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Original Article

Rosa canina L. Fruit Extracts Inhibit the Growth of Bacterial Triggers of some Autoimmune Inflammatory Diseases and Potentiate the ac*tivity of* Conventional Antibiotics

Yujun Wang[1], Yushu Liang[1], Ian Edwin Cock[1,2,*]
[1]School of Natural Sciences, Nathan Campus, Griffith University, Brisbane, AUSTRALIA.
[2]Environmental Futures Research Institute, Nathan Campus, Griffith University, Brisbane, AUSTRALIA.

ABSTRACT

Introduction: An increase in antibiotic resistance and a corresponding decrease in antimicrobial discovery have directed researchers towards alternative therapies, including plant based medicines. However, synergistic combinations of plant extracts with conventional antibiotics are a far more effective approach in overcoming resistance and potentiating the activity of antibiotics that are otherwise ineffective against resistant bacterial strains. **Methods:** The antibacterial activity of *Rosa canina* (*Rosehip*) 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 and HDF cell line viability. **Results:** The methanolic, water and ethyl acetate extracts showed good inhibitory activity against several microbes. However, combinations of the methanolic or aqueous extracts with conventional antibiotics proved significantly more effective in inhibiting the growth of *Proteus vulgaris*, *Klebsiella pneumonia* and *Acinetobacter baylyi* (bacterial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis respectively). In total, 4 synergistic interactions were noted. Notably, the methanolic extract restored significant growth inhibitory activity to chloramphenicol and tetracycline when tested in combination, thereby restoring their activity. **Conclusion:** Although the mechanisms of synergy are still unclear, studies indicate that compounds within *R. canina* may mimic the actions of resistance modifying agents, thus potentiating 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 and multiple sclerosis.

Key words: Synergy, Conventional antimicrobials, Interaction, Medicinal plants, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Drug combinations.

Correspondence:
Dr. Ian Edwin Cock
[1]School of Environment and Science, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Brisbane, Queensland 4111, AUSTRALIA.
[2]Environmental Futures Research Institute, Griffith University, 170 Kessels Rd, Nathan, Brisbane, Queensland 4111, AUSTRALIA.
Phone no: +61 7 37357637
E-mail: i.cock@griffith.edu.au
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INTRODUCTION

The improper and misuse of antibiotics has resulted in antibiotic resistance in a wide range of bacterial pathogens.[1] Furthermore, the discovery of new antimicrobial agents has decreased to no more than a few new antibiotics introduced to clinical use in the last decade.[1] Together, these factors have resulted in increased numbers of multi-resistant bacterial pathogens that are increasingly difficult to manage with the current range of antibiotic chemotherapies.[2] The development of alternative treatment methods is crucial and considered by the World Health Organisation (WHO) to be perhaps the biggest challenge currently facing medical science.[3] For a number of reasons reviewed elsewhere,[4] it is unlikely that the current methods of antibiotic discovery/development will be as successful in the future.

This is especially true for the development of new effective therapies to treat autoimmune inflammatory diseases. These are a group of debilitating diseases including rheumatoid arthritis (RA), ankylosing spondylitis (AS), lupus, Lyme disease, multiple sclerosis (MS), celiac disease and rheumatic fever (RV).[5] All of these diseases result from an abnormal immune response to self-tissue as a consequence of antigen challenge, often by bacterial pathogens. There is currently no cure for any of these diseases and the current treatment strategy is to alleviate the symptoms with analgesics and anti-inflammatory therapies. However, as RA, AS, MS and RV are induced in genetically people by bacterial pathogens, a more effective preventative treatment may be to target the growth of the specific trigger bacteria, thereby blocking the disease etiological events.[5] Whilst antibiotics are already available for the treatment of all of these bacteria, the development of resistant strains in recent years have decreased their efficacy towards some strains.[1] Furthermore, the prophylactic use of pure antibiotics over a long term would certainly induce resistance, thereby rendering the bacteria refractory to their actions. A better approach may be to use combinations of antibacterial components.[4]

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 extracts.[6] 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.[7,8] Combinational therapy is already preferred over mono-therapy to treat multiple life-threatening infectious diseases such as malaria, tuberculosis and HIV/AIDS due to its ability to target multiple facets of a disease and to curb resistance.[4] A combination of plant extracts/isolated compounds with conventional antibiotics may also prove to have an economic advantage.[6] Developing a new drug requires years of extensive and costly testing. However, combinational therapy can potentially restore an existing drug to a state of significantly reduced resistance, thereby bypassing the strenuous and expensive process of discovering new antimicrobial agents.[6] Further advantages of synergistic interactions include increased efficiency, reduced side effects, increased stability and bioavailability and the requirement for lower doses in comparison to synthetic alternatives.[6]

