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Interactive antimicrobial and toxicity profiles of conventional antimicrobials with Southern African medicinal plants

Z. Hübsch [a], R.L. Van Zyl [a], I.E. Cock [b,c], S.F. Van Vuuren [a,*]

[a] Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown 2193, South Africa
[b] Environmental Futures Centre, Nathan Campus Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, Australia
[c] Biomolecular and Physical Sciences, Nathan Campus Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, Australia

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ABSTRACT

Medicinal plant use plays an important role in the healthcare of many South Africans. Furthermore, in orthodox medicine, conventional antimicrobial agents are amongst the most commonly prescribed groups of drugs. Therefore, due to the prevalence of use of these two forms of healthcare, there is a high probability for their concurrent use. Thus, the aim of this study was to evaluate the interactive antimicrobial and toxicity profiles of six Southern African medicinal plants (*Agathosma betulina*, *Aloe ferox*, *Artemisia afra*, *Lippia javanica*, *Pelargonium sidoides* and *Sutherlandia frutescens*) when combined with seven conventional antimicrobials (ciprofloxacin, erythromycin, gentamicin, penicillin G, tetracycline, amphotericin B and nystatin). Antimicrobial activity was assessed using the minimum inhibitory concentration (MIC) assay against a range of pathogens and interactions were further classified using the sum of the fractional inhibitory concentration (∑FIC). Notable synergistic or antagonistic interactions were studied at various ratios (isobolograms). The toxicity of the individual samples, as well as the notable combinations, was assessed using the brine-shrimp lethality assay (BSLA) and the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on the HEK-293 human cell line. Of the 420 antimicrobial: plant combinations studied, 14.29% showed synergistic interactions, 7.56% antagonistic, 35.71% additive and 42.44% indifferent interactions. Some notable synergistic interactions (ciprofloxacin with *A. betulina* and *S. frutescens* against *Escherichia coli*) and antagonistic interactions (ciprofloxacin with *A. afra* organic extract against *Escherichia coli*) were identified. None of the notable combinations were found to show toxicity in the BSLA or MTT assay. In conclusion, the majority of combinations were found to have no notable interaction, alleviating some concern related to the concurrent use of these two forms of healthcare.

1. Introduction

Medicinal plants have been used for centuries as a source of medicine. The global importance of medicinal plants can be illustrated by the numerous conventional drugs that have been derived from plants and are currently used in clinical practice. Some examples of these drugs are quinine, atropine, opioids and taxol. In Africa, traditionally used medicinal plants play a vital role in the cultural heritage of the local people, with an estimated 60% of the population consulting traditional healers (Chinyama, 2009; Van Wyk et al., 2009). Approximately 3000 plants are used in traditional healing practices in South Africa by an estimated 200,000 traditional healers (Van Wyk et al., 2009). Popular Southern African medicinal plants, such as *Agathosma betulina*, *Aloe ferox*, *Artemisia afra*, *Lippia javanica*, *Pelargonium sidoides* and *Sutherlandia frutescens*, have been studied for their medicinal, antimicrobial and toxic properties (Table 1).

Interest in medicinal plant research has escalated, with the aim of identifying alternative antimicrobial therapies to overcome resistance (Aiyegoro and Okoh, 2009). There is, however, general consensus amongst the various studies, that plant derived antimicrobials possess a lower potency than conventional antimicrobials (Van Vuuren and Viljoen, 2011). Furthermore, antimicrobial resistance against conventional antimicrobials has been on the rise and has become a major public health concern. This has propelled research in the direction of combination therapies for enhanced efficacy. Many researchers have studied antimicrobial interactions between natural products, as well as combinations of natural products with conventional therapies. Websites now exist that are dedicated to herb–drug interactions (www.prescribeguide.com). Combinations of agents with antimicrobial properties that have already been investigated include combinations of various essential oils (Van Vuuren and Viljoen, 2006; Suliman et al., 2010) and conventional antimicrobial combinations with non-conventional antibiotics, such as anaesthetics (Gunics et al., 2000). Several studies investigating natural product combinations with conventional antimicrobials have already been conducted (Betoni et al., 2006; Rosato et al., 2007, 2008, 2009; D'Arrigo et al., 2010; Jarrar et al., 2010;

* Corresponding author. Tel.:+27 11 7172157; fax: +27 11 6424355.
E-mail address: Sandy.vanvuuren@wits.ac.za (S.F. Van Vuuren).

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Table 1
Medicinal plants investigated, with their traditional uses, evidence of toxicity and antimicrobial activity.

Plant, family and common names	Part used/mode of administration	Traditional medicinal uses	Known toxicity	Known antimicrobial activity[a]	References
Agathosma betulina (Berg.) Pillans, Rutaceae, buchu (Khoi, English), boegoe (Afrikaans), ibuchu (Xhosa).	Decoction or alcoholic tincture from leaves for gastrointestinal complaints. Infusions prepared from leaves ingested for kidney troubles. Buchu vinegar applied topically.	Kidney and urinary tract infections (UTI's), wounds, boils, rash, burns, gastrointestinal complaints, antibiotic protection of corpses.	No toxic effect on kidney cells (IC_{50} > 100 μg/ml). Allergic reactions have occurred.	Very weak activity against *E. coli, S. aureus, B. cereus, E. faecalis, K. pneumoniae, P. aeruginosa* and *C. neoformans.*	Watt and Breyer-Brandwijk (1962), Hutchings et al. (1996), Lis-Balchin et al. (2001), Moolla (2005), Moolla and Viljoen (2008), Van Wyk et al. (2009), Suliman et al. (2010), Van Wyk (2011).
Aloe ferox Mill., Asphodelaceae, bitteraalwyn, Kaapse aalwyn (Afrikaans), bitter aloe (English), umhlaba (Xhosa, Zulu, Sotho).	Fresh juice from leaves or decoctions and powders from leaves or roots applied topically or sniffed.	Ophthalmic inflammation, sexually transmitted infections, wounds, burns, sinusitis, conjunctivitis.	Joint weakness, partial paralysis, effects similar to curare poisoning, overdoses lead to nephritis, gastritis and pelvic congestion. No cytotoxicity at low doses.	Moderate to very weak activity against *C. albicans, Neisseria gonorrhoeae* and *Herpes simplex.*	Watt and Breyer-Brandwijk, 1962, Hutchings et al. (1996), Kambizi et al. (2007), Kambizi and Afolayan (2008), Van Wyk et al. (2009), Van Wyk (2011), Wintola et al. (2011).
Artemisia afra Jacq. ex. Willd., Asteraceae, umhlonyane (Xhosa, Zulu), lengana (Sotho, Tswana), als, alsem, wildeals (Afrikaans), african wormwood (English).	Infusion or decoction from leaves or roots for ingestion, poultice of leaves for topical application. Fumes from boiled leaves for inhalation.	Respiratory infections (coughs, colds, pneumonia, croup, whooping cough), gastrointestinal complaints, malaria, intestinal worms, boils.	Pulmonary oedema, haemorrhagic nephritis, degenerative liver changes, central nervous system effects due to thujone (hallucinations, confusion).	Moderate to very weak activity against *B. cereus, E. faecalis, S. aureus, E. coli, K. pneumoniae, C. albicans P. aeruginosa*, and *C. neoformans.*	Watt and Breyer-Brandwijk (1962), Hutchings et al. (1996), Huffman et al. (2002), Van Vuuren and Viljoen (2006), Mukinda and Syce (2007), Van Wyk et al. (2009), Suliman et al. (2010), Van Wyk (2011).
Lippia javanica (Burm. F.) Spreng., Verbenaceae, musukudu, bokhukhwane (Tswana), inzinziniba (Xhosa), umsuzwane (Zulu), mumara (Shona), fever tea (English), koorsbossie (Afrikaans).	Weak infusions prepared from leaves, twigs and roots made with milk or water, smoke inhalation or the direct application of leaves.	Respiratory infections (coughs, colds, bronchitis, influenza), skin infections, gastrointestinal complaints, malaria, measles, rashes, disinfecting anthrax-infected meat.	Photosensitivity but no other evidence of toxicity.	Moderate to very weak activity against *S. aureus, B. cereus, E. faecalis, E. coli, K. pneumoniae, C. albicans, P. aeruginosa* and *C. neoformans.*	Watt and Breyer-Brandwijk (1962), Hutchings et al. (1996), Huffman et al. (2002), Van Vuuren and Viljoen (2006), Van Wyk et al. (2009), Van Wyk (2011).
Pelargonium sidoides DC., Geraniaceae, umckaloabo (Zulu), silverleaf geranium (English), kalwerbossie (Afrikaans).	Root decoction or infusion made with milk or water for ingestion and topical application. Root can be chewed or powdered for ingestion with food.	Respiratory infections (bronchitis, sinusitis, influenza, pneumonia), sexually transmitted infections, gastrointestinal complaints, wounds.	Hepatotoxicity reports caused by *P. sidoides* ruled out.	Moderate to very weak activity against *Mycobacterium tuberculosis, S. aureus, S. pneumoniae, E. coli, K. pneumoniae, P. aeruginosa* and *Haemophilus influenzae.*	Watt and Breyer-Brandwijk (1962), Hutchings et al. (1996), Mukinda and Syce (2007), Van Wyk et al. (2009), Kolodziej (2011), Van Wyk (2011), Teschke et al. (2012).
Sutherlandia frutescens (L.) R. Br., Fabaceae, kankerbos (Afrikaans), cancer bush (English).	Strong decoctions or alcoholic tinctures made from leaves for internal or external use.	Respiratory infections (chronic bronchitis, colds, influenza), UTI's, wounds, gastrointestinal complaints, internal cancer and septicaemia.	No toxic effects on liver, kidney, muscles, lungs, bone and biochemical parameters found in mice given enormous doses. Considered safe due to long history of use in South Africa without reports of any toxicity. No toxic effects in healthy adults.	Moderate to very weak activity against *S. aureus* and other Staphylococcal spp., *E. faecalis* and *E. coli.*	Watt and Breyer-Brandwijk (1962), Hutchings et al. (1996), Seier et al. (2002), Katerere and Eloff (2005), Fu et al. (2008), Van Wyk et al. (2009), Van Wyk (2011).

