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Original Article

Antibacterial Activity and Toxicity Profiles of *Eclipta prostrata* (L.) L. Extracts and Conventional Antibiotics against Bacterial Triggers of Some Autoimmune Diseases

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ABSTRACT

Background: *Eclipta prostrata* (L.) L. has been used traditionally to treat numerous diseases, including many caused by bacterial pathogens. However, *E. prostrata* 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. prostrata* extracts and conventional antibiotics were studied and classified using the sum of the fractional inhibitory concentration (∑FIC). The toxicity of the individual samples and the combinations was assessed using the *Artemia* lethality assay (ALA). **Results:** *Eclipta prostrata* leaf extracts displayed notable antibacterial activity against the bacterial triggers of rheumatoid arthritis (*P. mirabilis* and *P. vulgaris*), ankylosing spondylitis (*K. pneumoniae*), and multiple sclerosis (*A. baylyi* and *P. aeruginosa*). The aqueous extract was a particularly good inhibitor of *Proteus* spp. growth. (MICs = 165µg/mL), whilst lower potency was noted against other bacterial pathogens. Furthermore, combining the extracts with conventional antibiotics resulted in potentiation of the inhibitory activity for some combinations, particularly those containing chloramphenicol as the antibiotic component. None of the individual components (nor the combinations) were toxic in the ALA assay. **Conclusion:** The *E. prostrata* methanolic, aqueous and ethyl acetate extracts displayed clinically relevant antibacterial activity against *P. mirabilis* and *P. vulgaris,* and lower potency against *K. pneumonia, A. baylyi* and *P. aeruginosa* when tested alone. The lack of toxicity of the extract and combinations indicates that *E. prostrata* extract and antibiotic combinations may provide leads in the development of new therapies to prevent and treat the autoimmune diseases rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis.

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

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INTRODUCTION

Autoimmune inflammatory disorders are a group of debilitating conditions including rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis that afflict genetically susceptible individuals.[1,2] There are no 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 progression through the use of disease modifying drugs. None of these treatments is ideal and prolonged usage of these drugs is often accompanied by unwanted side effects and toxicity.[1,2] There is a need to develop safer, more effective treatments for these conditions that 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. The major microbial trigger of rheumatoid arthritis has been identified as *Proteus* spp. (especially *Proteus mirabilis*), which are 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.[1,2] 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

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of the disease and reduce the severity of the symptoms once the disease has progressed.

Whilst antibiotics are available to treat infections of these bacteria, the development of multiple antibiotic resistant bacterial strains has rendered multiple clinical antibiotics of decreased efficacy, or in some cases, has rendered the antibiotics completely effective.[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,[3] it is unlikely that the current methods of antibiotic discovery/development will be as successful in the future. Instead, examination of traditional medicines with therapeutic properties may generate new drug leads for the development of new antibiotics. 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 plant phytochemicals often have much lower activity than the crude extract that they are derived from.[5] 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.[6-8] 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.[3,4] A combination of plant extracts/isolated compounds with conventional antibiotics may also prove to have an economic advantage.[5] 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. Further advantages of synergistic combinations include increased efficiency, reduced side effects, increased stability and bioavailability, and the requirement for lower doses in comparison to synthetic alternatives.[5]

Eclipta prostrata (L.) L. (commonly known as false daisy, bringaraja, yerba de tago, Gunta kalagaraku) is a small herbaceous plant of the family Asteraceae. It is native to warm temperate to tropical regions worldwide. Preparations prepared from the dried whole plant are used in the traditional medicine system Ayuverda to treat skin, respiratory, coronary and haemorragic diseases.[9] The same study also states that *E. prostrata* extracts are also used to retard hair loss, treat vitiligo and to enhance renal and kidney function. Based on its traditional uses, several studies have examined the pharmacological effects of this species, and have reported that it has anti-osteoporotic,[10,11] hepatoprotective,[12] anti-inflammatory and analgesic effects,[13,14] as well as anti-diabetic,[15,16] hypolipidemia[17,18] anti-tumour[19-22] and neuroprotective activities.[23] Several of these diseases are due to bacterial infections. Notably, several studies have also reported noteworthy antibacterial activity for multiple bacterial pathogens including *Bacillus subtilis* and *Pseudomonas aeruginosa*.[24] Additionally, alkaloids isolated from *E. prostrata* leaves inhibit the growth of *Escherichia coli, Shigella botdii, Staphylococcus aureus* and *Streptococcus faecalis*.[25] However, *E. prostrata* extracts are yet to be tested against the bacterial triggers of rheumatoid arthritis (*Proteus mirabilis*) and ankylosing spondylitis (*Klebsiella pneumoniae*). Furthermore, whilst *E. prostrata* extracts have been tested against one of the bacterial triggers of multiple sclerosis (*Pseudomonas aeruginosa*),[25] they are yet to be tested against another bacterial trigger of this disease (*Acinetobacter baylyi*). This study investigates the antimicrobial effects of *E. prostrata* leaf 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

