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Original Article

Antibacterial Activity and Toxicity Profiles of *Epilobium parviflorum* (Schreb.) Schreb. Extracts and Conventional Antibiotics against Bacterial Triggers of Some Autoimmune Diseases

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ABSTRACT

Background: *Epilobium parviflorum* (Schreb.) Schreb. has been used traditionally to treat prostate, bladder and kidney diseases, as well as inflammation. However, *E. parviflorum* extracts are yet to be tested for the ability to inhibit the growth of bacterial triggers of autoimmune diseases. **Materials and Methods:** Antimicrobial activity was assessed using disc diffusion and liquid dilution minimum inhibitory concentration (MIC) assays against a panel of bacterial triggers of some autoimmune diseases. Interactions between the *E. parviflorum* extracts and conventional antibiotics were studied and classified using the sum of the fractional inhibitory concentration (∑FIC). Notable synergistic interactions were further examined across a range of ratios using isobologram analysis. The toxicity of the individual samples and the combinations was assessed using the *Artemia* lethality assay (ALA) assay. **Results:** *Epilobium parviflorum* extracts displayed notable antibacterial activity against the bacterial trigger of rheumatoid arthritis (*P. mirabilis*), but were ineffective against *K. pneumoniae, A. baylyi, P. aeruginosa* and *S. pyogenes*. The methanolic and ethyl acetate extracts were particularly good inhibitors of *P. mirabilis* growth, with MIC values of 484 and 623µg/mL recorded respectively. Furthermore, combining the extracts with conventional antibiotics resulted in significant potentiation of the inhibitory activity for some combinations. Interestingly, all combinations containing chloramphenicol or ciprofloxacin produced either synergistic or additive effects against *P. mirabilis*. None of the individual components (nor the combinations) were toxic in the ALA assay. **Conclusion:** The *E. parviflorum* methanolic and ethyl acetate extracts displayed clinically relevant antibacterial activity against *P. mirabilis* when tested alone. Furthermore, the methanolic and aqueous extracts potentiated the activity of chloramphenicol and ciprofloxacin in combination. The lack of toxicity of the extract and combinations indicates that *E. parviflorum* methanolic and aqueous extract and antibiotic combinations may provide leads in the development of new therapies to prevent and treat rheumatoid arthritis.

Keywords: Medicinal plants, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Conventional antimicrobials, Synergy, Drug interaction, Toxicity.

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INTRODUCTION

The improper, overuse and misuse of antibiotics has resulted in the development of antibiotic resistance in many bacterial pathogens, rendering many common clinical antibiotics of limited efficacy against common pathogens.[1] In recent years, antibiotic resistance has risen rapidly, thereby creating multiple antibiotic resistant organisms that are becoming increasingly difficult to manage using the current range of available antibiotic chemotherapies.[2] Simultaneously, the discovery and development of new antibiotic therapies has steadily decreased to no more than a few antibiotics synthesised or discovered in the last decade.[3] The development of alternative treatment methods is crucial and is considered by the World Health Organisation (WHO) to be one of the biggest challenge facing medical science.[4] For a number of reasons reviewed elsewhere,[5] it is unlikely that the current methods of antibiotic discovery/development will be as successful in the future.

Examination of traditional medicines for natural compounds with therapeutic properties is a promising method of generating new drug leads for the development of new antibiotics. Despite