[a] Moderate antimicrobial activity = MIC of 1.00–3.00 mg/ml; very weak antimicrobial activity = MIC of ≥8.00 mg/ml.

Van Vuuren and Viljoen, 2011). Most of these studies focus on antibiotic combinations with common herbs such as *Rosmarinus officinalis*, *Origanum vulgare*, *Thymus vulgaris*, *Mentha piperita* and *Melaleuca alternifolia*. Some combination studies of natural products and conventional antimicrobials have also focused on the isolation of phytochemicals, such as phenols, tannins and flavonoids and evaluating these effects on antimicrobials, with many synergistic interactions having been identified (Sibanda and Okoh, 2007; Hemaiswarya et al., 2008; Jayaraman et al., 2010; Palaniappan and Holley, 2010). Various plants have been found to be synergistic enhancers for conventional antimicrobials, even if the plants do not possess antimicrobial activity themselves (Aiyegoro and Okoh, 2009). Adwan et al. (2010), as well as Van Vuuren and Viljoen (2011), highlight that the potentiating effect of plants on conventional antimicrobials has been neglected and this aspect requires further investigation.

Not only is it important to investigate these combinations to identify possible alternatives to overcome resistance, but combination studies also provide valuable information for use in the clinical setting, where natural product–drug interactions can occur. Many people in Southern Africa use both traditional and conventional medications concurrently (Van Wyk et al., 2009) without knowledge of the potential interactions which may occur. The lack of knowledge of interactions between natural products and conventional drugs, as well as the lack of reporting of natural product or traditional medicinal use to healthcare professionals can pose a serious risk to patient safety (Butterweck and Derendoff, 2012; Vieira and Huang, 2012). It has been acknowledged that even in some of the finest hospitals, traditional medicine is found to be used by patients in conjunction with conventional therapies [personal communication, Dr. Motlalepula Matsabisa, Director IKS Health Unit, Medical Research Council (MRC)]. The practice of combining traditional or natural products with conventional medicine has been found prevalent not only in Southern Africa, but also globally. In a study conducted in Western countries in 2005, 12.1–18.6% of the population indicated herbal drug use concomitantly with prescription drugs reaching 16% (Tindle et al., 2005; Singh and Levine, 2006). In Canada, 9–23.2% of the population indicated herbal drug use, with 5.3% confirming concurrent use with prescription drugs (Singh and Levine, 2006). A national survey performed in the United States of America, indicated that 72% of patients using herbal remedies were found to be additionally using prescription drugs. Furthermore, 84% of patients reported using over-the-counter medication in combination with natural products. Some patients preferentially combined these two forms of healthcare, with the belief that there would be an enhanced effect (Maizes and Dog, 2010).

There have been many instances where natural products have been used concurrently with conventional medicine and severe reactions have resulted. Well characterised interactions have been summarised by Vickers et al. (2001), where it was reported that several traditional/conventional medicine interactions are not yet well defined and it has been recommended that if patients are taking conventional medication, that traditional remedies should be used with caution. There is a misconception amongst many people that natural products are safe. Natural products still have the potential for severe interactions and many are not devoid of toxicity (Hermann and Von Richter, 2012; Markowitz and Zhu, 2012). With most of the studies to date focusing only on testing antimicrobial activity of the combinations, the identification of possible toxic effects of these combinations have been neglected. Many studies have, however, acknowledged the need for toxicological screening, not only of the individual plants, but also on combinations (Fennell et al., 2004; Adwan et al., 2009). Some medicinal plants at higher than therapeutic doses are toxic, as demonstrated in a review by Fennell et al. (2004). However, this toxicity is usually only evident when the medication is consumed in large quantities or for prolonged periods. Existing evidence of toxicity of the plants investigated in this current study has been summarised in Table 1.

In spite of the extensive studies that have already been reported on the interactions between natural products and conventional antimicrobials, no studies could be found that provides information of possible interactions of commercially relevant, Southern African medicinal plants in combination with conventional antimicrobials. This cannot be ignored, hence a comprehensive investigation of these interactions is warranted. Interactive profiles could have a considerable effect on conventional treatment regimens, particularly since most patients do not report traditional medicine use to healthcare providers. Therefore, the purpose of this study was to evaluate the interactive antimicrobial and toxicity profiles, when six Southern African medicinal plants were combined with seven conventional antimicrobial agents. The plants used in this investigation (Table 1) are all included in the 350 species classified as the most commonly used and traded medicinal plants in South Africa (Van Wyk et al., 2009) and are also in the top 16 plants classified as most commercially relevant in a review by Van Wyk (2011).

2. Material and methods

2.1. Sourcing and preparation of plant samples

A. betulina (batch VV 01/13/02/12) was purchased from the commercial trader, S. Chicken Naturals, Cape Town. *A. ferox* (voucher SVV-173) and *A. afra* (voucher SVV-172) were collected from the Walter Sisulu National Botanical Gardens, Gauteng. These plants were identified and harvested under the guidance of Andrew Hankey, Associate Curator, South African National Biodiversity Institute. *L. javanica* (voucher SVV-174) was identified and collected by Assoc. Prof. S.F. Van Vuuren from the wild population in Fairlands, Johannesburg. *P. sidoides* (batch 0212105) and *S. frutescens* (batch 0312010) were purchased from Parceval (Pty) Ltd. Pharmaceuticals, Cape Town. Certificates of analysis were received from Parceval (Pty) Ltd. Pharmaceuticals for these two plants, providing proof of purity. All plant harvesting occurred during the warm summer months and the plant material was received at the University of the Witwatersrand in March 2012. The plant parts analysed in this study were selected to be most closely related to the parts traditionally used.

Plant material was left to dry at room temperature for approximately seven days until completely dry, after which, it was ground into a fine powder using the high speed Fritsch Pulverisette grinder (Labotec). For organic extracts, the dried, macerated plant material was submerged in a mixture of dichloromethane and methanol (1:1) for 24 h at 37 °C in a shaker/incubator (Labcon). Thereafter, the liquid was filtered and the filtrate left in open glass bottles, under a fume hood, for the complete evaporation of solvent, leaving behind the solid extract. Aqueous extracts were prepared by submerging the macerated plant material in sterile distilled water for 24 h at 25 °C in a shaker/incubator. The liquid was then filtered and the filtrate stored at −80 °C before lyophilisation (Virtis). Aqueous extracts were left under ultra-violet light overnight to ensure the elimination of any microbial contamination. Extracts were stored in sealed sterile bottles, at room temperature and protected from light, until further analysis.

Essential oils from the aromatic plants (*A. afra*, *A. betulina* and *L. javanica*) were hydro-distilled, using a Clevenger-type apparatus. Round bottom flasks, with a 5 l capacity, were packed tightly with fresh, aerial plant material and approximately 800 ml of distilled water was added to each flask. The condensed essential oils were collected in amber, glass vials (Macherey-Nagel) to prevent evaporation, and stored at 4 °C until further analysis (Van Vuuren, 2007).

2.2. Toxicity studies

2.2.1. Brine-shrimp lethality assay

Artificial salt water was prepared by dissolving 32 g of Tropic Marine® Sea Salt in 1 l of distilled water, of which 500 ml was added to a bottomless, inverted plastic bottle. Dried, brine-shrimp (*Artemia*

franciscana) eggs (Ocean Nutrition™) were weighed out (0.5 g) and added to the salt water. To ensure a high hatch rate, a rotary pump was used to aerate the water and disperse the eggs, and the eggs exposed to a concentrated source of light from a lamp (220–240 V). The eggs were incubated under these conditions for 18–24 h, at ambient temperature. A volume of 400 µl salt water containing on average 40–60 live brine-shrimp was added to each well of a 48 well micro-titre plate. Thereafter, 400 µl sample (plant samples, antimicrobials or a combination of both, all diluted in distilled water or 1% dimethyl sulphoxide (DMSO) for organic extracts and essential oils) was added to triplicate wells. All samples were tested for toxicity at a concentration of 1 mg/ml, since a concentration above 1 mg/ml not resulting in brine-shrimp death was considered non-toxic for the assay (Bussmann et al., 2011). The negative control consisted of 32 g/l salt water and the positive control consisted of 1.6 mg/ml potassium dichromate (Fluka). The plates were observed under a light microscope (Olympus) (40× magnification) immediately after sample addition (at time 0) for any dead brine-shrimp, which would be excluded from percentage mortality calculations. Dead brine-shrimp were then counted after 24 and 48 h. Thereafter, a lethal dose of 50 µl of glacial acetic acid (100% v/v; Saarchem) was added to each well and a total dead brine-shrimp count undertaken. The percentage mortality was then calculated (Cock and Kalt, 2010). Samples providing a percentage mortality greater than 50% were considered toxic (Bussmann et al., 2011). These samples were then tested at concentrations of 1, 0.5, 0.25, 0.125, 0.063 and 0.031 mg/ml to obtain a log-sigmoid dose response curve, generated with GraphPad Prism® software (Version 5), from which the LC_{50} values were determined. The LC_{50} value represented the concentration of a test substance necessary to have a lethal effect on 50% of the brine-shrimp.

2.2.2. MTT cell proliferation assay

The human kidney epithelial (Graham or HEK-293) cells were cultured in Dulbecco's Modified Eagles Medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS) (Thermo Scientific), 1% non-essential amino acids (Sigma-Aldrich) and 1% penicillin/streptomycin/fungizone mixture (10,000 U penicillin/ml, 10,000 µg streptomycin/ml and 25 µg fungizone/ml) (Sigma-Aldrich). The cell line was maintained at 37 °C with 5% CO_2, in accordance with the methods by Mosmann (1983) and Van Zyl et al. (2006). A waiver for the use of the human kidney epithelial (Graham) cell line was obtained from the University of the Witwatersrand Human Research Ethics Committee (Reference W-CJ-120309-3).