Dried *Eclipta prostrata* (L.) L. leaves were purchased from Noodles Emporium, Australia. Voucher specimens are stored in the School of Environment and Science, Griffith University, Australia (voucher number EP-Bang)- A1-2017-1). Individual quantities (1g) of the plant material were weighed into separate tubes and 50mL 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 leaves 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 to determine the extraction yield and were dissolved in 10mL of deionised water (containing 1% DMSO).

Qualitative phytochemical analysis

Phytochemical analysis of the *E. prostrata* leaf extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved using standard assays.[26,27]

Antibacterial analysis

Conventional antibiotics

Penicillin-G (potency of 1440-1680µg/mg), chloramphenicol (≥98% purity by HPLC), erythromycin (potency ≥850µg/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 (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.[28-30] 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. 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 24 hr 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. prostrata* leaf extracts and the conventional antibiotics was initially assessed using a modified disc diffusion assay.[31,32] 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.[33] Furthermore, as microplate liquid dilution MIC assays are one of 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, and to approximate bacterial colonisation of solid surfaces.

Microplate liquid dilution MIC assay

A standard liquid dilution MIC assay[34-36] 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. 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.[37,38] 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:[33-35]

$FIC^{(i)}$ = MIC (a) in combination with (b)

MIC (a) independently

$FIC^{(ii)}$ = MIC (b) in combination with (a)

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).[33-35]

Artemia franciscana lethality assay (ALA)

Toxicity of the *E. prostrata* extracts, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.[39,40] 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 ± SEM of at least three independent experiments, each with internal triplicates (n=9). 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.

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *E. prostrata* 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	101	10.1	+++	+++	++	-	-	+	-	-	-	++	+	++	-	-
Water	68	6.8	+++	+++	+	-	-	+	-	-	-	+++	++	++	-	-
Ethyl Acetate	7	0.7	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Chloroform	39	3.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	12	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extractions of the dried *E. prostrata* leaf material (1g) with solvents of varying polarity yielded dried plant extracts ranging from 7mg (ethyl acetate extract) to 101mg (methanolic 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

Proteus mirabilis growth was susceptible to the methanolic, aqueous, ethyl acetate and chloroform *E. prostrata* extracts (ZOIs of 8.2, 7.2, 7.6 and 8 mm respectively), but was unaffected by the hexane extract (Figure 1a). Indeed, these extracts produced similar or better zones of inhibition (ZOIs) compared to the ampicillin control (7.4 mm), although the chloramphenicol control was a substantially stronger inhibitor of *P. mirabilis* growth (13.8 mm). In contrast, the hexane extract was completely devoid of *P. mirabilis* growth inhibitory activity. Similar trends and susceptibilities were noted for the inhibition of *P. vulgaris* growth by the *E. prostrata* extracts, with inhibition recorded for the methanolic, aqueous, ethyl acetate and chloroform extracts (8.6, 6.8, 8.2 and 8.8 mm respectively), whilst the hexane extract devoid of inhibitory activity (Figure 1b).

