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Original Article

The Interactive Antimicrobial Activity of Conventional Antibiotics and *Petalostigma* spp. Extracts Against Bacterial Triggers of some Autoimmune Inflammatory Diseases

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ABSTRACT

Introduction: An increase in antibiotic resistance and a corresponding decrease in antimicrobial discovery have directed researchers towards alternative therapies, including plant-based medicines. However, synergistic combinations of plant extracts with conventional antibiotics are a far more effective approach in overcoming resistance and potentiating the activity of antibiotics that are otherwise ineffective against resistant bacterial strains. **Methods:** In this study, *Petalostigma* spp. (native Australian medicinal plants) extracts were combined with a range of conventional antibiotics and tested against various microbial triggers of autoimmune diseases. The fruit and leaves were extracted separately with solvents of varying polarity and investigated for the ability to inhibit bacterial growth using disc diffusion and liquid dilution MIC techniques. **Results:** The methanolic and water extracts showed low to moderate inhibitory activity against several microbes. However, combinations of the mid-low polarity extracts with conventional antibiotics proved significantly more effective in inhibiting the growth of *Proteus mirabilis* and *Acinetobacter baylyi* (bacterial triggers of rheumatoid arthritis and multiple sclerosis respectively). In total, 14 different combinations proved to be synergistic. Notably, two antibiotics (chloramphenicol and erythromycin) with no inhibitory activity against *P. mirabilis* alone were shown to have substantial activity when tested in combination with *Petalostigma* spp. extracts. **Conclusion:** Although the mechanisms of synergy are still unclear, studies indicate that compounds within *Petalostigma* spp. may mimic the actions of resistance modifying agents, thus potentiating the activity of several antibiotics that are relatively ineffective alone. Isolation of these agents may be highly beneficial in drug design against several bacteria including the microbial triggers of rheumatoid arthritis and multiple sclerosis.
Key words: Synergy, Conventional antimicrobials, Interaction, Medicinal plants, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Drug combinations, Efflux pump inhibitor.

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INTRODUCTION

Autoimmune inflammatory disorders including ankylosing spondylitis, multiple sclerosis, rheumatoid arthritis and rheumatic fever are a group of debilitating conditions that afflict genetically susceptible individuals. They are triggered by exposure to specific exogenous antigens, with bacterial antigens being the most important. Whilst the triggers of some autoimmune diseases are still unknown, serotyping studies have identified the bacterial triggers of several of these conditions and the bacterial antigens responsible for the inducing an immune response. *P. mirabilis*, a normal part of the human gastrointestinal flora, has been identified as trigger of rheumatoid arthritis.[1] Similarly, *K. pneumoniae* can initiate ankylosing spondylitis in genetically susceptible people and both *A. baylyi* and *P. aeruginosa* can initiate the onset of multiple sclerosis.[1] The development of medicines targeting these specific bacterial triggers may prevent the disease onset. This would reduce the severity of the symptoms once the disease has progressed, as well as blocking tissue damage.

Antibiotic therapeutics that inhibit the growth of these bacteria already exist and their use for prophylactic treatment may be an attractive option. However, the improper and misuse of antibiotics has resulted in antibiotic resistance.[2] As a consequence, two main events have occurred in parallel throughout the last century. The discovery of antimicrobial agents has steadily decreased to no more than a few antibiotics synthesised or discovered in the last decade.[3] Simultaneously, antibiotic resistance has risen to an all-time high, creating multi-resistant organisms that are becoming increasingly difficult to manage using the current range of available antibiotic chemotherapies.[4] The development of alternative treatment methods is crucial and considered by the World Health Organisation (WHO) to

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be perhaps the biggest challenge facing medical science.[5] For a number of reasons reviewed elsewhere,[6] it is unlikely that the current methods of antibiotic discovery/development will be as successful in the future.

Traditional medicines have great potential for antimicrobial drug development. Despite this, relatively few plant derived antibiotic compounds are in common use. This is likely because synergistic interactions are often required to potentiate the antibacterial activity and purified compounds often have much lower activity than the crude extract.[7] A combinational approach that allows synergistic interaction between plant extracts (or pure plant compounds) and conventional antibiotics may be more effective in combatting bacterial pathogens, especially antibiotic resistant strains.[3,8,9] Combinational therapy is already preferred over mono-therapy in multiple life-threatening infectious diseases such as malaria, tuberculosis and HIV/AIDS due to its ability to target multiple facets of a disease and to curb resistance.[5] A combination of plant extracts/isolated compounds with conventional antibiotics may also prove to have an economic advantage.[7] Developing a new drug requires years of extensive and costly testing. However, combinational therapy can potentially restore an existing drug to a state of significantly reduced resistance, thereby bypassing the strenuous and expensive process of discovering new antimicrobial agents.[7] Further advantages of synergistic interactions include increased efficiency, reduced side effects, increased stability and bioavailability and the need for lower doses in comparison to synthetic alternatives.[7]

Petalostigma pubescens Domin and *Petalostigma triloculare* Mull. Arg. are endemic Australian plants that were used traditionally as antimicrobial and antiseptic agents by indigenous Australians.[10] Recent studies have demonstrated good antibacterial activity for both species against a panel of human pathogens.[11] However, these plants are yet to be tested against the bacterial triggers of rheumatoid arthritis (*Proteus mirabilis*), ankylosing spondylitis (*Klebsiella pneumoniae*), multiple sclerosis (*Acinetobacter baylyi*, *Pseudomonas aeruginosa*) and rheumatic fever (*Streptococcus pyogenes*).[12] Furthermore, *Petalostigma* spp. extracts are yet to be tested for bacterial growth inhibitory activity in combinational studies with conventional antibiotics. Therefore, this study investigates the antimicrobial effects of *P. pubescens* and *P. triloculare* and their ability to potentiate the growth inhibitory properties of conventional antibiotics against the bacterial triggers of some autoimmune inflammatory diseases.

MATERIALS AND METHODS

Plant source and extraction

Petalostigma pubescens Domin and *Petalostigma triloculare* Mull.Arg. leaves and fruit were collected from single trees in Toohey Forrest, Brisbane, Australia and identified by Dr Ian Cock, Griffith University. The *P. pubescens* tree is located at gps coordinates of -27° 33' 15.08" S, 153° 3' 18.63" E and the *P. triloculare* tree is located at gps coordinates of -27° 33' 15.90" S, 153° 3' 10.35" E. Voucher specimens of all specimens were deposited in the School of Natural Sciences, Griffith University, Australia. Voucher numbers of the *P. pubescens* leaves and fruit specimens are GUPPL-2016-2/10 and GUPPF-2016-2/10 respectively. The voucher numbers of *P. triloculare* leaves and fruit specimens are GUPTL-2016-2/10 and GUPTF-2016-2/10 respectively. The plant materials were thoroughly dried using a Sunbeam food dehydrator and the materials stored at -30°C 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 deionised water, methanol, chloroform, hexane or ethyl acetate 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 4°C on an orbital shaker at 20 revolutions per min. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum. The solvent extracts were air dried at room temperature in the shade. The aqueous extracts were lyophilised by freeze drying at -50°C. The resultant dried extracts were weighed and dissolved in 10 mL deionised water (containing 1 % DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the *Petalostigma* spp extracts were conducted by modified versions of previously described assays.[12,13] The modified assays are briefly outlined below.

Alkaloids

Two methods were used to test for the presence of alkaloids:

Mayers reagent test

A few drops of aqueous solution of hydrochloric acid and 500 µl Mayer's reagent was added to 200µL of each extract. Formation of a white precipitate was taken to indicate the presence of alkaloids.

Mayer's reagent: Mercuric chloride (1.358 g) was dissolved in 60 ml deionised water. Potassium Iodide (5.0 g) was dissolved in 10 ml deionised water. The mercuric chloride and potassium iodide solutions were mixed and made up to 100 ml with deionised water.

Wagners reagent test

A 200µL volume of each extract was treated with a few drops of dilute hydrochloric acid and 500µL Wagner's reagent. A reddish-brown flocculent precipitate indicated the presence of alkaloid.

Wagner's reagent: 1.27 g Iodine and 2 g Potassium Iodide were dissolved in 5 ml deionised water and made up to final volume 100 ml with deionised water.

Anthraquinones

Free anthraquinones were detected by adding a few drops of concentrated sulphuric acid to 500µL each extract extract, followed by the addition of 500µL of ammonia. A rose-pink colour indicates the presence of free anthraquinones. For combined anthraquinones, 450µL of each extract was individually added to 50µL concentrated HCl. A 500µL volume of chloroform was subsequently added and the colour was noted. The formation of a rose-pink colour indicates the presence of combined anthraquinones.

Cardiac glycosides

A volume of 500µL of each extract was individually added to 500µL glacial acetic acid. A few drops of 1% aqueous iron chloride and concentrated sulphuric acid were then carefully added. The presence of a red/brown ring of the interface or the formation of a green/blue colour throughout the solution indicates the presence of cardiac glycosides.

Flavonoids

Flavonoids were detected by adding 100µL of aqueous sodium hydroxide to 1mL of each extract. The development of an intense yellow colour indicated the presence of flavonoids. A volume of 100µL of concentrated HCl was subsequently added to the solution. Reversion to the original colour confirmed the presence of flavonoids.

Phenolic compounds

Phenolic compounds were detected by adding 200µL of extract to 2mL of 3% aqueous sodium carbonate. A volume of 200µL Folin-Ciocalteu reagent was subsequently added and the mixture was allowed to stand for 30 min at room temperature. The formation of blue/gray colour indicated the presence of phenolic groups.

Water soluble phenol test

Two drops of 1% ferric chloride were added separately to 500µL of the individual extracts. A red colour change indicates presence of water-soluble phenols.

Water insoluble phenol test

Dichloromethane (500µL), 3 drops of 1% ferric chloride and 1 drop of pyridine were added to 500µL of each extract in individual tubes and mixed. The presence of insoluble phenols was indicated by a colour change.

Phytosterols

Phytosterols were detected by adding three drops of acetic anhydride to 500µL of the extracts, followed by the addition of a few drops concentrated sulphuric acid. After 5 min the colour of the solution was noted. Formation of a blue/green colour indicated the presence of phytosterols.

