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Original Article

Interactive Antimicrobial and Toxicity Profiles of *Scaevola spinescens* R.Br. Extracts with Conventional Antibiotics

Baxter Blonk[1,2] , Ian E.Cock[1,2,*]

ABSTRACT

Background: *Scaevola spinescens* was traditionally used by Australian Aborigines to treat a variety of conditions including bacterial and viral infections, inflammation and cancer. Conventional antibiotics are amongst the most commonly prescribed groups of drugs in allopathic medicine. Therefore, these therapies may be used concurrently by practitioners of complementary medicine and there is a need to evaluate their effects in combination. **Methods:** Plant extracts were prepared using solvents of varying polarity and subjected to qualitative phytochemical screening analysis. Antimicrobial activity was assessed using disc diffusion and liquid dilution minimum inhibitory concentration (MIC) assays against a panel of pathogenic triggers of some autoimmune diseases. Interactions between the *S. spinescens* extracts and conventional antibiotics were studied and classified by determining the sum of the fractional inhibitory concentration (∑FIC). Synergistic interactions were further examined across a range of ratios using isobolograms analysis. The toxicity of the individual samples and of the combinations was assessed using the *Artemia* lethality assay (ALA) and an MTS HDF cell viability assays. **Results:** Methanolic, aqueous and ethyl acetate extracts showed moderate to good inhibitory activity against several bacterial pathogens known to trigger autoimmune inflammatory diseases in genetically susceptible individuals. However, combinations of the methanolic, aqueous, ethyl acetate and hexane extracts with conventional antibiotics proved significantly more effective in inhibiting the growth of *Klebsiellia pneumoniae* and *Streptococcus pyogenes* (bacterial triggers of ankylosing spondylitis and rheumatic fever respectively). In total, 4 combinations proved to be synergistic, all of which contained tetracycline as the conventional antibiotic component. Furthermore, all conventional antibiotics and *S. spinescens* leaf extracts were determined to be nontoxic when tested alone in the *Artemia* nauplii and HDF bioassays. Combining the extracts and antibiotics did not significantly affect the toxicity of the combinations. **Conclusion:** *S. spinescens* extracts were effective inhibitors of the growth of several bacterial triggers of autoimmune inflammatory diseases when tested alone. Additionally, the methanolic, aqueous and ethyl acetate extracts potentiated the activity of tetracycline against bacterial otherwise resistant to its actions. Isolation of the synergising compounds in these extracts may be beneficial in drug design against several bacteria including the microbial triggers of ankylosing spondylitis and rheumatic fever.

Key words: Australian plant, Maroon bush, Medicinal plants, Antibiotic resistance, Conventional antimicrobials, Synergy, Interaction, Toxicity

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INTRODUCTION

Recent increases in bacterial resistance to clinical antibiotics and a corresponding decrease in antibiotic discovery has made the development of new antibiotic therapies a high priority.[1] Traditional medicines have great potential for antimicrobial drug development and recent interest in medicinal plant research has escalated, with the aim of identifying alternative antibiotic therapies.[2] However, with several notable exceptions, plant-derived antimicrobials usually possess lower potency than conventional antimicrobials, possibly due to synergistic interactions between phytochemicals in plant extracts.[1] Therefore, combinational therapies may be more effective in overcoming resistance and potentiating the activity of conventional antibiotics that are otherwise ineffective against resistant bacterial strains. Several studies investigating combinations of conventional antimicrobials-with African,[3-4] Asian[5-6] and Australian traditional medicinal plants[7] have recently been published, highlighting the increased efficacy of some combinational antibiotic therapies. Several of these studies have identified plant extracts which synergistically enhance the activity of conventional antimicrobials, even when the plant extracts do not possess antimicrobial activity in isolation.[2,4,7-8]

Practitioners of complementary and alternative medicine frequently use traditional and allopathic medications concurrently without knowledge of

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the potential interactions and side effects which may occur. The lack of understanding of the potential interactions between natural/complementary medicines and allopathic drugs, may pose serious risks to patient safety.[9-10] Whilst accurate figures are not available, studies estimate that between 20%[11-12] and 72%[13] of patients in Western countries use herbal drugs concomitant with prescription drugs. Of further concern, estimates of the use of over-the-counter medications in combination with natural therapies is substantially higher and many patients combine these two forms of healthcare with the belief that there would be an enhanced effect.[13] Not surprisingly, there have been many instances where natural products have been used concurrently with conventional medicine and severe reactions have resulted.[14-15] Much more work is required to test the safety of allopathic and complementary drug combinations

Scaevola spinescens R.Br. (family Goodeniaceae) (commonly known as currant bush, maroon bush and fanflower) is a rigid scrubby bush that grows to 1m in height and is distributed throughout the drier inland areas of the Australian continent. It has short hair covered branchlets which are often covered in short, sharp spines. In the warmer summer months, the plant develops cream to yellow coloured flowers which later develop into small purple berries. *S. spinescens* was selected for this study due to its range of traditional therapeutic uses by the first Australians in the treatment of pathogenic disease.[16-17] An infusion of the roots was used to treat stomach pain and urinary tract diseases. A decoction of crushed stem was used to treat boils, rashes and skin disorders. The whole plant was burnt and the fumes inhaled to treat colds. Leaves and twigs were steamed and sores treated by exposure to this steam. *S. spinescens* root bark was used to treat cancer,[16] although their efficacy has yet to be verified in controlled laboratory studies. However, despite its range of traditional medicinal uses, the therapeutic properties of *S. spinescens* have not been extensively studied. Several recent studies have reported broad spectrum antibacterial activity of several *S. spinescens* extracts against a panel of 14 bacterial pathogens.[18-19] The antiviral activity of *S. spinescens* has also been reported. *S. spinescens* leaf extracts inhibit cytomegalovirus (CMV) late antigen production by more than 25% in human cells.[20] Another study confirmed antiviral activity for a methanolic *S. spinescens* extract using an MS2 bacteriophage model system.[19,21] These studies demonstrate the inhibitory potential of *S. spinescens* extracts against bacterial and viral pathogens. Our study aimed to extend these earlier studies by evaluating the growth inhibitory properties of *S. spinescens* leaf extracts against bacterial triggers of some autoimmune inflammatory diseases. Furthermore, the interactive antimicrobial and toxicity profiles of combinations of *S. spinescens* extracts and five conventional antibiotic drugs was examined.

MATERIALS AND METHODS

Sourcing and preparation of plant samples

Scaevola spinescens R.Br. leaves were supplied by Jeannie Crago of the Outback Books, Australia (a commercial supplier of *S. spinescens* tea). Voucher specimens are deposited in the School of Natural Sciences, Griffith University, Australia (voucher number GUSSB1-2009-1). The leaves were thoroughly dried using a Sunbeam food dehydrator and the materials stored at -30ºC until required. Prior to use, the plant materials were thawed and ground into a coarse powder. Individual quantities (1.5 g) of the ground plant material were weighed into separate tubes and 50 mL of methanol, deionised water, ethyl acetate, chloroform or hexane were added. All solvents were obtained from Ajax, Australia and were AR grade. The ground plant materials were extracted in each solvent for 24 h at 4ºC by gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum. The solvent extracts were air dried at room temperature. The aqueous extracts were lyophilised by freeze drying at -50ºC. The resultant dried extracts were weighed to determine yield and dissolved in 10 mL deionised water (containing 1 % DMSO).

Qualitative phytochemical analysis

Phytochemical analysis of the *S. spinescens* extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved as previously described.[22-23]

Antibacterial analysis

Conventional antibiotics

Penicillin-G (potency of 1440-1680 µg/mg), chloramphenicol (≥98 % purity by HPLC, erythromycin (potency ≥850 µg/mg), gentamicin (potency of 600 µg/mg), and tetracycline (≥95% purity by HPLC) were purchased from Sigma-Aldrich, Australia for use in the microplate liquid dilution assay. The conventional antibiotics were prepared in sterile deionised water at stock concentrations of 0.01 mg/mL and stored at 4ºC until use. For the disc diffusion studies, ampicillin (2 µg) and chloramphenicol (10 µg) standard discs were obtained from Oxoid Ltd., Australia and used as positive controls.

