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Original Article

Anti-microbial Activity of *Rubus idaeus* L. Leaf Extracts in Combination with Antibiotics against Bacterial Triggers of Selected Autoimmune Diseases

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ABSTRACT

Background: *Rubus idaeus* L. leaves have been used in traditional healing systems for the treatment of morning sickness, easing labour pains and for preventing miscarriage. Whilst few studies have examined the leaves of this species for therapeutic properties, the fruit have antibacterial activity against multiple bacterial pathogens. This study examines the growth inhibitory effects of *R. idaeus* leaf extracts, both alone and in combination with conventional antibiotics against bacterial triggers of selected autoimmune inflammatory diseases. **Results:** *Rubus idaeus* leaf extracts displayed noteworthy antibacterial activity against several bacterial triggers of autoimmune diseases. The methanolic, aqueous and ethyl acetate extracts were particularly good inhibitors of *P. mirabilis, A. baylyi* and *P. aeruginosa* growth, with MIC values as low as 53 µg/mL. Combining the extracts with conventional antibiotics potentiated the inhibitory activity for several combinations containing chloramphenicol or gentamicin against *A. baylyi*. Interestingly, the other combinations were generally non-interactive and no antagonistic interactions were detected, indicating that all combinations can be used without decreasing the efficacy of the therapy. None of the individual components (or the combinations) were toxic in the ALA assay. **Conclusion:** *Rubus idaeus* leaf extracts have potential for the prevention and treatment of rheumatoid arthritis and multiple sclerosis when used alone. Furthermore, some combinations of the extracts and conventional antibiotics had greater potency against the bacterial triggers of multiple sclerosis, indicating they may be beneficial for prophylactic therapy in individuals genetically predisposed to that disease.

Keywords: Medicinal plants, Synergy, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Drug combinations, Efflux pump inhibitor.

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INTRODUCTION

Specific exogenous antigens may trigger autoimmune inflammatory disorders such as ankylosing spondylitis, multiple sclerosis, rheumatic fever and rheumatoid arthritis in genetically susceptible people. In total, approximately 80 autoimmune diseases with varying susceptibility profiles have been reported.[1] For many of these diseases, the triggers are established, although the etiological events of other autoimmune diseases remain unknown. Whilst some of these diseases are triggered by environmental and dietary stimuli, the majority are triggered by bacterial and viral pathogens. There are currently no cures available for any of these diseases. Instead, the therapeutic approach is to administer anti-inflammatory drugs and analgesics to treat the symptoms. Whilst this approach decreases the patient's discomfort, tissue damage still occurs. Additionally, the prolonged used of anti-inflammatory drugs causes toxicity and numerous deleterious side effects.[2] Instead, targeting the trigger antigens of autoimmune diseases may block the disease etiology, thereby preventing the diseases and its downstream effects. Many of the pathogenic triggers of these diseases have already been identified by serotyping studies, allowing the use of chemotherapies targeting the diseases prevention.[1] *Proteus mirabilis* can trigger rheumatoid arthritis in genetically susceptible people, whilst *Klebsiella pneumoniae* may trigger ankylosing spondylitis.[3-5] Multiple sclerosis can be initiated in genetically susceptible people by *Acinetobacter baylyi* and *Pseudomonas aeruginosa* infections, and *Streptococcus pyogenes* may induce the onset of rheumatic fever.[3-5]

Prophylactic antibiotic therapies targeting these pathogens is an attractive option as it would prevent the disease onset, and thus the downstream symptoms. However, prolonged prophylactic

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antibiotic monotherapy use would result in the development bacterial resistance towards the therapy, thereby decreasing the efficacy of the antibiotic for future use.[6] Indeed, the misuse and overuse of antibiotics has already resulted in the development of bacterial pathogens with resistance to multiple antibiotic therapies.[6] These multi-resistant pathogens are becoming increasingly difficult to manage using the current range antibiotic chemotherapies and previously effective therapies are failing to address some infections.[6,7] This has resulted in dramatic increases in the mortality associated with some bacterial pathogens.[8] Furthermore, the discovery of new antimicrobial agents has rapidly declined in recent years, and few antibiotics synthesised or discovered in the last decade were introduced clinically during that period.[6] The development of safe and effective new antibiotic therapies is crucial and is considered by the World Health Organisation (WHO) to be perhaps the most urgent challenge facing medical science.[9] For a number of reasons reviewed elsewhere,[6] the current methods of antibiotic discovery/development are unlikely to provide an adequate pipeline of new antibiotics in the future and alternative drug discovery and treatment modalities may be required.

