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Original Article

Rheum palmatum L. Root Extracts Inhibit the Growth of Bacterial Triggers of Selected Autoimmune Inflammatory Diseases and Potentiate the Activity of Conventional Antibiotics

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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 may be a more effective approach in overcoming resistance and potentiating the activity of antibiotics that are otherwise ineffective against resistant bacterial strains. **Materials and Methods:** The antibacterial activity of *Rheum palmatum* L. (Turkey rhubarb) root extracts was investigated by disc diffusion and quantified by liquid dilution and solid phase MIC assays. The extracts were also combined with a range of conventional antibiotics and tested against various microbial triggers of autoimmune diseases. The ΣFIC values obtained from these assays were used to determine the class of combinational effects and isobologram analysis was used to determine the ideal synergistic ratio(s). Toxicity was evaluated by *Artemia* nauplii mortality and HDF cell viability assays. **Results:** The methanolic, aqueous and ethyl acetate *R. palmatum* root extracts showed noteworthy inhibitory activity against several microbial triggers of autoimmune inflammatory diseases, whilst the chloroform and hexane extracts were also good inhibitors of *P. aeruginosa* growth. Some combinations of the *R. palmatum* root extract and conventional antibiotics were substantially more effective in inhibiting bacterial growth. Three synergistic and nine additive interactions were noted. Notably, the methanolic extract restored significant growth inhibitory activity to penicillin and ampicillin when tested in combination against *Proteus* spp. In contrast, four antagonistic interactions were noted for combinations containing gentamicin (against *P. aeruginosa* and *S. pyogenes*), indicating that those combinations should be avoided when treating infections caused by those bacteria. **Conclusion:** *Rheum palmatum* extracts have potential as inhibitors of bacterial triggers of selected autoimmune inflammatory diseases. Furthermore, extract components may also potentiate the activity of two antibiotics that are relatively ineffective alone. Isolation of these agents may be beneficial in drug design against several bacteria, including the microbial triggers of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever.

Keywords: Synergy, Polygonaceae, Turkey rhubarb, Medicinal plants, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Drug combinations.

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INTRODUCTION

The overuse and misuse of antibiotics has induced resistance towards multiple antibiotics in a wide range of bacterial pathogens, whilst the discovery of new antibiotics has decreased dramatically in recent years.[1] As a result, many bacterial infections that were previously readily treatable are now difficult to manage using the current antibiotic repertoire and the development of novel antibiotic therapies is now one of the biggest challenges facing medical science.[2] Unfortunately, it is unlikely that the previous methods of antibiotic discovery will yield high numbers of new antibiotics in the future.[1] Furthermore, novel therapy modalities are required to treat chronic diseases due to the extended antibiotic treatment durations required.

Autoimmune inflammatory diseases are debilitating diseases that include rheumatoid arthritis (RA), ankylosing spondylitis (AS), multiple sclerosis (MS), and rheumatic fever (RV).[3] These diseases are caused by exposure to antigens inducing an abnormal immune response to self-tissue in afflicted individuals. There are no cure for any autoimmune inflammatory diseases. Instead, analgesics and anti-inflammatory drugs are generally used to mitigate the disease symptoms, whilst allowing the individuals immune system to overcome the infection. However, whilst that approach decreases the symptoms of RA, AS, MS and RV, it does not inhibit the tissue damage inherent to these diseases. A more effective method of autoimmune disease management may be to prevent infections of the bacterial triggers of these disease to block the disease etiology.[3] There are already antibiotics available that are effective inhibitors of all of the bacterial triggers of RA, AS, MS and RV, although recent increases in the incidence of multi-antibiotic resistant bacterial strains have decreased their efficacy, and several antibiotics are now ineffective against those strains.[1] Additionally, prolonged usage of pure antibiotics to treat chronic infections would induce further antibiotic resistant pathogen strains. A better approach may be to use complex plant-based preparations, or combinations of plant extracts and conventional antibiotics to increase efficacy, and to inhibit the development of antibiotic resistance.[1]

Traditional medicines have great potential for antimicrobial drug development. Despite this, relatively few plant derived antibiotic compounds are in common use clinically. This may be because synergistic interactions are often required to potentiate the antibacterial activity and purified compounds often have much lower activity than the crude extract.[4] 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.[5-6] Combinational therapies are already preferred over mono-therapy to treat multiple life-threatening infectious diseases such as malaria, tuberculosis and HIV/AIDS due to their ability to target multiple facets of a disease and to curb resistance.[1] Combinations of plant extracts/isolated compounds with

conventional antibiotics may also prove to have economic advantages.[4] 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 lengthy and expensive process of discovering new antimicrobial agents.[4] Furthermore, synergistic combinations may have increased efficiency, reduced side effects, increased stability and bioavailability and require lower doses in comparison to synthetic alternatives to achieve therapeutic outcomes.[5]

Rheum palmatum L. (commonly known as Turkey rhubarb, Chinese rhubarb, East Indian rhubarb; Family Polygonaceae) is a herbaceous perennial plant that is related to edible rhubarb (a hybrid of *Rheum rhabarbarum* L. and *Rheum rhaponticum* L.). It is easily distinguished from other *Rheum* spp. by its size (*R. palmata* grows to six feet tall, whereas other species grows to a maximum of two feet tall). *Rheum palmatum* is native to western China, northern Tibet and the Mongolian plateau, although it has been widely spread to areas including India and east Europe via early Chinese trade routes. Indeed, the roots of this species were prominently traded along the Silk Road as an herbal medicine. In ancient China, *R. palmata* roots were consumed for their laxative properties, and as a treatment for fever.[7] It has also been used as a poultice to treat oedema. However, it should be used with caution and has been associated with several toxic effects including nausea, abdominal pain, vomiting, diarrhoea, as well as damage to the liver, stomach and thyroid. It should also be avoided by pregnant women as it is a uterine stimulant and can cause miscarriage. Furthermore, whilst the roots of this species have traditional uses, the leaves contain high levels of oxalic acid and are toxic.[7] Notably, several of the diseases treated with *R. palmata* roots are caused by bacterial pathogens. However, despite its well documented traditional uses, there has been relatively little research into the antibacterial properties of *R. palmatum* roots.