Rosa canina L. (commonly known as dog rose) is wild rose species native to Europe, northwest Africa and western Asia. Following flowering, bright red fruit known as rose hips form. The rose hips are used for the production of jams, jellies, beverages and soups. They are also consumed as herbal infusions and may be eaten raw. Rose hips are high in ascorbic acid. Indeed, the fresh fruit may contain up to 1.5% ascorbic acid (by mass).[9] Relatively high levels of β-carotene, lutein, zeaxanthin and lycopene have also been detected in rose hips.[10,11] Many of these compounds have beneficial therapeutic properties in the treatment of arthritis, gout, urinary tract infections, fever, cold and as a diuretic and laxative.[11] Despite the interesting phytochemistry and its therapeutic properties, *R. canina* remains poorly investigated. Notably, a hydroalcoholic *R. canina* rose hip extract was recently reported to have good anti-inflammatory activity. Despite this, *R. canina* extracts are yet to be tested against the bacterial triggers of rheumatoid arthritis (*Proteus mirabilis*), ankylosing spondylitis (*Klebsiella pneumoniae*), multiple sclerosis (*Acinetobacter baylyi*, *Pseudomonas aeruginosa*) and rheumatic fever (*Streptococcus pyogenes*).[5] Furthermore, we were unable to find any studies testing the antibacterial activity of *R. canina* extracts in combination with conventional antibiotics. Therefore, this study was undertaken to investigate the antimicrobial effects of *R. canina* rose hip 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 *Rosa canina* L. fruit powder was obtained from Noodles Herbal Emporium, Australia and a voucher specimen (GU2017RP) was deposited in the School of Natural Sciences, Griffith University, Australia. Individual 1g masses of the ground rosehip 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 24h at 4°C with gentle shaking. The extracts were filtered through Whatman No. 54 filter paper under vacuum. The solvent extracts were air dried at room temperature in the shade. The aqueous extracts were lyophilised by freeze drying at -50°C. The resultant dried extracts were weighed to determine the extraction yield and then dissolved in 10mL deionised water (containing 1% DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the *R. canina* extracts for the presence of alkaloids, cardiac glycosides, flavonoids, phenolic compounds, phytosterols, saponins, tannins and triterpenoids was achieved as previously described.[12,13]

Antibacterial screening

Conventional Antibiotics

Penicillin-G (1440-1680μg/mg), chloramphenicol (≥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 discs (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.[5] 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 24h and were subcultured and maintained in nutrient broth at 4°C until use.

Evaluation of antibacterial activity

Antibacterial activity screening of the *R. canina* 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 allows for comparisons with other studies. A solid phase agar disc diffusion assay was also used in this study for comparison.

Microplate liquid dilution MIC assay

The MICs of the extracts were evaluated by standard methods.[15] All plates were incubated at 37°C for 24h. 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 6h 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.[12,13] 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. canina* extracts and the conventional antibiotics were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC) for each combination.[15] The FIC values for each component (a and b) were calculated using the following equations where a represents the plant extract sample and b represents the conventional antibiotic:

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

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

The ΣFIC was then calculated using the formula ΣFIC = FIC(a) + FIC(b). The interactions were classified as synergistic (ΣFIC ≤0.5), additive (ΣFIC >0.5-1.0), indifferent (ΣFIC >1.0-4.0) or antagonistic (ΣFIC >4.0).[15]

Varied ratio combination studies (isobolograms)

For each combination producing synergistic interactions, nine different ratios spanning the range 10:90 (extract:antibiotic) to 90:10 (extract:antibiotic) were tested. 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 MTT cellular proliferation assay was used to determine a cellular evaluation of toxicity.

Artemia franciscana Kellogg nauplii toxicity screening

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

Cellular viability assay

All *R. canina* extracts were also screened individually using a normal human primary dermal fibroblast (HDF) standard assay.[17] 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; Thermo Fisher 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 24h 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 3h. 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 38mg (*R. canina* hexane extract) to 312mg (aqueous *R. canina* 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)

P. mirabilis growth was inhibited by the mid to high polarity *R. canina* water, methanol and ethyl acetate extracts (Figure 1). The methanolic extract was the strongest *P. mirabilis* growth (as judged by ZOI), with a ZOI of 8.6±0.4mm. A volume of 10µL of this extract was infused into the disc, which equates to approximately 100µg of extract infused into the disc. The ZOI for this extract is substantially smaller than that of the ampicillin (13.3±0.6mm) and chloramphenicol controls (12.5±0.5 mm). However, it is noteworthy that these control antibiotics were tested pure and at relatively high doses (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 is an effective inhibitor of *P. mirabilis* growth and may be effective in the prevention and treatment of rheumatoid arthritis. The aqueous and ethyl acetate extracts had similar ZOIs to the methanolic extract. 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), although slightly smaller ZOIs were measured. As for *P. mirabilis*, the methanolic extract was the strongest

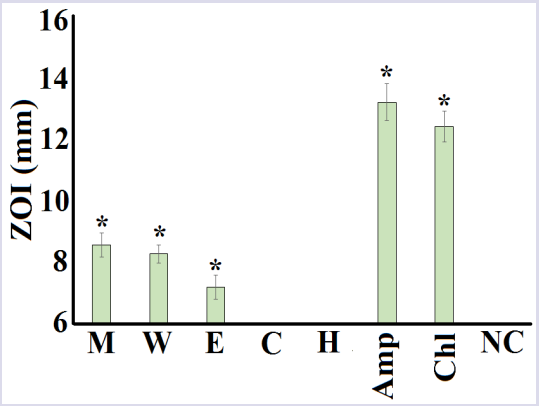

Figure 1: Antibacterial activity of R. canina extracts against *P. mirabilis* (ATCC21721) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of two repeats, each with internal triplicates (n=6) ± SEM. * indicates results that are significantly different to the negative control (P<0.01).

inhibitor of *P. vulgaris* growth (ZOI=7.7±0.3mm). The aqueous and ethyl acetate extracts had similar efficacy, with ZOIs of 7.3±0.3mm and 6.7±0.3mm respectively. In contrast, larger ZOIs were measured for the ampicillin (10.0±*0.5*mm) and chloramphenicol controls (11.3±*0.6*mm). All other extracts were ineffective at inhibiting *P. vulgaris* growth.

