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Original Article

Antibacterial Activity and Toxicity Profiles of Selected Medicinal Plant Extracts and Conventional Antibiotics against Bacterial Triggers of Some Autoimmune Diseases

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ABSTRACT

Background: *Nigella sativa* L., *Anongessius latifolia* (Roxb. ex DC.) Wall. ex Euill. and Perr. and shilajit have been used traditionally to treat numerous infectious diseases, including many caused by bacterial pathogens. However, extracts of these traditionally medicines have been poorly studied and are yet to be tested for the ability to inhibit the growth of bacterial triggers of multiple sclerosis and rheumatic fever. **Materials and Methods:** Antimicrobial activity of selected plant extracts was assessed using disc diffusion and liquid dilution minimum inhibitory concentration (MIC) assays against some bacterial triggers of multiple sclerosis and rheumatic fever. Interactions between the extracts and conventional antibiotics were studied and classified using the sum of the fractional inhibitory concentration (∑FIC). The toxicity of the individual samples and the combinations was assessed using the artemia lethality assay (ALA) assay. **Results:** The methanolic *A. latifolia* extract displayed notable antibacterial activity against the bacterial triggers of multiple sclerosis (*A. baylyi* and *P. aeruginosa*), and rheumatic fever (*S. pyogenes*). Furthermore, combining the methanolic *A. latifolia* extract with tetracycline and chloramphenicol resulted in potentiation of the inhibitory activity against *P. aeruginosa* and *S. pyogenes*. None of the individual components (nor the combinations) were toxic in the ALA assay. **Conclusion:** The *A. latifolia* methanolic displayed clinically relevant antibacterial activity against *A. baylyi, P. aeruginosa* and *S. pyogenes* when tested alone. Furthermore, that extract also potentiated the activity of tetracycline and chloramphenicol against some bacteria. The lack of toxicity of the extracts and combinations indicates that these combinations may provide leads in the development of new therapies to prevent and treat the autoimmune diseases multiple sclerosis and rheumatic fever.

Keywords: Medicinal plants, Black caraway, Multiple sclerosis, Rheumatic fever, Conventional antimicrobials, Synergy, Drug interaction, Toxicity.

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INTRODUCTION

Autoimmune inflammatory disorders are a group of debilitating conditions including rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever that afflict genetically susceptible individuals.[1,2] There are no cures for these disorders. Instead, current treatment strategies aim to alleviate the symptoms (particularly pain, swelling and inflammation) with analgesics and anti-inflammatory agents and/or to modify the disease process through the use of disease modifying drugs. None of these treatments is ideal as prolonged usage of these drugs is often accompanied by unwanted side effects and toxicity.[1,2] There is a need to develop safer, more effective treatments for these conditions which will not only alleviate the symptoms, but may also cure or prevent the disease. These autoimmune disorders may be triggered in susceptible individuals by specific microbial infections. Serotyping studies have identified several of the bacterial triggers of these conditions and the bacterial antigens responsible for the induction of an immune response. The major microbial triggers of multiple sclerosis have been identified *Acinetobacter baylyi* and *Pseudomonas aeruginosa*.[1,2] Similarly, *Streptococcus pyogenes* has been identified as a trigger for rheumatic fever in genetically susceptible people.[2] The development of antibiotic agents targeting specific bacterial triggers of these disorders would enable afflicted individuals to prevent the onset of these diseases and to reduce the severity of the symptoms once the disease has progressed.

Whilst antibiotics are available to treat infections of these bacteria, the development of multiple antibiotic resistant