Saponins

A 1mL volume of each extract was added individually to 1mL deionised water and shaken vigorously for 30 sec. The tubes were allowed to stand for 15min and the presence or absence of persistent frothing was noted. Persistent frothing indicated the presence of saponins.

Tannins

Tannins were detected by adding two drops of 1% aqueous ferric chloride reagent to 500µL of each extract. Formation of blue, blue-black, green or green-black colouration indicates the presence of tannins.

Triperpenoids

Triterpenoids were detected by slowly adding 1mL of extract to 400µL chloroform, followed by careful addition of 400µL concentrated sulphuric acid. Formation of a red/brown/purple colour at the interface indicated the presence of triterpenoids.

Antibacterial screening

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 and used for the microplate liquid dilution assay. All antibiotics were prepared in sterile deionised water at stock concentrations of 0.01 mg/mL and stored at 4°C until use. For the disc diffusion studies, ampicillin (2 µg) and chloramphenicol discs (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 auto-immune inflammatory diseases in genetically susceptible individuals.[12] Reference strains of *Proteus mirabilis* (ATCC21721), *Klebsiella pneumoniae* (ATCC31488), *Acinetobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Type Culture Collection, USA. A clinical isolate strain of *Streptococcus pyogenes* was 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 37°C for 24 h and were subcultured and maintained in nutrient broth at 4°C until use.

Evaluation of antibacterial activity

Antibacterial activity screening of the *Petalostigma* spp. extracts was assessed using a modified disc diffusion assay.[11] 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.[14] 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.[14] 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.[11] Graphs of the zone of inhibition versus concentration were plotted and MIC values were achieved using linear regression.

Petalostigma spp. extract-conventional antibiotic synergy studies

Fractional inhibitory concentration (FIC) assessment

Interactions between the *Petalostigma* spp. extracts and the conventional antibiotics were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC) for each combination.[15] 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).[15]

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 to determine the ideal ratios to induce synergy. All combinations were tested in duplicate in two independent experiments, providing four replicates for each combination ratio. The data is presented as the mean of four 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.

Toxicity screening

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 MTT cellular proliferation assay was used to determine a cellular evaluation of toxicity.

Artemia franciscana Kellogg nauplii toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4 mg/mL) and serially diluted in artificial seawater as a reference toxin. Toxicity of the *Petalostigma* spp. extracts, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.[15,16] The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Cellular viability assay

All *Petalostigma* spp. extracts and conventional antibiotics were screened individually towards normal human primary dermal fibroblasts (HDF). The HDF cells were obtained from American Type Culture Collection (ATCC PCS-201-012) and were cultured and maintained in Dulbecco's modified eagle medium (DMEM; ThermoFisher Scientific, Australia), supplemented with 10 % foetal calf serum (Life Technologies), 50 µg/mL streptomycin (Sigma-Aldrich, Australia) and 50 IU/mL penicillin (Sigm-Aldricha, Australia). The cells were maintained as monolayers in 75 mL flasks at 37°C, 5 % CO_2 in a humidified atmosphere until approximately 80% confluent. Once confluency was achieved, 1 mL of trypsin (Sigma, Australia) was added to the culture flasks and incubated at 37°C, 5 % CO_2 for 15 min to dislodge the HDF cells. The cell suspensions were then transferred to a 10 mL centrifuge tube and sedimented by centrifugation. The supernatant was discarded and the cells were resuspended in 9 mL of fresh media (lacking streptomycin and penicillin supplementation). Aliquots of the resuspended cells (70 µL, containing approximately 5000 cells) were added to individual wells of a 96 well plate. A volume of 30 µL of the test extracts or cell media (for the negative control) was added to individual wells and the plates were incubated at 37°C, 5% CO_2 for 24 h in a humidified atmosphere. All extracts were screened at 200 µg/mL. The cells were then washed in PBS (pH 7.2) to remove interference due to sample colour. A volume of 20 µL of Cell Titre 96 Aqueous One solution (Promega) was subsequently added to each well and the plates were incubated for a further 3 h. Absorbances were recorded at a test wavelength of 540 nm and a blank wavelength of 690nm using a Molecular Devices, Spectra Max M3 plate reader. 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 ± SEM 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 *Petalostigma* spp. plant materials (1.5 g) with solvents of varying polarity yielded dried plant extracts ranging from 93 ± 4 mg (*P. pubescens* fruit ethyl acetate extract) to 768 ± 10 mg (*P. pubescens* fruit methanolic 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.

Bacterial growth inhibition screening

Inhibition of a bacterial trigger of rheumatoid arthritis (*P. mirabilis*)

With some notable exceptions, *P. mirabilis* growth was particularly susceptible to the higher polarity aqueous and methanolic *Petalostigma* spp. extracts (Figure 1). The aqueous *Petalostigma* spp. leaf extracts were the exception to this trend, with only small zones of inhibition recorded. *P. pubescens* aqueous fruit extract was the best inhibitor of *P. mirabilis* growth, with a zone of inhibition of 18.4 ± *0.5* mm measured. Notably, both the *P. pubescens* aqueous and methanolic fruit extracts (17.9 ± *0.5* mm) produced larger zones of inhibition than the ampicillin (16.9 ± *0.4* mm) and chloramphenicol controls (16.5 ± *0.6* mm) (Figure 1a). *P. pubescens* fruit and *P. triloculare* leaf ethyl acetate extracts were also moderate inhibitors of *P. mirabilis* growth.

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

All *Petalostigma* spp. extracts inhibited the growth of *K. pneumoniae* (Figure 2) albeit, generally with lower efficacy than for *P. mirabilis* growth inhibition. With the exception of the aqueous *P. triloculare* leaf extract, the higher polarity aqueous and methanolic extracts were good growth inhibitors (inhibition zones >10 mm). The methanolic *P. triloculare* leaf extract was the best growth inhibitor, with an inhibition zone of 14 mm. This is noteworthy as this *K. pneumoniae* strain is resistant to β-lactam antibiotics (as evident from the low zone of inhibition seen for ampicillin in Figure 2). 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 zone of 22 ± 0.9 mm. The growth inhibition recorded for the lower polarity chloroform, hexane and ethyl acetate extracts were significantly lower than for the polar extracts. In total, 17 of the 20 *Petalostigma* spp. extracts induce larger zones of inhibition than seen for the ampicillin control.

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

The aqueous and methanolic extracts (with the exception of the aqueous *Petalostigma* spp. leaf extracts) were potent inhibitors of *A. baylyi* growth (Figure 3). The methanolic *P. pubescens* fruit extract was the most potent inhibitor of *A. baylyi* growth (18.4 ± 0.5 mm). Indeed, the inhibition zones produced by this extract were comparable with those of the chloramphenicol control and substantially higher than the ampicillin control. Similarly, the aqueous *P. pubescens* fruit extract was a good inhibitor of *A. baylyi* growth, with a zone of inhibition of 18.1 ± 0.7 mm. The aqueous and methanolic *P. triloculare* fruit extracts and the methanolic *P. triloculare* leaf extract inhibited the growth of *A. baylyi* with substantially greater efficacy than the ampicillin control, with zones of inhibition >15 mm

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

Extract		Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
P. pubescens leaf	W	270 ± 5	27 ± 1	+++	-	+++	-	++	++	-	-	-	++	+++	++	-
	M	460 ± 6	46 ± 1	+++	-	+++	-	++	+++	-	++	++	++	+++	++	-
	C	200 ± 4	20 ± 1	+	-	-	-	-	-	-	-	-	++	-	-	-
	H	103 ± 3	10 ± 1	+	-	+	-	+++	-	-	-	-	++	-	-	-
	E	200 ± 3	200 ± 1	+++	-	+++	-	-	+	-	-	-	+++	+++	++	-
P. pubescens fruit	W	615 ± 6	62 ± 1	+++	-	+++	-	+	+++	-	-	-	++	+++	++	-
	M	768 ± 10	77 ± 1	+++	-	+++	-	+	+++	+	-	-	+++	+++	++	-
	C	143 ± 7	14 ± 1	-	-	+	-	-	-	-	-	-	++	-	-	-
	H	110 ± 5	11 ± 1	++	-	+++	-	-	-	-	++	-	++	+	+++	-
	E	93 ± 4	9 ± 1	+++	-	++	-	+	++	-	-	-	+++	++	++	-
P. triloculare leaf	W	543 ± 7	54 ± 1	+++	-	+++	-	+++	+++	-	-	-	-	+++	++	-
	M	263 ± 2	26 ± 1	+++	-	+++	-	+++	+++	-	-	-	++	+++	++	-
	C	190 ± 4	19 ± 1	+	-	+	-	-	-	-	-	-	++	-	-	-
	H	163 ± 7	16 ± 1	+	-	+	-	+	-	-	-	-	++	-	-	-
	E	217 ± 7	22 ± 1	+++	-	+++	-	-	-	-	-	-	+++	+++	++	-
P. triloculare fruit	W	633 ± 4	63 ± 1	+++	-	+++	-	-	+++	++	-	-	-	+++	++	-
	M	698 ± 8	70 ± 1	+++	-	+++	-	+	+++	++	-	-	+++	+++	++	-
	C	185 ± 6	19 ± 1	+	-	+++	-	-	-	-	-	-	++	-	-	-
	H	115 ± 4	12 ± 1	+++	-	++	-	-	-	++	-	-	+++	+++	++	-
	E	120 ± 6	12 ± 1	+++	-	++	-	-	-	+	-	-	+++	+++	++	-

Table 1: Cont'd.

