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Original Article

Larrea tridentata (DC.) Coville Leaf Extracts Inhibit the Growth Gastrointestinal Bacterial Pathogens

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ABSTRACT

Introduction: *Larrea tridentata* (DC.) Coville leaves are used traditionally to treat diarrhoea and general bacterial infections. Despite the traditional uses, relatively few studies have examined the ability of *L. tridentata* leaf extracts to inhibit the growth of bacterial gastrointestinal pathogens. **Materials and Methods:** The growth inhibitory activity of *L. tridentata* leaf extracts was assessed against a panel of gastrointestinal bacterial pathogens by disc diffusion and liquid dilution minimum inhibitory concentration (MIC) methods. The toxicity of the extracts was evaluated by *Artemia* nauplii lethality assays. **Results:** *Larrea tridentata* extracts strongly inhibited the growth of several bacterial gastrointestinal pathogens, with MIC values as low as 86 µg/mL. The aqueous and chloroform extracts were particularly good inhibitors of several of the most virulent pathogens, although the methanolic and ethyl acetate extracts also displayed noteworthy activity against some pathogens. None of the *L. tridentata* extracts were toxic in the ALA toxicity assay, indicating that they are safe to use therapeutically. **Conclusion:** *Larrea tridentata* leaf extracts inhibited the growth of a panel of clinically relevant gastrointestinal pathogens. Thus, *L. tridentata* leaf extracts have potential in the treatment of diarrhoea and other gastrointestinal diseases.

Keywords: Chaparral, Creosote bush, Antibacterial activity, Gastrointestinal disease, Diarrhoea, *Bacillus cereus*, *Shigella sonnei*, *Staphylococcus aureau*.

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INTRODUCTION

Gastrointestinal illness is a major cause of illness globally. Indeed, the world Health Organization (WHO) estimates that approximately nine million children less than five years of age die from the effects of diarrhoea annually.[1] Very little has changed since that report was published and diarrhoea remains the leading killer of children globally, accounting for approximately 9% of all deaths among children under the age of five.[2] This equates to approximately 1400 young children dying each day from diarrhoea (~530,000 child mortalities annually). Whilst there are numerous antibiotics that are effective for the treatment of diarrhoea, many bacterial pathogens have developed resistance to conventional antibiotics in recent years, rendering them of lower effect, or even completely ineffective against resistant pathogens.[3] The gastrointestinal environment provides ideal conditions for the exchange of genetic material (including resistance genes) between bacteria. Many bacterial pathogens are now extremely (XDR) or totally drug resistant (TDR) to common clinical chemotherapies and there are now limited therapeutic options against those pathogens. There is an urgent need to develop new treatment options to combat these diseases through the development of novel drugs.

An examination of traditional plant-based therapies may highlight promising leads for antimicrobial drug development and there has recently been a substantial increase in interest in this field.[4-6] *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 several northern Mexico states (Coahuila, Chihuahua, Durango, Nuevo Leon, San Luis Potosi, Sonora and Zacatecas), as well as the south-western states of the United States of America (Arizona, California, Nevada, New Mexico, Texas, Utah). *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)[7,8] and cystitis.[7,9] Leaf infusions were considered particularly useful for ulcers, gastrointestinal distress and diarrhoea.[7,10] Furthermore, boiled *L. tridentata* leaves are applied to wounds and sores as an antiseptic.[7] Notably, many of these maladies are caused by bacterial pathogens, which indicates that *L. tridentata*

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leaf preparations may also inhibit the growth of other bacteria. However, despite the traditional uses of *L. tridentata* leaves, their growth inhibitory properties have not been extensively tested against many bacterial gastrointestinal pathogens.

Previous studies have identified and highlighted several phytochemicals with therapeutic properties from *L. tridentata* leaves.[7,11,12] In particularly, those studies highlighted several lignans including nordihydroguaiaretic acid (NDGA; Figure 1b), (7S,8S,70 S,80 S)-3,30,40 -trihydroxy-4-methoxy-7,70 –epoxylignan (Figure 1c), meso-(rel 7S,8S,70 R,80 R)-3,4,30,40 -tetrahydroxy7,70-epoxylignan (Figure 1d), and (E)-4,40 -dihydroxy-7,70 -dioxolign-8(80)-ene (Figure 1e). Multiple lignans have well-characterised antibacterial, antifungal, antiallergic, anti-inflammatory and analgesic properties[13] and may therefore have potential in the treatment of pathogenic gastrointestinal diseases. Additionally, multiple flavonoids including herbacetin 3,8,40-trimethyl ether (Figure 1f), 5,7,4'-trihyd-roxy-3,8-dimethoxyflavone (Figure 1g), apigenin (Figure 1h), dihydroisorhamnetin (Figure 1i), and isokempferide (Figure 1j) have also been identified in *L. tridentata* leaves.[12] The antibacterial activities of multiple flavonoids have also been well documented.[14] 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 bacterial gastrointestinal pathogens. This study quantifies the antibacterial properties of *L. tridentata* leaf extracts against a panel of gastrointestinal bacterial pathogens, and compares it to the effects of the extracts against some normal gastrointestinal bacterial flora species.

MATERIALS AND METHODS

Plant material and extraction

Dried and ground *Larrea tridentata* (DC.) Coville leaves were purchased from Noodles Emporium, Australia and were sourced from verified plants in North America. Voucher specimens are deposited in the School of Natural Sciences, Griffith University, Australia (voucher number Chap-A1A-2018-LTA). Individual 1 g masses of the ground leaves were extracted separately in 50 mL of methanol, deionised water, ethyl acetate, chloroform or hexane. All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The dried *L. tridentata* leaves were extracted for 24 hr at 4°C with gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54). The filtered solvent extracts were air dried at room temperature, whilst the aqueous extract was dried by freeze drying at -50°C. The dried extracts were subsequently weighed and resuspended in 10 mL of deionised water (containing 1% DMSO) and stored as aliquots at -30°C.