For experimental purposes, once confluency of the cells had been achieved, the trypsinised cells were re-suspended to a cell density of 0.5 million cells/ml. A volume of 180 µl of cell suspension was added to each well of a sterile micro-titre plate before being incubated at 37 °C for 6 h in a humidified environment with 5% CO_2. Samples were screened at 100 µg/ml, in triplicate, per plate and all samples tested in at least two independent experiments. A colour control for each sample (absent of cell suspension) was included, along with two wells of a 0% cell control (sample-free) and 14 wells of 100% cell suspension control (sample-free). Quinine and camptothecin (100 µg/ml and 1 mg/ml; Sigma-Aldrich) were included as the positive controls. The prepared plates were incubated at 37 °C for 44 h. At which time, a washing step was undertaken using PBS (pH 7.2), to ensure no interference by the plant sample colour with the MTT absorbance readings and to minimize any interaction with the MTT. Thereafter, 40 µl MTT solution (Sigma-Aldrich; 5 mg/ml) was added to each well and incubated for a further 4 h. DMSO was then added to each well to stop the reaction and to dissolve the formazan crystals.

The absorbance of the dissolved crystals was read using the Labsystems iEMS MF reader, at a test wavelength of 540 nm and reference wavelength of 690 nm. Percentage cellular viability was then calculated using the following equation, where "Abs" signifies absorbance, and all absorbance values used in the calculation were derived from deducting the absorbance value at 690 nm from the absorbance value at 540 nm ($Abs_{540} - Abs_{690}$) (Kamatou, 2006):

$$\%\text{Cell viability} = \frac{\text{Abs test sample} - (\text{Mean Abs control} - \text{Mean Abs blank}) \times 100}{(\text{Mean Abs control} - \text{Mean Abs blank})}.$$

2.3. Antimicrobial analysis

2.3.1. Minimum inhibitory concentration assays

Based on their prevalence to cause nosocomial infections, the following micro-organisms were studied; three Gram-positive bacteria, *Staphylococcus aureus* (American Type Culture Collection (ATCC) 25923), *Enterococcus faecalis* (ATCC 29212) and *Bacillus cereus* (ATCC 11778), three Gram-negative bacteria; *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27858), along with two yeasts; *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 14116). All micro-organisms were cultured in Tryptone Soya broth (TSB) (Oxoid) and kept viable by sub-culturing. Streak plates were prepared to ensure the purity of the culture, as well as for isolation of pure colonies for sub-culturing. The bacteria were incubated at 37 °C for 24 h and the yeasts at 25 °C for 48 h. A waiver for the use of these micro-organisms was obtained from the University of the Witwatersrand Human Research Ethics Committee (Reference W-CJ-130726-1).

The conventional antimicrobials included erythromycin (potency of ≥850 µg/mg), gentamicin (potency of 600 µg/mg), nystatin (potency of ≥4400 United States Pharmacopeia (USP) units/mg), penicillin G (potency of 1440–1680 units/mg), tetracycline [≥95% High Performance Liquid Chromatography (HPLC)], ciprofloxacin (≥98% HPLC) and amphotericin B (80% HPLC), which were all purchased from Sigma-Aldrich (South Africa). The antibiotics were prepared in sterile distilled water, to a concentration of 0.01 mg/ml and the antifungals were prepared to 0.1 mg/ml. Amphotericin B was initially solubilized in 1% (v/v) DMSO before further additions of sterile water.

The MIC assay was used to evaluate the antimicrobial activity of the plant samples and the conventional antimicrobials independently, followed by evaluation in combination. The guidelines for the micro-titre plate method, to determine the antibacterial activity of plant samples were in accordance with methods by Eloff (1998). The Clinical and Laboratory Standards Institute (CLSI) guidelines (2012) were followed when analysing the conventional antimicrobials.

Each well of the micro-titre plate was filled with 100 µl of sterilized distilled water. The individual plant samples and conventional antimicrobials were then introduced into the wells of the first row, as 100 µl for individual samples or 50 µl of each agent in the double combination. Plant samples were introduced at a starting concentration of 32 mg/ml in acetone (organic extracts) or sterile water (aqueous extracts). The conventional antimicrobials were introduced at a starting concentration of 0.01 mg/ml for antibiotics and 0.1 mg/ml for antifungal agents. The positive control, to ensure antimicrobial susceptibility of pathogens, consisted of 0.1 mg/ml amphotericin B or 0.01 mg/ml ciprofloxacin for yeasts and bacteria, respectively. A solvent control comprising of the acetone solvent whereby sterile water (used to replace sample) was diluted to 32 mg/ml was tested to ensure that the solvent used did not itself exhibit antimicrobial activity. A negative control was included (comprising of media and test organism), to ensure that the media was capable of supporting microbial growth. After the addition of the samples to the plate, the serial doubling dilution method was employed. The prepared micro-titre plates were then inoculated with the relevant pathogen, with each inoculum having a size of approximately 1×10^6 colony forming units (CFU)/ml. Plates, sealed with a sterile adhesive sealer, were then incubated at 25 °C for 48 h and 37 °C for 24 h for yeasts and bacteria, respectively.

After incubation, 40 µl of the colour indicator, 0.40 mg/ml ρ-iodonitrotetrazolium violet (INT; Sigma-Aldrich), was added to each

well, which turned purple-pink in the presence of microbial growth. The end point MIC value was then taken as the lowest concentration of test sample that resulted in the inhibition of growth, which was seen by the absence of the purple-pink colour of the indicator. All samples and their combinations were tested in at least duplicate. Extracts and essential oils were considered to exhibit noteworthy antimicrobial activity for MIC values <1 mg/ml and ≤2 mg/ml, respectively (Duarte et al., 2005; Rios and Recio, 2005; Van Vuuren, 2008).

2.3.2. Fractional inhibitory concentration (FIC) assessment

Interactions between the combinations of plant samples and conventional antimicrobials were further classified using the sum of the fractional inhibitory concentration (∑FIC). The FIC was calculated using the following equation, where (a) represents the plant sample and (b) the conventional antimicrobial sample (Van Vuuren and Viljoen, 2011);

$$FIC^{(i)} = \frac{MIC(a)\,\text{in combination with}\,(b)}{MIC(a)\,\text{independently}}$$

$$FIC^{(ii)} = \frac{MIC(b)\,\text{in combination with}\,(a)}{MIC(b)\,\text{independently}}.$$

The ∑FIC was then calculated using the equation: $\sum FIC = FIC^{(i)} + FIC^{(ii)}$. The interactions were classified as being synergistic for ∑FIC values of ≤0.5, additive (>0.5–1.0), indifferent (>1.0–≤4.0) or antagonistic (>4.0) (Van Vuuren and Viljoen, 2011). Tentative interpretations were included where the MIC value were greater than the highest concentration tested to provide an estimation of what the possible interactive profile for the combination could have been. These interpretations were not given a ∑FIC value, as only absolute values could be used in ∑FIC calculations.

2.3.3. Varied ratio combination studies (isobolograms)

For notable synergistic or antagonistic interactions, nine different ratios of the combination were prepared and the MIC values determined. The samples were combined at fixed concentrations of 0.01 or 0.1 mg/ml for antibiotics or antifungals, respectively, and 32 mg/ml for the plant sample, at various volume ratios (antimicrobial:plant), resulting in varied concentrations for each ratio (Table 2). Data points for each ratio studied were plotted on an isobologram using the GraphPad Prism® software (Version 5). The construction of isobolograms allowed for the identification of the agent (plant or antimicrobial sample) most responsible for the synergistic or antagonistic effects within the combination. Data points falling below and including the 0.5:0.5 line indicated synergy, while those above the 0.5:0.5 line, up to and including the 1.0:1.0 line indicated an additive interaction. Data points above the 1.0:1.0 line, up to and including the 4.0:4.0 line indicated a non-interactive or indifferent interaction and data points falling above the 4.0:4.0 line indicated antagonism (Van Vuuren and Viljoen, 2011).

3. Results and discussion

The percentage yield for each extract, as well as each essential oil was calculated and has been recorded in Table 3.

3.1. Toxicity studies

Two assays, namely the BSLA and the MTT assay were used to assess the toxicity of the individual samples and eight notable combinations (essential oil, aqueous and organic extracts of *A. betulina* and *A. afra* in combination with ciprofloxacin, and the aqueous and organic extracts of *S. frutescens* with ciprofloxacin), identified in the antimicrobial studies. The BSLA was undertaken for the preliminary toxicity screening; however, the MTT assay provided a cellular evaluation of toxicity.

3.1.1. Brine-shrimp lethality assay

All plant samples (extracts and oils) and antimicrobials were individually screened at 1 mg/ml. The extracts were only considered toxic if they induced percentage mortalities greater than 50% (LC_{50}) (Bussmann et al., 2011). Three individual plant samples were found to show toxicity, namely *A. betulina* essential oil, and the organic extracts of *L. javanica* and *S. frutescens*, demonstrating a percentage mortality of 100% (LC_{50}: 0.31 ± 0.03 mg/ml), 70.13 ± 5.29% (LC_{50}: 0.51 ± 0.03 mg/ml) and 82.69 ± 4.51% (LC_{50}: 0.45 ± 0.05 mg/ml), respectively. When tested individually, the antimicrobials demonstrated no toxicity in the BSLA (Table 4).

3.1.2. MTT cell proliferation assay

The plant samples and conventional antimicrobials, all individually screened at 100 μg/ml, demonstrated no toxicity toward the human kidney epithelial cells, however, two of the essential oils (*A. betulina* and *A. afra*) demonstrated a potential for toxicity with a cellular viability of 64.10 ± 6.29% and 68.28 ± 4.64%, respectively (Table 4).