One study reported noteworthy growth inhibitory effects of *R. palmatum* methanolic root extracts against a panel of bacterial pathogens including *Bacillus subtilus, Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Micrococcus roseus, Pseudomonas aeruginosa* and *Shigella sonnei*, with MIC values between 50 and 100µg/mL.[8] That study also reported similar antifungal activity against several fungal species. Despite these earlier studies, *R. palmatum* preparations are yet to be tested against many bacteria, including of the bacterial triggers of several autoimmune diseases. This study was undertaken to screen *R. palmatum* root extracts against some bacterial triggers of rheumatoid arthritis (*Proteus mirabilis* and *Proteus vulgaris*), ankylosing spondylitis (*Klebsiella pneumoniae*), multiple sclerosis (*Acinetobacter baylyi* and *Pseudomonas aeruginosa*) and rheumatic fever (*Streptococcus pyogenes*).[3] Furthermore, we were unable to find any studies testing the antibacterial activity of *R. palmatum* root extracts in combination with conventional antibiotics. Therefore, this study was undertaken to investigate the antimicrobial effects of *R. palmatum* root extracts 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

Certified *R. palmatum* L. root powder was obtained from Noodles Herbal Emporium, Australia and a voucher specimen (GU2018sTR1) was deposited in the School of Environment and Science, Griffith University, Australia. Individual 1g masses of the ground plant material were weighed into separate 50mL Falcon tubes and 50mL of methanol, deionised water, ethyl acetate, chloroform or hexane were individually added. All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The ground plant materials were extracted in each solvent for 24hr at 4°C with gentle shaking. The extracts were filtered through Whatman No. 54 filter paper under vacuum and 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 to determine the extraction yield and then dissolved in 10mL deionised water (containing 1% DMSO).

Qualitative Phytochemical Studies

Phytochemical analysis of the *R. palmatum* root extracts for the presence of alkaloids, cardiac glycosides, flavonoids, phenolic compounds, phytosterols, saponins, tannins and triterpenoids was achieved as previously described.[9-11]

Antibacterial Screening

Conventional Antibiotics

Penicillin-G (1440-1680µg/mg), chloramphenicol (≥98% purity), ampicillin (≥98% purity), erythromycin (≥850µg/mg), gentamycin (600µg/mg), and tetracycline (≥95% purity) 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 0.01mg/mL and stored at 4°C until use. For the disc diffusion studies, ampicillin (10µg) and chloramphenicol (10µg) standard discs were obtained from Oxoid Ltd., Australia and used as positive controls.

Bacterial Cultures

All bacterial strains were selected based on their ability to trigger autoimmune inflammatory diseases in genetically susceptible individuals.[3] Reference strains of *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC21719), *Klebsiella pneumoniae* (ATCC31488), *Acinetobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Type Culture Collection, USA. A clinical isolate strain of *Streptococcus pyogenes* was obtained from the School of Environment and Science 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 24hr and were subcultured and maintained in nutrient broth at 4°C until use.

Evaluation of Antibacterial Activity

Antibacterial activity screening of the *R. palmatum* extracts was assessed using a modified disc diffusion assay.[12-13] Ampicillin (10µg) and chloramphenicol discs (10µg) were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10µL of distilled water (containing 1% DMSO) 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 facilitates comparisons with other studies. A solid phase agar disc diffusion assay was also used in this study for comparison as it more accurately represents the growth patterns of the bacteria on solid surfaces.

Microplate Liquid Dilution MIC Assay

The MICs of the extracts were evaluated by standard methods.[15-16] All plates were incubated at 37°C for 24hr. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, Australia and dissolved in sterile deionised water to prepare a 0.2mg/mL INT solution. A 40µL volume of this solution was added into all wells and the plates were incubated for a further 6hr at 37°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.[9-10] Graphs of the zone of inhibition versus Ln concentration were plotted and MIC values were achieved using linear regression.

Sum of Fractional Inhibitory Concentration (ΣFIC) Assessment

Interactions between the *R. palmatum* root extracts and the conventional antibiotics were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC) for each combination.[15,17] 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).[15,17]

Varied Ratio Combination Studies (Isobolograms)

For each combination producing synergistic interactions, nine different ratios spanning the range 10:90 (extract:antibiotic) to 90:10 (extract:antibiotic) were tested. All combinations were tested in duplicate in 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 indicate synergy; those above the 0.5:0.5 line, up to and including the 1.0:1.0 line indicate an additive interaction; data points above the 1.0:1.0 line indicate indifferent interaction.

