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Original Article

Larrea tridentata (DC.) Coville Leaf Extracts Inhibit the Bacterial Triggers of Some Autoimmune Inflammatory Diseases

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ABSTRACT

Background: *Larrea tridentata* leaves are used traditionally to treat bacterial infections and inflammation. Despite the traditional uses, relatively few studies have examined the ability of *L. tridentata* leaf extracts to inhibit the growth of the bacterial triggers of autoimmune inflammatory diseases. **Materials and Methods:** *Larrea tridentata* extracts were screened for growth inhibitory activity against bacterial triggers of selected autoimmune inflammatory diseases and the activity was quantified using disc diffusion and liquid dilution MIC assays. Toxicity was evaluated using the *Artemia* nauplii cytotoxicity assay. **Results:** *Larrea tridentata* extracts strongly inhibited the growth of several bacterial triggers of autoimmune diseases, although they were ineffective inhibitors of *A. baylyi* growth. The methanolic and ethyl acetate extracts were particularly good inhibitors of *Proteus* spp. and *Klebsiella pneumoniae* growth, with LD MIC values between 32 and 375 µg/mL. The methanolic and ethyl acetate extracts were also particularly good inhibitors of *Streptococcus pyogenes* (LD MICs 63-125 µg/mL). None of the *L. tridentata* extracts were toxic in the ALA toxicity assay. **Conclusion:** *Larrea tridentata* leaf extracts inhibit the growth of some bacterial triggers of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever. Further *in vivo* studies to determine the anti-inflammatory and antibacterial mechanisms are warranted.

Keywords: Chaparral, Creosote bush, Antibacterial activity, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Rheumatic fever, Lignin.

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INTRODUCTION

Exposure of the immune system to specific antigens may stimulate the production of self-reactive antibodies in some individuals, resulting in a range of autoimmune inflammatory diseases, including rheumatoid arthritis (RA), ankylosing spondylitis (AS), multiple sclerosis (MS) and rheumatic fever (RF).[1-3] Some autoimmune diseases are triggered by environmental and dietary stimuli (e.g. the gluten stimulus in Celiac's disease).[1] However, other autoimmune inflammatory diseases are triggered by bacterial pathogens. Targeting these autoimmune antigenic triggers may block the etiology of the disease in genetically susceptible people, as well as preventing the downstream immunological and inflamatory events of these diseases. Such preventative therapies may therefore be particularly effective against autoimmune inflammatory diseases. Notably, the antigenic triggers of several autoimmune inflammatory diseases have already been identified via serotyping and genotyping studies,[1] allowing novel chemotherapies to be developed that target the diseases' etiology. *Proteus mirabilis* is a bacterial trigger of RA rheumatoid arthritis in genetically susceptible people,[4] *Klebsiella pneumoniae* may trigger AS,[5] *Acinetobacter baylyi* and *Pseudomonas aeruginosa* infections can initiate MS,[6] whilst *Streptococcus pyogenes* can induce rheumatic fever in individuals with specific genetic markers.[7]

An examination of traditional plant-based therapies may highlight promising leads for the treatment of inflammation and autoimmune inflammatory diseases. Plant medicinal usage is reasonably well documented for some cultures.[1,8] although ethnobotanical studies are lacking for other ethnic groups. *Larrea tridentata* (DC.) Coville (Family Zygophyllaceae; commonly called chaparral, creosote bush, greasewood) is an evergreen shrub (Figure 1a) that is native to the Chihuahuan, Mojave and Sonoran desert regions of the south-western states of the United States of America (Arizona, California, Nevada, New Mexico, Texas, Utah), as well as several northern Mexico states (Coahuila, Chihuahua, Durango, Nuevo Leon, San Luis Potosi, Sonora and

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Zacatecas). It is an evergreen shrub that grows to 3 meters tall, with dark green lanceolate resinous leaves and yellow flowers to 25 mm in diameter (Figure 1b). *Larrea tridentata* leaves are used in the traditional medicine of several native American groups to treat multiple pathogenic diseases, including several urinary tract and sexually transmitted infections, tuberculosis, varicella (chicken pox)[9-11] Leaf infusions were considered particularly useful for ulcers, gastrointestinal distress and diarrhoea.[9,12] Additionally, boiled *L. tridentata* leaves are applied to wounds and sores as an antiseptic.[9] Notably, many of these maladies are caused by bacterial pathogens, which indicates that *L. tridentata* leaf preparations may also inhibit the growth of other bacteria. Interestingly, *L. tridentata* leaves are also reputed to have anti-inflammatory and analgesic properties,[9,10] and thus they may also be useful for treating the later phase events of autoimmune inflammatory diseases. However, despite the traditional uses of *L. tridentata* leaves, their growth inhibitory properties have not been extensively tested against the bacterial triggers of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis or rheumatic fever.

