Validating the *in vitro* antimicrobial activity of *Artemisia afra* in polyherbal combinations to treat respiratory infections

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Abstract

Artemisia afra is one of the most widely used medicinal plants in African traditional medicine and is commonly administered in polyherbal combinations to treat respiratory infections. Focussing on plant volatiles, the aim of this study was to provide scientific evidence for the antimicrobial activity of *A. afra* (principle plant) in combination with essential oils from three medicinal aromatic plants; *Agathosma betulina*, *Eucalyptus globulus* and *Osmitopsis asteriscoides*. *In vitro* minimum inhibitory concentration (MIC) assays were undertaken on four pathogens (*Enterococcus faecalis* ATCC 29212, *Moraxella catarrhalis* ATCC 23246, *Klebsiella pneumoniae* NCTC 9633 and *Cryptococcus neoformans* ATCC 90112) to determine antimicrobial efficacy of the oils and their combinations. The fractional inhibitory concentration (FIC) and isobolograms were used to interpret pharmacodynamic interactions such as synergy, antagonism or additive profiles. The antimicrobial activity of the individual oils mostly displayed moderate activity. Predominantly, additive interactions were noted. The most prominent synergistic interaction (FIC value of 0.5) was observed when *A. afra* was combined with *O. asteriscoides* in the 8:2 ratio (eight parts *A. afra* with two parts *O. asteriscoides*) against *C. neoformans*. No antagonistic interactions were evident.

Keywords: *Agathosma betulina*; *Artemisia afra*; Antimicrobial; *Eucalyptus globulus*; Essential oil; *Osmitopsis asteriscoides*; Synergistic and additive interactions

1. Introduction

Artemisia afra Jacq. ex Willd., commonly referred to as African wormwood, is one of the most widely used traditional medicines in southern Africa (Watt and Breyer-Brandwijk, 1962; Graven et al., 1992; Thring and Weitz, 2006; Van Wyk et al., 2009). This highly aromatic indigenous medicinal plant is abundantly distributed throughout the mountainous regions of the South Western Cape and extends northwards, through the Limpopo Province (South Africa) and into tropical east Africa (Van Wyk et al., 2009). Due to its natural abundance and wide spectrum of medicinal use (Watt and Breyer-Brandwijk, 1962; Von Koenen, 1996; Van Wyk and Wink, 2004; Thring and Weitz, 2006; Van Wyk et al., 2009), it has been the preferred treatment by traditional healers. *A. afra* is well known for the treatment of upper and lower respiratory tract infections, where symptoms include chills, coughs, throat infections, colds, fever, inflammation of the throat, bronchitis and blocked nose. Treatment is administered, either by inserting fresh leaves into the nostril or by the administration of infusions, decoctions, in steam inhalation and/or used as a gargle (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; Van Wyk and Wink, 2004; Van Wyk et al., 2009).

A. afra also exhibits potent pharmacological activities including antimicrobial, antioxidant, CNS-effects (sedative, antidepressant), cardiovascular, and spasmolytic activity which has been well documented and reviewed recently by Liu et al. (2009). In relation to the anti-infective properties, *A. afra* has shown inhibitory activity against some Gram-positive and Gram-negative bacteria, fungi, as well as protozoa (Graven et al., 1992; Bruneton, 1995; Poswal and Witbooi, 1997; Mangena and Muyima, 1999; Huffman et al., 2002; Muyima et al., 2002; Van Vuuren and Viljoen, 2006; Viljoen et al., 2006a; Van

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Vuuren, 2007). A recent report on the anti-mycobacterial activity of *A. afra* (MIC value of 1.57 mg/ml against *Mycobacterium smegmatis*) demonstrated potential activity against respiratory pathogens (Mativandlela et al., 2008). Even though the antimicrobial activity of *A. afra* has been thoroughly studied using disc diffusion, dilution and time-kill assays, validation on the combined use with other plant species is lacking. This is surprising, as the use of *A. afra* in combination with other medicinal plants has been widely documented in the ethnobotanical literature (Table 1). Based on the ethnobotanical data summarised in Table 1, the oils from selected aromatic plants (*Agathosma betulina*, *Eucalyptus globulus,* and *Osmitopsis asteriscoides*) were investigated to determine if synergistic antimicrobial interactions were apparent when combined with *A. afra*.