Toxicity Screening

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

Artemia franciscana Kellogg Nauplii Toxicity Screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4mg/mL) and serially diluted in artificial seawater as a reference toxin. Toxicity of the *R. palmatum* root extracts, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.[18-19] The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Cellular Viability Assay

All extracts were also screened individually using a normal human primary dermal fibroblast (HDF) standard assay.[20-21] Briefly, 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, Australia), 50µg/mL streptomycin (Sigma-Aldrich, Australia) and 50 IU/mL penicillin (Sigm-Aldricha, Australia) at 37°C, 5% CO_2 in a humidified atmosphere. Individual 70µL volumes of culture media (containing approximately 5000 cells) were added to wells of a 96 well plate and 30µL of the test extracts or cell media (for the negative control) was added to each well. The plates were incubated at 37°C, 5% CO_2 for 24hr in a humidified atmosphere. All extracts were screened at 200µg/mL. The cells were then washed in PBS (pH 7.2) to remove interference due to sample colour. A 20µL volume of Cell Titre 96 Aqueous One solution (Promega) was added to each well and the plates were incubated for a further 3hr. Absorbances were recorded at a test wavelength of 540nm 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 ranged from 124mg (*R. palmatum* hexane root extract) to 238mg (methanolic *R. palmatum* extract) (Table 1). Qualitative phytochemical screening (Table 1) showed that the higher polarity solvents (methanol and water) extracted the greatest mass and widest diversity of phytochemical classes.

Bacterial Growth Inhibition Screening

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

Proteus mirabilis growth was inhibited by the mid to high polarity *R. palmatum* root water, methanol and ethyl acetate extracts (Figure 1). The methanolic extract was the strongest inhibitor of *P. mirabilis* growth (as judged by ZOI), with a ZOI of 13.3mm. A volume of 10µL of this extract was infused into the disc, which equates to approximately 147µg of extract infused into the disc. Notably, the *P. mirabilis* strain screened in this study was unaffected by anmpicillin and the ZOI for this extract is similar to that of the chloramphenicol control (14.2mm). Notably, the chloramphenicol control antibiotic was pure and was tested and at relatively high dose (10µg/disc). In contrast, the extracts were crude mixtures and the antimicrobial compounds would be expected to account for a small % of the total extract mass. Therefore, the methanolic extract was considered to be a particularly effective

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *R. palmatum* root 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	Phytosterols	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
M	147	14.7	+++	+++	+++	-	+	-	-	-	-	+++	+	++	-
W	150	15.0	+++	++	+++	-	+	-	-	-	-	+++	+	+	-
E	54	5.4	+	++	-	-	-	-	-	-	-	+	-	-	-
C	53	5.3	-	-	+	-	-	-	-	-	-	-	-	-	-
H	33	3.3	-	-	+	-	-	-	-	-	-	-	-	-	-

+++ 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.

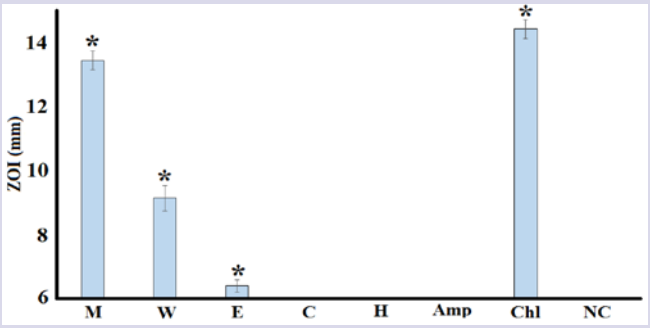

Figure 1: Antibacterial activity of *R. palmatum* root extracts against *P. mirabilis* (ATCC21721) measured as zones of inhibition (ZOI; mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of three independent repeats, each with internal triplicates (n=9) ± SEM. * indicates results that are significantly different to the negative control (p<0.01).

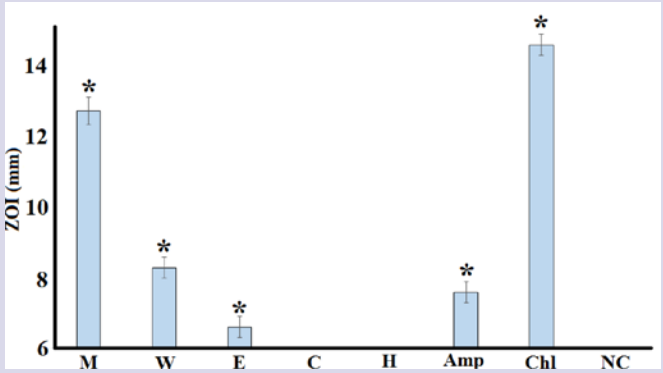

Figure 2: Antibacterial activity of *R. palmatum* root extracts against *P. vulgaris* (ATCC21719) measured as zones of inhibition (ZOI; mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of three independent repeats, each with internal triplicates (n=9) ± SEM. * indicates results that are significantly different to the negative control (p<0.01).

inhibitor of *P. mirabilis* growth and may be effective in the prevention and treatment of rheumatoid arthritis. The aqueous and ethyl acetate extracts had substantially smaller ZOIs than the methanolic extract, (9.3 and 6.7mm respectively), although this inhibition was still noteworthy. In contrast, the chloroform and hexane extracts were completely ineffective against *P. mirabilis* growth. Similar inhibitory trends were noted for *P. vulgaris* growth (Figure 2). As for *P. mirabilis*, the methanolic extract was the strongest inhibitor of *P. vulgaris* growth (ZOI 13.7mm). The ZOI measured for the methanolic extract was comparable to the ZOI measured for the pure chloramphenicol control and substantially bigger than those noted for the ampicillin control (7.6mm). Whilst also displaying inhibitory activity, the aqueous and ethyl acetate extracts had substantially lower efficacy than the methanolic extract, with ZOIs of 8.3 and 6.6mm respectively. All other extracts were ineffective at inhibiting *P. vulgaris* growth.

