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Original Article

Lythrum salicaria L. Extracts Down-Regulate Pro-Inflammatory Cytokine Release and Inhibit the Bacterial Triggers of Some Autoimmune Inflammatory Diseases

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ABSTRACT

Background: *Lythrum salicaria* L. has been used traditionally to treat a variety of conditions including bacterial infections and inflammation. Despite this, only preliminary studies have examined *L. salicaria* extracts for therapeutic properties relevant to these traditional uses. **Materials and Methods:** Dried *L. salicaria* whole plant material was extracted with solvents of various polarities and tested for the ability to modulate the release of IFN-γ, IL-6 and TNF-α from RAW 264.7 cells using a BD™ cytometric bead array assay. The ability of the extracts to inhibit the growth of some bacterial triggers of selected autoimmune inflammatory diseases was also quantified by disc diffusion and liquid dilution MIC assays. The ΣFIC of extract/antibiotic combinations was determined and used to classify the class of interaction. Toxicity was evaluated using *Artemia* nauplii and HDF cytotoxicity assays. **Results:** Methanolic and aqueous *L. salicaria* extracts substantially reduced the release of IFN-γ, IL-6 and TNF-α from RAW 264.7 cells in both PMA/ionomycin and LPS stimulatory models. Additionally, the methanolic, aqueous and ethyl acetate extracts strongly inhibited the growth of several bacterial triggers of autoimmune diseases, with MIC values as low as 240 µg/mL against some bacteria. Furthermore, combining the extracts with conventional antibiotics resulted in significant potentiation of the inhibitory activity for some combinations, with two synergistic and 19 additive combinations detected. Notably, three antagonistic combinations were also noted. None of the individual components (nor the combinations) were toxic in the ALA or MTS assays. **Conclusion:** *Lythrum salicaria* extracts down-regulate the release of IFN-γ, IL-6 and TNF-α from RAW 264.7 cells and inhibit the growth of bacterial triggers of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever. Further *in vivo* testing and studies to determine the anti-inflammatory and antibacterial mechanisms and to identify the bioactive principles in the *L. salicaria* extracts is warranted.

Keywords: Medicinal Plants, Immunomodulation, Pro-Inflammatory Cytokines, Synergy, Rheumatoid Arthritis, Ankylosing Spondylitis, Multiple Sclerosis, Antibacterial Activity.

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INTRODUCTION

Autoimmune inflammatory diseases are a group of more than 80 debilitating conditions that include rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever.[1,2] There are currently no cures available for any of these diseases. Instead, the therapeutic approach is administered anti-inflammatory drugs and analgesics to treat the symptoms. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and analgesics are most frequently used for these purposes. However, whilst this approach decreases the patient's discomfort, it does not significantly affect disease progression and tissue damage may still occur. Additionally, the prolonged used of anti-inflammatory drugs causes toxicity and numerous deleterious side effects.[3] For example, the prolonged usage of NSAIDs that inhibit cyclooxygenase-2 (COX-2) is often associated with increased incidences of myocardial infarction.[4] A multi-faceted approach that not only addresses the inflammatory disease symptoms, but also targets the etiology of these diseases may be a substantially more effective therapy and would also allow for prophylactic usage to prevent the onset of the disease, thereby protecting against tissue damage as well as decreasing the inflammatory symptoms.

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There are no common causes for the autoimmune inflammatory diseases, although all occur when the host's immune system produces self-reactive antibodies in response to exposure to specific antigens. Whilst some of these diseases are triggered by environmental and dietary stimuli, the majority are triggered by bacterial and viral pathogens. Targeting the antigenic triggers of the autoimmune diseases may block the disease etiology, thereby also preventing the downstream effects of the disease. Many of the pathogenic triggers of these diseases have already been identified by serotyping studies, allowing the use chemotherapies targeting the diseases prevention.[1] *Proteus mirabilis* can trigger rheumatoid arthritis in genetically susceptible people,[5] whilst *Klebsiella pneumoniae* may trigger ankylosing spondylitis.[6] Multiple sclerosis can be initiated in genetically susceptible people by *Acinetobacter baylyi* and *Pseudomonas aeruginosa* infections[7] and *Streptococcus pyogenes* may induce the onset of rheumatic fever.[8]

A re-examination of ethnobotany and traditional plant-based medicines is a promising approach for the development of novel chemotherapies to treat autoimmune inflammatory diseases. Plant-derived medicines were used to treat both inflammation and pathogenic disease prior to the development of modern therapeutics.[1,9] Several traditional healing systems (e.g. Ayurveda and Traditional Chinese Medicine (TCM)) are still commonly used, particularly in developing populations. Many plant medicines contain several well characterised compounds with therapeutic properties. The presence of multiple compounds, which target different aspects of complex inflammatory diseases and function via different mechanisms would increase the efficacy of the therapy and would also decrease the induction to later recalcitrance to the therapy, thereby extending its useful lifetime.[10]

Lythrum salicaria L. (family Lythraceae; commonly known as purple loosestrife, blooming Sally, purple willow-weed, rainbow weed) is an herbaceous perennial plant that is native to Europe, Asia, northwestern Africa and southeastern Australia. It grows up to 2 tall by 1.5 m wide and consists of numerous erect red-purple stems growing from a single woody root mass and has red-purple flower clusters. *Lythrum salicaria* was selected for this study due to its long history of traditional uses to treat a wide range of diseases and ailments (as reviewed by.[11] It is particularly well known for its uses to treat haemorrhages and to stem bleeding, as well as its uses to treat diarrhoea, dysentery and other gastrointestinal disorders. It is also used to treat infected wounds and to promote wound healing. Notably, many of these conditions are caused by bacterial pathogens and several studies have verified the antibacterial properties of *L. salicaria* preparations against multiple bacteria including *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*,[12] *Bacillus cereus, Mycobacterium smegmatis* and *Micrococcus luteus*.[13] However, those studies only tested a single extract concentration and MIC values were not determined, making comparisons with other studies difficult.

Additionally, *L. salicaria* preparations have also been used traditionally to treat inflammatory and related conditions, including rheumatism,[11] intestinal inflammation and benign prostatic hyperplasia.[11] Several previous studies have reported anti-inflammatory activity for *L. salicaria* preparations using *in vivo* models.[14] The authors of that study reported noteworthy anti-inflammatory activity for the extract at relatively high doses (200 mg/kg). In contrast to the acetylsalicylic acid control used in that study, the *L. salicaria* extract also did not cause gastric ulceration. Further studies demonstrated that an aqueous *L. salicaria* extract induced decreased cellular release of IL-8 and identified salicarinins and ellagitannins as being responsible for this activity.[15] Our study aimed to extend these earlier studies by evaluating the immune-modulatory properties of *L. salicaria* extracts against three pro-inflammatory cytokines, as well as examining the ability of the extracts to inhibit the growth of some bacterial triggers of selected autoimmune inflammatory diseases. Furthermore, the interactive antimicrobial and toxicity profiles of combinations of *L. salicaria* extracts and conventional antibiotics was examined.