A. betulina (P.J. Bergius) Pillans, belonging to the Rutaceae family, is more commonly known as "buchu". It is an aromatic, round leafed perennial shrub, with a long-standing reputation of commercial importance (Van Wyk, 2008). The essential oil is known to have therapeutic properties particularly for treating respiratory conditions such as the treatment of fever, coughs, colds, and flu (Simpson, 1998; Moolla, 2006; Viljoen et al., 2006b; Moolla and Viljoen, 2008).

E. globulus Labill. is commonly known as blue gum and belongs to the family Myrtaceae. It is a very large tree (up to 60 m in height) with a characteristic shedding bark (Horn et al., 1964; Van Wyk and Wink, 2004). The essential oil is colourless or pale yellow, and it has an aromatic camphoraceous odour (Watt and Breyer-Brandwijk, 1962). Although used extensively as an indigenous medicinal plant in Australia and Tasmania (Jordan et al., 1993; Dutkowski and Potts, 1999; Van Wyk and Wink, 2004), *E. globulus* is also used in South Africa, as a household remedy for colds as well as an ingredient in herbal nasal preparations (Watt and Breyer-Brandwijk, 1962; Felhaber, 1997). *E. globulus* essential oil has been widely studied and said to have a broad spectrum of antibacterial and antifungal activity (Tewari and Akhila, 1985; Zakarya et al., 1993; Cimanga et al., 2002; Sartorelli et al., 2007; Ghalem and Mohamed, 2008a,b).

O. asteriscoides (P.J. Bergius) Less. from the Asteraceae family, is commonly referred to as bels (Van Wyk et al., 2009). It is a vigorous, branched glabrous shrub, which has a strong camphor odour (Bremer, 1972; Van Wyk et al., 2009). The leaves are oblong and glandular, having numerous diminutive oil-containing glands on the surface (Van Wyk et al., 2009). This Cape-Dutch remedy has been traditionally used to treat various ailments ranging from inflammation, cuts, swellings chest complaints, intestinal disorders, fevers to respiratory related illnesses (Watt and Breyer-Brandwijk, 1962; Scott et al., 2004; Van Wyk et al., 2009).

2. Material and methods

2.1. Plant collection and distillation of essential oils

Leaves and stems of *A. afra* were collected from the Klipriviersburg Nature Reserve in Klipriviersburg, South of Johannesburg. *O. asteriscoides* leaves were collected from Hermanus, Cape Town. *A. betulina* leaves were purchased from Warren Chem Specialties (South Africa). *E. globulus* leaves were collected from Cresta, North Western Johannesburg, Gauteng. All plant collections were carried out in the summer months of 2007.

Table 1
Traditional use of *A. afra* in combination with other plant species for the treatment of respiratory complaints.