Interestingly, previous research has identified and highlighted several phytochemicals with therapeutic properties from *L. tridentata* leaves.[9,13,14] In particularly, those studies highlighted several lignans including nordihydroguaiaretic acid (NDGA; Figure 1c), (7S,8S,70 S,80 S)-3,30,40-trihydroxy-4-methoxy-7,70-epoxylignan (Figure 1d), meso-(rel 7S,8S,70 R,80 R)-3, 4, 30, 40-tetrahydroxy 7,70-epoxylignan (Figure 1e) and (E)-4,40-dihydroxy-7,70-dioxolign-8(80)-ene (Figure 1f). Multiple lignans have well-characterised antibacterial, antifungal, antiallergic, anti-inflammatory and analgesic properties[15] and may therefore have potential against several phases of autoimmune inflammatory diseases. Additionally, multiple flavonoids including herbacetin 3,8,40-trimethyl ether (Figure 1g), 5,7,4'-trihyd-roxy-3,8-dimethoxyflavone (Figure 1h), apigenin (Figure 1i), dihydroisorhamnetin (Figure 1j), and isokempferide (Figure 1k) have also been identified in *L. tridentata* leaves.[14] The antioxidant, antibacterial, anti-inflammatory and analgesic activities of multiple flavonoids have also been well documented.[16] Despite the traditional uses of *L. tridentata* leaves and their known phytochemistry, the growth-inhibitory properties of *L. tridentata* leaves have not been extensively tested against the bacterial triggers of RA, AS, MS or RF. This study aimed to address this gap in the literature by quantifying the antibacterial activity of the *L. tridentata* leaf extracts against *Proteus mirabilis. Klebsiella pneumoniae, Acinitobacter baylyi, Pseudomonas aeruginosa* and *Streptococcus pyogenes*.

MATERIALS AND METHODS

Sourcing and preparation of plant samples

The dried and ground *Larrea tridentata* (DC.) Coville leaves 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 Chap-A1A-2018-LTA). Individual quantities (1 g) of the dried leaves were weighed into separate tubes and 50 mL of methanol, deionised water, ethyl acetate, chloroform or hexane was 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 and were then filtered through Whatman No. 54 filter paper under vacuum. The solvent extracts were subsequently air dried at room temperature, whilst the aqueous extract was lyophilised by freeze drying at -50°C. The resultant dried extracts were weighed and dissolved in 10 mL of deionised water (containing 1% DMSO) and stored as aliquots at -30°C until use.

Qualitative phytochemical studies

Qualitative phytochemical analysis of the *L. tridentata* leaf extracts to detect the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids and evaluate their relative abundances was conducted using standard assays.[17-19]

Antibacterial analysis

Conventional antibiotics

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

Bacterial cultures

The bacterial pathogens screened in this study were selected for study as they are all triggers autoimmune inflammatory diseases in genetically susceptible individuals.[1,2] Reference strains of *Proteus mirabilis* (ATCC21721), *Klebsiella pneumoniae* (ATCC31488), *Acinetobacter baylyi* (ATCC33304), *Pseudomonas aeruginosa* (ATCC39324) and *Streptococcus pyogenes* (ATCC12384) were purchased from American Type Culture Collection, USA. Clinical isolates of *K. pneumoniae, P. aeruginosa* and *S. pyogenes* were obtained from the School of Environment and Science teaching laboratory at Griffith University, Australia. All bacteria were cultured at 37°C in nutrient broth (Oxoid Ltd., Australia) for 24 hr and maintained in nutrient broth at 4°C until use. Streaked nutrient agar (Oxoid Ltd., Australia) plates were prepared to the manufactures specifications and were tested in parallel to ensure the purity of all bacterial cultures.