Extract		Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
P. pubescens leaf	W	270 ± 5	27 ± 1	+++	-	+++	-	++	++	-	-	-	++	+++	++	-
	M	460 ± 6	46 ± 1	+++	-	+++	-	++	+++	-	++	++	++	+++	++	-
	C	200 ± 4	20 ± 1	+	-	-	-	-	-	-	-	-	++	-	-	-
	H	103 ± 3	10 ± 1	+	-	+	-	+++	-	-	-	-	++	-	-	-
	E	200 ± 3	200 ± 1	+++	-	+++	-	-	+	-	-	-	+++	+++	++	-
P. pubescens fruit	W	615 ± 6	62 ± 1	+++	-	+++	-	+	+++	-	-	-	++	+++	++	-
	M	768 ± 10	77 ± 1	+++	-	+++	-	+	+++	+	-	-	+++	+++	++	-
	C	143 ± 7	14 ± 1	-	-	+	-	-	-	-	-	-	++	-	-	-
	H	110 ± 5	11 ± 1	++	-	+++	-	-	-	-	++	-	++	+	+++	-
	E	93 ± 4	9 ± 1	+++	-	++	-	+	++	-	-	-	+++	++	++	-
P. triloculare leaf	W	543 ± 7	54 ± 1	+++	-	+++	-	+++	+++	-	-	-	-	+++	++	-
	M	263 ± 2	26 ± 1	+++	-	+++	-	+++	+++	-	-	-	++	+++	++	-
	C	190 ± 4	19 ± 1	+	-	+	-	-	-	-	-	-	++	-	-	-
	H	163 ± 7	16 ± 1	+	-	+	-	+	-	-	-	-	++	-	-	-
	E	217 ± 7	22 ± 1	+++	-	+++	-	-	-	-	-	-	+++	+++	++	-
P. triloculare fruit	W	633 ± 4	63 ± 1	+++	-	+++	-	-	+++	++	-	-	-	+++	++	-
	M	698 ± 8	70 ± 1	+++	-	+++	-	+	+++	++	-	-	+++	+++	++	-
	C	185 ± 6	19 ± 1	+	-	+++	-	-	-	-	-	-	++	-	-	-
	H	115 ± 4	12 ± 1	+++	-	++	-	-	-	++	-	-	+++	+++	++	-
	E	120 ± 6	12 ± 1	+++	-	++	-	-	-	+	-	-	+++	+++	++	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. W = aqueous extract; M = methanolic extract; C = chloroform extract; H = hexane extract; E = ethyl acetate extract.

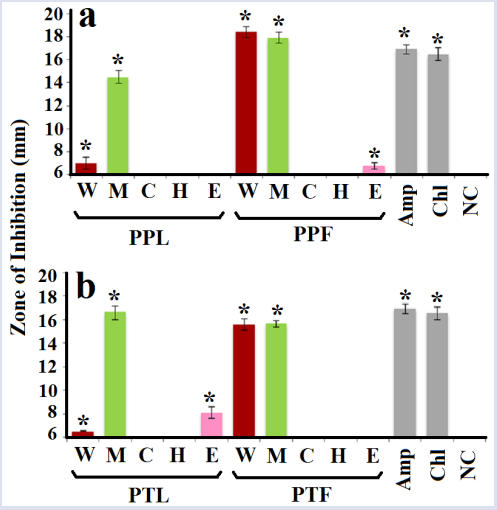

Figure 1: Antibacterial activity of *P. pubescens* (a) and *P. triloculare* (b) fruit and leaf extracts against *P. mirabilis* (ATCC: 21721) measured as zones of inhibition (mm). PP = *P. pubescens*; PT = *P. triloculare;* L = leaf; F = fruit; W = aqueous extract; M = methanolic extract; C = chloroform extract; H = hexane extract; E = ethyl acetate extract. Positive control = Amp (ampicillin 2µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of at least six replicates (two repeats) ± SEM. * indicates results that are significantly different to the negative control ($P<0.01$).

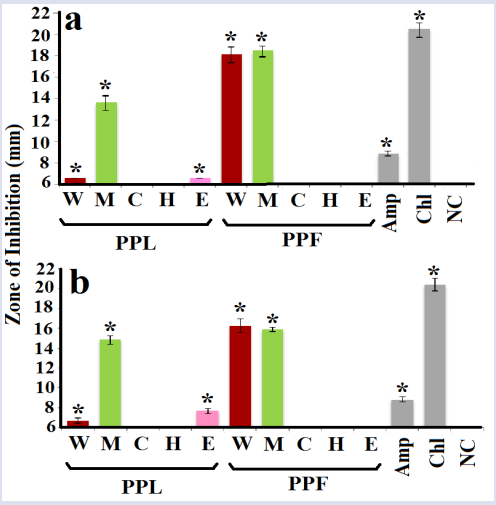

Figure 3: Antibacterial activity of *P. pubescens (a)* and *P. triloculare (b)* fruit and leaf extracts against *A. baylyi* (ATCC: 33304) measured as zones of inhibition (mm). PP = *P. pubescens*; PT = *P. triloculare;* L = leaf; F = fruit; W = aqueous extract; M = methanolic extract; C = chloroform extract; H = hexane extract; E = ethyl acetate extract. Positive control = Amp (ampicillin 2µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of at least six replicates (two repeats) ± SEM. * indicates results that are significantly different to the negative control ($P<0.01$).

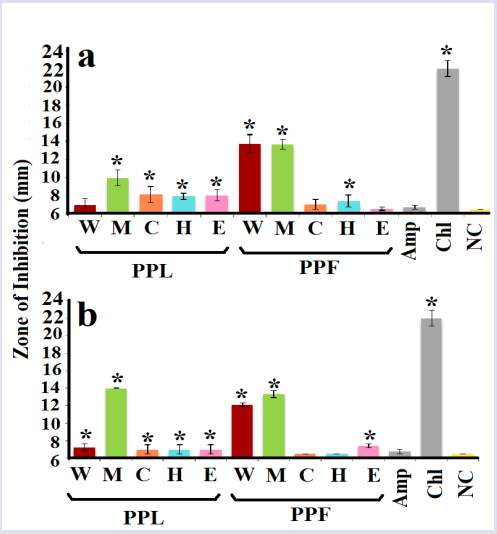

Figure 2: Antibacterial activity of *P. pubescens* (a) and *P. triloculare* (b) fruit and leaf extracts against *K. pneumoniae* (ATCC: 31488) measured as zones of inhibition (mm). PP = *P. pubescens*; PT = *P. triloculare;* L = leaf; F = fruit; W = aqueous extract; M = methanolic extract; C = chloroform extract; H = hexane extract; E = ethyl acetate extract. Positive control = Amp (ampicillin 2µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of at least six replicates (two repeats) ± SEM. * indicates results that are significantly different to the negative control ($P<0.01$).

(compared to 8.8 ± *0.3* mm for the ampicillin control) (Figure 3b). With the exception of the ethyl acetate *Petalostigma* spp. leaf extracts, all of the less polar (hexane, chloroform and ethyl acetate) extracts were completely devoid of *A. baylyi* growth inhibitory activity.

With the exception of *P. pubescens* fruit ethyl acetate and *P. triloculare* fruit hexane extracts, all extracts inhibited the growth of *P. aeruginosa* (Figure 4). The *P. pubescens* fruit methanolic extract was the best inhibitor of *P. aeruginosa* growth, with a zone of inhibition of 16 ± *0.5* mm. This was particularly noteworthy as the *P. aeruginosa* strain tested in this study was resistant to both the ampicillin and chloramphenicol controls, each inducing only 6.5 mm zones of inhibition. Furthermore, the methanolic and aqueous extracts of *Petalostigma* spp. fruit and the methanolic *P. triloculare* leaf extract also had zones of inhibition >12 mm. Notably, all of the *P. pubescens* leaf extracts (including the less polar hexane, chloroform and ethyl acetate extracts) had zones of inhibition >7 mm. Our studies therefore indicate that the methanolic *P. pubescens* fruit extract was the most effective inhibitor of both bacterial triggers of multiple sclerosis.

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

All *P. pubescens* extracts inhibited *S. pyogenes* growth (Figure 5a). Similarly, with the exception of the chloroform and hexane extracts, all of the *P. triloculare* extracts also inhibited the growth of *S. pyogenes,* albeit often with lower efficacies (Figure 5b). The aqueous *Petalostigma* spp. fruit extracts were substantially better *S. pyogenes* growth inhibitors (>12mm zones of inhibition) compared to the corresponding leaf extracts. The methanolic *P. triloculare* leaf extract was the strongest

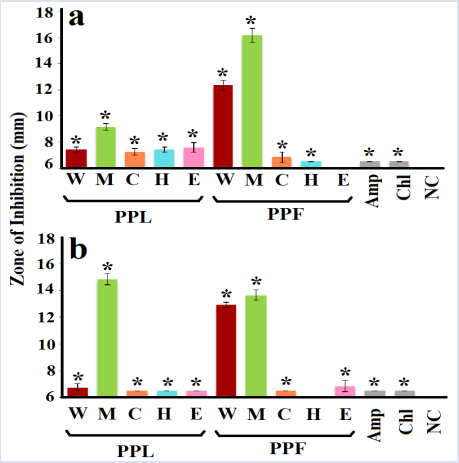

Figure 4: Antibacterial activity of *P. pubescens* (a) and *P. triloculare* (b) fruit and leaf extracts against *P. aeruginosa* (ATCC: 39324) measured as zones of inhibition (mm). PP = *P. pubescens*; PT = *P. triloculare;* L = leaf; F = fruit; W = aqueous extract; M = methanolic extract; C = chloroform extract; H = hexane extract; E = ethyl acetate extract. Positive control = Amp (ampicillin 2µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of at least six replicates (two repeats) ± SEM. * indicates results that are significantly different to the negative control (*P*<0.01).

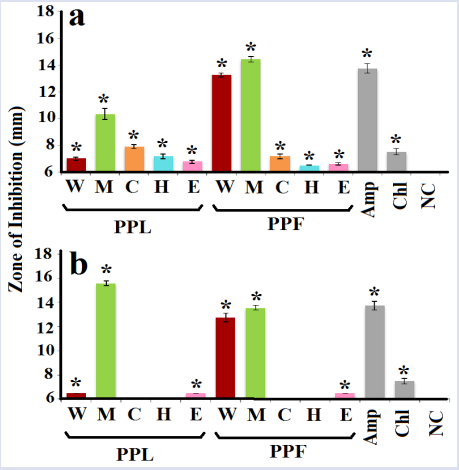

Figure 5: Antibacterial activity of *P. pubescens (a)* and *P. triloculare (b)* fruit and leaf extracts against a clinical isolate of *S. pyogenes* measured as zones of inhibition (mm). PP = *P. pubescens*; PT = *P. triloculare;* L = leaf; F = fruit; W = aqueous extract; M = methanolic extract; C = chloroform extract; H = hexane extract; E = ethyl acetate extract. Positive control = Amp (ampicillin 2µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of at least six replicates (two repeats) ± SEM. * indicates results that are significantly different to the negative control (*P*<0.01).

inhibitor of *S. pyogenes* growth with an inhibition zone of 15.6 ± *0.5 mm. The methanolic P. pubescens* fruit extract was similarly potent (14.4 ± 0.5 mm). Indeed, the methanolic extracts of both the *P. triloculare* leaf and the *P. pubescens* fruit had greater relative activity than the ampicillin (13.8 ± 0.9 mm) and chloramphenicol controls (7.5 ± 0.6 mm).