A re-examination of ethnobotany and traditional plant-based medicines is a promising approach for antimicrobial drug development. Plant-derived medicines were commonly used to treat pathogenic disease prior to the modern era of antibiotic discovery, which began with the discovery of penicillin.[6] Ancient cultures had a good understanding of plant species with medicinal properties and much of that traditional knowledge was associated with the treatment and eradication of bacterial pathogens. Indeed, several traditional healing systems (e.g., Ayurveda and Traditional Chinese Medicine (TCM) are still in common use and are often well documented, making species selection for screening relatively simple. Furthermore, some plant medicines contain multiple well characterised antibacterial compounds. The presence of multiple antibacterial compounds (which often function via different mechanisms) not only increases the efficacy of the therapy, but also decreases the possibility of the therapy inducing further bacterial resistance.[6]

Despite the widespread use of traditional medicines, surprisingly few plant preparations or isolated compounds are commonly used as antimicrobial therapies in allopathic medicine. This may be due to the preference for monotherapies by allopathic medicine. Traditional medicines often require synergistic interactions between components in the formulation to potentiate the antibacterial activity of the medicine. Purified compounds may be substantially less potent than the crude extract they are derived from.[10] A combinational therapy model that utilises synergistic interactions between conventional antibiotics and plant extracts (or pure plant compounds) may therefore be more effective for the treatment of bacterial diseases, especially against antibiotic resistant bacteria strains[11,12] Indeed, combinational therapies are already preferred over mono-therapies for the treatment of several life-threatening infectious diseases including malaria, tuberculosis and HIV/AIDS as different components in the combinational therapy target multiple facets of a disease, thereby increasing the efficacy of the therapy and reducing the development of further resistance[6,10] As the development of new drugs may require years of costly clinical trials, therapies containing combinations of conventional antibiotics and plant extracts/isolated compounds may also have economic advantages.[10] Combinational therapies may potentially block bacterial resistance mechanisms, thereby restoring the efficacy of an antibiotic therapy.

Rubus idaeus L. (family Rosaceae) (commonly known as raspberry or red raspberry; Figure 1a) is native to Europe and northern Asia, although it has been naturalised and is now grown widely in temperate regions globally. The fruit of this plant is an important food crop and is known for its high antioxidant content.[13,14] It is particularly rich in ascorbic acid (Figure 1b) and folate, as well as the anthocyanins cyanidin-3-sophoroside (Figure 1c), cyanidin-3-2(G)-glucosylrutinoside (Figure 1d) and cyaniding-3-glucoside (Figure 1e).[15] They are also relatively rich in the ellagitannins ellagic acid (Figure 1f), sanguiin H-6 and lambertianin C and gallotannins.[16] The fruit is traditionally used as a nutritious food and there are few records of it traditional medicinal use. However, multiple screening studies have reported that the fruit have antibacterial[17] and anticancer bioactivities,[18] and are useful for preventing cardiovascular disease and type 2 diabetes mellitus.[19] In contrast, the traditional use of *R. idaeus* leaves is relatively well documented, particularly for the treatment of pregnancy-related ailments including morning sickness, easing labour pains and for preventing miscarriage.[20] Despite this, the pharmacological effects of *R. idaeus* leaves is relatively poorly studied and most examinations of the leaves have largely only examined the phytochemical composition,[21] without linking the constituents with biological properties. Our study aimed to screen *R. idaeus* leaves for the ability to inhibit the growth of some bacterial triggers of the autoimmune diseases rheumatoid arthritis (*Proteus mirabilis*), ankylosing spondylitis (*Klebsiella pneumoniae*), multiple sclerosis (*Acinetobacter baylyi* and *Pseudomonas aeruginosa*) and rheumatic fever (*Streptococcus pyogenes*).[3-5] Furthermore, the interactive antimicrobial and toxicity profiles of combinations of *R. idaeus* leaf extracts and six conventional antibiotic drugs was examined.

MATERIALS AND METHODS

Sourcing and preparation of plant samples

Dried and milled *Rubus idaeus* L. leaf material was purchased from Noodles Emporium, Australia. Voucher specimens are deposited in the School of Natural Sciences, Griffith University, Australia (voucher number RasLS1A-2017-1/1). Individual 1 g

masses of the ground plant material were weighed into separate tubes and 50 mL of methanol, deionised water, ethyl acetate chloroform or hexane were added. All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The ground plant materials were extracted in each solvent for 24 hr at 4°C by gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum. The solvent extracts were air dried at room temperature in the shade. The aqueous extracts were lyophilised by freeze drying at -50°C. The resultant dried extracts were weighed and dissolved in 10 mL deionised water (containing 1% DMSO).

Qualitative phytochemical analysis

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

Anti-bacterial analysis

Conventional antibiotics

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

Bacterial cultures

All bacterial strains were selected based on their ability to trigger autoimmune inflammatory diseases in genetically susceptible individuals.[3-5] Reference strains of *Proteus mirabilis* (ATCC21721), *Klebsiella pneumoniae* (ATCC31488), *Acinetobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Type Culture Collection, USA. A clinical isolate strain of *Streptococcus pyogenes* was obtained from the School of 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