MATERIALS AND METHODS

Sourcing and preparation of plant samples

Lythrum salicaria L. whole plant material was -provided by Greg Jardine of Dr Red Nutraceuticals, Australia and was supplied as dried plant material. Voucher specimens are deposited in the School of Environment and Science, Griffith University, Australia (voucher number PLS_WP-2017a). Prior to use, the plant material was ground into a coarse powder and individual 1 g masses 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 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 extraction yield and solubilised in 10 mL deionised water (containing 1% DMSO).

Qualitative phytochemical analysis

Phytochemical analysis of the *L. salicaria* extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved as previously described.[16,17]

Analysis of immune-modulatory activity

RAW 264.7 cells were obtained from American Type Culture Collection (ATCC; USA) and were cultured on Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10

% heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, MA, USA) containing 100 U/mL penicillin and 100 µg/mL streptromycin (Invitrogen) at 37°C under a 5% CO_2 atmosphere. The immunomodulatory effects of the *L. salicaria* extracts was assessed using unstimulated RAW 264.7 cells, as well as cells stimulated with Phorbol 12-Myristate 13-Acetate (PMA) and ionomycin, or cells stimulated with lipopolysaccharide (LPS) by standard methods.[18] For each stimulatory assay, approximately 100,000 cells in 100 µL media were seeded into the wells of round-bottom 96-well culture plate. Unstimulated PBMCs were treated in triplicate with 10 µg/mL of cyclosporine A (Sigma-Aldrich, Castle Hill, NSW, Australia), 100 µg/mL of each extract, or remained untreated. The RAW cells were also stimulated using a mixture of PMA (Sigma, Australia; 50 ng/mL) and ionomycin (1 µg/mL eBioscience, San Diego, CA, USA). The RAW cells were also concurrently activated with LPS (10 ng/mL) (Sigma-Aldrich, Australia) to evaluate the regulation of myeloid-associated cytokines. The stimulated RAW cells were treated with 100 µg/mL of the plant extracts, or 10 µg/mL of the cyclosporine control, or remained untreated. All incubation plates were placed in an incubator for 24 hr at 37°C under a 5% CO_2 atmosphere. Following the incubation period, the plates were centrifuged and the culture supernatants were collected for cytokine analysis.

BD™ Cytometric Bead Array

Interferon gamma (IFN-γ), Interleukin 6 (IL-6) and tumour necrosis factor (TNF) from the RAW 264.7 cell culture supernatant were quantified using BD™ Mouse Th1/Th2/Th17 Cytometric Bead Array kit (CBA) (BD™ Biosciences, San Jose, CA, USA). The CBA assays were performed according to the manufacturer's instruction and run using a five laser Special Order LSRFortessa™ with HTS (BD, Franklin Lakes, NJ, USA). Cytokine concentrations (pg/mL) were calculated based on the sample MFI compared to the cytokine standard curves. Bar graphs and statistics were produced using GraphPad Prism version 7.02 (GraphPad Software Inc, La Jolla, CA, USA).

Antibacterial 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), ciprofloxacin ((≥98% purity by HPLC) 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 (10 µg) and chloramphenicol discs (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.[1,2] 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 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 *L. salicaria* extracts and the conventional antibiotics was initially assessed using a modified disc diffusion assay.[19] Ampicillin (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.[20] 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[20] 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 plate. The plant samples and conventional antibiotics (100 µL) 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 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.[19] 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.[21,22]

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

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

The $^{\Sigma}$FIC was then calculated using the equation: $^{\Sigma}FIC=FIC^{(i)}+FIC^{(ii)}$. The interactions were classified as being synergistic for $^{\Sigma}$FIC values of ≤ 0.5, additive (>0.5-1.0), indifferent (>1.0-≤4.0) or antagonistic (>4.0).[21,22] Tentative interpretations were included where the MIC value was greater than the highest concentration tested to provide an estimation of what the possible interactive profile for the combination could have been. These interpretations were not given a $^{\Sigma}$FIC value, as only absolute values could be used in $^{\Sigma}$FIC calculations and not 'greater than' values.

Toxicity screening

The toxicity of the *L. salicaria* extracts was assessed using 2 assays. *Artemia* nauplii lethality assays (ALA) were used as a rapid preliminary toxicity screen, whilst an MTS cellular proliferation assay was used for a determination of cytotoxicity.

Brine-shrimp lethality assay

The toxiciy of the *L. salicaria* extracts, conventional antibiotics and the reference toxin were assessed using a modified *Artemia franciscana* nauplii lethality assay (ALA).[23] 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.

Cellular viability assay

The extracts were also screened for toxicity towards normal human primary dermal fibroblasts (HDF; ATCC PCS-201-012) using standard assays.[24] Briefly, all extracts were screened following 24 hr exposure to the extracts at 200 µg/mL, with incubation at 37°C and 5 % CO_2 in a humidified atmosphere. Following the initial incubation, 20 µL of Cell Titre 96 Aqueous One solution (Promega) was added to each well and the plates were incubated for a further 3 hr. Absorbances were recorded at a test wavelength of 540 nm and a blank wavelength of 690 nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed three time, each with internal triplicates (n=9). Triplicate controls were included on each plate. The % cellular viability of each test was calculated using the following formula:

$$\%\ \text{cellular viability} = \frac{\text{Abs test sample} - (\text{mean Abs control} - \text{mean Abs blank})}{(\text{mean Abs control} - \text{mean Abs blank})}$$

Cellular viability ≤50% of the untreated control indicated toxicity, whereas extracts or controls with >50% untreated control viability were deemed to be nontoxic.

Statistical analysis

Data is expressed as the mean±SEM of at least three independent experiments. One-way ANOVA was used to calculate statistical significance between the negative control and treated groups with a p<0.01 considered to be statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extractions of the *L. salicaria* extracts (1 g) with solvents of varying polarity yielded dried plant extracts ranging from 92 mg (*L. salicaria* hexane extract) to 184 mg (*L. salicaria* 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.

Analysis of immunomodulatory activity

The immunomodulatory effects of the *L. salicaria* extracts were assessed using unstimulated cells and 2 different stimulatory conditions. Firstly, the cytokine levels of unstimulated RAW 264.7 cells were tested and used as a negative control to determine basal levels of cytokine release (Figure 1). CsA was included in the assay as a positive control for immunosuppression as it blocks immune cell activation cytokine release pathways.[18] Notably,

none of the extracts or the CsA control elicited any cytokine release above that of the untreated control.

The immunomodulatory activity of the extracts against RAW 264.7 cells was evaluated following stimulation with PMA/ionomycin (Figure 2). Notably, the methanolic and aqueous *L. salicaria* extracts significantly suppressed the release of the proinflammatory IFN-γ, IL-6 and TNF-α from the RAW 264.7 cells. The CsA control also suppressed the release of all of the proinflammatory cytokines, although the suppression of IL-6 was not statistically significant. In contrast, the ethyl acetate, chloroform and hexane extracts were ineffective inhibitors of IFN-γ, IL-6 and TNF-α, with levels similar to the untreated control recorded for those extracts. The general immunosuppressive potency of the extracts and CsA control against PMA/ionomycin-stimulated RAW 264.7 cells was CsA>PLM>PLW.

