species is unusual, interesting and very promising. Furthermore, to the best of our knowledge, no previous reports have been dedicated to the antimicrobial activity of the essential oil of *C. guidotti*, a *Commiphora* sp. demonstrating broad-spectrum noteworthy activity against all

pathogens tested except *E. coli*. Previous biofilm antimicrobial studies have been undertaken on *B. papyrifera* and *B. rivae* oils and demonstrated a considerable antimicrobial activity with inhibition percentages ranging from 59·1 to 99·0% against a range of pathogens (Schillaci *et al.* 2008). Another study was conducted on the antimicrobial effects of the oleo-gum resin of *B. papyrifera* against *Staph. aureus*, demonstrating MIC values of 500 μg ml^{-1} (Abdallah *et al.* 2009). This value is lower than that of the MIC identified for the essential oil and thus suggests that the extract is more effective than the essential oil when tested against specific micro-organisms.

The chemical composition of these oils is congruent with that reported previously in literature. Noteworthy antimicrobial activity was evident for 80% of the frankincense and myrrh essential oil samples, with the most noteworthy efficacy identified for *C. myrrha* oil. Although the antimicrobial activities for some of these species have been previously investigated, the significance of the use of frankincense and myrrh species in combination has demonstrated largely noteworthy activity against a wide range of test micro-organisms. The $\sum$FIC analysis indicated that these oils have some favourable antimicrobial interactions (11·1% synergy and 41·7% additive effects) when placed in combination, with no antagonism identified for any of these samples studied. Thus, more than half of the combinations (52·8%) possess advantageous and positive results, further justifying the traditional use of these oils in combination. The isobolograms of selected samples against *B. cereus* further demonstrate, particularly with the *B. papyrifera* and *C. myrrha* combination, the synergistic effects when various combinations are investigated. The historical and antimicrobial importance of these oils can thus not be ignored as efficacies independently and in combination prove extremely promising as antimicrobial agents.

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