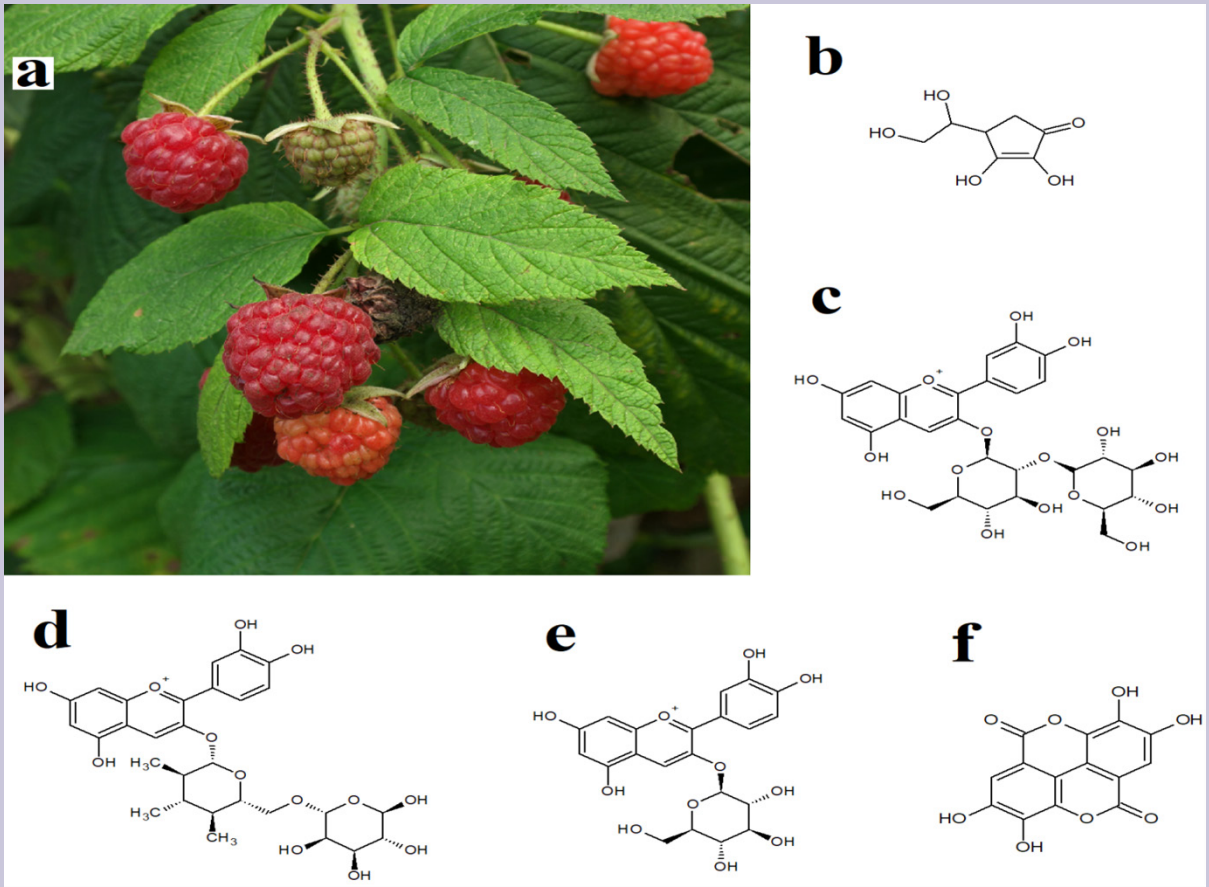

Figure 1: *R. idaeus* (a) leaves and fruit, as well as the chemical structures of (b) ascorbic acid, (c) cyanidin-3-sophoroside, (d) cyanidin-3-2(G)-glucosylrutinoside, (e) cyaniding-3-glucoside, and (f) ellagic acid.

cultures were incubated at 37°C for 24 hr and were sub cultured and maintained in nutrient broth at 4°C until use.

Evaluation of bacterial susceptibility to growth inhibition

The susceptibility of the bacteria to the *R. idaeus* leaf extracts and the conventional antibiotics was initially assessed using a modified disc diffusion assay.[24] Ampicillin (2 µg), tetracycline (10 µg) and chloramphenicol discs (10 µg) were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using two methods. A liquid dilution MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay.[25,26] Furthermore, as microplate liquid dilution MIC assays are perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method allows for comparisons with other studies. A solid phase agar disc diffusion assay was also used in this study for comparison.

Microplate liquid dilution MIC assay

A standard liquid dilution MIC assay[25] was used to evaluate the antimicrobial activity of the plant samples and the conventional antimicrobials independently, and in combinations. Briefly, 100 µL of sterilized distilled water was dispensed into each well of 96 well micro-titre plates. The plant samples and conventional antibiotics (100 µL) were then added into separate wells of the first row of the plate. Plant samples were introduced at a starting concentration of 32 mg/mL whilst the conventional antibiotics were introduced at a starting concentration of 0.01 mg/mL. A negative control (nutrient broth), a sterile control (without bacteria) and a sample-free culture control (to ensure the media was capable of supporting microbial growth) were included on all plates. After addition of the test samples to the plate, each was serially diluted by doubling dilution. The relevant bacterial culture inoculum (100 µL) was then added to all wells of the plate except the sterile control wells. Each inoculum contained approximately $1x10^6$ Colony Forming Units (CFU)/mL. All plates were subsequently incubated at 37°C. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, Australia and dissolved in sterile deionised water to prepare a 0.2 mg/mL INT solution. A 40 µL volume of this solution was added into all wells and the plates were incubated for a further 6 hr at 30°C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

Disc diffusion MIC assay

The minimum inhibitory concentrations (MIC) of the extracts was also evaluated by disc diffusion assay as previously described.[26-28] Graphs of the Zone of Inhibition (ZOI) versus Ln of the concentration were plotted and MIC values were achieved using linear regression.

