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Original Research Article

Interactive antimicrobial and toxicity profiles of *Pittosporum angustifolium* Lodd. extracts with conventional antimicrobials**Baxter Blonk^{1,2}, Ian E. Cock^{1,2}**

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ABSTRACT

Objective: *Pittosporum angustifolium* Lodd. is used to treat a variety of pathogenic diseases and inflammation by Australian aborigines. Practitioners of complementary medicine frequently use herbal medicines concurrently with conventional antibiotics. There is a need to evaluate their effects in combination.

Methods: The bacterial growth inhibitory activity of *P. angustifolium* leaf extracts was assessed against a panel of pathogenic triggers of some autoimmune diseases by standard disc diffusion and liquid dilution minimum inhibitory concentration (MIC) methods.

Combinational effects between the extracts and conventional antimicrobials were classified using the sum of the fractional inhibitory concentration. Synergistic interactions were further assessed across a range of ratios by isobologram analysis. The toxicity of the individual samples and combinations was evaluated by *Artemia* lethality and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium human dermal fibroblast cell viability assays.

Results: *P. angustifolium* leaf extracts strongly inhibited the growth of several bacterial triggers of autoimmune diseases. The methanolic, aqueous and ethyl acetate extracts were particularly good inhibitors of *Proteus mirabilis* and *Klebsiella pneumonia* growth (MIC = 26 and 57 µg/mL respectively). Some combinations of the extracts and conventional antibiotics significantly potentiated the combined inhibitory activity compared to the individual components. Of the 250 combinations studied, approximately 0.02% showed synergistic interactions, 49.6% were additive, 46.8% showed indifferent interactions and antagonism occurred in only 0.02% of the combinations. Interestingly, all of the synergistic and antagonistic combinations contained tetracycline as their antibiotic component.

Conclusion: *P. angustifolium* leaf extracts inhibit the growth of pathogenic triggers of some autoimmune diseases. Some extracts also potentiated the activity of conventional antibiotics, without significantly affecting the toxicity of the combination.

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1. Introduction

Despite their initial efficacy, recent increase in bacterial resistance to clinical antibiotics has made the development of new antibiotic therapies a high priority [1]. A parallel decrease in the introduction of new antibiotic therapies in recent years has further compounded this problem. As a result, interest in re-evaluating medicinal plants for new antibiotic chemotherapies has escalated substantially in recent years [2]. However, whilst the bacterial growth inhibitory activity of some plant extracts and essential oils is promising, isolated plant-derived antimicrobial compounds usually possess lower potency than the currently available antimicrobial regimen [3]. Individual compounds in plants often act synergistically to substantially potentiate the activity of the combination compared to that of the individual components. Combinational approaches may prove to be more effective in overcoming antibiotic resistance and may even allow conventional antibiotics to again be effective against resistant bacterial strains. Several studies have already investigated combinations of natural product and conventional antimicrobials and have reported promising results [3–9]. Several plant species have been identified that substantially potentiate the activity of conventional antimicrobials, even when the plants do not possess antimicrobial activity alone [2,3,8]. Other studies have screened combinations of purified phenols, tannins or flavonoids with conventional antibiotics and have identified several synergistic formulations of medical importance [10–13]. It is important to investigate these interactive effects to identify possible alternatives to overcome resistance. Further, combinational studies may also provide valuable information for clinical settings, where complementary therapies may be used concurrently with allopathic drugs.

Consumers of complementary and alternative medicine (CAM) frequently use CAM therapies and allopathic medications concurrently without knowledge or understanding of the potential interactions and counter-indications. A lack of understanding of natural medicine/conventional drugs interactions, may pose serious risks to patient safety, especially when the patient does not report the use of natural medicines to their healthcare professional [14,15]. It is of concern that approximately 20% of the population in Western countries use CAMs in combination with prescription drugs [16,17]. Indeed, a recent study into the use of CAMs in Canada estimated that 23% of the population regularly use herbal products [17]. This study also reported that over 5% of the population used CAMs concurrently with prescription drugs [17]. A similar survey in the United States reported that 72% of patients used herbal remedies whilst also taking prescription drugs [18]. That study also reported that 84% of the surveyed patients used over-the-counter medications concurrently with natural therapies. However, severe reactions may occur when natural therapies and conventional medicine are used concurrently [19,20]. Further work is urgently needed to test the safety of allopathic and CAM combinations.

Like synthetic drugs, natural products may induce severe interactions and are not devoid of toxicity [21,22]. Furthermore, combining drug therapies may result in counter-indications and toxicities. Despite this, drug combination studies generally focus on the efficacy of the herb and drug combinations. Identification of toxic effects of the combinations is often neglected. Despite extensive studies reporting interactions between herbal medicines/natural products and other allopathic therapies [19], there are limited reports of interactions between traditional medicinal plants and conventional antimicrobials. Such interactions may have considerable effects on the efficacy and safety of the conventional treatment regimens. A comprehensive investigation of these interactions is therefore warranted for any medicinal plant material.

Pittosporum angustifolium Lodd. (family Pittosporaceae; formerly known as *Pittosporum phylliraeoides*; commonly known as weeping *Pittosporum*, cattlebush, Gumbi Gumbi,

Gumby Gumby, Cumbi Cumbi and native apricot) was selected for this study, due to its range of traditional therapeutic uses by the first Australians in the treatment of pathogenic disease [23,24]. Decoctions and infusions prepared from the leaves are reputed to inhibit a variety of bacterial, fungal and viral pathogens [23,25]. A decoction of the fruit was also used to treat eczema and pruritus. Anecdotal reports have also associated *P. angustifolium* leaf preparations with anticancer properties [26], and this species is sometimes referred to as “Queensland anticancer tree.” Furthermore, *P. angustifolium* extracts have been reported to have moderate cytotoxic activity towards A427 lung cancer cells [27]. Despite its range of traditional medicinal uses, the phytochemistry and therapeutic potential of *P. angustifolium* remain to be rigorously studied. One study reported that *P. angustifolium* leaf extracts inhibited Ross River virus-induced cytopathicity by more than 25%, but were ineffective against poliovirus and cytomegalovirus. A more recent study reported broad-spectrum antibacterial activity of *P. angustifolium* leaf extracts against a panel of enteric bacteria [25]. This study aimed to extend these earlier studies by evaluating the growth inhibitory properties of *P. angustifolium* leaf extracts against bacterial triggers of some autoimmune inflammatory diseases. Furthermore, the interactive antimicrobial and toxicity profiles of combinations of *P. angustifolium* extracts and five conventional antibiotic drugs are reported for the first time.