Plants in combination	Uses	Administration	References
A. afra and *E. globulus*	Respiratory complaints	Crushed leaves or steam from infusions are inhaled or decoctions are taken	Watt and Breyer-Brandwijk, 1962; Hutchings et al. (1996)
A. afra and *A. betulina*	Respiratory complaints	Herbal wine	Watt and Breyer-Brandwijk, (1962)
A. afra and *Zanthoxylum capense*	The Europeans and Africans use it in febrile conditions, and it is used as a treatment for colds	A decoction and an infusion of the leaf is used	Watt and Breyer-Brandwijk (1962)
A. afra and *O. asteriscoides*	Respiratory complaints	Tincture	Watt and Breyer-Brandwijk (1962)
A. afra, E. globulus and *Leonotis microphylla*	Fever, chest infections and digestive disturbances	Infusion	Watt and Breyer-Brandwijk (1962)
A. afra, Z. capense and *Allium sativum*	Respiratory complaints	Decoction	Watt and Breyer-Brandwijk (1962)
A. afra and *Lippia javanica*	Fevers, respiratory complaints, measles and as a prophylactic against lung inflammations	Infusion, taken with milk	Watt and Breyer-Brandwijk (1962)
A. afra, O. asteriscoides and *E. globulus*	Respiratory complaints	Infusion, tincture	Watt and Breyer-Brandwijk (1962); Van Wyk et al. (2009)
A. afra and *Tetradenia riparia* and salt	Coughs	Decoctions	Hutchings et al. (1996)
A. afra and *Alepidea amatymbica*	Colds and flu	Leaves and root/rhizome	Jäger (2003)
A. afra and *Warburgia salutaris*	Acute bronchitis, coughs from colds or flu, fever	Leaves and bark	Felhaber (1997); Jäger (2003)
A. afra, *A. amatymbica* and *Leonotis leonurus*	Asthma	Leaves, root and leaves	Felhaber (1997)
A. afra, *W. salutaris* and *Acorus calamus*	Chronic bronchitis and emphysema	Leaves, bark and rhizome	Felhaber (1997)

Voucher specimens for each specimen (*A. afra* — SFVV14 and SFVV61, *O. asteriscoides* — SFVV23, *A. betulina* — SFVV13, and *E. globulus* — SFVV33) were deposited in the Department of Pharmacy and Pharmacology, University of the Witwatersrand. Plant identification was confirmed by A. Viljoen. As many of the traditional uses and preparation of the remedies allude to the volatile constituents (e.g. inhalation therapy and preparation of tinctures) the essential oils from each plant were distilled by conventional hydro-distillation in a Clevenger-type apparatus. Leaves and stems were heated, the essential oils condensed in a cooling system and thereafter decanted after 3 h.

2.2. Antimicrobial susceptibility tests

All microbiological techniques, culture and media preparation were undertaken as described in the CLSI/NCCLS (2003) guidelines. Reference microbial strains (*Enterococcus faecalis* ATCC 29212, *Moraxella catarrhalis* ATCC 23246, *Klebsiella pneumoniae* NCTC 9633 and *Cryptococcus neoformans* ATCC 90112) were selected on the basis of their respiratory pathogenesis (Bannister et al., 2000; Verduin et al., 2002). Strains were obtained from the National Health Laboratory Services (NHLS) and sub-cultured from stock agar plates, where purity was confirmed. The bacteria were cultured in Tryptone Soya broth (TSB) for 24 h. The yeast (*C. neoformans*), was incubated for 48 h. Cultures were prepared for micro-dilution assays using a 1:100 dilution, yielding an approximate inoculum size of 1×10^6 colony forming units (CFU)/ml). The minimum inhibitory concentration (MIC) micro-dilution assay was carried out, as described by Van Vuuren and Viljoen (2009). Starting concentrations of all essential oils at 128 mg/ml in acetone were prepared. Conventional antimicrobials i.e. ciprofloxacin for the bacteria and amphotericin B for the yeast (*C. neoformans*) were included in all repetitions to confirm the antimicrobial susceptibility.

2.3. Fractional inhibitory concentration (FIC)

As described by Van Vuuren (2007), the minimum inhibitory concentration (MIC) data was used to calculate the fractional inhibitory concentration (FIC) using the following equation:

$$FIC_I = \frac{MIC\ (a)\ in\ combination\ with\ (b)}{MIC\ (a)\ independently}$$

$$FIC_{II} = \frac{MIC\ (a)\ in\ combination\ with\ (b)}{MIC\ (b)\ independently}.$$

(a) represents *A. afra* and (b) represents either *A. betulina*, *E. globulus* or *O. asteriscoides*. The sum of the FIC, known as the FIC index was calculated as:

$$FIC_{index} = FIC_I + FIC_{II}.$$

The FIC index was adapted from Odds (2003) and modified to include an additive interpretation (Schelz et al., 2006; Iten et al., 2009). This was used to determine the correlation between the two plant essential oils and may be classified as either synergistic (≤ 0.5), additive ($>0.5-1.0$), non-interactive ($>1.0-\leq 4.0$) or antagonistic (>4.0).

