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Can rooibos (*Aspalathus linearis*) tea have an effect on conventional antimicrobial therapies?

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ABSTRACT

One of the most commonly consumed and commercially relevant herbal teas in South Africa is rooibos tea, prepared from the plant, *Aspalathus linearis* (Burm. F.) Dahlg. In orthodox medicine, antimicrobial agents are amongst the most commonly prescribed groups of drugs and thus there is a considerable possibility for the concurrent use of these drugs with the highly popular beverage, rooibos tea. Therefore, the aim of this study was to investigate the interactive antimicrobial and toxicity profiles of *A. linearis* (aqueous and organic extract), when combined with seven conventional antimicrobials (ciprofloxacin, erythromycin, gentamicin, penicillin G, tetracycline, amphotericin B and nystatin). The antimicrobial activity of *A. linearis* was evaluated, independently and in combination, using the minimum inhibitory concentration (MIC) assay against two yeasts, three Gram-positive and three Gram-negative bacteria. The interactions were further evaluated using the sum of the fractional inhibitory concentration ($\sum$FIC) assessment. Combinations demonstrating notable synergistic or antagonistic interactions were investigated in various ratios (isobolograms). The toxicity of *A. linearis* extracts and antimicrobials was assessed independently and in combination, using the brine-shrimp lethality assay (BSLA), and the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay on the human HEK-293 cell line. *A. linearis* (aqueous and organic extract) with penicillin G demonstrated the most notable interactions, when tested against the Gram-positive bacteria, with $\sum$FIC values ranging from 0.01 (synergistic) to 0.94 (additive). Varied ratio studies of this combination were most synergistic against *Staphylococcus aureus*. Four antagonistic combinations were identified against the Gram-negative bacteria and yeasts. In the BSLA, no combinations were identified to be toxic. However, in the MTT assay, two combinations were found to demonstrate a possible toxic effect (*A. linearis* aqueous and organic extract with nystatin), with inhibitory effects of 73.76 ± 3.36% and 56.88 ± 6.61%, respectively, thus warranting further *in vivo* studies.

1. Introduction

South Africa is culturally diverse, with various medicinal systems being practised. Very often, beverages such as wine, beer and tea prepared from plants are consumed not only for their cultural relevance, but for nutritional or medicinal purposes too (Van Wyk and Gericke, 2000). In South Africa, many medicinal plants have been consumed as herbal teas for decades (Watt and Breyer-Brandwijk, 1932; Van Wyk and Gericke, 2000; McGaw et al., 2007; Bhat and Moskovitz, 2009; Van Wyk et al., 2009). Some plants from which herbal teas are prepared include *Leyssera gnaphalioides* (Hongertee), *Plecostachys serpyllifolia* (Hottentots tee), and *Viscum capense* (Cape Mistletoe), with the more well-known plants such as *Artemisia afra*, *Lippia javanica* and *Sutherlandia frutescens* often being consumed as teas for medicinal purposes. The most commercially relevant teas in South Africa are prepared from plants such as *Athrixia phylicoides* (bush tea or Zulu tea), *Cyclopia* spp. (honeybush tea) and *Aspalathus linearis* (rooibos tea) (Van Wyk and Gericke, 2000). Of these plants, *A. linearis* has been the most successful in commercialisation, with a large international demand (Joubert et al., 2008).

Commercialisation of rooibos started as early as 1904 by Benjamin Ginsberg, where the tea was marketed as the famous South African brand, “Eleven O'Clock”. Mass cultivation, however, only started in the early 1930s. Even though *A. linearis* only occurs naturally in the Cederberg area of the Western Cape, South Africa (Dahlgren, 1968), exporting of the tea to international markets generates millions of rand per year for the country. The international market for rooibos tea far outweighs the local demand, as shown by data compiled by the South African Rooibos Council, where only 18% of the annual yield of rooibos, both fermented and unfermented, produced in 2007, was for

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the South African market. The remaining 82% of the annual production for 2007 was for export to international markets, namely Germany (53%), the Netherlands (11%), United Kingdom (7%), Japan (6%), and the United States of America (5%) (Joubert et al., 2008). It has been estimated that annual yields of rooibos tea, for both the local and the international market range between 4,000,000 kg and 9,000,000 kg (Van Wyk and Gericke, 2000).

Rooibos tea is not only consumed for the enjoyment of its taste and aroma, but also for its medicinal properties. The tea has been found to provide relief for allergies, dermatological problems, asthma, infantile colic and other gastrointestinal complaints, such as nausea and heartburn (Joubert et al., 2008; Van Wyk et al., 2009). It has also been reported that rooibos tea can improve appetite, reduce tension and improve sleep (Morton, 1983). Rooibos tea is not well-known by the general public for its antimicrobial activity, but there have been some studies confirming the inhibitory effects of rooibos against certain microorganisms, such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Streptococcus mutans* and *Candida albicans* (Nakano et al., 1997; Schepers, 2001; Almajano et al., 2008; Coetzee et al., 2008).

The medicinal properties have drawn extensive attention, with studies on the anti-oxidant, antimutagenic, anti-allergenic, vasodilatory and dermatological effects of rooibos tea. A review by Joubert et al. (2008), on South African herbal teas highlights numerous studies which have focused on the possible health benefits of rooibos tea, such as the nitric oxide induced-vasodilatory effects (Persson et al., 2006); or the immunomodulatory effects, through increased antibody production (Kunishiro et al., 2001); as well as the bronchodilatory, antispasmodic and blood pressure lowering effects demonstrated *in vivo* (Khan and Gilani, 2006).