2. Material and methods

2.1. Sourcing and preparation of plant samples

P. angustifolium auct. non (DC) Benth. leaf plant material was collected by Philip Higson of the Queensland Bush Foods Association, Australia from verified trees outside Blackall, Australia. The leaves were collected at global position system coordinates of $-24^{\circ} 27' 33.27''$ S, $145^{\circ} 32' 56.26''$ E. Voucher specimens are deposited in the School of Natural Sciences, Griffith University, Australia (voucher number: GUPAB1-2014-1/1). The leaves were thoroughly dried using a Sunbeam food dehydrator (model Dt5600) and the dried material was stored at -30°C . The plant material was thawed and ground into a coarse powder prior to use. Individual quantities (1.5 g) of the ground plant material were weighed into separate Falcon tubes, and 50 mL of deionised water, methanol, chloroform, hexane or ethyl acetate were added. All solvents were analytical reagent (AR) grade and were obtained from Ajax Fine Chemicals, Australia. All extractions were performed for 24 h, at 4°C , with gentle shaking. Particulate material was removed by filtration through Whatman No. 54 filter paper. The solvent extracts were air-dried at room temperature, whilst the aqueous extract was lyophilised. Once dried, the extracts were weighed to determine yield and were then dissolved in 10 mL deionised water (containing 1% dimethyl sulfoxide).

2.2. Qualitative phytochemical analysis

Phytochemical analysis of the *P. angustifolium* extracts for the presence of alkaloids, cardiac glycosides, flavonoids, phenolic compounds, phytosterols, saponins, tannins and triterpenoids used standard assays [28,29].

2.3. Antibacterial analysis

2.3.1. Conventional antibiotics

Penicillin G (1440–1680 $\mu\text{g}/\text{mg}$), chloramphenicol ($\geq 98\%$ purity), erythromycin (≥ 850 $\mu\text{g}/\text{mg}$), gentamicin (600 $\mu\text{g}/\text{mg}$) and tetracycline ($\geq 95\%$ purity) were purchased from Sigma-Aldrich, Australia. All antibiotics were prepared as 0.01 mg/mL stocks in sterile deionised water.

2.3.2. Bacterial cultures

The bacterial species examined in this study were selected because they are known triggers of autoimmune inflammatory diseases [30]. Reference strains of *Acinetobacter baylyi*

(ATCC33304), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721) and *Pseudomonas aeruginosa* (ATCC39324) were obtained from American Type Culture Collection (ATCC), USA. The School of Natural Sciences teaching laboratory at Griffith University kindly provided the clinical strain of *Streptococcus pyogenes* used in this study. All bacteria were individually cultured to log phase in nutrient broth (Oxoid Ltd., Australia). Streak nutrient agar (Oxoid Ltd., Australia) plates were cultured in parallel to ensure the purity of all bacterial cultures and to allow for subculturing. All cultures were incubated at 37 °C for 24 h and were maintained in nutrient broth at 4 °C.

2.3.3. Evaluation of bacterial susceptibility to growth inhibition

Bacterial susceptibility to the *P. angustifolia* extracts, and to the conventional antibiotics, was assessed by standard disc diffusion (DD) methods [25]. Standard discs of ampicillin (2 µg) and chloramphenicol (10 µg) were purchased from Oxoid Ltd., Australia for use as positive controls. Sterile filter paper discs were infused with 10 µL of distilled water and included as a negative control.

2.4. Minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) for each extract was determined using liquid dilution (LD) MIC assays and solid phase agar DD assays.

2.4.1. Microplate LD MIC assay

A standard LD MIC assay [31] was used to evaluate the bacterial growth inhibitory activity of the extracts and conventional antibiotics, both independently and in combinations. Briefly, log phase bacterial cultures were diluted to produce a McFarlands inoculation culture [31]. A 100 µL volume of sterilized nutrient broth was dispensed into all wells of a 96-well microtitre plate. A 100 µL volume of the plant extracts or conventional antibiotics was dispensed into separate wells of the top row of the plate. A negative control (nutrient broth), sterile control (broth without bacteria) and a sample-free culture control (to ensure the media was capable of supporting microbial growth) were also included on all plates. Each test sample or control was serially diluted down each column on the plate by doubling dilution. The assay culture inoculum (100 µL, containing approximately 1×10^6 colony-forming units/mL) was then added to all wells except the sterile control wells and incubated overnight at 37 °C. p-Iodonitrotetrazolium violet (INT, Sigma-Aldrich, Australia) was dissolved in sterile deionised water to a concentration of 0.2 mg/mL. A volume of 40 µL of the INT solution was added into all wells and the plate was incubated for a further 6 h at 37 °C. The MIC was visually determined as the lowest dose at which colour development was inhibited.

2.4.2. DD MIC assay

The MIC of each extract was also quantified using the DD assay [29]. Graphs of the zone of inhibition (ZOI) versus natural logarithm concentration were plotted and MIC values were calculated by linear regression.

2.5. Extract-conventional antibiotic interaction studies

2.5.1. Fractional inhibitory concentration assessment

Interactions between the plant samples and conventional antimicrobials were detected and classified via determination of the sum of the fractional inhibitory concentration (Σ FIC). The FIC was calculated using the following equation, where (a) represents the plant sample and (b) the conventional antimicrobial sample [3]:

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

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

The Σ FIC was then calculated using the equation: Σ FIC = $FIC^{(i)} + FIC^{(ii)}$. The interactions were classified as being synergistic for Σ FIC values of ≤ 0.5 , additive (> 0.5 and ≤ 1.0),

indifferent (> 1.0 and ≤ 4.0) or antagonistic (> 4.0) [3].

2.5.2. Varied ratio combination studies (isobolograms)

When synergistic interactions were detected, varied ratios of the combination were prepared (Table 1) and the MIC value of each ratio was determined. These data were plotted on an isobologram using the GraphPad Prism® (Version 5, GraphPad Software, La Jolla California, USA). Isobologram analysis allows for the determination of which component (plant extract or conventional antibiotic) contributed most to the synergistic potentiation detected for the combination. Data points falling below the 0.5:0.5 line indicated synergy, whilst those above the 0.5:0.5 line, but below the 1.0:1.0 line, indicated an additive interaction. Data points above the 1.0:1.0 line, but below the 4.0:4.0 line indicated a noninteractive or indifferent interaction, whilst data points falling above the 4.0:4.0 line indicated antagonism [3].

2.6. Toxicity studies

Two assays were used to evaluate and quantify the toxicity of the extracts. The *Artemia* lethality assay was utilised for rapid preliminary toxicity assessment. An 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based cellular proliferation assay was used as a cellular evaluation of the extracts' toxicity.