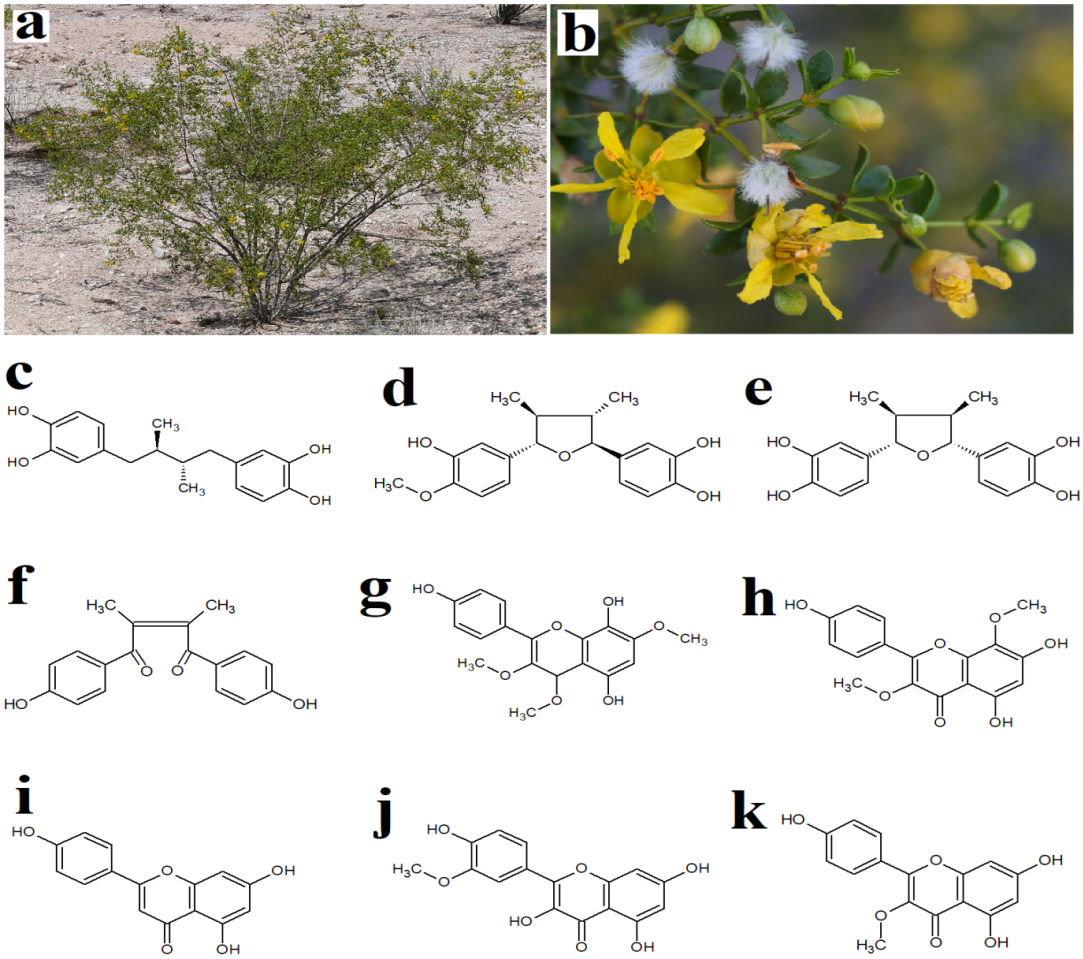

Figure 1: *Larrea tridentata* (a) whole plant and (b) flowers, as well as the lignin compounds (c) nordihydroguaiaretic acid, (d) (7S,8S,70 S,80 S)-3,30,40-trihydroxy-4-methoxy-7,70-epoxylignan, (e) meso-(rel 7S,8S,70 R,80 R)-3,4,30,40 -tetrahydroxy7,70-epoxylignan, (f) (E)-4,40-dihydroxy-7,70-dioxolign-8(80)-ene, and the flavonoids (g) herbacetin 3,8,40-trimethyl ether, (h) 5,7,4′-trihyd-roxy-3,8-dimethoxyflavone, (i) apigenin, (j) dihydroisorhamnetin, (k) isokempferide.

Evaluation of bacterial susceptibility to growth inhibition

Bacterial susceptibility to the *L. tridentata* extracts and the conventional antibiotics was assessed using standard disc diffusion assays.[20] Ampicillin (10 µg), chloramphenicol discs (10 µg) and tetracycline discs (10 µg) were obtained from Oxoid Ltd., Australia and included in the assays as positive controls. Filter discs infused with 10 µL of distilled water were included as a negative control.

Minimum Inhibitory Concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using liquid dilution MIC assays and solid phase agar disc diffusion assays.

Microplate liquid dilution MIC assay

The antibacterial activity of the individual *L. tridentata* extracts and conventional antibiotics was quantified using standard liquid dilution MIC assays.[21-23] Briefly, 100 µL of sterilized distilled water was first dispensed into each well of 96 well micro-titre plate. The *L. tridenta* extracts or conventional antibiotics (100 µL) were then individually dispensed into the first row of the plate. Nutrient broth (negative control) and a sterile control (media without bacteria) were included on all plates to verify that the assay was functioning correctly. Each test well was then serially diluted down each column by doubling serial dilution. Bacterial culture (100 µL containing approximately $1x10^6$ Colony Forming Units (CFU)/mL) was then added to all wells of the plate (excluding the sterile control wells) and incubated at 37°C for 24 hr. The colourimetric indicator *p*-iodonitrotetrazolium violet (INT) (Sigma-Aldrich, Australia) was prepared in sterile deionised water as a 0.2 mg/mL INT stock solution. Following the incubation, 40 µL of the INT stock solution was dispensed into all wells and the plates were incubated for 6 hr at 30°C to allow full colour development. The lowest dose at which colour development was completely inhibited was classified as the MIC of the test.

Disc diffusion MIC assay

The minimum inhibitory concentration (MIC) of each extract was also quantified by disc diffusion assay.[24,25] Graphs of the zone of inhibition (ZOI) versus Ln concentration were plotted and MIC values were calculated by linear regression.

Toxicity screening

Toxicity evaluations of the *L. tridentata* extracts, conventional antibiotics and the reference toxin were assessed using adapted *Artemia franciscana* nauplii lethality zssays (ALA).[26] 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 following 24 and 48 hr exposure and is expressed as a % of the untreated control. The LC_{50} for each treatment was calculated using Probit analysis.

Statistical analysis

All data is expressed as the mean±SEM of 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.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extractions of the *L. tridentata* plant material (1 g) with solvents of varying polarity yielded dried plant extracts ranging from 27 mg (*L. tridentata* hexane extract) to 240 mg (*L. tridentata* methanolic extract) (Table 1). Qualitative phytochemical screening showed that the higher polarity solvents (methanol and water) extracted the greatest amount and widest diversity of phytochemical classes.

Bacterial growth inhibition

Inhibition of the growth of the bacterial triggers of rheumatoid arthritis (Proteus spp.*)*

Proteus mirabilis growth was particularly susceptible to the higher polarity aqueous and methanolic *L. tridentata* extracts, with ZOIs of 9.7-9.9 mm (Figure 2a). This inhibition was noteworthy as the zones of inhibition (ZOIs) produced by these extracts were substantially greater than the ZOIs produced by ampicillin and tetracycline (8 and 8.2 mm respectively). In contrast, the *P. mirabilis* strain tested in this study was highly susceptible to chloramphenicol, with a ZOI of 14.3 mm. The medium polarity chloroform and ethyl acetate *L. tridentata* extracts were also good inhibitors of *P. mirabilis* growth, with ZOIs of 8.3 and 8 mm respectively. In contrast, the hexane extract was completely ineffective against *P. mirabilis*. Similar trends were noted for the *L. tridentata* extracts against *P. vulgaris* growth (Figure 2b), although this bacterium was slightly more susceptible to the extracts than *P. mirabilis* was. The methanolic extract had the most potent apparent growth inhibitory activity, with a ZOI of 10.5 mm.