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bacterial strains has rendered multiple clinical antibiotics of decreased efficacy, or in some cases, has rendered the antibiotics completely effective.[3] The development of alternative treatment methods is crucial and is considered by the World Health Organisation (WHO) to be one of the biggest challenges facing medical science.[4] For a number of reasons reviewed elsewhere,[3] it is unlikely that the current methods of antibiotic discovery/development will be as successful in the future. Instead, examination of traditional medicines for natural compounds with therapeutic properties may generate new drug leads for the development of new antibiotics. Despite this, relatively few plant-derived antibiotic compounds are in common use clinically. This may be because synergistic interactions are often required to potentiate the antibacterial activity and purified plant phytochemicals often have much lower activity than the crude extract(s) that they are derived from.[5] A combinational approach that allows synergistic interaction between plant extracts (or pure plant compounds) and conventional antibiotics may be more effective in combatting bacterial pathogens, especially in antibiotic resistant strains.[6-8] Combinational therapy is already preferred over mono-therapy in multiple life-threatening infectious diseases such as malaria, tuberculosis and HIV/AIDS due to its ability to target multiple facets of a disease and to curb resistance.[3,4] A combination of plant extracts/isolated compounds with conventional antibiotics may also prove to have an economic advantage.[5] Developing a new drug requires years of extensive and costly testing. However, combinational therapy can potentially restore an existing drug to a state of significantly reduced resistance, thereby bypassing the lengthy and expensive process of discovering new antibiotic agents. Further advantages of synergistic interactions include increased efficiency, reduced side effects, increased stability and bioavailability and the requirement for lower doses in comparison to synthetic alternatives.[5]

Two plants and a rock exudate were selected for screening in this study due to their widespread uses in traditional medicine. *Nigella sativa* L. (family Ranunculaceae; synonyms *Nigella cretica* Mill., *Nigella indica* Roxb., *Nigella truncata* Viv.; common names black caraway, black cumin) is widely used throughout the world in traditional medicine. It is particularly well known in South Asian healing systems Ayurveda, Siddha and Unani for its anti-hypertensive, anti-diarrhoeal, antibacterial and analgesic properties, as well as its beneficial effects against skin diseases.[9] Several studies have confirmed the antibacterial properties of *N. sativa* seed extracts against multiple bacteria. One study that used a disc diffusion assay reported inhibitory activity against *Staphylococcus aureus*.[10] However, that study screened a very high concentration extract (300 mg/mL) and did not determine an MIC value, making comparisons with other studies impossible. As MIC values >5 mg/mL are generally classified as non-inhibitory in disc diffusion assays[11,12] and as only a single, high concentration was tested, it is not possible to classify the antibacterial properties of the seeds on the basis of that study. A subsequent study tested similar extracts against a Methicillin Resistant *S. aureus* (MRSA) strain and reported strong inhibitory activity (MIC 200-500 μg/mL).[13] Another study screened *N. sativa* seed extracts against an extended panel of 16 gram negative and 6 g positive bacteria,[14] although MIC values were also not reported in that study.

Anongessius latifolia (Roxb. ex DC.) Wall. ex Euill. and Perr. (family Combretaceae; common name axlewood) has also been used traditionally to treat bacterial infections. Bark extracts of this species are used in several Indian traditional medicine systems to treat a wide variety of gastrointestinal and skin infections.[15] Notably, *A. latifolia* bark extracts have been reported to inhibit the growth of *Bacillus subtilis, S. aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumonia, Proteus mirabilis* and *Micrococcus* spp.[16] Unfortunately that study reports zones of inhibition in a well diffusion assay, making comparisons with other studies difficult. Substantially more studies are required to validate the traditional uses of this species and to quantify its efficacy against important human bacterial pathogens.

Shilajit (also known as Mumijo) is a viscous black-brown tar or resin that exudes from rocks in high mountainous regions, particularly in the Himalayas. Whilst it is similar in appearance to an inorganic tar, shilajit is instead produced from the decomposition of latex/resin-bearing plants including *Euphorbia royleana* Boiss. and *Trifolium repens* L.[17,18] It is used in traditional Indian healing systems as a tonic, laxative, expectorant, diuretic, immunomodulatory, anti-hypertensive and as an analgesic. It also has antibacterial properties when applied to the skin.[18] Most frequently, shilajit is used as a component of herbal combinations and it is believed that it may function as an adjuvant. Despite the widespread use of *N. sativa* seeds, *A. latifolia* bark and shilajit in the treatment of bacterial infections, they have been relatively poorly examined for their antibacterial activities. Our study aims to extend the previous studies by screening extracts of these traditional medicines against some bacterial triggers of multiple sclerosis (*Acinetobacter baylyi* and *Pseudomonas aeruginosa*[1]) and rheumatic fever (*Streptococcus pyogenes*[2]). Furthermore, unlike many of the earlier studies examining the antibacterial activity of these traditional medicines, our study also quantifies the antibacterial potency by two methods, and evaluates the toxicity of the extracts.