2.6.1. Brine-shrimp lethality assay

Toxicity of the *P. angustifolia* extracts, conventional antibiotics and controls was assessed using a modified *Artemia franciscana* Kellogg nauplii lethality assay [32,33]. Briefly, 400 μ L of artificial seawater containing 40–60 live *A. franciscana* nauplii was added to each well of a 48-well microtitre plate. Individual 400 μ L volumes of the test samples (diluted plant extracts, antimicrobials or combinations) were added to the wells and incubated at (25 ± 1) °C. Negative controls (32 g/L artificial seawater; Sigma Australia) and positive controls (1 mg/mL potassium dichromate [$K_2Cr_2O_7$; AR grade, Chem-Supply, Australia]) were included on all plates. After 24 hours of exposure, the number of moribund *A. franciscana* nauplii was counted and the percentage mortality was calculated [33,34]. The median lethal dose (LD_{50}) value was determined as the concentration of a test substance necessary to have a lethal effect on 50% of the *A. franciscana* nauplii. The results are expressed as the mean of three independent experiments, each with internal triplicates ($n = 9$).

2.6.2. MTS cellular viability assay

The *P. angustifolia* extracts and conventional antibiotics were screened against normal human primary dermal fibroblasts (HDFs; American Type Culture Collection [ATCC PCS-201-012]) using conventional assays [35]. Briefly, aliquots of the HDFs (70 μ L, containing approximately 5000 cells) were added to individual wells of a 96-well plate. The test extracts or controls (30 μ L) were added to individual wells and the plates were incubated at 37 °C with 5% CO_2 for 24 h in a humidified atmosphere. All extracts were initially tested at 200 μ g/mL. Following a wash in phosphate-buffered saline (pH 7.2) to remove interference due to sample colour, 20 μ L of CellTiter 96 Aqueous One solution (Promega) was added to all wells and the plates were incubated at 37 °C for a further 3 h. Absorbances were recorded at a test wavelength of 540 nm and a blank wavelength of 690 nm using a Spectra Max M3 plate reader (Molecular Devices, Sunnyvale, CA, USA). All tests were performed three times, each with internal triplicates ($n = 9$). The percentage of cellular viability of each test was calculated using the following formula:

$$\text{Percentage of cellular viability} = \frac{A_{\text{test sample}} - (\text{mean } A_{\text{control}} - \text{mean } A_{\text{blank}})}{(\text{mean } A_{\text{control}} - \text{mean } A_{\text{blank}})}$$

A refers to absorbance. Less than 50% cellular viability indicated toxicity, whereas tests with $> 50\%$ untreated control viability were defined as nontoxic.

2.7. Statistical analysis

Data are expressed as the mean $\pm$ standard deviation of at least three independent experiments with three internal triplicates ($n = 9$). One-way analysis of variance was used to calculate statistical significance between the negative control and treated groups with a P value < 0.01 considered to be statistically significant.

3. Results

3.1. Extraction yields and qualitative phytochemical screening

Solvent extraction of the *P. angustifolium* leaves (1.5 g) yielded dried plant extracts ranging from 55 mg (*P. angustifolium* leaf hexane extract) to 255 mg (*P. angustifolium* leaf aqueous extract; Table 1). The qualitative phytochemical screenings (Table 2) determined that the methanol and water extracts were the most efficient solvents, extracting the highest masses and greatest diversity of phytochemical classes.

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

Extract	Solvent				
	Methanol	Water	Ethyl acetate	Chloroform	Hexane
Mass of dried extracted material (mg)	245	255	65	115	55
Concentration of extract (mg/mL)	24.5	25.5	6.5	11.5	5.5
Phenols					
Total phenolics	++	+++	—	—	—
Water soluble	+	+	—	—	—
Water insoluble	+	+++	—	—	—
Cardiac glycosides (Keller-Kiliani test)	—	—	—	—	—
Saponins (froth persistence)	++	+++	+	—	—
Triterpenes (Salkowski test)	++	++	—	—	—
Phytosterols (Acetic Anhydride test)	—	—	—	—	—
Alkaloids					
Meyers test	—	—	—	—	—
Wagners test	—	—	—	—	—
Flavanoids					
Shinoda test	++	+++	+	+	—
Kumar test	++	+++	+	+	—
Tannins (Ferric Chloride test)	—	+	—	—	—
Anthraquinones					
Free	—	—	—	—	—
Combined	—	—	—	—	—

+++ : a large response; ++ : a moderate response; + : a minor response; — : no response in the assay.

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

Volume ratio of extract: antibiotic (μ L)	Concentration of plant extract in combination (mg/mL)	Concentration of antibiotic ^a in combination (μ g/mL)
90:10	90.00	3.20
80:20	80.00	6.40
70:30	70.00	9.60
60:40	60.00	12.80
50:50	50.00	16.00
40:60	40.00	19.20
30:70	30.00	22.40
20:80	20.00	25.60
10:90	10.00	28.80

^a means penicillin G, chloramphenicol, erythromycin, gentamicin or tetracycline.

3.2. Bacterial growth inhibition screening

3.2.1 Inhibition of *Proteus* spp. (rheumatoid arthritis triggers) growth

P. mirabilis growth was generally most susceptible to the higher polarity aqueous and methanolic *P. angustifolium* leaf extracts and less susceptible to the mid to lower polarity extracts (Fig. 1a). Indeed, ZOI of approximately 14 mm and 15 mm were recorded for the

methanolic and aqueous extracts, respectively, against the clinical *P. mirabilis* strain. A further trend was also evident: whilst the extracts were strong inhibitors of both *P. mirabilis* strains, the clinical isolate was generally the more susceptible strain. The chloroform extract was the exception to both of these trends, recording the largest ZOI of all the extracts, and inhibiting the reference bacterial strain substantially more strongly than the clinical strain (as determined by ZOI diameter). Notably, this extract also produced larger ZOI than both of the reference antibiotics (ampicillin and chloramphenicol) tested in this study. The *P. angustifolium* leaf ethyl acetate extract was also a moderate inhibitor of *P. mirabilis* growth. In contrast, the hexane extract was a poor inhibitor of the growth of both *P. mirabilis* strains. *Proteus vulgaris* growth was substantially less susceptible to the *P. angustifolium* leaf extracts than *P. mirabilis* was (Fig. 1b). The *P. angustifolium* methanolic leaf extract was the best growth inhibitor, albeit with ZOI < 8 mm. All other extracts were determined to be relatively weak inhibitors of *P. vulgaris* growth (ZOIs < 7 mm). Indeed, the hexane extract was completely ineffective in inhibiting *P. vulgaris* growth. Notably, this *P. vulgaris* strain was relatively unaffected by ampicillin (ZOI < 7 mm), indicating that this is an ampicillin-resistant strain. In contrast, chloramphenicol was highly effective (ZOI = 16 mm).