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 to evaluate their relative abundances was conducted using standard assays.[15,16]

Antibacterial screening

Conventional antibiotics

Chloramphenicol (≥98% purity), ciprofloxacin (≥98 % purity by HPLC), erythromycin (≥850 µg/mg), gentamicin (600 µg/mg), penicillin-G (1440-1680 µg/mg) and tetracycline (≥95% purity) were purchased from Sigma-Aldrich, Australia. All antibiotics were prepared as 0.01 mg/mL stocks in sterile deionised water. For the disc diffusion studies, pre-loaded ampicillin (10 µg), chloramphenicol (10 µg) and tetracycline (10 µg) susceptibility discs (Oxoid Ltd., Australia) were used as positive controls.

Test microorganisms

All media was obtained from Oxoid Ltd., Australia and prepared as per the manufacturer's instructions. Reference strains of *Aeromonas hydrophila* (ATCC7966), *Bacillus cereus* (ATCC14579), *Bacillus megaterium* (ATCC14581), *Eneteobacter cloacea* (ATCC13047), *E. coli* (ATCC0157 and ATCCK12), *Shigella sonnei* (ATCC 25931), and *Staphylococcus aureus* (ATCC 25923) were purchased from American Tissue Culture Collection, USA. The clinical strains of *Alcaligenes faecalis, Salmonella newport, Salmonella salford, Staphylococcus epidermidis* and *Yersinia entercolitica* were kindly provided by the School of Environment and Science teaching laboratory, Griffith University. All bacterial strains were individually cultured to log phase in nutrient broth (Oxoid Ltd., Australia). Streak nutrient agar (Oxoid Ltd., Australia) plates were cultured in parallel to ensure the purity of all bacterial cultures and to allow for sub-culturing. All cultures were incubated at 37°C for 24 hr and were maintained in nutrient broth at 4°C. The antibacterial test conditions conformed to CLSI standardised methods.[17]

Antimicrobial activity screening

The *L. tridentata* leaf extracts were initially screened undiluted using a standard disc diffusion assay as previously described.[18,19] Briefly, individual bacterial suspensions (100 µL) was spread evenly onto nutrient agar plates and 6 mm filter paper discs infused with individual extracts were placed onto the surface of the plates. The plates were incubated at 37°C for 24 hr and the diameters of the zones of inhibition (ZOI) were measured to the closest whole mm and expressed as the mean values (± SEM) of three independent experiments, each with internal triplicate measurements (n=9). Standard discs of ampicillin (10 µg), chloramphenicol (10 µg) and tetracycline (10 µg) (Oxoid Ltd., Australia) and discs infused with

10 µL of distilled water (containing 1% DMSO) were included on each agar plate as positive and negative controls.

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

A standard liquid dilution MIC assay[20,21] was used to evaluate the bacterial growth inhibitory activity of the extracts and conventional antibiotics. Briefly, log phase bacterial cultures were diluted to produce a McFarlands inoculation culture.[20,21] A 100 µL volume of sterilized nutrient broth was dispensed into all wells of a 96 well micro-titre plate and 100 µL of the plant extracts or conventional antibiotics were then dispensed into separate wells of the top row of the plate. A negative control (nutrient broth), sterile control (broth without bacteria) and a sample-free culture control (to ensure the media was capable of supporting microbial growth) were also included on all plates. Each test column was serially diluted by doubling dilution. Relevant bacterial test culture inoculums (100 µL, containing approximately $1x10^6$ colony forming units (CFU)/mL) were then added to all wells except the sterile control wells and incubated overnight at 37°C. p-Iodonitrotetrazolium violet (INT; Sigma-Aldrich, Australia) was dissolved in sterile deionised water to give a stock concentration of 0.2 mg/mL. A volume of 40 µL of the stock INT solution was dispensed into all wells and the plate was incubated for a further 6 hr at 37°C. The MIC was visually determined as the lowest dose at which colour development was inhibited.

Disc diffusion MIC assay

The minimum inhibitory concentration (MIC) of each extract was also quantified by disc diffusion assay.[22,23] Graphs of the zone of inhibition (ZOI) versus Ln concentration were plotted and MIC values were calculated by linear regression.

Artemia franciscana nauplii toxicity screening

Toxicity was evaluated using an *A. franciscana* nauplii (brine shrimp) lethality assay, modified for testing plant extracts.[24,25] Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in distilled water as a 1.6 mg/mL solution and used as a reference toxin in the *Artemia franciscana* nauplii bioassay. *Artemia franciscana* nauplii were incubated in the presence of the test extracts, reference toxin or artificial seawater (negative control) at 25 ± 1°C under artificial light (1000 Lux). All treatments were performed three times in triplicate (n = 9). The number of dead were counted in each well at 24 hr and the remaining live nauplii were then sacrificed and the total number of nauplii in each well were counted and used to calculate the % mortality per well. LC_{50} values were calculated for each treatment using probit analysis.

Statistical analysis

Data are 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 control and treated groups, with p values <0.01 considered to be statistically significant.

RESULTS

Extraction yields and qualitative phytochemical screening

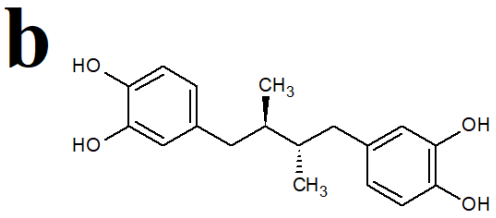
Extractions of the *L. tridentata* plant material (1g) with solvents of varying polarity yielded dried plant extracts ranging from 27 mg (*L. tridentata* hexane extract) to 240 mg (*L. tridentata* methanolic extract) (Table 1). Qualitative phytochemical screening showed that the higher polarity solvents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

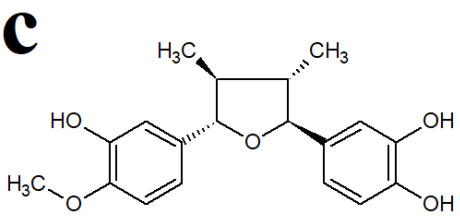
Antibacterial activity

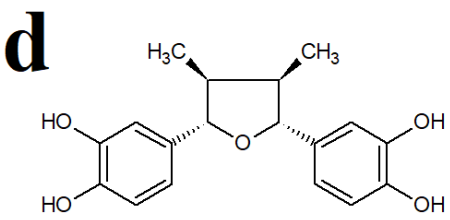
Inhibition of the growth of gastrointestinal Gram-negative rod bacteria