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 *Petalostigma* spp. extracts inhibited all of the bacteria tested and were more potent in comparison to the corresponding less polar extracts. The MIC values of the conventional antibiotic controls were only determined for the liquid dilution assay. Commercially manufactured discs with set amounts of antibiotics loaded were used for the disc diffusion assay and thus the zones of only single doses was recorded for the disc diffusion MIC assay. Gentamicin was the most potent antibiotic (as judged by its MIC) and inhibited the widest range of bacterial species. Notably, the *P. aeruginosa* strain used in these studies was particularly resistant to all of antibiotics, with the exception of gentamicin. Furthermore, with the exception of *P. mirabilis*, all of the other bacterial strains were resistant to penicillin.

The MIC values determined for the *Petalostigma* spp. extracts compare relatively well between the disc diffusion and liquid dilution assays with a few notable exceptions e.g. inhibition of *S. pyogenes* growth by *P. triloculare* leaf aqueous (DD >10000 µg/mL; LD 344 µg/mL). All bacterial species were generally more susceptible to *P. pubescens* fruit than to *P. triloculare* fruit extracts (based on MIC values). In contrast, *P. pubescens* and *P. triloculare* leaf extracts had similar levels of antimicrobial activity. The growth of *P. mirabilis* was inhibited by methanolic *P. pubescens* leaf (DD MIC 1329 µg/mL; LD MIC 1469 µg/mL) and fruit extracts (DD MIC 1069 µg/mL; LD MIC 2594 µg/mL) and by the methanolic *P. triloculare* leaf extract (DD MIC 1273 µg/mL; LD MIC 1500 µg/mL), although these values indicate only low to moderate activity. Aqueous *P. pubescens* fruit and *P. triloculare* leaf extracts were moderate inhibitors of *P. mirabilis* growth. Aqueous *P. pubescens* leaf (DD MIC >10000 µg/mL; LD MIC 750 µg/mL) and *P. triloculare* leaf extracts (DD MIC 2940 µg/mL; LD MIC 750 µg/mL) were also moderate *K. pneumoniae* growth inhibitors. *P. pubescens* leaf ethyl acetate and *P. triloculare* leaf methanolic extracts were also moderate *K. pneumoniae* growth inhibitors. Similarly, the growth of *A. baylyi* was inhibited by methanolic extracts of *P. pubescens* fruit (DD MIC 1313 µg/mL; LD MIC 2594 µg/mL), *P. triloculare* leaf (DD MIC 1441 µg/mL; LD MIC 3000 µg/mL) and the aqueous *P. pubescens* fruit extract (DD MIC 1687 µg/mL; LD MIC 3938 µg/mL) with low to moderate potency. The strongest growth inhibitor of *P. aeruginosa* was the methanolic *P. pubescens* fruit extract (DD MIC 6757 µg/mL; LD MIC 2406 µg/mL), although these values indicate only low potency. Similarly, the aqueous *P. triloculare* leaf, methanolic *P. pubescens* leaf (DD MIC 1843 µg/mL; LD MIC 750 µg/mL), methanolic *P. pubescens* fruit (DD MIC 1247 µg/mL; LD MIC 656 µg/mL) and methanolic *P. triloculare* leaf extracts (DD MIC 1969 µg/mL; LD MIC 453 µg/mL) were also moderate growth inhibitors of *S. pyogenes* growth.

Fractional inhibitory concentration (FIC) assessment Combinational effects on a bacterial trigger of rheumatoid arthritis *(P. mirabilis)*

A wide range of interactions was evident for combinations of the *Petalostigma* spp. extracts with conventional antibiotics when tested against *P. mirabilis* (Table 3). Approximately 18% of the combinations

Table 2: Disc diffusion and liquid dilution MIC values for *P. pubescens* and *P. triloculare* fruit and leaf extracts against *P. mirabilis*, *K. pneumoniae*, *A. baylyi*, *P. aeruginosa* and *S. pyogenes* growth (µg/mL).

EXRACT	*P. mirabilis* (ATCC: 33304)		*K. pneumoniae* (ATCC: 31488)		*A. baylyi* (ATCC: 21721)		*P. aeruginosa* (ATCC: 39324)		*S. pyogenes*	
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC
PLW	>10000	3000	>10000	750	>10000	3000	8918	3250	6504	1810
PLM	1329	1469	4627	2938	2049	2938	8994	3000	1843	750
PLC	-	-	>10000	-	-	-	5986	-	2189	5250
PLH	-	-	>10000	-	-	-	3430	-	2079	-
PLE	-	6000	6022	1500	>10000	2000	5442	6000	>10000	2250
PFW	1478	3938	2214	3938	1687	3938	4741	3940	2128	3938
PFM	1069	2594	2183	2594	1313	2594	6757	2406	1247	656
PFC	-	-	5225	-	-	-	>10000	-	>10000	-
PFH	-	-	3430	-	-	-	>10000	-	>10000	-
PFE	8000	2000	8000	-	-	-	-	-	8000	1500
TLW	>10000	1500	2940	750	2832	3000	>10000	8000	>10000	344
TLM	1273	1500	2812	1500	1441	3000	3287	3688	1969	453
TLC	-	-	4503	-	-	-	>10000	-	-	-
TLH	-	-	3752	-	-	-	>10000	-	-	4000
TLE	>10000	5250	4002	5250	5994	-	>10000	-	>10000	1500
TFW	3879	4625	>10000	9250	4385	9250	>10000	4438	8138	2344
TFM	2382	5313	5127	>10000	4416	5313	7558	2500	5253	2563
TFC	-	-	>10000	-	-	-	>10000	-	-	-
TFH	-	-	>10000	-	-	-	-	-	-	3250
TFE	-	-	>10000	-	-	-	>10000	-	>10000	3500
Positive controls										
Penicillin	ND	2.5	ND	-	ND	-	ND	-	ND	-
Chloramphenicol	ND	-	ND	1.25	ND	2.5	ND	-	ND	-
Gentamicin	ND	1.25	ND	0.31	ND	0.31	ND	0.63	ND	0.63
Erythromycin	ND	-	ND	-	ND	2.5	ND	-	ND	-
Tetracycline	ND	-	ND	1.25	ND	1.25	ND	-	ND	2.5
Negative control	ND	-	ND	-	ND	-	ND	-	ND	-

P = *P. pubescens*; T = *P. triloculare*; L = leaf; F = fruit; W = water; M = methanol; C = chloroform; H = hexane; E = ethyl acetate. DD = disc diffusion; LD = liquid dilution. - indicates no inhibition at any dose tested.

Numbers indicate the mean DD MIC and LD MIC values of triplicate determinations, expressed in µg/mL.

ND = MIC could not be determined as only a single dose was tested.

produced additive effects, whilst the majority of combinations were non-interactive. Notably, the methanolic *P. triloculare* leaf extract produced additive interactions with all conventional antibiotic combinations against *P. mirabilis*. However, combinations of gentamicin with either *P. pubescens* leaf ethyl acetate extract (Σ FIC = 6) or *P. triloculare* leaf hexane extract (Σ FIC = 4.5) were antagonistic against *P. mirabilis* growth inhibition. This is noteworthy and indicates that these combinations should be avoided as chemotherapeutic options in the treatment of *P. mirabilis* infections. A total of seven synergistic interactions were observed between *Petalostigma* spp. extracts and conventional antibiotic combinations. Interestingly, three of these synergies occurred when penicillin-G was combined with the low to mid polarity *P. pubescens* fruit hexane (Σ FIC = 0.31), *P. pubescens* fruit ethyl acetate (Σ FIC = 0.50) and *P. triloculare* leaf ethyl acetate extracts (Σ FIC = 0.50). Similarly, combinations of chloramphenicol with *P. pubescens* fruit hexane (Σ FIC = 0.25), *P. triloculare* leaf hexane (Σ FIC = 0.25) and *P. triloculare* leaf ethyl acetate extracts (Σ FIC = 0.31) also resulted in synergy. The combination of *P. pubescens* fruit hexane extract and erythromycin also proved synergistic against the growth of *P. mirabilis* (Σ FIC = 0.25). Therefore, a general trend was noted: most synergies occurred either with penicillin or chloramphenicol when in combination with lower polarity extracts against *P. mirabilis*. This contrasts with the earlier studies into the growth inhibitory activity of the *Petalostigma* spp. alone, where the higher polarity extracts were generally the most potent bacterial growth inhibitors.

Table 3: Σ FIC values of *Petalostigma* spp. extracts in combination with conventional antibiotics against *P. mirabilis* (ATCC 21721).

	Penicillin	Chloramphenicol	Gentamicin	Erythromycin	Tetracycline
PLW	1.50	*0.56*	2.00	1.13	1.13
PLM	1.13	1.03	1.25	1.03	1.03
PLC	2.50	-	1.13	-	-
PLH	2.50	-	1.13	-	-
PLE	-	*0.63*	6.00	2.50	2.50
PFW	1.25	1.06	1.50	1.06	1.06
PFM	1.13	*0.52*	*0.63*	0.52	0.52
PFC	2.50	-	0.84	-	-
PFH	**0.31**	**0.25**	1.13	**0.25**	-
PFE	**0.50**	*0.63*	1.50	2.50	2.50
TLW	1.25	1.06	1.50	1.06	1.06
TLM	*0.56*	*0.52*	*0.63*	*0.52*	*0.52*
TLC	2.50	-	1.13	-	-
TLH	*0.63*	**0.25**	4.50	-	-
TLE	**0.50**	**0.31**	1.50	*0.63*	*0.63*
TFW	1.25	1.06	1.50	1.06	1.50
TFM	1.25	1.06	1.50	1.06	1.06
TFC	*0.63*	-	1.13	-	-
TFH	2.50	-	1.13	-	-
TFE	*0.63*	-	1.13	-	-

P = *P. pubescens*; T = *P. triloculare*; L = leaf; F = fruit; W = water; M = methanol; C = chloroform; H = hexane; E = ethyl acetate. - indicates that the Σ FIC could not be determined.