2.4. Isobologram construction

While the FIC data gives an indication of the interaction between two plant combinations, a more in-depth approach (graphical isobolograms) is presented, which considers the different ratios at which the two plant samples were combined (Van Vuuren, 2007). Combinations of *A. afra* with either *A. betulina*, *E. globulus* or *O. asteriscoides* were prepared 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 for all plant combinations. Isobolograms were constructed using Graphpad Prism® to present the mean MIC values of the combinations as ratios, according to the equation;

$$X = \frac{MIC\ value\ of\ combination\ of\ essential\ oils}{MIC\ value\ of\ essential\ oil\ independently}$$

$$Y = \frac{MIC\ value\ of\ combination\ of\ essential\ oils}{MIC\ value\ of\ essential\ oil\ independently}$$

The isobolograms were interpreted by examining the data points for each ratio in relation to the MICs for the oils independently. The interpretation of the FIC index was used to analyse interactions and thus ratio points falling below and/or on the 0.5 line of the isobologram were interpreted as synergistic. Points between 0.5 and/on the 1.0 line were interpreted as additive and points above the 1.0 line were defined as either non-interactive (>1.0 and 4.0) or antagonistic (>4.0). Positive controls (ciprofloxacin for the bacteria and amphotericin B for *C. neoformans*) were included in all repetitions to confirm the antimicrobial susceptibility. Negative controls were included to confirm sterility and effect of solvents. All tests were undertaken at least in duplicate. Separate experimental procedures were undertaken on selected combinations to ensure uniformity of results.

3. Results and discussion

3.1. Antimicrobial susceptibility tests

The antimicrobial activity of the individual oils mostly displayed moderate activity (Table 2). The MIC values for *A. afra* ranged between 2.6 and 9.3 mg/ml, depending on the pathogen studied. The MIC values of *E. globulus* and *O. asteriscoides* essential oils ranged from 1.3 to 8.0 mg/ml and 0.6 to 8.0 mg/ml respectively. *A. betulina* displayed poor antimicrobial activity (MIC 6.0–16.0 mg/ml) activity. *C. neoformans* was the most sensitive pathogen against all tested oils.

Table 2
MIC (mg/ml) values of *A. afra*, *A. betulina*, *O. asteriscoides* and *E. globulus* against four pathogens.

Essential oil plant sample	Test micro-organism			
	M. catarrhalis ATCC 23246	*K. pneumoniae* NCTC 9633	*E. faecalis* ATCC 29212	*C. neoformans* ATCC 90112
A. afra	8.0	9.3	8.7	2.6
A. betulina	8.0	16.0	8.0	6.0
E. globulus	8.0	8.0	8.0	1.3
O. asteriscoides	8.0	8.0	8.0	0.6
Controls ciprofloxacin/amphotericin B	6.3 E-03	2.3 E-04	6.3 E-04	3.1 E-03

The antimicrobial activity of *A. afra* essential oils has previously been reported (Graven et al., 1992; Gundidza, 1993; Mangena and Muyima, 1999; Huffman et al., 2002; Scott et al., 2004; Van Vuuren and Viljoen, 2006; Viljoen et al., 2006a; Van Vuuren, 2007; Liu et al., 2009). Many of these studies only reported results obtained using the disc diffusion assay. This, together with chemotypic variation previously noted for *A. afra* (Viljoen et al., 2006a) makes it difficult to compare results. However, the results are mostly congruent with our previous studies in which we used the same chemotype (Van Vuuren and Viljoen, 2006; Viljoen et al., 2006a; Van Vuuren, 2007). The antimicrobial activity of *A. betulina* essential oil has been reported by Lis-Balchin et al. (2001), Viljoen et al. (2006b) and Moolla and Viljoen (2008). Where comparable, the results confer favourably with previous studies. *E. globulus* essential oil has been widely studied and said to have a broad spectrum of antibacterial and antifungal activity