The methanolic, aqueous and ethyl acetate extracts also inhibited the growth of *K. pneumonia*, albeit with smaller zones of inhibition, indicating lower inhibitory activity (ZOIs of 7.2, 6.4, 6.7 mm; Figure c). In contrast, the lower polarity chloroform and hexane extracts were devoid of bacterial growth inhibitory activity. Thus, it is likely that the antibacterial component(s) in the *E. prostrata* leaf extracts are mid to high polarity. It is likely that the compounds responsible for inhibiting the growth of *A. baylyi* may also be relatively polar as only the methanolic and aqueous extracts inhibited the growth of that bacterium, with ZOIs of 6.8 and 6.3mm respectively (Figure 1d). However, the small ZOIs (<7mm for all extracts) indicates that these extracts have relatively weak activity against that bacterium. In contrast, all of the *E. prostrata* extracts inhibited *P. aeruginosa* growth. Indeed, the largest ZOI was recorded with the hexane extract, indicating that different, lower polarity compounds may contribute to the growth inhibitory activity against *P. aeruginosa*. It is noteworthy that the *A. baylyi* and *P. aeruginosa* strain tested in this study was relatively resistant to the ampicillin control. Indeed, *P. aeruginosa* was completely resistant to this antibiotic, whilst a ZOI of 7.2mm was measured against *A. baylyi*. Similarly, previous studies in our group have reported that this bacterial strain is resistant to several other antibiotics, as well as to other plant extracts with reported antibacterial activity.[41-43]