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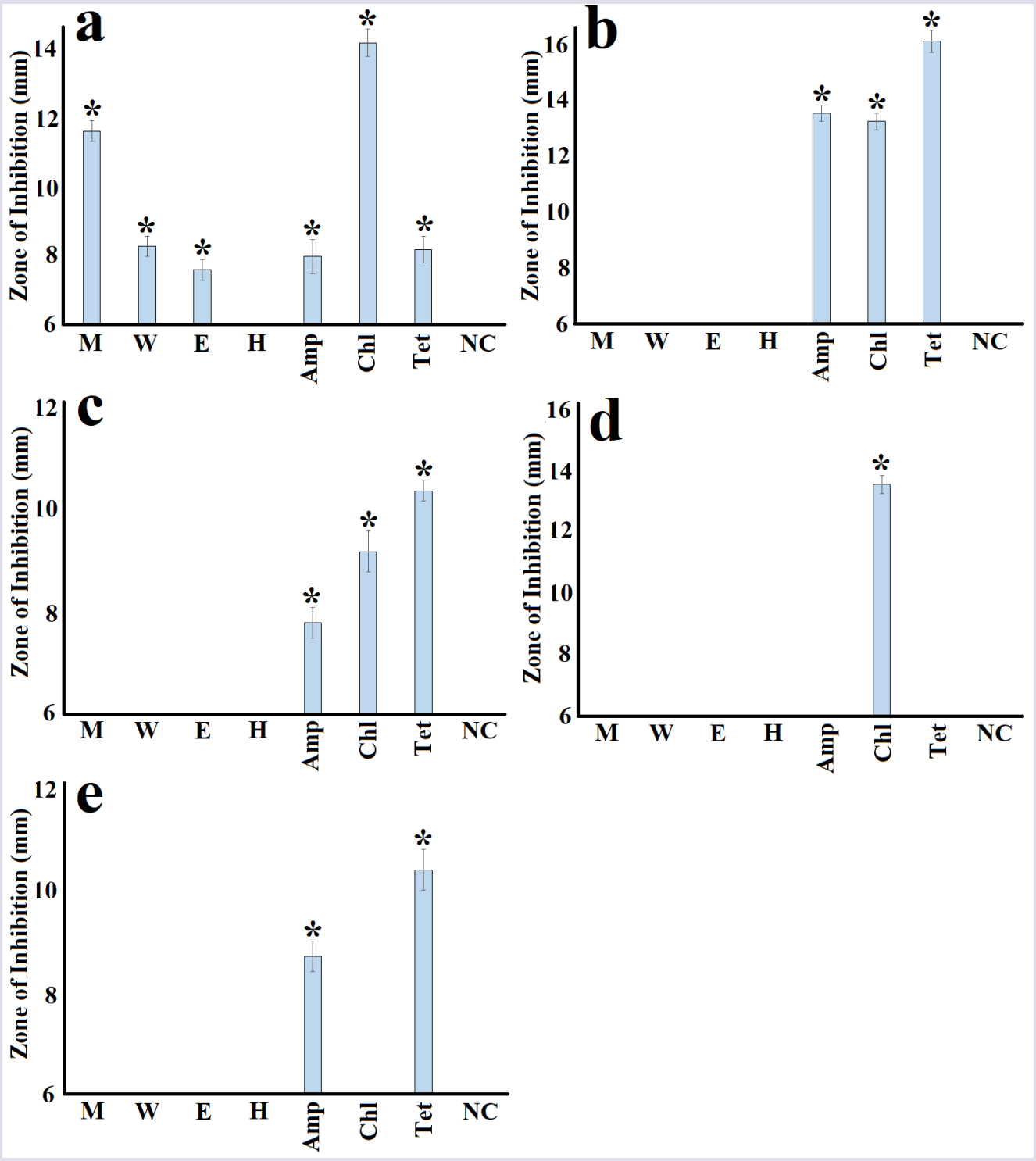

Figure 1: Antibacterial activity of *E. parviflorum* extracts extracts against (a) *P. mirabilis* (ATCC21721); (b) *K. pneumoniae* (ATCC31488); (c) *A. baylyi* (ATCC33304); (d) *P. aeruginosa* (ATCC: 39324); and (e) *S. pyogenes* clinical strain, measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; H = hexane extract; Amp = ampicillin (2µg); Chl = chloramphenicol (10µg); Tet = tetracycline (10µg); NC = negative control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates ± S.D. * indicates results that are significantly different to the negative control ($P<0.01$).

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 plant phytochemicals often have much lower activity than the crude extract that they are derived from.[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 in antibiotic resistant strains.[2,7,8] Combinational therapy is already preferred over mono-therapy in multiple life-threatening infectious diseases such as malaria, tuberculosis and HIV/AIDS due to its ability to target multiple facets of a disease and to curb resistance.[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 lengthy and expensive process of discovering new antibiotic 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]

Epilobium parviflorum (Schreb.) Schreb. (Synonym: *Chamaenerion parviflorum*; common names hoary willowherb, smallflower hairy willowherb) is a small herbaceous plant of the family Onagraceae. It is widely distributed across most of Europe and also occurs in parts of northern Africa, southern and western Asia and northern America. Extracts prepared from *E. parviflorum* are used in traditional European medicine to treat disorders of the prostrate gland, kidneys and bladder, and are frequently used to treat benign prostatic hyperplasia.[9] Additionally several published studies have reported anti-inflammatory[10,11] and antimicrobial properties.[12-14] However, *E. parviflorum* 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*).[15-17] Furthermore, *E. parviflorum* extracts are yet to be tested for bacterial growth inhibitory activity in combinational studies with conventional antibiotics. Therefore, this study investigates the antimicrobial effects of *E. parviflorum* 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

Sourcing and preparation of plant samples

The *Epilobium parviflorum* (Schreb.) Schreb. above ground plant material used in this study were purchased from Noodles Emporium, Australia. Voucher specimens are deposited in the School of Natural Sciences, Griffith University, Australia (Voucher number EPAI-A1-2018-1A). Individual quantities (1g) of the plant material were weighed into separate tubes and 50mL of methanol, deionised water, ethyl acetate or hexane were added. All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The seeds were extracted in each solvent for 24 hr at 4°C with 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 10mL of deionised water (containing 1% DMSO).