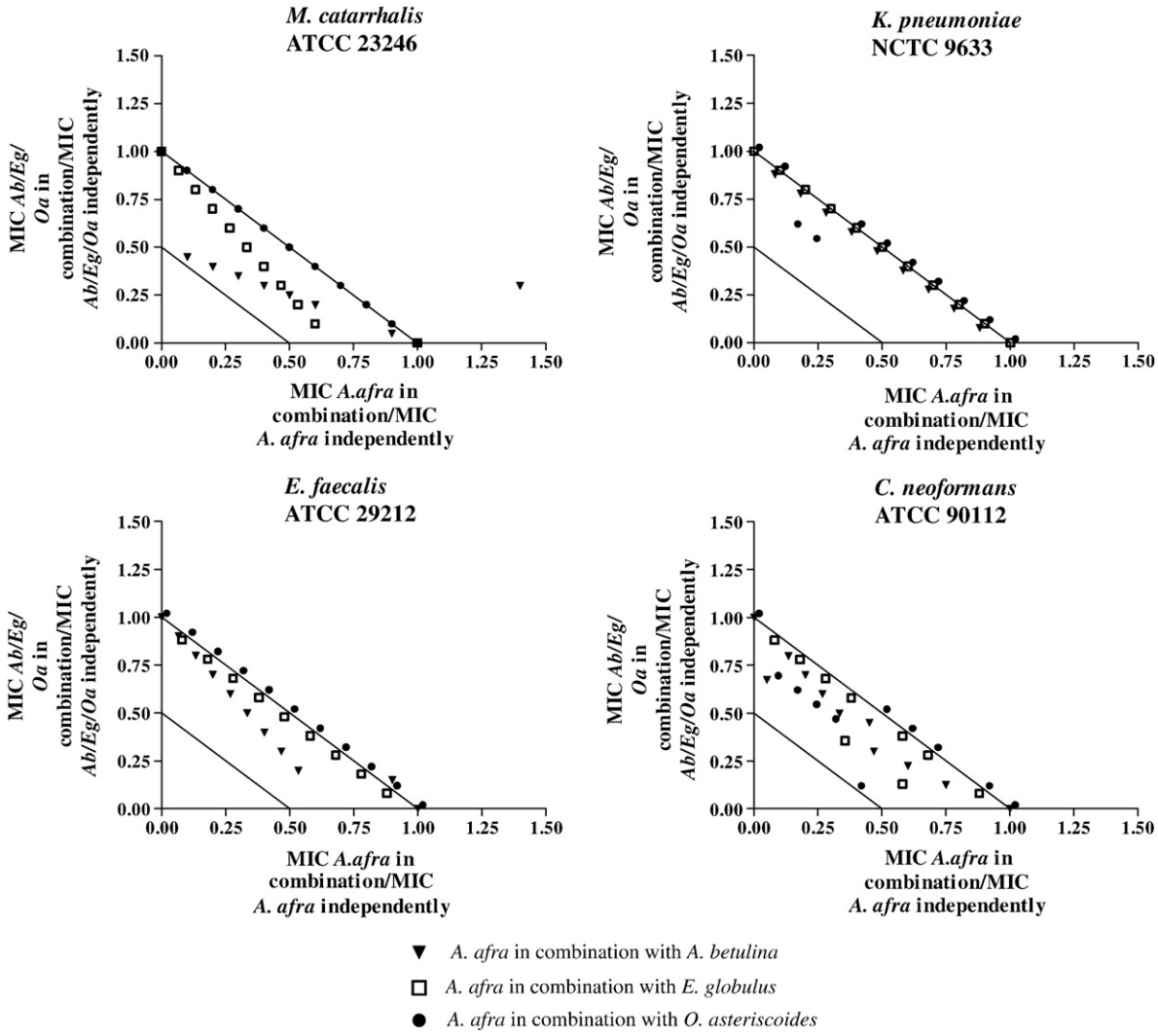

Fig. 1. Isobologram representation of the combinations of *A. afra* with *A. betulina*, *A. afra* with *E. globulus,* and *A. afra* with *O. asteriscoides* against four pathogens.