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

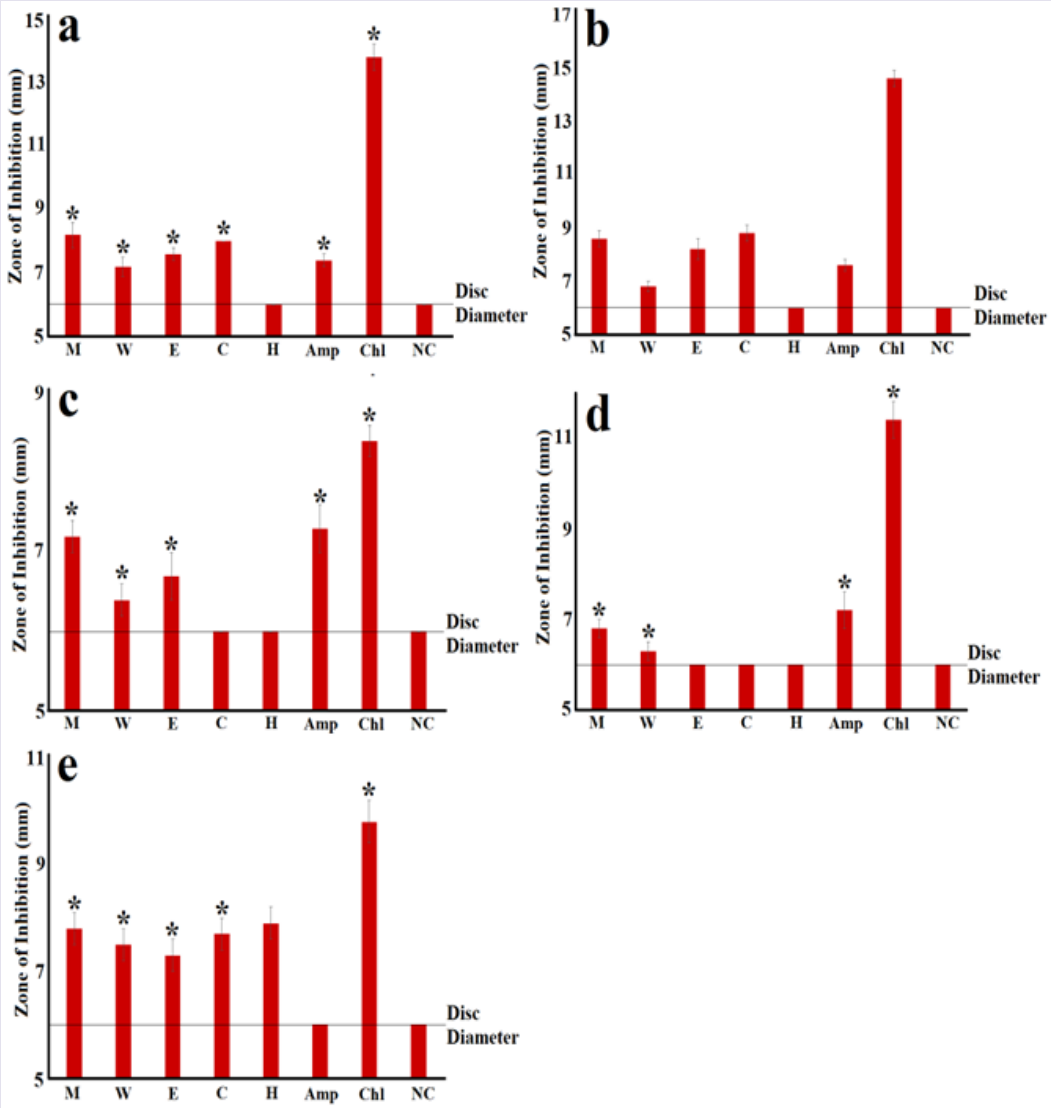

Figure 1: Antibacterial activity of *E. prostrata* leaf extracts against (a) *P. mirabilis* (ATCC21721); (b) *P. vulgaris* (ATCC21719); (c) *K. pneumoniae* (ATCC31488); (d) *A. baylyi* (ATCC33304); and (e) *P. aeruginosa* (ATCC: 39324), measured as zones of inhibition (mm). M = methanolic extract; W = 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 ± SEM; * indicates results that are significantly different to the negative control (p<0.01).

2). Consistent with the antibacterial disc screening assays, all bacterial strains tested were susceptible to the *E. prostrata* extracts, although only weak inhibitory activity was recorded against *P. aeruginosa* and *A. baylyi*. However, as previously noted, those bacterial strains were particularly resistant to the antibiotic controls. Indeed, *P. aeruginosa* growth was completely unaffected by penicillin-G, erythromycin and chloramphenicol in the liquid dilution assay. The growth of this bacterium was only inhibited by tetracycline, with an MIC of 2.5µg/mL. However, this MIC value indicates that this bacterium is also resistant to tetracycline as MIC values >1µg/mL in this assay are indicative of resistance.[33,34] Similarly, the *E. prostrata* extracts and the antibiotic controls were relatively weak inhibitors of *A. baylyi* growth. Indeed, penicillin-G and tetracycline were completely ineffective against this bacterium. Furthermore, whilst erythromycin inhibited *A. baylyi* growth, MIC values substantially >1µg/mL were measured (MIC values >1µg/mL is considered resistant for pure antibiotics in this assay).[33,34] As both *A. baylyi* and *P. aeruginosa* can trigger multiple sclerosis in genetically susceptible people,[1,2] it is unlikely that these extracts would be strong bacterial growth inhibitors of the bacterial triggers of multiple sclerosis. Therefore, they may be of limited use for the prevention and treatment of that disease.

Table 2: Disc diffusion (DD) and liquid dilution (LD) MIC values (µg/mL) for *E. prostrata* leaf extracts against microbial triggers of some autoimmune inflammatory diseases.

Extract	*P. mirabilis*		*P. vulgaris*		*K. pneumoniae*		*A. baylyi*		*P. aeruginosa*	
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC
M	630	363	>5000	630	2523	1460	>5000	2523	2423	1080
W	220	165	480	165	1650	886	>5000	1250	2500	1256
E	1307	245	360	245	1340	1027	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-
H	>5000	-	-	-	-	-	-	-	-	-
Controls										
Penicillin-G	ND	-	ND	-	ND	-	ND	-	ND	-
Erythromycin	ND	-	ND	-	ND	-	ND	3.3	ND	-
Tetracycline	ND	-	ND	3.3	ND	-	ND	-	ND	2.5
Chloramphenicol	ND	3.3	ND	1.67	ND	1.67	ND	0.83	ND	-

M = methanol extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane; DD = disc diffusion; LD = liquid dilution; - indicates no inhibition at any dose tested.

The *Proteus* spp. were substantially more susceptible to the *E. prostrata* extracts compared to *A. baylyi* and *P. aeruginiosa*. The potency of aqueous extract was particularly noteworthy, with MIC values of 165µg/mL against both *Proteus* spp. tested. The methanolic and ethyl acetate extracts also had noteworthy activity against the *Proteus* spp., with MIC values generally substantially <650µg/mL. In contrast, the chloroform and hexane extracts were completely devoid of activity. As *Proteus* spp. can trigger rheumatoid arthritis in genetically susceptible people,[1,2] the *E. prostrata* leaf extracts (particularly the ethyl acetate extract) may be useful for the prevention of this disease, as well as other diseases caused by these bacteria.