Bacterial cultures

All bacterial strains were selected based on their ability to trigger autoimmune inflammatory diseases in genetically susceptible individuals.[24] Reference strains of *Proteus mirabilis* (ATCC21721),*Proteus vulgaris* (ATCC21719), *Klebsiella pneumoniae* (ATCC31488), *Acinetobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Type Culture Collection, USA. Clinically isolated strains of of *P. mirabilis*, *K. pneumoniae*, *A. baylyi, P. aeruginosa* and *Streptococcus pyogenes* were obtained from the School of Natural Sciences teaching laboratory, Griffith University, Australia. All bacteria were cultured in nutrient broth (Oxoid Ltd., Australia). Streak nutrient agar (Oxoid Ltd., Australia) plates were tested in parallel to ensure the purity of all bacterial cultures and for sub-culturing. All bacterial cultures were incubated at 37ºC for 24 h and were sub cultured and maintained in nutrient broth at 4ºC until use.

Evaluation of bacterial susceptibility to growth inhibition

The susceptibility of the bacteria to the *S. spinescens* extracts and the conventional antibiotics was initially assessed using a modified disc diffusion assay.[25]Ampicillin (2 µg) and chloramphenicol discs (10 µg) were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using two methods. A liquid dilution MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay.[26] Furthermore, as microplate liquid dilution MIC assays are perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method allows for comparisons with other studies. A solid phase agar disc diffusion assay was also used in this study for comparison.

Microplate liquid dilution MIC assay

The MICs of the extracts were evaluated by standard methods.[26] Briefly, overnight bacterial cultures were added drop wise to fresh nutrient broth and the turbidity was visually adjusted to produce a McFarlands number 1 standard culture. This was subsequently diluted 1 in 50 with nutrient broth, resulting in the MIC assay inoculum culture. A volume of 100

µL sterile broth was added to all wells of a 96 well plate. Test extracts or control antibiotics (100 µL) were then added to the top row of each plate and 1 in 2 serial dilutions were prepared in each column of wells by transferring 100 µL from the top well to the next well in each column, etc. A growth control (without extract) and a sterile control (without inoculum) were included on each plate. A volume of 100 µL of bacterial culture inoculum was added to all wells except the sterile control wells. All plates were incubated at 37°C for 24 h. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, Australia and dissolved in sterile deionised water to prepare a 0.2 mg/mL INT solution. A 40 µL volume of this solution was added into all wells and the plates were incubated for a further 6 h at 30°C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

Disc diffusion MIC assay

The minimum inhibitory concentrations (MIC) of the extracts was also evaluated by disc diffusion assay as previously described.[23,25] Graphs of the zone of inhibition versus concentration were plotted and MIC values were achieved using linear regression.

Extract-conventional antibiotic interaction studies

Fractional inhibitory concentration (FIC) assessment

Interactions between the *S. spinescens* extracts and the conventional antibiotics were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC) for each combination.[3,7] The FIC values for each component (a and b) were calculated using the following equations where a represents the plant extract sample and b represents the conventional antibiotic:

$$FIC(a) = \left(\frac{MIC[a \text{ in combination with b}]}{MIC[a \text{ independently}]}\right)$$

$$FIC(b) = \left(\frac{MIC[b \text{ in combination with a}]}{MIC[b \text{ independently}]}\right)$$

The ΣFIC was then calculated using the formula ΣFIC = FIC(a) + FIC(b). The interactions were classified as synergistic (ΣFIC <0.5), additive (ΣFIC >0.5-1.0), indifferent (ΣFIC >1.0-4.0) or antagonistic (ΣFIC >4.0).[3,7]

Varied ratio combination studies (isobolograms)

For each combination producing synergistic interactions, nine different ratios spanning the range 10:90 (extract:antibiotic) to 90:10 (extract:antibiotic) were tested. All combinations were tested in duplicate in three independent experiments, providing six replicates for each combination ratio. The data is presented as the mean of six replicates. Data points for each ratio examined were plotted on a isobologram and this was used to determine optimal combination ratios to obtain synergy. Data points on or below the 0.5:0.5 line indicated synergy; 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 indicated indifferent interaction.[3,7]

Toxicity studies

Two assays were used to assess the toxicity of the individual samples. The *Artemia* lethality assay (ALA) was utilised for rapid preliminary toxicity screening, whereas the MTS cellular proliferation assay was used to determine a cellular evaluation of toxicity.

Artemia franciscana Kellogg nauplii toxicity screening

Toxicity of the *S. spinescens* extracts, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.[27-28] Samples providing a percentage mortality greater than 50% were considered toxic.[28] These samples were also serially diluted and tested across the concentration range 1- 0.032 mg/mL to obtain a log-sigmoid dose response curve, generated with GraphPad Prism® software (Version 5), from which the LD_{50} values were determined.

Human dermal fibroblasts (HDF) cellular viability assay

The HDF cells used in this study were from American Type Culture Collection (ATCC PCS-201-012). The *S. spinescens* extracts (200 µg/mL) and conventional antibiotics were screened individually towards normal human primary dermal fibroblasts (HDF) using standard methods.[29] Quinine (Sigma, Australia) was included on each plate as a positive control. All tests were performed in at least triplicate and triplicate controls were included on each plate. The % cellular viability of each test was calculated using the following formula:

$$\%\ \text{cellular viability} = \frac{\text{Abs test sample} - (\text{mean Abs control} - \text{mean Abs blank})}{(\text{mean Abs control} - \text{mean Abs blank})}$$

Cellular viability ≤50% of the untreated control indicated toxicity, whereas extracts or controls with >50% untreated control viability were deemed to be nontoxic.

Statistical analysis

Data is expressed as the mean ± standard deviation of at least three independent experiments. One way ANOVA was used to calculate statistical significance between the negative control and treated groups, with a P value <0.01 considered to be statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extractions of the *S. spinescens* plant materials (1.5 g) with solvents of varying polarity yielded dried plant extracts ranging from 85 mg (*S. spinescens* leaf hexane extract) to 232 mg (*S. spinescens* leaf chloroform extract) (Table 1). Qualitative phytochemical screening (Table 1) showed that the higher polarity solvents (methanol and water) extracted the greatest amount and widest diversity of phytochemical classes. Both extracts contained moderate to high levels of phenolics (water soluble phenolics only), flavanoids and tannins with lower levels of saponins present in both extracts. Alkaloids were only detected in the methanol extract by the Meyer test, and only in low relative abundance. The ethyl acetate, chloroform and hexane extracts all only had detectable levels of water insoluble phenols and only a low response was seen for each solvent.

Bacterial growth inhibition screening

Inhibition of a bacterial triggers of rheumatoid arthritis *(P. mirabilis and P. vulgaris)*

P. mirabilis growth was susceptible to all *S. spinescens* leaf extracts, although it was particularly susceptible to the higher polarity aqueous and methanolic *S. spinescens* extracts and generally less susceptible to the lower polarity extracts (Figure 1a). Indeed, zones of inhibition of approximately 11.3 ± 0.6 mm and 12.1 mm ± 0.6 mm were recorded for the methanolic against the reference and clinical *P. mirabilis* strains respectively. Similarly, 10.6 ± 0.9 mm and 9.6 ± 0.6 mm were recorded for the aqueous extract against the reference and clinical strains respectively. The *S. spinescens* leaf ethyl acetate and chloroform extracts were

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *S. scaevola* leaf extracts.

Extract	Mass of Dried Extracted Material (mg)	Conventration of ectract (mg/mL)	Phenols			Cardiac Glycosides	Saponins	Triterpenes	Phytosterols	Alkaloids		Flavanoids		Tannins	Anthraquinones	
			Total Phenolics	Water Soluble	Water Insoluble	Keller-Kiliani Test	Froth Persistence	Salkowski Test	Acetic Anhydride Test	Meyers Test	Wagners Test	Shinoda Test	Kumar test	Ferric Chloride Test	Free	Combined
Methanol	116	11.6	+++	+++	++	-	+	-	-	+	+	+++	+++	+++	-	-
Water	210	21	+++	+++	++	-	+	-	-	-	-	+++	+++	+++	-	-
Ethyl Acetate	160	16	-	-	-	-	-	-	-	-	-	+	+	+	-	-
Chloroform	232	23.2	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Hexane	85	8.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

also good inhibitors of *P. mirabilis* growth, with inhibition zones generally in the range 9-10.5 mm. In contrast, both *P. mirabilis* strains displayed only low susceptibility to the hexane extract.