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

The methanolic and aqueous *R. palmatum* root extracts also inhibited the growth of *K. pneumonia*, albeit with much smaller ZOIs than measured for the *Proteus* spp. (Figure 3). The methanolic extract was the strongest growth inhibitor, with a ZOIs of 6.8mm, whilst the aqueous extract gave a slightly smaller ZOI. These ZOIs were substantially smaller than those of the ampicillin (8.6mm) and chloramphenicol controls (12.2mm). All other extracts were completely ineffective against *K. pneumonia*, although it is noteworthy that those extracts were tested at a substantially lower concentration (3.3-5.5µg/mL) than the methanolic and aqueous *R. palmatum* root extracts, which may account for its lack of apparent inhibitory activity against this bacterium. As *K. pneumoniae*

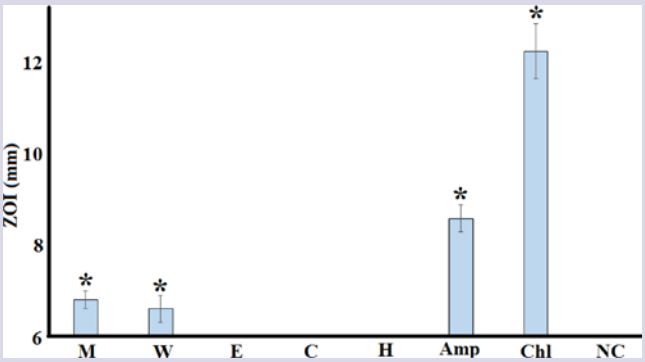

Figure 3: Antibacterial activity of *R. palmatum* root extracts against extracts against *K. pneumoniae* (ATCC31488) measured as zones of inhibition (ZOI; mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of three independent repeats, each with internal triplicates (n=9) ± SEM. * indicates results that are significantly different to the negative control (p<0.01).

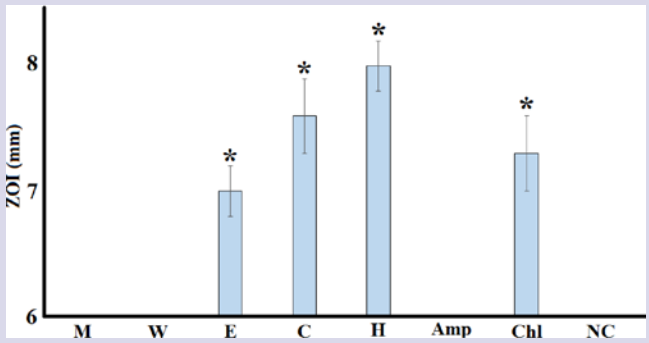

Figure 5: Antibacterial activity of *R. palmatum* root extracts against *P. aeruginosa* (ATCC39324) measured as zones of inhibition (ZOI; mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of three independent repeats, each with internal triplicates (n=9) ± SEM. * indicates results that are significantly different to the negative control (p<0.01).

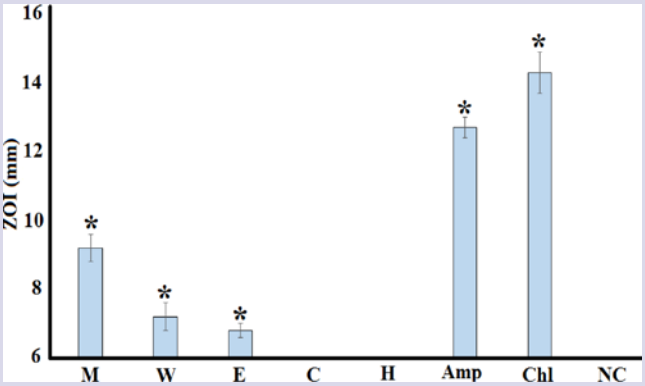

Figure 4: Antibacterial activity of *R. palmatum* root extracts against *A. baylyi* (ATCC33304) measured as zones of inhibition (ZOI; mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of three independent repeats, each with internal triplicates (n=9) ± SEM. * indicates results that are significantly different to the negative control (p<0.01).

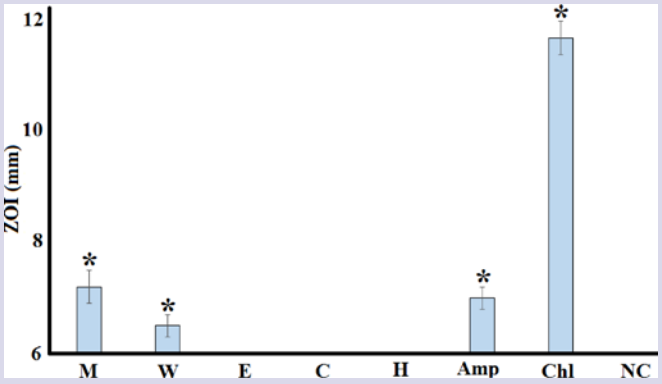

Figure 6: Antibacterial activity of *R. palmatum* root extracts against a clinical isolate of *S. pyogenes* measured as zones of inhibition (ZOI; mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of three independent repeats, each with internal triplicates (n=9) ± SEM. * indicates results that are significantly different to the negative control (p<0.01).

can induce ankylosing spondylitis in genetically susceptible individuals,[3] the methanolic and aqueous *R. palmatum* root extracts may be beneficial in the prevention and treatment of that disease.