Additionally, the suppression of selected pro-inflammatory cytokines was determined for RAW 264.7 cells stimulated with LPS (Figure 3). Interestingly, a different immunomodulatory pattern was observed compared to the PMA/ionomycin stimulated cells, indicating that the extracts contain a combination of immunomodulatory compounds that regulate different immunological and inflammatory pathways. In contrast to the PMA/ionomycin stimulated cells, the CsA control and the *L. salicaria* extracts did not significantly affect the release of

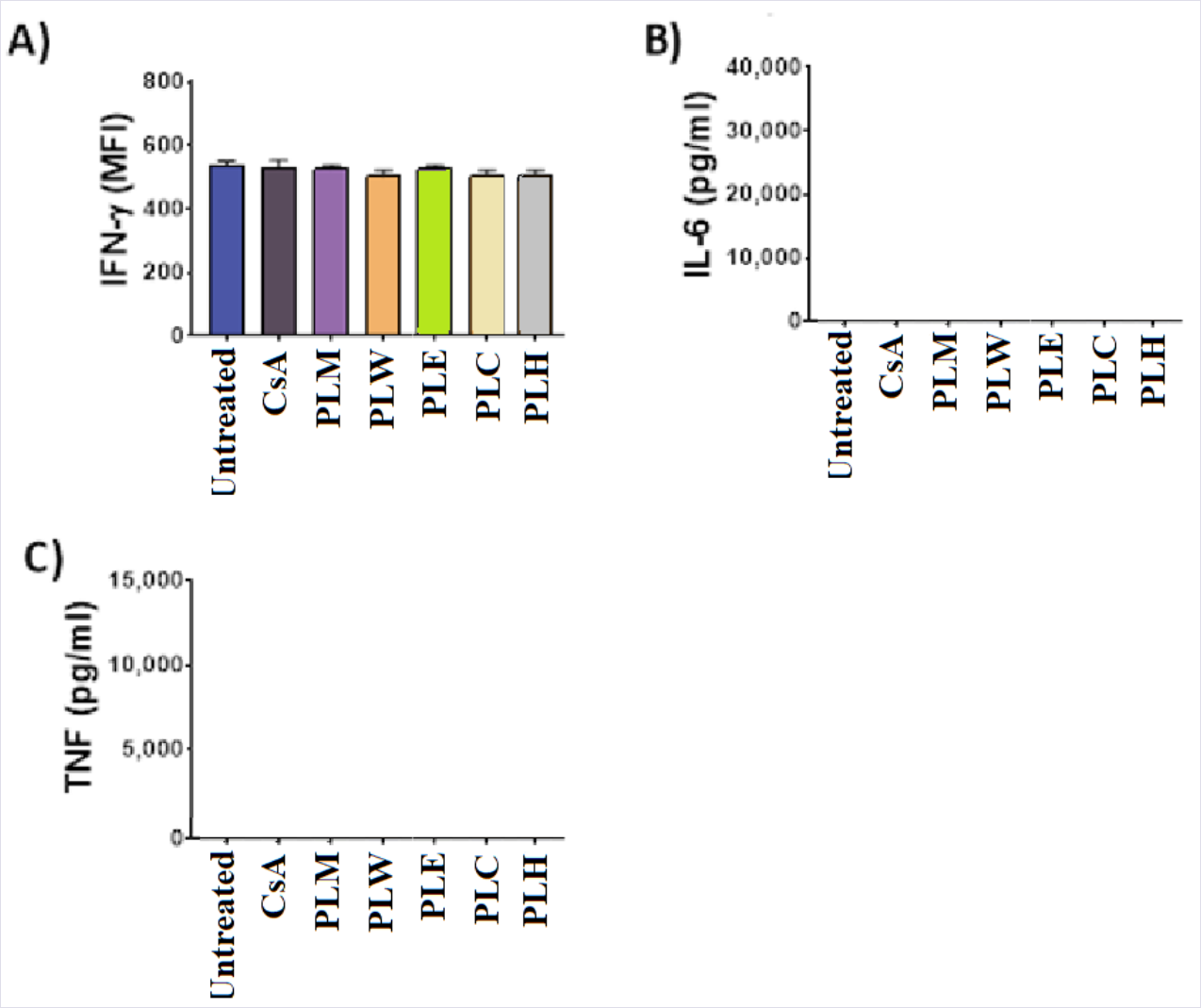

Figure 1: Cytokine secretions from unstimulated RAW 264.7 cells. Unstimulated RAW 264.7 cells were activated were treated with extracts, cyclosporine A (positive control) or left untreated (negative control) for 24 hr. Cytokine secretions in cell culture supernatant were measured as pg/mL or mean fluorescence intensity (MFI) using BD™ Cytometric Bead Array analysis. The data is displayed as the mean of three replicates±SEM. Student's t-test *p* values *=<0.05; **=<0.01; ***=<0.001. CsA= cyclosporine; PLM=Purple Loosestrife Methanolic extract; PLW=Purple Loosestrife Water extract; PLE=Purple Loosestrife Ethyl acetate extract; PLC=Purple Loosestrife Chloroform extract; PLH=Purple Loosestrife Hexane extract.

IFN-γ from the LPS activated RAW 264.7 cells. In contrast, IL-6 and TNF-α secretion into the media was significantly reduced by exposure to the methanolic and aqueous extracts. The most substantial suppression was observed for IL-6, particularly for the aqueous *L. salicaria* extracts and by the CsA control.

Bacterial growth inhibition screening

Proteus mirabilis growth was particularly susceptible to the higher polarity aqueous and methanolic *L. salicaria* extracts and generally less susceptible to the lower polarity extracts (Figure 4a). 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, these 3 extracts all produced larger ZOIs than both of the reference antibiotics 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 *L. salicaria*