Qualitative phytochemical analysis

Phytochemical analysis of the *E. parviflorum* extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved using standard assays.[18,19]

Antibacterial analysis

Conventional antibiotics

Penicillin-G (potency of 1440-1680µg/mg), chloramphenicol (≥98% purity by HPLC), ciprofloxacin (≥98%), erythromycin (potency ≥850µg/mg), gentamicin (potency of 600µg/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 0.01mg/mL and stored at 4°C until use. For the disc diffusion studies, ampicillin (2µg), tetracycline (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.[15-17] Reference strains of *Proteus mirabilis* (ATCC21721), *Klebsiella pneumoniae* (ATCC31488), *Acinetobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Type Culture Collection, USA. A clinical isolate strain of *Streptococcus pyogenes* was obtained from the School of Natural Sciences teaching laboratory, Griffith University, Australia. All bacteria were cultured in nutrient broth (Oxoid Ltd., Australia). Streak nutrient agar (Oxoid Ltd., Australia) plates were tested in parallel to ensure the purity of all bacterial cultures and for sub-culturing. All bacterial cultures were incubated at 37°C for 24hr and were subcultured and maintained in nutrient broth at 4°C until use.

Evaluation of bacterial susceptibility to growth inhibition

The susceptibility of the bacteria to the *E. parviflorum* extracts and the conventional antibiotics was initially assessed using a modified disc diffusion assay.[20,21] Ampicillin (2 µg), tetracycline (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 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.[22] 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[22,23] was used to evaluate the antimicrobial activity of the plant samples and conventional antimicrobials independently and in combinations. Briefly, 100µL of sterilized distilled water was dispensed into each well of 96 well micro-titre plate. The plant samples and conventional antibiotics (100µL) were then added into separate wells of the first row of the plate. The *E. parviflorum* extracts were introduced at a starting concentration of 32mg/mL whilst the conventional antibiotics were introduced at a starting concentration of 0.01mg/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 serial dilution. The relevant bacterial culture inoculum (100µL) 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 0.2mg/mL INT solution. A 40µL 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.[24,25] Graphs of the zone of inhibition (ZOI) versus Ln of the concentration were plotted and MIC values were calculated using linear regression.

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 (∑FIC). The FIC was calculated using the following equation, where (a) represents the plant sample and (b) the conventional antimicrobial sample.[26,27]

$$\frac{\text{MIC (a) in combination with (b)}}{\text{MIC (a) independently}}$$

$$\frac{\text{MIC (b) in combination with (a)}}{\text{MIC (b) independently}}$$

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).[26,27]

Varied ratio combination studies (isobolograms)

For the identified synergistic interactions, nine different ratios of the combination were prepared and the MIC values determined. The samples were combined in various from 10:90 to 90:10 (extract: antimicrobial). Data points for each ratio were plotted on an isobologram using the GraphPad Prism® software (Version 5). The construction of isobolograms allowed for the identification of the agent (plant or antimicrobial sample) most responsible for the synergistic effects within the combination. Data points falling below the 0.5:0.5 line indicated synergy, while those above the 0.5:0.5 line, but below the 1.0:1.0 line indicated an additive interaction. Data points above the 1.0:1.0 line, but below the 4.0:4.0 line indicated a non-interactive or indifferent interaction, and data points falling above the 4.0:4.0 line indicated antagonism.[22]

Artemia franciscana lethality assay (ALA)

Toxicity of the *E. parviflorum* extracts, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.[28,29] 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. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Statistical analysis

Data is expressed as the mean ± S.D. 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 *E. parviflorum* plant material (1g) with solvents of varying polarity yielded dried plant extracts ranging from 11mg (*E. parviflorum* extracts ethyl acetate and hexane extracts) to 172mg (*E. parviflorum* extracts aqueous extract) (Table 1). Qualitative phytochemical screening (Table 1) showed that the higher polarity solvents (methanol and water) extracted the greatest amount and widest diversity of phytochemical classes.

Bacterial growth inhibition screening

Only *P. mirabilis* growth was susceptible to the *E. parviflorum* extracts extracts in the disc diffusion screening assays (Figure 1a). The methanol extract was a particularly potent inhibitor of *P. mirabilis* growth, with a ZOI of approximately 11.7mm. This compares favourably to the antibiotic controls included in this assay. Indeed, this extract produced considerably stronger inhibition of this bacterium than either ampicillin or tetracycline (on the basis of the size of the ZOI), which produced ZOIs of ~8mm and 8.2mm respectively. In contrast, the chloramphenicol control was substantially more effective (ZOI~14.3mm) than the extracts or other antibiotic controls. The aqueous and ethyl acetate extracts were also noteworthy inhibitors of the growth of this bacterium, with ZOIs of 8.3 and 7.6mm respectively. Notably, the extracts tested in this screening assay were initially tested at the concentration at which they were prepared to provide an approximation of the concentration at which the *E. parviflorum* extracts would be used traditionally. Therefore, the aqueous extract was tested at approximately 2.1 times the concentration than the methanolic extract and 15.6-fold higher the ethyl acetate and hexane extracts. Therefore, it is likely that the methanolic, ethyl acetate and hexane extracts may be much stronger inhibitors of *P. mirabilis* growth than is evident in this screening study. In contrast to the *P. mirabilis* susceptibility results, all *E. parviflorum* extracts extracts were completely ineffective inhibitors of *K. pneumoniae* (Figure 1b), *A. baylyi* (Figure 1c), *P. aeruginosa* (Figure 1d) and *S. pyogenes* growth (Figure 1e).