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

With the exception of the hexane extract, all *P. angustifolium* leaf extracts inhibited *K. pneumoniae* growth (Fig. 1c). However, the inhibitory efficacy was substantially lower than seen for *P. mirabilis*. In general, the reference *K. pneumoniae* strain was more sensitive to the *P. angustifolium* leaf extracts than was the clinical strain. The methanolic extract produced the greatest growth inhibition (ZOI $\approx$ 10 mm for the reference strain of *K. pneumoniae*). Notably, resistance against β -lactam antibiotics was noted for both *K. pneumoniae* strains. In contrast, both *K. pneumoniae* strains were highly susceptible to chloramphenicol, with ZOIs > 16 mm against both strains. The growth inhibition recorded for ethyl acetate and chloroform extracts was significantly lower than for the methanolic extract, whilst the hexane was completely ineffective against both *K. pneumoniae* strains.

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

The aqueous *P. angustifolium* leaf extract was the most effective *A. baylyi* growth inhibitor against the reference and clinical strains (8.0 mm and 7.2 mm respectively; Fig. 1d). However, these ZOIs are indicative of only low to moderate activity. Similarly, the chloroform, ethyl acetate, methanol and hexane extracts each produced small ZOIs against both *A. baylyi* strains. In contrast, the ampicillin (ZOI = 10.2 ± 0.4 mm) and chloramphenicol (ZOI = 12 mm) controls were good inhibitors of the reference *A. baylyi* strain. The clinical *A. baylyi* strain was substantially more resistant to ampicillin and chloramphenicol (ZOIs of 7.8 ± 0.4 mm and 9.6 ± 0.4 mm respectively).

All *P. angustifolium* extracts inhibited *P. aeruginosa* growth for both strains (Fig. 1e). The aqueous *P. angustifolium* leaf extract was the best *P. aeruginosa* growth inhibitor, with ZOIs = 7.9 ± 0.6 mm and 9.6 ± 0.6 mm for reference and clinical *P. aeruginosa* strains respectively. This is a noteworthy finding as both *P. aeruginosa* strains were resistant to ampicillin and chloramphenicol (each inducing ZOIs in the range of 6.5–7.5 mm).

Furthermore, the methanolic and ethyl acetate *P. angustifolium* leaf extracts also inhibited *P. aeruginosa* growth, albeit with ZOIs = 7.7–8.3 mm, indicative of only low to moderate inhibition. Both *P. aeruginosa* strains were substantially less affected by chloroform and hexane extracts (ZOIs generally < 6.5 mm). Our study therefore indicates that the aqueous *P. angustifolium* leaf extract was the most effective inhibitor of both bacterial triggers of multiple sclerosis. The methanolic and ethyl acetate extracts also had noteworthy growth inhibitory activity.

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

The methanolic, aqueous and chloroform *P. angustifolium* leaf extracts also inhibited *S. pyogenes* growth, albeit with small ZOI, indicative of only low efficacy (Fig. 1f). The ethyl acetate and hexane extracts were completely ineffective as *S. pyogenes* growth inhibitors. The methanolic *P. angustifolium* leaf extract was the strongest inhibitor of *S. pyogenes* growth. However, this extract only produced ZOI of (7.3 ± 0.3) mm, indicating low growth inhibitory activity. Interestingly, this *S. pyogenes* strain was relatively resistant to the chloramphenicol control, but susceptible to ampicillin (ZOIs of 7.6 mm and 14.6 mm respectively).

Fig. 1. Antibacterial activity of *Pittosporum angustifolium* leaf extracts against (a) *Proteus mirabilis* (ATCC21721) and clinical isolate strains; (b) *Proteus vulgaris* (ATCC21719); (c) *Klebsiella pneumoniae* (ATCC31488) and clinical strain; (d) *Acinetobacter baylyi* (ATCC33304) and clinical strain; (e) *Pseudomonas aeruginosa* (ATCC39324) and clinical strain; (f) *Streptococcus 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 (2 μ g); Chl: chloramphenicol (10 μ g); NC: negative control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates (two repeats) $\pm$ standard deviation. * $P < 0.01$, vs negative control.

3.3. Determination of MIC

The antimicrobial efficacy of the extracts was further quantified by the LD and DD MIC assays (Table 3). Consistent with the antibacterial screening assays, the methanol and water *P. angustifolium* leaf extracts inhibited the growth of all of the bacterial triggers of the tested autoimmune diseases. As MIC values of ampicillin and chloramphenicol were determined in the DD assay using discs preloaded with a fixed amount of antibiotic, we were only able to determine MICs for the conventional antibiotics in the LD assay. Notably, gentamicin was the most potent antibiotic against all bacterial species. Interestingly, the bacterial strains tested were generally resistant to all conventional antibiotics tested, except gentamycin. *A. baylyi* and *P. aeruginosa* were also resistant to gentamicin, making them resistant to all of the antibiotics tested.

The MIC values determined for the *P. angustifolium* leaf extracts were consistent between both MIC assays. The methanolic *P. angustifolium* leaf extract was a strong *P. mirabilis* growth inhibitor (LD MICs of 38 µg/mL and 26 µg/mL against the reference and clinical bacterial strains respectively). The aqueous extract (LD MICs of 570 µg/mL and 505 µg/mL against the reference and clinical bacterial strains, respectively) and the ethyl acetate extract (LD MICs of 440 µg/mL and 563 µg/mL against the reference and clinical bacterial strains, respectively) were also strong *P. mirabilis* growth inhibitors. The *P. angustifolium* leaf chloroform extract was a low to moderate inhibitor of *P. mirabilis* (LD MICs of 1250 µg/mL and 750 µg/mL against the reference and clinical bacterial strains, respectively). In contrast, *P. vulgaris* was substantially more resistant to the *P. angustifolium* leaf extracts, with MIC values generally substantially > 1000 µg/mL.