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

The *L. tridentata* extracts also strongly inhibited the growth of *K. pneumoniae* (Figure 3). In contrast to inhibition of the

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *L. tridentata* 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	240	24	+++	++	+	-	+	-	-	-	-	++	+++	++	-	-
Water	150	15	+++	++	++	-	+	-	-	-	-	+++	+++	++	-	-
Ethyl Acetate	50	5	+	+	-	-	-	-	-	-	-	++	+	+	-	-
Chloroform	55	5.5	+	+	-	-	-	-	-	-	-	+	+	+	-	-
Hexane	27	2.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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

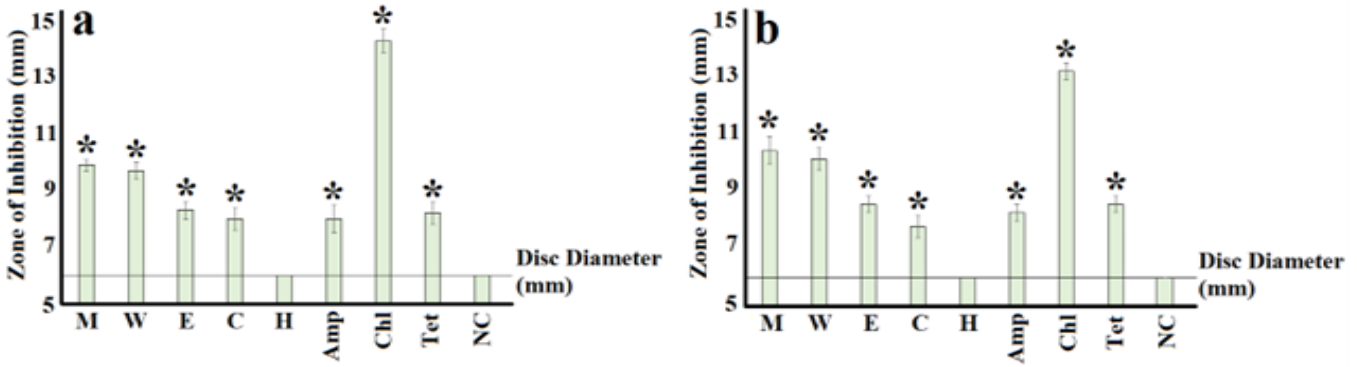

Figure 2: Antibacterial activity of the *L. tridentata* extracts against (a) *P. mirabilis* (ATCC21721) and (b) *P. vulgaris* (ATCC21719) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); Tet=tetracycline (10 µg); NC=negative control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n=9)±SEM * indicates results that are significantly different to the negative control (p<0.01).

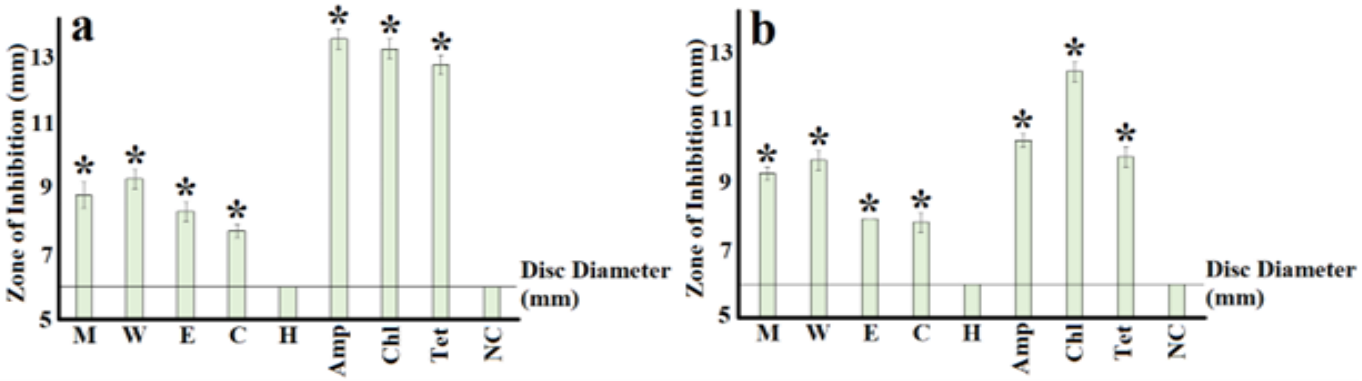

Figure 3: Antibacterial activity of the *L. tridentata* extracts against (a) *K. pneumoniae* (ATCC31488) and (b) *K. pneumoniae* (clinical isolate) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); Tet=tetracycline (10 µg); NC=negative control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n=9)±SEM * indicates results that are significantly different to the negative control (p<0.01).

Proteus spp., the growth of both *K. pneumoniae* strains was most susceptible to the aqueous *L. tridentata* extract, with ZOIs of 9.3 and 9.8 mm against the reference and clinical isolate strains respectively. The methanolic extract was also a strong growth inhibitor (ZOIs of 8.8 and 9.4 mm against the reference and clinical isolate strains). The medium polarity ethyl acetate and chloroform extracts were also good *K. pneumoniae* growth inhibitors (7.7-8.3 mm), whilst the hexane extract was ineffective against both strains. As *K. pneumoniae* can trigger ankylosing spondylitis in genetic susceptible individuals, these results indicate that the high polarity *L. tridentata* extracts in particular (and the medium polarity to a lesser extent) have potential for use in the prevention and treatment of ankylosing spondylitis, as well as other diseases caused by *K. pneumoniae*.