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

The methanolic, *R. palmatum* root extract was also a noteworthy inhibitor of *A. baylyi* growth, with a ZOI of 9.3mm (Figure 4). This *A. baylyi* strain was highly susceptible to ampicillin and chloramphenicol, with ZOIs of 11.6 and 14.3mm respectively. The aqueous and ethyl acetate extracts also inhibited *A. baylyi* growth, although the small ZOIs (7.4 and 6.8mm respectively) indicate only low inhibitory activity. In contrast, the *P. aeruginosa* strain tested in this study was completely resistant to the higher polarity methanolic and aqueous *R. palmatum* root extracts, but was inhibited by the low to mid-polarity extracts (Figure 5). Notably, this was a particularly resistant bacterium. It was also completely resistant to the ampicillin control and displayed only low sensitivity towards chloramphenicol, with a ZOI of ~7.3mm. Thus, the *R. palmatum* root extracts may be useful in preventing the onset of multiple sclerosis as *A. baylyi* and *P. aeruginosa* can induce multiple sclerosis in genetically susceptible people.[3]

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

Streptococcus pyogenes growth was inhibited by the methanolic and aqueous *R. palmatum* root extracts, albeit with relatively small ZOIs (Figure 6). The ethyl acetate, chloroform and hexane extracts were completely ineffective at inhibiting the growth of this bacterium. The

methanolic extract was the strongest growth inhibitor, albeit with a relatively small ZOI (7.3mm). Notably, this inhibition was slightly better than that of ampicillin (7.0mm). This is noteworthy as the ampicillin control was pure and was tested at relatively high doses (10µg/disc). In contrast, *S. pyogenes* was substantially more susceptible to chloramphenicol (~12.7mm). The water extract also inhibited *S. pyogenes* growth, albeit with a substantially smaller ZOI (6.6mm), indicating low to moderate growth inhibitory activity. As *S. pyogenes* can trigger rheumatic fever in genetically susceptible people,[3] the *R. palmatum* root methanolic extract (and to a lesser extent, the aqueous extract) may be effective in the prevention and treatment of this disease (and other diseases caused by this bacterium).

Quantification of Minimum Inhibitory Concentration (MIC)

The relative antimicrobial strength of the extracts 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, the higher polarity methanol and water *R. palmatum* root extracts were generally most effective at inhibiting the growth of the bacterial triggers of the selected autoimmune diseases. The MIC values of the conventional antibiotic controls were only determined for the liquid dilution assay. Commercially manufactured discs with fixed amounts of antibiotics loaded were used for the disc diffusion assay and thus the zones of only single doses were recorded. Gentamicin was generally the most potent antibiotic (as judged by its MIC) and was the only antibiotic to inhibit the growth of all of the bacterial species tested. Notably, the *P. aeruginosa* strain used in these studies was resistant to all of antibiotics except gentamicin. Furthermore, with the exception of *P. mirabilis* and *P. vulgaris*, all of the other bacterial strains were completely resistant to penicillin.

The MIC values determined for the *R. palmatum* root extracts compare relatively well between the disc diffusion and liquid dilution assays, although the MIC values were generally lower in the liquid dilution assay, with some notable exceptions. All bacterial species except *P. aeruginosa* were most susceptible to the methanolic and aqueous extracts. The growth of *P. mirabilis* was inhibited by methanolic (LD MIC 68µg/mL), aqueous (LD MIC 582µg/mL) and ethyl acetate extracts (LD MIC 625µg/mL), with MIC values that indicate noteworthy growth inhibitory activity. Similar, albeit slightly higher MIC values were also determined for these extracts against *P. vulgaris*. Therefore, these extracts may be useful in the prevention and treatment of rheumatoid arthritis. The methanolic extract was also had noteworthy inhibitory activity against *K. pneumoniae* growth (LD MIC 867µg/mL). Similar efficacy was seen for the aqueous extract (LD MIC 867µg/mL) and thus both the methanolic and aqueous extracts may also be useful in the prevention and treatment of ankylosing spondylitis. The methanolic, aqueous and ethyl acetate extracts also inhibited the growth of *A. baylyi* (LD MIC values of 459, 1879 and 686µg/mL), *P. aeruginosa* (LD MIC values of 230, 939 and 194µg/mL). The chloroform and hexane extracts were also good inhibitors of *P. aeruginosa* growth (LD MIC values of 128 and 96µg/mL respectively), indicating all of the *R. palmatum* root extracts may be useful in treating and preventing multiple sclerosis. Similarly, the inhibition of *S. pyogenes* by the methaniolic and aqueous extracts was noteworthy (LD MICs of 230 and 939µg/mL respectively).

Effects of extract and antibiotic combinations: ΣFIC assessment

Combinations of the *R. palmatum* root extracts with conventional antibiotics were tested against each bacteria to determine the classes of interactions for these combinations (Table 3). ΣFIC values could not be determined for many of the combinations as one or both of the components in the combination were ineffective against the tested bacterium when tested alone. Many of the effective combinations were determined to be non-interactive. Whilst these combinations have no additional benefit over the individual monotherapies alone, the lack of antagonism indicates that taking these therapies in combination would not have detrimental effects. This is important information as allopathic and complementary therapies are often taken concurrently.

Three combinations also produced synergistic effects (methanol extract and penicillin against both *Proteus* spp.; methanol and ampicillin against *P. mirabilis*), with ΣFIC values of 0.43, 0.38 and 0.48 respectively. As these combinations have substantially enhanced effects compared to either component alone, they would be beneficial for the treatment and prevention of rheumatoid arthritis (and other diseases causes by *Proteus* spp.). Additionally, nine additive combinations were identified. Notably, all of these contained penicillin, ampicillin or tetracycline as the antibiotic component. As these combinations have greater efficacy than either component alone, they would also be beneficial in treating infections of these bacteria.