3.2. Antimicrobial studies

The MIC results for the antimicrobial studies undertaken on the individual samples have been recorded in Tables 5.1 and 5.2, respectively. All conventional antimicrobials fell within the break point expectation ranges (Andrews, 2004; CLSI, 2012), except for tetracycline against *E. faecalis* and *P. aeruginosa*, where a reduced susceptibility was noted, possibly due to emerging resistance by the strain tested. The individual plant samples demonstrated mostly weak antimicrobial activity, which is in accordance with the literature (Table 1). *L. javanica* demonstrated the best activity, where noteworthy susceptibility was observed against six of the eight tested pathogens. The organic extract of *L. javanica* also

Table 2
The concentration ratios used for antimicrobial and plant sample combination studies.

Volume ratio of antimicrobial: plant sample (μl)	Concentration of antibacterial[a] in combination (μg/ml)	Concentration of antifungal[b] in combination (μg/ml)	Concentration of plant sample[c] in combination (mg/ml)
90:10	9.00	90.00	3.20
80:20	8.00	80.00	6.40
70:30	7.00	70.00	9.60
60:40	6.00	60.00	12.80
50:50	5.00	50.00	16.00
40:60	4.00	40.00	19.20
30:70	3.00	30.00	22.40
20:80	2.00	20.00	25.60
10:90	1.00	10.00	28.80

[a] Ciprofloxacin/erythromycin/gentamicin/penicillin G/tetracycline.
[b] Amphotericin B/nystatin.
[c] Samples include all the essential oils, organic and aqueous extracts of the plants indicating notable interactions with conventional antimicrobials (results of which have been provided in the form of isobolograms).

Table 3
Percentage yield values for all the plant samples investigated.

Plant	Plant part used in analysis	Percentage yield (% w/w)		
		Essential oil	Aqueous extract	Organic extract
Agathosma betulina	Leaves	1.54	1.43	4.80
Aloe ferox	Leaves	NA	4.14	2.99
Artemisia afra	Leaves and twigs	0.32	9.91	8.16
Lippia javanica	Leaves	0.69	8.31	11.16
Pelargonium sidoides	Roots (tubers)	NA	7.84	3.18
Sutherlandia frutescens	Leaves	NA	11.42	5.89

NA = plant not aromatic in nature and hence no essential oil could be distilled, or in the case of the aromatic plant, *P. sidoides*, an insufficient quantity of essential oil could be obtained from the roots.

demonstrated the lowest MIC of 0.25 mg/ml against *S. aureus*, compared to the other tested plant samples (Table 5.2).

A total of 420 conventional antimicrobial:medicinal plant combinations were tested for interactive antimicrobial activity (Tables 6.1, 6.2 and 6.3). Of the 420 combinations, 14.29% were synergistic, 7.56% antagonistic, 35.71% additive and 42.44% were indifferent or non-interactive in nature. A few notable synergistic and antagonistic interactions were identified in this current study, such as the combinations of ciprofloxacin with *A. betulina* (essential oil, aqueous and organic extracts), *A. afra* (essential oil and organic extract) and *S. frutescens* (organic extract) showing synergistic interactions against *E. coli*. In contrast, *A. afra* aqueous extract demonstrated a significant antagonistic interaction with ciprofloxacin against *E. coli*.

3.3. Notable combinations

3.3.1. Ciprofloxacin in combination with A. betulina

The combination of *A. betulina* with ciprofloxacin provided a notable interactive profile, when tested against *E. coli*, which is most commonly the cause of UTI's. In orthodox medicine, fluoroquinolones, such as ciprofloxacin, have been used in the treatment of UTI's for many years (Merck Manual, 2006; SAMF, 2012). In traditional medicine, *A. betulina* is very often ingested orally, as an aqueous infusion or alcoholic tincture, for the treatment of UTI's (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; Van Wyk et al., 2009). The aqueous extract of *A. betulina* showed a promising synergistic effect in combination with ciprofloxacin, when tested against *E. coli*. The MIC values of the aqueous extract (90 μg/ml) and ciprofloxacin (0.03 μg/ml) in combination (Table 6.2) were well below the MIC values for the agents when tested individually (≥8.00 mg/ml for the aqueous extract and 0.08 μg/ml for ciprofloxacin) (Tables 5.2 and 5.1, respectively), thereby demonstrating a tentative ∑FIC interpretation of synergy.

When the organic extract of *A. betulina* was combined with ciprofloxacin and tested against *E. coli*, a tentative ∑FIC interpretation of synergy was also identified. As observed with the aqueous extract: ciprofloxacin combination, the MIC values for the agents in combination (Table 6.2) were well below the MIC values of the agents when tested individually (Tables 5.1 and 5.2), thereby demonstrating a synergistic interaction. The essential oil of *A. betulina* in combination with ciprofloxacin, when tested against *E. coli*, demonstrated a tentative

Table 4
Mortality (%) and cell death (%) results for samples tested individually in the BSLA and MTT assay, respectively (n = 6).

Sample		Mortality ± S.D. (%)[a]		Cell death ± S.D. (%)[b]
		After 24 h:	After 48 h:	After 48 h:
Antimicrobials	Ciprofloxacin	0.00	0.00	0.10 ± 0.01
	Erythromycin	0.00	0.00	0.10 ± 0.01
	Gentamicin	1.12 ± 0.58	8.99 ± 0.33	0.10 ± 0.01
	Penicillin G	0.00	0.00	0.10 ± 0.01
	Tetracycline	0.00	6.67 ± 1.16	0.10 ± 0.01
	Amphotericin B	0.00	0.00	5.93 ± 3.41
	Nystatin	0.00	0.00	0.10 ± 0.01
Essential oils	*A. betulina*	100.00 ± 0.00	100.00 ± 0.00	35.90 ± 6.29
	A. afra	0.00	1.39 ± 0.58	31.72 ± 4.64
	L. javanica	0.58 ± 0.52	1.17 ± 0.71	0.10 ± 0.01
Aqueous extracts	*A. betulina*	0.00	0.00	0.10 ± 0.01
	A. ferox	0.00	0.00	0.10 ± 0.01
	A. afra	0.00	0.00	0.10 ± 0.01
	L. javanica	0.00	1.43 ± 0.58	0.10 ± 0.01
	P. sidoides	0.00	3.45 ± 0.58	0.10 ± 0.01
	S. frutescens	0.00	0.00	0.10 ± 0.01
Organic extracts	*A. betulina*	0.00	0.00	0.10 ± 0.01
	A. ferox	0.00	0.00	0.10 ± 0.01
	A. afra	0.00	0.00	0.10 ± 0.01
	L. javanica	0.00	70.13 ± 5.29	0.10 ± 0.01
	P. sidoides	0.00	0.00	0.10 ± 0.01
	S. frutescens	13.46 ± 0.58	82.69 ± 4.51	0.10 ± 0.01
Controls	Quinine	0.00[a,b]	0.00[a]	71.38 ± 4.73[a]
			11.76 ± 1.00[b]	0.10 ± 0.01[b]
	Camptothecin	0.00[a]	2.08 ± 0.58[a]	76.07 ± 2.94[a]
		30.00 ± 2.00[b]	100.00 ± 0.00[b]	0.10 ± 0.01[b]
	Potassium dichromate	100.00 ± 0.00[c]		NT

S.D. = standard deviation; NT = control not tested in the assay. Cell death (%) = 100 − cell viability (%).
[a] Tested at a concentration of 1 mg/ml.
[b] Tested at a concentration of 100 μg/ml.
[c] Tested at a concentration of 1.6 mg/ml.

Table 5.1
Minimum inhibitory concentration (MIC) values (μg/ml) for all conventional antimicrobials, when tested individually.

Test organism	Antibiotics					Antifungals	
	Cip	Ery	Gen	Pen	Tet	Amp	Nys
S. aureus (ATCC 25923)	0.47	0.31	1.88	≥2.50	0.23	NT	NT
B. cereus (ATCC 11778)	0.63	0.31	≥2.50	≥2.50	0.16	NT	NT
E. faecalis (ATCC 29212)	1.25	1.25	≥2.50	≥2.50	≥2.50	NT	NT
E. coli (ATCC 25922)	0.08	NA	≥2.50	NT	1.25	NT	NT
K. pneumoniae (ATCC 13883)	0.63	NA	≥2.50	NT	1.25	NT	NT
P. aeruginosa (ATCC 27853)	0.16	NA	0.31	NT	≥2.50	NT	NT
C. albicans (ATCC 10231)	NT	NT	NT	NT	NT	1.56	2.34
C. neoformans (ATCC 14116)	NT	NT	NT	NT	NT	0.39	1.56

Cip = ciprofloxacin; Ery = erythromycin; Gen = gentamicin; Pen = penicillin G; Tet = tetracycline; Amp = amphotericin B; Nys = nystatin; NT = micro-organism is not susceptible to the antimicrobial; ≥2.50 = antimicrobial samples were not tested at higher concentrations for the determination of a MIC value.

synergistic interaction (Table 6.2); however, this interaction would not be relevant for the treatment of urinary tract infections, since the essential oil is not used traditionally in this manner of oral ingestion.

Since the combination between *A. betulina* (essential oil, aqueous and organic extracts) and ciprofloxacin against *E. coli* provided such a notable synergistic profile, the combinations were tested at varying ratios. Most ratios were found in the synergistic or additive region of Fig. 1, with only four ciprofloxacin: *A. betulina* ratios (9:1; 8:2; 7:3 and 3:7) of the organic extract and one ratio (7:3) (refer to Table 2, for ratio concentrations) of the essential oil, indicating an indifferent interaction. The identified synergistic interactions could possibly lead to more effective treatment of UTI's and reverse the resistance of *E. coli* toward ciprofloxacin, however, further *in vivo* testing would be warranted to support such claims.

When the combinations of ciprofloxacin with *A. betulina* (essential oil, aqueous and organic extracts) were tested for toxicity, none of the combinations were found to show toxicity, with a 0.00% mortality and cell viability no less than 100% in the BSLA and MTT assays, respectively.