The methanolic, aqueous and ethyl acetate *P. angustifolium* leaf extracts were also good *K. pneumoniae* growth inhibitors (LD MIC values generally < 500 µg/mL). The ethyl acetate extract was particularly potent against the clinical *K. pneumoniae* strain, with a LD MIC of 57 µg/mL. The aqueous, methanolic and ethyl acetate extracts were also good *A. baylyi* growth inhibitors (MIC values of 850, 1187 and 572 µg/mL against the reference bacterial strain). Similarly, though slightly higher, MICs were determined against the clinical strain. The ethyl acetate extract was the strongest *P. aeruginosa* growth inhibitor (MIC values of 275 and 330 µg/mL for the reference and clinical strains, respectively). The aqueous extract (LD MICs of 776 µg/mL and 585 µg/mL against the reference and clinical *P. aeruginosa* strains, respectively) was also a good inhibitor of *P. aeruginosa* growth, whilst the methanolic extract was a moderate growth inhibitor (LD MICs of 1750 µg/mL and 1660 µg/mL for the reference and clinical strains, respectively). The *P. angustifolium* leaf hexane extract was completely devoid of growth inhibitory activity against all bacterial strains, with MIC values > 10,000 µg/mL against all bacterial species.

Table 3. DD and LD MIC values ($\mu\text{g/mL}$) for *Pittosporum angustifolium* leaf extracts against microbial triggers of some autoimmune inflammatory diseases.

Bacterial species	Methanol extract		Aqueous extract		Ethyl acetate extract		Chloroform extract		Hexane extract		Control					
											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
<i>P. mirabilis</i> (ATCC33304)	48	38	625	570	587	440	1918	1250	> 10,000	> 10,000	2.5	1.25	3.3	2.5	0.63	–
<i>P. mirabilis</i> clinical isolate	29	26	648	505	426	563	671	754	> 10,000	> 10,000	2.5	1.25	3.3	2.5	0.63	–
<i>P. vulgaris</i> (ATCC21719)	1824	860	2460	1575	3600	1855	> 10,000	> 10,000	> 10,000	> 10,000	2.5	1.25	3.3	1.25	1.25	–
<i>K. pneumoniae</i> (ATCC31488)	845	620	458	294	251	157	903	1450	–	–	3.3	1.9	1.9	1.9	0.3	–
<i>K. pneumoniae</i> clinical isolate	487	308	366	192	29	57	3485	2488	–	–	2.5	1.9	1.6	1.9	0.3	–
<i>A. baylyi</i> (ATCC21721)	1650	1187	1240	850	836	572	> 10,000	> 10,000	> 10,000	> 10,000	3.3	2.5	2.5	1.25	1.25	–
<i>A. baylyi</i> clinical isolate	1868	1465	1420	1126	1140	892	> 10,000	> 10,000	> 10,000	> 10,000	3.3	3.3	2.5	1.25	1.25	–
<i>P. aeruginosa</i> (ATCC39324)	1558	1750	1432	776	341	275	5680	3387	> 10,000	> 10,000	3.3	1.25	3.3	1.25	1.25	–
<i>P. aeruginosa</i> clinical isolate	1736	1660	725	585	428	330	> 10,000	> 10,000	> 10,000	> 10,000	3.3	1.25	3.3	2.5	1.25	–
<i>S. pyogenes</i> clinical isolate	3250	1563	> 10,000	3287	–	–	> 10,000	> 10,000	–	–	3.3	2.5	3.3	2.5	0.63	–

DD: disc diffusion; LD: liquid dilution; MIC: minimum inhibitory concentration; Pen: penicillin G; Chlor: chloramphenicol; Eryth: erythromycin; Tetra: tetracycline; Gent: gentamycin; NC: negative control; –: no inhibition at any dose tested. *P. mirabilis*: *Proteus mirabilis*; *P. vulgaris*: *Proteus vulgaris*; *K. pneumoniae*: *Klebsiella pneumoniae*; *A. baylyi*: *Acinetobacter baylyi*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *S. pyogenes*: *Streptococcus pyogenes*.

3.4. FIC assessment

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

Twenty-six (52%) of the *P. angustifolium* leaf extract and conventional antibiotic combinations produced additive effects, when tested against the reference and clinical *P. mirabilis* strains (Table 4). Similarly, 20 combinations (80%) produced additive interactions when tested against *P. vulgaris*. As these combinations produced effects greater than either the individual extract or conventional antibiotic components, these combinations would be beneficial in the prevention and treatment of rheumatoid arthritis. Indeed, combinations containing the methanolic extract were highly potent, with 20–30 µg/mL MIC values. All of the other combinations were noninteractive. 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.

3.4.2. 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*. The majority (56%) of the combinations were noninteractive. Whilst no added benefit is gained from using these combinations, it is safe to use these extracts and antibiotics together without compromising the activity of either component. A further 16 (32%) of the combinations produced additive effects. Several of these combinations were potent inhibitors of *K. pneumoniae* growth. For example, the combination of the ethyl acetate extract with either penicillin or gentamycin each yielded an MIC value of 29 µg/mL against the clinical *K. pneumoniae* strain, and slightly higher against the reference strain. Similarly, the combination of chloramphenicol and the aqueous extract produced an MIC value of 62 µg/mL against the clinical *K. pneumoniae* strain. 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.

Three synergistic interactions were also noted for combinations of the *P. angustifolium* leaf extracts and conventional antibiotics against the growth of *K. pneumoniae* (Table 4). These interactions are particularly noteworthy and produced substantially increased efficacy compared with that of either component alone. Combining tetracycline with the ethyl acetate *P. angustifolium* leaf extract produced the greatest efficacy (MIC of 12 µg/mL against the clinical *K. pneumoniae* strain). This combination may therefore be very useful for the treatment of *K. pneumoniae* infections. Further synergistic interactions were detected when tetracycline was combined with either the methanolic or aqueous *P. angustifolium* leaf extract (MIC values of 71 and 44 µg/mL respectively against the clinical *K. pneumoniae* strain). Notably, all of the synergistic combinations against *K. pneumoniae* were against the clinical strain of the bacterium. A very different trend was noted for the reference *K. pneumoniae* strain. Indeed, some of the same combinations that were synergistic against the clinical strain were antagonistic against the reference strain. In particular, combinations of tetracycline with either methanolic or ethyl acetate *P. angustifolium* leaf extract induced antagonistic effects and should be avoided in treatment of this bacterium. These results emphasise the need for caution when using combinational therapies and highlight the need to screen against multiple bacterial strains. If the susceptibility of a particular bacterial strain is not known when treating *K. pneumoniae*-induced disease, it may be prudent to avoid using the combination to avoid further reducing the efficacy of the treatment. However, use of the aqueous extract-tetracycline combination may still be warranted, as it produced a synergistic interaction against the clinical *K. pneumoniae* strain and was noninteractive against the reference strain, and therefore did not reduce its efficacy. However, testing against other *K. pneumoniae*

strains is warranted to confirm that no other antagonistic effects are detected. Of further note, the interactive differences between these *K. pneumoniae* strains indicate that the tetracycline resistance mechanism may be different between the two strains and mechanistic studies are required for further elucidation.