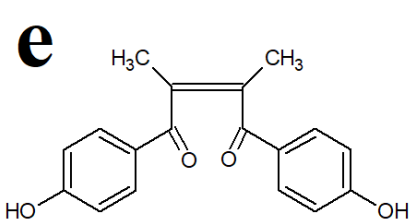
The Gram-negative rod bacteria screened in this study were selected as most of them are relatively common causes of food poisoning (Figure 2).[26] In contrast, *A. faecalis* (Figure 2a) and *E. cloaceae* (Figure 2c) were included in the study as they are normal and non-pathogenic components of the human gastrointestinal flora. Notably, *E. cloaceae* was completely resistant to the ethyl acetate, chloroform and hexane extracts, and only relatively small zones of inhibition (ZOIs) were measured when the methanolic and aqueous extracts were tested (6.6 and 7.2 mm respectively) (Figure 2c). The effects of treatments for gastrointestinal illnesses on the normal gastrointestinal microflora is an important consideration when considering food poisoning and diarrhoeal chemotherapies. Therapies that affect normal gastrointestinal balance may allow potentially pathogenic bacterial species to compete effectively against beneficial non-pathogenic species, thereby inducing further illness. Thus, the relative lack of inhibitory activity of the *L. tridentata* leaf extracts against *E. cloaceae* indicates that they may not adversely affect the levels of this bacterium in the gastrointestinal tract. This bacterium was sensitive to the control antibiotics (particularly chloramphenicol, with a ZOI of 11.7 mm), indicating that the assay was functioning correctly.

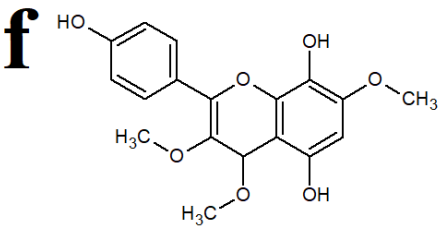
In contrast, the *L. tridentata* leaf extracts had substantial inhibitory effects on the growth of *A. faecalis* (Figure 2a). This bacterium is also a normal component of the human gastrointestinal microbiome and is generally considered non-pathogenic, although it is an opportunistic bacteria and can induce illness in immunocompromised people.[26] The methanolic and aqueous

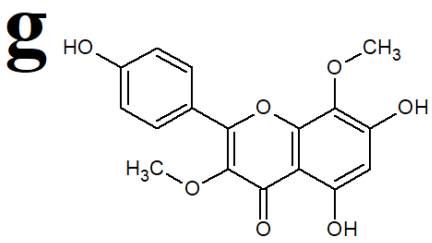


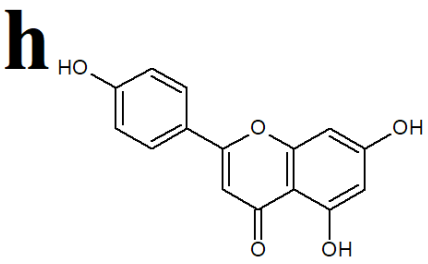


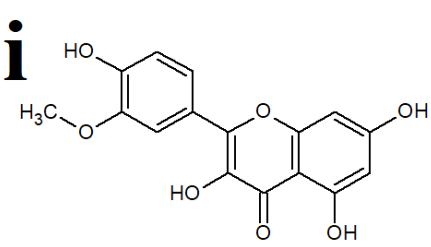












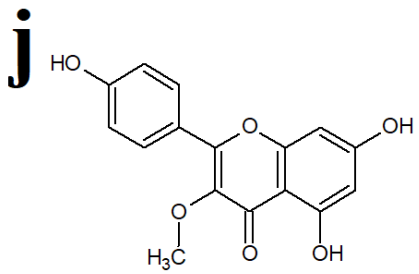


Figure 1: (a) *Larrea tridentata*, as well as the lignin compounds (b) nordihydroguaiaretic acid, (c) (7S,8S,70 S,80 S)-3,30,40-trihydroxy-4-methoxy-7,70-epoxylignan, (d) meso-(rel 7S,8S,70 R,80 R)-3,4,30,40 -tetrahydroxy7,70-epoxylignan, (e) (E)-4,40-dihydroxy-7,70-dioxolign-8(80)-ene, and the flavonoids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

extracts were particularly good inhibitors of *A. faecalis* growth, with ZOIs of 9 and 11 mm respectively. The strong inhibitory effects of these extracts against this bacterium may impact on their usefulness in the treatment of gastrointestinal diseases and further studies are required to examine the effects in mixed culture systems, and their effects against other non-pathogenic gastrointestinal microflora species. Interestingly, the *A. faecalis* strain examined in this study was resistant to ampicillin and tetracycline, but was highly sensitive to chloramphenicol (ZOI = 10.4 mm).

Notably, the *L. tridentata* leaf extracts displayed substantial inhibitory activity against many Gram-negative rod bacteria

associated with gastrointestinal illness. The methanolic, aqueous, ethyl acetate and chloroform extracts were all good inhibitors of both the ATCC0157 (Figure 2d) and ATCC K12 strains (Figure 2e) of *E. coli* examined in this study, with ZOIs generally between 8 and 9 mm. In contrast, these *E. coli* strains were completely resistant to ampicillin, indicating that the *L. tridentata* leaf extracts may be useful in inhibiting the growth of antibiotic-resistant pathogens. In contrast, both *E. coli* strains were sensitive to chloramphenicol (MICs of 14.5 and 13.3 mm respectively) and tetracycline (MICs of 8.2 and 8.6 mm respectively), confirming that the assay was functioning correctly. Whilst *E. coli* is a normal component of the gastrointestinal microflora, some strains are highly virulent and are major causes of food poisoning in humans.[26] Thus, these results highlight the potential of the *L. tridentata* leaf extracts in treating *E. coli* related food poisoning.