3.3.2. Ciprofloxacin in combination with A. afra

The organism, *E. coli*, is commonly responsible for infectious gastrointestinal complaints, which could arise from eating contaminated food or drinking contaminated water. In rural areas, these complaints are often treated with the medicinal plant, *A. afra*, in comparison to fluoroquinolone or ciprofloxacin usage, in orthodox medicine (Merck Manual, 2006; SAMF, 2012). The essential oil and organic extract of *A. afra* in combination with ciprofloxacin displayed synergistic interactions against *E. coli* (∑FIC of 0.27 for both combinations) (Table 6.2). In contrast, the aqueous extract combination demonstrated an antagonistic interaction with ciprofloxacin against *E. coli* (∑FIC of 8.55) (Table 6.2). *A. afra* is most commonly consumed orally as an aqueous infusion (herbal tea) for the treatment of gastrointestinal complaints and hence the antagonistic interaction noted here may warrant caution and require further pharmacokinetic studies to further investigate the mechanism of the interaction.

The combination of essential oil, aqueous or organic extract with ciprofloxacin, was tested in varied ratios against *E. coli*, since these combinations showed variance in interactive profiles, ranging from synergistic to highly antagonistic interactions. In the varied ratio studies (Fig. 2), the ∑FIC evaluation of antagonism for the aqueous extract combination (Table 5.2) was supported by the ratio containing the equal volumes (5:5). Similarly, the ∑FIC evaluation of synergy for the organic extract combination, as well as the essential oil combination was supported in the varied ratio study (Fig. 2). Even though the ratio containing equal volumes of each agent for this combination was found to be synergistic, some ratios were found in the antagonistic region for both the organic extract and essential oil combinations when combined with ciprofloxacin (Fig. 2). Therefore, combinations of

Table 5.2
Minimum inhibitory concentration (MIC) values (mg/ml) for all plant samples, when tested individually.

Sample	*S. aureus* (ATCC 25923)			*B. cereus* (ATCC 11778)			*E. faecalis* (ATCC 29212)			*E. coli* (ATCC 25922)			*K. pneumoniae* (ATCC 13883)			*P. aeruginosa* (ATCC 27853)			*C. albicans* (ATCC 10231)			*C. neoformans* (ATCC 14116)		
	Aq	Org	EO	Aq	Org	EO	Aq	Org	EO	Aq	Org	EO	Aq	Org	EO	Aq	Org	EO	Aa	Org	EO	Aq	Org	EO
A. betulina	≥8.00	2.00	**2.00**	≥8.00	**0.75**	**0.63**	≥8.00	2.00	≥8.00	≥8.00	≥8.00	≥8.00	≥8.00	≥8.00	≥8.00	≥8.00	4.00	4.00	6.00	3.00	**2.00**	3.00	**0.75**	**0.75**
A. ferox	≥8.00	4.00	NA	≥8.00	3.00	NA	≥8.00	≥8.00	NA	≥8.00	≥8.00	NA	≥8.00	≥8.00	NA	6.00	6.00	NA	≥8.00	2.00	NA	≥8.00	≥8.00	NA
A. afra	2.00	**0.50**	**2.00**	≥8.00	**0.38**	**2.00**	≥8.00	2.00	≥8.00	3.00	3.00	3.00	4.00	2.00	≥8.00	2.00	1.50	4.00	4.00	1.50	**1.00**	1.00	**0.75**	**0.75**
L. javanica	4.00	**0.25**	**1.50**	≥8.00	≥8.00	**1.50**	≥8.00	1.00	3.00	2.00	1.00	**2.00**	≥8.00	1.00	3.00	2.00	4.00	**2.00**	**0.75**	1.00	**1.50**	1.00	**0.38**	**0.38**
P. sidoides	2.00	1.50	NA	2.00	1.50	NA	1.00	2.00	NA	≥8.00	≥8.00	NA	≥8.00	≥8.00	NA	2.00	1.50	NA	1.50	2.00	NA	1.00	1.50	NA
S. frutescens	≥8.00	2.00	NA	≥8.00	**0.75**	NA	≥8.00	4.00	NA	≥8.00	2.00	NA	≥8.00	≥8.00	NA	≥8.00	4.00	NA	>8.00	3.00	NA	≥8.00	1.00	NA
Ciprofloxacin	0.00047			0.00063			0.00125			0.00008			0.00063			0.00016			NA			NA		
Amphotericin B	NA			NA			NA			NA			NA			NA			0.00156			0.00039		
Solvent or media	≥8.00			≥8.00			≥8.00			≥8.00			≥8.00			≥8.00			≥8.00			≥8.00		

≥8.00 = samples were not tested further at higher concentrations for the determination of an MIC; Aq = Aqueous extract; Org = Organic extract; EO = Essential oil; NA = Not applicable as no essential oil tested or micro-organism is not susceptible to the antimicrobial; **bold highlight** = noteworthy antimicrobial activity.

Table 6.1
MIC (μg/ml) and ∑FIC values for the plant: antibiotic combinations, against the Gram-positive bacterial strains.

Combination	*S. aureus* (ATCC 25923)						*B. cereus* (ATCC 11778)						*E. faecalis* (ATCC 29212)					
	Aq AM	∑FIC (Int.)	Org AM	∑FIC (Int.)	EO AM	∑FIC (Int.)	Aq AM	∑FIC (Int.)	Org AM	∑FIC (Int.)	EO AM	∑FIC (Int.)	Aq AM	∑FIC (Int.)	Org AM	∑FIC (Int.)	EO AM	∑FIC (Int.)
A. betulina + ciprofloxacin	2000	1.59	2000	2.34	1000	1.18	1000	T	500	0.92	1000	2.10	3000	T	2000	1.50	1500	T
	0.63	(IND)	0.63	(IND)	0.32	(IND)	0.32	(ADD)	0.16	(ADD)	0.32	(IND)	0.94	(IND)	0.63	(IND)	0.47	(ADD)
A. betulina + erythromycin	1000	1.16	2000	3.03	1000	1.53	1000	T	500	1.19	380	0.99	3000	T	≥4000	T	2000	T
	0.32	(IND)	0.63	(IND)	0.32	(IND)	0.32	(IND)	0.16	(IND)	0.12	(ADD)	0.94	(IND)	≥1.25	(IND)	0.63	(ADD)
A. betulina + gentamicin	≥4000	1.17	1500	1.00	1000	0.67	2000	T	2000	T	≥4000	T	≥4000	T	1000	T	≥4000	T
	≥1.25	(IND)	0.47	(ADD)	0.32	(ADD)	0.63	(*SYN*)	0.63	(*SYN*)	≥1.25	(ADD)	≥1.25	(ADD)	0.32	(ADD)	≥1.25	(ADD)
A. betulina + penicillin G	≥4000	T	2000	1.25	1000	0.63	≥4000	T	1000	1.46	750	1.29	≥4000	T	1500	0.94	≥4000	T
	≥1.25	(ADD)	0.63	(IND)	0.32	(ADD)	≥1.25	(ADD)	0.32	(IND)	0.23	(IND)	≥1.25	(ADD)	0.47	(ADD)	≥1.25	(ADD)
A. betulina + tetracycline	500	0.76	500	0.95	500	0.95	190	T	190	0.63	130	**0.46**	≥4000	T	1000	T	≥4000	T
	0.16	(ADD)	0.16	(ADD)	0.16	(ADD)	0.12	(ADD)	0.06	(ADD)	0.04	(*SYN*)	≥1.25	(ADD)	0.32	(ADD)	≥1.25	(ADD)
A. ferox + ciprofloxacin	≥4000	T	1000	0.93	NA		≥4000	T	2000	1.67	NA		2000	T	2000	T	NA	
	≥1.25	(IND)	0.32	(ADD)			≥1.25	(IND)	0.63	(IND)			0.63	(ADD)	0.63	(ADD)		
A. ferox + erythromycin	1000	T	1000	1.28	NA		500	T	500	**0.34**	NA		2000	T	2000	T	NA	
	0.32	(IND)	0.32	(IND)			0.16	(ADD)	0.08	(*SYN*)			0.63	(ADD)	0.63	(ADD)		
A. ferox + gentamicin	≥4000	T	1500	1.00	NA		3000	T	≥4000	T	NA		≥4000	T	≥4000	T	NA	
	≥1.25	(ADD)	0.47	(ADD)			0.94	(ADD)	≥1.25	(ADD)			≥1.25	(ADD)	≥1.25	(ADD)		
A. ferox + penicillin G	2000	T	2000	0.75	NA		2000	T	750	**0.35**	NA		≥4000	T	1500	0.94	NA	
	0.63	(ADD)	0.63	(ADD)			0.63	(ADD)	0.24	(*SYN*)			≥1.25	(ADD)	0.47	(ADD)		
A. ferox + tetracycline	≥4000	T	750	1.23	NA		≥4000	T	190	0.63	NA		≥4000	T	≥4000	T	NA	
	≥1.25	(*ANT*)	0.24	(IND)			≥1.25	(*ANT*)	0.06				≥1.25	(ADD)	≥1.25	(ADD)		
A. afra + ciprofloxacin	≥4000	T	500	1.34	2000	2.34	≥4000	T	500	1.57	1000	1.01	≥4000	T	2000	1.50	2000	T
	≥1.25	(*ANT*)	0.16	(IND)	0.63	(IND)	≥1.25	(IND)	0.16	(IND)	0.32	(IND)	≥1.25	(IND)	0.63	(IND)	0.63	(ADD)
A. afra + erythromycin	1000	1.53	500	1.52	1000	1.53	750	T	250	0.92	500	0.77	≥4000	T	2000	1.50	2000	T
	0.32	(IND)	0.16	(IND)	0.32	(IND)	0.23	(ADD)	0.08	(ADD)	0.16	(ADD)	≥1.25	(IND)	0.63	(IND)	0.63	(ADD)
A. afra + gentamicin	≥4000	T	1000	2.17	1000	0.67	≥4000	T	1000	T	≥4000	T	≥4000	T	1000	T	≥4000	T
	≥1.25	(IND)	0.32	(IND)	0.32	(ADD)	≥1.25	(IND)	0.32	(*SYN*)	≥1.25	(IND)	≥1.25	(ADD)	0.32	(ADD)	≥1.25	(ADD)
A. afra + penicillin G	1500	0.94	2000	*4.25*	2000	1.25	750	T	1500	*4.40*	1000	0.63	1000	T	2000	1.25	2000	T
	0.47	(ADD)	0.63	(*ANT*)	0.63	(IND)	0.24	(*SYN*)	0.47	(*ANT*)	0.32	(ADD)	0.32	(*SYN*)	0.63	(IND)	0.63	(ADD)
A. afra + tetracycline	1000	1.89	500	1.70	500	0.95	190	T	130	0.59	190	**0.48**	≥4000	T	1000	T	≥4000	T
	0.32	(IND)	0.16	(IND)	0.16	(ADD)	0.06	(*SYN*)	0.04	(ADD)	0.06	(*SYN*)	≥1.25	(ADD)	0.32	(ADD)	≥1.25	(ADD)
L. javanica + ciprofloxacin	2000	1.84	100	0.51	500	0.67	2000	T	130	T	500	0.58	≥4000	T	1000	1.26	750	**0.44**
	0.63	(IND)	0.05	(ADD)	0.16	(ADD)	0.63	(IND)	0.04	(*SYN*)	0.16	(ADD)	≥1.25	(IND)	0.32	(IND)	0.24	**(SYN)**
L. javanica + erythromycin	1000	1.28	500	2.52	1000	1.70	500	T	20	T	190	**0.32**	2000	T	250	**0.32**	3000	1.75
	0.32	(IND)	0.16	(IND)	0.32	(IND)	0.16	(ADD)	0.005	(*SYN*)	0.06	(*SYN*)	0.63	(ADD)	0.08	**(SYN)**	0.94	(IND)
L. javanica + gentamicin	≥4000	T	130	0.54	750	0.63	≥4000	T	130	T	≥4000	T	≥4000	T	130	T	2000	T
	≥1.25	(IND)	0.04	(ADD)	0.24	(ADD)	≥1.25	(ADD)	0.04	(*SYN*)	≥1.25	(IND)	≥1.25	(ADD)	0.04	**(SYN)**	0.63	(ADD)
L. javanica + penicillin G	≥4000	T	750	3.09	1000	0.80	2000	T	750	T	1000	0.80	2000	T	2000	2.25	2000	0.92
	≥1.25	(IND)	0.23	(IND)	0.32	(ADD)	0.63	(ADD)	0.24	(*SYN*)	0.32	(ADD)	0.63	(ADD)	0.63	(IND)	0.63	(ADD)
L. javanica + tetracycline	1000	1.64	380	2.04	500	1.03	250	T	20	T	130	**0.34**	≥4000	T	500	T	≥4000	T
	0.32	(IND)	0.12	(IND)	0.16	(IND)	0.08	(ADD)	0.005	(*SYN*)	0.04	(*SYN*)	≥1.25	(ADD)	0.16	(ADD)	≥1.25	(IND)
P. sidoides + ciprofloxacin	750	0.89	750	1.01	NA		500	**0.50**	380	**0.44**	NA		1500	1.88	1000	0.76	NA	
	0.24	(ADD)	0.24	(IND)			0.16	(*SYN*)	0.12	(*SYN*)			0.47	(IND)	0.32	(ADD)		
P. sidoides + erythromycin	500	0.77	500	0.85	NA		259	**0.39**	190	**0.32**	NA		1500	1.88	1500	1.13	NA	
	0.16	(ADD)	0.16	(ADD)			0.08	(*SYN*)	0.06	(*SYN*)			0.47	(IND)	0.47	(IND)		
P. sidoides + gentamicin	500	**0.34**	500	**0.42**	NA		1000	T	1000	T	NA		2000	T	1500	T	NA	
	0.16	(*SYN*)	0.16	(*SYN*)			0.32	(*SYN*)	0.32	(*SYN*)			0.63	(IND)	0.47	(ADD)		
P. sidoides + penicillin G	500	**0.32**	250	**0.20**	NA		2000	1.25	750	0.60	NA		1000	1.13	380	**0.24**	NA	
	0.16	(*SYN*)	0.08	(*SYN*)			0.63	(IND)	0.24	(ADD)			0.32	(IND)	0.12	(*SYN*)		
P. sidoides + tetracycline	250	**0.48**	500	1.03	NA		250	0.63	190	0.51	NA		1000	T	1000	T	NA	
	0.08	(*SYN*)	0.16	(IND)			0.08	(ADD)	0.06	(ADD)			0.32	(IND)	0.32	(ADD)		
S. frutescens + ciprofloxacin	≥4000	T	1000	1.18	NA		1000	T	750	1.38	NA		2000	T	1000	0.51	NA	
	≥1.25	(IND)	0.32	(IND)			0.32	(ADD)	0.24	(IND)			0.63	(ADD)	0.32	(ADD)		