Rooibos tea is consumed on a daily basis by many people throughout the world (Jaganyi and Ndlovu, 2001). Previously, tea consumption was only popular in eastern countries. Now the western populations are also enjoying the health benefits offered by teas (Almajano et al., 2008; Chan et al., 2010). Since rooibos tea is such a popular beverage, as indicated by the data compiled by the South African Rooibos Council, the possibility for concurrent consumption with conventional medicine is considerable. Rooibos tea is commonly consumed by ill people as a form of comfort. It is also a beverage that is often consumed in the mornings with breakfast, when many conventional antimicrobials need to be taken, which results in the possibility for concurrent use.

Herb–drug interactions have become of great clinical importance, with websites being dedicated to this issue (www.prescribeguide.com). Previous studies on the interactions between various teas and commercial pharmaceutical drugs (Yamazaki, 1996; Isogai et al., 2001; Zhao et al., 2001; Tiwari et al., 2005; Matsuda et al., 2007) indicate that there should also be equal importance placed on tea–drug interactions in the clinical setting, as there is on herb–drug interactions. Many studies have focused on the effects of various plants when consumed in combination with conventional antibiotics, which was demonstrated in a review by Van Vuuren and Viljoen (2011). However, only a few scientific articles could be found on the effects of rooibos tea on conventional drugs when consumed in conjunction. One such study was by Matsuda et al. (2007), where it was found that rooibos increased cytochrome P450 enzyme (CYP3A4) activity in the intestine of rats, which resulted in the reduced efficacy of the benzodiazepine, midazolam. A review by Mertens-Talcott et al. (2006), also reported that rooibos induced CYP450 hepatic enzymes, which resulted in the increased metabolism of many conventional drugs, such as calcium channel blockers used in the treatment of cardiovascular disease; and statins used for the treatment of hypercholesterolaemia. These findings clearly demonstrate that there is a potential for rooibos to interact with conventional drugs, due to its effect on hepatic enzymes.

Since rooibos tea is consumed so freely and regularly, there is an assumption that it is safe and free of toxicity. It has been acknowledged by McGaw et al. (2007), that potential toxicity of regularly consumed beverages is an important consideration. There have been no reports documenting the toxicity seen with rooibos consumption, at the normal consumed concentrations of the tea (Joubert et al., 2008). However, the toxicological aspect of rooibos tea, when consumed in large doses, has been investigated to some extent. A study by Marnewick et al. (2011), demonstrated that chronic consumption of rooibos caused no adverse effects in the kidney or liver of humans.

The toxic effect of rooibos tea when consumed in conjunction with conventional drugs also needs to be addressed. A few previous studies have mentioned the possibility of toxicity when rooibos is consumed together with conventional drugs, due to the ability of rooibos tea to inhibit hepatic CYP450 enzymes, resulting in reduced metabolism of conventional drugs when consumed in conjunction (Jang et al., 2004). However, no studies were identified, where the effects of *A. linearis* combined with conventional antimicrobial agents have been investigated. Therefore, the aim of this study was to determine the interactive antimicrobial and toxicity profiles of *A. linearis* (aqueous and organic extract), when placed in combination with seven conventional antimicrobial agents.

2. Materials and methods

2.1. Sourcing and preparation of plant samples

A. linearis leaves (4 kg super grade, pasteurised, fermented leaves) were donated by Rooibos Limited, Clanwilliam, Cape Town. The leaves were provided in the form in which the tea is commercially sold, ensuring that the plant samples were as closely related to that which is used by consumers. The leaves were harvested from shrubs growing in the Clanwilliam region of the Cederberg area. Harvesting occurred during the warm summer months and the plant material was received at the University of the Witwatersrand in March 2012.

The dry leaves were ground into a fine powder using a high speed Fritsch Pulverisette grinder (Labotec). The aqueous extract of *A. linearis*, which mimics the traditional form of consumption, was prepared by submerging the ground plant material in sterile distilled water and left in a platform shaker/incubator, for 24 h at 25 °C. The liquid was then filtered and the filtrate stored at −80 °C before lyophilisation (Virtis). The lyophilised aqueous extract was then left under ultra-violet light overnight to ensure the elimination of any microbial contamination. The organic extract of *A. linearis* was also prepared, even though it is not used traditionally in this form. This is to ensure the testing of a concentrated form of extract prepared from the plant, which will ensure the extraction of both polar and non-polar compounds. To prepare the organic extract, the plant material was submerged in a mixture of dichloromethane and methanol (1:1), for 24 h at 37 °C, in a platform 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 the solvent, leaving behind the solid organic extract. The percentage yields were calculated at 1.37% and 1.98% for the aqueous and organic extract respectively, after which the extracts were stored in sealed sterile bottles, at room temperature and protected from light, until further analysis.

2.2. Toxicity studies

2.2.1. Brine-shrimp lethality assay

Artificial sea water was prepared by dissolving 16 g of Tropic Marine® Sea Salt in 500 ml of distilled water, which was then dispensed into an inverted, bottomless plastic bottle. Dried, brine-shrimp (*Artemia franciscana*) eggs (Ocean Nutrition™) (0.5 g), purchased from a local pet store, were added to the salt water. To ensure a high hatch rate, the salt water was aerated with a rotary pump (Kiho) and a constant source of light and warmth from a lamp (220–240 V) was provided. The eggs were incubated under these conditions, at 25 °C for 18–24 h.