Salmonella spp. are one of the most common causes of food poisoning in humans, particularly from eggs and milk, as well as from incorrectly stored meat or poultry products.[26] Notably, the methanolic and aqueous *L. tridentata* leaf extracts were good inhibitors of *S. newport* (ZOIs of 8.4 and 9 mm for the methanolic and aqueous extracts respectively; Figure 2f) and *S. salford* (ZOIs of 9.5 and 7.5 mm for the methanolic and aqueous extracts respectively; Figure 2g), highlighting their potential in the treatment of salmonellosis. Both of the *Salmonella* spp. screened were relatively resistant to ampicillin and tetracycline, with only relatively small ZOIs measured. In contrast, both species were susceptible to the chloramphenicol control, with ZOIs of 9.2 and 9.9 mm measured for *S. newport* and *S. salford* respectively, confirming that the assay was functioning correctly.

Aeromonas hydrophilia is another relatively common cause of food poisoning in humans.[26] This bacterium produces aerolysin (a cytotoxic enterotoxin), as well as several proteins that induce gastroenteritis and diarrhoea. Whilst the methanolic and aqueous *L. tridentata* leaf extracts inhibited the growth of *A. hydrophilia*, the relatively small ZOIs indicate relatively weak inhibitory activity (Figure 2b). Thus, the *L. tridentata* leaf extracts may be of limited use against *A. hydrophilia*-induced gastrointestinal illness. However, *A. hydrohilia* is resistant to most common used clinical antibiotics, making it difficult to treat.[26] Indeed, our studies confirmed that the strain screened herein was completely resistant to ampicillin and tetracycline in the disc diffusion assay, although it was highly susceptible to chloramphenicol. It is therefore possible that the *L. tridentata* leaf extracts may have potential in the treatment of *A. hydrophilia*-induced gastrointestinal illness. The *L. tridentata* leaf extracts were also completely ineffective inhibitors of *Y. entercolitica* growth (Figure 2h), and would therefore not be useful for the treatment of yersiniosis.

Inhibition of the growth of gastrointestinal Gram-negative cocci bacterial pathogens

The Gram-negative coccus bacterium *Shigella sonnei* was included in this study as it is also a relatively common cause of food poisoning in humans.[26] The methanolic and aqueous *L. tridentata* leaf extract were particularly strong inhibitors of *S.*

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 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	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.