MATERIALS AND METHODS

Sourcing and preparation of plant samples

The dried *Nigella sativa* L. seeds and *Anongessius latifolia* (Roxb.) ex DC.) Wall. and Perr, bark and the shilajit powder used in this study was a kind gift from Prof Luay Rashan, Dhofar University, Oman. Individual 1 g masses of each material were weighed into separate tubes and 50 mL of AR grade methanol (Ajax Fine Chemicals, Australia) or deionised water were added. The powders were extracted for 24 hr at 4ºC with gentle shaking

and the extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum. The methanolic extracts were air dried at room temperature in the shade. The aqueous extracts were lyophilised by freeze drying at -50ºC. The resultant dried extracts were weighed to determine the extraction yield and were dissolved in 10 mL of deionised water (containing 1% DMSO).

Qualitative phytochemical analysis

Phytochemical analysis of the extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved using standard assays.[19-21]

Antibacterial analysis

Conventional antibiotics

Chloramphenicol (≥98% purity by HPLC), erythromycin (potency ≥850 µg/mg), tetracycline (≥95% purity by HPLC), gentamicin (potency of 600 µg/mg) and ciprofloxacin (≥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 4ºC until use. For the disc diffusion studies, ampicillin (10 µg), tetracycline (10 µg) and chloramphenicol (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 multiple sclerosis or rheumatic fever in genetically susceptible individuals.[1,2] Reference strains of *Acinetobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Type Culture Collection, USA. A *Streptococcus pyogenes* clinical isolate strain was provided by the School of Environment and 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 37ºC for 24 hr and were subcultured and maintained in nutrient broth at 4ºC until use.

Evaluation of bacterial susceptibility to growth inhibition

The susceptibility of the bacteria to the extracts and the conventional antibiotics was initially assessed using a modified disc diffusion assay.[22,23] 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.[24] 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[25-27] was used to evaluate the antimicrobial activity of the extracts and conventional antimicrobials independently and in combinations. Briefly, 100 µL of sterilized distilled water was dispensed into each well of 96 well micro-titre plates. The plant samples and conventional antibiotics (100 µL) were then added into separate wells of the first row of the plate. The conventional antibiotics were introduced at a starting concentration of 0.01 mg/mL. A negative control (nutrient broth), a sterile control (without bacteria) and a sample-free culture control (to ensure the media was capable of supporting microbial growth) were included on all plates. After addition of the test samples to the plate, each was serially diluted by doubling serial dilution. The relevant bacterial culture inoculum (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 37ºC. 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 30ºC. 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.[28,29] Graphs of the zone of inhibition (ZOI) versus Ln of the concentration were plotted and MIC values were calculated using linear regression.

Fractional Inhibitory Concentration (FIC) assessment

Interactions between combinations of the extracts and conventional antibiotics were further classified using the sum of the fractional inhibitory concentration (ΣFIC). The FIC was calculated using the following equation, where (a) represents the extract and (b) the conventional antimicrobial sample:[24-26]

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

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

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).[24-26]

Artemia franciscana Lethality Assay (ALA)

Toxicity of the extracts, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.[30,31] 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 LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Statistical analysis

Data is expressed as the mean±SEM. of at least three independent experiments, each with internal triplicates (n=9). One-way ANOVA was used to calculate statistical significance between the negative control and treated groups with a p<0.01 considered to be statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extractions of the dried plant material (1 g) with solvents of varying polarity yielded dried plant extracts ranging from 128 mg (aqueous *Anongessius latifolia* leaf extract) to 225 mg (methanolic *Nigella sativa* seed extract) (Table 1). Qualitative phytochemical screening (Table 1) showed that the higher polarity solvents (methanol and water) extracted the greatest amount and widest diversity of phytochemical classes.