Quantification of minimum inhibitory concentration (MIC)

The relative level of antimicrobial activity was further evaluated by determining the MIC values using two methods: the liquid dilution MIC assay and the disc diffusion MIC assay (Table 2). Consistent with the antibacterial disc screening assays, only *P. mirabilis* was susceptible to the *E. parviflorum* extracts, whilst all of the other bacteria tested were unaffected. The methanolic and ethyl acetate extracts were a particularly good inhibitors of *P. mirabilis* growth, with LC_{50} values of 484 and 623µg/mL respectively. Therefore, these extracts would be useful for preventing and treating rheumatoid arthritis in genetically susceptible people. The aqueous extract also displayed *P. mirabilis* growth inhibitory activity, albeit with a substantially higher MIC indicative of moderate activity (1834µg/mL). Notably, both the methanol and ethyl acetate extracts would contain mid polarity compounds, whereas the aqueous extract would contain exclusively higher polarity compounds, indicating that lower polarity compounds may be responsible (at least in part) for the inhibitory activity against *P. mirabilis*. The inhibitory activity of the *E. parviflorum* extracts against this bacterium were particularly interesting as the *P. mirabilis* strain tested in our study was resistant to multiple

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *E. parviflorum* 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	81	8.1	+++	++	+	-	+	-	-	-	-	++	+++	++	-	-
Water	172	17.2	+++	++	++	-	+	-	-	-	-	+++	+++	++	-	-
Ethyl Acetate	11	1.1	+	+	-	-	-	-	-	-	-	++	+	+	-	-
Hexane	11	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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

Table 2: Disc diffusion (DD) and liquid dilution (LD) MIC values (µg/mL) for *E. parviflorum* extracts against *P. mirabilis*.

-	Test	DD MIC	LD MIC
Extracts	M	624	484
	W	3255	1834
	E	850	623
	H	-	-
Controls	Penicillin	ND	-
	Chloramphenicol	ND	1.25
	Erythromycin	ND	-
	Tetracycline	ND	-
	Gentamicin	ND	0.63
	Ciprofloxacin	ND	0.63

M = methanol extract; W = water extract; E = ethyl acetate extract; H = hexane; DD = disc diffusion; LD = liquid dilution; ND indicates that the MIC was not determined in the DD assay as only a single dose was tested; - indicates no inhibition was noted at any concentration. Bacteria with MIC values >1µg/mL for pure antibiotics are considered resistant to that antibiotic.

antibiotics. Indeed, it was completely resistant to penicillin-G, erythromycin and tetracycline as these antibiotics did not inhibit bacterial growth at any concentration tested. Similarly, an MIC value of 1.25µg/mL was measured for chloramphenicol against *P. mirabilis*. As MIC values >1µg/mL for pure antibiotics indicate antibiotic resistance in this assay,[30,31] it was determined that this bacterium was only appreciably susceptible to gentamicin and ciprofloxacin of the antibiotic controls tested. The good activity of the *E. parviflorum* extracts against the bacterium indicates that it may therefore be particularly useful in preventing and treating rheumatoid arthritis.

In contrast, all of the other bacteria tested were unaffected by the *E. parviflorum* extracts (Figures 1b-e). Notably, several other studies have screened these same bacterial strains previously and have reported all of them to have multi-drug antibiotic resistance.[32-34] The *P. aeruginosa* strain has been reported to be particularly resistant to most conventional antibiotics. Notably, some of the extracts could only be tested at low concentrations. The ethyl acetate and hexane extracts were extracted at low concentrations (280 and 780µg/mL respectively). Therefore, testing of these extracts was only possible using maximum concentrations of 70 and 195µg/mL respectively in the assay. As MIC values <1000µg/mL are considered noteworthy in this assay, it is possible that these extracts may be useful growth inhibitors of the other bacterial species if tested at higher concentrations. Further studies are planned to test this hypothesis.