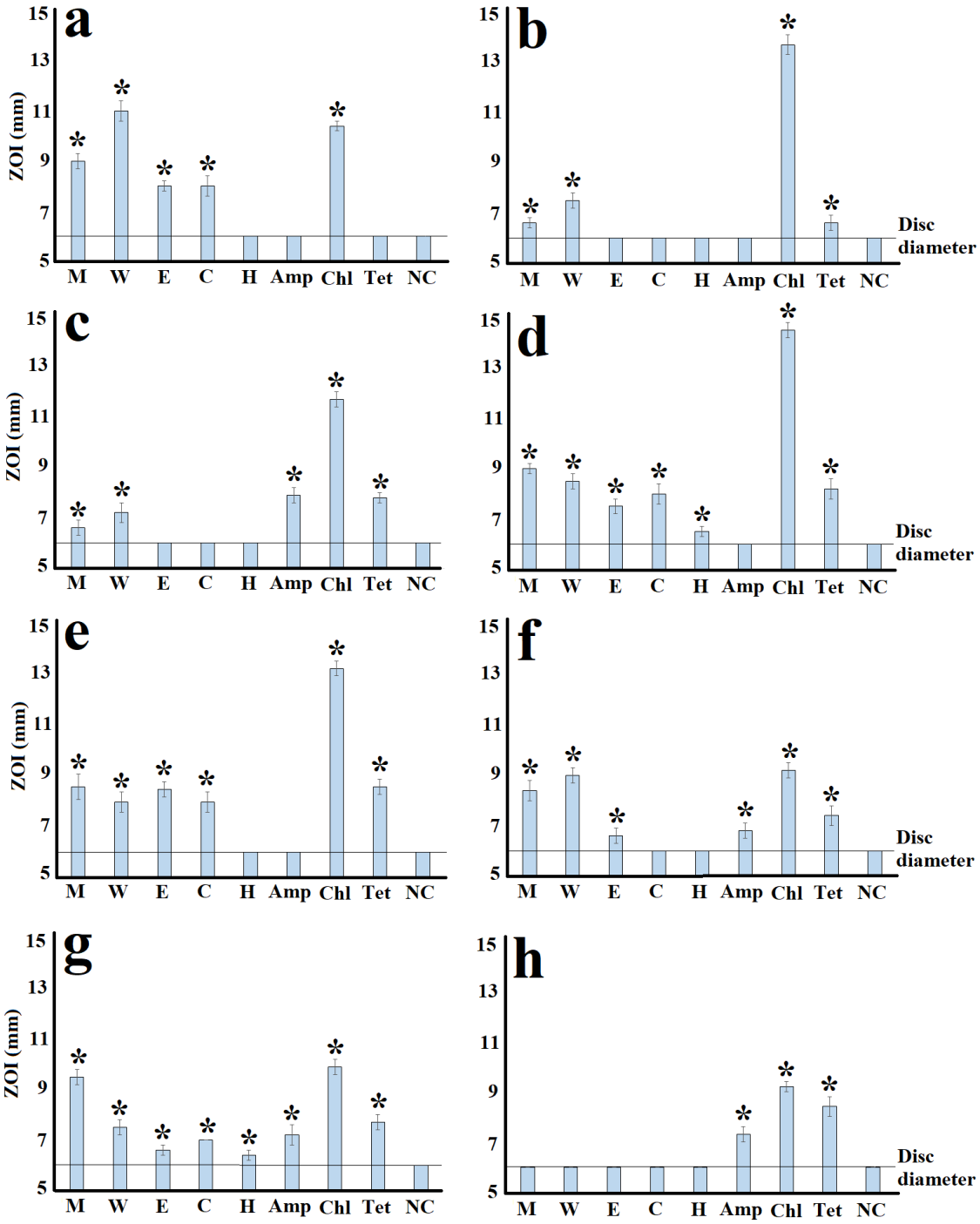


Figure 2: Antibacterial activity of the *L. tridentata* leaf extracts against the Gram-negative rod bacteria (a) *A. faecalis* (clinical strain), (b) *A. hydrophilia* (ATCC7966), (c) *E. cloacea* (ATCC13047), (d) *E. coli* (ATCC0157), (e) *E. coli* (ATCC K12), (f) *S. newport* (clinical strain), (g) *S. salford* (clinical strain), and (h) *Y. entercolitica* (clinical strain) measured as zones of inhibition (mm). ZOI = zone of inhibition; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. Positive controls: Amp = ampicillin (10 µg); Chl = chloramphenicol (10 µg); Tet = tetracycline (10 µg); Negative control (NC) = water. Results are expressed as mean ZOIs of at three replicates, each with internal triplicates ($n = 9$) ± SEM. * indicates results that are significantly different to the negative control ($p < 0.01$).

sonnei growth, with a ZOI of 9 and 12 mm respectively (Figure 3). The ethyl acetate and chloroform extracts also significantly inhibited *S. sonnei* growth, albeit with substantially smaller ZOIs (7.2 and 7.3 mm respectively), indicating lower growth inhibitory activity. However, it is noteworthy that inhibition in this assay is reliant on the diffusion on molecules in the aqueous agar gel environment, which is dependent upon the polarity of the extract components. As ethyl acetate and chloroform are medium to low polarity extracts, measurement of ZOIs in this assay may underestimate the growth inhibitory activity in liquid

environments (such as the gestrointestinal tract), and testing in liquid assays (such as the liquid dilution MIC results presented later in this report) is also required. Notably, the *S. sonnei* strain screened herein was resistant to ampicillin and tetracycline, further highlighting the potential of the *L. tridentata* leaf extracts for the treatment of shigellosis, particularly against antibiotic resistant strains. In contrast, *S. sonnei* was highly susceptible to chloramphenicol (ZOI = 13.8 mm).

Inhibition of the growth of gastrointestinal gram positive bacteria

Bacteria of the genus *Bacillus* can also cause food poisoning and it can be particularly difficult to control their growth in stored food. Whilst refrigeration of food inhibits the growth of many pathogenic bacteria, *Bacillus* spp. are psychrotropic and readily grow at reduced temperatures.[26] Indeed, the retardation of the growth of other bacteria below 5°C, provides a selective advantage for psychrotropic pathogens and *Bacillus*-induced gastrointestinal disease is particularly relevant in refrigerated foods. Additionally, *Bacillus* spp. form heat-resistant endospores, making food processing methods such as pasteurisation relatively ineffective. Therefore, treatments that are effective against *Bacillus* spp. may be particularly useful, both therapeutically and as a method of food preservation. Noteworthy growth inhibitory activity was noted for the methanolic and aqueous *L. tridentata* leaf extracts against *B. cereus* (Figure 4a) and *B. megaterium* (Figure 4b), with ZOIs generally ≥8 mm). The ethyl acetate and chloroform extracts also inhibited the growth of both *Bacillus* spp., although the relatively small ZOIs (<7 mm), indicates only low to moderate activity. Notably, whilst all of the conventional antibiotic controls tested in this study inhibited the growth of both *Bacillus* spp., only relatively small ZOIs were recorded. Thus, the *L. tridentata* leaf extracts may be particularly useful against antibiotic-resistant *Bacillus* strains.

Staphylococcus spp. (particularly *S. aureus*) are particularly common sources of food-bourne illness.[26,27] The *L. tridentata* extracts were also effective inhibitors of *S. aureus* (Figure 4c) and *S. epidermidis* growth (Figure 4d). The methanolic and aqueous extracts displayed the strongest apparent growth inhibitory activity against both *Staphylococcus* spp., with ZOIs generally >8 mm. In contrast, *S. aureus* and *S. epidermidis* were substantially less susceptible to the ethyl acetate and chloroform extracts (ZOIs generally ~7 mm). The hexane extract was completely ineffective against both *Staphylococcus* spp. Notably, the *Staphylococcus* strains screened herein were both resistant to the ampicillin and tetracycline controls, with only small ZOIs recorded. In contrast, *S. aureus* and *S. epidermidis* were both highly susceptible to chloramphenicol (ZOIs of 13.8 and 8 mm respectively). Therefore, the inhibitory activity of the *L. tridentata* extracts (especially the methanolic and aqueous extracts) indicates that these extracts may be particularly useful for treating *Staphylococcus*-induced