Bacterial growth inhibition screening

Acinetobacter baylyi growth was particularly susceptible to the methanolic *A. latifolia* bark extract, with a zone of inhibition (ZOI) of 9.5 mm measured (Figure 1). The inhibition of *A. baylyi* growth by the aqueous *A. latifolia* extract was also noteworthy (ZOI=8.6 mm), albeit significantly less potent (as judged by ZOIs). The *A. baylyi* growth inhibition by the *A. latifolia* extracts compares favourably to the inhibition by the positive control. Indeed, the ampicillin control produced a 7.2 mm ZOI. In contrast, *A. baylyi* was highly susceptible to the chloramphenicol control, with a ZOI of 11.4 mm. Notably the antibiotic controls are pure compounds, whereas the extracts are crude mixtures and the antibiotic components in these extracts may account for only a low percentage of the extracted material. Therefore, the inhibition by the *A. latifolia* extracts may be particularly useful for controlling *A. baylyi* infections. In contrast, the methanolic

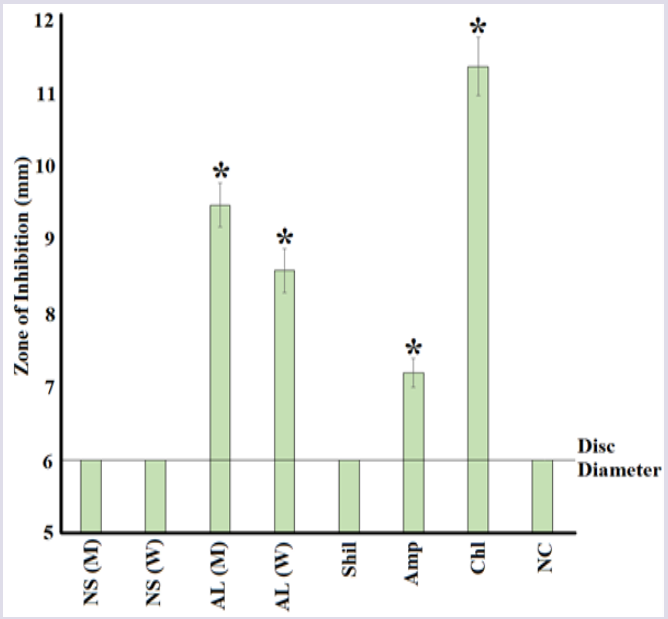

Figure 1: Antibacterial activity of selected extracts against *A. baylyi* (ATCC33304) measured as zones of inhibition (mm). M=Methanolic extract; W=aqueous extract; NS=*N. sativa*; AL=*A. latifolia*; Shil=Shilajit; Amp=Ampicillin (10 µg); Chl=Chloramphenicol (10 µg); NC=Negative control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates±SEM. * indicates results that are significantly different to the negative control (p<0.01).

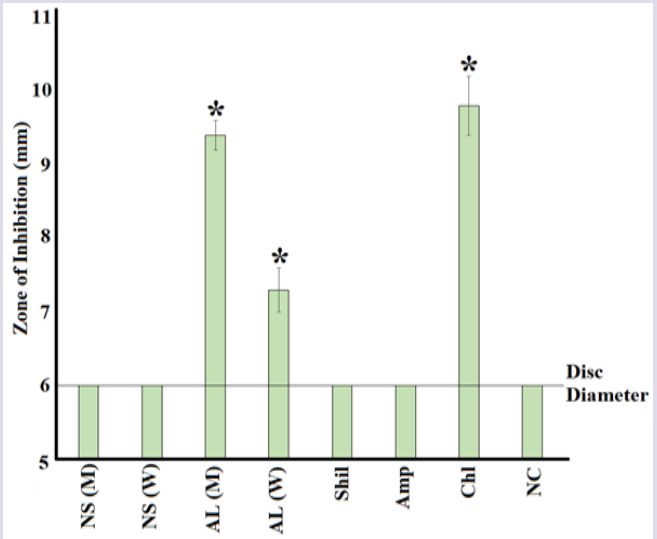

Figure 2: Antibacterial activity of selected extracts against *P. aeruginosa* (ATCC39324) measured as zones of inhibition (mm). NS=M=Methanolic extract; W=aqueous extract; NS=*N. sativa*; AL=*A. latifolia*; Shil=Shilajit; Amp=Ampicillin (10 µg); Chl=Chloramphenicol (10 µg); NC=Negative control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates±SEM. * indicates results that are significantly different to the negative control (p<0.01).

and aqueous *N. sativa* extracts, and the shiljara suspension were completely ineffective against *A. baylyi*.