P. vulgaris growth (Figure 1b) was substantially less susceptible to the *S. spinescens* leaf extracts than *P. mirabilis* was. The higher polarity methanolic *S. spinescens* leaf extract was again the best growth inhibitor, with zones of inhibition of 8.2 ± 0.3 mm. The chloroform extract was slightly less effective, with zones of inhibition of 7.4 ± 0.2 mm. All other extracts were substantially less effective *P. vulgaris* growth inhibitors, with inhibition generally zones ≤7mm. notably, this *P. vulgaris* strain was also resistant to ampicillin, with only small zones of inhibition (>7mm) recorded. In contrast, this bacterium was particularly susceptible to chloramphenicol, with a 16mm zone of inhibition.

Inhibition of a bacterial trigger of ankylosing spondylitis *(K. pneumoniae*

All *S. spinescens* leaf extractsexcept the hexane extract inhibited the growth of both *K. pneumoniae* strains (Figure 1c) albeit, generally with lower efficacy than measured for *P. mirabilis* growth inhibition. The reference bacterial strain was generally more sensitive to the *S. spinescens* leaf extracts than the clinical strain was, and the higher polarity methanolic extract was the best growth inhibitor (inhibition zones 8.5 ± 0.5 mm against the reference *K. pneumoniae* strain). Interestingly, both the reference and clinical *K. pneumoniae* strains were resistant to β-lactam antibiotics. Indeed, the ampicillin zone of inhibition was not significantly different to that of the negative control. In contrast, this bacterium was highly susceptible to chloramphenicol, with an inhibition zones>16 mm against both strains. The aqueous *S. spinescens* leaf extract was also a good inhibitor of *K. pneumoniae,* with inhibition zones of 8.3 ± 0.3 mm and 7.6 ± 0.3 mm against the reference and clinical bacterial strains respectively. The growth inhibitory activity recorded for the mid to lower polarity ethyl acetate and chloroform extracts was slightly lower than this and the hexane was completely devoid of inhibitory activity against both strains.

Inhibition of bacterial triggers of multiple sclerosis (*A. baylyi* and *P. aeruginosa*)

The aqueous *S. spinescens* leaf extract was the best inhibitor of the growth of both *A. baylyi* strains (8.5 ± 0.5 mm and 7.9± 0.3 mm respectively) (Figure 1d). Similarly, the methanol, ethyl acetate and chloroform extracts produced zones of inhibition generally >7.5 mm against both *A. baylyi* strains.Whilst the hexane extract also inhibited the growth of both *A. baylyi* strains, the small zones of inhibition (≤6.6 mm) are indicative of only weak growth inhibitory activity. In contrast, both the ampicillin (10.2 ± 0.4mm inhibition zones) and chloramphenicol (12 mm inhibition zones) controls were good inhibitors of the reference bacterial strain. In contrast, the *A. baylyi* clinical strain was substantially less susceptible to the ampicillin and chloramphenicol controls, with inhibition zones of 7.8 ± 0.4 mm and 9.6 ± 0.4 mm respectively.

All of the *S. spinescens* leaf extracts inhibited the growth of both the reference and clinical *P. aeruginosa* strains (Figure 1e). The methanolic *S. spinescens* leaf extract was the best inhibitor of *P. aeruginosa* growth, with zones of inhibition of 7.8 ± 0.4 mm and 8.3 ± 0.3 mm against the reference and clinical strains respectively. This was particularly noteworthy as the *P. aeruginosa* strain tested in this study was resistant to both the ampicillin and chloramphenicol controls, each inducing zones of inhibition generally in the range 6.5-7.5 mm. Furthermore, the aqueous, ethyl acetate and chloroform *S. spinescens* leaf extracts were also relatively good inhibitors of *P. aeruginosa* growth, each producing zones of inhibition generally in the7-8 mm range. Both *P. aeruginosa* strains were substantially less susceptible to the hexane extracts, with zones of inhibi-

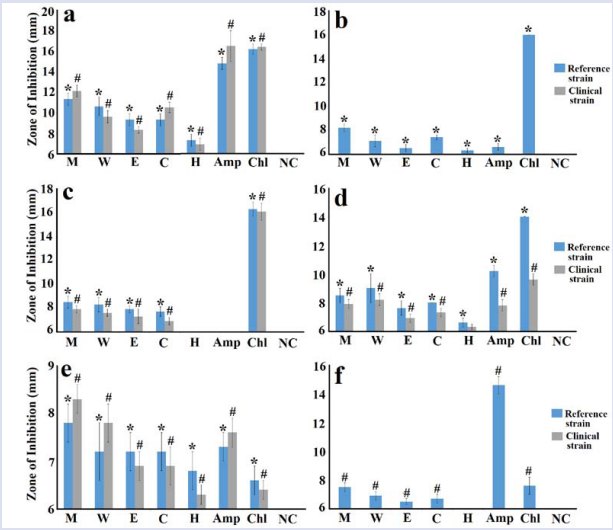

Figure 1: Antibacterial activity of *S. spinescens* leaf extracts against. (a) *P. mirabilis* (ATCC21721) and clinical isolate strains; (b) *P. vulgaris* (ATCC21719); (c) *K. pneumoniae* (ATCC31488) and clinical strain; (d)*A. baylyi* (ATCC33304) and clinical strain; (e) *P. aeruginosa* (ATCC: 39324) and clinical strain; (f) *S. pyogenes* clinical strain, measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2µg); Chl = chloramphenicol (10µg); NC = negative control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates (two repeats) ± S.D. * and # indicate results that are significantly different to the negative control (P<0.01) for the reference and clinical strains respectively.

tion of approximately 6.5 mm. Our studies therefore indicate that the methanolic and aqueous *S. spinescens* leaf extracts were the most effective inhibitors of both bacterial triggers of multiple sclerosis, although the ethyl acetate and chloroform extracts also had noteworthy growth inhibitory activity.

Inhibition of the bacterial trigger of rheumatic fever (*S. pyogenes*)

The methanolic, aqueous, ethyl acetate and chloroform *S. spinescens* leaf extracts inhibited *S. pyogenes* growth, albeit generally only with small zones of inhibition indicative of low efficacy (Figure 1f).The hexane extracts were completely devoid of antibacterial activity. The methanolic *S. spinescens* leaf extract was the strongest inhibitor of *S. pyogenes* growth, although even this extract only producedinhibition zones of 7.5± 0.3 mm. This *S. pyogenes* strain was also relatively resistant to chloramphenicol, but susceptible to ampicillin, with zones of inhibition of approximately 7.6 and 14.6 mm respectively.

Quantification of minimum inhibitory concentration (MIC)

The relative level of antimicrobial activity was further evaluated by determining the MIC values using two methods: the liquid dilution MIC assay and the disc diffusion MIC assay (Table 2). Consistent with the antibacterial screening assays, each of the higher polarity methanol and water *S. spinescens* leaf extracts inhibited all of the bacteria tested and they were more potent in comparison to the corresponding lower polarity extracts. The MIC values of the conventional antibiotic controls were only determined for the liquid dilution assay as commercial discs containing a fixed mass of antibiotic were used in the disc diffusion assay. Thus, the zones of only single doses was recorded for that assay and we were unable to determine MIC values. Gentamicin was the most potent

Table 2: Disc diffusion (DD) and liquid dilution (LD) MIC values (µg/mL) for *S. spinescens* leaf extracts against microbial triggers of some autoimmune inflammatory diseases.