(Zakarya et al., 1993; Cimanga et al., 2002; Sartorelli et al., 2007; Ghalem and Mohamed, 2008a,b). A recent study indicated potent antimicrobial activity (51.4–85.6 μg/ml) against five pathogens including methicillin-resistant *Staphylococcus aureus* (Tohidpour et al., 2010). The MIC values for *E. globulus* reported here were modest in comparison. The antimicrobial activity of *O. asteriscoides* is well known, even though different methodologies were used to determine antimicrobial activity (Huffman et al., 2002; Viljoen et al., 2003; Scott et al., 2004).

The essential oil compositions for these species have previously been reported and are thus not elaborated on here (Viljoen et al., 2003, 2006a,b; Maciel et al., 2010).

3.2. Fractional inhibitory concentration (FIC) and isobologram construction

The MIC results for all combinations of *A. afra* with *E. globulus*, *A. afra* with *O. asteriscoides* as well the combination of *A. afra* with *A. betulina,* against the four pathogens are shown as isobolograms (Fig. 1). The calculated FIC data used to plot the ratios are presented in Table 3. All positive and negative controls were within acceptable ranges and responded in accordance with the test procedures. With the combination of *A. afra* and *A. betulina* (Fig. 1), predominantly additive interactions were noted for all pathogens studied. No antagonism was observed and only three combinations (8:2 and 7:3 ratios with *M. catarrhalis* and 9:1 with *E. faecalis* where *A. afra* was dominant) displayed non-interactive antimicrobial interactions. The *A. afra*:*A. betulina* interaction displaying the most prominent additive interaction was observed with the pathogen *M. catarrhalis* where close to synergistic interactions (0.6) were observed for two ratios (2:8 and 1:9, where *A. betulina* essential oil was dominant).

For the combination of *A. afra* with *E. globulus* (Fig. 1; Table 3), additive interactions at FIC value 1.0 were mostly noted for all ratios against all the pathogens studied. It is only with the pathogen *M. catarrhalis* that lower additive values (0.7–0.9) were observed. As with the *A. afra*:*A. betulina* combination, no antagonism was observed. Furthermore, non-interactive combinations were not evident.

When *A. afra* was combined with *O. asteriscoides*, additive interactions with FIC values of 1.0 were evident in all ratios for *M. catarrhalis, K. pneumoniae* and *E. faecalis.* Two exceptions were noted for the pathogen *K. pneumoniae* where ratios 3:7 and 2:8 (*O. asteriscoides* is dominant) showed minor variations (0.8) of additivity. The most pronounced synergistic combination of all *A. afra* combinations tested in this study was the 8:2 ratio (8 parts *A. afra* with 2 parts *O. asteriscoides*) against *C. neoformans* displaying an FIC value of 0.5.

It is well known that medicinal plants have been used since antiquity to treat common infectious diseases and it has long been acknowledged that essential oils exhibit antimicrobial properties. Moreover, the use in combination to potentiate antimicrobial activity has been addressed in a number of studies. A disc diffusion study carried out by Geda (1995) on various essential oil combinations commonly used in India revealed synergistic interactions for selected combinations. Other studies

Table 3
FIC values of *A. afra*, *A. betulina*, *O. asteriscoides*, and *E. globulus* against four pathogens at various ratios.