For the preparation of the 48-well micro-titre plates, 400 μl salt water containing 40–60 live brine-shrimp was added to each well along with 400 μl sample (plant samples, antimicrobials or a combination of both). All the latter samples were studied at a final concentration of 1 mg/ml in triplicate per plate, with plates being duplicated. A concentration of above 1 mg/ml was considered non-toxic for the assay (Bussmann et al., 2011). The negative toxic-free control consisted of 32 g/l artificial sea water that mimicked the natural environment for the brine-shrimp, to support their growth and survival. The positive control consisted of 1.6 mg/ml potassium dichromate, which is a known highly toxic compound (Sigma-Aldrich). Dead brine-shrimp were counted after 24 and 48 h by viewing plates under a light microscope (Olympus) at 40× magnification. After which, a lethal dose of acetic acid (Saarchem; 100% (v/v); 50 μl) was added to each well, for a final count to be undertaken such as to calculate the percentage mortality (Cock and Kalt, 2010). A percentage mortality of 50% or greater was considered biologically toxic and further varied concentrations would need to be prepared to determine the dose or concentration required to have a lethal effect on 50% of the brine-shrimp (LD_{50}) from a log-sigmoid dose–response curve generated by GraphPad Prism® (Version 5) software (Bussmann et al., 2011).

2.2.2. The 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

2.2.2.1. Cell culturing and trypsinisation. 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). Experimental culture media was similar to complete culture media, however, it did not contain the antimicrobials. In accordance with the methods followed by Mosmann (1983) and van Zyl et al. (2006), the cells were maintained at 37 °C in 5% CO_2 and when confluency of the cells was reached, the cells were trypsinised to use in the cell proliferation assay or to reseed a culture. 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).

2.2.2.2. The MTT cell proliferation assay. Of the 0.5 million cells/ml suspension, 180 μl was added to each well of a sterile 96-well micro-titre plate (VWR International). To ensure that the cells adhered to the well surface before addition of the samples, the plates were incubated at 37 °C for 6 h, in a humidified environment with 5% CO_2. The samples and their combinations were all screened at 100 μg/ml and tested in triplicate wells, with a colour control (absent of cell suspension) included for each sample. A cell-free, sample-free control consisting only of culture media was also included. Positive controls of quinine and camptothecin (Sigma-Aldrich) were included for comparison to the untreated, 100% cell suspension control. The prepared plates were then incubated at 37 °C for a further 44 h. A washing step was then undertaken using phosphate buffered saline (pH 7.20; PBS), to ensure no interference of plant sample colour in absorbance readings and sample–MTT interaction. Thereafter, 40 μl sterile 5.00 mg/ml MTT (Sigma-Aldrich) solution was added to each well and plates were incubated for a further 4 h. A volume of 170 μl of the supernatant was then removed from each well, ensuring no disruption of the adherent cells. This was replaced with dimethyl sulphoxide (DMSO) for the discontinuation of the reaction and to solubilise the formazan crystals.

The absorbance of dissolved crystals was then read (Labsystems iEMS MF reader) at a test wavelength of 540 nm and reference wavelength of 690 nm, connected to a computer with Ascent® software. Percentage cell 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):

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

Samples were further tested at a concentration of 1 mg/ml for comparison with the BSLA; however, the colour of the plant samples resulted in numerous washing steps to prevent the interference of sample colour in the absorbance readings. Excessive washing compromised cell viability and therefore these results were not taken into account.

2.3. Antimicrobial analysis

2.3.1. Minimum inhibitory concentration (MIC) assays

To represent the three main classes of micro-organisms, three Gram-positive bacteria; *S. aureus* (American Type Culture Collection (ATCC) 25923), *Enterococcus faecalis* (ATCC 29212) and *Bacillus cereus* (ATCC 11778), three Gram-negative bacteria; *Klebsiella pneumoniae* (ATCC 13883), *E. coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27858), and lastly two yeasts; *C. albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 14116) were selected. The pathogens were selected on the basis of their prevalence in nosocomial infections and their causality of infections, such as gastrointestinal tract, respiratory tract and skin infections, for which the selected medicinal plants are traditionally used. 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. 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 selected conventional antimicrobials included erythromycin (potency of ≥850 μg/mg), gentamicin (potency of 600 μg/mg), nystatin (potency of ≥4400 United States Pharmacopoeia (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 antimicrobials were prepared in sterile distilled water, while the antifungal, amphotericin B, was dissolved in 1% (v/v) DMSO in sterile water.

The MIC assay was used to evaluate the antimicrobial activity of *A. linearis* aqueous and organic extracts, when tested individually as well as in combination with the conventional antimicrobials. The micro-titre plate method to determine the antimicrobial activity of plant samples were in accordance with methods by Eloff (1998). The Clinical and Laboratory Standards Institute (CLSI) guidelines (2012) were also consulted when analysing conventional antimicrobial drugs.

Each well of the micro-titre plate was filled with 100 μl of sterilized distilled water and samples introduced into the wells of the first row as 100 μl for individual samples or 50 μl of each sample in the double combinations. Plant samples were introduced at starting concentrations 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 and 0.1 mg/ml for antibiotics and antifungals, respectively. All samples and plant: antimicrobial combinations were tested in at least duplicate. The positive control (to confirm antimicrobial susceptibility) was either ciprofloxacin (0.01 mg/ml) or amphotericin B (0.1 mg/ml) for bacteria and yeasts, respectively. Three negative controls were used, one of which was external to plating and consisted of a media control, to ascertain sterility of the media (TSB). Included was the negative culture control of TSB alone, which was absent of a sample with antimicrobial activity, to allow for the

growth of the microbe. The third negative control was the solvent control, which consisted of acetone (32 mg/ml) for organic extract testing. The solvent control was included to ascertain whether the diluent had any antimicrobial effects. A serial doubling dilution was undertaken after all samples had been plated and the plates then inoculated with the relevant pathogen, ensuring an inoculum 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, ρ-iodonitrotetrazolium violet (INT; 0.4 mg/ml; Sigma-Aldrich) was added to each well, which turned purple-pink in the presence of microbial growth. The end point MIC value was therefore taken as the first clear well within a column, which represented the lowest concentration of test sample that inhibited microbial growth.