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

Only the methanol and hexane *R. canina* extracts inhibited the growth of *K. pneumoniae* (Figure 3). The methanolic extract was the strongest growth inhibitor, albeit with a relatively small ZOI (7.5±0.5mm) compared with that seen for the *Proteus* spp. The hexane extracts also inhibited *K. pneumoniae* growth, although the ZOI (6.7±0.3mm) is indicative of only weak antibacterial activity. As *K. pneumoniae* can induce ankylosing spondylitis in genetically susceptible individuals,[5] these extracts may be beneficial in the prevention and treatment of that disease. In contrast, this bacterium was highly susceptible to the ampicillin and chloramphenicol controls, with inhibition zones of 11.7±0.6mm and 9.8±0.4mm respectively. No growth inhibition was detected for the mid to lower polarity chloroform and ethyl acetate extracts.

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

Only the methanolic *R. canina* extract inhibited *A. baylyi* growth, albeit with a small ZOI (6.8±0.3mm), indicative of weak inhibitory activity (Figure 4). However, it is noteworthy that this bacterium was also relatively resistant to ampicillin (ZOI=6.8±0.3mm). In contrast, this *A. baylyi* strain was highly susceptible to chloramphenicol (ZOI=11.3±0.6mm). Similarly, only the methanolic extract inhibited *P. aeruginosa* growth (Figure 5). However, the 6.6±0.3mm ZOI measured for this extract indicated only weak growth inhibitory activity. However, the *P. aeruginosa* strain tested in this study was resistant to both the ampicillin and chloramphenicol controls, each inducing zones of inhibition of only approximately 6.5mm. Thus, the methanolic *R. canina* extract may still be useful in prevention and treating *P. aeruginosa* infections. As *A. baylyi* and *P. aeruginosa* can both induce multiple sclerosis in genetically susceptible people,[5] the methanolic *R. canina* extract may be useful in the prevention and treatment of that disease, as well as other illnesses caused by these bacteria.

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

S. pyogenes growth was inhibited by the methanolic and aqueous *R. canina* extracts (Figure 6). All other extracts were completely ineffective at inhibiting the growth of this bacterium. The methanolic extract was particularly potent, with a ZOI of 9.3±0.6mm. Indeed, the methanolic extract had stronger relative activity than the ampicillin (8.2±0.4mm) and chloramphenicol controls (6.8±0.3mm). This is particularly noteworthy as both positive controls are pure compounds and were tested at relatively high doses (10µg/disc). The water extract also inhibited *S. pyogenes* growth, albeit with substantially smaller ZOIs indicative of only low to moderate growth inhibitory activity. As *S. pyogenes* can trigger rheumatic fever in genetically susceptible people,[5] the *R. canina* 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, each of the higher polarity methanol and water *R. canina* extracts were generally effective at inhibiting the growth of all of the bacteria tested. The lack of inhibition of *K. pneumonia* by the aqueous extract was the only exception to this trend. The MIC values of the conventional antibiotic controls were only determined for the liquid dilution assay. Commercially manufactured discs with set amounts of antibiotics loaded were used for the disc diffusion assay and thus the zones of only single doses were recorded. Gentamycin was the most potent antibiotic (as judged by its MIC) and inhibited the widest range of bacterial species. Indeed, all bacterial species tested were susceptible to this antibiotic. Notably, the *P. aeruginosa* strain used in these studies was resistant to all of antibiotics except gentamycin. Furthermore, with the exception of *P. mirabilis* and *P. vulgaris*, all of the other bacterial strains were completely resistant to penicillin.

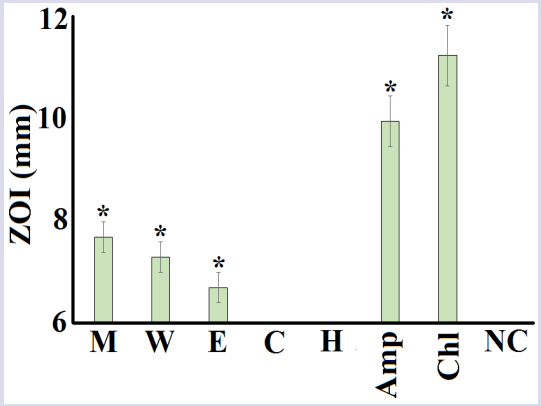

Figure 2: Antibacterial activity of *R. canina* extracts against *P. vulgaris* (ATCC21719) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of two repeats, each with internal triplicates (*n*=6) ± SEM. * indicates results that are significantly different to the negative control (*P*<0.01).