Synergy (bold text) = ≤ 0.5; Additive (italics) = > 0.5-1.0; Indifferent (no highlighting) = > 1.0 - ≤ 4; Antagonistic (underlined text) = > 4.0

Numbers indicate the mean FIC values of 4 determinations

Table 4: Σ FIC values of *Petalostigma* spp. extracts in combination with conventional antibiotics against *K. pneumoniae* (ATCC 31488).

	Penicillin	Chloramphenicol	Gentamicin	Erythromycin	Tetracycline
PLW	1.03	1.25	2.00	1.03	1.25
PLM	1.06	1.50	3.00	1.06	1.50
PLC	-	4.50	1.03	-	*0.56*
PLH	-	4.50	1.03	-	*0.56*
PLE	1.06	3.00	3.00	2.13	1.50
PFW	1.06	1.50	3.00	1.06	1.50
PFM	1.03	1.25	2.00	1.03	1.25
PFC	-	1.13	1.03	-	*0.56*
PFH	-	1.13	2.06	-	*0.56*
PFE	-	1.13	2.06	-	*0.56*
TLW	1.03	1.25	2.00	1.03	1.25
TLM	1.03	1.25	2.00	1.03	1.25
TLC	-	4.50	1.03	-	4.50
TLH	-	*0.56*	4.13	-	4.50
TLE	2.50	1.50	4.50	2.50	0.75
TFW	1.13	-	2.50	*0.56*	-
TFM	1.13	-	2.50	*0.56*	-
TFC	-	*0.56*	1.03	-	*0.84*
TFH	-	1.13	1.03	-	*0.56*
TFE	-	1.13	1.03	-	*0.56*

P = *P. pubescens*; T = *P. triloculare*; L = leaf; F = fruit; W = water; M = methanol; C = chloroform; H = hexane; E = ethyl acetate. - indicates that the Σ FIC could not be determined.

Synergy (bold text) = ≤ 0.5; Additive (italics)= > 0.5-1.0; Indifferent (no highlighting) = > 1.0 - ≤ 4; Antagonistic (underlined text) = > 4.0

Numbers indicate the mean FIC values of duplicate determinations

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

No synergistic interactions were noted for combinations of the *Petalostigma* spp. extracts and conventional antibiotics against the growth of *K. pneumoniae* (Table 4). Approximately 13% of the combinations were additive, indicating that these combinations may still be beneficial due to increased growth inhibitory efficacies. The majority of these additive interactions involved combinations of the less polar chloroform, hexane and ethyl acetate extracts with tetracycline. Interestingly, five of these less polar extracts seemed to be antagonistic when combined with other antibiotics. Chloramphenicol in conjunction with either *P. pubescens* leaf (chloroform and hexane) or *P. triloculare* leaf chloroform extracts yielded antagonistic interactions. Similarly, gentamicin in combination with hexane and ethyl acetate *P. triloculare* leaf extracts were also antagonistic. Combinations of tetracycline and *P. triloculare* leaf chloroform and hexane extracts also produced antagonistic interactions against *K. pneumoniae* growth inhibition. Thus, these combinations should be avoided as chemotherapeutic options against *K. pneumoniae* infections.

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

A range of interactions were observed between *Petalostigma* spp. extracts and conventional antibiotics against *A. baylyi* (Table 5). Four of the combinations were antagonistic. Three of these antagonistic combinations involved combinations of gentamicin with either with *P. pubescens* leaf water and ethyl acetate extracts or with aqueous *P. triloculare* leaf extract. The combination of the *P. pubescens* leaf ethyl acetate extract with erythromycin was also determined to be antagonistic. Thus, these combinations should be avoided for the treatment of *A. baylyi* infections. Of the other combinations, approximately 23% were additive and nearly half of those were in combination with tetracycline. Thus, these combinations may be beneficial due to increased growth inhibitory efficacies. Of greater interest, a total of seven synergistic combinations were observed. Interestingly,

Table 5: Σ FIC values of *Petalostigma* spp. extracts in combination with conventional antibiotics against *A. baylyi* (ATCC 33304).

	Penicillin	Chloramphenicol	Gentamicin	Erythromycin	Tetracycline
PLW	1.13	1.50	5.00	1.50	**0.50**
PLM	1.06	1.25	3.00	1.25	**0.38**
PLC	-	2.50	1.03	2.50	1.13
PLH	-	2.50	1.03	*0.63*	1.13
PLE	2.50	-	4.50	4.00	**0.38**
PFW	1.06	1.25	3.00	1.25	**0.38**
PFM	1.03	1.13	2.00	1.13	*0.63*
PFC	-	2.50	*0.52*	2.50	1.13
PFH	-	2.50	1.03	*0.63*	*0.56*
PFE	-	0.63	1.03	2.50	*0.56*
TLW	1.13	*0.75*	5.00	1.50	**0.50**
TLM	*0.53*	*0.63*	1.50	*0.63*	*0.75*
TLC	-	2.50	1.03	2.50	*0.56*
TLH	-	2.50	2.06	2.50	*0.56*
TLE	-	*0.63*	2.06	2.50	**0.28**
TFW	*0.56*	*0.75*	2.50	*0.75*	**0.50**
TFM	1.06	1.25	3.00	1.25	*0.75*
TFC	-	2.50	1.03	*0.63*	*0.56*
TFH	-	2.50	1.03	2.50	*0.84*
TFE	-	2.50	1.03	2.50	*0.56*

P = *P. pubescens*; T = *P. triloculare*; L = leaf; F = fruit; W = water; M = methanol; C = chloroform; H = hexane; E = ethyl acetate. - indicates that the Σ FIC could not be determined.

Synergy (bold text) = ≤ 0.5; Additive (italics)= > 0.5-1.0; Indifferent (no highlighting) = > 1.0 - ≤ 4; Antagonistic (underlined text) = > 4.0

Numbers indicate the mean FIC values of 4 determinations

Table 6: Σ FIC values of *Petalostigma* spp. extracts in combination with conventional antibiotics against *P. aeruginosa* (ATCC 33304).

	Penicillin	Chloramphenicol	Gentamicin	Erythromycin	Tetracycline
PLW	1.13	1.13	3.00	1.13	*0.56*
PLM	1.13	1.06	2.00	1.06	1.06
PLC	*1.00*	*1.00*	1.06	-	-
PLH	*0.63*	*1.00*	1.06	-	-
PLE	1.56	2.50	10.00	2.50	*0.63*
PFW	1.06	1.06	2.00	1.06	1.06
PFM	1.03	1.03	3.00	1.03	1.03
PFC	-	-	*0.53*	-	-
PFH	-	-	1.06	-	-
PFE	-	-	1.06	-	-
TLW	*0.63*	*0.63*	2.50	*0.62*	1.13
TLM	1.06	1.06	2.00	1.06	1.03
TLC	-	-	1.06	-	-
TLH	-	-	1.06	-	-
TLE	-	-	8.50	-	-
TFW	1.06	1.06	2.00	1.06	1.06
TFM	1.03	1.03	1.50	1.03	1.03
TFC	-	-	2.13	-	-
TFH	-	-	2.50	-	-
TFE	-	-	2.50	-	-

P = *P. pubescens*; T = *P. triloculare*; L = leaf; F = fruit; W = water; M = methanol; C = chloroform; H = hexane; E = ethyl acetate. - indicates that the Σ FIC could not be determined.

Synergy (bold text) = ≤ 0.5; Additive (italics)= > 0.5-1.0; Indifferent (no highlighting) = > 1.0 - ≤ 4; Antagonistic (underlined text) = > 4.0

Numbers indicate the mean FIC values of duplicate determinations.

all synergistic interactions occurred when extracts were combined with tetracycline against *A. baylyi*. As a general trend, the higher polarity (water and methanol) to mid-polarity (ethyl acetate) extracts interacted synergistically with tetracycline. These include aqueous (Σ FIC = 0.5), methanolic (Σ FIC = 0.38) and ethyl acetate (Σ FIC = 0.38) *P. pubescens* leaf extracts; the aqueous *P. pubescens* fruit extract (Σ FIC = 0.38); the aqueous (Σ FIC = 0.5) and ethyl acetate (Σ FIC = 0.28) *P. triloculare* leaf extracts; and the aqueous *P. triloculare* fruit extract (Σ FIC = 0.5).

Table 6 summarises the interactions of *Petalostigma* spp. extracts and conventional antibiotics against *P. aeruginosa*. Several extracts interacted additively with all of the conventional antibiotics tested, producing a total of seven additive combinations. In contrast, the *P. pubescens* leaf ethyl acetate extract and the *P. triloculare* leaf ethyl acetate extract were antagonistic when combined with gentamicin. A further general trend from these studies indicated that most antagonistic results occurred when gentamicin was used in combination with *Petalostigma* spp. leaf extracts, with some notable exceptions. Thus, the combination of gentamycin and any *Petalostigma* spp. leaf extract should be avoided.

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

The interactive antimicrobial interactions of *Petalostigma* spp. extracts with various conventional antibiotics against *S. pyogenes* are summarised in Table 7. A total of 12 combinations were categorised as being additive interactions and thus may be beneficial in treating *S. pyogenes* infections. However, the *P. triloculare* fruit hexane extract was antagonistic in combination with tetracycline and thus this combination should be avoided as a chemotherapeutic option to treat *S. pyogenes* infections. This was the only antagonistic result determined against *S. pyogenes*, indicating that all other combinations will not counter-indicate with the inhibitory properties of the conventional antibiotics and instead may have limited beneficial effects.

Varied ratio combination studies (isobolograms) Synergistic interactions with penicillin-G

Three combinations of *Petalostigma* spp. extract with penicillin-G were identified as inducing synergistic interactions (Figure 6). Notably, all of

Table 7: Σ FIC values of *Petalostigma* spp. extracts in combination with conventional antibiotics against *S. pyogenes*.