2.3.2. Fractional inhibitory concentration (FIC) assessment

Combinations of *A. linearis* (aqueous and organic extract) with the conventional antimicrobial were further assessed using the sum of the fractional inhibitory concentration (∑FIC), which would allow for the classification of the type of interaction occurring. 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):

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

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

The ∑FIC was calculated using the equation; $\sum FIC = FIC^{(i)} + FIC^{(ii)}$. Depending on the values obtained, the interactions could then be classified as being synergistic ∑FIC (≤0.5), additive (>0.5–1.0), indifferent (>1.0–≤4.0) or antagonistic (>4.0) in nature (Van Vuuren and Viljoen, 2011). Tentative interpretations were included, where the MIC value of one of the individual agents in the combination was greater than the highest concentration tested (>8 mg/ml). Tentative interpretations provided an indication of the possible interactive profile for the combination. An ∑FIC value was not given since the calculation can only be undertaken on absolute values and results having 'greater than' values could not be considered in the calculation.

2.3.3. Varied ratio combination studies (isobolograms)

For notable synergistic or antagonistic interactions that were observed in the ∑FIC evaluation, a varied ratio study was undertaken to determine which agent within the combination was most responsible for the interactive profile observed. Nine different ratios of the combination were prepared and the MIC values determined. Table 1 demonstrates the concentrations for each ratio tested. Data points for each ratio studied were plotted on an isobologram using the GraphPad Prism® software (Version 5). Data points falling below the 0.5:0.5 line indicated synergy, while those above the 0.5:0.5 line but below the 1.0:1.0 line indicated an additive effect. Data points above the 1.0:1.0 line, but below the 4.0:4.0 line indicated an indifferent interaction and those falling above the 4.0:4.0 line indicated antagonism (Van Vuuren and Viljoen, 2011).

Table 1
The concentration ratios used for antimicrobial and plant sample combination studies.

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

[a] Ciprofloxacin/erythromycin/gentamicin/penicillin G/tetracycline.
[b] Amphotericin B/nystatin.

3. Results and discussion

3.1. Toxicity analysis

3.1.1. Brine shrimp lethality assay

The BSLA was used as a preliminary toxicity screening tool to identify any possible toxicity of *A. linearis* aqueous and organic extracts, independently, as well as in combination with the selected conventional antimicrobials. Independently, both the aqueous and organic extracts demonstrated no toxic effects at 1 mg/ml after 24 and 48 h (Table 2). When tested individually, most of the conventional antimicrobials were not toxic against the brine-shrimp, except for gentamicin and tetracycline both killing less than 10% of the brine-shrimp after 48 h (Table 2). These two antimicrobials were still not considered toxic in the BSLA, since the mortality rate at 1 mg/ml was below 50% compared to the positive control, potassium dichromate. A similar safety profile was noted when the aqueous and organic extract of *A. linearis* was combined with the conventional antimicrobials, except for three combinations. Namely, the combinations between *A. linearis* aqueous extract and ciprofloxacin (31.58 ± 1.53%), *A. linearis* aqueous extract and gentamicin (4.35 ± 0.58%) and *A. linearis* organic extract with erythromycin (6.67 ± 0.58%) (Table 2). However, the degree of mortality observed does not warrant classifying the interactions as toxic and should not have had an influence on the antimicrobial inhibitory effect observed with these interactions. Neither should they have significant effect on the patient taking the combination.

3.1.2. MTT cell proliferation assay

As in the brine-shrimp assay, the individual aqueous and organic extract of *A. linearis* demonstrated no toxicity in the MTT assay (Table 2). Similarly, no toxicity was observed for the individual conventional antimicrobials, with all antimicrobials demonstrating a cell viability of approximately 100%, except for amphotericin B (cell viability of 94.07 ± 3.41%) (Table 2). To account for possible interactions between extracts and the MTT, appropriate controls and washing steps were included. Increased formazan production was noted for some of the samples, which could have been due to either increased mitochondrial activity or increased cell numbers.

The lack of toxicity for *A. linearis* on human kidney epithelial cells is in accordance with findings by Marnewick et al. (2003), who reported that an aqueous extract of fermented and unfermented rooibos displayed no toxic effects on rat kidneys when provided as the only drinking fluid for the rats over a 10 week period. Later, Marnewick et al. (2011) also found that fermented rooibos had no adverse effects on kidney function in 83 human male and female participants from Cape Town, when six cups of rooibos tea were consumed per day for six weeks. Even though some of the kidney function indicators were increased after the test period, all were still within reference ranges, therefore supporting the safety of short term rooibos consumption.

The combinations of *A. linearis* (aqueous and organic extract) with six of the seven conventional antimicrobials, demonstrated no potentiation or induction of toxicity (Table 2). However, the combination of both the *A. linearis* aqueous and organic extracts with the antifungal agent, nystatin, demonstrated a possible toxic effect, with cell growth being inhibited by 73.76 ± 3.36% and 56.88 ± 6.61%, respectively. Nystatin has been shown to cause cellular toxicity towards J774

Table 2
Mortality (%) and cell death (%) results for the BSLA and the MTT assay, respectively.