Bacterial Species	Methanol Extract		Aqueous Extract		Ethyl Acetate Extract		Chloroform Extract		Hexane Extract		Controls							
											Pen		Chlor		Eryth		Tetra	Gent	NC
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	LD MIC		LD MIC		LD MIC		LD MIC	LD MIC	LD MIC
P. mirabilis **(ATCC: 33304)**	510	1000	751	1280	938	2000	3038	3000	2200	3000	2.5		1.25		3.3		2.5	0.63	-
P. mirabilis **clinical isolate**	293	575	563	675	931	2000	2878	3000	2700	3000	2.5		1.25		3.3		2.5	0.63	-
P. vulgaris **(ATCC21719)**	581	1125	880	1250	4000	4000	5400	6000	2204	3000	2.5		1.25		3.3		1.25	1.25	-
K. pneumoniae **(ATCC: 31488)**	575	836	1683	625	251	200	>10000	>10000	-	6000	3.3		1.9		1.9		1.9	0.3	-
*K. pneumoniae***clinical isolate**	575	915	1860	625	500	320	>10000	>10000	-	6000	2.5		1.9		1.6		1.9	0.3	-
A. baylyi **(ATCC: 21721)**	983	620	1133	410	426	617	1750	>10000	2766	3000	3.3		2.5		2.5		1.25	1.25	-
*A. baylyi***clinical isolate**	725	408	988	325	400	553	2750	>10000	1567	2500	3.3		3.3		2.5		1.25	1.25	-
P. aeruginosa **(ATCC: 39324)**	200	184	1432	781	440	200	642	600	1255	3000	3.3		1.25		3.3		1.25	1.25	-
P. aeruginosa **clinical isolate**	425	568	1653	1896	523	200	1318	1600	1527	3000	3.3		1.25		3.3		2.5	1.25	-
S. pyogenes **clinical isolate**	3250	1562	4018	2575	1693	1358	5750	5521	-	3000 -	3.3		2.5		3.3		2.5	0.63	-

M = methanol extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane; DD = disc diffusion; LD = liquid dilution; Pen = penicillin-G; Chlor = chloramphenicol; Eryth = erythromycin; Tetra = tetracycline; Gent = gentamycin. - indicates no inhibition at any dose tested.

Table 3: MIC (µg/ml) and ∑FIC values for the *S. spinescens* extract: antibiotic combinations, against some bacterial triggers of selected autoimmune inflammatory diseases.

	P.mirabilis				*P. vulgaris*		*K. pneumoniae*				*A. baylyi*				*P. aeruginosa*				*S. pyogenes*	
	ATCC33304		Clinical isolate		ATCC21719		ATCC31488		Clinical isolate		ATCC21721		Clinical isolate		ATCC39324		Clinical isolate		Clinical isolate	
	MIC	ΣFIC (Int)	MIC	ΣFIC (Int)	MIC	ΣFIC (Int)	MIC	ΣFIC (Int)	MIC	ΣFIC (Int)	MIC	ΣFIC (Int)	MIC	ΣFIC (Int)	MIC	ΣFIC (Int)	MIC	ΣFIC (Int)	MIC	ΣFIC (Int)
M + Pen	625	1.25 (IND)	288	1.0 (ADD)	563	1.0 (ADD)	418	1.0 (ADD)	458	1.0 (ADD)	465	1.5 (IND)	204	1.0 (ADD)	138	1.5 (IND)	284	1.0 (ADD)	1953	2.5 (IND)
M + Chlor	750	1.5 (IND)	359	1.25 (IND)	703	1.25 (IND)	1045	2.5 (IND)	1144	2.5 (IND)	465	1.5 (IND)	204	1.0 (ADD)	138	1.5 (IND)	284	1.0 (ADD)	1953	2.5 (IND)
M + Eryth	750	1.5 (IND)	288	1.0 (ADD)	563	1.0 (ADD)	418	1.0 (ADD)	458	1.0 (ADD)	310	1.0 (ADD)	204	1.0 (ADD)	138	1.5 (IND)	284	1.0 (ADD)	1953	2.5 (IND)
M + Tetra	375	0.75 (ADD)	216	0.75 (ADD)	563	1.0 (ADD)	443	1.06 (ADD)	197	**0.43 (SYN)**	657	2.12 (IND)	408	2.0 (IND)	161	1.75 (IND)	355	1.25 (IND)	484	0.62 (ADD)
M + Gent	750	1.5 (IND)	288	1.0 (ADD)	563	1.0 (ADD)	418	1.0 (ADD)	458	1.0 (ADD)	310	1.0 (ADD)	204	1.0 (ADD)	138	1.5 (IND)	284	1.0 (ADD)	1953	2.5 (IND)
W + Pen	800	1.25 (IND)	338	1.0 (IND)	625	1.0 (ADD)	469	1.5 (IND)	469	1.5 (IND)	308	1.5 (IND)	163	1.0 (ADD)	976	2.5 (IND)	1422	1.5 (IND)	1931	1.5 (IND)
W + Chlor	960	1.5 (IND)	422	1.25 (IND)	344	0.55 (ADD)	938	2.0 (IND)	469	1.5 (IND)	308	1.5 (IND)	163	1.0 (ADD)	488	1.25 (IND)	1422	1.5 (IND)	1931	1.5 (IND)
W + Eryth	960	1.5 (IND)	338	1.0 (ADD)	625	1.0 (ADD)	469	1.5 (IND)	469	1.5 (IND)	205	1.0 (ADD)	163	1.0 (ADD)	488	1.25 (IND)	1422	1.5 (IND)	1931	1.5 (IND)
W + Tetra	960	1.5 (IND)	253	0.75 (ADD)	625	1.0 6(ADD)	234	0.75 (ADD)	144	**0.46 (SYN)**	435	2.12 (IND)	167	1.03 (IND)	781	2.0 (IND)	1422	1.5 (IND)	1442	1.12 (IND)
W + Gent	960	1.5 (IND)	338	1.0 (ADD)	625	1.0 (ADD)	469	1.5 (IND)	469	1.5 (IND)	205	1.0 (ADD)	163	1.0 (ADD)	488	1.25 (IND)	1422	1.5 (IND)	1931	1.5 (IND)
E + Pen	1120	1.12 (IND)	1000	1.0 (ADD)	2000	1.0 (ADD)	100	1.0 (ADD)	160	1.0 (ADD)	463	1.5 (IND)	277	1.0 (ADD)	100	1.0 (ADD)	150	1.5 (IND)	679	1.0 (ADD)
E + Chlor	1500	1.5 (IND)	1250	1.25 (IND)	2500	1.25 (IND)	102	1.02 (IND)	99	0.62 (ADDD)	463	1.5 (IND)	277	1.0 (ADD)	100	1.0 (ADD)	150	1.5 (IND)	679	1.0 (ADD)
E + Eryth	1500	1.5 (IND)	1000	1.0 (ADD)	2000	1.0 (ADD)	100	1.0 (ADD)	160	1.0 (ADD)	309	1.0 (ADD)	277	1.0 (ADD)	100	1.0 (ADD)	150	1.5 (IND)	679	1.0 (ADD)
E + Tetra	1500	1.5 (IND)	750	0.75 (ADD)	2000	1.0 (ADD)	100	1.0 (ADD)	56	**0.35 (SYN)**	654	2.12 (IND)	570	2.06 (IND)	125	1.25 (IND)	150	1.5 (IND)	720	1.06 (IND)
E + Gent	1500	1.5 (IND)	1000	1.0 (ADD)	2000	1.0 (ADD)	100	1.0 (ADD)	160	1.0 (ADD)	309	1.0 (ADD)	277	1.0 (ADD)	100	1.0 (ADD)	100	1.0 (ADD)	679	1.0 (ADD)
C + Pen	3375	2.25 (IND)	1500	1.0 (ADD)	3000	1.0 (ADD)	>5000	1.0 (ADD)	>5000	1.0 (ADD)	>5000	1.5 (IND)	>5000	1.0 (ADD)	300	1.0 (ADD)	800	1.0 (ADD)	2761	1.0 (ADD)
C + Chlor	2250	1.5 (IND)	1875	1.25 (IND)	3750	1.25 (IND)	>5000	2.5 (IND)	>5000	1.25 (IND)	>5000	1.5 (IND)	>5000	1.0 (ADD)	300	1.0 (ADD)	800	1.0 (ADD)	2761	1.0 (ADD)
C + Eryth	1500	1.0 (ADD)	1500	1.0 (ADD)	3000	1.0 (ADD)	>5000	1.0 (ADD)	>5000	1.0 (ADD)	>5000	1.0 (ADD)	>5000	1.0 (ADD)	300	1.0 (ADD)	800	1.0 (ADD)	2761	1.0 (ADD)
C + Tetra	1500	1.0 (ADD)	1500	1.0 (ADD)	3000	1.0 (ADD)	>5000	1.06 (ADD)	>5000	1.06 (IND)	>5000	2.12 (IND)	>5000	2.06 (IND)	750	2.5 (IND)	1000	1.25 (IND)	2926	1.06 (IND)
C + Gent	1500	1.0 (ADD)	1500	1.0 (ADD)	3000	1.0 (ADD)	>5000	1.0 (ADD)	>5000	1.0 (ADD)	>5000	1.0 (ADD)	>5000	1.0 (ADD)	300	1.0 (ADD)	800	1.0 (ADD)	2761	1.0 (ADD)
H + Pen	1875	1.25(IND)	1500	1.0 (ADD)	1500	1.0 (ADD)	3000	1.0 (ADD)	3000	1.0 (ADD)	2250	1.5 (IND)	1250	1.0 (ADD)	1500	1.0 (ADD)	1500	1.0 (ADD)	1500	1.0 (ADD)
H + Chlor	2250	1.5 (IND)	1875	1.25 (IND)	1875	1.25 (IND)	>5000	2.5 (IND)	3750	1.25 (IND)	2250	1.5 (IND)	1250	1. (ADD)	1500	1.0 (ADD)	1500	1.0 (ADD)	1500	1.0 (ADD)
H + Eryth	1500	1.0 (ADD)	1500	1.0 (ADD)	1500	1.0 (ADD)	3000	1.0 (ADD)	3000	1.0 (ADD)	1500	1.0 (ADD)	1250	1.0 (ADD)	1500	1.0 (ADD)	1500	1.0 (ADD)	1500	1.0 (ADD)
H + Tetra	1500	1.0 (ADD)	1500	1.0 (ADD)	1500	1.0 (ADD)	3180	1.06 (IND)	3180	1.06 (IND)	3180	2.12 (IND)	2575	2.06 (IND)	3750	2.5 (IND)	1875	1.25 (IND)	675	**0.45 (SYN)**
H + Gent	1500	1.0 (ADD)	1500	1.0 (ADD)	1500	1.0 (ADD)	3000	1.0 (ADD)	3000	1.0 (ADD)	1500	1.0 (ADD)	1250	1.0 (ADD)	1500	1.0 (ADD)	1500	1.0 (ADD)	1500	1.0 (ADD)