Extract-conventional antibiotic interaction studies

Fractional Inhibitory Concentration (FIC) assessment

Interactions between the combinations of plant samples and conventional antimicrobials were further classified using the sum of the fractional inhibitory concentration ($^{\Sigma}$FIC). The FIC was calculated using the following equation, where (a) represents the plant sample and (b) the conventional antimicrobial sample[10,25]

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

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

The ∑FIC was then calculated using the equation: $\sum FIC = FIC^{(i)} + FIC^{(ii)}$. The interactions were classified as being synergistic for ∑FIC values of ≤0.5, additive (>0.5-1.0), indifferent (>1.0-≤4.0) or antagonistic (>4.0).[10]

Brine-shrimp lethality assay

The toxicity of the *R. idaeus* leaf extracts, conventional antibiotics and the reference toxin were assessed using a modified *Artemia franciscana* nauplii lethality assay (ALA).[29-31] Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4 mg/mL) and serially diluted in artificial seawater as a reference toxin. The mortality induction of all tests and controls was assessed at 24 and 48 hr and expressed as a % of the untreated control. The LC_{50} for each treatment was calculated using Probit analysis.

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 $p<0.01$ considered to be statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extractions of the *R. idaeus* leaf extracts (1 g) with solvents of varying polarity yielded dried plant extracts ranging from 27 mg (*R. idaeus* leaf hexane extract) to 187 mg (*R. idaeus* leaf aqueous extract) (Table 1). Qualitative phytochemical screening showed

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

Extract	Mass of Dried Extracted Material (mg)	Conventration of ectract (mg/mL)	Phenols			Cardiac Glycosides	Saponins	Triterpenes	Phytosterols	Alkaloids		Flavanoids		Tannins	Anthraquinones	
			Total Phenolics	Water Soluble	Water Insoluble	Keller-Kiliani Test	Froth Persistence	Salkowski Test	Acetic Anhydride Test	Meyers Test	Wagners Test	Shinoda Test	Kumar test	Ferric Chloride Test	Free	Combined
Methanol	123	12.3	+++	+++	++	-	+	-	-	-	-	+++	++	++	-	-
Water	187	19.7	+++	+++	+	-	+	-	-	-	-	+++	++	++	-	-
Ethyl Acetate	28	2.8	++	++	-	-	-	-	-	-	-	+	+	+	-	-
Chloroform	78	7.8	+	+	-	-	-	+	-	-	-	-	-	-	-	-
Hexane	27	2.7	-	-	-	-	-	+	-	-	-	-	-	-	-	-

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

that the higher polarity solvents (methanol and water) extracted the greatest amount and widest diversity of phytochemical classes.

Bacterial growth inhibition screening

Proteus mirabilis growth was particularly susceptible to the higher polarity aqueous, ethyl acetate and methanolic *R. idaeus* leaf extracts and generally was not susceptible to the lower polarity chloroform and hexane extracts (Figure 2a). Indeed, zones of inhibition of approximately 10.6 and 12.6 mm were recorded for the methanolic and aqueous extracts respectively against *P. mirabilis*. The ethyl acetate also produced noteworthy inhibition of *P. mirabilis* growth, albeit with a substantially smaller ZOI than measured for the methanolic or aqueous extracts. Notably, all three extracts produced larger ZOIs than both of the reference anti-biotics ampicillin and tetracycline (8 and 8.2 mm respectively). In contrast, the chloramphenicol control was a strong inhibitor of *P. mirabilis* growth, with a ZOI of 14.3 mm. The lower polarity chloroform and hexane extracts were completely devoid of inhibitory activity, indicating that the major antibacterial components may be polar. Similarly, the methanolic, aqueous and ethyl acetate *R. idaeus* extracts inhibited the growth of *K. pneumoniae* (Figure 2b), albeit generally with substantially lower efficacy than for *P. mirabilis* growth inhibition. As noted for *P. mirabilis* inhibition, the higher polarity methanolic and aqueous extracts were the best growth inhibitor (inhibition zones of 7.3 and 7.6mm respectively). The ethyl acetate extract produced slightly smaller ZOIs (~7 mm). In contrast, this bacterium was substantially more susceptible to the positive antibiotic controls. The noteworthy growth inhibitory activity of the methanolic, aqueous and ethyl acetate extracts against both *P. mirabilis* (a bacterial trigger of rheumatoid arthritis) and *K. pneumonia* (a trigger of ankylosing spondylitis) indicate that they may be useful for the prevention and treatment of these diseases, as well as other diseases that these bacteria cause.

The methanolic *R. idaeus* leaf extract was the best inhibitor of the growth of *A. baylyi* (a bacterial trigger of multiple sclerosis in genetically susceptible people), with a ZOI of ~9 mm (Figure 2c). The aqueous and ethyl acetate extracts also produced smaller ZOIs against. *baylyi* (7.3 and 6.3 mm respectively). In contrast, the chloramphenicol and tetracycline controls produced relatively large ZOIs (9.2 and 10.4 mm respectively). The ampicillin control was a less potent *A. baylyi* inhibitor (on the basis of ZOI), with a 7.8mm ZOI. The mid to high polarity *R. idaeus* leaf extracts also inhibited the growth of another bacterial trigger of multiple sclerosis, *P. aeruginosa*. Indeed, ZOIs of 9.6, 11.3 and 8.2 mm were measured for the methanolic, aqueous and ethyl acetate extracts respectively (Figure 2d). This inhibition is particularly noteworthy as the *P. aeruginosa* strain tested in this study was completely resistant to both the ampicillin and tetracycline controls. Our studies therefore indicate that the methanolic and aqueous *R. idaeus* leaf extract (and to a lesser extent, the ethyl