S. frutescens + erythromycin	1500	T	1000	1.53	NA	500	T	250	0.59	NA	2000	T	1000	0.51	NA	
	0.47	(IND)	0.32	(IND)		0.16	(ADD)	0.08	(ADD)		0.63	(ADD)	0.32	(ADD)		
S. frutescens + gentamicin	2000	T	250	**0.17**	NA	≥4000	T	2000	T	NA	≥4000	T	2000	T	NA	
	0.63	(ADD)	0.08	(SYN)		≥1.25	(ADD)	0.63	(IND)		≥1.25	(ADD)	0.63	(ADD)		
S. frutescens + penicillin G	≥4000	T	≥4000	T	NA	3000	T	750	1.10	NA	≥4000	T	1000	**0.38**	NA	
	≥1.25	(ADD)	≥1.25	(IND)		0.94	(ADD)	0.24	(IND)		≥1.25	(ADD)	0.32	(SYN)		
S. frutescens + tetracycline	1000	T	500	0.95	NA	190	T	190	0.63	NA	≥4000	T	2000	T	NA	
	0.32	(IND)	0.16	(ADD)		0.06	(SYN)	0.06	(ADD)		≥1.25	(ADD)	0.63	(ADD)		

Aq = aqueous extract MIC value; Org = organic extract MIC value; EO = essential oil MIC value; AM = antimicrobial MIC value; Int = Interaction; NA = no essential oil tested; T = no absolute value could be calculated and therefore only a tentative interpretation is provided; **SYN** = synergistic interaction; ADD = additive interaction; IND = indifferent interaction; *ANT* = antagonistic interaction.

ciprofloxacin and *A. afra* are highly dose-dependent. Interestingly, the ratio of 3:7 (ciprofloxacin: *A. afra*) was found in the antagonistic region, for all three plant sample types (essential oil, aqueous and organic extracts) prepared from *A. afra* (Fig. 2), thus indicating that a higher concentration of *A. afra* may be responsible for the antagonism noted.

When testing the toxicity of the combination of ciprofloxacin with *A. afra* (essential oil, aqueous and organic extracts), none of the combinations were found to show toxicity in either the BSLA or MTT assay, compared to the positive controls at either 24 or 48 h of exposure. In the BSLA, the essential oil and aqueous extract combination with ciprofloxacin showed a 1.25 ± 1.00% and 0.00% mortality after 48 h, respectively. The organic extract combination with ciprofloxacin demonstrated a 2.13 ± 0.58% mortality within the first 24 h of exposure, with no further death occurring thereafter. These mortality rates were not considered significant enough for a varied ratio toxicity study to be undertaken, since mortality rates were well below 50%.

3.3.3. Ciprofloxacin in combination with S. frutescens

The combination of ciprofloxacin with *S. frutescens* demonstrated a notable synergistic profile against *E. coli* (Table 6.2). As with *A. betulina*, *S. frutescens* is a medicinal plant commonly used in the treatment of UTI's. Therefore it was of interest to observe the potential of this combination (Merck Manual, 2006; SAMF, 2012). Since *S. frutescens* is commonly ingested orally as an alcoholic tincture for the treatment of UTI's, results obtained from the organic extract in combination would most closely depict the possible interactions between ciprofloxacin and *S. frutescens*, when consumed in the traditional form. The organic extract of *S. frutescens* when combined with ciprofloxacin showed a favourable synergistic interaction against *E. coli* ($\sum$FIC of 0.28) (Table 6.2). *S. frutescens* can also be consumed as a herbal tea, therefore the combination with the aqueous extract was also evaluated in varied ratios (Table 5.2).

When examining the various mixtures, most ratios for both the aqueous and organic extract combinations with ciprofloxacin were found in the additive region (Fig. 3). Three ciprofloxacin: *S. frutescens* ratios (6:4; 5:5 and 3:7) (refer to Table 2, for ratio concentrations) for the organic extract combination were found below or on the 0.5:0.5 line, thereby demonstrating a synergistic interaction. Only one ciprofloxacin: *S. frutescens* ratio point (1:9) for the organic extract was found in the indifferent region. Four of the ratios (7:3; 3:7; 2:8; 1:9) for the aqueous extract combination were found in the indifferent region (Fig. 3).

When the combinations of ciprofloxacin and *S. frutescens* (aqueous and organic extracts) were tested for toxicity in the BSLA, none of the combinations were found to show toxicity. The aqueous extract combination showed a 0.00% mortality, however, the organic extract combination demonstrated a 2.55 ± 0.58% and 39.49 ± 2.08% mortality, after 24 and 48 h, respectively. However, the mortalities were still below 50% and therefore not considered toxic in nature for the BSLA. Similarly, in the MTT assay the combinations demonstrated no toxicity compared to the positive controls.

3.4. General discussion of medicinal plant:conventional antimicrobial combinations

A review by Van Vuuren and Viljoen (2011), documented numerous combinations of plants with conventional antimicrobials. A summary of the results for many combination studies were given, where most often, synergy had been reported. In the review, no studies were found where conventional antimicrobials were investigated in combination with the Southern African medicinal plants selected for analysis in this study. This further demonstrates the lack of information pertaining to interactive Southern African medicinal plant:antimicrobial combinations and thus highlights the need for the scientific investigation of these combinations. A previous study was found where *S. frutescens* in combination with antiretroviral medication reduced the efficacy of the antiretroviral drugs (Mills et al., 2005). Fasinu et al. (2013b) also found the potential

Table 6.2
MIC (μg/ml) and ∑FIC values for the plant: antibiotic combinations, against the Gram-negative bacterial strains.