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

The *L. tridentata* extracts were also screened for growth inhibitory activity against two bacterial triggers of multiple sclerosis (*A. baylyi, P. aeruginosa*). All extracts were completely ineffective inhibitors of *A. baylyi* (Figure 4a). In contrast, *A. baylyi* growth was inhibited by all of the conventional antibiotics, with ZOIs of 7.8, 9.2 and 10.4 mm for ampicillin, chloramphenicol and tetracycline respectively. In contrast, the methanolic and chloroform extracts inhibited the growth of both *P. aeruginosa* strains, although the reference strain (Figure 4b) was substantially less susceptible to the extracts than the clinical isolate strain was (Figure 4c). All of the other extracts were completely ineffective inhibitors of *P. aeruginosa* growth. Both strains of *P. aeruginosa* were also strongly resistant to ampicillin and tetracycline, with no ZOIs evident for either strain. Notably, previous studies have confirmed that these *P. aeruginosa* strains are resistant to these and other antibiotics.[27] Therefore, the inhibitory activity noted for the methanolic and chloroform extracts may indicate that these extracts may be particularly useful for preventing and treating multiple sclerosis (and other diseases caused by *P. aeruginosa* infections). Both *P. aeruginosa* strains were highly susceptible to chloramphenicol (10.4-13.6 mm), confirming that the assay was functioning correctly.

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

With the exception of the hexane extract, all of the *L. tridentata* leaf extracts also inhibited *S. pyogenes* growth (Figure 5). The higher polarity methanolic (ZOIs of 8.4-8.7 mm) and aqueous extracts (ZOIs of 8.8-9 mm) were the strongest inhibitors of the growth

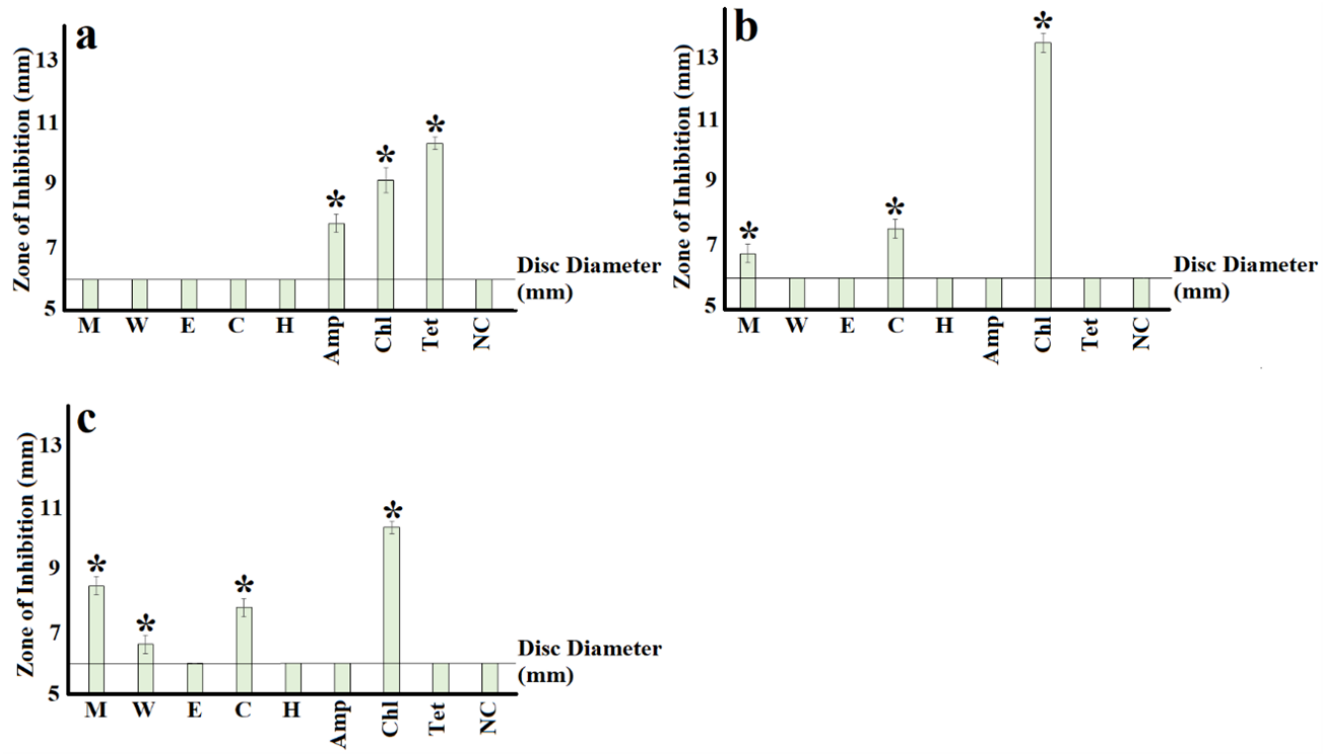

Figure 4: Antibacterial activity of the *L. tridentata* extracts against (a) *A. baylyi* (ATCC33304), *P. aeruginosa* (ATCC39324) and (c) *P. aeruginosa* (clinical isolate) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); Tet=tetracycline (10 µg); NC=negative control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n=9)±SEM * indicates results that are significantly different to the negative control (p<0.01).