The *A. latifolia* extracts were also effective inhibitors of *P. aeruginosa* growth (Figure 2). The methanolic *A. latifolia* extracts was a substantially better inhibitor of *P. aeruginosa* growth than the corresponding aqueous extract (ZOIs of 9.4 and 7.3 mm respectively). All other extracts were completely devoid of *P. aeruginosa* growth inhibitory activity (as judged by ZOIs). It is noteworthy that the *P. aeruginosa* strain tested in this study was relatively resistant to the control antibiotics. Indeed, the ampicillin control was completely inactive against this bacterium. Similarly, previous studies in our group have reported that this bacterial strain is resistant to several other antibiotics, as well as to other plant extracts with reported antibacterial activity.[32-34] A different trend was noted for *S. pyogenes* inhibition (Figure 3). Whilst the methanolic *A. latifolia* bark extract was the most potent inhibitor of the growth of this bacterium, the *N. sativa* extracts also inhibited the growth, albeit with substantially smaller ZOIs (~7 mm). As noted for the inhibition profiles of the other bacteria, shilajit was completely ineffective against *S. pyogenes* growth.

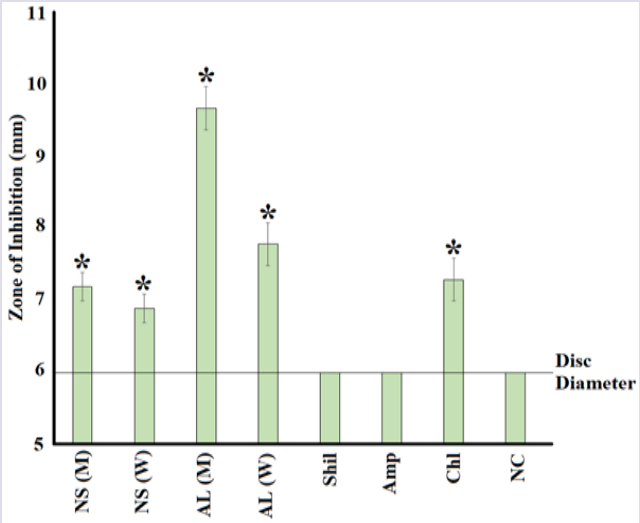

Figure 3: Antibacterial activity of selected extracts against *S. pyogenes* (clinical isolate strain) measured as zones of inhibition (mm). M=Methanolic extract; W=aqueous extract; NS=*N. sativa*; AL=*A. latifolia*; Shil=Shilajit; Amp=Ampicillin (10 µg); Chl=Chloramphenicol (10 µg); NC=Negative control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates±SEM. * indicates results that are significantly different to the negative control (p<0.01).

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 and disc diffusion MIC assays (Table 2). All bacterial strains tested were susceptible to the *A. latifolia* extracts, with the methanolic extract generally being a much better inhibitor of bacterial growth than the aqueous extract. The methanolic *A. latifolia* extract was a particularly good inhibitor of *P.*

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

Extract	Mass of Dried Extracted Material (mg)	Concentration of extract (mg/mL)	Phenols			Cardiac	Saponins	Triterpenes	Phytosterols	Alkaloids		Flavonoids		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
Nigella sativa (M)	225	22.5	+++	+++	++	-	-	+	-	-	-	+++	+	++	-	-
Nigella sativa (W)	160	16	+++	+++	++	-	-	-	-	-	-	++	+	++	-	-
Anongessius latifolia (M)	144	14.4	+++	+++	+	-	-	+	-	-	-	+++	++	++	-	-
Anongessius latifolia (W)	128	12.8	+++	+++	-	-	-	-	-	-	-	+++	++	++	-	-
Shilajit (W)	230	23	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay; M=Methanolic extract; W=aqueous extract/solution.

Table 2: Disc diffusion (DD) and liquid dilution (LD) MIC values (µg/mL) of the extracts against microbial triggers of multiple sclerosis and rheumatic fever.