M = methanolic extract; W = aqueous extract; E = ethyl acetate extract C = chloroform extract; H = hexane extract; Pen = penicillin-G; Chlor = chloramphenicol; Eryth = erythromycin; Tetracycline = tetracycline; Gent = gentamycine; Int. = Interaction; **SYN = synergistic interaction**; ADD = additive interaction; IND = indifferent interaction; *ANT* = antagonistic interaction.

antibiotic (as judged by its MIC). Indeed, most of the bacterial strains tested were partially resistant to all of the conventional antibiotics except gentamycin. The *A. baylyi* and *P. aeruginosa* reference and clinical strainswerealso resistant to gentamicin, making them partially resistant to all of the antibiotics tested.

The MIC values determined for the *S. spinescens* leaf extracts compare relatively well between the disc diffusion and liquid dilution assays. The growth of *P. mirabilis* was relatively strongly inhibited by the methanolic and aqueous *S. spinescens* leaf extracts, with MIC's≤1000 µgmL against both the reference and clinical bacterial strains. The ethyl acetate extract chloroform and hexane *S. spinescens* leaf extracts each inhibited *P. mirabilis* growth with low activity (generally ≤2000 µg/mL). *P. vulgaris* was substantially more resistant to the *S. spinescens* leaf extracts, with substantially higher MIC values than determined against *P. mirabilis*.

The methanolic, aqueous and ethyl acetate *S. spinescens* leaf extracts were also good inibitors of *K. punemoniae* growth with MIC values substantially <1000 µg/mL against both the reference and clinical *K. pnneumoniae* strains. The ethyl acetate extract was a particularly potent inhibitor of the growth of the clinical *K. pneumoniae* strain with LD MIC values of 200 and 320 µg/mL against the reference and clinical strains respectively. The methanolic, aqueous and ethyl acetate extracts were moderate to good inhibitors of *A. baylyi* growth, with MIC values of 408, 325 and 553 µg/mL against the clinical bacterial strain, and slightly higher MIC values determined against the reference strain. The methanolic and ethyl acetate extracts were the strongest inhibitors of *P. aeruginosa* growth, with MIC values of 184 and 200 µg/mL respectively determined against there ference bacterial strain. The aqueous and chloroform extracts (LD MIC's of 781 µg/mL and 600 µg/mL against the reference *P. aeruginosa* strain respectively) were also a good inhibitors of *P. aeruginosa* growth. Interestingly, the clinical *P. aeruginosa* strains was substantially more resistant to the *S. spinescens* leaf extracts, with MIC values against this strain generally more than double the MIC values determined for the reference strain. In contrast, the MIC values determined for the *S. spinescens* leaf extracts against *S. pyogenes* (generally ≥1500 µg/mL) are indicative of only low to moderate growth inhibitory activity.

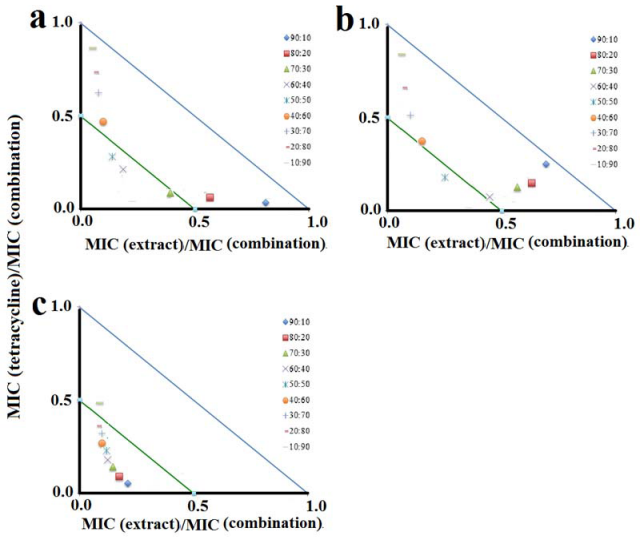

Figure 2: Isobologram for combinations of tetracycline with S. spinescens leaf.

(a) methanolic extract, (b) aqueous extract and (c) ethyl acetate extract, tested at various ratios against the clinical strain of K. pneumoniae. Results represent mean MIC values of four replicates. Ratio = % extract: % antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic (Σ FIC < 0.5). Any points between the 0.5:0.5 and 1.0:1.0 lines are deemed additive (Σ FIC > 0.5-1.0).

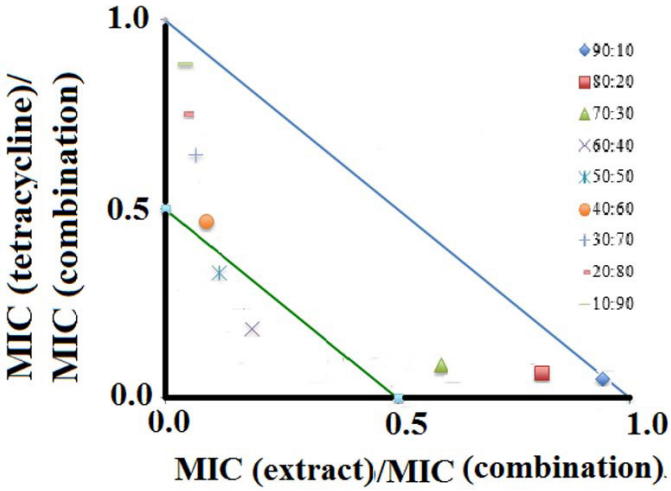

Figure 3: Isobologram for combinations of tetracycline with *S. spinescens* leaf hexane extract tested at various ratios against the clinical strain of *S. pyogenes*. Results represent mean MIC values of four replicates. Ratio = % extract: % antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic (Σ FIC ≤ 0.5). Any points between the 0.5:0.5 and 1.0:1.0 lines are deemed additive (Σ FIC > 0.5-1.0).

Fractional inhibitory concentration (FIC) assessment

Combinational effects on a bacterial trigger of rheumatoid arthritis *(Proteus spp.)*

Twenty six (52%) of the *S. spinescens* leaf extracts and conventional antibiotic combinations produced additive effects when tested against the reference and clinical *P. mirabilis* strains (Table 3). Similarly, 14 combinations (56%) also produced additive interactions when tested against *P. vulgaris*. As these combinations produce effects greater than either the individual extract or conventional antibiotic components, they would be beneficial in the prevention and treatment of rheumatoid arthritis. All of the other combinations produced indifferent interactions. Whilst these combinations provide no added benefit over that of the individual components alone, the components do not antagonise each other's effects and are therefore safe to use concurrently without risk of lessening the efficacy of either component.