of both the reference (Figure 5a) and clinical isolate *S. pyogenes* strains (Figure 5b). This inhibition compared favourably to that of the ampicillin (ZOIs of 8 and 7.6 mm against the reference and clinical isolate strains). Furthermore, both *S. pyogenes* strains were completely resistant to chloramphenicol at the dose tested, indicating that these extracts may be useful in preventing and treating rheumatic fever in genetically susceptible people, as well as treating other diseases caused by this bacterium. In contrast, tetracycline was a strong inhibitor of *S. pyogenes* growth, with ZOIs of 10.4 and 8.8 mm against the reference and clinical isolate strains respectively). Noteworthy ZOIs were also noted for the he ethyl acetate and chloroform extracts, whilst the hexane extract was completely ineffective against both *S. pyogenes* strains.

Quantification of Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of the *L. tridentata* extracts and conventional antibiotics was further evaluated by determining the MIC values using liquid dilution MIC assays (Table 2). MIC values>1 µg/mL for the pure conventional antibiotic standards have previously been defined as indicative of antibiotic resistance.[21-23] Notably, all of the bacterial strains tested were resistant to penicillin-G, chloramphenicol and erythromycin. Additionally, with the exception of both *Proteus* spp. and the reference *K. pneumoniae* strain, the bacteria triggers of autoimmune diseases were also resistant to tetracycline. Gentamycin was the most effective conventional antibiotic, with all bacteria susceptible (MIC values 0.31-0.63 µg/mL). Ciprofloxacin was also effective against all bacterial strains except the *Proteus* spp. and both *P. aeruginosa* strains.

The *L. tridentata* extracts displayed noteworthy growth inhibitory activity against several of the bacterial strains, although the hexane extract was ineffective against all bacteria (Table 2). The ethyl acetate *L. tridentata* extract was the most potent inhibitor of the *Proteus* spp. (LD MICs 63-125 µg/mL) and both *K. pneumoniae* strains (LD MICs of 32 µg/mL against both strains), although the methanolic, aqueous and chloroform extracts (LD MICs of 63-375 µg/mL) were also good inhibitors of *Proteus* spp. and *K. pneumoniae* growth. In contrast, the chloroform extract was the best inhibitor of *P. aeruginosa* growth, with MIC values of 500 µg/mL against both strains. Whilst the methanolic and aqueous extracts also inhibited *P. aeruginosa* growth, the MIC values determined for those extracts were substantially >1000 µg/mL, indicating only low to moderate inhibitory activity. The *L. tridentata* chloroform extract was also the strongest inhibitor of both *S. pyogenes* strains (MIC=63 µg/mL). Similar MIC values were also noted for the aqueous extract (MICs of 100 and 63 µg/mL against the reference and clinical isolate strains) against *S. pyogenes*. The methanolic and ethyl acetate extracts were also good inhibitors of *S. pyogenes* growth, albeit with slightly higher

Table 2: MIC values of the *L. tridentata* extracts and conventional antibiotics (µg/mL) against some bacterial triggers of selected autoimmune anti-inflammatory diseases.

Extract or Antibiotic DD MIC		*P. mirabilis* (ATC 21721)		*P. vulgaris* (ATCC 21719)		*K. pneumoniae* (ATCC 31488)		*K. pneumoniae* (clinical isolate)		*A. baylyi* (ATCC 33304)		*P. aeruginosa* (ATCC 39324)		*P. aeruginosa* (clinical isolate)		*S. pyogenes* (ATCC 12384)		*S. pyogenes* (clinical isolate)	
		LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	
Extracts	M	240	250	186	375	300	63	365	63	-	-	2580	2000	1580	1000	626	500	578	500
	W	500	250	330	250	313	125	426	125	-	-	-	-	3268	2000	1250	100	1168	63
	E	78	63	65	125	71	32	128	32	-	-	-	-	-	-	258	125	188	125
	C	500	250	372	250	500	250	725	125	-	-	528	500	623	500	125	63	100	63
	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Conventional Antibiotics	Pen	ND	1.25	ND	1.25	ND	2.5	ND	2.5	ND	2.5	ND	1.25	ND	2.5	ND	2.3	ND	3.3
	Chl	ND	3.3	ND	2.5	ND	2.5	ND	1.25	ND	2.5	ND	1.25	ND	1.25	ND	1.25	ND	2.5
	Ery	ND	2.5	ND	1.25	ND	1.25	ND	1.25	ND	1.25	ND	1.25	ND	2.5	ND	2.5	ND	3.3
	Tet	ND	0.63	ND	0.63	ND	0.63	ND	1.25	ND	1.25	ND	1.25	ND	1.25	ND	1.25	ND	2.5
	Cip	ND	1.25	ND	1.25	ND	0.63	ND	0.63	ND	0.63	ND	1.25	ND	1.25	ND	0.63	ND	0.63
	Gent	ND	0.63	ND	0.63	ND	0.31	ND	0.63	ND	0.31	ND	0.63	ND	0.63	ND	0.31	ND	0.63

DD MIC=MIC in the disc diffusion assay; LD MIC=MIC in the liquid dilution assay; M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; Pen=penicillin-G; Chl=chloramphenicol; Ery=erythromycin; Tet=tetracycline; Cip=ciprofloxacin; Gent=gentamycin; - indicates no inhibition at any dose tested; ND=no MIC was determined as the antibiotic was tested at a single dose only in the DD assay.