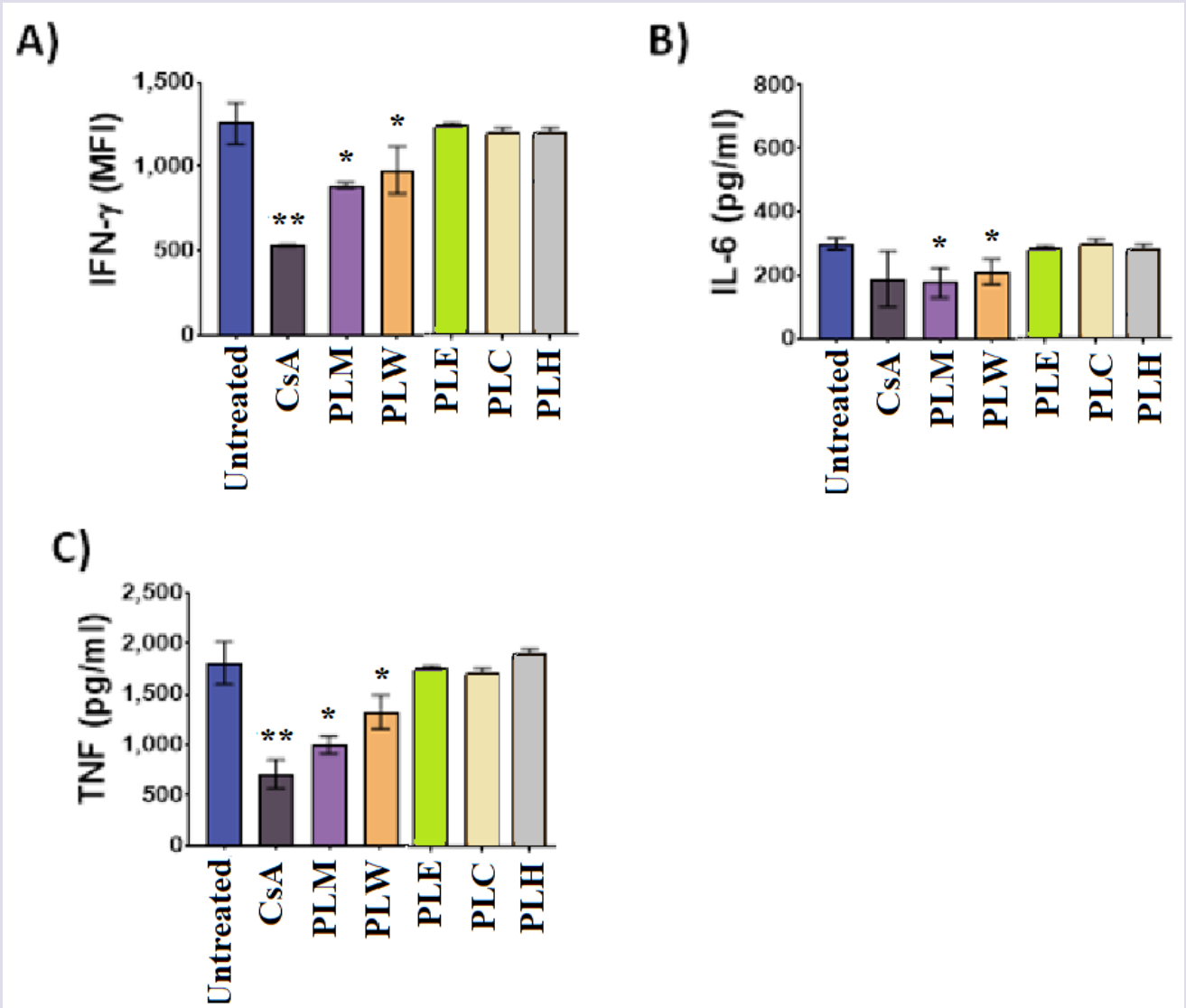

Figure 2: Cytokine secretions from PMA and ionomycin-activated RAW 264.7 cells. RAW 264.7 cells were activated with a cocktail containing 50 ng of PMA and 1 µg ionomycin and treated with extracts, cyclosporine A (positive control) or left untreated (negative control) for 24 hr. Cytokine secretions in cell culture supernatant were measured as pg/mL or mean fluorescence intensity (MFI) using BD™ Cytometric Bead Array analysis. The data is displayed as the mean of three replicates±SEM. Student's t-test *p* values *=<0.05; **=<0.01; ***=<0.001. CsA=Cyclosporine; PLM=Purple Loosestrife Methanolic extract; PLW=Purple Loosestrife Water extract; PLE=Purple Loosestrife Ethyl acetate extract; PLC=Purple Loosestrife Chloroform extract; PLH=Purple Loosestrife Hexane extract.