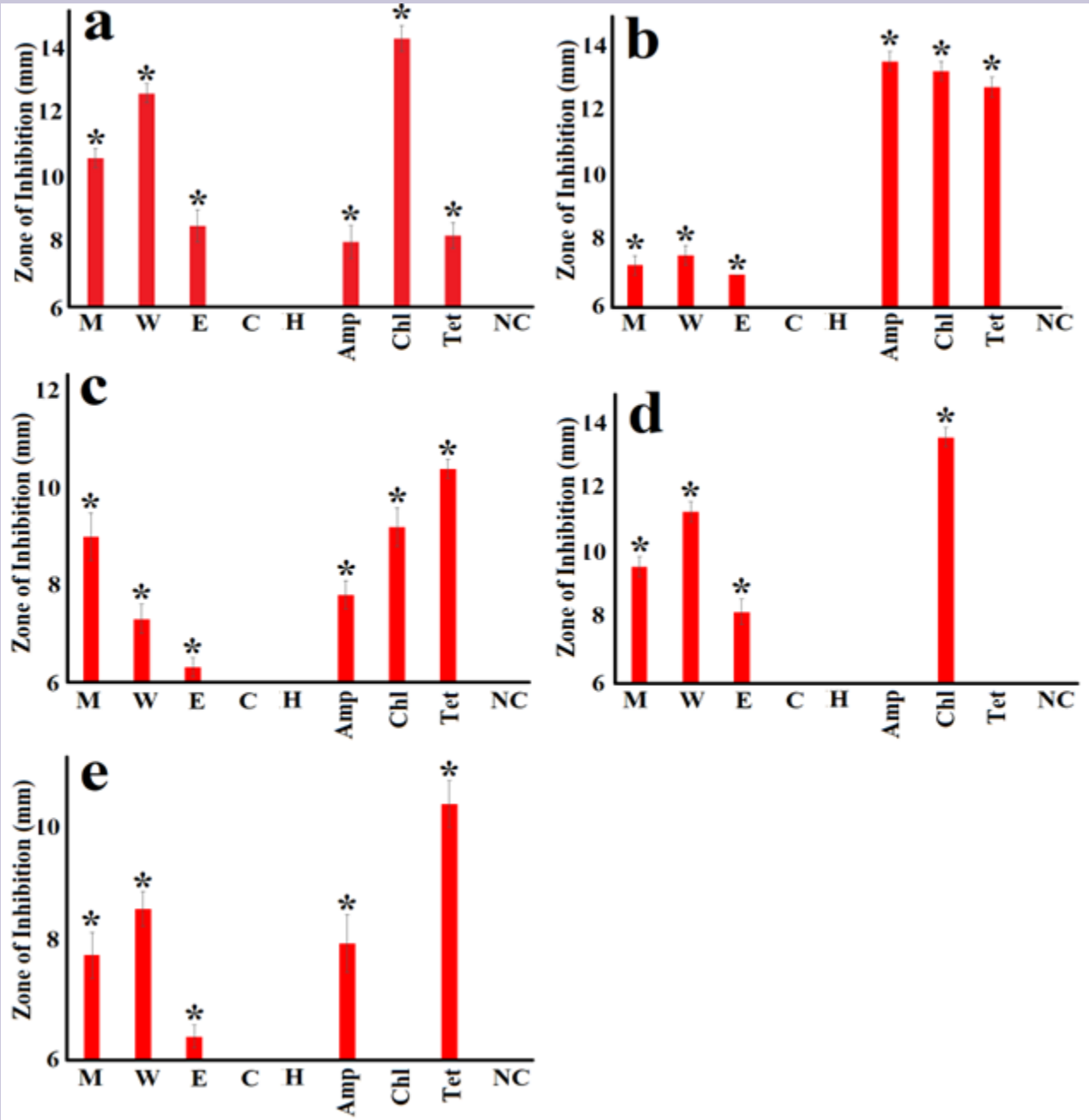

Figure 2: Anti-bacterial activity of *R. idaeus* leaf extracts against (a) *P. mirabilis* (ATCC21721); (b) *K. pneumoniae* (ATCC31488); (c) *A. baylyi* (ATCC33304); (d) *P. aeruginosa* (ATCC: 39324); (e) *S. pyogenes* clinical strain, measured as zones of inhibition (mm). M=Methanolic extract; W=aqueous extract; E=Ethyl acetate extract; C=Chloroform extract; H=Hexane extract; Amp=ampicillin (2 µg); Chl=Chloramphenicol (10 µg); Tet=Tetracycline (10 µg); NC=Negative Control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates (two repeats)±SEM; * indicates results that are significantly different to the negative control ($p<0.01$).

acetate extract) were the most effective inhibitor of both bacterial triggers of multiple sclerosis.