	Penicillin	Chloramphenicol	Gentamicin	Erythromycin	Tetracycline
PLW	1.06	1.06	2.00	1.06	1.25
PLM	1.02	1.02	1.25	1.02	1.06
PLC	2.50	2.50	1.25	2.50	-
PLH	-	-	1.06	-	2.50
PLE	1.13	1.13	3.00	1.13	0.75
PFW	*0.53*	*0.53*	-	*0.53*	*0.63*
PFM	1.01	1.01	1.13	1.01	1.03
PFC	-	-	1.06	-	2.50
PFH	-	-	2.13	-	*0.62*
PFE	1.13	1.13	3.00	1.13	*0.75*
TLW	1.02	1.02	1.25	1.01	1.06
TLM	1.01	1.01	1.13	1.01	1.03
TLC	-	-	*0.53*	-	2.50
TLH	2.50	*0.63*	1.25	2.50	-
TLE	1.06	1.06	2.00	1.06	1.25
TFW	1.03	1.03	1.50	1.03	1.13
TFM	1.03	1.03	1.50	1.03	1.13
TFC	-	-	1.06	-	2.50
TFH	2.50	2.50	1.25	2.50	4.00
TFE	*0.63*	*0.63*	2.50	*0.63*	-

P = *P. pubescens*; T = *P. triloculare*; L = leaf; F = fruit; W = water; M = methanol; C = chloroform; H = hexane; E = ethyl acetate. - indicates that the ΣFIC could not be determined.

Synergy (bold text) = ≤ 0.5; Additive (italics)= > 0.5-1.0; Indifferent (no highlighting) = > 1.0 - ≤ 4; Antagonistic (underlined text) = > 4.0

Numbers indicate the mean FIC values of duplicate determinations

Table 8: LC_{50} values determined for *Petalostigma* spp. fruit and leaf extracts in the *Artemia* nauplii and HDF bioassays following 24 h exposure.

Species		Extract	LC_{50} value (µg/mL)	
		ALA	HDF assay	
Petalostigma pubescens	Leaf	Water	661	-
		Methanol	749	-
		Chloroform	-	-
		Hexane	-	-
		Ethyl Acetate	750	-
	Fruit	Water	733	-
		Methanol	735	-
		Chloroform	-	-
		Hexane	-	-
		Ethyl Acetate	750	-
Petalostigma triloculare	Leaf	Water	750	-
		Methanol	1135	-
		Chloroform	-	-
		Hexane	-	-
		Ethyl Acetate	1505	-
	Fruit	Water	750	-
		Methanol	750	-
		Chloroform	-	-
		Hexane	-	-
		Ethyl Acetate	750	-

- indicates that less than 50% mortality was induced by the extract at all concentrations tested.

100% mortality was induced by potassium dichromate (positive control) and none by the sea water (negative control) at 1000 µg/mL.

these synergistic combinations occurred when tested against *P. mirabilis* growth. These combinations were further examined using isobologram analysis across a range of extract: penicillin-G ratios to identify the ideal ratios to obtain synergy. Interestingly, all of the ratios of *P. pubescens* fruit hexane extract and penicillin-G worked synergistically as *P. mirabilis* growth inhibitors (Figure 6a). Similarly, all combination ratios of the *P. pubescens* fruit ethyl acetate extract and penicillin-G produced synergistic interactions (Figure 6b). Thus, all ratios of these combinations would be beneficial to enhance *P. mirabilis* 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 rheumatoid arthritis), the ideal extract: penicillin-G ratio may be 90:10. However, when used for the treatment of acute infections (e.g. urinary tract infections), the ratio which maximises the efficacy of the treatment (i.e. the 10:90 ratio) may be the preferred option.

In contrast, the *P. triloculare* leaf ethyl acetate extract in combination with penicillin-G (Figure 6c) had a wider range of results. Only the ratios between 10-40% *P. triloculare* leaf ethyl acetate extract and 60-90% penicillin-G produced synergistic interactions. In addition, five out of the nine different ratios produced additive interactions. However, whilst 20:80, 30:70 and 40:60 extract: antibiotic ratios are classified as synergistic interactions, they were on the cut off between synergy and additive interactions. Therefore, 10% of *P. triloculare* leaf (ethyl acetate) extract and 90% penicillin-G (10:90) was deemed to be the best combination ratio for synergistic *P. mirabilis* growth inhibition.

Synergistic interactions with chloramphenicol

Three combinations of *Petalostigma* spp. extract with chloramphenicol induced synergistic interactions for *P. mirabilis* growth inhibition (Figure 7). Interestingly, all ratios of *P. pubescens* fruit hexane extract and chloramphenicol produced synergistic inhibition against *P. mirabilis* growth (Figure 7a). Similarly, all ratios of the *P. triloculare* leaf hexane extract and

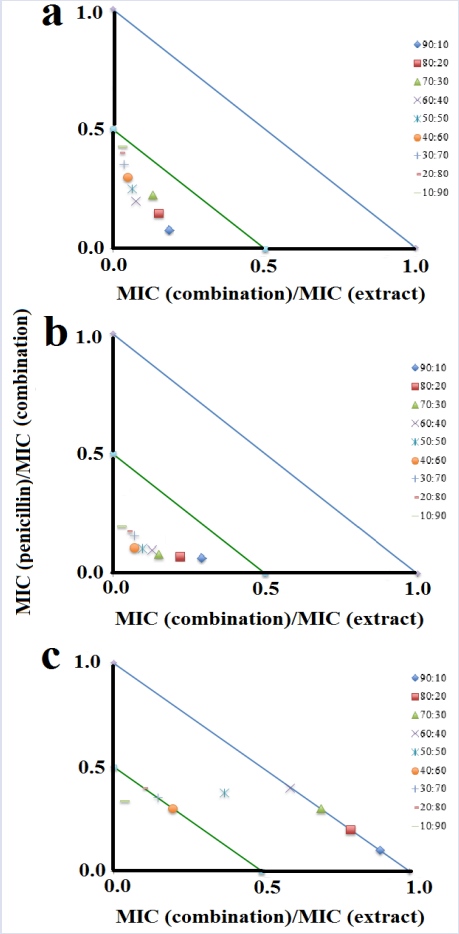

Figure 6: Isobologram for combinations of penicillin-G and (a) *P. pubescens* fruit hexane extract, (b) *P. pubescens* fruit ethyl acetate extract, (c) *P. triloculare* leaf ethyl acetate extract tested at various ratios against *P. mirabilis* (ATCC: 21721). Results represent mean MIC values of four replicates. Ratio = % extract: % antibiotic. Ratios lying on or underneath the green line are considered to be synergistic (Σ FIC ≤ 0.5). Any points between the green and blue line or on the blue line are deemed additive (Σ FIC > 0.5-1.0).

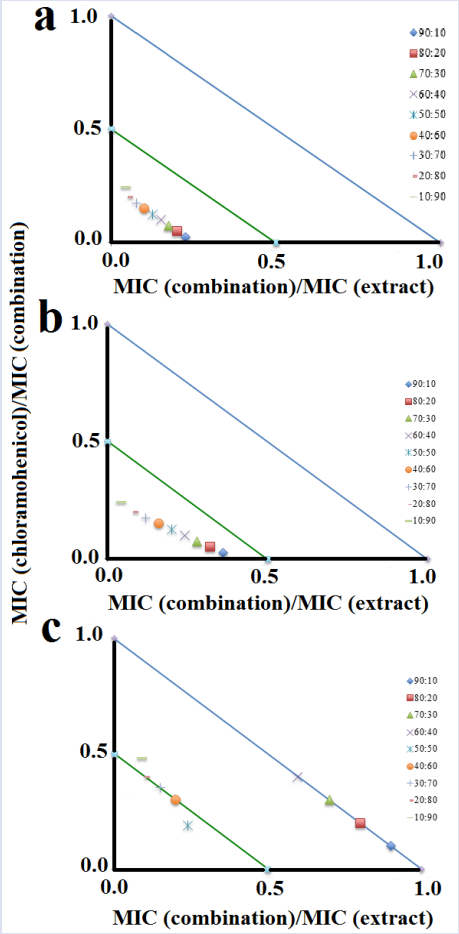

Figure 7: Isobologram for combinations of chloramphenicol and (a) *P. pubescens* fruit hexane extract, (b) *P. triloculare* leaf hexane extract, (c) *P. triloculare* leaf ethyl acetate extract tested at various ratios against *P. mirabilis* (ATCC: 21721). Results represent mean MIC values of four replicates. Ratio = % extract: % antibiotic. Ratios lying on or underneath the green line are considered to be synergistic (Σ FIC ≤ 0.5). Any points between the green and blue line or on the blue line are deemed additive (Σ FIC > 0.5-1.0).

chloramphenicol produced synergistic growth inhibition of *P. mirabilis* (Figure 7b). Therefore all ratios would be beneficial treatment options to enhance *P. mirabilis* growth inhibition. However, for long term prophylactic treatment, 90% extract and 10% chloramphenicol may be the ideal ratio for long term usage, whilst the 10:90 ratio may be preferential for the treatment of acute infections.

The combination of *P. triloculare* leaf ethyl acetate extract and chloramphenicol produced a wide range of results (Figure 7c). Four additive ratios were detected. Furthermore, combinations of 20-50% extract and 50-80% antibiotic acted synergistically against *P. mirabilis* growth. The ideal synergistic ratio for the treatment and prevention of rheumatoid arthritis (i.e. the treatment that minimises the amount of chloramphenicol used) was identified to be 50% *P. triloculare* leaf ethyl acetate extract and 50% chloramphenicol. In contrast, the preferred ratio for acute infections (the highest antibiotic % to maximise the efficacy of the treatment) is the 20% *P. triloculare* leaf ethyl acetate extract and 80% chloramphenicol ratio.

Synergistic interactions with erythromycin

The antimicrobial interactions between the *P. pubescens* fruit hexane extract and erythromycin (Figure 8) follows a similar trend to that observed for the combination of the *Petalostigma* extracts with either penicillin-G or chloramphenicol. Nearly all of the combinations of extract: antibiotic were synergistic. The 10:90 and 20:80 ratios produced additive interactions. All other ratios were synergistic inhibitors of *P. mirabilis* growth. The ideal synergistic ratio for growth inhibition of *P. mirabilis* in the treatment and prevention of rheumatoid arthritis is the 90% *P. pubescens* fruit (hexane) extract:10% erythromycin ratio, whilst the 30:70 ratio may be preferred for the treatment of acute infections.