Combination	*E. coli* (ATCC 25922)						*K. pneumoniae* (ATCC 13883)						*P. aeruginosa* (ATCC 27853)					
	Aq AM	∑FIC (Int.)	Org AM	∑FIC (Int.)	EO AM	∑FIC (Int.)	Aq AM	∑FIC (Int.)	Org AM	∑FIC (Int.)	EO AM	∑FIC (Int.)	Aq AM	∑FIC (Int.)	Org AM	∑FIC (Int.)	EO AM	∑FIC (Int.)
A. betulina + ciprofloxacin	90	T	50	T	70	T	1500	T	750	T	190	T	500	T	250	0.56	500	1.13
	0.03	(*SYN*)	0.02	(*SYN*)	0.02	(*SYN*)	0.47	(ADD)	0.23	(*SYN*)	0.06	(*SYN*)	0.16	(IND)	0.08	(ADD)	0.16	(IND)
A. betulina + gentamicin	≥4000	T	≥4000	T	≥4000	T	1000	T	2000	T	≥4000	T	750	T	500	0.65	500	0.65
	≥1.25	(ADD)	≥1.25	(ADD)	≥1.25	(ADD)	0.32	(*SYN*)	0.63	(*SYN*)	≥1.25	(ADD)	0.23	(ADD)	0.16	(ADD)	0.16	(ADD)
A. betulina + tetracycline	≥4000	T	≥4000	T	≥4000	T	≥4000	T	3000	T	≥4000	T	≥4000	T	≥4000	T	≥4000	T
	≥1.25	(IND)	≥1.25	(IND)	≥1.25	(IND)	≥1.25	(IND)	0.94	(IND)	≥1.25	(IND)	≥1.25	(ADD)	≥1.25	(IND)	≥1.25	(IND)
A. ferox + ciprofloxacin	2000	T	50	T	NA		≥4000	T	3000	T	NA		1500	3.19	750	1.57	NA	
	0.63	(*ANT*)	0.02	(*SYN*)			≥1.25	(IND)	0.94	(IND)			0.47	(IND)	0.23	(IND)		
A. ferox + gentamicin	≥4000	T	≥4000	T	NA		3000	T	≥4000	T	NA		≥4000	T	≥4000	T	NA	
	≥1.25	(ADD)	≥1.25	(ADD)			0.94	(ADD)	≥1.25	(ADD)			≥1.25	(*ANT*)	≥1.25	(*ANT*)		
A. ferox + tetracycline	≥4000	T	≥4000	T	NA		≥4000	T	2000	T	NA		2000	T	≥4000	T	NA	
	≥1.25	(IND)	≥1.25	(IND)			≥1.25	(IND)	0.63	(ADD)			0.63	(ADD)	≥1.25	(IND)		
A. afra + ciprofloxacin	2000	*8.55*	70	**0.27**	70	**0.27**	3000	2.24	750	0.75	1000	T	1500	3.69	250	0.67	500	1.13
	0.63	(*ANT*)	0.02	(*SYN*)	0.02	(*SYN*)	0.94	(IND)	0.23	(ADD)	0.32	(ADD)	0.47	(IND)	0.08	(ADD)	0.16	(IND)
A. afra + gentamicin	≥4000	T	2000	0.92	≥4000	T	2000	T	1000	T	≥4000	T	1500	2.27	500	0.85	500	0.65
	≥1.25	(IND)	0.63	(ADD)	≥1.25	(IND)	0.63	(ADD)	0.32	(ADD)	≥1.25	(ADD)	0.47	(IND)	0.16	(ADD)	0.16	(ADD)
A. afra + tetracycline	≥4000	T	≥4000	T	≥4000	T	≥4000	T	3000	2.25	≥4000	T	2000	T	1000	T	≥4000	T
	≥1.25	(IND)	≥1.25	(IND)	≥1.25	(IND)	≥1.25	(IND)	0.94	(IND)	≥1.25	(IND)	0.63	(IND)	0.32	**(SYN)**	≥1.25	(IND)
L. javanica + ciprofloxacin	500	2.25	70	**0.32**	30	**0.14**	3000	T	130	**0.19**	1500	1.25	1000	2.50	250	0.56	750	1.82
	0.16	(IND)	0.02	(*SYN*)	0.01	(*SYN*)	0.94	(ADD)	0.04	(*SYN*)	0.47	(IND)	0.32	(IND)	0.08	(ADD)	0.23	(IND)
L. javanica + gentamicin	≥4000	T	3000	2.38	≥4000	T	≥4000	T	250	T	2000	T	≥4000	T	250	**0.32**	500	0.77
	≥1.25	(IND)	0.94	(IND)	≥1.25	(IND)	≥1.25	(ADD)	0.08	(*SYN*)	0.63	(ADD)	≥1.25	(*ANT*)	0.08	(*SYN*)	0.16	(ADD)
L. javanica + tetracycline	≥4000	T	2000	2.50	2000	1.50	3000	T	1500	1.88	≥4000	T	3000	T	500	T	1500	T
	≥1.25	(IND)	0.63	(IND)	0.63	(IND)	0.94	(IND)	0.47	(IND)	≥1.25	(IND)	0.94	(IND)	0.16	(*SYN*)	0.47	(ADD)
P. sidoides + ciprofloxacin	1000	T	50	T	NA		3000	T	2000	T	NA		≥4000	T	≥4000	T	NA	
	0.32	(*ANT*)	0.02	(*SYN*)			0.94	(ADD)	0.63	(IND)			≥1.25	(*ANT*)	≥1.25	(*ANT*)		
P. sidoides + gentamicin	≥4000	T	≥4000	T	NA		1000	T	500	T	NA		≥4000	T	≥4000	T	NA	
	≥1.25	(ADD)	≥1.25	(ADD)			0.32	(*SYN*)	0.16	(*SYN*)			≥1.25	(*ANT*)	≥1.25	(*ANT*)		
P. sidoides + tetracycline	≥4000	T	≥4000	T	NA		≥4000	T	≥4000	T	NA		1000	T	1500	T	NA	
	≥1.25	(IND)	≥1.25	(IND)			≥1.25	(IND)	≥1.25	(IND)			0.32	(ADD)	0.47	(IND)		
S. frutescens + ciprofloxacin	500	T	50	**0.28**	NA		≥4000	T	2000	T	NA		2000	T	250	0.56	NA	
	0.16	(IND)	0.02	(*SYN*)			≥1.25	(IND)	0.63	(IND)			0.63	(*ANT*)	0.08	(ADD)		
S. frutescens + gentamicin	≥4000	T	≥4000	T	NA		≥4000	T	≥4000	T	NA		≥4000	T	380	0.84	NA	
	≥1.25	(ADD)	≥1.25	(IND)			≥1.25	(ADD)	≥1.25	(ADD)			≥1.25	(*ANT*)	0.23	(ADD)		
S. frutescens + tetracycline	≥4000	T	3000	2.25	NA		≥4000	T	3000	T	NA		≥4000	T	1500	T	NA	
	≥1.25	(IND)	0.94	(IND)			≥1.25	(IND)	0.94	(IND)			≥1.25	(ADD)	0.47	(ADD)		

Aq = aqueous extract MIC value; Org = organic extract MIC value; EO = essential oil MIC value; AM = antimicrobial MIC value; Int. = Interaction; NA = no essential oil tested; T = no absolute value could be calculated and therefore only a tentative interpretation is provided; **SYN** = synergistic interaction; ADD = additive interaction; IND = indifferent interaction; *ANT* = antagonistic interaction.

Table 6.3
MIC (μg/ml) and ∑FIC values for the plant: antifungal combinations, against the yeasts.

Combination	*C. albicans* (ATCC 10231)						*C. neoformans* (ATCC 14116)					
	Aq AM	∑FIC (Int.)	Org AM	∑FIC (Int.)	EO AM	∑FIC (Int.)	Aq AM	∑FIC (Int.)	Org AM	∑FIC (Int.)	EO AM	∑FIC (Int.)
A. betulina + amphotericin B	≥4000	T	1500	3.51	2000	*5.01*	130	1.04	250	2.33	130	1.17
	≥12.50	(*ANT*)	4.69	(IND)	6.25	(*ANT*)	0.39	(IND)	0.78	(IND)	0.39	(IND)
A. betulina + nystatin	380	0.56	1000	1.67	1000	1.84	380	0.88	500	1.67	380	1.26
	1.17	(ADD)	3.13	(IND)	3.13	(IND)	1.17	(ADD)	1.56	(IND)	1.17	(IND)
A. ferox + amphotericin B	3000	T	1500	3.51	NA		≥4000	T	130	1.02	NA	
	9.38	(*ANT*)	4.69	(IND)			≥12.50	(*ANT*)	0.39	(IND)		
A. ferox + nystatin	≥4000	T	1000	1.84	NA		≥4000	T	250	0.53	NA	
	≥12.50	(*ANT*)	3.13	(IND)			≥12.50	(*ANT*)	0.78	(ADD)		
A. afra + amphotericin B	3000	*6.76*	2000	*5.34*	1500	*4.51*	190	1.69	190	1.75	250	2.33
	9.38	(*ANT*)	6.25	(*ANT*)	4.69	(*ANT*)	0.59	(IND)	0.59	(IND)	0.78	(IND)
A. afra + nystatin	750	1.19	380	0.75	1000	2.34	190	0.57	500	1.67	500	1.67
	2.35	(IND)	1.17	(ADD)	3.13	(IND)	0.59	(ADD)	1.56	(IND)	1.56	(IND)
A. linearis + amphotericin B	≥4000	T	1500	3.51	NA		750	T	130	1.09	NA	
	≥12.50	(*ANT*)	4.69	(IND)			2.35	(*ANT*)	0.39	(IND)		
A. linearis + nystatin	1500	T	1000	1.67	NA		1500	T	190	0.51	NA	
	4.69	(IND)	3.13	(IND)			4.70	(IND)	0.59	(ADD)		
L. javanica + amphotericin B	500	1.67	750	2.25	2000	*5.34*	130	1.13	190	2.00	100	1.02
	1.56	(IND)	2.35	(IND)	6.25	(*ANT*)	0.39	(IND)	0.59	(IND)	0.30	(IND)
L. javanica + nystatin	190	**0.50**	380	0.88	2000	4.00	250	0.75	190	0.88	250	1.16
	0.59	**(SYN)**	1.17	(ADD)	6.25	(IND)	0.78	(ADD)	0.59	(ADD)	0.78	(IND)
P. sidoides + amphotericin B	750	2.00	1500	3.76	NA		130	1.13	190	1.63	NA	
	2.34	(IND)	4.69	(IND)			0.39	(IND)	0.59	(IND)		
P. sidoides + nystatin	500	1.00	750	1.38	NA		250	0.75	190	0.51	NA	
	1.56	(ADD)	2.35	(IND)			0.78	(ADD)	0.59	(ADD)		
S. frutescens + amphotericin B	≥4000	T	100	**0.22**	NA		≥4000	T	750	*6.76*	NA	
	≥12.50	(*ANT*)	0.30	(*SYN*)			≥12.50	(*ANT*)	2.35	(*ANT*)		
S. frutescens + nystatin	≥4000	T	1000	1.67	NA		≥4000	T	500	1.50	NA	
	≥12.50	(*ANT*)	3.13	(IND)			≥12.50	(*ANT*)	1.56	(IND)		