Combinational effects on a bacterial trigger of ankylosing spondylitis *(K. pneumoniae)*

A variety of combinational effects were noted when extract-antibiotic combinations were tested against *K. pneumoniae*. Interestingly, three synergistic interactions were noted when tetracycline was combined with either the methanolic, aqueous or ethyl acetate extracts. Interestingly, all of the synergistic combinations against *K. pneumoniae* were against the clinical strain of the bacterium.These combinations were potent inhibitors of *K. pneumoniae* growth. Combinations of tetracycline with the *S. spinescens* methanolic, aqueous and ethyl acetate extracts yielded MIC values of 56 µg/mL respectively against the clinical *K. pneumoniae* strain. The majority (58%) of the combinations were additive. Thus, these combinations would also be beneficial in the prevention and treatment of ankylosing spondylitis (and other *K. pneumoniae* infections) as they increase the efficacy of the therapy without either component antagonising the effects of the other component.The remaining 18 combinations (36%) produced indifferent effects. Whilst no added benefit would be gained from using these combinations, it is safe to use these extracts and antibiotics together without compromising the activity of either component.

Table 4: Mortality (%) and cellular viability (%) results for extracts and conventional antibiotics tested individually and as combinations in the ALA and MTS cell viability assays respectively

	Sample	Mortality ± SD (%) [a]		Cell viability ± SD (%) [b]
		After 24 hrs:	After 48 hrs:	After 24 hrs:
Antimicrobials	Penicillin G	1.8 ± 1.4	4.3 ± 2.4	98.3 ± 3.4
	Chloranphenicol	2.7 ± 1.3	5.6 ± 3.3	102.2 ± 3.7
	Erythromycin	1.2 ± 0.6	5.8 ± 2.3	97.7 ± 5.5
	Tetracycline	2.4 ± 1.5	5.1 ± 2.8	95.8 ± 4.7
	Gentamicin	3.1 ± 1.8	6.7 ± 2.6	94.7 ± 4.6
Extracts	M	5.1 ± 1.8	11.6 ± 2.5	93.7 ± 6.5
	W	4.3 ± 2.0	10.4 ± 3.2	95.6 ± 5.8
	E	2.9 ± 1.6	8.8 ± 3.5	102.5 ± 4.2
	C	4.7 ± 2.5	11.0 ± 3.9	93.1.8 ± 5.5
	H	3.3 ± 1.6	6.4 ± 3.2	101.9±4.6
Combinations	M + Penicillin G	3.5 ± 2.0	9.7 ± 3.8	91.2 ± 5.9
	M + Chloramphenicol	7.1 ± 4.6	17.8. ± 4.4	86.7 ± 5.5
	M + Erythromycin	3.8 ± 2.9	10.5 ± 3.7	89.9 ± 4.7
	M + Tetracycline	6.3 ± 2.7	13.4 ± 4.4	87.8 ± 4.0
	M + Gentamicin	8.3 ± 3.5	22.6 ± 5.1	82.9 ± 5.5
	W + Penicillin G	4.7 ± 3.1	10.6 ± 39	98.6 ± 5.3
	W + Chloramphenicol	5.5 ± 2.7	12.7 ± 3.8	91.4 ± 5.6
	W + Erythromycin	3.9 ± 2.9	9.4 ± 3.5	94.3 ± 3.8
	W + Tetracycline	7.7 ± 3.5	15.6 ± 4.2	89.8 ± 4.1
	W + Gentamicin	8.5 ± 4.0	19.2 ± 3.8	86.4 ± 5.1
	E + Penicillin G	2.2 ± 1.0	8.5 ± 3.6	103.4 ± 4.7
	E + Chloramphenicol	4.6 ± 3.2	10.5 ± 3.8	93.8 ± 4.0
	E + Erythromycin	4.0 ± 2.8	9.7 ± 4.5	97.1 ± 5.7
	E + Tetracycline	5.8 ± 4.0	12.7 ± 5.3	91.2.2 ± 3.3
	E + Gentamicin	9.2 ± 4.6	26.5 ± 4.2	85.4 ± 4.6
	C + Penicillin G	4.2 ± 3.7	9.3 ± 2.7	93.9 ± 4.4
	C + Chloramphenicol	6.2 ± 3.9	15.2 ± 4.0	87.8.2 ± 5.2
	C + Erythromycin	3.2 ± 1.7	7.8 ± 3.8	103.7 ± 4.6
	C + Tetracycline	6.8 ± 3.5	16.2 ± 3.9	88.4.6 ± 4.6
	C + Gentamicin	9.0 ± 4.0	22.6 ± 4.1	85.7 ± 7.2
	H + Penicillin G	2.5 ± 2.0	8.8 ± 4.2	101.9 ± 3.8
	H + Chloramphenicol	5.1 ± 2.4	11.4 ± 3.5	95.8 ± 5.4
	H + Erythromycin	4.4 ± 3.2	10.5 ± 3.5	95.7± 6.0
	H + Tetracycline	6.8 ± 2.8	14.7 ± 3.8	92.2 ± 4.7
	H + Gentamicin	7.5 ± 3.6	11.7 ± 4.0	93.6 ± 5.3
Controls	Deionised water	2.7 ± 1.7	3.6 ± 2.5	96.8 ± 5.7
	Quinine	2.3 ± 1.1[a]	4.6 ± 2.7a	31.4 ± 4.8 [b]
	Potassium dichromate	100.00 ± 0.00 [a]		NT

a = mortality in the Artemia nauplii assay b = cell viability in the HDF assay; NT = not tested.

Combinational effects on bacterial triggers of multiple sclerosis *(A. baylyi* and *P. aeruginosa)*

No synergistic interactions were detected between the *S. spinescens* leaf extracts and conventional antibiotics against *A. baylyi* (Table 3). Thirty (60%) of the combinations were additive and would therefore be beneficial in preventing and treating *A. baylyi* infections. Of the additive combinations, those containing the aqueous extract were generally the strongest *A. baylyi* growth inhibitors,with MIC values of approximately 160 µg/mL for combinations with all conventional antibiotics against the clinical *A. baylyi* strain. Indifferent interactions accounted for a further 20 (40%) of the combinations. Whilst no additional benefit would be gained by taking these therapies concurrently, they would not reduce each other's efficacy.

Neither synergistic nor antagonistic interactions were detected against the reference and clinical strains of *P. aeruginosa* (Table 3). All interactions against these bacterial strains were either addivive or indifferent. ΣFIC values indicative of additive interactions were determined for twenty five of the combinations (50%). Several of these had low MIC values and would therefore be particularly beneficial in the prevention and treatment of *P. aeruginosa* associated disease, including multiple sclerosis. Indeed, MIC values generally 100-300 µg/mL were calculated for the methanolic and ethyl acetate *S. spinescens* leaf extracts in combination with all the conventional antibiotics against both the reference and clinical *P. aeruginosa* strains. Similarly, combinations containing the aqueous extract were also good inhibitors of the reference *P. aeruginosa* growth, albeit with slightly higher MIC values (500-1000 µg/mL for most combinations). All of these combinations have enhanced efficacy and would be beneficial for inhibiting *P. aeruginosa* growth. The remaining twenty five (50%) of the combinations produced indifferent combinational effects. These therapies could therefore be used concurrently without decreasing the efficacy of either component, although no added benefit would be obtainedfrom these combinations.

Combinational effects on a bacterial trigger of rheumatic fever *(S. pyogenes)*

The interactive antimicrobial interactions of *S. spinescens* leaf extracts with the conventional antibiotics against *S. pyogenes* are summarised in Table 3. One combination (tetracycline in combination with thehexane extract) produced a synergistic effect. The decreased MIC value of this combination (675 µg/mL) compared to the extracts when tested alonehighlights the potential of this combination in treating *S.pyogenes* induced diseases, including rheumatic fever. Thirteen (52%) further combinations were categorised as being additive interactions, and thus may also be beneficial in treating *S. pyogenes* infections. All other combinations (44%) were classified as being indifferent.