Extract	*A. baylyi*		*P. aeruginosa*		*S. pyogenes*	
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC
Nigella sativa (M)	-	-	-	-	2253	1560
Nigella sativa (W)	-	-	-	-	3550	1840
Anongessius latifolia (M)	1683	1150	1086	825	1008	780
Anongessius latifolia (W)	2030	1496	2237	1850	1746	1445
Shilajit (W)	-	-	-	-	-	-
Controls						
Erythromycin	ND	1.25	ND	3.3	ND	3.3
Tetracycline	ND	2.5	ND	1.25	ND	2.5
Chloramphenicol	ND	1.25	ND	1.25	ND	2.5
Ciprofloxacin	ND	0.62	ND	1.36	ND	0.63
Gentamicin	ND	0.31	ND	0.62	ND	0.63

M=Methanol extract; W=Water extract; DD=Disc Diffusion; LD=Liquid Dilution; - indicates no inhibition at any dose tested; ND=an MIC was not determined in the DD assay.

Table 3: ΣFIC values for the extracts and conventional antibiotic combinations against susceptible bacteria.

Bacteria	Extract	Erythromycin	Tetracycline	Chloramphenicol	Ciprofloxacin	Gentamicin
A. baylyi	AL (M)	1.46	1.85	1.38	4.11	3.25
		(IND)	(IND)	(IND)	(ANT)	(IND)
	AL (W)	1.58	2.13	1.5	3.83	2.92
		(IND)	(IND)	(IND)	(IND)	(IND)
P. aeruginosa	AL (M)	1.38	0.45	0.55	1.29	3.62
		(IND)	(SYN)	(ADD)	(IND)	(IND)
	AL (W)	1.54	1.13	1.66	2.17	2.22
		(IND)	(IND)	(IND)	(IND)	(IND)
S. pyogenes	NS (M)	3.27	2.73	0.87	3.13	3.62
		(IND)	(IND)	(ADD)	(IND)	(IND)
	NS (W)	3.55	2.25	1.24	2.85	3.88
		(IND)	(IND)	(IND)	(IND)	(IND)
	AL (M)	2.24	0.37	0.73	4.07	2.18
		(IND)	(SYN)	(ADD)	(ANT)	(IND)
	AL (W)	1.63	1.16	1.82	2.48	2.67
		(IND)	(IND)	(IND)	(IND)	(IND)

M=Methanolic extract; W=aqueous extract; NS=N. sativa; AL=A. latifolia; Shil=Shilajit; SYN=synergy; ADD=Additive Interaction; IND=Indifferent Interaction; ANT=Antagonism.

aeruginosa growth, with an MIC of 825 µg/mL (in the liquid dilution assay). This is particularly noteworthy as this bacterial strain is a multi-drug resistant strain. Indeed, as MIC values for pure antibiotics in this assay >1 µg/mL have been defined as indicative of resistance,[24] this *P. aerogenosa* strain was determined to be resistant to all of the conventional antibiotics tested except gentamicin. Previous studies in our group have also confirmed the resistance of this bacterium to these (and other) antibiotics.[35-37] Similarly, the methanolic *A. latifolia* bark extract also had noteworthy inhibitory activity against *A. baylyi*, albeit with higher MIC values (1150 µg/mL). As both *A. baylyi* and *P. aeruginosa* can trigger multiple sclerosis in genetically susceptible people,[1,2] these extracts may be useful for the prevention and treatment of this disease. The *N. sativa* and shilajit extracts were completely ineffective against these bacteria.