Aq = aqueous extract MIC value; Org = organic extract MIC value; EO = essential oil MIC value; AM = antimicrobial MIC value; Int. = Interaction; NA = no essential oil tested; T = no absolute value could be calculated and therefore only a tentative interpretation is provided; ***SYN*** = synergistic interaction; ADD = additive interaction; IND = indifferent interaction; *ANT* = antagonistic interaction.

for *S. frutescens* to interact with the conventional drug, midazolam, where it was found that the plant had the ability to delay the production of midazolam metabolites, resulting in a 40% reduction in clearance.

Some other Southern African medicinal plants have demonstrated the potential for interactions with conventional drugs due to their effects on metabolic enzymes. For example, *Hypoxis hemerocallidea* (African potato) has been shown to modulate the CYP3A4 enzyme (Mills et al., 2005). Fasinu et al. (2013a) found that the aqueous extract of *H. hemerocallidea* has the potential to modulate other CYP450 enzymes too. Another Southern African medicinal plant showing interactive potential, is *Harpagophytum procumbens* (devil's claw), which has been found to have an effect on the CYP3A4 enzyme. Instead of the enzyme induction as seen with the previously mentioned examples, devil's claw inhibits the enzyme, thereby resulting in prolonged activity of conventional drugs metabolised by this enzyme, which could result in an increased risk of adverse effects and toxicity. An example is the combination of devil's claw together with warfarin, resulting in purpura (Fugh-Berman, 2000; Van den Bout-Van den Beukel et al., 2006).

Ciprofloxacin was one of the antimicrobials most commonly associated with a positive interactive potential, which was also demonstrated in this current study. Ahmad and Aqil (2006) tested ciprofloxacin in combination with crude extracts of 15 Indian medicinal plants, where the combinations showed synergistic effects when tested against enteric bacteria. Van Vuuren et al. (2009) evaluated the interactions between ciprofloxacin and the essential oils of *M. alternifolia*, *T. vulgaris*, *M. piperita* and *R. officinalis*, using the micro-dilution assay, against various pathogens. In the study, a varied interactive profile was seen, which included synergistic, antagonistic and additive interactions. It was found that the interactions were very much dependant on the ratios in which the agents were combined and ultimately dependent on the final concentrations used. The previous studies show that ciprofloxacin:plant containing combinations mostly demonstrated synergistic profiles, which was also noted in this current study, where ciprofloxacin was found to provide the most notable combinations with the selected medicinal plants.

4. Conclusions

The majority of the conventional antimicrobials in combination with commercially relevant medicinal plants used in Southern Africa

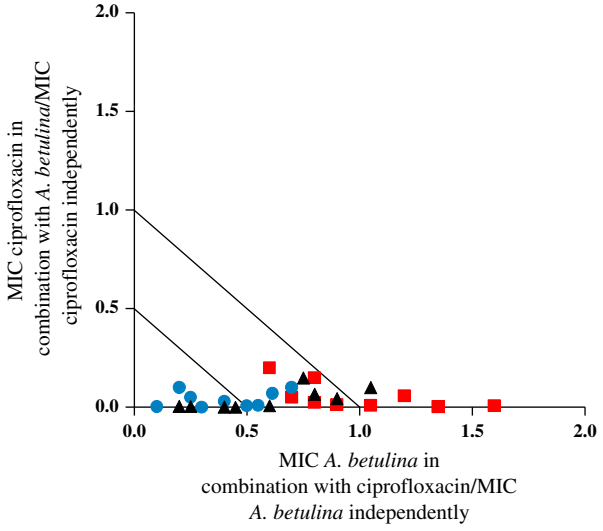

Fig. 1. Isobologram for *Agathosma betulina* (● = aqueous extract; ■ = organic extract; ▲ = essential oil) in combination with ciprofloxacin, when tested at various ratios, against *Escherichia coli*.

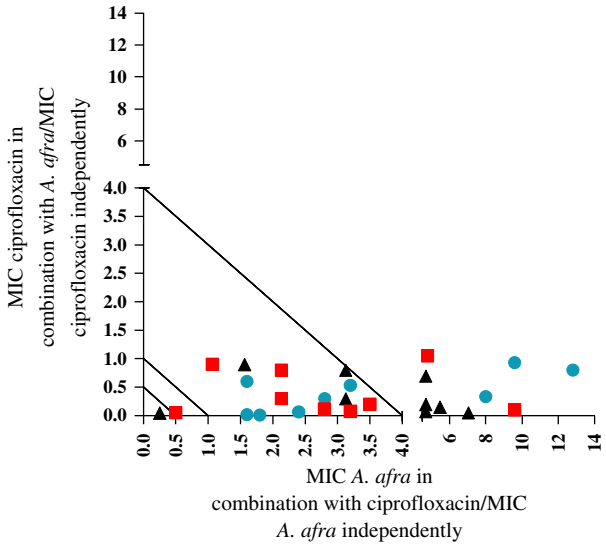

Fig. 2. Isobologram for *Artemisia afra* (● = aqueous extract; ■ = organic extract; ▲ = essential oil) in combination with ciprofloxacin, when tested at various ratios, against *Escherichia coli*.

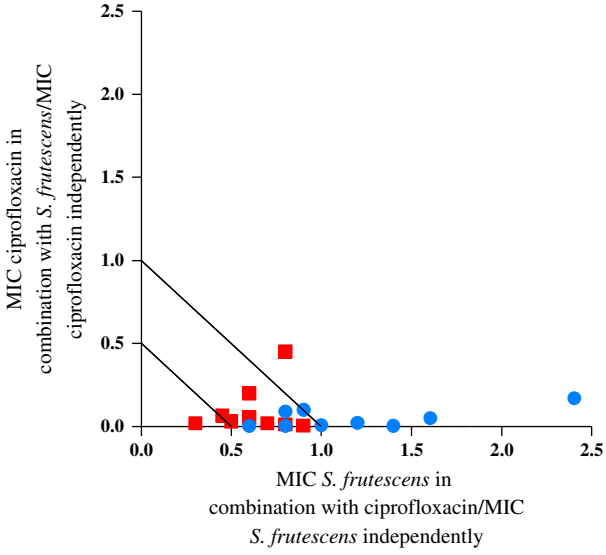

Fig. 3. Isobologram for *Sutherlandia frutescens* (● = aqueous extract; ■ = organic extract) in combination with ciprofloxacin, when tested at various ratios, against *Escherichia coli*.

demonstrated indifferent interactive profiles (42.44%), followed by additive interactions (35.71%); which alleviate some concerns related to concurrent use of the two forms of healthcare, since these interactions are not associated with any advantages or disadvantages. Synergy was seen for 14.29% of the antimicrobial:medicinal plant combinations studied. The implications of a synergistic interaction include enhanced efficacy, thereby allowing lower dose administration, with reduced side effects and possibly reduced antimicrobial resistance (Van Vuuren and Viljoen, 2011). Of the 420 antimicrobial:medicinal plant combinations tested, 7.56% demonstrated antagonistic interactions. The implications of an antagonistic interaction include a reduction in the efficacy of conventional antimicrobials, thereby increasing the burden placed on healthcare systems. In most combination studies found in the literature, synergistic interactions are emphasized, with the reporting of antagonism being neglected. In the current study, a few antagonistic interactions were identified, with the most considerable antagonism seen with the aqueous extract of *A. afra* with ciprofloxacin against *E. coli*, which could have an impact on the treatment of gastrointestinal complaints caused by *E. coli*.

None of the conventional antimicrobials (independently), and none of the notable combinations investigated demonstrated toxicity in the BSLA and MTT assays. However, some of the individual plant samples demonstrated toxicity in the BSLA, with a mortality of 100 ± 0.00%, 70.13 ± 5.29% and 82.69 ± 4.51% for *A. betulina* (essential oil), *L. javanica* (organic extract) and *S. frutescens* (organic extract), respectively. In the MTT assay, the essential oils of *A. betulina* and *A. afra* demonstrated some toxicity, however, this was not considered significant enough to determine IC_{50} values.

Future recommendations include further *in vivo* investigations for the combinations demonstrating notable synergistic or antagonistic interactions, to support the *in vitro* findings. In addition, studies to determine the possible mechanism of action resulting in the observed interaction are also warranted, as well as the determination of active compounds within plant material responsible for the interactions.

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