Fractional inhibitory concentration (FIC) assessment

Three combinations (33%) of the *E. parviflorum* extracts and conventional antibiotic combinations that inhibited *P. mirabilis* growth produced synergistic effects when tested together (Table 3). This is particularly noteworthy as these combinations have substantially higher growth inhibitory activity than either component separately (>4 times higher activity). Therefore, these combinations would be particularly useful in preventing and treating rheumatoid arthritis (and other illnesses caused by *P. mirabilis* infections). Additionally, three combinations (33%) produced additive effects against *P. mirabilis*. As these combinations produce effects greater than either the individual extract or conventional antibiotic components, these combinations would also be beneficial in the prevention and treatment of rheumatoid arthritis. All of the other inhibitory combinations were non-interactive. Whilst these combinations provide no added benefit over that of the individual components alone, the components do not antagonise each other's effects and are therefore safe to use concurrently without risk of lessening the efficacy of either component.

Varied ratio combination studies (isobolograms)

Three synergistic combinations were detected against *P. mirabilis*. One of these interactions was in a combination containing chloramphenicol (with the aqueous extract), whereas two combinations contained ciprofloxacin and either the methanolic or aqueous extracts. These combinations were further examined using isobologram analysis across a range of extract-antibiotic ratios to identify the ideal ratios to obtain synergy (Figure 2). For the combination containing chloramphenicol and the methanolic extract, synergy occurred for ratios of ≥30% extract, up to ≤60% extract (Figures 2a). Ratios outside this range produced additive effects and would thus also be beneficial for inhibiting *P. mirabilis* growth. These results indicate that extract component(s) may be competing with the antibiotic component for interaction with the bacterial resistance mechanism (e.g. an efflux pump or antibiotic inactivating enzyme). Therefore, as the ratio of extract increases, the extract component(s) outcompete the antibiotic for interaction with the resistance mechanism, effectively blocking its ability to inhibit the antibiotics effects. Thus, the methanolic extracts may contain competitive inhibitors of the bacterial resistance protein(s), although the specific mechanism is yet to be determined. A different trend was evident for both combinations containing ciprofloxacin as the antibiotic component (Figures

Table 3: ΣFIC values for the *E. parviflorum* extracts and conventional antibiotic combinations against *P. mirabilis*.

-	M	W	E	H
Chloramphenicol	0.76	0.28	0.85	-
	(ADD)	(SYN)	(ADD)	
Gentamicin	1.5	2.0	2.2	-
	(IND)	(IND)	(IND)	
Ciprofloxacin	0.25	0.09	0.93	-
	(SYN)	(SYN)	(ADD)	

M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; H = hexane extract; SYN = synergistic interaction; *ADD = additive interaction*; IND = indifferent interaction.

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

	Sample	Mortality ± SD (%)	
		After 24 hrs:	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
	Ciproflaxacin	5.5 ± 2.0	8.3 ± 2.1
Extracts	M	10.6 ± 2.5	35.9 ± 3.3
	W	9.4 ± 2.7	26.2 ± 3.8
	E	5.3 ± 2.4	13.2 ± 2.6
	H	6.2 ± 2.4	12.7 ± 1.8
Combinations	M + Chl	9.8 ± 3.4	33.7 ± 3.4
	M + Gent	10.7 ± 2.5	30.8 ± 3.7
	M + Cip	14.2 ± 3.6	38.5 ± 2.0
	W + Chl	7.3 ± 2.6	15.5 ± 3.1
	W + Gent	5.8 ± 3.8	17.4 ± 3.1
	W + Cip	10.7 ± 3.5	35.4 ± 3.8
	E + Chl	8.4 ± 2.9	22.6 ± 3.5
	E + Gent	12.7 ± 3.3	33.0 ± 3.9
	E + Cip	16.3 ± 3.4	38.6 ± 4.0
	H + Chl	6.4 ± 2.7	14.6 ± 3.3
	H + Gent	8.7 ± 3.1	19.2 ± 3.9
	H + Cip	7.6 ± 2.4	17.5 ± 3.1
Controls	Deionised water	2.7 ± 1.7	3.6 ± 2.5
	Potassium dichromate	100.00 ± 0.00[a]	

Potassium dichromate was tested at a concentration of 1000µg/mL; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; H = hexane extract; Chl = chloramphenicol; Gent = gentamicin; Cip = ciprofloxacin; SD = standard deviation. Results represent means ± SD of 3 independent experiments, each preformed in triplicate (n = 9).