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		*A. faecalis*		*A. hydrophilia*		*B. cereus*		*B. megaterium*		*E. cloacea*		*E. coli 0157*		*E. coli K12*		*S. newport*		*S. salford*		*S. aureus*		*S. epidermidis*		*S. sonneii*		*Y. entercolitica*	
		DD	LD	DD	LD	DD	LD	DD	LD	DD	LD	DD	LD	DD	LD	DD	LD	DD	LD	DD	LD	DD	LD	DD	LD	DD	LD
Extracts	M	2240	1860	3630	2375	1463	705	2286	1630	>5000	>5000	946	705	1138	650	2256	1560	1626	1410	772	529	926	705	>5000	2820	-	-
	W	1422	755	1860	1280	1032	405	860	260	>5000	1880	1562	926	1865	1160	2766	1860	2543	1866	983	286	1156	488	463	86	-	-
	E	1250	940	-	-	>5000	>5000	-	-	-	-	880	463	922	470	>2500	>2500	>2500	>2500	588	351	453	239	1586	940	-	-
	C	514	254	-	-	2203	1550	246	125	-	-	472	286	500	325	-	-	1566	1173	511	254	684	254	1080	702	-	-
	H	-	-	-	-	-	-	-	-	-	-	>5000	>5000	-	-	-	-	>5000	>5000	-	-	-	-	-	-	-	-
Conventional Antibiotics	Pen	ND	3.3	ND	3.3	ND	2.5	ND	2.5	ND	2.5	ND	-	ND	-	ND	3.3	ND	2.3	ND	3.3	ND	3.3	ND	-	ND	2.5
	Chl	ND	1.3	ND	1.3	ND	1.3	ND	1.3	ND	1.3	ND	1.3	ND	1.3	ND	2.5	ND	1.3	ND	1.3	ND	1.3	ND	1.3	ND	1.3
	Ery	ND	1.3	ND	1.3	ND	1.3	ND	1.3	ND	2.5	ND	2.5	ND	2.5	ND	1.3	ND	2.5	ND	1.3	ND	1.3	ND	2.5	ND	2.5
	Tet	ND	3.3	ND	3.3	ND	1.3	ND	1.3	ND	0.6	ND	1.3	ND	1.3	ND	1.3	ND	1.3	ND	1.3	ND	1.3	ND	1.3	ND	1.3
	Cip	ND	1.3	ND	1.3	ND	1.3	ND	1.3	ND	0.6	ND	1.3	ND	1.3	ND	1.3	ND	0.6	ND	1.3	ND	0.6	ND	1.3	ND	1.3
	Gen	ND	0.3	ND	0.3	ND	0.6	ND	0.6	ND	0.3	ND	0.6	ND	0.6	ND	0.6	ND	0.6	ND	0.3	ND	0.3	ND	0.6	ND	0.6

DD = MIC in the disc diffusion assay; LD = 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; Gen = 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. Noteworthy antibacterial activity is highlighted within the table.

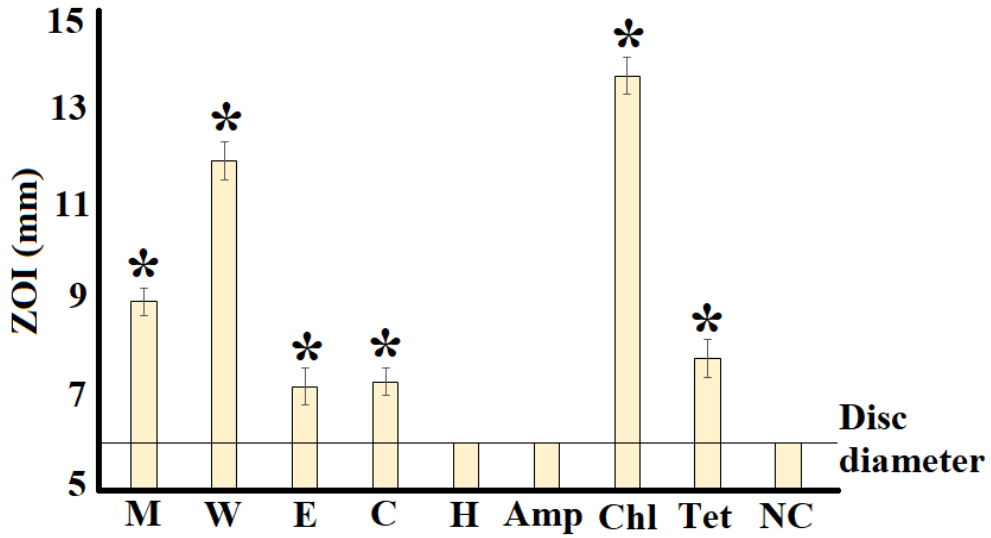


Figure 3: Antibacterial activity of the *L. tridentata* leaf extracts against the Gram-negative coccus bacteria *S. sonnei* (ATCC 25931) measured as zones of inhibition (mm). ZOI = zone of inhibition; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. Positive controls: Amp = ampicillin (10 µg); Chl = chloramphenicol (10 µg); Tet = tetracycline (10 µg); Negative control (NC) = water. Results are expressed as mean ZOIs of at three replicates, each with internal triplicates (n = 9) ± SEM. * indicates results that are significantly different to the negative control ($p < 0.01$).

gastrointestinal illness, including against antibiotic-resistant *Staphylococcus* 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 and solid-phase MIC assays (Table 2). MIC values >1 µg/mL for the pure conventional antibiotic standards have previously been defined as indicative of antibiotic resistance.[20,28] Notably, all of the bacterial strains tested were resistant to penicillin-G, chloramphenicol and erythromycin. Additionally, with the exception of *E. cloaceae*, all bacterial gastrointestinal pathogens were also resistant to tetracycline. Gentamycin was the most effective conventional antibiotic, with all bacteria susceptibile (MIC values 0.3-0.6 µg/mL). Ciprofloxacin was also effective against all bacterial strains except *E. cloaceae*.

The *L. tridentata* extracts displayed noteworthy growth inhibitory activity against several bacterial strains, although the hexane extract was ineffective against all bacterial pathogens (Table 2). The chloroform *L. tridentata* extract was the most potent inhibitor of the majority of bacterial pathogens, with LD MICs as low as 125 µg/mL recorded against *B. megaterium*. The relatively low polarity of this solvent indicates that some of the antibacterial *L. tridentata* extract components are of relatively low polarity. The methanolic, aqueous and ethyl acetate extracts were also good inhibitors of many of the gastrointestinal bacterial pathogens. Indeed, the lowest MIC value (86 µg/mL) was determined for the aqueous *L. tridentata* leaf extract against *S. sonnei,* indicating that polar phytochemicals may be responsible for growth inhibitory activity against *S. sonneii*. It is therefore likely that different compounds are responsible for the inhibitory activity in different bacterial cells.