Ratios[a]		Pathogens											
		M. catarrhalis ATCC 23246			*K. pneumoniae* NCTC 9633			*E. faecalis* ATCC 29212			*C. neoformans* ATCC 90112		
A	B	*A. afra*[A] and *A. betulina*[B]	*A. afra*[A] and *E. globulus*[B]	*A. afra*[A] and *O. asteriscoides*[B]	*A. afra*[A] and *A. betulina*[B]	*A. afra*[A] and *E. globulus*[B]	*A. afra*[A] and *O. asteriscoides*[B]	*A. afra*[A] and *A. betulina*[B]	*A. afra*[A] and *E. globulus*[B]	*A. afra*[A] and *O. asteriscoides*[B]	*A. afra*[A] and *A. betulina*[B]	*A. afra*[A] and *E. globulus*[B]	*A. afra*[A] and *O. asteriscoides*[B]
10	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
9	1	1.0	0.7	1.0	1.0	1.0	1.0	1.1	1.0	1.0	0.9	1.0	1.0
8	2	1.8	0.7	1.0	1.0	1.0	1.0	0.7	1.0	1.0	0.8	0.8	0.5
7	3	1.7	0.8	1.0	1.0	1.0	1.0	0.8	1.0	1.0	0.8	1.0	1.0
6	4	0.8	0.8	1.0	1.0	1.0	1.0	0.8	1.0	1.0	0.9	1.0	1.0
5	5	0.8	0.8	1.0	1.0	1.0	1.0	0.8	1.0	1.0	0.8	0.8	1.0
4	6	0.7	0.9	1.0	1.0	1.0	1.0	0.9	1.0	1.0	0.9	1.0	0.8
3	7	0.7	0.9	1.0	1.0	1.0	0.8	0.9	1.0	1.0	0.9	1.0	0.8
2	8	0.6	0.9	1.0	1.0	1.0	0.8	0.9	1.0	1.0	0.9	1.0	0.8
1	9	0.6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.7	1.0	0.8
0	10	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[a] Where Ratio A represent parts of *A. afra* and B represents parts of either *A. betulina, E. globulus* or *O. asteriscoides*. Interpretative values: synergistic (≤0.5), additive (>0.5–1.0), no interaction (>1.0–≤4.0) or antagonistic (>4.0).

include the combination of *Syzygium aromaticum* L. (clove) and *Rosmarinus officinalis* L. (rosemary) essential oils, where time-kill methodology was used to demonstrate additive, synergistic and antagonistic effects depending on the micro-organism tested (Fu et al., 2007). A combination of *Thymus vulgaris* L. (thyme) and *Pimpinella anisum* L. (anise) essential oil using broth micro-dilution methods reported varying efficacies depending on pathogen tested (Al-Bayati, 2008).

Even though a number of interactive studies have been undertaken, few have incorporated isobolograms, which have been used to describe synergy since 1978 (Berenbaum, 1978). Only recently, have studies incorporated isobologram representation for plant-based interactions (Kamatou et al., 2006; Iten et al., 2009; Van Vuuren and Viljoen 2009; Van Vuuren et al., 2009). Other than Kamatou et al. (2006) wherein *Salvia chamelaeagnea* and *Leonotis leonurus* was combined and tested for interactive antimicrobial principles, and the combination of *A. afra* with *Lippia javanica* essential oils by Van Vuuren (2007), no other studies have been undertaken on combinations of aromatic South African medicinal plants.

Although the mechanism of action of plant combinations have not been adequately explored, there is the thought that when used in combination, plants may provide many more compounds that act at multiple target sites and therefore act synergistically to increase effectiveness (Al-Bayati, 2008; Biavatti, 2009). One must also take cognisance of the traditional method of administration for many indigenous aromatic medicinal plants, which is the burning of plant material whereby the volatile components are released and inhaled. The mode of administration allows biologically active compounds to diffuse through the alveoli of the lungs and into the bloodstream (Mohagheghzadeh et al., 2006; Braithwaite and Viljoen, 2008). Furthermore, the lungs (which are predominantly affected in respiratory infections) are then the first target for therapy. The predominant additive interactions noted in the combination of *A. afra* with *A. betulina*, *E. globulus* or *O. asteriscoides* (Table 3; Fig. 1) lend credibility to their traditional use as a combined treatment against respiratory infections. Although only one synergistic interaction was observed, it was interesting to note that no antagonism and very little non-interaction was evident, making these combinations a viable option to explore further. Whether the aromatic plant combinations studied here provide an additive effect when administered clinically, should be investigated further.

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