Synergistic interactions with tetracycline

Seven different combinations of *Petalostigma* spp. extract and tetracycline were identified as synergistic against the growth of *A. baylyi*. Of these, four combinations contained *P. pubescens* extracts (Figure 9) and three combinations contained *P. triloculare* extracts (Figure 10). Therefore,

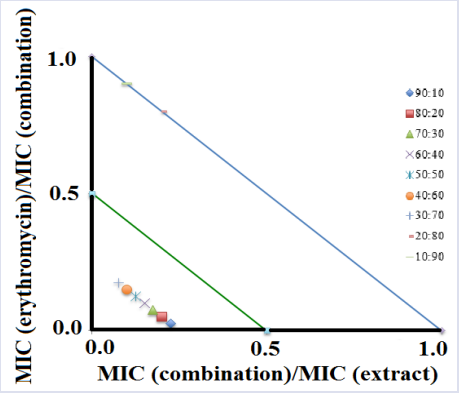

Figure 8: Isobologram for combinations of erythromycin and *P. pubescens* fruit hexane extract tested at various ratios against *P. mirabilis* (ATCC: 21721). Results represent mean MIC values of four replicates. Ratio = % extract: % antibiotic. Ratios lying on or underneath the green line are considered to be synergistic (Σ FIC ≤ 0.5). Any points between the green and blue line or on the blue line are deemed additive (Σ FIC > 0.5-1.0).

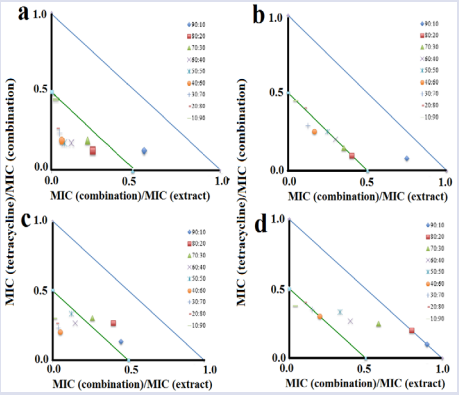

Figure 9: Isobologram for combinations of tetracycline and *P. pubescens* extracts tested at various ratios against *A. baylyi* (ATCC: 33304): (a) aqueous leaf extract, (b) methanolic leaf extract, (c) leaf ethyl acetate extract, (d) aqueous fruit extract. Results represent mean MIC values of four replicates. Ratio = % extract: % antibiotic. Ratios lying on or underneath the green line are considered to be synergistic (Σ FIC ≤ 0.5). Any points between the green and blue line or on the blue line are deemed additive (Σ FIC > 0.5-1.0).

various different ratios of these combinations were tested in order to identify the ideal ratio to obtain synergy. Nearly all of the ratios of *P. pubescens* leaf water extract with tetracycline acted synergistically against the growth of *A. baylyi* (Figure 9a). The 90% extract:10% tetracycline ratio was the only exception to this trend. The ideal synergistic ratio for growth inhibition of *A. baylyi* in the treatment and prevention of multiple sclerosis is 80% extract and 20% tetracycline, whilst the 10:90 ratio may be preferred for the treatment of acute *A. baylyi* infections. Similarly, the *P. pubescens* methanolic leaf extract was also determined to interact synergistically in combination with tetracycline at nearly all ratios. Indeed, only the 90 % extract, 10% tetracycline combination was non-synergistic. Instead, this combination produced an additive interaction (Figure 9b). Thus, the ideal combination ratios are the same as for the *P. pubescens* leaf water extract: tetracycline combination.

The ratios of *P. pubescens* leaf ethyl acetate extract in conjunction with tetracycline which interact synergistically were also determined (Figure 9c). Ratios between 10-60% extract and 40-90% antibiotic produced synergistic interactions. The 60% *P. pubescens* leaf ethyl acetate extract and 40% of tetracycline combination was determined to be the best ratio for synergistically inhibiting the growth of *A. baylyi* for longer term therapy whilst a 10% extract, 90% tetracycline ratio may be preferred for the treatment of acute *A. baylyi* infections. Similarly, the *P. pubescens* fruit water extract also interacted synergistically in combination with tetracycline in some ratios, yet additively against *A. baylyi* growth in other ratios. Ratios between 10-40% extract and 60-90% antibiotic were determined to produce synergistic interactions (Figure 9d). The preferred ratio to produce a synergistic interaction to inhibit the growth of *baylyi* in the treatment and prevention of multiple sclerosis is 10% of *P. pubescens* fruit water extract in combination with 90% of tetracycline. The 10% extract, 90% tetracycline ratio is preferred for the treatment of acute *A. baylyi* infections.

Figure 10a represents the interaction between *P. triloculare* leaf water extract in combination with tetracycline against *A. baylyi* growth. With a few notable exceptions (90:10 and 80:20, extract: tetracycline ratios), the majority of the extract: antibiotic combination ratios interacted synergistically for inhibiting the growth of *A. baylyi*. Similarly, all combinations of *P. triloculare* leaf ethyl acetate extract with tetracycline interacted synergistically for *A. baylyi* growth inhibition except the 90:10 and 80:20, extract: tetracycline ratios (Figure 10b). Thus 70% *P. pubescens* fruit water extract with 30% of tetracycline was deemed the preferred combination ratio to produce a synergistic interaction to inhibit the growth of *baylyi* in the treatment and prevention of multiple sclerosis. The 10% extract, 90% tetracycline ratio is preferred for the treatment of acute *A. baylyi* infections. In contrast, combinations of ≤ 50 % *P. triloculare* fruit water extract and ≥ 50% of tetracycline produced synergistic interactions for *A. baylyi* growth inhibition (Figure 10c). The best ratio for long term inhibition of *A. baylyi* growth was determined to be a mixture of 50% *P. triloculare* (fruit-water) extract with 50% tetracycline, whereas 10% extract with 90% tetracycline was deemed the best ratio for acute *A. baylyi* infections.

Quantification of toxicity

No LC_{50} values were determined for the chloroform and hexane extracts of either species as <50 % mortality was seen in all tested concentrations (Table 8). As extracts with LC_{50} values <1000 µg/ml towards *Artemia* nauplii have previously been defined as being toxic in this assay,[15,16] all chloroform and hexane extracts were deemed to be nontoxic. With the exception of aqueous and ethyl acetate extracts of *P. triloculare* leaf, all other *Petalostigma* spp. extracts were considered to be moderately toxic. Although the *A. franciscana* nauplii lethality assay is generally quite robust, it is noteworthy that brine shrimp are quite sensitive to changes in pH.[17] Acidic pH can suppress the rate of mitochondrial protein synthesis and potentially be fatal to the growth and development of nauplii. The presence of phenolic acids in the high-mid polarity extracts could potentially provide falsely high toxicity estimations. Indeed, previous studies have reported that extracts high in ascorbic acid can provide fallacious toxicity determinations.[18] Thus, this assay may have overestimated the toxicity of these extracts. In contrast, all plant extracts and conventional antibiotics demonstrated a lack of toxicity towards normal human primary dermal fibroblasts, with cellular viability for all tests substantially

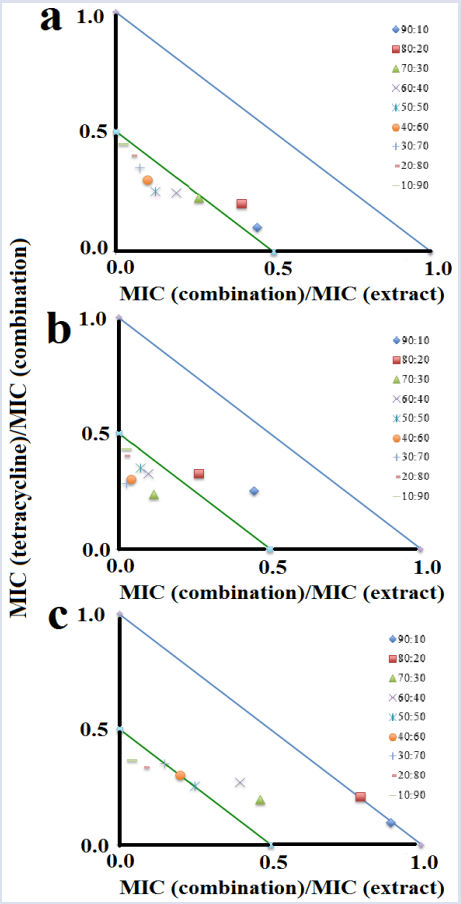

Figure 10: Isobologram for combinations of tetracycline and *P. triloculare* extracts tested at various ratios against *A. baylyi* (ATCC: 33304): (a) aqueous leaf fruit extract, (b) leaf ethyl acetate extract, (c) aqueous fruit extract. Results represent mean MIC values of four replicates. Ratio = % extract: % antibiotic. Ratios lying on or underneath the green line are considered to be synergistic (Σ FIC ≤ 0.5). Any points between the green and blue line or on the blue line are deemed additive (Σ FIC > 0.5-1.0).

>50% of the untreated control. All extracts were therefore deemed to be either nontoxic or of only low-moderate toxicity.

DISCUSSION

This study investigated the ability of *Petalostigma* spp. to inhibit the growth of some bacterial triggers of autoimmune inflammatory diseases, both alone and in combination with conventional antibiotics. Several *Petalostigma* spp. extracts were identified as effective bacterial growth inhibitors. In particular, the aqueous and methanolic extracts were strong inhibitors of *K. pneumoniae* and *S. pyogenes* growth, with MIC values with MICs as low as 344 µg/mL. However, the MIC values of most of the *Petalostigma* spp. extracts was substantially above 1000 µg/mL and are thus indicative of only low to moderate inhibitory activity. Whilst a detailed investigation of the phytochemistry of the *Petalostigma* spp. 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. Interestingly, nearly all of the water and methanolic extracts of *Petalostigma* spp. had relatively high abundances in flavonoids, tannins, phenols, triterpenoids and free anthraquinones. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.[19] 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.[20] Similarly, multiple tannins have broad spectrum antibacterial activity via a variety of intra and extracellular mechanisms, including the precipitation of microbial proteins.[21] Phenolics are toxic to microorganisms via enzyme inhibition mechanisms, possibly through non-specific interaction with proteins or by reaction with sulfhydryl groups.[22] Quinones can complex irreversibly with nucleophilic amino acids in proteins, generally leading to inactivation of the protein and loss of function, thus triggering microbial death.[22] Triterpenoids also have antibacterial activity, although the inhibitory mechanism is yet to be identified.[23] It is 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 *Petalostigma* spp. growth inhibition.