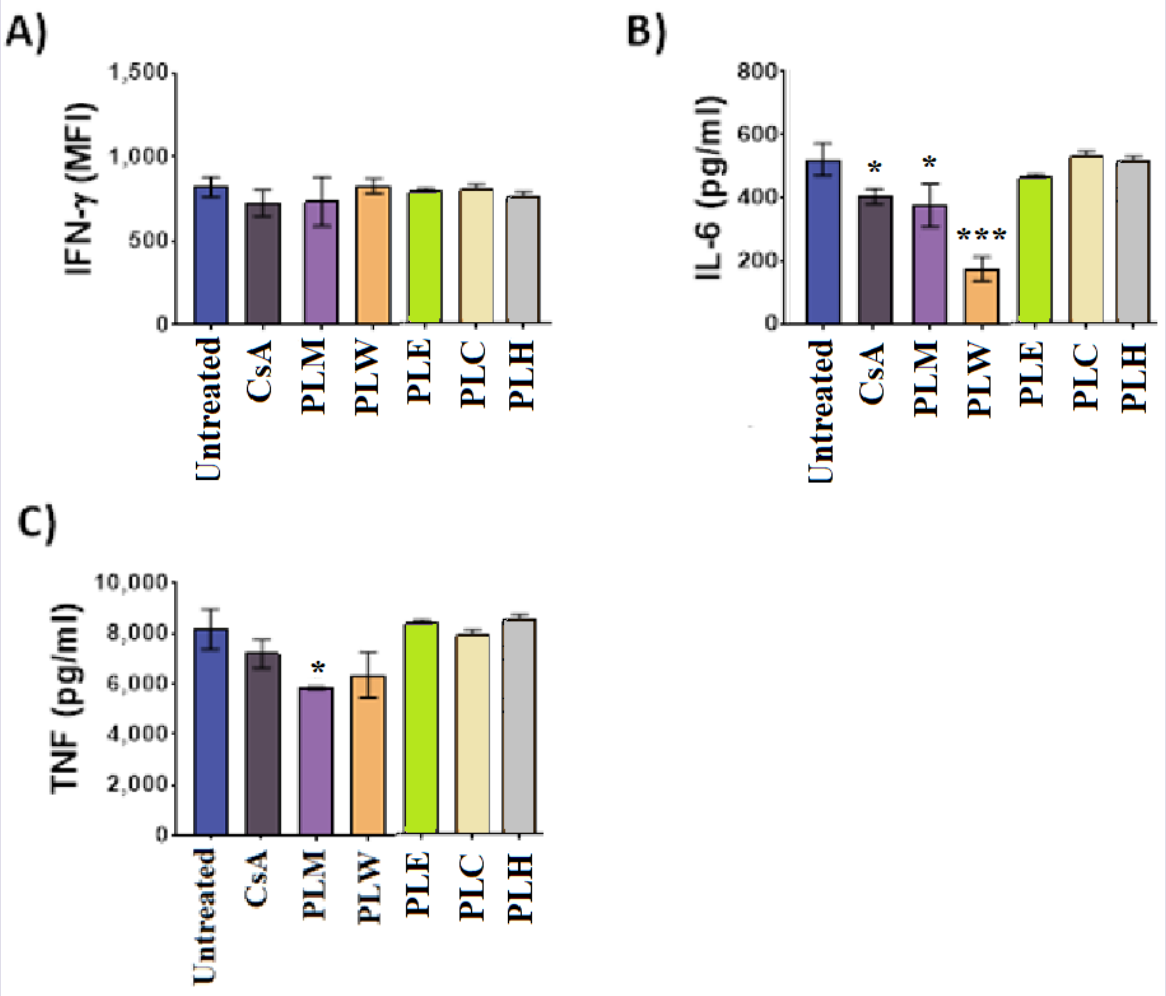

Figure 3: Cytokine secretions from lipopolysaccharide-activated RAW 264.7 cells. RAW 264.7 cells were activated with 100 ng lipopolysaccharide and treated with extracts, cyclosporine A (positive control) or left untreated (negative control) for 24 hr. Cytokine secretions in cell culture supernatant were measured as pg/mL or mean fluorescence intensity (MFI) using BD™ cytometric bead array analysis. The data is displayed as the mean of three replicates±SEM . Student's t-test *p* values *=<0.05; **=<0.01; ***=<0.001. CsA= Cyclosporine; PLM=Purple Loosestrife Methanolic extract; PLW=Purple Loosestrife Water extract; PLE=Purple Loosestrife Ethyl acetate extract; PLC=Purple Loosestrife Chloroform extract; PLH=Purple Loosestrife Hexane extract.

extracts inhibited the growth of *K. pneumoniae* (Figure 4b) 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 7.5 mM 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 *L. salicaria* 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 4c). 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 7.8 mM ZOI. The mid to high polarity *L. salicaria* 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 4d). This inhibition is particularly noteworthy as the *P. aeruginosa* strain tested in this study was

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

Extract	Mass of Dried Extracted Material (mg)	Concentration of extract (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	156	15.6	+++	++	+	-	++	-	-	-	-	+++	+++	+	-	-
Water	184	18.4	+++	+++	++	-	++	-	-	-	-	+++	+++	+	-	-
Ethyl Acetate	125	12.5	++	++	-	-	+	-	-	-	-	+	+	-	-	-
Chloroform	110	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	92	9.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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

completely resistant to both the ampicillin and tetracycline controls. Our studies therefore indicate that the methanolic and aqueous *L. salicaria* extract (and to a lesser extent, the ethyl acetate extract) were effective inhibitors of both bacterial triggers of multiple sclerosis.

The methanolic, aqueous and ethyl acetate *L. salicaria* extracts also inhibited *S. pyogenes* growth, albeit with small zones of inhibition, indicating lower efficacy (Figure 4e). The chloroform and hexane extracts were completely devoid of antibacterial activity. The aqueous *L. salicaria* extract was the strongest inhibitor of *S. pyogenes* growth, with a ZOI of 8.6 mM 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 methanolic extracts may be useful for preventing and treating rheumatic fever in antibiotic resistant *S. pyogenes* strains.

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 screening assays, each of the higher polarity methanol and water *L. salicaria* extracts inhibited all of the bacteria. The ethyl acetate extract also inhibited the growth of all of the bacteria, albeit with substantially lower potency than seen for the methanolic and aqueous extracts. In contrast, the chloroform and hexane extracts were completely ineffective against all bacteria tested. 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 zones of only single doses was recorded for that assay and we were unable to determine MIC values. In the liquid dilution assay, MIC values >1 µg/mL for pure compounds indicates antibiotic resistance.[20-22] Gentamicin was the most potent antibiotic (as judged by its MIC and the number of bacteria susceptible to it), although ciprofloxacin was also highly effective. Indeed, both gentamicin and ciprofloxacin were effective against all except one of the bacterial strains tested each.

The MIC values determined for *L. salicaria* extracts compare relatively well between the disc diffusion and liquid dilution assays. The growth of *P. mirabilis* was strongly inhibited by the methanolic and aqueous *L. salicaria* extracts (LD MIC's of 450

µg/mL and 240 µg/mL respectively). The ethyl acetate extract (LD MIC=900 µg/mL) also produced noteworthy inhibition of *P. mirabilis*, whilst the chloroform and hexane extracts were completely ineffective (results not shown). Similarly, the methanolic, aqueous and ethyl acetate *L. salicaria* extracts also inhibited *K. punemoniae* growth, although inhibition by the ethyl acetate extract (MIC=840 µg/mL) was substantially stronger than the other extracts (MICs of 1800 and 1680 µg/mL for the methanolic and aqueous extracts respectively). As *P. mirabilis* and *K. pneumoniae* can trigger rheumatoid arthritis and ankylosing spondylitis respectively in genetically susceptible people,[1] the methanolic, aqueous and ethyl acetate *L. salicaria* extracts may be useful in the prevention and treatment of these diseases and other illnesses caused by these bacteria.

The methanolic and aqueous *L. salicaria* extracts were also good inhibitors of *A. baylyi* growth, with MIC values of 900 and 840 µg/mL respectively. The aqueous and ethyl acetate extracts *L. salicaria* extracts were also strong inhibitors of *P. aeruginosa* growth, with MIC values of 240 and 420 µg/mL respectively. The methanolic extract (MIC=1200 µg/mL) was also a noteworthy inhibitor of *P. aeruginosa* growth (1200 µg/mL) These results are particularly interesting as this strain of *P. aeruginosa* was determined to be resistant to all of the conventional tested except gentamicin and previous studies[19,22] have also reported that this bacterium is resistant to these (and other) antibiotics. As *A. baylyi* and *P. aeruginosa* can trigger multiple sclerosis in genetically susceptible people, these extracts may be particularly useful for preventing the onset of this disease and treating it once it occurs.

The *S. pyogenes* clinical isolate strain screened in our study was also a particularly resistant strain. Indeed, this strain was resistant to all of the antibiotics tested except gentamicin and ciprofloxacin. This is interesting as β-lactam antibiotics (including penicillin)