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

	Sample	Mortality±SD (%)	
		After 24 hr	After 48 hr
Antibiotics	Erythromycin	1.8±1.4	4.3±2.4
	Tetracycline	2.7±1.3	5.6±3.3
	Chloramphenicol	3.7±2.6	8.3±2.4
	Ciprofloxacin	3.7±0.6	8.5±2.3
	Gentamicin	4.2±1.5	9.1±2.8
Extracts	NS (M)	24.6±3.6	68.3±3.8
	NS (W)	14.6±3.1	46.8±2.9
	AL (M)	8.5±3.1	21.9±3.6
	AL (W)	7.8±2.9	18.8±3.6
	Shil	10.4±3.7	21.5±3.2
Combinations	NS (M)+Erythromycin	32.1±3.2	73.4±3.5
	NS (M)+Tetracycline	22.6±3.0	55.7±3.6
	NS (M) +Chloramphenicol	34.6±2.9	78.4±3.8
	NS (M)+Ciprofloxacin	32.5±3.8	69.7±4.1
	NS (M)+Gentamicin	37.4±2.7	81.3±2.8
	NS (W)+Erythromycin	21.3±4.6	45.2± 3.4
	NS (W)+Tetracycline	17.6±2.3	33.7±4.3
	NS (W) +Chloramphenicol	18.8±3.9	41.2±3.8
	NS (W)+Ciprofloxacin	20.6±2.4	37.9±3.6
	NS (W)+Gentamicin	18.6±3.9	33.5±2.7
	AL (M)+Erythromycin	15.7±3.8	34.5±3.6
	AL (M)+Tetracycline	9.3±3.5	26.2±3.4
	AL (M) +Chloramphenicol	12.6	31.4±3.9
	AL (M)+Ciprofloxacin	14.1±3.3	37.2±4.1
	AL (M)+Gentamicin	12.8±3.5	29.2±3.8
	AL (W)+Erythromycin	17.4±4.6	37.5±3
	AL (W)+Tetracycline	9.2±3.7	27.4±3
	AL (W) +Chloramphenicol	12.8±3.2	30.4±2.8
	AL (W)+Ciprofloxacin	11±3.6	23.4±3.5
	AL (W)+Gentamicin	9.2±3.2	20.4±3.1
	Shil+Erythromycin	18.3±4.4	36.4±2.8
	Shil+Tetracycline	8.7±4.6	15.7±4
	Shil +Chloramphenicol	15.8±3.6	34.9±3.2
	Shil+Ciprofloxacin	13.8±3.6	17.9±3.7
	Shil+Gentamicin	11.4±3.2	16.7±2.8
Controls	Deionised water	2.7 ±1.7	3.6 ±2.5
	Potassium dichromate	100.00±0.00	

Potassium dichromate was tested at a concentration of 1000 µg/mL; M=Methanolic extract; W=aqueous extract; NS=Nigella sativa; AL=Anongessius latifolia; Shil=Shilfara; SD=Standard Deviation. Results represent means±SEM of 3 independent experiments, each preformed in triplicate (n=9).

The *A. latifolia* methanolic extract was also a good inhibitor of *S. pyogenes* growth. As *S. pyogenes* can trigger rheumatic fever in genetically susceptible people, this extract may also be useful in preventing and treating that disease (and other diseases caused by *S. pyogenes* infections). The aqueous *A. latifolia* extract, as well as both *N. sativa* extracts, also inhibited *S. pyogenes* growth and therefore may also be useful in the prevention and treatment of this disease. However, the higher MIC values of those extracts against *S. pyogenes* growth indicate that they may be substantially less effective than the *A. latifolia* methanolic extract, although this needs to be verified using *in vivo* assays. The shilajit extract was completely ineffective at inhibiting *S. pyogenes* growth.

Fractional Inhibitory Concentration (FIC) assessment

Two of the combinations tested in this study produced synergistic effects when tested together against *P. aeruginosa* and *S. pyoneges* (Table 3). Notably, both of these combinations contained the methanolic *A. latifolia* bark extract as the extract component, and tetracycline as the antibiotic component. As tetracycline resistance is most frequently produced by tetracycline-specific efflux pumps,[12] it is likely that *A. latifolia* methanolic extract contains inhibitors of that pump. Additionally, three combinations had additive effects in the assay. All of these contained chloramphenicol as the antibiotic component, indicating that the extracts may be counteracting a common resistance mechanism shared between the *P. aeruginosa* and *S. pyogenes* strains screened in our study. Whilst these combinations would not be as effective as synergistic combinations, they are still an improvement on using either the antibiotic or the extract alone. It would therefore be beneficial to use these combinations in the prevention and treatment of multiple sclerosis and rheumatic fever. Interestingly, the majority of additive combinations also contained the methanolic *A. latifolia* extract. The exception was the additive combination of chloramphenicol and the methanolic *N. sativa* extract against *S. pyogenes*. Two antagonistic interactions were also noted against *A. baylyi* and *S. pyogenes* (both containing the methanolic *A. latifolia* extract and ciprofloxacin). Therefore, these combinations should be avoided as antibiotic therapies. All of the other inhibitory combinations were non-interactive. Whilst these combinations provide no added benefit over that of the individual components alone, the components do not antagonise each other's effects and are therefore safe to use concurrently without risk of lessening the efficacy of either component.