The combinational studies with conventional antibiotics were of greater interest. Several combinations displayed substantially greater potential as therapeutic agents against bacterial triggers of rheumatoid arthritis and multiple sclerosis than the extracts or antibiotics did alone. A total of 14 synergistic combinations were identified in this study with seven synergistic combinations each noted against both *P. mirabilis* and *A. baylyi*. 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.[15] Seven combinations of plant extracts had synergistic activity with penicillin-G, chloramphenicol and erythromycin against the growth of *P. mirabilis*. Notably, *P. mirabilis* was initially completely non-susceptible to chloramphenicol, erythromycin and half of the extracts that proved to be synergistic in the combinational studies. Thus, this study identified combinations of plant extracts and antibiotics which may repurpose relatively ineffective antibiotics and greatly enhance their efficacy, even against otherwise resistant bacterial strains. All of the extracts in synergistic combinations against *P. mirabilis* were mid-low polarity (hexane or ethyl acetate) suggesting the presence of a common active compound or class of compounds that may be responsible for the synergistic effects. Furthermore, several of these hexane extracts 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.[15] However, the isobolograms of *P. pubescens* fruit-hexane and *P. triloculare* leaf-hexane uniformly produced synergistic interactions despite the antibiotic tested. Furthermore, most of the hexane extracts were synergistic with antibiotics at concentrations as low as 10%, further emphasising the efficacy of the extracts. Such a trend is consistent with irreversible mechanisms such as clavulanic acid/β-lactam antibiotic combinations[23] and future studies will aim at testing the synergistic mechanism and whether it is due to irreversible events. In contrast, other synergistic extract: antibiotic combinations produced a wider range of interactions, including synergistic, additive and indifferent interactions. This is more consistent with reversible competition between the extract component(s) and the conventional antibiotic for binding to an effector.[24]

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 utilised to rapidly remove antibiotics that have entered the bacterial cells, thus rendering them resistant to the effects of the antibiotic.[25,26] 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 and several examples have previously been reported.[26] Isoflavones isolated from *Lupinus argenteus* potentiate the activity of the natural plant antibiotic berberine as well as the synthetic fluoroquinoline antibiotic, norfloxacin as inhibitors of *S. aureus* growth.[26] That study reported that the isoflavone allows a greater concentration of berberine in the bacteria by inhibiting the efflux mechanism (MDR pump). Similarly, *Mezoneuron benthamianum* and *Securinega virosa* extracts act as efflux pump inhibitors for fluoroquinolone, tetracycline and erythromycin in resistant strains of *S. aureus* (MRSA).[27] As a consequence, the *M. benthamianum* ethanol extract and chloroform extract of *S. virosa* reduce the MIC (minimum inhibitory concentration) of norfloxacin against *S. aureus* by a factor of 4.

In our study, *P. mirabilis* was completely non-susceptible to chloramphenicol, tetracycline and erythromycin, with only a low susceptibility to penicillin. All of these antibiotics are susceptible to resistance due to efflux pumps.[3,28] A single pump can provide bacteria with resistance to a wide array of chemically and structurally diverse antibiotics and it is not uncommon for an organism to code for more than one efflux pump.[3,28] It is therefore imperative to identify agents that can block the efflux mechanism (efflux pump inhibitors - EPIs) or alter the process of efflux and in so doing, extend the life of existing antibacterial drugs. Plants produce various secondary metabolites that are used as defense mechanisms against pathogenic invaders. Some plants produce antimicrobials which, along with other compounds, inhibit the efflux of those antimicrobials from a bacterial cell. There are currently no EPI/antimicrobial drug combinations on the market, although research into identifying potential EPIs is ongoing.[28] The synergistic interaction against *P. mirabilis* in our study suggests the possibility of a common EPI in the *P. pubescens* fruit (hexane and ethyl acetate) and *P. triloculare* (hexane and ethyl acetate) extracts that could be inhibiting a MDR efflux pump in the microbe.

Alternatively (or in addition to MDR efflux pumps), the *P. mirabilis* strain used in our study may have acquired genes encoding for reduced-affinity penicillin-binding protein 2a (PBP2a) (rendering β-lactam antibiotics ineffective).[29] It is likely that as penicillin binding proteins are a group of protein enzymes, these phytochemicals may form nonspecific interaction and affect the bacterial cell biosynthesis. The *P. pubescens* fruit and *P. triloculare* leaf hexane extracts may also contain a β-lactamase inhibitor. β-lactamases are the major defense of gram-negative bacteria against β-lactam antibiotics.[30] Clavulanic acid is an irreversible β-lactamase inhibitor, which in combination with β-lactam antibiotics can block the bacterial antimicrobial resistance mechanism. The *Petalostigma* spp. hexane extracts-β-lactam antibiotic combinations have inhibitory profiles similar with those of clavulanic acid-β-lactam combinations.[24] Neither clavulanic acid[24] nor the *Petalostigma* spp. hexane extracts had appreciable intrinsic antimicrobial activity when tested on their own. The *Petalostigma* spp. hexane extracts were consistently synergistic against *P. mirabilis* growth, even at low levels of antibiotic, suggesting the possibility of a compound that behaves similarly to clavulanic acid (i.e. an irreversible/competitive inhibitor). Further studies are required to identify whether extract compounds mirror the chemical and biological characteristics of clavulanic acid (i.e. the presence of a β-lactam ring).

Seven *Petalostigma* spp. extracts were synergistic against *A. baylyi* when tested in combination with tetracycline against *A. baylyi*. All of these extracts were extracted using mid-high polarity solvents. It is likely that these extracts have overlapping phytochemical profile and the potentiator components may be common components of all of these extracts. However, the compound(s) which induce the synergistic effects may be in greater amounts in the *P. pubescens* (leaf-water) and *P. triloculare* (leaf-ethyl acetate) extracts in comparison to the other extract as they consistently produced synergistic interactions, even with low extract ratios. Efflux pumps are the main bacterial resistance mechanism which renders tetracycline inactive.[31] A total of nine multidrug efflux systems have been identified in *Acinetobacter* spp. including Tet (A), a potent tetracycline efflux protein.[32] Our studies suggest the possibility that certain EPIs may be common to all of the mid-high polarity extracts that were synergistic against *A. baylyi* in this study. In addition to potentiation activity, it is also likely that the extracts may themselves be participating in the inhibitory action against *A. baylyi* growth. In order to identify the active compound(s) that is/are inducing synergy, bioactive driven separation and identification studies and/or metabolomic profiling studies are required.

Ultimately, the preparation of combinations of *Petalostigma* spp. 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 *Petalostigma* spp. 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.[33] 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

A further trend was evident in our study: most of the extract-antibiotic combinations which did not produce synergistic effects, generally did not greatly affect the efficacy of the antibiotic i.e. they appear to not counter-indicate with the antibiotics tested in this study. This is important as many users of herbal and traditional medicines self-diagnose/treat, often with multiple therapies concurrently. Thus, an understanding of drug herbal medicine interactions is important. Only 3.2% of the combinations tested produced antagonistic interactions with the conventional antibiotics against the bacteria tested. Most of the antagonistic combinations occurred when leaf extracts (mid-low polarity) were tested in conjunction with gentamycin, with a few exceptions. Therefore, it is likely that an inhibitory molecule may be common to mid-low polarity extracts. These are important findings and highlight combinations which should be avoided. Interestingly, previous studies indicate that antagonistic combinations of plant extracts with gentamicin are not uncommon.[34] Therefore, caution must be exercised if using *Petalostigma* spp. extracts in conjunction with gentamicin, especially against *K. pneumoniae.*

CONCLUSION

The results of this study demonstrate the potential of *Petalostigma* spp. extracts in inhibiting the growth of some bacterial triggers of autoimmune inflammatory diseases. The aqueous and methanolic extracts were moderate inhibitors of several microbes. However, the therapeutic potential of the *Petalostigma* spp. extracts was far more apparent when tested in combination with conventional antibiotics as potentiators. The mid-low polarity extracts were substantially better potentiators of conventional antibiotics in inhibiting the growth of *Proteus mirabilis* and *Acinetobacter baylyi* than the higher polarity extracts. Although the mechanisms of synergy are still unclear, studies indicate that compounds within *Petalostigma* spp. could potentially mimic the actions of a resistance modifying agent, thus potentiating the activity of several antibiotics that are relatively ineffective alone. Therefore, a combinational approach not only increases the effectiveness of drugs, but can potentially reduce the side effects and lessen the potential to develop drug resistant pathogens.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide; **LC_{50}:** The concentration required to achieve 50 % mortality; **MIC:** Minimum inhibitory concentration.

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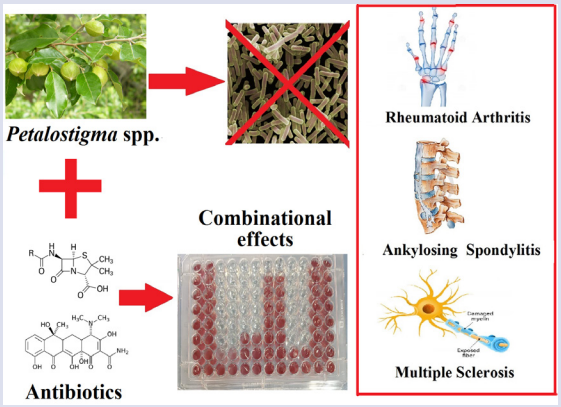
GRAPHICAL ABSTRACT



SUMMARY

- *Petalostigma* spp. methanolic and water extracts were screened for growth inhibitory activity against several microbes.
- Liquid dilution and disc diffusion techniques were used to quantify the efficacy of the extracts and compare them to conventional antibiotics.
- The extracts were also tested in combination with conventional antibiotics to determine whether the extracts cold potentiate the antibiotic activity.
- All synergistic combinations were tested in varying ratios of the antimicrobial components to determine the ideal ratios for therapeutic usage.
- Toxicity was evaluated using the *Artemia* nauplii bioassay.

ABOUT AUTHORS

Dr. Ian Cock leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin including *Aloe vera*, South Asian and South American tropical fruits, as well as Australia plants including *Scaevola spinescens, Pittosporum phylliraeoides, Terminalia ferdinandiana* (Kakadu plum), Australian Acacias, Syzygiums, *Petalostigmas* and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in nearly 200 scientific publications in a variety of peer reviewed journals.

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