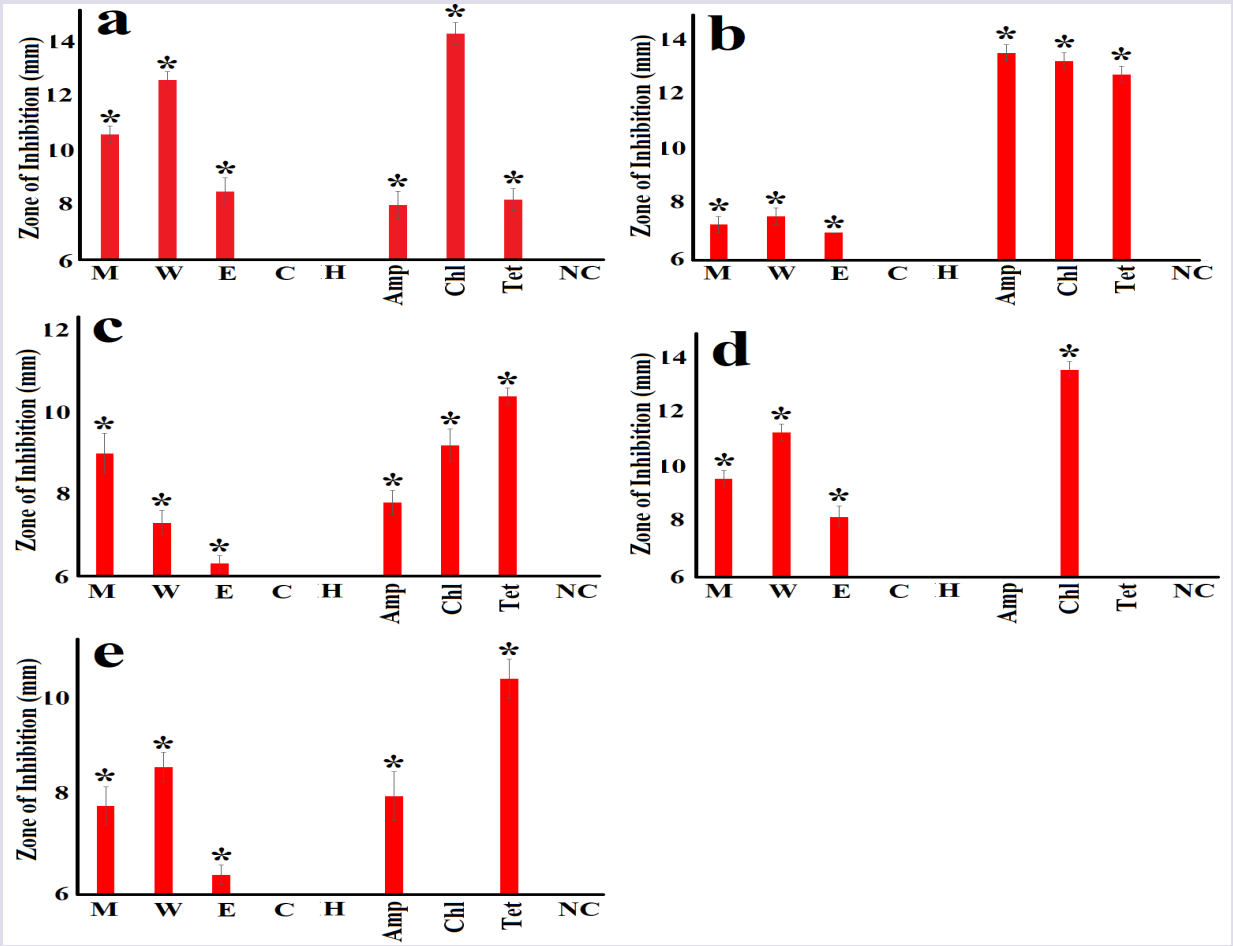

Figure 4: Antibacterial activity of *L. salicaria* extracts against (a) *P. mirabilis* (ATCC21721); (b) *K. pneumoniae* (ATCC31488); (c) *A. baylyi* (ATCC33304); (d) *P. aeruginosa* (ATCC: 39324); (e) *S. pyogenes* clinical strain, measured as zones of inhibition (mm). M=Methanolic extract; W=Aqueous extract; E=Ethyl acetate extract; C=Chloroform extract; H=Hexane extract; Amp=Ampicillin (10 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).

are generally still considered effective against more Streptococcal strains.[10] However, there are increasing reports of β-lactam resistant strains of *S. pyogenes*[25-28] and effective new therapies are urgently needed. Notably, the aqueous *L. salicaria* extract was a noteworthy inhibitor of the growth of the clinical *S. pyogenes* strain (MIC=840 µg/mL). As *S. pyogenes* infections can trigger the onset of rheumatic fever in genetically susceptible people, the aqueous *L. salicaria* extract may be effective at preventing the onset of this disease (and other diseases caused by this bacterium) and treating it once it occurs.

Combinational effects: Fractional inhibitory concentration (FIC) assessment

Of the eighteen *L. salicaria* extracts and conventional antibiotic combinations tested against *P. mirabilis*, the majority (13 combinations) were non-interactive (Table 3). Whilst no added benefit would be gained from using these combinations, it is safe to use these extracts and antibiotics together without compromising the activity of either component. A single combination (aqueous extract in combination with erythromycin) produced synergy and would therefore have substantially increased inhibitory activity towards this bacterium than either of the individual components. An additional four combinations produced additive effects when tested against *P. mirabilis*. As these combinations also produce effects substantially greater than either the individual extract or conventional antibiotic components, they would also be beneficial in the prevention and treatment of rheumatoid arthritis. Notably, no antagonistic interactions were noted against *P. mirabilis*, indicating that the methanolic, aqueous and ethyl acetate *L. salicaria* extracts are safe to use in combination with all of the conventional antibiotics tested in our study without decreasing their efficacy.

A variety of combinational effects were noted when extract-antibiotic combinations were tested against *K. pneumoniae*. Twelve of the extract-antibiotic combinations were non-interactive, indicating that it is safe to use these extracts and antibiotics together without compromising the activity of either component. A further four combinations produced additive effects. Interestingly, the majority of these contained tetracycline as the antibiotic component. As bacterial resistance to tetracycline is usually due to efflux pumps,[10,29,30] these results may indicate that the extracts contain tetracycline efflux inhibitory compounds, although this is yet to be verified. A further additive combination containing ciprofloxacin and the ethyl acetate extract was also detected. As this bacterium was susceptible to ciprofloxacin (Table 2; MIC=0.63 µg/mL), it is unlikely that the extract components are overcoming a ciprofloxacin resistance mechanism. Instead, it is likely that the extracts components affect a different target to ciprofloxacin. As the additive combinations produce effects that are greater than that of either individual component, these combinations may be useful for the prevention and treatment of ankylosing spondylitis. Notably, two combinations produced antagonistic results. Both of these combinations contained erythromycin as the antibiotic component. Thus, *L. salicaria* extracts should be avoided for the treatment of *K. pneumoniae* infections by individuals prescribed erythromycin.

Three additive interactions were also noted for combinations of the *L. salicaria* extracts and conventional antibiotics against the growth of *A. baylyi* (Table 3). Four further additive interactions were detected against *P. aeruginosa*. Notably, nearly all of the additive interactions against both *A. baylyi* and *P. aeruginosa* occurred in combinations that contained either chloramphenicol or erythromycin as the antibiotic component of the combination. As *A. baylyi* and *P. aeruginosa* can trigger multiple sclerosis in genetically susceptible people, these combinations may be beneficial in preventing and treating that disease in genetically susceptible people. Of further note, the combination of the methanolic *L. salicaria* extract and tetracycline induced

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

Bacterial Species	Methanol Extract		Aqueous Extract		Ethyl Acetate Extract		Controls					
							Pen	Chlor	Eryth	Tet	Gent	Cip
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	LD MIC	LD MIC	LD MIC	LD MIC	LD MIC	LD MIC
P. mirabilis	540	450	327	240	1053	900	1.25	3.3	2.5	0.63	1.25	0.63
K. pneumoniae	2658	1800	2420	1680	1280	840	2.5	2.5	1.25	0.3	0.31	0.63
A. baylyi	1487	900	1084	840	4126	3360	2.5	2.5	1.25	1.25	0.31	0.63
P. aeruginosa	1602	1200	485	240	726	420	1.25	1.25	1.25	1.25	0.63	1.25
S. pyogenes	1449	1150	1126	840	>5000	1680	3.3	2.5	3.3	2.5	0.63	0.63

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.

antagonistic effects against *A. baylyi* and should be avoided in treatments targeting this bacterium. These results emphasise the need for caution when using combinational therapies.