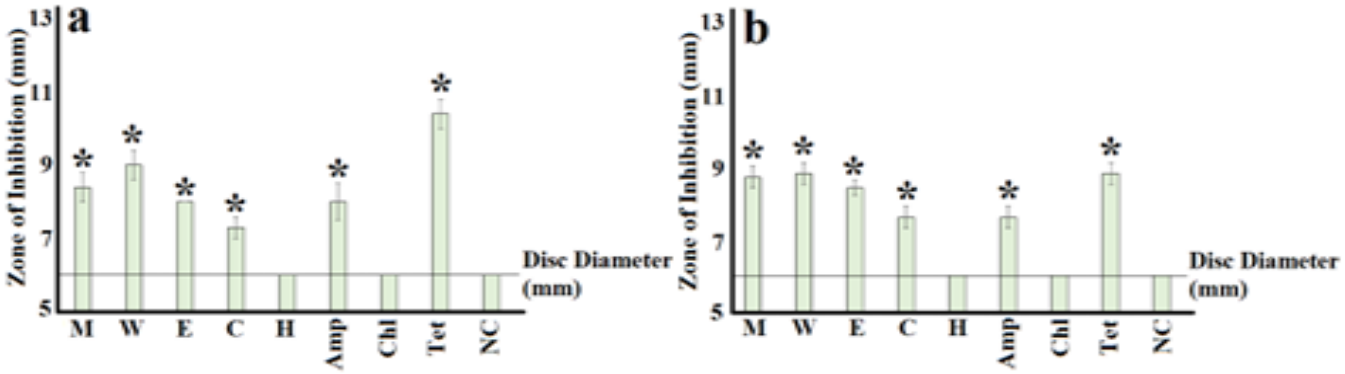

Figure 5: Antibacterial activity of the *L. tridentata* extracts against (a) *S. pyogenes* (ATCC12384) and (b) *S. pyogenes* (clinical isolate) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); Tet=tetracycline (10 µg); NC=negative control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n=9)±SEM * indicates results that are significantly different to the negative control (p<0.01).

MIC values (500 and 125 µg/mL for the methanolic and ethyl acetate extracts respectively).

Toxicity studies

All of the *L. tridentata* extracts (1000 µg/mL) and the conventional antibiotics (10 µg/mL) were tested individually in the *Artemia* lethality assay (ALA) (Table 3). The compounds were only considered toxic if they induced percentage mortalities greater than 50% (LC_{50}) following 24 hr of exposure to the *Artemia* nauplii.[27] All of the conventional antibiotics and *L. tridenta* extracts induced substantially <50% mortality. Therefore, all extracts and antibiotics were deemed nontoxic. In contrast, the positive control potassium dichromate induced 100% mortality in the ALA, indicating that the assay was functioning correctly.

DISCUSSION

This study evaluated and quantified the growth inhibitory properties of *L. tridentata* leaf extracts against bacterial triggers of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever.[1,2] There are currently no widely available effective cures for any of these autoimmune inflammatory diseases. Instead, current therapies target the disease symptoms (particularly inflammation and pain) using anti-inflammatory drugs (particularly non-steroidal anti-inflammatory drugs (NSAIDs)) and analgesics. Whilst these treatment modalities alleviate the patient's discomfort, they do not alter the progression of the disease, nor do they decrease the resultant damage to self-tissue. Additionally, prolonged anti-inflammatory drug usage induces toxicity and numerous negative side effects.[28] Indeed, prolonged use of cyclooxygenase-2 (COX-2) inhibitors may increase the incidence of myocardial infarction.[29] Chemotherapies that reduce inflammation as well as blocking the diseases' etiology, may be substantially more effective in preventing and treating these diseases. Such prophylactic treatment may limit tissue degradation in genetically susceptible people, as well as substantially decreasing the later phase inflammatory symptoms caused by these diseases.

Interestingly, few previous studies have focussed on disease prevention via inhibiting the trigger pathogens, although some several recent studies have examined these early-phase events.[20-23] The current study focussed on inhibiting the onset of some selected autoimmune diseases by inhibiting the growth of their bacterial triggers. *Proteus mirabilis* can induce the production of self-reactive antibodies in people genetically susceptible to rheumatoid arthritis.[1] *Klebsiella 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] Whilst several previous studies have screened *L. tridentata* extracts for antibacterial activity, most studies have concentrated on other bacterial species.[30-33] Methanolic *L. tridentata* leaf extracts similar to the extracts tested in our study have been reported to have noteworthy growth inhibitory activity towards multiple *Staphylococcus* spp. (including methicillin-resistant strains), with MIC values 31-188

Table 3: Mortality (%) of the conventional extracts, *L. tridentata extracts* and combinations in the ALA assay.

Test compound or combination		ALA Mortality±SEM (%)	
After 24 hr:		After 48 hr:	After 48 hr:
Conventional Antibiotics	Pen	1.8±1.4	4.3±2.4
	Chl	2.7±1.3	5.6±3.3
	Ery	1.2±0.6	5.8±2.3
	Tet	2.4±1.5	5.1±2.8
	Cip	4.4±2.6	8.3±3.2
	Gent	3.1±1.8	6.7±2.6
Extracts	M	17.3±3.5	34.6±3.4
	W	8.3±2.8	25.6±3.6
	E	5.3±2.4	12.6±3.7
	C	11.4±3	27.9±4.4
	H	4.4±2.8	15.5±3.1
Controls	PC	6.7±2.9	25.2±4.4
	NC	4.9±1.9	17.6±2.7

Results represent means±SEM of 3 independent experiments, each preformed in triplicate (n=9). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; Pen=penicillin-G; Chl=chloramphenicol; Ery=erythromycin; Tet=tetracycline; Cip=ciprofloxacin; Gent=gentamycin; PC=positive control (potassium dichromate); NC=negative (seawater) control.