3.4.3. Combinational effects against *A. baylyi* and *P. aeruginosa*

No synergistic interactions were detected between the *P. angustifolium* leaf extracts in combination with the conventional antibiotics against either *A. baylyi* strain (Table 4). Twenty-five (50%) of the tested combinations produced additive interactions. As they have increased efficacy, these combinations would be beneficial in preventing and treating *A. baylyi* infections. Of the combinations with additive effects, those containing ethyl acetate leaf extract were generally the strongest inhibitors of *A. baylyi* growth. Indeed, combinations of the ethyl acetate extract with penicillin, chloramphenicol, erythromycin, tetracycline and gentamycin produced MIC values of 429, 429, 286, 606 and 286 µg/mL, respectively, against the reference *A. baylyi* strain. All other additive combinations generally produced MIC values > 500 µg/mL against both bacterial strains. Indifferent interactions were determined for a further 24 combinations (48% of the total tested combinations). No additional benefit would be gained by combining these therapies. However, combining the extracts and the antibiotic components would not decrease the efficacy of either individual component. One antagonistic interaction was also noted between tetracycline and the aqueous *P. angustifolium* leaf extract. Thus, this combination should be avoided for the treatment of *A. baylyi* infections.

No synergistic or antagonistic interactions were detected for any combination tested against either *P. aeruginosa* strain (Table 4). All combinations produced either additive or indifferent effects against these bacterial strains. Additive interactions were determined for a further thirty-one combinations (62%). Notably, several of these combinations produced particularly low MIC values. Those combinations would be particularly beneficial in the prevention and treatment of multiple sclerosis, as well as other *P. aeruginosa*-associated diseases. Indeed, MIC values in the range of 130–200 µg/mL were calculated for the ethyl acetate *P. angustifolium* leaf extract in combination with all of the conventional antibiotics against both *P. aeruginosa* strains. Similarly, all combinations which contained the aqueous extract were stronger inhibitors of *P. aeruginosa* growth than either component separately (MIC values of 300–400 µg/mL for most combinations). As these combinations have enhanced efficacy compared to the individual components, their use as combinational therapies would be beneficial for treating *P. aeruginosa* infections. The remaining 19 (38%) of the combinations had effects that were not significantly different from those of the individual components and were therefore classed as indifferent interactions. Whilst they provide no added benefit over the monotherapies, these combinations could be used concurrently without decreasing the efficacy of either component.

C + Gent	625	(ADD)	377	(ADD)	5000	(ADD)	1088	(ANT)	1244	(IND)	5000	(IND)	5000	(IND)	1694	(IND)	5000	(IND)	5000	(SYN)
		1.00		>	1.00	1.50		1.00		>	1.00	>	1.00	>		5000		1.00		>
H + Pen	>	(ADD)	>	(ADD)	5000	(ADD)	–	(IND)	–	(ADD)	5000	(ADD)	5000	(ADD)	>	(ADD)	>	(ADD)	5000	(IND)
		1.12		>	1.00	1.00		1.00		>	1.00	>	1.00	>		1.00		>		5000
H + Chlor	5000	(IND)	5000	(ADD)	5000	(ADD)	–	(ADD)	–	(ADD)	5000	(ADD)	5000	(ADD)	5000	(ADD)	>	(ADD)	5000	(ADD)
		1.15		>	1.25	>		1.25		>	1.50	>	1.00	>		1.00		>		5000
H + Eryth	5000	(IND)	5000	(IND)	5000	(IND)	–	(IND)	–	(IND)	5000	(IND)	5000	(ADD)	5000	(ADD)	>	(ADD)	5000	(ADD)
		1.00		>	1.00	>		1.00		>	1.00	>	1.00	>		1.00		>		5000
H + Tetra	5000	(ADD)	5000	(ADD)	5000	(ADD)	–	(ADD)	–	(ADD)	5000	(ADD)	5000	(ADD)	5000	(ADD)	>	(ADD)	5000	(ADD)
		1.00		>	1.00	>		1.00		>	1.06	>	1.12	>		1.03		>		2.50
H + Gent	>	(ADD)	>	(ADD)	5000	(ADD)	–	(IND)	–	(IND)	5000	(IND)	5000	(IND)	5000	(IND)	>	(IND)	5000	(IND)
		1.00		>	1.00	>		1.00		>	1.00	>	1.00	>		1.00		>		1.00
	5000	(ADD)	5000	(ADD)	5000	(ADD)		(ADD)		(ADD)	5000	(ADD)	5000	(ADD)	5000	(ADD)		(ADD)		(ADD)

MIC: minimum inhibitory concentration; Σ FIC: sum of the fractional inhibitory concentration; M: methanolic extract; W: aqueous extract; E: ethyl acetate extract; C: chloroform extract; H: hexane extract; Pen: penicillin G; Chlor: chloramphenicol; Eryth: erythromycin; Tetra: tetracycline; Gent: gentamycin; Int: interaction; SYN: synergistic interaction; ADD: additive interaction; IND: indifferent interaction; ANT: antagonistic interaction.

3.4.4. Combinational effects against *S. pyogenes*

The interactive effects of *P. angustifolium* extracts in combination with the conventional antibiotics against *S. pyogenes* are summarised in Table 4. Two combinations (tetracycline in combination with either methanolic or chloroform extract) produced synergistic effects. The tetracycline-chloroform extract is of limited benefit as, despite substantially increased efficacy for the combination, the MIC value is high and indicates that the combination still has low inhibitory activity. In contrast, a substantially lower MIC value (367 µg/mL) was determined for the tetracycline-methanolic extract combination, highlighting the potential of this combination for the treatment of *S. pyogenes*-induced diseases, including rheumatic fever. Nine (36%) further combinations were determined to have additive interactions. These combinations may also be beneficial in treating *S. pyogenes* infections. All of the other combinations (56%) were classified as being indifferent.