One synergistic interaction was detected between the *L. salicaria* extracts and conventional antibiotics against *S. pyogenes*, in the aqueous extract-tetracycline combination (Table 3). Additionally, 4 further combinations produced additive effects and would therefore also be beneficial in treating *S. pyogenes* infections. Notably, the majority of the potentiating combinations (both synergistic and additive) contained the aqueous *L. salicaria* extract, with either chloramphenicol, erythromycin or tetracycline as the antibiotic component of the combination. Indifferent interactions accounted for all of the remaining combinations. Whilst no additional benefit would be gained by taking these therapies concurrently, they would not reduce each other's efficacy. No antagonistic interaction was also noted, indicating that the *L. salicaria* extracts are safe to use in combination with this panel of antibiotics without decreasing the efficacy of either component.

Table 3: Combinational effects determined as ΣFIC values for the plant: antibiotic combinations, against some bacterial triggers of selected autoimmune inflammatory diseases.

Bacterial Species	Extract	Antibiotics					
		Pen	Chlor	Eryth	Tet	Gent	Cip
P. mirabilis	M	1.46	1.5	1.06	1.06	1.73	1.02
		(IND)	(IND)	(IND)	(IND)	(IND)	(IND)
	W	1.08	0.56	0.4	0.56	1.25	1.42
		(IND)	(ADD)	(SYN)	(ADD)	(IND)	(IND)
	E	1.25	1.13	0.56	0.56	1.11	1.01
		(IND)	(IND)	(ADD)	(ADD)	(IND)	(IND)
K. pneumoniae	M	2.2	1.5	4.5	0.56	1.38	1.42
		(IND)	(IND)	(ANT)	(ADD)	(IND)	(IND)
	W	2.58	1.91	6.16	0.83	1.45	1.42
		(IND)	(IND)	(ANT)	(ADD)	(IND)	(IND)
	E	2.36	1.21	3.33	0.54	1.13	0.71
		(IND)	(IND)	(IND)	(ADD)	(IND)	(ADD)
A. baylyi	M	3.22	0.75	1.13	4.12	3.32	2.84
		(IND)	(ADD)	(IND)	(ANT)	(IND)	(IND)
	W	2.1	0.71	0.65	1.44	3.04	2.55
		(IND)	(ADD)	(ADD)	(IND)	(IND)	(IND)
	E	2.94	1.1	2.5	2.87	2.76	2.84
		(IND)	(IND)	(IND)	(IND)	(IND)	(IND)
P. aeruginosa	M	1.27	0.88	0.75	1.06	1.52	1.01
		(IND)	(ADD)	(ADD)	(IND)	(IND)	(IND)
	W	1.1	1.13	1.03	1.03	1.47	0.71
		(IND)	(IND)	(IND)	(IND)	(IND)	(ADD)
	E	1.57	0.63	1.06	1.06	1.3	1.42
		(IND)	(ADD)	(IND)	(IND)	(IND)	(IND)
S. pyogenes	M	1.7	0.84	1.18	1.02	2.16	1.83
		(IND)	(ADD)	(IND)	(IND)	(IND)	(IND)
	W	1.49	0.68	0.77	0.37	3.63	1.44
		(IND)	(ADD)	(ADD)	(SYN)	(IND)	(IND)
	E	1.66	1.07	1.16	0.73	2.48	1.88
		(IND)	(IND)	(IND)	(ADD	(IND)	(IND)

M=methanolic extract; W=aqueous extract; E=ethyl acetate extract C=chloroform extract; H=hexane extract; Pen=penicillin-G; Chlor=chloramphenicol; Eryth=erythromycin; Tetracycline=tetracycline; Gent=gentamycine; Cip=ciprofloxacin; Int.=SYN=synergistic interaction; ADD = additive interaction; IND=indifferent interaction; ANT=antagonistic interaction.

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.[23] When tested individually, the antimicrobials demonstrated no toxicity in the ALA (Table 4). Similarly, none of the *L. salicaria* 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 nontoxic. In contrast, the positive control potassium dichromate induced 100 % mortality in the ALA.

DISCUSSION

This study aimed to evaluate *L. salicaria* extracts as chemotherapies for the prevention and treatment selected autoimmune inflammatory diseases (RA, AS, MS, RF). There are no cures for any of these diseases and instead the current therapies aim to alleviate the inflammatory symptoms, thereby increasing the patient's comfort. However, that approach is not ideal as it still allows tissue damage to occur. Thus, a more holistic approach that not only increases the patients' comfort, but also targets the disease's etiology may be a better therapeutic option for the prevention and treatment of autoimmune inflammatory diseases. To provide an initial evaluation of the ability of the extracts to decrease inflammatory effects, our study examined the effects of the *L. salicaria* extracts on some pro-inflammatory cytokines. Specifically, we tested the ability of the extracts to down-regulate the secretion of IFN-γ, IL-6 and TNF from RAW 264.7 murine macrophages. We selected a CBA murine multiplex system to quantify the levels of these cytokines in cell culture media as this is a high-throughput sensitive assay that allows accurate quantification down to 10-30 pg/mL concentration levels. Cyclosporine A (CsA) was included in these assays as a positive control of immune-modulation. CsA is a cytotoxic immunosuppressive drug commonly used for the prevention of graft-versus-host disease (GVHD) in organ transplantation.[18] This drug blocks transcription of T cell cytokine genes related to activation, such as IL-2 and therefore suppresses the activity of these cells. To the best of our knowledge, this is the first study to report the immunological effects of *L. salicaria* extracts.

Our study demonstrates that methanolic and aqueous *L. salicaria* extracts significantly down-regulate the release of three pro-inflammatory cytokines in two stimulatory models. Indeed, the level of inhibition of IFN-γ (Figure 2a) and TNF-α (Figure 2c) in PMA/ionomycin-activated RAW 264.7 cells was similar to that seen for 10 µg/mL CsA and was substantially better than CsA for IL-6 (Figure 2b). A different trend was evident for the LPS-induced RAW 264.7 cells. Whilst no substantial effects were evident for either the *L. salicaria* extracts or the CsA control for IFN-γ (Figure 3a), the methanolic and aqueous *L. salicaria* extracts substantially down-regulated the levels of IL-6 (Figure 3b) and TNF-α (Figure 3c) secreted by the RAW 264.7 cells. These results indicate that the methanolic and aqueous *L. salicaria* extracts modulate both cellular immunity (in the PMA/ionomycin-activated RAW 264.7 cells) and innate myeloid immunity (in the LPS-stimulated cells). Thus, the extracts may be useful for addressing the downstream immune and inflammatory symptoms that occur for autoimmune inflammatory diseases. However, screening against an extended panel of pro- and anti-inflammatory cytokines is warranted to obtain a more complete understanding of the immune-modulatory effects of the extracts. Additionally, our studies were performed using *in vitro* assay models. Future studies are required to investigate bioavailability factors and to determine whether the extracts have similar effects *in vivo*.