Notably, four antagonistic combinations were also identified. All of these antagonistic interactions were against either *P. aeruginosa* or *S. pyogenes* and were in combinations that contained gentamicin as the antibiotic component. These are important results as they indicate that those combinations should be avoided when treating multiple sclerosis or rheumatic fever, or other infections caused by those bacteria.

Varied ratio combination studies (isobolograms)

The combination of the methanolic *R. palmatum* root extract with penicillin (Figure 7a) or ampicillin (Figure 7b) against *P. mirabilis*, as well as the methanolic extract and penicillin combination against *P. vulgaris* (Figure 7c) induced synergistic interactions and therefore these combinations were further investigated by isobologram analysis across a range of ratios to determine the ideal combination compositions to obtain synergy. All combination ratios containing 40-60% of the methanolic extract produced synergistic interactions in combination with penicillin against *P. mirabilis* (Figure 7a). Thus, these combination ratios would be beneficial to enhance *P. mirabilis* growth inhibition. However, bacteria would be less likely to develop resistance when combinations are used in ratios that 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 methanolic extract:penicillin ratio may be 60:40. 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 40:60 ratio) may be the preferred option.

Isobologram analysis for the combination of methanolic *R. palmatum* root extract and ampicillin also showed synergistic interactions against *P. mirabilis* in combinations containing 50 and 60% extract ratios (Figure 7b). All other combination ratios were additive. Therefore, the combination containing 60% methanolic *R. palmatum* root extract and 40% ampicillin was deemed the best combination ratio for prophylactic treatment to prevent rheumatoid arthritis, as well as decreasing the possibility of further increasing bacterial resistance to β-lactam antibiotics.

Similarly, the methanolic extract and penicillin combination was synergistic against *P. vulgaris* and was therefore also examined by isobologram analysis. All combination ratios containing 30-60% extract were synergistic against that bacterium. Thus, each of those combination ratios would be beneficial to enhance *P. mirabilis* growth inhibition, although the combination containing 60% extract and 40% penicillin is preferred for prophylactic prevention of rheumatoid arthritis, whilst the 30% extract and 70% penicillin combination may be preferred for acute *P. mirabilis* infections.

Table 2: Disc diffusion and liquid dilution MIC values for the *R. palmatum* extracts against *P. mirabilis, P. vulgaris, K. pneumoniae, A. baylyi, P. aeruginosa* and *S. pyogenes* growth (µg/mL).

	Proteus mirabilis		*Proteus vulgaris*		*Klebsiella pneumoniae*		*Acinetobacter baylyi*		*Pseudomanas aeruginosa*		*Streptococcus pyogenes*	
	DD	LD	DD	LD	DD	LD	DD	LD	DD	LD	DD	LD
M	128	68	159	84	1173	867	1473	459	-	230	883	230
W	1165	582	1228	720	1240	905	1879	1879	-	939	1054	939
E	1058	625	1122	683	-	-	1526	686	277	194	-	-
C	-	-	-	-	-	-	-	-	145	128	-	-
H	-	-	-	-	-	-	-	-	122	96	-	-
Positive Controls												
Pen	ND	2.5	ND	1.l25	ND	-	ND	-	ND	-	ND	-
Chl	ND	-	ND	-	ND	1.25	ND	2.5	ND	-	ND	-
Amp	ND	2.5	ND	2.5	ND	-	ND	-	ND	-	ND	-
Gent	ND	1.5	ND	1.25	ND	0.31	ND	0.31	ND	0.63	ND	0.63
Ery	ND	-	ND	-	ND	-	ND	2.5	ND	-	ND	-
Tet	ND	-	ND	2.5	ND	1.25	ND	1.25	ND	-	ND	2.5

M = methanol; W = water; E = ethyl acetate; C = chloroform; H = hexane; Pen = penicillin; Chl = chloramphenicol; Mmp = ampicillin; Gent = gentamicin; Ery = erythromycin; Tet -= tetracyclin; 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.

Table 3: ΣFIC values of *R. palmatum* root extracts in combination with conventional antibiotics against *P. mirabilis, P. vulgaris, K. pneumoniae, A. baylyi, P. aeruginosa* and *S. pyogenes*.

		Penicillin	Chloramphenicol	Ampicillin	Gentamycin	Erythromycin	Tetracycline
P. mirabilis	M	**0.43**	-	**0.48**	2.14	-	-
	W	*0.78*	-	*0.88*	2.58	-	-
	E	1.12	-	1.36	2.9	-	-
P. vulgaris	M	**0.38**	-	*0.86*	2.4	-	1.73
	W	*0.88*	-	1.2	2.52	-	2.21
	E	1.32	-	1.52	3.12	-	1.96
K. pneumoniae	M	-	1.65	-	1.46	-	1.35
	W	-	1.84	-	1.93	-	1.17
A. baylyi	M	-	*0.72*	-	3.86	2.72	*0.62*
	W	-	2.68	-	2.79	2.85	1.17
	E	-	*0.55*	-	3.44	1.94	*0.59*
P. aeruginosa	M	-	-	-	4.18	-	-
	W	-	-	-	2.88	-	-
	E	-	-	-	3.45	-	-
	C	-	-	-	4.76	-	-
	H	-	-	-	5.68	-	-
S. pyogenes	M	-	-	-	4.38	-	*0.74*
	W	-	-	-	2.78	-	1.36

M = methanol; W = water; E = ethyl acetate; C = chloroform; H = hexane. - indicates that the ΣFIC could not be determined. M = methanol; W = water; E = ethyl acetate; C = chloroform; H = hexane; - indicates that the ΣFIC could not be determined; Synergy (blue/bold highlighting) = ≤ 0.5; *Additive (green/italics highlighting)* = > 0.5-1.0; Indifferent (no highlighting) = > 1.0 - ≤ 4; Antagonistic (red/underlined highlighting) = > 4.0. Numbers indicate the mean ΣFIC values of 4 determinations.