All of the *L. tridentata* leaf extracts were ineffective against *E. cloaceae*. This is a noteworthy finding as *E. cloaceae* is a normal component of the normal human gastrointestinal microbiome.[26] Anti-diarrhoeal chemotherapies should ideally inhibit the growth of pathogenic bacteria, without upsetting the balance of the normal gastrointestinal microbiome. Decreases in the levels of normal and beneficial bacteria may provide selective advantages for pathogenic bacteria to grow by removing competing bacterial species. This indicates that the *L. tridentata* leaf extracts may be particularly useful for treating gastrointestinal disease, without inducing further negative effects, although this remains to be verified *in vivo. Alcaligenes faecalis* was also included in this study as it is also generally considered non-pathogenic (although it can cause gastrointestinal distress in immunocompromised people).[26] In contrast to the *E. cloaceae* susceptibility profile, *A. faecalis* was significantly inhibited by the aqueous, ethyl acetate and chloroform extracts. This may impact on the usefulness of these extracts to treat gastrointestinal diseases clinically. Further studies are required to evaluate the effects of *L. tridentata* leaf extracts against other bacterial components of the gastrointestinal microbiome, and to evaluate the effects *in vivo*.

Quantification of toxicity

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 % following 24 hours of exposure to the *Artemia* nauplii.[24,25] 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

Diarrhoea remains a leading killer of children, accounting for 8% of all death among children under the age of 5 annually.[29] Indeed, more than half a million children in this age group die each year from diarrhoeal disease,[1,29] which equates to over 1400 deaths per day. Furthermore, as bacterial pathogens develop further resistance to conventional antibiotics, medicines are expected to have reduced efficacy against diarrhoeal diseases and corresponding increases in mortality rates are expected.[3,30,31] There is an urgent need to develop new therapeutic options to combat these illnesses, either through the development of novel drug molecules, or by utilizing new therapy regimens that enable (or re-purpose) previously effective antibiotics that have lost their potency due to the emergence of highly resistant infections.

Plants are a promising source of new therapies to treat gastrointestinal diseases and numerous traditional medicines have been reported to be effective in the treatment of gastrointestinal illness.[32-34] *Larrea tridentata* leaves been used for thousands of years by several native American groups to treat multiple pathogenic illnesses, gastrointestinal distress and diarrhoea.[7,10] However, the antibacterial properties of *L. tridentata* leaf extracts have not been extensively reported and much more work is required. This report aimed to address this gap in the literature by screening *L. tridentata* leaf extracts against clinically relevant bacterial gastrointestinal pathogens, thereby evaluating their potential for the treatment of gastrointestinal illness in humans.

The bacteria screened in this study were selected because they are either causes of food poisoning and diarrhoea, or are normal components of the human gastrointestinal microbiome. *Staphylococcus* spp. (especially *S. aureus*) are common sources of food borne diseases globally.[26,27] *Bacillus* spp.[26,35] *A. hydrophilia*[26] and *E. coli*[36] produce and secrete enterotoxins and other proteins, which may cause gastroenteritis and diarrheal diseases. Notably, many of these toxins are heat stable and are thus not readily destroyed by heat treatments/pasteurisation. *Staphylococcus* spp. were also included in this study as they are one of the most common