Our study also tested the ability of the *L. salicaria* extracts to inhibit the etiological events of the selected autoimmune diseases. It has been established that exposure of genetically susceptible people to *P. mirabilis* can induce the production of self-reactive antibodies.[1] Similarly, *K. pneumoniae* can induce ankylosing spondylitis, *A. baylyi* and *P. aeruginosa* can induce multiple sclerosis and *S. pyogenes* can induce rheumatic fever in genetically susceptible people.[1,2] Blocking the onset of the autoimmune diseases not blocks the inflammatory symptoms, but also reduces the tissue damage associated with these diseases. Interestingly, relatively few studies to date have focussed on preventing the disease etiology, although several recent studies have begun to examine these early-phase events.[19,22] Interestingly, the mid to higher polarity ethyl acetate, methanol and water extracts were good inhibitors of the growth of all of the bacterial triggers of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever. Therefore, the methanolic and aqueous *L. salicaria* extracts were effective against both the etiological and down-stream inflammatory events of these diseases and may be particularly useful in their prevention and treatment.

The combinational studies also demonstrated potentiation of several extract/antibiotic combinations, indicating that these may also be effective against antibiotic resistant bacterial strains. Future studies are required to screen the *L. salicaria* extracts against extended panels of bacterial strains with different antibiotic susceptibility/resistance profiles. The implications of the enhanced antibacterial activity of the potentiating combinations include enhanced efficacy, allowing lower doses to be administered, thereby 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, extending the useful lifetime of the therapy.[10] However, the majority of combinations

Table 4: Mortality (%) and cellular viability (%) results for extracts and conventional antibiotics tested individually and as combinations in the ALA and MTS cell viability assays respectively.

Sample		ALA Mortality±SEM (%)[a]		HDF Cytotoxicity (24 hr)
		After 24 hr:	After 48 hr:	
Antimicrobials	Penicillin G	1.8±1.4	4.3±2.4	98.3±3.4
	Chloranphenicol	2.7±1.3	5.6±3.3	102.2±3.7
	Erythromycin	1.2±0.6	5.8±2.3	97.7±5.5
	Tetracycline	2.4±1.5	5.1±2.8	95.8±4.7
	Gentamicin	3.1±1.8	6.7±2.6	93.6±2.7
	Ciprofloxacin	4.4±2.6	8.3±3.2	94.7 ±4.6
Extracts	M	6.2±3.7	21.3±3.7	81.2±6.3
	W	4.5±2.8	15.7±3.6	85.3±5.5
	E	2.4±0.8	7.3±2.1	98.3±4.6
	C	4.7±2.2	12.7±3.6	87.8±6.1
	H	1.8±1.0	5.2±2.8	102.4 ±3.9
Combinations	M+Penicillin G	4.7±2.5	18.5±4.2	84.6±3.7
	M+Chloramphenicol	6.9±4.3	24.6±3.5	80.5±3.8
	M+Erythromycin	4.2±2.6	15.7±2.6	88.3±3.1
	M+Tetracycline	5.7±1.9	17.8±3.7	87.7±4.8
	M+Gentamicin	8.3±2.4	22±3.7	93.9±7.2
	M+Ciprofloxacin	7.8±2.6	28.3±4.2	76.9±4.6
	W+Penicillin G	4.3±2.8	12.8±3.2	93.6±2.7
	W+Chloramphenicol	4.8±3.1	13.5±4.1	89.5±4.5
	W+Erythromycin	3.6±2.4	10.8±2.4	92.7±3.0
	W+Tetracycline	5.6±2.8	17.5±3.1	89.0±3.5
	W+Gentamicin	8.7±2.8	25.6±4.2	84.8±4.8
	W+Ciprofloxacin	7.4±3.3	20.5±3.3	91.6±3.2
	E+Penicillin G	2.7±1.3	14.6±2.7	95.6±3.4
	E+Chloramphenicol	4.2±2.8	16.5±3.5	92.7±3.9
	E+Erythromycin	3.2±2.6	9.3±3.8	96.2±4.7
	E+Tetracycline	5.5±3.8	18.1±5.2	89.2±3.7
	E+Gentamicin	7.7±2.8	20.4±3.9	83.2±3.9
	E+Ciprofloxacin	7.7±5.0	22.7±4.6	87.2±6.1

a=Tested at a concentration of 1 mg/mL; b=Tested at a concentration of 200 µg/mL; M =methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; SD=standard deviation; NT=control not tested in the assay. Results represent means±SD of 3 independent experiments, each preformed in triplicate (n=9).

were determined to produce indifferent interactions. Whilst these combinations may have limited added benefit over using the conventional antibiotic (or extract) alone, they do alleviate some concerns related to concurrent use of the 2 forms of healthcare as these interactions indicate that neither therapy is reducing the efficacy of the other therapy.

In most published combination studies, synergistic interactions are generally emphasized, with the reporting of antagonism generally 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, three of *L. salicaria* extract/conventional antibiotic combinations demonstrated antagonistic interactions. Whilst this rate of antagonism is a relatively low, it is perhaps as important a finding as the synergistic interactions as it determines combinations which should be avoided. Further studies are required to examine the mechanism(s) of these interactions to better tailor combinations to treat different diseases.

None of the *L. salicaria* extract or conventional antibiotics demonstrated toxicity in the ALA and MTS assay when tested

independently. Similarly, all combinations were nontoxic 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 *L. salicaria* extracts may perhaps also not be surprising as this plant has been used therapeutically for hundreds of years (as reviewed in.[11] However, to the best of our knowledge, there is a lack of rigorous toxicity studies for *L. salicaria* preparations. The lack of toxicity of the combinations also further validates 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 nontoxic in complex biological systems.

CONCLUSION

Whilst the findings reported herein indicate the potential of *L. salicaria* extracts to treat the immune and inflammatory stages, as well as the etiological events of these autoimmune 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 needed to determine the active and potentiating compound(s) in the *L. salicaria* 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; **HDF:** Human dermal fibroblasts; **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; **ΣFIC:** The sum of the fractional inhibitory concentration; **ZOI:** Zone of inhibition.

SUMMARY

• *Lythrum salicaria* L. extracts were screened for the ability to modulate secretion of IFN-γ, IL-6 and TNF-α from RAW 264.7 cells using a BD™ cytometric bead array assay.

• The ability to inhibit the growth of some bacterial triggers of selected autoimmune inflammatory diseases was quantified by liquid dilution and disc diffusion assays.

• The extracts were also tested in combination with conventional antibiotics to determine whether the extracts cold potentiate the antibiotic activity.

• Toxicity of the extracts was evaluated using the *Artemia nauplii* and HDF cytotoxicity bioassays.

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