The methanolic, aqueous and ethyl acetate *R. idaeus* leaf extracts also inhibited *S. pyogenes* growth, albeit with small zones of inhibition indicative of low efficacy (Figure 2e). The chloroform and hexane extracts were completely devoid of antibacterial activity. The aqueous *R. idaeus* leaf extract was the strongest inhibitor of *S. pyogenes* growth, with a ZOI of 8.6mm measured. Notably, this *S. pyogenes* strain was partially resistant to ampicillin and completely resistant to chloramphenicol, yet susceptible to tetracycline (as judged by the ZOI). Thus, the aqueous and

Table 2: Disc Diffusion (DD) and Liquid Dilution (LD) MIC values (µg/mL) for *R. idaeus* extracts against microbial triggers of some autoimmune inflammatory diseases.

Bacterial Species	*P. mirabilis*		*K. pneumoniae*		*A. baylyi*		*P. aeruginosa*		*S. pyogenes*	
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC
Extracts										
M	256	74	1870	1460	129	100	410	250	1630	1528
W	528	228	2256	1866	1349	620	462	310	2186	1840
E	450	155	>2000	>2000	546	53	387	210	>2000	>2000
C	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-
Antibiotic controls										
Pen	ND	-	ND	-	ND	-	ND	-	ND	2.5
Chlor	ND	1.25	ND	1.25	ND	2.5	ND	2.5	ND	-
Eryth	ND	2.5	ND	-	ND	-	ND	-	ND	-
Tet	ND	-	ND	2.5	ND	-	ND	-	ND	-
Gent	ND	1.25	ND	0.63	ND	1.25	ND	0.31	ND	0.63
Cip	ND	0.63	ND	1.25	ND	1.25	ND	1.25	ND	1.25

M=Methanol extract; W=Water extract; E=Ethyl acetate extract; C=Chloroform extract; H=Hexane; DD=Disc Diffusion; LD=liquid dilution; Pen=penicillin-G; Chlor=Chloramphenicol; Eryth=Erythromycin; Tet=Tetracycline; Gent=Gentamycin; Cip=Ciprofloxacin. - indicates no inhibition at any dose tested; ND=MIC not determined.

Table 3: ΣFIC values for the *R. idaeus* leaf extracts and conventional antibiotic combinations against selected autoimmune bacteria.

	P. mirabilis			*K. pneumoniae*		*A. baylyi*			*P. aeruginosa*			*S. pyogenes*	
	M	W	E	M	W	M	W	E	M	W	E	M	W
Pen	-	-	-	-	-	-	-	-	-	-	-	1.76 (IND)	1.83 (IND)
Chlor	1.78 (IND)	1.45 (IND)	1.28 (IND)	2.15 (IND)	1.82 (IND)	0.92 (ADD)	1 (IND)	0.94 (ADD)	1.55 (IND)	1.38 (IND)	0.97 (ADD)	-	-
Eryth	2.2 (IND)	2.63 (IND)	1.87 (IND)	-	-	-	-	-	-	-	-	-	-
Tet	-	-	-	1.47 (IND)	1.83 (IND)	1.25 (IND)	1.67 (IND)	1.15 (IND)	-	-	-	-	-
Gent	2.63 (IND)	2.75 (IND)	2.2 (IND)	1.86 (IND)	2.27 (IND)	0.72 (ADD)	0.73 (ADD)	0.55 (ADD)	1.6 (IND)	2.4 (IND)	1.34 (IND)	1.47 (IND)	1.88 (IND)
Cip	1.15 (IND)	1.37 (IND)	1.44 (IND)	2.35 (IND)	1.89 (IND)	1.25 (IND)	1.9 (IND)	1.42 (IND)	1.52 (IND)	1.19 (IND)	1.24 (IND)	1.28 (IND)	1.44 (IND)

M=Methanolic extract; W=aqueous extract; E=Ethyl acetate extract; H=Hexane extract; Pen=Penicillin; Chlor=Chloramphenicol; Eryth=Erythromycin; Tet=Tetracycline; Gent=Gentamycin; Cip=Ciprofloxacin; ADD = Additive interaction; IND=Indifferent interaction; - unable to determine ΣFIC as at least one component was inactive.

methanolic extracts may still be useful for preventing and treating rheumatic fever.

Quantification of Minimum Inhibitory Concentration (MIC)

The relative level of anti-microbial activity was further evaluated by determining the MIC values using two methods: the liquid dilution MIC assay and the disc diffusion MIC assay (Table 2). Consistent with the antibacterial screening assays, each of the higher polarity methanol, aqueous and ethyl acetate *R. idaeus* leaf extracts inhibited all of the bacteria tested and they were more potent in comparison to the corresponding lower polarity extracts. The MIC values of the conventional antibiotic controls were only determined for the liquid dilution assay as commercial discs containing a fixed mass of antibiotic were used in the disc diffusion assay. Thus, the zone of only single doses was recorded

Table 4: Mortality (%) assessment for extracts and conventional antibiotics tested individually and as combinations in the *Artemia* nauplii lethality assay.