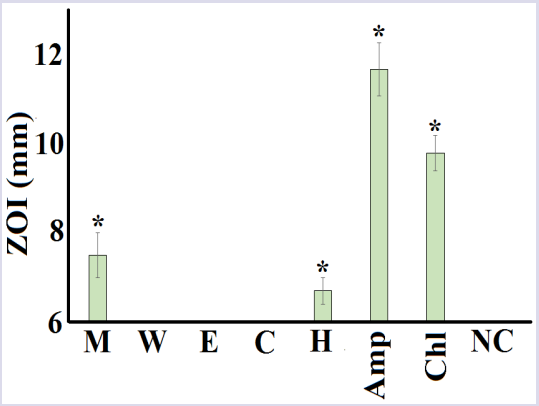

Figure 3: Antibacterial activity of *R. canina* extracts against extracts against *K. pneumoniae* (ATCC31488) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of two repeats, each with internal triplicates (*n*=6) ± SEM. * indicates results that are significantly different to the negative control (*P*<0.01).

The MIC values determined for the *R. canina* extracts compare relatively well between the disc diffusion and liquid dilution assays with few exceptions. All bacterial species were generally most susceptible to the methanolic extract, although the ethyl acetate extract had similar efficacy towards the *Proteus* spp. (based on MIC values). The growth of *P. mirabilis* was inhibited by methanolic (DD MIC 200µg/mL; LD MIC 150µg/mL) and ethyl acetate extracts (DD MIC 200µg/mL; LD MIC 185µg/mL) with MIC values that indicate strong growth inhibitory activity. The aqueous extract was also a strong inhibitor of this bacterium (DD MIC 700µg/mL; LD MIC 525µg/mL). 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 a good inhibitor of *K. pneumoniae* growth (DD MIC 870µg/mL; LD MIC 650µg/mL). Similarly, the hexane extract was a potent inhibitor of this bacterium (DD MIC 164µg/mL; LD MIC 82µg/mL) and thus may also be useful in the prevention and treatment of ankylosing spondylitis. Whilst the methanolic and aqueous also inhibited the growth of *A. baylyi, P. aeruginosa* and *S. pyogenes*, the MIC values were generally >1000µg/mL, indicating only low to moderate potency.

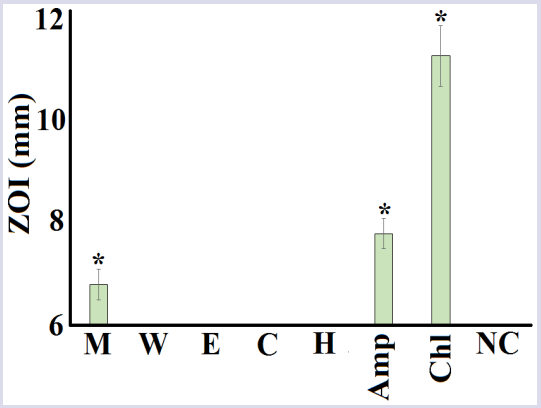

Figure 4: Antibacterial activity of *R. canina* extracts against *A. baylyi* (ATCC33304) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of two repeats, each with internal triplicates (n=6) ± SEM. * indicates results that are significantly different to the negative control (P<0.01).

Fractional inhibitory concentration (FIC) assessment

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

A range of interactions was evident for combinations of the *R. canina* extracts with conventional antibiotics when tested against *P. mirabilis* and *P. vulgaris* (Table 3). ΣFIC values could not be determined for the majority of the combinations as one or both of the components in the combination were in effective against the tested bacterium. Of the effective combinations, the majority of were non-interactive (approximately 79% of the inhibitory combinations). 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 often allopathic and complementary therapies may be taken concurrently. Notably, the methanolic extract induced synergistic effects in combination with tetracycline (ΣFIC=0.37). This combination may therefore be useful in the prevention and treatment of rheumatoid arthritis. The aqueous and ethyl acetate extracts also potentiated the activity of tetracycline, albeit to a lesser extent than the methanol extract, producing additive effects. Therefore, these combinations may also be useful in preventing and treating rheumatoid arthritis, as well as any other disease caused by *Proteus* spp. infections (e.g. urinary tract infections).

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

Two synergistic interactions were noted for combinations of the *R. canina* extracts and conventional antibiotics against the growth of *K. pneumonia* (Table 3). Interestingly, both of these combinations contained the methanolic extract and either chloramphenicol or tetracycline (ΣFIC

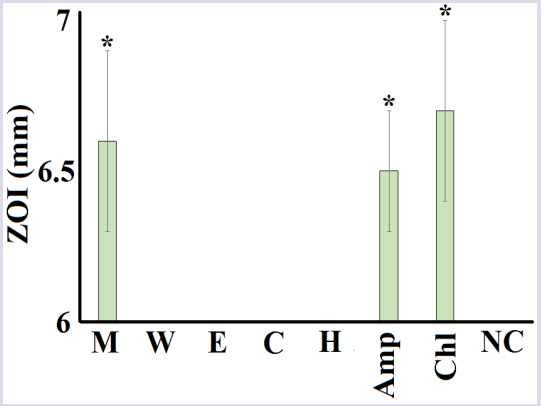

Figure 5: Antibacterial activity of *R. canina* extracts against *P. aeruginosa* (ATCC39324) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of two repeats, each with internal triplicates (n=6) ± SEM. * indicates results that are significantly different to the negative control (P<0.01).