µg/mL.[33] Notably, several of these studies report relatively high MIC values against other bacteria. One study reported MIC values of 6.25 mg/mL against *Clavibacter michiganesis* subsp. *michiganensis* and *Pseudomonas syringae*.[31] Notably, as many studies classify MIC values >1 mg/mL as low activity,[32-34] the *L. tridentata* extracts examined in that study would generally be considered to have only low antibacterial activity. Whilst the bacteria tested in that study are different to the species screened in our study, *P. syringae* is the same genus as *P. aeruginosa* that was tested in our study. Notably, only low to moderate activity (albeit with substantially lower MIC values) were also recorded against *P. aeruginosa* in our study.

Additionally, several previous studies have isolated lignin and flavonoid compounds from *L. tridentata* leaf extracts and screened them for antibacterial activity. One study identified the lignans dihydroguaiaretic acid, 2,4-epi-larreatricin, 3, 3′-demethoxy-6-O-demethylisoguaiacin) and the flavonoids 4,5,4′-dihydroxy-3,7,8,3′-tetramethoxyflavone; 5,5,4′-dih ydroxy-3,7,8-trimethoxyflavone; 6,5,4′-dihydroxy-7-methoxyflavone and 7, 5, 8, 4′-trihydroxy-3,7-dimethoxyflavone and screened them for antibacterial activity.[32] Notably, whilst all these compounds inhibited bacterial growth, most of them had relatively low inhibitory activity, with MIC values>50 µg/mL reported. That study reported that 3′-demethoxy-6-O -demethylisoguaiacin had the greatest antibacterial activity against antibiotic sensitive and resistant *S. aureus* (MIC 25 µg/mL), *Enterococcus faecalis* (MIC 12.5 µg/mL), *Escherichia coli* (MIC 50 µg/mL), *E. cloacae* (MIC 12.5 µg/mL) and MDR strains of *M. tuberculosis* (MIC 12.5 µg/mL). Unfortunately, we were unable to find studies that screened ′-demethoxy-6-O-demethylisoguaiacin against the bacterial triggers of RA, AS, MS or RF and future studies are required to screen for antibacterial activity against those pathogens.

Our study only tested the potential of the *L. tridentata* extracts to inhibit the etiology of some autoimmune diseases by blocking the bacterial triggers. The direct effects of the extracts on inflammation were not tested. However, several *L. tridentata* leaf compounds have substantial anti-inflammatory effects.[15] One study demonstrated that NDGA inhibits 12-O-tetradecanoylphorbol-13-acetate induced inflammation in a murine model by inhibiting hydrogen peroxidase production and lipid peroxidation, as well as increasing glutathione levels and the activity of cellular antioxidant enzymes.[35] Whilst we were unable to find studies evaluating the anti-inflammatory activity of the other lignans detected in *L. tridentata* extracts, a large number of studies have examined the anti-inflammatory activity of several other lignans [reviewed in.[36] Several lignans inhibit the production and/or release of pro-inflammatory cytokines,[36-39] whilst upregulating the production of anti-inflammatory cytokines.[40-42] Therefore, *L. tridentata* lignans may be particularly useful in the prevention and treatment of RA, AS, MS and RF as they block both the trigger events, as well as the later phase effects, although this has yet to be verified *in vivo*. Further studies are required to determine whether the lignans and flavonoids identified in *L. tridentata* extracts compounds also have beneficial effects towards other aspects of these diseases. None of the *L. tridentata* extracts tested in this study were toxic in the ALA toxicity assay. However, further *in vitro* toxicity studies using other human cell lines are required to verify the safety of these extracts prior to clinical usage. Future studies should also use *in vivo* toxicity assays to confirm the safety of these compounds and combinations in complex biological systems.

CONCLUSION

Whilst the findings reported herein indicate the potential of *L. tridentata* leaves to inhibit the etiological events of RA, AS, MS and RF, further *in vivo* investigations are required to support these *in vitro* findings. Furthermore, studies to determine the therapeutic mechanisms of the extracts are warranted. Additionally, further studies are required to determine whether *L. tridentata* extracts can also affect other phases of the progession of these autoimmune diseases.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ABBREVIATIONS

ALA: Brine-shrimp lethality assay; **DMSO:** Dimethyl sulfoxide; **INT:** ρ-iodonitrotetrazolium chloride; **LC_{50}:** Concentration of sample necessary to have a lethal effect on 50% of test organisms or cells; **MIC:** Minimum inhibitory concentration; **ZOI:** Zone of inhibition.

SUMMARY

- *Larrea tridenta* extracts were screened for growth inhibitory activity in the disc diffusion assay against bacterial triggers of some autoimmune diseases.
- The ability of the extracts to inhibit the growth of the bacteria was quantified by disc diffusion and liquid dilution MIC assays.
- Toxicity of the individual compounds and combinations was evaluated using the *Artemia* nauplii bioassay.

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