Substantially lower growth inhibitory activity was noted when the *prostrata* leaf extracts were tested against *K. pneumoniae*. Whilst the aqueous extract had noteworthy activity (MIC = 886µg/mL), higher MIC values indicative of moderate activity were measured for the methanolic and ethyl acetate extracts (MIC values of 1460 and 1027µg/mL respectively). Therefore, as this bacterium can trigger ankylosing spondylitis in genetically susceptible people,[1,2] the aqueous *E. prostrata* leaf extract (and to lesser extent, the methanolic and ethyl acetate extracts) may be useful in the prevention and treatment of this disease, as well as other diseases caused by this bacterium.

Fractional inhibitory concentration (FIC) assessment

None of the combinations of the *E. prostrata* extracts and conventional antibiotics produced synergistic effects when tested together against any of the bacteria tested (Table 3). However, three combinations had additive effects against *P. mirabilis*. Whilst these combinations would not be as effective as synergistic combinations, they are still an improvement on using either the antibiotic or the extract alone. It would therefore be beneficial to use these combinations in the prevention and treatment of rheumatoid arthritis. Interestingly, all of the additive combinations contained chloramphenicol as the antibiotic component. 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.

Toxicity evaluation

All plant extracts and antibiotics were individually screened at 1000µg/mL in the ALA assay (Table 4). 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.[39,40] When tested individually, the antimicrobials demonstrated no toxicity in the ALA. Similarly, none of the *E. prostrata* extracts produced mortality above 50% following 24hr exposure. Additionally, when the extract-antibiotic combinations were tested in the ALA, none of them produced mortality >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

This study investigated the ability of *E. prostrata* extracts to inhibit the growth of some bacterial triggers of auto-immune inflammatory diseases, both alone and in combination with conventional antibiotics. *Eclipta prostrata* was selected for this study as it is traditionally used to treat multiple illnesses, including several diseases caused by bacterial pathogens.[1,2] Furthermore, previous studies have reported antibacterial properties for *E.*

Table 3: ΣFIC values for the *E. prostrata* leaf extract and conventional antibiotic combinations against susceptible bacteria.

Bacteria	Extract	Penicillin-G	Chloramphenicol	Erythromycin	Tetracycline
P. mirabilis	M	-	0.6 (ADD)	-	-
	W	-	0.83 (ADD)	-	-
	E	-	0.77 (ADD)	-	-
	C	-	-	-	-
	H	-	-	-	-
P. vulgaris	M	-	1.74 (IND)	-	2.73 (IND)
	W	-	1.25 (IND)	-	1.12 (IND)
	E	-	1.56 (IND)	-	1.85 (IND)
	C	-	-	-	-
	H	-	-	-	-
K. pneumoniae	M	-	3.25 (IND)	-	-
	W	-	1.66 (IND)	-	-
	E	-	2.78 (IND)	-	-
	C	-	-	-	-
	H	-	-	-	-
A. baylyi	M	-	2.55 (IND)	1.46 (IND)	-
	W	-	1.73 (IND)	1.12 (IND)	-
	E	-	-	-	-
	C	-	-	-	-
	H	-	-	-	-
P. aeruginosa	M	-	-	-	1.2 (IND)
	W	-	-	-	1.37 (IND)
	E	-	-	-	-
	C	-	-	-	-
	H	-	-	-	-

M = methanolic extract; W = aqueous extract;E = ethyl acetate extract; C = chloroform extract; H = hexane extract; ADD = additive interaction; IND = indifferent interaction; - = an ΣFIC could not be determined as at least one component of the combination was inactive