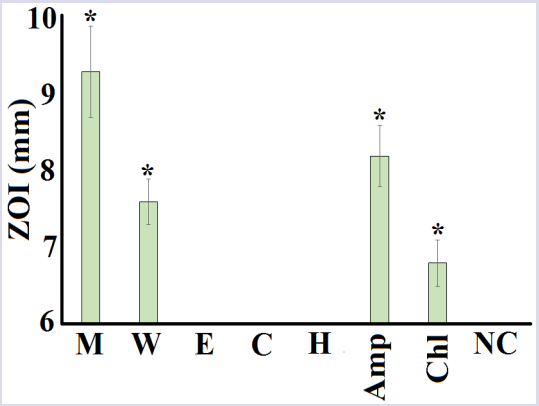

Figure 6: Antibacterial activity of *R. canina* extracts against a clinical isolate of *S. pyogenes* measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg)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

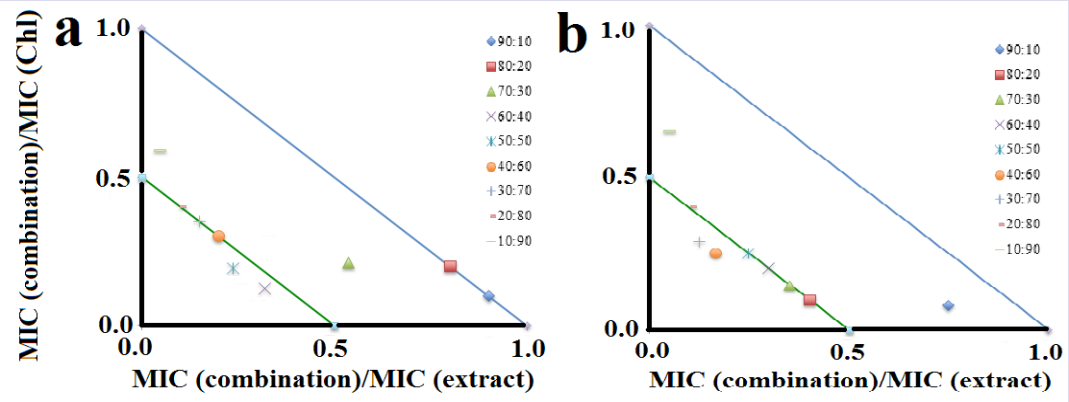

Figure 7: Isobologram for combinations of chloramphenicol and methanolic *R. canina* extract against (a) *K. pneumonia* and (b) *A. baylyi*. Results represent mean FIC values of four replicates. Ratio = % extract:% antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic (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). Chl = chloramphenicol.

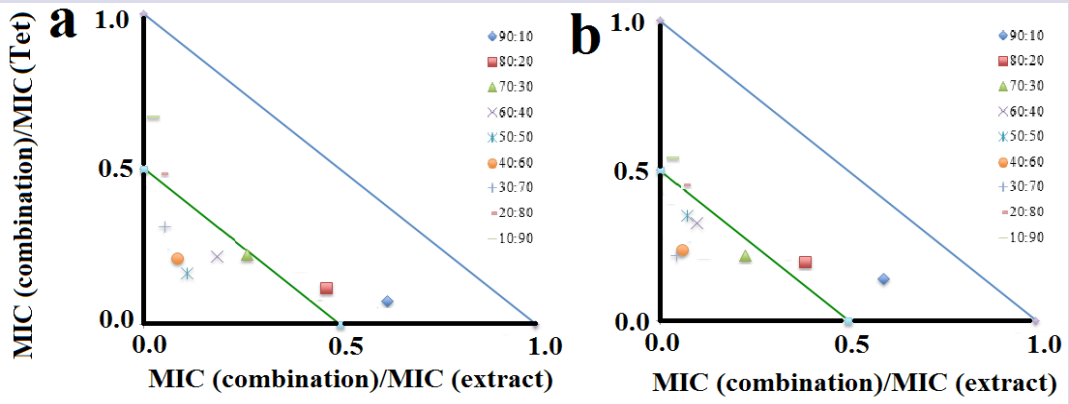

Figure 8: Isobologram for combinations of tetracycline and methanolic *R. canina* extract against (a) *P. vulgaris* and (b) *K. pneumonia*. Results represent mean FIC values of four replicates. Ratio = % extract:% antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic (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). Tet = tetracycline.

0.46 and 0.29 respectively). These combinations may therefore be effective in the prevention and treatment of ankylosing spondylitis (and other diseases caused by *K. pneumonia*). Combination of the same antibiotics with the hexane extract produced additive effects, indicating that these combinations may also be beneficial in the treatment of those diseases due to their increased growth inhibitory efficacies compared to the individual components. The majority of the other combinations were either non-interactive or ineffective.