3.5. Varied ratio combination studies (isobolograms)

3.5.1. Synergistic interactions against *K. pneumoniae*

Synergistic effects were noted for three combinations against the clinical strain of *K. pneumoniae*. Interestingly, all of these combinations included tetracycline. These combinations were tested across a range of extract-antibiotic ratios using isobologram analysis to identify the best ratios to produce synergy (Fig. 2). Interestingly, all of the methanolic *P. angustifolium* leaf extracts synergised the activity of tetracycline, even at low ratios (Fig. 2a). Indeed, all combinations containing $\geq 20\%$ and $\leq 80\%$ methanolic *P. angustifolium* leaf extract resulted in synergy. Ratios outside this range produced additive effects and would also be beneficial as *K. pneumoniae* growth inhibitors. Similarly, the aqueous (Fig. 2b) and ethyl acetate (Fig. 2c) *P. angustifolium* leaf extracts also produced synergistic interactions in combination with tetracycline at most of the ratios tested (generally between 30% and 80% extract). Thus, all ratios of these combinations would enhance the inhibitory activity against *K. pneumoniae* growth. As bacteria are less likely to develop resistance when the amount of conventional antibiotic used is minimised, the ideal extract to tetracycline ratio may be 80:20 for long-term prophylactic treatment (as would be required to prevent and treat rheumatoid arthritis). However, when used for the treatment of acute infections (e.g., urinary tract infections), the 20:80 ratio may be the preferred ratio option to maximise the efficacy of the treatment.

Fig. 2. Isobologram for combinations of tetracycline with *Pittosporum angustifolium* leaf methanolic extract (a), *aqueous* extract (b) and *ethyl acetate* extract (c) tested at various ratios against the clinical strain of *Klebsiella pneumoniae*. Results represent mean MIC values of four replicates. Ratio = percentage of extract:percentage of antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic ($\Sigma\text{FIC} \leq 0.5$). Any points between the 0.5:0.5 and 1.0:1.0 lines are deemed additive ($\Sigma\text{FIC} > 0.5-1.0$). ΣFIC : sum of the fractional inhibitory concentration; MIC: minimum inhibitory concentration.

3.5.2. Synergistic interactions against *S. pyogenes*

The methanolic and chloroform *P. angustifolium* leaf extracts produced synergistic effects against *S. pyogenes* growth when combined with tetracycline (Fig. 3). The methanolic extract-tetracycline combinations containing 50%–70% ratios of the methanolic *P. angustifolium* leaf extract produced synergistic interactions against *S. pyogenes* growth (Fig. 3a). Similarly, combinations containing 40%–60% of the chloroform extract of *P. angustifolium* leaf and tetracycline produced synergistic growth inhibition of *S. pyogenes* (Fig. 3b). Furthermore, all other ratios of these extracts combined with tetracycline yielded additive interactions. As all combination ratios of these extracts and tetracycline had substantially greater activity than the individual components alone, all combinations would be beneficial to inhibit *S. pyogenes* growth. However, the combinations producing synergistic interactions are preferred as they would provide maximal benefit. For long-term prophylactic treatment, combinations containing 70% or 60% extract (for the methanolic and chloroform extracts, respectively) and tetracycline may be the ideal ratios, as these ratios minimise the levels of tetracycline, thereby decreasing the possibility that the bacteria will develop further resistance to the antibiotic. In contrast, the 10:90 ratio may be preferential for the treatment of acute infections.

Fig. 3. Isobologram for combinations of tetracycline with *Pittosporum angustifolium* leaf. (a) Methanolic extract and (b) chloroform extract, tested at various ratios against the clinical strain of *Streptococcus pyogenes*. Results represent mean MIC values of four replicates. Ratio = percentage of extract:percentage of antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic ($\Sigma\text{FIC} \leq 0.5$). Any points between the 0.5:0.5 and 1.0:1.0 lines are deemed additive ($\Sigma\text{FIC} > 0.5-1.0$). ΣFIC : sum of the fractional inhibitory concentration; MIC: minimum inhibitory concentration.

3.6. Toxicity studies

3.6.1. *Artemia* lethality assay

All plant extracts and antibiotics were initially screened individually at 1 mg/mL. The extracts were only deemed to be toxic if they induced $\geq 50\%$ mortality (LD_{50}) in the *Artemia* nauplii after 24 hours of exposure. All of the conventional antibiotics were nontoxic when tested individually in the *Artemia* lethality assay (Table 5). Similarly, all *P. angustifolium* extracts produced mortality that was not significantly different from the negative control. When tested in combination in the *Artemia* lethality assay, none of the extract-antibiotic combinations induced mortality that was significantly different from the negative controls. No single component or combination induced $> 50\%$ mortality. Therefore, all combinations and individual components were deemed nontoxic. The positive control, potassium dichromate, induced 100% mortality in the *Artemia* lethality assay, indicating that the assay was functioning correctly.

3.6.2. MTS cell viability assay

The plant extracts and conventional antibiotics were screened against HDF at 200 $\mu\text{g/mL}$ in the cell viability assay. In this assay, extracts producing $< 50\%$ cell viability at 200 $\mu\text{g/mL}$ are deemed to be toxic [33]. None of the extracts or conventional antibiotics displayed $< 50\%$ HDF viability. Therefore, all extracts and conventional antibiotics were deemed to be nontoxic (Table 5). Similarly, all combinations provided substantially $> 50\%$ cell viability and were therefore also deemed to be nontoxic. In contrast, exposure to the positive control (quinine) reduced HDF cell viability by approximately 70%.

Table 5. Mortality and cellular viability results for extracts and conventional antibiotics tested individually and as combinations in the *Artemia* lethality assay and MTS cell viability assay respectively.

Sample	Mortality (%) ^a		Cell viability (%) ^b
	After 24 h	After 48 h	After 24 h
Antimicrobials			
Penicillin G	1.8 $\pm$ 1.4	4.3 $\pm$ 2.4	98.3 $\pm$ 3.4
Chloranphenicol	2.7 $\pm$ 1.3	5.6 $\pm$ 3.3	102.2 $\pm$ 3.7
Erythromycin	1.2 $\pm$ 0.6	5.8 $\pm$ 2.3	97.7 $\pm$ 5.5
Tetracycline	2.4 $\pm$ 1.5	5.1 $\pm$ 2.8	95.8 $\pm$ 4.7
Gentamicin	3.1 $\pm$ 1.8	6.7 $\pm$ 2.6	94.7 $\pm$ 4.6
Extracts			