2b and 2c). Those combinations were synergistic across a wider range of ratios, even at very low levels of the extract. Indeed, for the combination containing the methanolic extract, all ratios except the 10% extract ratio produced synergy against *P. mirabilis* growth. A similar trend was noted for the aqueous extract-ciprofloxacin combination, although a minimum of 30% of the extract was required in the combination to induce synergy. The ability to counteract the effects of the bacterial resistance mechanism, even when only low extract % is present, indicates that these extracts contain components that may function

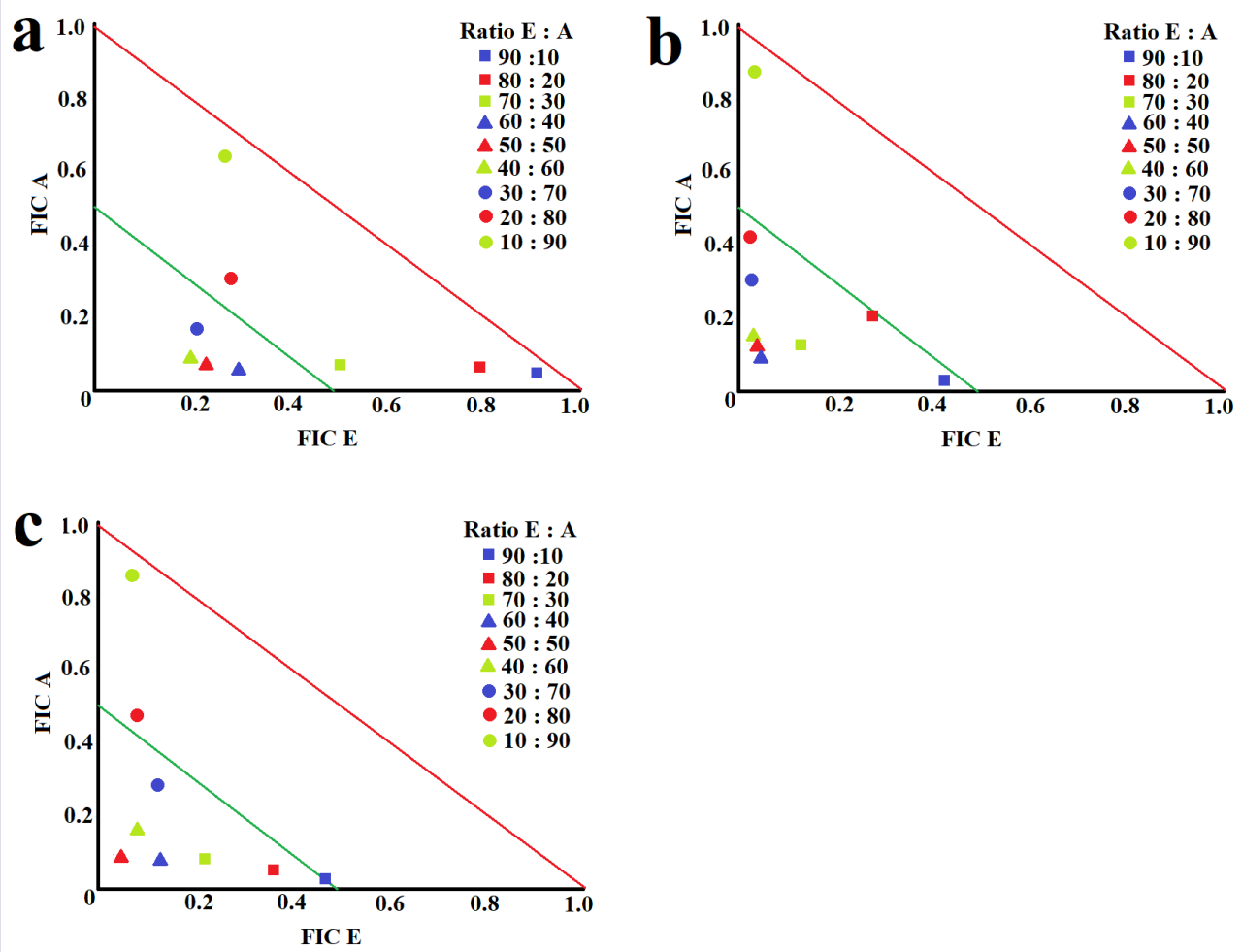

Figure 2: Isobolograms of extract and antibiotic combinations with *E. parviflorum* extracts: (a) aqueous extract and chloramphenicol, (b) methanolic extract and ciprofloxacin and (c) aqueous extract and ciprofloxacin, tested at various ratios against the *P. mirabilis*. Results represent mean FIC values of four replicates. E = extract; A = antibiotic; ratio = % extract-antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic (Σ FIC ≤ 0.5). Any points between the 0.5:0.5 and 1.0:1.0 lines are deemed additive (Σ FIC > 0.5-1.0).

via irreversible mechanisms, and the levels of potentiating compounds is higher in the methanolic extract compared to the aqueous extract. A similar trend has been reported for the combination of amoxycillin and clavulanic acid in the clinical antibiotic therapy Augmentin®.[35] Indeed, the profile of the synergistic ratios of the Augmentin® components is the basis of the 15% clavulanic acid: 85% amoxicillin composition of that chemotherapeutic. As all combinations of the methanolic extract and ciprofloxacin >10% extract (or >20% for the aqueous extract combination) produced synergy, both of these combination ratios would be beneficial to enhance *P. mirabilis* growth inhibition. However, bacteria would be less likely to develop resistance when combinations are used in ratios that minimise the amount of conventional antibiotic used. Thus, for long term prophylactic treatment (as would be required to prevent and treat rheumatoid arthritis), the ideal extract-antibiotic ratio may be 90:10 for the ciprofloxacin-methanolic extract combination, and 80:20 for the ciprofloxacin-aqueous extract combination respectively. However, when used for the treatment of acute infections (e.g. urinary tract infections), the ratio that maximises the efficacy of the treatment (i.e. the 20:80 or 30:70 ratio for the methanolic and aqueous extract containing combinations respectively) may be the preferred option.