	Sample or combination	Mortality (% ± S.D.)[a]		Cell death (% ± S.D.)[b]
		After 24 h:	After 48 h:	After 48 h:
Antimicrobials	Ciprofloxacin	0.00	0.00	0.1 ± 0.01
	Erythromycin	0.00	0.00	0.1 ± 0.01
	Gentamicin	1.12 ± 0.58	8.99 ± 0.33	0.1 ± 0.01
	Penicillin	0.00	0.00	0.1 ± 0.01
	Tetracycline	0.00	6.67 ± 1.16	0.1 ± 0.01
	Amphotericin	0.00	0.00	5.93 ± 3.41
	Nystatin	0.00	0.00	0.1 ± 0.01
Aqueous extract	*A. linearis*	0.00	0.00	0.1 ± 0.01
	A. linearis + ciprofloxacin	0.00	31.58 ± 1.53	0.1 ± 0.01
	A. linearis + erythromycin	0.00	0.00	0.1 ± 0.01
	A. linearis + gentamicin	0.00	4.35 ± 0.58	0.1 ± 0.01
	A. linearis + penicillin	0.00	0.00	0.1 ± 0.01
	A. linearis + tetracycline	0.00	0.00	0.1 ± 0.01
	A. linearis + amphotericin	0.00	0.00	0.1 ± 0.01
	A. linearis + nystatin	0.00	0.00	73.76 ± 3.36
Organic extract	*A. linearis*	0.00	0.00	0.1 ± 0.01
	A. linearis + ciprofloxacin	0.00	0.00	0.1 ± 0.01
	A. linearis + erythromycin	0.00	6.67 ± 0.58	0.1 ± 0.01
	A. linearis + gentamicin	0.00	0.00	0.1 ± 0.01
	A. linearis + penicillin	0.00	0.00	0.1 ± 0.01
	A. linearis + tetracycline	0.00	0.00	0.1 ± 0.01
	A. linearis + amphotericin	0.00	0.00	0.1 ± 0.01
	A. linearis + nystatin	0.00	0.00	56.88 ± 6.61
Controls	Camptothecin	0.00[a]	2.08 ± 0.58[a]	76.07 ± 2.94[a]
		30.00 ± 2.00[b]	100.00[b]	0.1 ± 0.01[b]
	Quinine	0.00[a,b]	0.00[a]	71.38 ± 4.73[a]
			11.76 ± 1.00[b]	0.1 ± 0.01[b]
	Potassium dichromate		100.00[c]	NA

NA = control not tested in or relevant to the assay; S.D. = standard deviation.
[a] Final concentration of 1 mg/ml.
[b] Final concentration of 100 μg/ml.
[c] Tested at a concentration of 1.6 mg/ml.

macrophages and to lyse red blood cells, and at a concentration 100 fold lower than tested in this study, did not affect the viability of RAW 264.7 leukaemia cells (Tzimogianni et al., 1989). The possibility for nystatin to interact with rooibos to cause nephrotoxicity is unlikely, since nystatin has negligible absorption into the systemic circulation, thus passing the renal excretory pathway (SAMF, 2012). Intravenously administered nystatin is known to possess toxicity and as such is now only administered topically, where the drug penetrates the surface layer (epidermis), but does not transverse to the blood stream (Sheppard and Lampiris, 2012; LIFE, 2013). Topical adverse effects have been reported to include hypersensitivity, skin irritation and pruritis (Sheppard and Lampiris, 2012). Nystatin entering the gastrointestinal tract is excreted unchanged, however, the possibility for other cellular interactions in the gastrointestinal tract should be observed. The possibility of rooibos facilitating increased uptake of nystatin through the gastrointestinal wall into the systemic circulation should also be considered. An *in vitro* study by Tarirai et al. (2012) using Caco-2 cell monolayers and excised porcine jejunum tissue, has already shown that rooibos tea can have an effect on the intestinal absorptive profile of the conventional drug, cimetidine. As such, extended *in vivo* studies on the pharmacokinetic and pharmacodynamic properties of the combinations are warranted.

3.2. Antimicrobial analysis

3.2.1. Antimicrobial activity of individual samples

The antimicrobial activities of the conventional antimicrobials are already well-known. Antimicrobial activity of these agents was, however, still evaluated independently in this study, to provide the necessary data for the overall interactive evaluation. Testing these antimicrobials independently also validated the reliability of the micro-dilution assay, to ensure that the MIC values obtained in this study were in accordance with break point expectations (Andrews, 2004; CLSI, 2012). The MIC values obtained were all congruent with the break point expectations; except for tetracycline against *E. faecalis* and *P. aeruginosa*, where these two strains demonstrated an enhanced susceptibility towards tetracycline. Break point expectations are derived from published antimicrobial analysis of samples against various pathogens, providing a range for the expected MIC value of a specific antimicrobial agent against a pathogen.

When *A. linearis* was investigated individually for antimicrobial activity, no noteworthy antimicrobial activity was identified for both the aqueous and organic extracts. Noteworthy antimicrobial activity of extracts was defined as MIC values of <1.00 mg/ml (Ríos and Recio, 2005; Van Vuuren, 2008; Ncube et al., 2012). The lack of noteworthy antimicrobial activity demonstrated by *A. linearis* was expected, since rooibos tea is more commonly known for its antimutagenic and antioxidant activity, rather than for its antimicrobial activity (Van Wyk et al., 2009). Many studies have investigated the antimicrobial properties of green and black tea (Toda et al., 1989; Diker et al., 1991; Fukai et al., 1991; Diker and Hascelik, 1994; Yeo et al., 1995), however, very little has been done on the antimicrobial properties of rooibos. Two studies that investigated the antimicrobial activity of extracts of *A. linearis* were conducted by Schepers (2001) and Coetzee et al. (2008). Schepers (2001) tested the soluble solids of unfermented and fermented rooibos extract at concentrations varying from 0.50 g/l to 5.00 g/l, against a range of pathogens. The growth of the pathogens in the presence of the extracts was determined spectrophotometrically. It was found that rooibos had an inhibitory effect on *E. coli*, *S. aureus* and *B. cereus* growth after 12 h. For the soluble solids from the unfermented rooibos, the percentage decrease in growth ranging from 10.20 to 35.10% for *E. coli*, 5.70 to 50.10% for *S. aureus* and 9.40% to 47.50% for *B. cereus*, when tested at concentrations ranging from 0.50 to 5.00 g/l. The soluble solids from the fermented rooibos demonstrated a percentage decrease in growth of 14.30–69.00% for *E. coli*, 8.20–90.8% for *S. aureus* and 14.40–80.30% for *B. cereus*, when tested at concentrations ranging from 0.50 to

5.00 g/l. These three pathogens were also included in the current study, with both the aqueous and organic extracts displaying the best antimicrobial activity against *E. coli* (MIC value of 1.50 mg/ml). Coetzee et al. (2008) also identified the inhibitory effects of rooibos extracts against *E. coli*, but the extracts were tested at very high concentrations and the inhibitory effect was only seen at a concentration of 10 mg/ml, therefore cannot be considered noteworthy as an antimicrobial.