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

A range of interactions were observed between *R. canina* extracts and conventional antibiotics against *A. baylyi* (Table 3). The combination of the methanolic extract with chloramphenicol resulted in a synergistic interaction (ΣFIC=0.5). A further two combinations were additive in combination with tetracycline. Thus, these combinations may be beneficial due to their increased growth inhibitory efficacies. These combinations include methanolic (ΣFIC = 0.6) and aqueous (ΣFIC = 0.8) extracts in combination with tetracycline. As *A. baylyi* is one of the bacterial triggers of multiple sclerosis,[5] combinations may be beneficial in the prevention and treatment of that disease. Table 3 also summarises the interactions of the *R. canina* extracts and conventional antibiotics against *P. aeruginosa*. Most combinations were ineffective at inhibiting *P. aeruginosa* growth and therefore ΣFIC values could not be determined. The methanolic and aqueous extracts in combination with gentamycin were non-interactive. Whilst there is no added benefit in combining these therapies, their concurrent use would not decrease the activity of either component and therefore they may be safely used in combination without decreasing the efficacy of the treatment.

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

The combinational antimicrobial effects of the *R. canina* extracts with various conventional antibiotics against *S. pyogenes* are summarised in Table 3. No synergistic effects were detected for any combination. The only additive combination detected was for the methanolic extract and tetracycline (ΣFIC=0.8). As this combination produces increased efficacy compared to either monotherapy alone, it may be beneficial in treating *S. pyogenes* infections. This combination may therefore be useful in the prevention of rheumatic fever in genetically susceptible individuals,

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

Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
M	108	10.8	+++	++	+++	-	++	++	-	-	-	+++	++	-	-
W	312	31.2	+++	++	++	-	++	+	-	+	+	+++	++	-	-
E	81	8.1	+	+	-	-	-	-	-	-	-	++	-	-	-
C	46	4.6	+	-	+	-	+	-	-	-	-	+	-	-	-
H	38	3.8	+	-	+	-	-	+	-	-	-	-	-	-	-

+++ 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.

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

EXRACT	*P. mirabilis* (ATCC33304)		*P. vulgaris* (ATCC21719)		*K. pneumoniae* (ATCC31488)		*A. baylyi* (ATCC21721)		*P. aeruginosa* (ATCC39324)		*S. pyogenes*	
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC
M	200	150	450	320	870	650	1850	1360	>5000	2710	790	1360
W	700	525	1080	868	ND	-	ND	>5000	ND	>5000	>5000	3900
E	200	185	586	365	ND	-	ND	-	ND	-	ND	-
C	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
H	ND	-	ND	-	164	82	ND	-	ND	-	ND	-
Positive controls												
Penicillin	ND	2.5	ND	1.25	ND	-	ND	-	ND	-	ND	-
Chloramphenicol	ND	-	ND	-	ND	1.25	ND	2.5	ND	-	ND	-
Gentamycin	ND	1.25	ND	1.25	ND	0.31	ND	0.31	ND	0.63	ND	0.63
Erythromycin	ND	-	ND	-	ND	-	ND	2.5	ND	-	ND	-
Tetracycline	ND	-	ND	2.5	ND	1.25	ND	1.25	ND	-	ND	2.5
Negative control	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-

M = methanol; W = water; E = ethyl acetate; C = chloroform; H = hexane. 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.

as well as impetigo, streptococcal throat infections etc. It is noteworthy that this bacterial strain displayed substantial resaistance to most of the conventional antibiotics. Indeed, only gentamycin and tetracycline inhibited the growth of this bacterium although the relatively high MIC for tetracycline (2.5µg/mL) indicates only low efficacy. Thus, it appears that this *S. pyonenes* strain is also resistant to tetracycline. Perhaps of greater interest, the combination of the methanolic extract and gentamycin was antagonistic. This combination should be avoided as a chemotherapeutic option to treat *S. pyogenes* infections. This was the only antagonistic result determined against *S. pyogenes*, indicating that all other combinations will not counter-indicate with the inhibitory properties of the conventional antibiotics.

Varied ratio combination studies (isobolograms)

Synergistic interactions with chloramphenicol

The combination of the methanolic *R. canina* extract and chloramphenicol induced synergistic interactions against *K. pneumonia* (Figure 7a) and *A. baylyi* (Figure 7b). These combinations were further examined using isobologram analysis across a range of extract:chloramphenicol

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

		Penicillin	Chloramphenicol	Gentamycin	Erythromycin	Tetracycline
P. mirabilis	M	1.5	-	2	-	-
	W	-	-	1.25	-	-
	E	2.5	-	1.13	-	-
	C	-	-	-	-	-
	H	-	-	-	-	-
P. vulgaris	M	1.2	-	2.6	-	**0.37**
	W	2.2	-	3.4	-	*0.57*
	E	1.4	-	2.9	-	*0.94*
	C	-	-	-	-	-
	H	-	-	-	-	-
K. pneumonia	M	-	**0.46**	2.7	-	**0.29**
	W	-	-	-	-	-
	E	-	-	-	-	-
	C	-	-	-	-	-
	H	-	*0.8*	3.5	-	*0.55*
A. baylyi	M	-	**0.5**	3.2	1.4	*0.6*
	W	-	2	3.5	1.8	*0.8*
	E	-	-	-	-	-
	C	-	-	-	-	-
	H	-	-	-	-	-
P. aeruginosa	M	-	-	1.6	-	-
	W	-	-	2.4	-	-
	E	-	-	-	-	-
	C	-	-	-	-	-
	H	-	-	-	-	-
S. pyogenes	M	-	-	4	-	*0.8*
	W	-	-	2.8	-	1.1
	E	-	-	-	-	-
	C	-	-	-	-	-
	H	-	-	-	-	-