Varied ratio combination studies (isobolograms)

Synergistic interactions against a bacterial trigger of ankylosing spondylitis

Three synergistic combinations were detected against the clinical strain of *K. pneumoniae*. Notably, all of these combinations included tetracycline as the conventional antibiotic component. These combinations were further examined using isobologram analysis across a range of extract:tetracycline ratios to identify the ideal ratios to obtain synergy (Figure 2). In each isobologram, the data more closely aligned with the y (antibiotic) axis, indicating that the growth inhibitory activity was mainly due to tetracycline and the extract was potentiating its activity. For combinations containing the methanolic (Figure 2a) and aqueous extracts (Figure 2b), the range of combination ratios inducing synergistic interactions was narrow. Indeed, for combinations containing the aqueous extract, only the 50% extract, 50% tetracycline combination was synergistic. Similarly, only combinations containing 50% or 60% of the methanolic extract were synergistic whilst all other combination ratios produced additive interactions.In contrast, nearly all of the ethyl acetate *S. spinescens* leaf extract synergised the activity of tetracycline, even at low ratios (Figure 2c). Indeed, all combinations containing ≥20% ethyl acetate *S. spinescens* leaf extract resulted in synergy, whilst the ratio containing 10% produced additive effects and would thus also be beneficial for inhibiting *K. pneumoniae* growth. This is a particularly interesting result as the ability for the extract to synergise the antibiotic, even at very low levels, may indicate that an extract component(s) may be functioning via irreversible mechanisms (Ilanka *et al*. 2018; Cheesman *etal*. 2017). Thus, all ratios containing ≥20% ethyl acetate *S. spinescens* leaf extract would be beneficial to enhance *K. pneumoniae* growth inhibition. However, bacteria would be less likely to develop resistance when combinations are used in ratios which minimise the amount of conventional antibiotic used. Thus, for long term prophylactic treatment (as would be required to prevent and treat ankylosing spondylitis), the ideal extract:tetracycline ratio may be 90:10. However, when used for the treatment of acute infections, the ratio which maximises the efficacy of the treatment (i.e. the 20:80 ratio) may be the preferred option.

Synergistic interactions against a bacterial trigger of rheumatic fever

The hexane *S. spinescens* leaf extract induced synergistic interactions in combination with tetracyclineagainst *S. pyogenes* growth inhibition (Figure 3). Only combinations containing 50or 60% of the*S. spinescens* hexane leaf extract produced synergistic inhibition against *S. pyogenes*-growth. All other ratios of the hexane extract and tetracycline produced additive interactions. As all ratios of these extracts potentiated the activity of tetracycline, all combinations would be beneficial to inhibit *S. pyogenes* growth. However, the synergistic interactions would provide maximal benefit. For long term prophylactic treatment, the combination containing 60 % hexane extract and 40% tetracycline may be the ideal ratio as this ratio minimises the level of tetracycline and thus decreases the possibility of developing further resistance to the antibiotic.

Toxicity studies

Two assays (ALA and the MTS cell viability assay) were used to assess the toxicity of the individual extracts and conventional antibiotics, as well as extract – antibiotic combinations. The ALA was undertaken for the preliminary toxicity screening whilst the MTS cell viability assay provided a cellular evaluation of toxicity.

Artemia lethality assay (ALA)

All plant extracts and antibiotics were individually screened at 1 mg/mL in the assay. The extracts were only considered toxic if they induced percentage mortalities greater than 50% (LD_{50}) following 24 h of exposure to the *Artemia* nauplii.[28] When tested individually, the antibiotics demonstrated no toxicity in the ALA (Table 4). Similarly, none of the *S. spinescens* extracts produced mortality or cell viability significantly different to that of the negative control.When tested together in the ALA, none of the extract-antibiotic combinations produced mortality significantly different to the negative controls, and no single component or combination induced >50% mortality. Therefore, all combinations and individual components were deemed nontoxic. In contrast, the positive control potassium dicrhromate induced 100% mortality in the ALA.

MTS cell viability assay

The plant extracts and conventional antibiotics were also each individually screened at 200 µg/mL against HDF in the cell viability assay. In this assay, extracts which produce <50% cell at 200 µg/mL are deemed to be toxic.[29] None of the extracts or conventional antibiotics displayed <50%HDF viability and thus all were deemed to be non-toxic (Table 4). Similarly, all combinations provided substantially >50% cell viability and

were thus also deemed to be toxic. In contrast, exposure to the positive control (quinine) reduced HDF cell viability by approximately 70%.

DISCUSSION

This study investigated the ability of *S. spinescens* extracts to inhibit the growth of some bacterial triggers of autoimmune inflammatory diseases, both alone and in combination with conventional antibiotics. Several *S. spinescens* extracts were identified as effective bacterial growth inhibitors. The ethyl acetate extract was a particularly good growth inhibitor of *K. pneumoniae, A. baylyi* and *P. aeruginosa* (MIC values generally <500 µg/mL), but was substantially less potent against *Proteus* spp. and *S. pyogenes*. However, the MIC values of most of the *S. spinescens* extracts was substantially above 1000 µg/mL and are thus indicative of only low to moderate inhibitory activity. This antibacterial profile is consistent with that of a previous study from our group which also reported moderate to good growth inhibitory activity against a panel of bacterial species, and substantially lower inhibitory activity against other species.[18] Our previous study only measured antibacterial efficacy using a disc diffusion MIC assay. Disc diffusion assays often underestimate the antibacterial efficacy of plant extracts for several reasons:[30] As the disc diffusion method is reliant on the diffusion of a molecule through the aqueous environment of an agar gel, the results may be affected by the solubility of the extract compounds in the aqueous environment. Polar compounds that are highly soluble in water would be expected to diffuse easily in the gel, whereas less soluble compounds would not diffuse as readily and thus be concentrated around the disc. Diffusion of extract molecules within a gel is also affected by the size of those molecules. Thus the diffusion of large, complex phytochemicals (eg. complex tannins) within agar gels would also be retarded and may provide a false idea of the efficacy of an extract. In the current study, we also determined antibacterial efficacy of the *S. spinescens* leaf extracts using a liquid dilution MIC assay as this is considered to be a more accurate and reproducible method.[26] Furthermore, as this method is perhaps most widely used to quantify antibiotic strength, it allows for comparison between studies.

Whilst a detailed investigation of the phytochemistry of the *S. spinescens* extracts was beyond the scope of this study, the qualitative phytochemical studies highlighted several phytochemical classes that may contribute to the bacterial growth inhibitory activity. The methanolic and aqueous extracts had relatively high abundances in polyphenolics, flavonoids and tannins. The ethyl acetate extract contained similar classes of compounds, albeit at lower relative abundances. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.[31] This has been attributed to a variety of mechanisms, including their ability to complex with extracellular and soluble proteins and as well as bacterial cell walls.[32]Similarly, multiple tannins have broad spectrum antibacterial activity via a variety of intra- and extracellular mechanisms, including the precipitation of microbial proteins.[33] Phenolics are toxic to microorganisms via enzyme inhibition mechanisms, possibly through non-specific interaction with proteins or by reaction with sulfhydryl groups.[34] It is also likely that other phytochemical classes may also contribute to the growth inhibitory properties of these extracts. Therefore, phytochemical evaluation studies and bioactivity driven isolation of active components are required to evaluate the mechanism of the *S. spinescens* extracts growth inhibition. The combinational studies with conventional antibiotics were perhaps of greater interest. Several combinations displayed substantially greater potential as therapeutic agents against bacterial triggers of ankylosing spondylitis and rheumatic fever than the extracts or antibiotics did alone. Four synergistic combinations were identified in this study, with three synergistic combinations noted against both *K. pneumoniae* and a single synergistic combination against *S. pyogenes*. Notably, all of these combinations contained tetracycline as the conventional antibiotic component. The implications of a synergistic interaction include enhanced efficacy, thereby allowing lower dose administration, with reduced side effects and possibly reduced antimicrobial resistance, or conversely greater efficacy with administration of the same dosage.[8] Notably, both of these bacteria were initially resistant to tetracycline. Thus, this study identified combinations of plant extracts and antibiotics which may repurpose tetracycline and greatly enhance its efficacy, even against otherwise resistant bacterial strains. All of the *S. spinescens* leaf extracts in synergistic combinations were mid-high polarity (methanolic, aqueous or ethyl acetate) suggesting the presence of a common active compound or class of compounds that may be responsible for the synergistic effects. Furthermore, the ethyl acetate extract-tetracycline combination induced synergy at nearly all ratios of extract:antibiotic. This is surprising as previous studies with other plant species generally report that different ratios tend to provide a mix of interactions, generally with additive, indifferent and a few synergistic interactions.[3] Such a trend is consistent with irreversible mechanisms such as those of clavulanic acid/β-lactam antibiotic combinations[1] and future studies will aim at testing the synergistic mechanism and whether it is due to irreversible events. In contrast, all other synergistic extract-tetracycline combinations produced a wider range of interactions, including synergistic and additive interactions. This is more consistent with reversible competition between the extract component(s) and the conventional antibiotic for binding to an effector.[35]