	Sample	Mortality±SD (%)	
		After 24 hr:	After 48 hr:
Antimicrobials	Penicillin G	1.8±1.4	4.3±2.4
	Chloranphenicol	2.7±1.3	5.6±3.3
	Erythromycin	1.2±0.6	5.8±2.3
	Tetracycline	2.4±1.5	5.1±2.8
	Gentamicin	3.1±1.8	6.7±2.6
	Ciprofloxacin	5.5±2.0	8.3±2.1
Extracts	M	8.4±2.8	27.3±3.2
	W	7.1±2.3	22.5±3.2
	E	4.2±1.6	10.5±1.9
Combinations	M+Pen	7.7±3.6	32.4±2.8
	M + Chloro	10±2.8	41.4±2.5
	M + Eryth	8.6±2	30.7±2.6
	M + Tet	11.3±1.9	33.8±3.1
	M + Gent	8.7±2.3	27.6±4.4
	M + Cip	10.4±3	33.8±2.9
	W + Pen	5.3±2.4	18.3±2.7
	W + Chloro	8.7±2.4	27.4±3.1
	W + Eryth	7.9±2.4	24.8±2.0
	W + Tet	10.1±3.3	34.1±3.6
	W + Gent	5. ±3.6	15.7±2.6
	W + Cip	8.9±3.6	24.7±4.1
	E + Pen	4.7±2.6	19.3±3.4
	E + Chloro	6.6±2.6	24.7±2.5
	E + Eryth	7.7±2.4	24.1±2.2
	E + Tet	14.6±1.9	32.7±3.2
	E + Gent	7.6±3.5	23.1±4.2
	E + Cip	10.4±2.8	29.6±4.2
Controls	Deionised water	2.7 ±1.7	3.6 ±2.5
	Potassium dichromate	100.0±0.0	

Potassium dichromate was tested at a concentration of 1000 µg/mL; M=Methanolic extract; W=aqueous extract; E=Ethyl acetate extract; Chloro=Chloramphenicol; Eryth=Erythromycin; Tet=Tetracycline; Gent=Gentamicin; Cip=Ciprofloxacin; SD=Standard Deviation. Results represent means±SEM of 3 independent experiments, each preformed in triplicate (n = 9).

for that assay and we were unable to determine MIC values. Gentamicin was the most potent antibiotic (as judged by its MIC). Indeed, most of the bacterial strains tested were partially resistant to all of the conventional antibiotics except gentamicin. The *P. mirabilis* and *A. baylyi* strains were also resistant to gentamicin, making them resistant to all of the antibiotics tested. The MIC values determined for *R. idaeus* leaf extracts compare relatively well between the disc diffusion and liquid dilution assays. The growth of *P. mirabilis* was strongly inhibited by the methanolic *R. idaeus* leaf extract (LD MIC=74 µg/mL). The aqueous extract (LD MIC=228 µg/mL) and the ethyl acetate extract (LD MIC=155 µg/mL) were also strong *P. mirabilis* growth inhibitors. The *R. idaeus* leaf extracts also displayed noteworthy inhibition of *A. baylyi* (MICs of 100, 620 and 53 µg/mL for the methanolic, aqueous and ethyl acetate extracts respectively) and *P. aeruginosa* (MICs of 250, 310 and 210 µg/mL for the methanolic, aqueous and ethyl acetate extracts respectively). Thus, these extracts may be useful in the prevention and treatment of rheumatoid arthritis (which may be induced by *P. mirabilis*) and multiple sclerosis (which can be triggered by *A. baylyi* and *P. aeruginosa*).

In contrast, substantially lower potencies were noted against *K. pneumoniae* (MIC values of 1460 and 1866 µg/mL for the methanolic and aqueous extracts respectively) and *S. pyogenes* (MIC values of 1528 and 1840 µg/mL for the methanolic and aqueous extracts respectively). These MIC values indicate only low to moderate growth inhibitory activity. Therefore, the *R. idaeus* leaf extracts may be of limited use for the prevention and treatment of ankylosing spondylitis (which may be triggered in genetically susceptible individuals by *K. pneumoniae*) and rheumatic fever (which can be triggered by *S. pyogenes*). However, it is noteworthy that both of these bacteria were particularly antibiotic-resistant strains, and were only susceptible to gentamicin (MIC values>1 µg/mL for pure antibiotics is considered resistant in this assay).[23-26] Thus, future studies are required to screen *R. idaeus* leaf extracts against an extended panel of *K. pneumoniae* and *S. pyogenes* strains to obtain a better understanding of their potential against ankylosing spondylitis and rheumatic fever (and other diseases caused by these bacteria).

Combinational effects: Fractional Inhibitory Concentration (FIC) assessment

Whilst the *R. idaeus* leaf extracts yielded promising antibacterial effects when tested alone against some bacteria, combining the extracts with conventional antibiotics has the potential to potentiate the growth inhibitory activity compared to that of the individual components. Therefore, combinations of the extracts and antibiotics were tested and the Σ_{FIC} values were calculated to determine the classes of interaction (Table 3). Only combinations where both components were effective growth inhibitors are included in that table as it was not possible to calculate Σ_{FIC} values for other combinations. Interestingly, none of the combinations tested were synergistic against any of the tested bacteria. However, six of the 47 combinations (~13%) produced additive effects (Table 3). Whilst the potentiation of activity is not as pronounced for additive interactions compared to synergy, the potency of the extracts is enhanced and therefore these combinations is preferential compared to the individual components. Notably, five of these additive interactions were against *A. baylyi*, with another additive interaction recorded against *P. aeruginosa*. Therefore, these combinations may be beneficial for the prevention and treatment of multiple sclerosis as they provide greater efficacy than either the extract or antibiotic components alone. Notably, all of the other combinations tested were non-interactive and no antagonistic combinations were detected. Whilst non-interactive combinations provide no added benefit over that of the individual components alone, the components do not antagonise each other's effects and are therefore safe to use concurrently without risk of lessening the efficacy of either component.