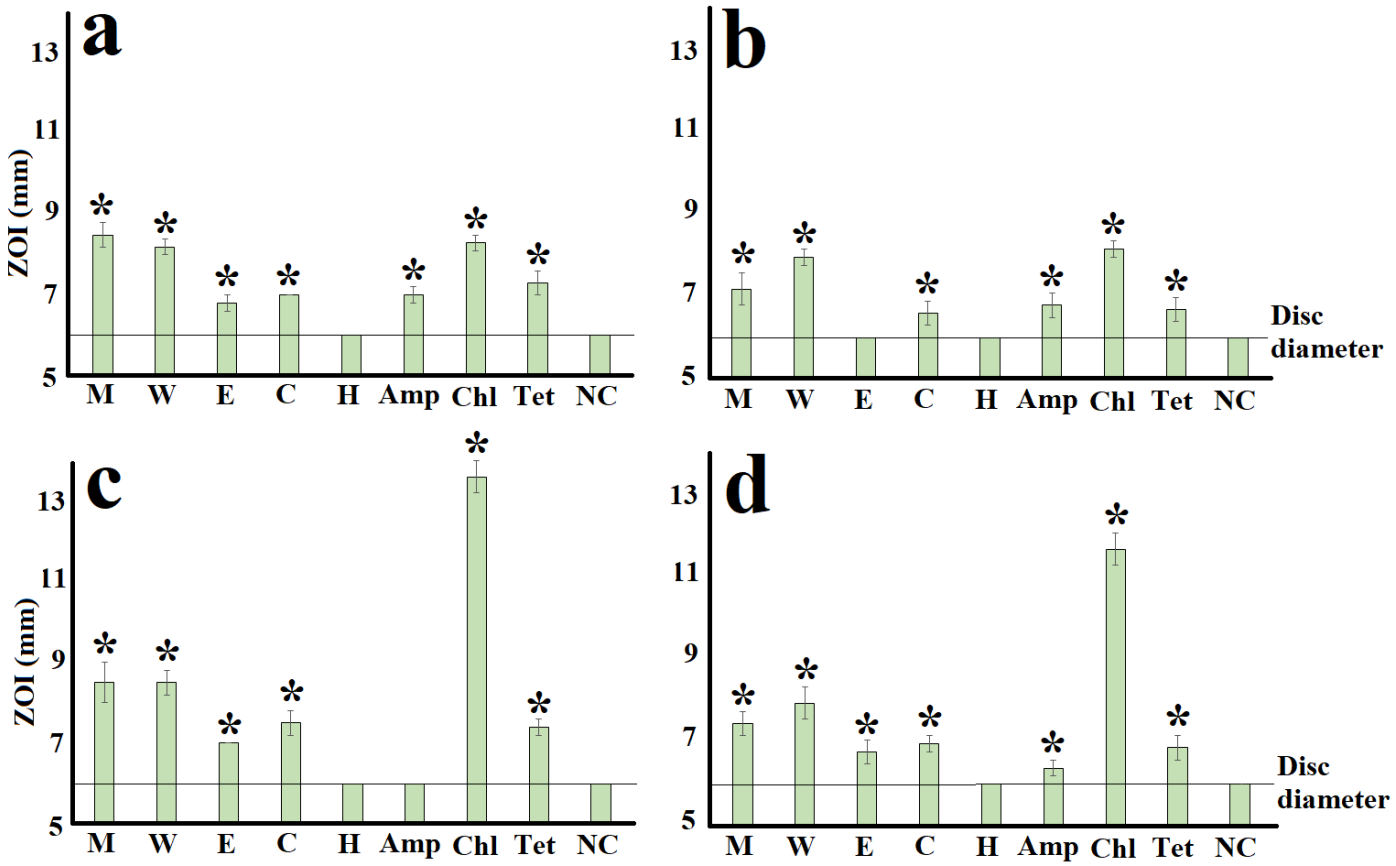


Figure 4: Antibacterial activity of the *L. tridentata* leaf extracts against the gram positive bacteria (a) *B. cereus* (ATCC14579), (b) *B. megaterium* (ATCC14581), (c) *S. aureus* (ATCC25923), (d) *S. epidermidis* (clinical strain) measured as zones of inhibition (mm). ZOI = zone of inhibition; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. Positive controls: Amp = ampicillin (10µg); Chl = chloramphenicol (10µg); Tet = tetracycline (10µg); Negative control (NC) = water. Results are expressed as mean ZOIs of at three replicates, each with internal triplicates ($n = 9$) ± SEM. * indicates results that are significantly different to the negative control ($p < 0.01$).

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:
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.

source of food poisoning worldwide.[26,27] Notably, *S. aureus* and *S. epidermidis* were substantially inhibited by the *L. tridentata* leaf extracts, despite these bacterial strains being resistant to most of the conventional antibiotics tested. Notably, with the exception of *Y. entercolitica*, all pathogenic bacteria screened in our study were inhibited by 2 or more of the *L. tridentata* leaf extracts, with potent activity recorded for all extracts except the hexane extract. However, the susceptibility profile varied greatly between bacterial species. For example, *Bacillus megaterium* was particularly susceptible to the chloroform *L. tridentata* leaf extract (LD MIC = 125 µg/mL), whilst the aqueous extract was most effective against *S. sonnei* (LD MIC = 86 µg/mL).

The ability of the *L. tridentata* leaf extracts to inhibit the growth of psychrotrophic bacteria in our study was particularly interesting. Food is frequently stored at temperatures below 5°C to retard bacterial growth. However, even psychrotrophic bacteria (e.g. *A. hydrophilia, E. coli* and *Bacillus* spp.) readily grow at these temperatures, thereby compromising the safety of cold-stored foods.[35-38] Therefore, antibacterial agents that inhibit the growth of psychrotrophic bacteria may be particularly useful. Notably, the *L. tridentata* leaf extracts significantly inhibited the growth of all of the psychrotrophic bacteria tested in this study. The growth inhibitory activity of the *L. tridentata* leaf extracts against spore forming *Bacillus* spp. was also noteworthy. Notably, our study focussed on the growth inhibitory properties of the *L. tridentata* leaf extracts on aerobic gastrointestinal bacterial pathogens. However, the anaerobic spore forming bacteria *Clostridium botulinium* also causes substantial incidences of food poisoning and is of considerable concern due to the severity of the symptoms caused by botulism poisoning.[27] Future studies into the potential of *L. tridentata* leaf extracts to treat food poisoning and gastrointestinal illness should also screen the extracts against anaerobic gastrointestinal pathogens, including *C. botulinium*.

The individual antibiotic extract components in the *L. tridentata* leaf extracts were not identified in this study. However, the qualitative screening studies noted several commonalities between the extracts with greatest bacterial growth inhibitory activity, corresponding to extracts that generally contained relatively high levels of tannins and flavonoids. The potent antibacterial activities of tannin compounds are well known. Indeed, strong growth inhibitory activity against a broad spectrum of bacterial species has been reported for numerous gallotannins.[39] Furthermore, several antibacterial mechanisms for gallotannins have been reported, including binding cell surface lipotoichoic acid and proline-rich cell surface proteins, and via inhibition of glucosyltransferase enzymes.[40,41] Elligitannins are also highly potent inhibitors of bacterial growth and function via several antibiotic mechanisms, including interaction with cytoplasmic oxidoreductases, and by disrupting bacterial cell walls.[39] Thus, it is likely that *L. tridentata* leaf extract tannins may contribute to the antibacterial activity of the extracts reported in our study. However, it is likely that other phytochemical classes may also contribute to the growth inhibitory properties of these extracts, either via direct antibacterial activity, or as potentiators of the activity of other components. Further phytochemical evaluation studies and bioactivity driven isolation of *L. tridentata* leaf extract components is required to further evaluate the mechanism(s) of bacterial growth inhibition.

Notably, none of the *L. tridentata* leaf extracts were toxic when tested in the *Artemia franciscana* nauplii toxicity assay, although further testing in cell line studies is also required before these extracts are considered for clinical usage. Furthermore, our studies only examined the effects of the *L. tridentata* leaf extracts against planktontic bacteria. Biofilms, which occurs when bacteria attach to tissue surfaces (e.g. the gastrointestinal lumen) are generally substantially more difficult to treat than planktonic infections. The biofilms structure renders it resistant to the host's immune system and protects the individual bacteria in the biofilm from exogenous antibiotics.[42] This aspect of bacterial colonisation *in vivo* may greatly affect susceptibility and this requires substantially more attention. As bacteria in biofilms generally have MIC values at least an order of magnitude higher than in liquid cultures, it would be interesting to test whether the *L. tridentata* leaf extracts also block biofilm formation of the bacterial pathogens examined in this report and future studies are planned to examine this.

CONCLUSION

The results of this study demonstrate the potential of *L. tridentata* leaf extracts as inhibitors of the growth of some bacterial species associated with gastrointestinal disaease and diarrhoea. Additionally, the *L. tridentata* leaf extracts were nontoxic, further indicating their suitability for therapeutic usage. Further studies are required to isolate the inhibitory compounds and identify their antimicrobial mechanisms.

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SUMMARY

- *Larrea tridentata* leaf extracts were screened for growth inhibitory activity in the disc diffusion assay against gastrointestinal bacterial pathogens.
- 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 *L. tridentata* leaf extracts was evaluated using the *Artemia* nauplii bioassay.

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