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.

ratios to identify the ideal ratios to obtain synergy. All combination ratios containing 20-60% of the methanolic extract produced synergistic interactions against *K. pneumonia* (Figure 7a). Thus, these combination ratios would be beneficial to enhance *K. pneumonia* growth inhibition. However, bacteria would be less likely to develop resistance when combinations are used in ratios which minimise the amount of conventional antibiotic used. Thus, for long term prophylactic treatment (as would be required to prevent and treat ankylosing spondylitis), the ideal extract:chloramphenicol ratio may be 60:40. However, when used for the treatment of acute infections (e.g. lung infections), the ratio which maximises the efficacy of the treatment (i.e. the 20:80 ratio) may be the preferred option.

Similarly, the methanolic *R. canina* extract in combination with chloramphenicol produced synergy against *A. baylyi* across a wide range of ratios (Figure 7b). Only the ratios containing between 10-20% methanolic *R. canina* extract, or the combination containing 90% extract, produced additive effects. All other combination ratios were synergistic. However, whilst combinations containing 50-80% extract are classified as

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

Extract	LC_{50} value (µg/mL)	
	ALA	HDF assay
M	1280	-
W	1850	-
E	-	-
C	-	-
H	-	
PC	48	-

- indicates that less than 50% mortality was induced by the extract at all concentrations tested. 100% mortality was induced by potassium dichromate (positive control) and none by the sea water (negative control) at 1000 µg/mL. ALA = Atremia 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.

synergistic interactions, they were on the cut off between synergy and additive interactions. Therefore, the combination containing 40% methanolic *R. canina* extract and 60% chloramphenicol was deemed to be the best combination ratio for prophylactic treatment to prevent multiple sclerosis, as well as decreasing the possibility of further increasing bacterial resistance to chloramphenicol.

Synergistic interactions with tetracycline

Combinations of the methanolic *R. canina* extract and tetracycline induced synergistic interactions against *P. vulgaris* (Figure 8a) and *K. pneumonia* (Figure 8b). All combination containing 30-70% of the extract were synergistic against both of these bacteria. Therefore, these ratios would be beneficial as treatment options to enhance the growth inhibitory activity against *P. vulgaris* and *K. pneumonia*. However, the 70% extract and 30% tetracycline combination may be the ideal ratio for long prophylactic treatment, whilst the combination containing 30% extract may be preferential for the treatment of acute infections.

Quantification of toxicity

No LC_{50} values were determined for the ethyl acetate, chloroform or hexane extracts as <50 % mortality was seen in all tested concentrations (Table 4). In contrast, LC_{50} values of 1280 and 1850µg/ml were determined for the methanolic and aqueous extracts respectively. As extracts with LC_{50} values <1000 µg/ml towards *Artemia* nauplii have previously been defined as being toxic in this assay,[16] 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 either nontoxic.

DISCUSSION

This study investigated the ability of *R. canina* extracts to inhibit the growth of some bacterial triggers of autoimmune inflammatory diseases, both alone and in combination with conventional antibiotics. Several *R. canina* extracts were identified as effective bacterial growth inhibitors. The methanolic and aqueous extracts were particularly strong inhibitors of *P. mirabilis, P. vulgaris* and *K. pneumoniae* growth, with MIC values as low as 150µg/mL. Whilst these extracts also inhibited the growth of *A. baylyi, P. aeruginosa* and *S. pyogenes*, the MIC values were generally substantially >1000µg/mL and are thus indicative of only low to moderate inhibitory activity. Whilst a detailed investigation of the phytochemistry of the *R. canina* extracts was beyond the scope of this study, the qualitative phytochemical studies highlighted several phytochemical classes that may contribute to the bacterial growth inhibitory activity. Interestingly, the methanolic and aqueous *R. canina* extracts had relatively high abundances in polyphenolics, flavonoids, tannins, triterpenoids and saponins. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.[11] 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.[18] Similarly, multiple tannins have broad spectrum antibacterial activity via a variety of intra- and extra-cellular mechanisms, including the precipitation of microbial proteins.[19] Triterpenoids also have antibacterial activity, although the inhibitory mechanism is yet to be identified.[20] 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. canina* extracts growth inhibitory activity.

The studies combining the extracts with conventional antibiotics were of perhaps greater interest. Several combinations displayed substantially greater potential as therapeutic agents against bacterial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis than the extracts or antibiotics alone. Four synergistic combinations were identified in this study, with two synergistic combinations each noted in combinations of the methanolic *R. canina* extract in combination with either chloramphenicol or tetracycline. The implications of a synergistic interaction include enhanced efficacy, thereby allowing lower dose administration, with reduced side effects and possibly reduced antimicrobial resistance, or conversely greater efficacy with administration of the same dosage.[15] Notably, the bacteria against which synergistic combinations were detected (*P. vulgaris, K. pneumonia* and *A. baylyi*) were initially resistant to chloramphenicol and tetracycline. Thus, this study identified combinations of plant extracts and antibiotics which may repurpose relatively ineffective antibiotics and greatly enhance their efficacy, even against otherwise resistant bacterial strains. All of the extracts in synergistic combinations were high polarity extracts (methanol or water) suggesting the presence of a common active compound or class of compounds that may be responsible for the synergistic effects. Future studies are required to elucidate the potentiating mechanism(s) for the synergistic and additive combinations detected in our study.