Toxicity studies

All plant extracts and antibiotics were individually screened at 1 mg/mL in the *Artemia* lethality assay (ALA). The extracts were only considered toxic if they induced percentage mortalities greater than 50% (LD_{50}) following 24 hr of exposure to the *Artemia* nauplii.[29-31] When tested individually, the antimicrobials demonstrated no toxicity in the ALA (Table 4). Similarly, none of the *R. idaeus* extracts produced mortality or cell viability significantly different to that of the negative control. When tested together in the ALA, none of the extract-antibiotic combinations produced mortality significantly different to the negative controls, and no single component nor combination induced >50% mortality. Therefore, all combinations and individual components were deemed to be non-toxic. In contrast, the potassium dichromate positive control induced 100% mortality in the ALA.

DISCUSSION

This study investigated the ability of *R. idaeus* leaf extracts to inhibit the growth of some bacterial triggers of auto-immune inflammatory diseases, both alone and in combination with conventional antibiotics. *Rubus idaeus* was selected for this study as it is traditionally used in several traditional healing systems, yet its biological properties have been relatively poorly examined. Whilst the fruit has been reported to have noteworthy antibacterial activity,[17] *R. idaeus* leaf extracts are yet to be rigorously examined for antibacterial properties. To the best of our knowledge, no previous studies has tested *R. idaeus* leaf extracts for the ability to inhibit the growth of the bacterial triggers of autoimmune inflammatory diseases. Several extracts were identified as effective growth inhibitors against *P. mirabilis, A. baylyi* and *P. aeruginosa* growth, with clinically relevant potency. The methanolic and ethyl acetate extracts had the strongest inhibitory activity against all bacteria, indicating that they may be particularly useful in preventing and treating rheumatoid arthritis, and multiple sclerosis (as well as other infections caused by these bacteria) when used by alone. The extracts also inhibited the growth of *K. pneumoniae* and *S. pyogenes*, albeit at substantially lower potency. Therefore, the extracts may also prevent and treat ankylosing spondylitis and rheumatic fever, although higher doses of these extracts may be required for clinical effects.

The majority of the conventional antibiotic and *R. idaeus* leaf extract combinations demonstrated indifferent interactions. Whilst these combinations have limited added benefit over the conventional antibiotic (or extract) alone, they do alleviate some concerns related to concurrent use of the two forms of healthcare as these interactions indicate that neither therapy is reducing the efficacy of the other therapy. Additionally, several combinations (particularly those containing chloramphenicol of gentamicin) displayed additive effects against *A. baylyi*. The implications of these potentiated interactions include enhanced efficacy, thereby allowing lower doses to be administered, thus reducing any side effects of the chemotherapy.[10] Of further benefit, bacterial

exposure to lower levels of the conventional antibiotics would decrease the induction of further antibiotic resistance.[6]

In most published combination studies, synergistic interactions are emphasized, with the reporting of antagonism being neglected. However, co-therapy of drugs which have antagonistic interaction would reduce the efficacy of both therapies, thereby increasing the burden placed on the healthcare system. Notably, none of the *R. idaeus* leaf extract-conventional antibiotic combinations tested displayed antagonistic interactions. This is an important finding as it determines combinations that should be avoided for chemotherapeutic use.

None of the *R. idaeus* leaf extract or conventional antibiotics demonstrated toxicity in the ALA and MTS assay when tested independently. Similarly, all combinations were non-toxic in both assays, indicating their potential for therapeutic use. The non-toxicity of the conventional antibiotics is hardly surprising as these drugs have a long history of therapeutic use and their lack of toxicity has previously been verified in clinical trials. The lack of toxicity determined for the *R. idaeus* leaf extract may perhaps also not be surprising as this plant has been used therapeutically for hundreds of years. However, to the best of our knowledge, there is a lack of rigorous toxicity studies for *R. idaeus* leaf extracts. The lack of toxicity of the combinations also indicates their potential for therapeutic usage. However, further *in vitro* studies using other human cell lines are required to verify their safety. Furthermore, *in vivo* testing is also required to confirm that the extracts and combinations retain efficacy and remain non-toxic in complex biological systems.

CONCLUSION

Whilst the findings reported herein indicate the potential of *R. idaeus* leaf extracts (particularly in combination with chloramphenicol or gentamicin against *A. baylyi*) as preventative and therapeutic options against bacterial triggers of some autoimmune inflammatory diseases, further *in vivo* investigations are required to support these *in vitro* findings. Furthermore, studies to determine the possible mechanism of action resulting in the observed interaction are warranted, and bioactivity driven compound isolation and/or metabolomics studies are also required to determine the active compound(s), as well as those responsible for the antibiotic potentiation, within the *R. idaeus* leaf extracts.

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ABBREVIATIONS

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

SUMMARY

- *Rubus idaeus* leaf extracts inhibit the growth of several bacterial triggers of selected autoimmune diseases.
- The methanolic, aqueous and ethyl acetate extracts were good growth inhibitors against all bacteria.
- Several combinations of the *R. idaeus* extracts and conventional antibiotics had greater activity against *A. baylyi* than the extract or antibiotic components individually.
- No antagonistic combinations were noted.
- All *R. idaeus* leaf extracts were non-toxic.

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