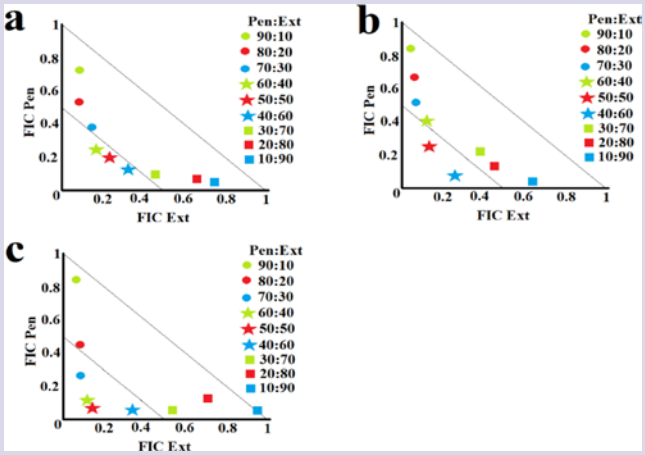

Figure 7: Isobologram analysis of combinations containing the methanolic *R. palmatum* root extract with (a) penicillin and (b) ampicillin against *P. mirabilis*, or (c) the methanolic *R. palmatum* root extract in combination with penicillin against *P. vulgaris*. Results represent mean FIC values of four replicates. Ratio = % extract:% antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic (ΣFIC ≤0.5). Any points between the 0.5:0.5 and 1.0:1.0 lines are deemed additive (ΣFIC >0.5-1.0). Pen = penicillin: Ext = methanolic extract.

Table 4: LC_{50} values determined for *R. palmatum* root extracts in the *Artemia* nauplii and HDF bioassays following 24 hr exposure.

Extract	LC_{50} value (µg/mL)	
	ALA	HDF assay
M	1436	-
W	1785	-
E	-	-
C	-	-
H	-	
PC	56	NT

- indicates that less than 50% mortality was induced by the extract at all concentrations tested. ALA = *Artemia* nauplii toxicity assay; HDF = human dermal fibroblast toxicity assay; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NT = Not tested.

Quantification of Toxicity

No LC_{50} values were determined for the ethyl acetate, chloroform or hexane *R. palmatum* root extracts as <50% mortality was seen in all tested concentrations (Table 4). In contrast, LC_{50} values of 1436 and 1785µg/ml were determined for the methanolic and aqueous extracts respectively. As extracts with LC_{50} values <1000µg/ml towards *Artemia* nauplii have previously been defined as being toxic in this assay,[18-19] all extracts were deemed to be nontoxic. Furthermore, all plant extracts demonstrated a lack of toxicity towards normal human primary dermal fibroblasts, with cellular viability for all tests substantially >50% of the untreated control. All extracts were therefore deemed to be nontoxic.

DISCUSSION

This study investigated the ability of *R. palmatum* root extracts to inhibit the growth of some bacterial triggers of autoimmune inflammatory diseases, both alone and in combination with conventional antibiotics. Several *R. palmatum* root extracts were effective bacterial growth inhibitors. The methanolic extract was a particularly strong inhibitor of bacterial growth, with MIC values as low as 68µg/mL (against *P. mirabilis*). Whilst a detailed investigation of the phytochemistry of the *R. palmatum* root extracts was beyond the scope of this study, qualitative phytochemical analysis highlighted several phytochemical classes that may contribute to the bacterial growth inhibitory activity. Interestingly, the methanolic and aqueous *R. palmatum* root extracts had relatively high abundances of polyphenolics, flavonoids and anthraquinones, as well as lower levels of tannins and saponins. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.[22-25] This has been attributed to a variety of mechanisms, including their ability to complex with extracellular and soluble proteins, as well as bacterial cell walls.[25] Similarly, multiple tannins have broad spectrum antibacterial activity via a variety of intra- and extra-cellular mechanisms, including the precipitation of microbial proteins.[26] 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 the active components are required to evaluate the mechanism of the *R. palmatum* root extract's growth inhibitory activity.

The studies combining the extracts with conventional antibiotics also highlighted other therapeutic options. In particular, combinations containing methanolic *R. palmatum* root extract and either penicillin or ampicillin produced synergistic effects against *P. mirabilis* and *P. vulgaris*, despite this bacterium being resistant to both of these antibiotics. It appears that the methanolic extract functioned as a resistance-modifying agent, inhibiting bacterial resistance mechanisms and allowing the antibiotic to function with improved efficacy. This is particularly interesting and highlights an effective treatment modality against antibiotic-resistant *Proteus* spp. infections. As several multi-antibiotic strains of these bacterium have been reported,[3,9,16-17] this is a particularly interesting and relevant finding. Furthermore, several additive combinations were also noted. Whilst the extract does not increase the activity of the antibiotics to as great an extent in these combinations as in synergistic combinations, the potency of the treatment is still substantially increased compared to either monotherapy when used separately. Thus, these additive combinations would also be beneficial when used to treat infections of those bacteria.