3.2.2. Combination studies

3.2.2.1. Fractional inhibitory concentration assessment. A total of 56 combinations were evaluated and ∑FIC calculations recorded in Tables 3.1–3.3 for the Gram-positive, Gram-negative bacteria and yeasts, respectively. Of these combinations, 19.64% were synergistic, 7.14% were antagonistic, 19.64% were additive and 53.57% were found to be indifferent in nature. The indifferent interactions noted between the majority of the combinations of the *A. linearis* extracts with the conventional antimicrobials, alleviate some concern related to their concurrent use, as no advantage or disadvantage is associated with this type of interaction.

The most synergistic interactions were found against the Gram-positive bacteria, when *A. linearis* was combined with penicillin G, where the combination was most synergistic against *S. aureus* (Table 3.1). It is highly likely that one of the constituents of the extracts could have potentiated the uptake and antimicrobial effect of the antibiotic. Studies have found that epigallocatechin-3-gallate (EGEG), a compound commonly found in many teas, has a synergistic effect with the β-lactam antibiotics, such as penicillin, and their derivatives, when tested against methicillin-resistant *S. aureus* (MRSA) (Yamazaki, 1996; Zhao et al., 2001; Hu et al., 2002). EGEG is not found in rooibos tea (Almajano et al., 2008), but further studies are warranted to identify the compound/s and mechanism of action by which this strong synergistic interaction is facilitated. It has been reported that penicillin G has been found to possess a mostly potentiating or synergistic effect when in combination with other plants, such as *Catha edulis*, where a fourfold potentiation of penicillin G against *Fusobacterium nucleatum* was seen (Al-hebshi et al., 2006). *Rhus coriaria*, *Sacropoterium spinosum* and *Rosa damasecena* were also found to be synergistic in combination with penicillin G, when tested against three clinical strains of *P. aeruginosa* (Adwan et al., 2010). Penicillin G has also shown strong potentiating activity when in combination with some plant compounds, such as eugenol, thymol and carvacrol, when tested against *E. coli*, *S. aureus*, *Streptococcus pyogenes* and *Salmonella typhimurium*. The strongest synergistic effect was seen against *S. aureus* ($^{\sum}$FIC of 0.11) when carvacrol and penicillin G were combined (Palaniappan and Holley, 2010). In the current study, the strongest synergistic effect for the combination of *A. linearis* extracts and penicillin G was also seen against *S. aureus* (Table 3.1). Antagonistic interactions were minimal, with only four antagonistic ∑FIC values identified when *A. linearis* was combined with ciprofloxacin, gentamicin and amphotericin B. No antagonism was noted against the tested Gram-positive pathogens (Tables 3.2 and 3.3).

In a study by Tiwari et al. (2005), the combined antimicrobial activities of green and black tea extracts with chloramphenicol, gentamicin, methicillin or nalidixic acid were found to be mostly synergistic against enteropathogens. This could mean that tea drinkers experience an enhanced therapeutic effect of an antimicrobial agent, thereby recovery from an infection may be more rapid.

3.2.2.2. Varied ratio combination studies (isobolograms). Rooibos tea is not consumed in fixed concentrations. People vary the number of cups of tea they consume per day. Also, the concentration of active ingredients varies depending on how long the teabag is left to draw, thereby varying the strength of the tea. Variations in season, cultivation, harvesting and leaf preparation may result in a changed concentration of active ingredients. To determine whether different concentrations of rooibos influence the interaction when in combination with an antimicrobial agent, the combinations were assessed at various ratios. The combinations demonstrating the most notable synergistic interactions in the ∑FIC evaluation were tested against the Gram-positive pathogens when *A. linearis* (aqueous and organic extract) was combined with penicillin G. Thus, varied ratios of these combinations were analysed against the three Gram-positive bacteria, namely *B. cereus*, *E. faecalis* and *S. aureus*.

Table 3.1
MIC (μg/ml) and ∑FIC values for the combination of *A. linearis* with the various antibiotics, against the Gram-positive pathogens.