Toxicity evaluation

All plant extracts and antibiotics were individually screened at 1000 µg/mL in the ALA assay (Table 4). The extracts were only considered toxic if they induced percentage mortalities greater than 50% (LC_{50}) following 24 hr of exposure to the artemia nauplii.[30,31] When tested individually, the antimicrobials demonstrated no toxicity in the ALA. Similarly, none of the extracts produced mortality above 50% following 24 hr exposure. Additionally, when the extract-antibiotic combinations were tested in the ALA, none of them produced mortality >50% mortality. Therefore, all combinations and individual components were deemed nontoxic. In contrast, the positive control potassium dichromate induced 100% mortality in the ALA.

DISCUSSION

This study investigated the ability of *N. sativa* seed extracts, *A. latifolia* bark extracts and aqueous shilfara suspensions to inhibit the growth of some bacterial triggers of multiple sclerosis and rheumatic fever, both alone and in combination with conventional antibiotics. These traditional medicines were selected for this study as they are traditionally used to treat multiple pathogenic illnesses, including several diseases caused by bacteria.[9,10,13-18] Furthermore, previous studies have reported antibacterial properties for *N. sativa* and *A. latifolia* extracts against multiple bacteria.[9,10,13-18] However, to the best of our knowledge, none of these previous studies has tested similar extracts for the ability to inhibit the growth of the bacterial triggers of multiple sclerosis or rheumatic fever. The methanolic *A. latifolia* extract were identified as a particularly effective growth inhibitor against all of the bacteria screened herein, with clinically relevant potency, indicating that it may be particularly useful in preventing and treating multiple sclerosis and rheumatic fever in genetically susceptible people (as well as other infections caused by these bacteria) when used by alone.

The combinational studies combining the extracts with conventional antibiotics also yielded interesting results. Several combinations displayed enhanced growth inhibitory activity against *P. aeruginosa* and *S. pyogenes* compared to that of either the extract or antibiotic components alone. Notably, all of the potentiating combinations contained the methanolic *A. latifolia* extract. Two synergistic (in combinations containing tetracycline) and four additive combinations (in combinations containing chloramphenicol) were noted. The implications of this potentiation include enhanced efficacy, the requirement for lower dose administration and a reduction in side effects, as well as possibly reduced antimicrobial resistance.[3] Also importantly, two combinations (containing ciprioflоxacin and the *A. latifolia* methanolic extract) produced antagonistic effects and therefore should be avoided.

None of the extracts or conventional antibiotics demonstrated toxicity in the ALA assay when tested independently. Similarly, all combinations were nontoxic, indicating their potential for therapeutic use. The non-toxicity of the conventional antibiotics is hardly surprising as these drugs have a long history of therapeutic use and their lack of toxicity has previously been verified in clinical trials. The lack of toxicity determined for the extracts is also not surprising, as they have long been used in several traditional medicine systems to treat pathogenic diseases.[9,10,13-18] The lack of toxicity of the combinations in our study also confirms

their potential for therapeutic usage. However, further *in vitro* studies using human cell lines are required to verify their safety. Furthermore, *in vivo* testing is also required to confirm that the extracts and combinations retain efficacy and remain nontoxic in complex biological systems.

CONCLUSION

Whilst the findings reported herein support the therapeutic properties of the methanolic *A. longifoli*a bark extract as a preventative and therapeutic option against multiple sclerosis and rheumatic fever, further *in vivo* investigations are required to support these *in vitro* findings. Furthermore, studies to determine the possible mechanism of action resulting in the observed interaction are warranted, and bioactivity driven compound isolation and/or metabolomics studies are also required to determine the active compound(s) within the extracts.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ALA: Artemia franciscana lethality assay; **DMSO:** Dimethyl sulfoxide; **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.

SUMMARY

• Selected medicinal plant extracts were screened for the ability to block the growth of a panel of bacterial triggers of selected autoimmune diseases.

• The antibacterial activity was quantified by determining the MIC values of each extract.

• The extracts were also screened in combination with conventional antibiotics and the class of interaction was evaluated by ΣFIC analysis.

• Toxicity of the extracts was determined using the Artemia nauplii toxicity bioassay.

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