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

	Sample	Mortality ± SEM (%)	
		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
Extracts	M	12.4 ± 2.6	33.2 ± 2.8
	W	10.3 ± 2.5	27.5 ± 3.5
	E	9.7 ± 2.7	23.4 ± 2.8
	C	10.3 ± 2.8	22.7 ± 2.9
	H	11.6 ± 3.1	23.2 ± 3.2
Combinations	M + Pen	24.2 ± 3.1	52.6 ± 3.7
	W + Pen	18.3 ± 2.9	32.7 ± 2.9
	E + Pen	15.9 ± 2.8	41.2 ± 3
	C + Pen	17.3 ± 2.8	44.6 ± 3.8
	H + Pen	14.8 ± 2.8	37.9 ± 3.4
	M + Chl	22.4 ± 3.8	57.2 ± 4.2
	W + Chl	19.4 ± 2.4	42.6 ± 3.3
	E + Chl	17.8 ± 3.2	41.4 ± 3.7
	C + Chl	20.5 ± 3	39.7 ± 2.6
	H + Chl	14.5 ± 3.3	37.4 ± 3.6
	M + Eryth	12.6 ± 3.3	35.1 ± 3
	W + Eryth	10.2 ± 3.5	30.2 ± 2.4
	E + Eryth	15.7 ± 3.7	29.4 ± 2.8
	C + Eryth	12.4 ± 3.5	31.7 ± 3.2
	H + Eryth	10.6 ± 2.4	22.5 ± 3.5
	M + Tet	12.6 ± 3.3	27.8 ± 3.4
	W + Tet	8.5 ± 3	18 ± 2.8
	E + Tet	10.8 ± 2.5	23.1 ± 3.7
	C + Tet	11.2 ± 3.5	24.8 ± 2.9
	H + Tet	8.6 ± 2.9	19.4 ± 3.6
Controls	Deionised water	2.7 ± 1.7	3.6 ± 2.5
	Potassium dichromate	100.00 ± 0.00	

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; Eryth = erythromycin; Tet = tetracycline; SEM = standard error of the mean. Results represent means ± SEM of 3independent experiments, each preformed in triplicate (n = 9).

prostrata extracts against multiple bacteria.[24,25] However, the previous studies did not test *E. prostrata* 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, P. vulgaris, K. pneumonia, A. baylyi* and *P. aeruginosa*, with clinically relevant potency. The aqueous extract had the strongest inhibitory activity against all bacteria, indicating that it may be particularly useful in preventing and treating rheumatoid arthritis, and (to a lesser extent) ankylosing spondylitis and multiple sclerosis, as well as other infections caused by these bacteria, when used by alone.

The combinational studies combining the *E. prostrata* extracts with conventional antibiotics also yielded interesting results. Several combinations displayed enhanced potential as therapeutic agents against *P. mirabilis* compared with the inhibitory activity

of either the extract or antibiotic componentss alone. Indeed, three additive combinations were noted, with all of these containing chloramphenicol (in combination with either the methanolic, aqueous or ethyl acetate extracts). The implications of this potentiation include enhanced efficacy, the requirement for lower dose administration and a reduction in side effects, as well as possibly reduced antimicrobial resistance.[3] Importantly, none of the combinations produced antagonistic effects. This is an important finding as it indicates that it is safe to use the *E. prostrata* extracts and conventional antibiotics in combination without decreasing the efficacy of either component.

None of the *E. prostrata* extracts or conventional antibiotics demonstrated toxicity in the ALA assay when tested independently. Similarly, all combinations were non-toxic, 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. prostrata* extract may also be unsurprising as they have long been used in several traditional medicine systems to treat pathogenic diseases.[9-25] The lack of toxicity of the combinations in our study also confirms 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 non-toxic in complex biological systems.

CONCLUSION

Whilst the findings reported herein support the therapeutic properties of the *E. prostrata* extracts as preventative and therapeutic options against rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis, 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) within the *E. prostrata* 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; **ΣFIC:** The sum of the fractional inhibitory concentration.

SUMMARY

- *Eclipta prostrata* extracts were tested for iantibacterial activity against some bacterial triggers of autoimmune diseases.
- The antibacterial activity was quantified by MIC determination.
- The higher polarity extracts displayed noteworthy inhibitory activity on their own.
- The extracts were also tested in combination with conventional antibiotics.
- The extracts potentiated the activity of some antibiotics.
- The extracts were non-toxic (both alone and in combinations with antibiotics) in the *Artemia* lethality assay.

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