Toxicity evaluation

All plant extracts and antibiotics were individually screened at 1000µg/mL in the assay. The extracts were only considered toxic if they induced percentage mortalities greater than 50% (LC_{50}) following 24 hr of exposure to the *Artemia* nauplii.[35] When tested individually, the antimicrobials demonstrated no toxicity in the ALA (Table 4). Similarly, none of the *E. parviflorum* extracts produced mortality 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 non-toxic. In contrast, the positive control potassium dichromate induced 100% mortality in the ALA.

DISCUSSION

Autoimmune inflammatory disorders are a group of debilitating conditions including rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever that afflict genetically susceptible individuals. There is no current cures for these disorders. Instead, current treatment strategies aim to alleviate the symptoms (particularly pain, swelling and inflammation) with analgesics and anti-inflammatory agents and/or to modify the disease process through the use of disease modifying drugs. None of these treatments is ideal as prolonged usage of these drugs is often accompanied by unwanted side effects and toxicity.[15, 16] There is a need to develop safer, more effective treatments for these conditions which will not only alleviate the symptoms, but may also cure or prevent the disease. These autoimmune disorders may be triggered in susceptible individuals by specific microbial infections. Serotyping studies have identified several of the bacterial triggers of these conditions and the bacterial antigens responsible for the induction of an immune response.[15,16] The major microbial trigger of rheumatoid arthritis has been identified as *Proteus mirabilis*, a normal part of the human gastrointestinal flora. Similarly, *Klebsiella pneumoniae* has been shown to initiate ankylosing spondylitis and *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis.[15,16] The development of antibiotic agents targeting specific bacterial triggers of autoimmune inflammatory disorders would enable afflicted individuals to target these microbes and thus prevent the onset of the disease and reduce the severity of the symptoms once the disease has progressed.

This study investigated the ability of *E. parviflorum* extracts to inhibit the growth of some bacterial triggers of auto-immune inflammatory diseases, both alone and in combination with conventional antibiotics. *Epilobium parviflorum* was selected for this study as it is traditionally used to treat inflammation, as well as several illness caused by bacterial pathogens.[9-14] Furthermore, previous studies have reported antibacterial properties for *E. parviflorum* extracts against multiple bacteria.[12-14] However, to the best of our knowledge, none of these previous studies has tested *E. parviflorum* 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*, although, all extracts were completely ineffective against all of the other bacterial strains tested. Thus, the *E. parviflorum* extracts tested in our study only have clinically relevant potency against *P. mirabilis* if used alone. The methanolic extract had the strongest inhibitory activity, with an MIC of 484µg/mL, although the ethyl acetate also had noteworthy activity (MIC=623µg/mL). This indicates that these extracts may be useful in preventing and treating rheumatoid arthritis and other infections of this bacterium when used by itself. However, the combinational studies combining the *E. parviflorum* extracts with conventional antibiotics were of greater interest. Several combinations displayed substantially enhanced potential as therapeutic agents against *P. mirabilis* than either the extracts or antibiotics alone. Indeed, three synergistic combinations were noted, with two of these containing ciprofloxacin (in combination with either the methanolic or aqueous extracts). The implications of these synergistic interactions include enhanced efficacy, the requirement for lower dose administration and a reduction in side effects, as well as possibly reduced antimicrobial resistance.[5] Indeed, the use of synergistic combinations may ultimately enhance or repurpose ineffective drugs with greater efficacy.

Bacteria have developed numerous resistance mechanisms to block or inhibit the effects of antibiotics. A common method is through the use of multi-drug resistant (MDR) efflux pumps which are encoded chromosomally and utilized to rapidly remove antibiotics that have entered the bacterial cells, thus rendering them resistant to the effects of the antibiotic.[36,37] A single pump may allow the bacteria to escape several types of antibiotics. 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 useful tools in combination with some previously ineffective/resistance prone antibiotic compounds.[37] Isoflavones isolated from *Lupinus argenteus* Pursh potentiate the activity of the natural plant antibiotic berberine as well as the synthetic fluoroquinoline antibiotic, norfloxacin as inhibitors of *S. aureus* growth.[37] That study reported that the isoflavone allows a greater concentration of berberine to accumulate in the bacteria by inhibiting the efflux mechanism (MDR pump). Similarly, *Mezoneuron benthamianum* (Roxb.) Benth. and *Securinega virosa* Leandri extracts act as efflux pump inhibitors for fluoroquinolone, tetracycline and erythromycin in resistant strains of *S. aureus* (MRSA).[36] 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 approximately 4.