		***S. aureus* (ATCC 25923)**				***B. cereus* (ATCC 11778)**				***E. faecalis* (ATCC 29212)**			
Combination	**Sample type**	**Indiv MIC**	**Combine MIC**	**∑FIC**	**Int**	**Indiv MIC**	**Combine MIC**	**∑FIC**	**Int**	**Indiv MIC**	**Combine MIC**	**∑FIC**	**Int**
A. linearis + ciprofloxacin	Aq	≥ 8000	2000	NA	IND	≥ 8000	2000	NA	IND	3000	2000	1.17	IND
	Cip	0.47	0.63			0.63	0.63			1.25	0.63		
	Org	3000	500	0.51	ADD	2000	1000	1.01	IND	2000	1500	1.13	IND
	Cip	0.47	0.16			0.63	0.32			1.25	0.47		
A. linearis + erythromycin	Aq	≥ 8000	2000	NA	IND	≥ 8000	1000	NA	IND	3000	4000	NA	IND
	Ery	0.32	0.63			0.32	0.32			1.25	1.25		
	Org	3000	2000	2.70	IND	2000	500	0.77	ADD	2000	≥ 4000	NA	IND
	Ery	0.32	0.63			0.32	0.16			1.25	≥ 1.25		
A. linearis + gentamicin	Aq	≥ 8000	≥ 4000	NA	ADD	≥ 8000	3000	NA	IND	3000	≥ 4000	NA	IND
	Gen	1.88	≥ 1.25			≥ 2.50	0.94			≥ 2.50	≥ 1.25		
	Org	3000	1000	**0.50**	**SYN**	2000	500	NA	**SYN**	2000	500	NA	**SYN**
	Gen	1.88	0.32			≥ 2.50	0.16			≥ 2.50	0.16		
A. linearis + penicillin G	Aq	≥ 8000	30	NA	**SYN**	≥ 8000	250	NA	**SYN**	3000	1000	**0.46**	**SYN**
	Pen	≥ 2.50	0.01			≥ 2.50	0.08			≥ 2.50	0.32		
	Org	3000	30	**0.01**	**SYN**	2000	130	**0.08**	**SYN**	2000	1500	0.94	ADD
	Pen	≥ 2.50	0.01			≥ 2.50	0.04			≥ 2.50	0.47		
A. linearis + tetracycline	Aq	≥ 8000	1000	NA	IND	≥ 8000	250	NA	ADD	3000	3000	NA	IND
	Tet	0.23	0.32			0.16	0.08			≥ 2.50	0.94		
	Org	3000	500	0.87	ADD	2000	190	**0.48**	**SYN**	2000	2000	NA	IND
	Tet	0.23	0.16			0.16	0.06			≥ 2.50	0.63		

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference; Aq = aqueous extract; Org = organic extract; Indiv = MIC for individual samples; Combine = MIC for samples when in combination; Int = interaction classification; NA = where ≥MIC values are observed, an absolute ∑FIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; and SYN = synergistic interaction.

Table 3.2
MIC (μg/ml) and ∑FIC values for the combination of *A. linearis* with the various antibiotics, against the Gram-negative pathogens.

		***E. coli* (ATCC 25922)**				***K. pneumoniae* (ATCC 13883)**				***P. aeruginosa* (ATCC 27853)**			
Combination	**Sample type**	**Indiv MIC**	**Combine MIC**	**∑FIC**	**Int**	**Indiv MIC**	**Combine MIC**	**∑FIC**	**Int**	**Indiv MIC**	**Combine MIC**	**∑FIC**	**Int**
A. linearis + ciprofloxacin	Aq	1500	1000	*4.67*	*ANT*	4000	500	0.64	ADD	≥ 8000	1500	3.13	IND
	Cip	0.08	0.32			0.63	0.16			0.16	0.47		
	Org	1500	500	2.33	IND	3000	190	**0.16**	**SYN**	3000	500	1.17	IND
	Cip	0.08	0.16			0.63	0.06			0.16	0.16		
A. linearis + gentamicin	Aq	1500	≥ 4000	NA	IND	4000	2000	NA	ADD	≥ 8000	1500	1.71	IND
	Gen	≥ 2.50	≥ 1.25			≥ 2.50	0.63			0.32	0.47		
	Org	1500	3000	2.38	IND	3000	2000	NA	ADD	3000	≥ 4000	NA	*ANT*
	Gen	≥ 2.50	0.94			≥ 2.50	0.63			0.32	≥ 1.25		
A. linearis + tetracycline	Aq	1500	≥ 4000	NA	IND	4000	≥ 4000	NA	IND	≥ 8000	1500	NA	**SYN**
	Tet	1.25	≥ 1.25			1.25	≥ 1.25			≥ 2.50	0.47		
	Org	1500	3000	2.75	IND	3000	≥ 4000	NA	IND	3000	1500	NA	ADD
	Tet	1.25	0.94			1.25	≥ 1.25			≥ 2.50	0.47		

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference; Aq = aqueous extract; Org = organic extract; Indiv = MIC for individual samples; Combine = MIC for samples when in combination; Int = interaction classification; NA = where ≥ MIC values are observed, an absolute ∑FIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; SYN = synergistic interaction; and *ANT* = antagonistic interaction.

For the combination of *A. linearis* aqueous extract and penicillin G (Fig. 1a), all ratios were found to be either synergistic or additive in nature, with no ratios falling above the 1.0:1.0 line. The penicillin G: *A. linearis* aqueous extract ratio, consisting of equal volumes of each agent in the combination (50:50 μl; antimicrobial: plant sample) (refer to Table 1 for ratio concentrations) was found to be synergistic against all three Gram-positive pathogens. When the extract and antimicrobial were combined in equal volumes (50:50 μl), the concentration of rooibos in the ratio was 16 mg/ml (Table 1). Schepers (2001) found that the average concentration of a cup of rooibos tea is 2 mg/ml, therefore, the ratio consisting of equal volumes of extract and antimicrobial, would require eight cups of tea to be consumed with 5.00 μg/ml of penicillin G for the strong synergistic interaction to occur (Table 1). The combination of *A. linearis* (aqueous extract) and penicillin G in varied ratios was mostly synergistic against *S. aureus*, with six of the nine ratios being highly synergistic (Fig. 1a), which supports the ∑FIC evaluation. The three penicillin G: *A. linearis* ratios for the aqueous extract combination that were not synergistic against *S. aureus*, were the 3:7; 2:8 and 1:9 ratios, indicating that a high ratio of *A. linearis* aqueous extract is preferred to optimise the antimicrobial action of penicillin G.