Microbes have developed numerous resistance mechanisms to avoid the effects of antibiotics. One main method is through the use of multi-drug resistant (MDR) efflux pumps which are encoded chromosomally and utilized to rapidly remove antibiotics that have entered the bacterial cells, thus rendering them resistant to the effects of the antibiotic.[1] A single pump may allow the bacteria to escape several types of antimicrobials. When these efflux pumps are inhibited, the intracellular concentration of antibiotic will increase, allowing the treatment to once again be effective. Interestingly, many plants possess MDR pump inhibitors in order to enhance the activity of their own natural antimicrobial compounds. Such MDR pump inhibitors become great tools when used in combination with some previously ineffective/resistance prone antibiotic compounds.[1] Surprisingly, there are currently no EPI/antimicrobial drug combinations on the market and much more research is needed in this area. Synergy was only detected in this study in combinations containing tetracycline, against tetracycline resistant bacteria. Efflux pumps are the main bacterial resistance mechanism which renders tetracycline inactive.[36] A total of nine multidrug efflux systems have been identified including Tet (A), a potent tetracycline efflux protein.[36] It is therefore possible that *S. spinescens* extract component(s) may be inhibiting one or more tetracycline efflux pumps, thereby blocking tetracycline efflux from the cell and allowing the antibiotic to inhibit bacterial protein synthesis. However, further studies are required to confirm this.

The preparation and usage of combinations of *S. spinescens* extract/compound with conventional antibiotic will depend on the nature of the pathogen and of the disease treated. In general, combinations of antibiotic with pure *S. spinescens* derived compounds would be preferred for acute infections as they are much less complex, easier to standardize and have lower chances of unwanted side effects. The use of crude extracts in these preparations is also effective and may still be acceptable to treat some diseases. However, when treating chronic illness, or using a combinational approach to prevent illness (as would be required in preventing autoimmune inflammatory diseases), the use of a pure potentiator compound in combination with the antibiotic may not be desirable. Continuous exposure of bacteria to a pure antibiotic (or to a combination of a single antibiotic and single potentiator) is likely to induce resistance to one or both of the compounds in the bacteria. Indeed, some *E.coli* strains

are now resistant to amoxicillin-clavulanic acid combinations.[1] However, crude plant extracts often contain numerous antibacterial compounds which may affect multiple bacterial targets. Thus, using a plant extract (rather than pure plant compounds) in combination with an antibiotic is less likely to result in resistant bacteria. Indeed, we were unable to find any reports of any bacteria developing resistance to a crude plant extract. For this reason, when recommending preferred combination ratios throughout this study, we have recommended two different ratios for acute and chronic conditions. The lowest extract:highest antibiotic ratio which produced synergy has been deemed as the ideal ratio for treating acute bacterial infections, whilst we deemed the highest extract:lowest antibiotic ratio which produced synergy to be preferred for preventing and treating chronic disease

All of the combinations which were not synergistic were either indifferent or additive interactions. Although these combinations did not provide any significant benefit by enhancing the efficacy of the antibiotics, they also did not antagonise the antibiotic. Thus, co-administration of the extracts with the conventional antibiotics in these combinations will not lessen the efficacy of the conventional therapies. This is important information as many individuals self-medicate with herbal and traditional medicines and it is therefore important to understand how these medicines interact. There is a common misconception amongst consumers that all natural products are safe. However, like synthetic drugs, natural products may induce severe interactions and are not devoid of toxicity.[37-38] Natural product combinational studies generally focus on the efficacy of the drug and drug combinations, and identification of possible toxic effects of these combinations has been neglected. Despite extensive studies reporting interactions between herbal medicines and natural products when used with conventional antimicrobials,[14] there are limited reports of interactions between traditional medicinal plants and conventional antimicrobials. Interactions between such combinations may have considerable effects on the efficacy of conventional treatment regimens, as many patients do not report their concurrent usage of traditional medicines to their healthcare providers. Hence, a comprehensive investigation of these interactions is warranted for any plant material with therapeutic uses. None of the *S. scaevola* leaf extracts or conventional antibiotics, displayed toxicity in either the ALA or HDF MTS assays. Similarly, all combinations were nontoxic in both assays, confirming their potential for therapeutic use. The non-toxicity of the conventional antibiotics is hardly surprising as these drugs have a long history of therapeutic use and their lack of toxicity has previously been verified in clinical trials. The lack of toxicity determined for the *S. spinescens* leaf extracts may perhaps also not be surprising as this plant has been used by Australian Aborigines therapeutically for perhaps thousands of years. However, there is a relative lack of prior reports of rigorous toxicity studies for *S. spinescens*. Whilst the lack of toxicity detected for the combinations indicate their potential for therapeutic usage, further *in vitro* studies using other human cell lines are required to verify their safety. Furthermore, *in vivo* testing is also required to confirm that the extracts and combinations retain efficacy and remain nontoxic in complex biological systems.

CONCLUSION

The majority of the conventional antibiotic and *S. spinescens* leaf extract combinations demonstrated additive orindifferent interactions. Whilst these combinations may have limited added benefit compared with using the conventional antibiotic (or extract) alone, they do alleviate some concerns related to concurrent use of the two forms of healthcare as these interactions indicate that neither therapy is reducing the efficacy of the other therapy. Synergy was seen for 4 of the antimicrobial: medicinal plant combinations studied. The implications of these synergistic interaction include enhanced efficacy, thereby allowing lower doses to be administered, thus reducing any side effects of the chemotherapy. Of further benefit, bacterial exposure to lower levels of the conventional antibiotics would decrease the induction of further antibiotic resistance.[1] Whilst the findings reported here indicate the potential of *S. spinescens* leaf extracts (particularly in combination with tetracycline) as preventative and therapeutic options against bacterial triggers of some autoimmune inflammatory diseases, further *in vivo* investigations are required to support these *in vitro* findings. Furthermore, studies to determine the possible mechanism of action resulting in the observed interaction are warranted, and bioactivity driven compound isolation and/or metabolomics studies are also required to determine the active compound(s), as well as those responsible for the antibiotic potentiation, within the *S. spinescens* leaves.

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CONFLICT OF INTEREST

Both authors report no conflicts of interest.

ABBREVIATIONS

ALA: Brine-shrimp lethality assay; **DMSO**: Dimethyl sulfoxide; **HDF:** Human dermal fibroblasts; **INT**: ρ-iodonitrotetrazolium chloride; **LD_{50}**: Dose of sample necessary to have a lethal effect on 50% of test organisms or cells; **MIC**: Minimum inhibitory concentration; **ΣFIC:** The sum of the fractional inhibitory concentration.

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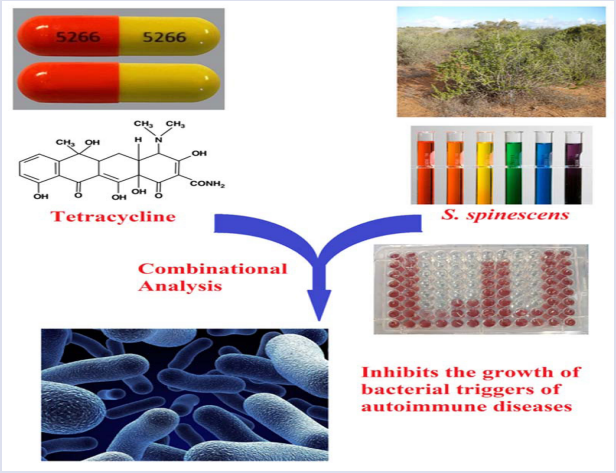
GRAPHICAL ABSTRACT



SUMMARY

- Methanolic, aqueous and ethyl acetate *S. spinescens* leaf extracts inhibited the growth of some bacterial triggers of autoimmune diseases. The methanol and ethyl acetate extracts were particularly potent inhibitors of *P. aeruginosa* growth (MICs 184 and 200 µg/mL respectively).The aqueous and methanolic extracts also synergised the activity of the tetracycline against *K. pneumonia* and *S. pyogenes*. All *S. spinescens* extracts were nontoxic in the *Artemia* and HDF assays.Combining the extracts and conventional antibiotics did not significantly alter the toxicity of the combination.

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