Furthermore, most of the extract-antibiotic combinations tested in this study 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. Furthermore, the use of these additional therapies is often without their physician's knowledge. However, exceptions were noted for some combinations containing gentamicin against *P. aeruginosa* or *S. pyogenes*. This is an important finding and highlights that combinations containing gentamicin should be avoided when treating infections of these bacteria. An understanding of drug herbal medicine interactions is important to avoid such counter-indications. Interestingly, previous studies indicate that antagonistic combinations of plant extracts with gentamicin are not uncommon.[27]

Microbes have developed numerous resistance mechanisms to avoid the effects of antibiotics. One common method is through the use of multi-drug resistant (MDR) efflux pumps, which are encoded chromosomally and are used to rapidly remove antibiotics that have entered the bacterial cells, thus rendering them resistant to the effects of the antibiotic.[28-29] 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 multi-drug

resistance (MDR) pump inhibitors in order to enhance the activity of their own natural antimicrobial compounds. Such MDR pump inhibitors are effective in combination with some previously ineffective/resistance prone antibiotic compounds and several examples have previously been reported.[30] Isoflavones isolated from *Lupinus argenteus* Pursh potentiate the activity of the natural plant antibiotic berberine as well as the synthetic fluoroquinoline antibiotic, norfloxacin as inhibitors of *S. aureus* growth.[30] That study reported that the isoflavone allows a greater concentration of berberine to occur inside the bacteria by inhibiting the efflux mechanism (MDR pump). Similarly, *Mezoneuron benthamianum* Baill. and *Securinega virosa* (Roxb. Ex Willd) Baill. extracts act as efflux pump inhibitors for fluoroquinolone, tetracycline and erythromycin in resistant strains of *S. aureus* (MRSA).[31] 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, all bacterial species were resistant to penicillin, chloramphenicol, ampicillin, erythromycin and tetracycline, with only low susceptibility or complete resistance to each antibiotic. All of these antibiotics are susceptible to resistance due to efflux pumps.[30,32] 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.[30, 32] 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.[30] The synergistic interactions in our study suggests the possibility of a common EPI in the *R. palmatum* root extracts that could be inhibiting a MDR efflux pump in these bacteria.

Alternatively (or in addition to MDR efflux pumps), the bacteria screened in our study may have acquired genes encoding for reduced-affinity penicillin-binding protein 2a (PBP2a) (rendering β-lactam antibiotics ineffective).[31] As penicillin binding proteins are a group of protein enzymes, these phytochemicals may form nonspecific interactions and affect the bacterial cell wall biosynthesis. The *R. palmatum* root extracts may also contain a β-lactamase inhibitor. β-lactamases are the major defense of gram-negative bacteria against β-lactam antibiotics.[33] For example, clavulanic acid is an irreversible β-lactamase inhibitor, which in combination with β-lactam antibiotics can block the bacterial antimicrobial resistance mechanism.[34] 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).

Ultimately, the preparation of combinations of *R. palmatum* root extracts (or purified compounds) with conventional antibiotic will depend on the nature of the pathogen and of the disease treated. In general, combinations of antibiotic with pure *R. palmatum* root 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 preferred. 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.[35] 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 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 that produced synergy to be preferred for preventing and treating chronic disease.

CONCLUSION

The results of this study demonstrate the potential of the *R. palmatum* root extracts in inhibiting the growth of some bacterial triggers of autoimmune inflammatory diseases. Extract components may also potentiate the activity of antibiotics that are relatively ineffective alone. Therefore, a combinational approach not only increases the effectiveness of these antibiotics, but also may also potentially reduce the side effects and reduce the development of drug resistant pathogens. Isolation of the bioactive and potentiating compounds may be beneficial in drug design against several bacteria including the microbial triggers of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ABBREVIATIONS

ALA: *Artemia* lethality assay; **DMSO:** Dimethyl sulfoxide; **EPI:** Efflux pump inhibitor; **FIC:** Fractional inhibitory concentration; **HDF:** Human dermal fibroblasts; **LC_{50}:** The concentration required to achieve 50 % mortality; **MIC:** Minimum inhibitory concentration; **MDR:** Multi-drug resistant; **ZOI:** Zone of inhibition.

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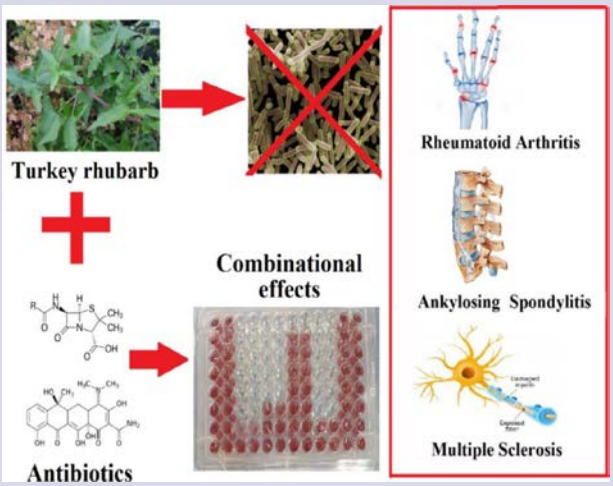
PICTORIAL ABSTRACT



SUMMARY

- *Rheum palmatum* root extracts were screened for the ability to block the growth of a panel of bacterial triggers of autoimmune inflammatory diseases.
- The antibacterial activity was quantified by determining the MIC values of each extract.
- The extracts were also tested in combination with conventional antibiotics and the class of interaction was determined
- Synergistic combinations were screened at various ratios to determine the ideal ratios to provide synergy.
- Toxicity of *R. palmatum* extracts was determined using the *Artemia* nauplii and HDF cell viability toxicity bioassays.

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 250 publications in a variety of peer reviewed journals.

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