M	6.2 ± 3.7	21.3 ± 3.7	81.2 ± 6.3
W	4.5 ± 2.8	15.7 ± 3.6	85.3 ± 5.5
E	2.4 ± 0.8	7.3 ± 2.1	98.3 ± 4.6
C	4.7 ± 2.2	12.7 ± 3.6	87.8 ± 6.1
H	1.8 ± 1.0	5.2 ± 2.8	102.4 ± 3.9
Combinations			
M + penicillin G	4.7 ± 2.5	18.5 ± 4.2	84.6 ± 3.7
M + chloramphenicol	6.9 ± 4.3	24.6 ± 3.5	80.5 ± 3.8
M + erythromycin	4.2 ± 2.6	15.7 ± 2.6	88.3 ± 3.1
M + tetracycline	5.7 ± 1.9	17.8 ± 3.7	87.7 ± 4.8
M + gentamicin	7.8 ± 2.6	28.3 ± 4.2	76.9 ± 4.6
W + penicillin G	4.3 ± 2.8	12.8 ± 3.2	93.6 ± 2.7
W + chloramphenicol	4.8 ± 3.1	13.5 ± 4.1	89.5 ± 4.5
W + erythromycin	3.6 ± 2.4	10.8 ± 2.4	92.7 ± 3.0
W + tetracycline	5.6 ± 2.8	17.5 ± 3.1	89.0 ± 3.5
W + gentamicin	7.4 ± 3.3	20.5 ± 3.3	84.8 ± 4.8
E + penicillin G	2.7 ± 1.3	14.6 ± 2.7	95.6 ± 3.4
E + chloramphenicol	4.2 ± 2.8	16.5 ± 3.5	92.7 ± 3.9
E + erythromycin	3.2 ± 2.6	9.3 ± 3.8	96.2 ± 4.7
E + tetracycline	5.5 ± 3.8	18.1 ± 5.2	89.2 ± 3.7
E + gentamicin	7.7 ± 5.0	22.7 ± 4.6	83.2 ± 3.9
C + penicillin G	4.6 ± 2.4	12.3 ± 3.0	94.6 ± 3.1
C + chloramphenicol	5.5 ± 3.6	14.7 ± 2.9	91.2 ± 4.3
C + erythromycin	2.8 ± 2.1	9.8 ± 4.5	102.6 ± 4.2
C + tetracycline	6.4 ± 4.2	17.8 ± 3.8	91.6 ± 3.2
C + gentamicin	8.9 ± 4.6	21.0 ± 3.5	87.2 ± 6.1
H + penicillin G	3.7 ± 2.7	10.6 ± 4.8	103.2 ± 4.7
H + chloramphenicol	4.9 ± 2.7	13.0 ± 3.8	96.3 ± 5.1
H + erythromycin	4.1 ± 3.4	11.8 ± 3.5	93.9 ± 7.2
H + tetracycline	5.8 ± 2.6	12.3 ± 4.6	91.6 ± 3.8
H + gentamicin	3.3 ± 2.6	6.9 ± 3.7	94.1 ± 4.8
Control			
Deionised water	2.7 ± 1.7	3.6 ± 2.5	96.8 ± 5.7
Quinine	2.3 ± 1.1 ^a	4.6 ± 2.7 ^a	31.4 ± 4.8 ^b
Potassium dichromate	100.00 ± 0.00 ^a		NT

^a: tested at a concentration of 1 mg/mL; ^b: tested at a concentration of 200 µg/mL; M: methanolic extract; W: aqueous extract; E: ethyl acetate extract; C: chloroform extract; H: hexane extract; NT: control not tested in the assay; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium. Results represent mean ± standard deviation of 3 independent experiments, each performed in triplicate ($n = 9$).

4. Discussion

The majority of the conventional antibiotic and *P. angustifolium* extract combinations demonstrated additive interactive profiles (49.6%). Indifferent interactions (46.8%) were also particularly common. Whilst these combinations may have limited added benefit over using the conventional antibiotic (or extract) alone, they show that neither therapy is reducing the efficacy of the other. Synergy was seen for 0.02% of the antimicrobial and medicinal plant combinations. These synergistic interactions show enhanced efficacy, and could allow lower doses to be administered to reach the same effect, thus reducing side effects of the chemotherapy [3]. Exposure of bacteria to lower levels of the conventional antibiotics could decrease further induction of antibiotic resistance [1].

In most published combination studies, synergistic interactions are emphasized, with the reporting of antagonism being neglected. However, cotherapy with drugs that have antagonistic interaction would reduce the efficacy of both therapies, thereby increasing the

burden placed on the healthcare system. Notably, of the 250 combinations of *P. angustifolium* leaf extracts and conventional antibiotics tested, only 4 (0.02%) demonstrated antagonistic interactions. Whilst this rate of antagonism is low, these findings are as important as the synergistic interactions, as they indicate combinations that should be avoided. Interestingly, all of the antagonistic interactions were present in combinations containing tetracycline. The reference strain of *K. pneumoniae* was particularly prone to antagonism when exposed to either the methanolic, ethyl acetate or chloroform extract in combination with tetracycline, whilst synergy was evident in the corresponding clinical *K. pneumoniae* strain when treated with the same *P. angustifolium* leaf extract-conventional antibiotic combinations. This is a particularly interesting finding and may indicate that the two strains have different tetracycline resistance mechanisms. Further studies are needed to examine these mechanisms to better tailor combinations to different diseases. This finding also highlights the need to test several strains of the same bacterial species to ensure that the therapy is relevant across a range of strains of the pathogen.

None of the *P. angustifolium* leaf extracts or conventional antibiotics demonstrated toxicity in the *Artemia* lethality assay or MTS assay when tested independently [34,35]. Similarly, all combinations were nontoxic in both assays, indicating their potential for therapeutic use. The nontoxicity 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 from *P. angustifolium* leaf extracts may, perhaps, also not be surprising, as this plant has been used therapeutically by Australian aborigines for thousands of years. However, to the best of our knowledge, there is a lack of rigorous toxicity studies for *P. angustifolium*. We are aware of only a single study that reported a lack of toxicity for *P. phylloroides* (a previous taxonomic classification for *P. angustifolium*) leaf extracts [25]. The lack of toxicity of the combinations also indicates their potential for therapeutic usage. However, 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.

5. Conclusions

The findings reported here indicate the potential of *P. angustifolium* leaf extracts (particularly in combination with tetracycline) as preventative and therapeutic options against bacterial triggers of some autoimmune inflammatory diseases. However, 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 interactions are warranted, and bioactivity-driven compound isolation and/or metabolomics studies are required to determine the active compound(s), as well as those responsible for the antibiotic potentiation within the *P. angustifolium* leaves. Furthermore, as the extracts used in this study were produced using organic solvents, there may be the perception that these extracts contain residual solvents and this may decrease the public's perception of their safety. Future studies should focus on greener methods of extraction (such as supercritical fluid extraction) to increase the perception of safety. These extraction methods should also allow for a substantial scale-up in production.

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Competing interests

The authors declare that they have no competing interests.

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