Notably, all of the synergistic combinations noted in our study contained either chloramphenicol or ciprofloxacin as the antibiotic component. Both of these antibiotics are susceptible to resistance due to efflux pumps[5] and therefore it is possible that the *E. parviflorum* extracts may contain one or more efflux pump inhibitors (EFIs). 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.[5] It is therefore imperative to identify agents that can block the efflux mechanism or alter

the process of efflux to extend the life of multiple antibacterial drugs. Plants produce various secondary metabolites that are used as defense compounds against pathogenic invaders. Some plants produce antimicrobials which, along with other compounds, inhibit the efflux of those antimicrobials from a bacterial cell. *Berberis* spp. are known for their production of the antimicrobial alkaloid berberine.[38] However, they also produce an inhibitor of a *Staphylococcus aureus* efflux pump, identified as 5-methoxyhydnocarpin (5-MHC). 5-MHC induces a significantly decreased MIC for berberine against *S. aureus*, thereby greatly potentiating its efficacy. Recently, many other natural plant based phytoconstituents have been identified as potential efflux pump inhibitors.[38] Similarly, methanolic extracts of *Punica granatum* L. have synergistic activity with chloramphenicol, gentamicin, ampicillin, tetracycline and oxacillin against strains of MRSA and MSSA.[39] The same extracts also either inhibit the MDR efflux pump NorA or enhance the influx of the antibiotics. Therefore, methanolic *P. granatum* extracts may dramatically enhance the activity of various different antibiotics including chloramphenicol, potentially, extending the lifespan of the antibiotic.[5] Furthermore, baicalein extracted from the leaves of *Scutellaria baicalensis* Georgi also has the potential to inhibit NorA efflux pump and thus potentiate the activity of gentamicin.[40] Carnosic acid from *Rosmarinus officinalis* L. has been shown to successfully potentiate the activity of erythromycin by the inhibition of MDR pumps.[2] Similar MDR efflux pump inhibitors may also be present in the *E. parviflorum* extracts. There are currently no EPI/antimicrobial drug combinations on the market, although research into identifying potential EPIs is ongoing.[5,38]

Interestingly, both of the synergistic interactions containing ciprofloxacin (in combination with the methanolic or aqueous extracts) occurred with ratios as low as 20% extract to 80% antibiotic, emphasising the efficacy of these extracts. Such a trend is consistent with irreversible mechanisms such as that of clavulanic acid/β-lactam antibiotic combinations[5,41] and future studies will aim at testing the synergistic mechanism of these combinations. In contrast, other synergistic extract-antibiotic combinations (chloramphenicol and aqueous extract) produced a wider range of interactions at different ratios, including synergistic and additive effects. This is more consistent with reversible competition between the extract component(s) and the conventional antibiotic for binding to an effector.[5] Of the combinations which were not synergistic, 33% were additive and would therefore provide additional benefit compared to using either therapy alone. A further 33% of the antibacterial combinations produced indifferent effects. Although these combinations did not provide any significant benefit by enhancing the efficacy of the antibiotics, they also did not counter-indicate with the antibiotic. Thus, co-administration of the extracts with the conventional antibiotics in these combinations will not lessen the efficacy of the conventional therapies. This information is important as many individuals self-medicate with herbal and traditional medicines and it is therefore important to understand how these medicines interact. Notably, none of the combinations were antagonistic to the action of the conventional antibiotics.

None of the *E. parviflorum* extracts or conventional antibiotics demonstrated toxicity in the ALA assay when tested independently. Similarly, all combinations were nontoxic, 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 *E. parviflorum* extract may also be unsurprising as they have long been used in several traditional medicine systems to treat inflammation and rheumatic conditions.[9-14] However, to the best of our knowledge, there is a lack of rigorous toxicity studies for *E. parviflorum* extracts. The lack of toxicity of the combinations in our study also indicates their potential for therapeutic usage. However, further *in vitro* studies using human cell lines are required to verify their safety. Furthermore, *in vivo* testing is also required to confirm that the extracts and combinations retain efficacy and remain nontoxic in complex biological systems.

CONCLUSION

Whilst the findings reported here indicate the potential of *E. parviflorum* extracts (particularly in combination with ciprofloxacin or chloramphenicol) as preventative and therapeutic options against *P. mirabilis*, 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 *E. parviflorum* extracts.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ALA: brine-shrimp lethality assay; **DMSO:** dimethyl sulfoxide; **FIC:** fractional inhibitory concentration; **INT:** ρ-iodonitrotetrazolium chloride; **LC_{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.

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