Microbes have developed numerous resistance mechanisms to avoid the effects of antibiotics. One main method is through the use of multi-drug resistant (MDR) efflux pumps which are encoded chromosomally and are used to rapidly remove antibiotics that have entered the bacterial cells, thus rendering them resistant to the effects of the antibiotic.[20,21] A single pump may allow the bacteria to escape several types of antimicrobials. When these efflux pumps are inhibited, the intracellular concentration of antibiotic will increase, allowing the treatment to once again be effective. Interestingly, many plants possess MDR pump inhibitors in order to enhance the activity of their own natural antimicrobial compounds. Such MDR pump inhibitors become effective tools when used in combination with some previously ineffective/resistance prone antibiotic compounds and several examples have previously been reported.[22] Isoflavones isolated from *Lupinus argenteus* potentiate the activity of the natural plant antibiotic berberine as well as the synthetic fluoroquinoline antibiotic, norfloxacin as inhibitors of *S. aureus* growth.[22] 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* and *Securinega virosa* extracts act as efflux pump inhibitors for fluoroquinolone, tetracycline and erythromycin in resistant strains of *S. aureus* (MRSA).[23] 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-G, chloramphenicol, 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.[22-28] A single pump can provide bacteria with resistance to a wide array of chemically and structurally diverse antibiotics and it is not uncommon for an organism to code for more than one efflux pump.[22,28] It is therefore imperative to identify agents that can block the efflux mechanism (efflux pump inhibitors - EPIs) or alter the process of efflux and in so doing, extend the life of existing antibacterial drugs. Plants produce various secondary metabolites that are used as defense mechanisms against pathogenic invaders. Some plants produce antimicrobials which, along with other compounds, inhibit the efflux of those antimicrobials from a bacterial cell. There are currently no EPI/antimicrobial drug combinations on the market, although research into identifying potential EPIs is ongoing.[22] The synergistic interactions in our study suggests the possibility of a common EPI in the *R. canina* methanolic and aqueous 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).[29] It is likely that as penicillin binding proteins are a group of protein enzymes, these phytochemicals may form nonspecific interaction and affect the bacterial cell wall biosynthesis. The *R. canina* extracts may also contain a β-lactamase inhibitor. β-lactamases are the major defense of gram-negative bacteria against β-lactam antibiotics.[30] Clavulanic acid is an irreversible β-lactamase inhibitor, which in combination with β-lactam antibiotics can block the bacterial antimicrobial resistance mechanism.[31] 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. canina* 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. canina* 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.[32] 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 which produced synergy to be preferred for preventing and treating chronic disease

A further trend was evident in our study: most of the extract-antibiotic combinations which did not produce synergistic effects, generally did not greatly affect the efficacy of the antibiotic i.e. they appear to not counter-indicate with the antibiotics tested in this study. This is important as many users of herbal and traditional medicines self-diagnose/treat, often with multiple therapies concurrently. Thus, an understanding of drug herbal medicine interactions is important. Only a single combination tested in this study produced antagonistic interactions with the conventional antibiotics (against *S. pyogenes* in conjunction with gentamycin). This is an important finding and highlights that this combination should be avoided when treating *S. pyogenes* infections. Interestingly, previous studies indicate that antagonistic combinations of plant extracts with gentamycin are not uncommon.[33]

CONCLUSION

The results of this study demonstrate the potential of the *R. canina* extracts in inhibiting the growth of some bacterial triggers of autoimmune inflammatory diseases. The aqueous and methanolic extracts were good inhibitors of several microbes. However, the therapeutic potential of the *R. canina* extracts was far more apparent when tested in combination with conventional antibiotics as potentiators. Although the potentiation mechanisms are still unclear, compounds within *R. canina* extracts may mimic the actions of resistance modifying agents, thus potentiating the activity of several antibiotics that are relatively ineffective alone. Therefore, a combinational approach not only increases the effectiveness of drugs, but may also potentially reduce the side effects and reduce the development of drug resistant pathogens.

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ABBREVIATIONS

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

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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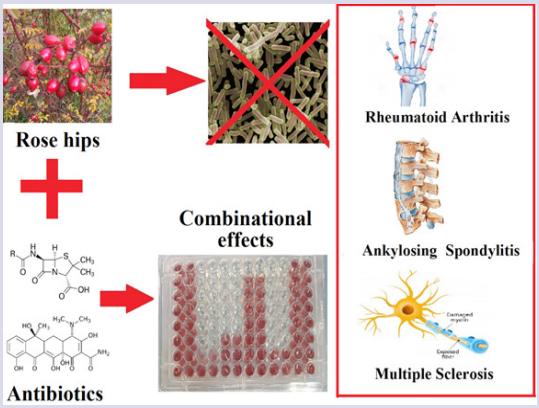
PICTORIAL ABSTRACT



SUMMARY

- *R. canina* rose hip 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 the *B. acerifolius* leaf and flower 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 200 publications in a variety of peer reviewed journals.