For the combination of the organic extract of *A. linearis* and penicillin G, all ratios were found to be either synergistic or additive, except for two ratios against *B. cereus*, which were identified as being indifferent. Interestingly, again, the penicillin G: *A. linearis* organic extract ratio, consisting of equal volumes of each agent in the combination (50:50 μl) (refer to Table 1, for ratio concentrations) was found to be synergistic against all three Gram-positive pathogens. The organic extract combination, similar to that of the aqueous extract combination with penicillin G in varied ratios, was most synergistic against *S. aureus*, where all nine of the tested ratios were found below the 0.5:0.5 line (Fig. 1b).

4. Conclusions

This study has shown that rooibos tea when taken in combination with conventional antimicrobials results in interactions that are mostly indifferent and additive in nature. This alleviates some of the concern related to the concurrent use of rooibos tea with antimicrobials. Some notable interactions were identified in this study, which would require further *in vivo* studies for confirmation. The most synergistic interactions were identified for the combinations with penicillin G against the Gram-positive pathogens. Synergistic interactions are very often considered advantageous, as it could result in the enhanced efficacy of the conventional drugs, allowing for a dose reduction and fewer side effects. Therefore, the synergy observed supports the statement that tea and antibiotic combinations could be useful in reducing drug-resistance problems, due to the potentiating effect (Abascal and Yarnell, 2002).

Table 3.3
MIC (μg/ml) and ∑FIC values for the combination of *A. linearis* with the various antifungal agents, against the yeasts.

		***C. albicans* (ATCC 10231)**				***C. neoformans* (ATCC 14116)**			
Combination	**Sample type**	**Indiv MIC**	**Combine MIC**	**∑FIC**	**Int**	**Indiv MIC**	**Combine MIC**	**∑FIC**	**Int**
A. linearis + amphotericin B	Aq	≥ 8000	≥ 4000	NA	*ANT*	≥ 8000	750	NA	*ANT*
	Amp	1.56	≥ 12.50			0.39	2.35		
	Org	3000	1500	3.51	IND	1500	130	1.09	IND
	Amp	1.56	4.69			0.39	0.39		
A. linearis + nystatin	Aq	≥ 8000	1500	NA	IND	≥ 8000	1500	NA	IND
	Nys	2.34	4.69			1.56	4.70		
	Org	3000	1000	1.67	IND	1500	190	0.51	ADD
	Nys	2.34	3.13			1.56	0.59		

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference; Aq = aqueous extract; Org = organic extract; Indiv = MIC for individual samples; Combine = MIC for samples when in combination; Int = interaction classification; NA = where ≥MIC values are observed, an absolute ∑FIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; and *ANT* = antagonistic interaction.

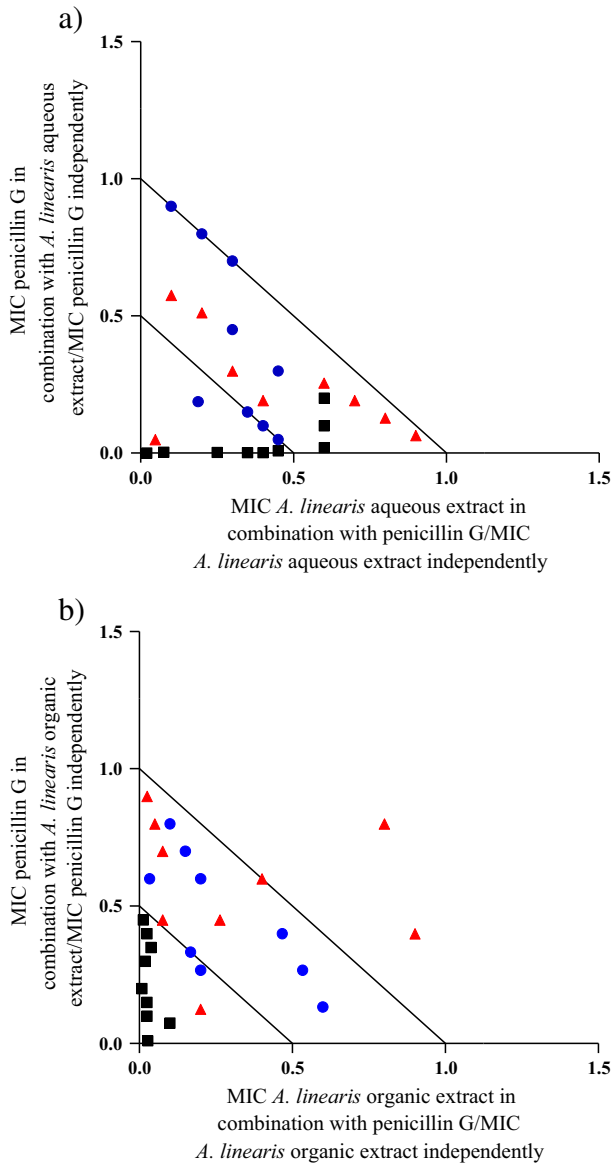

Fig. 1. Isobologram for *A. linearis* aqueous (a) and organic (b) extract in combination with penicillin G, when tested at various ratios against the Gram-positive micro-organisms (■ = *S. aureus*; ▲ = *B. cereus*; ● = *E. faecalis*).

Rooibos, independently and in combination with the conventional antimicrobials showed no toxic effects in the BSLA and MTT assay, except for the combination of *A. linearis* with nystatin in the latter assay. Nystatin on its own should not cause systemic effects as it is not absorbed across the gastrointestinal tract (SAMF, 2012), but in combination with *A. linearis*, it is possible that the cellular or systemic uptake of nystatin could be facilitated by a component/s in the extract.

Future recommendations include mechanism of action studies regarding the combinations. The current study has demonstrated the potential for tea–drug interactions and therefore emphasises the need for further tea–drug interaction studies to be conducted with other conventional drugs. Future tea–drug *in vivo* studies are also warranted, to support the *in vitro* findings, particularly for the combination of *A. linearis* with nystatin.

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