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Original Article

Antibacterial Activity and Toxicity Profiles of *Artemisia annua* L. Extracts and Conventional Antibiotics against Bacterial Triggers of Some Autoimmune Diseases

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ABSTRACT

Background: *Artemisia annua* L. has been used traditionally to treat numerous infectious diseases, including many caused by bacterial pathogens. However, *A. annua* extracts are yet to be tested for the ability to inhibit the growth of bacterial triggers of autoimmune diseases. **Methods:** Antimicrobial activity was assessed using disc diffusion and liquid dilution minimum inhibitory concentration (MIC) assays against a panel of bacterial triggers of some autoimmune diseases. Interactions between the *A. annua* extracts and conventional antibiotics were studied and classified using the sum of the fractional inhibitory concentration (ΣFIC). The toxicity of the individual samples and the combinations was assessed using the *Artemia* lethality assay (ALA) assay. **Results:** *Artemisia annua* leaf extracts displayed notable antibacterial activity against the bacterial triggers of rheumatoid arthritis (*P. mirabilis* and *P. vulgaris*), ankylosing spondylitis (*K. pneumoniae*), and one of the triggers of multiple sclerosis (*A. baylyi*), although they were ineffective against *P. aeruginosa*. The ethyl acetate extracts were particularly good inhibitors of *Proteus* spp. growth, whilst the chloroform extract was the best inhibitor of *K. pneumoniae* (on the basis of MICs). Furthermore, combining the extracts with conventional antibiotics resulted in potentiation of the inhibitory activity for some combinations, particularly those containing chloramphenicol as the antibiotic component. None of the individual components (nor the combinations) were toxic in the ALA assay. **Conclusion:** The *A. annua* methanolic and ethyl acetate extracts displayed clinically relevant antibacterial activity against *P. mirabilis* and *P. vulgaris,* whilst the chloroform *A. annua* extract had the best activity against *K. pneumoniae* when tested alone. The lack of toxicity of the extract and combinations indicates that *A. annua* extract and antibiotic combinations may provide leads in the development of new therapies to prevent and treat the autoimmune diseases rheumatoid arthritis and ankylosing spondylitis.

Key words: Medicinal plants, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, 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 and multiple sclerosis that afflict genetically susceptible individuals.[1,2] There are no cures for these disorders. Instead, current treatment strategies aim to alleviate the symptoms (particularly pain, swelling and inflammation) with analgesics and anti-inflammatory agents and/or to modify the disease 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 trigger of rheumatoid arthritis has been identified as *Proteus* spp. (especially *Proteus mirabilis*), which are a normal part of the human gastrointestinal flora. Similarly, *Klebsiella pneumoniae* has been shown to initiate ankylosing spondylitis and *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis.[1,2] The development of antibiotic agents targeting specific bacterial triggers of autoimmune inflammatory disorders would enable afflicted individuals to target these microbes and thus prevent the onset of the disease and reduce the severity of the symptoms once the disease has progressed.

Whilst antibiotics are available to treat infections of these bacteria, the development of multiple antibiotic resistant bacterial strains has rendered multiple clinical antibiotics of decreased efficacy, or in some cases, has rendered the antibiotics completely effective.[3] The development of alternative treatment methods is crucial and is considered by the World Health Organisation (WHO) to be one of the biggest 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 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]

Artemisia annua L. (synonym *Artemisia chamomilla* C. Winkl.; common names sweet wormwood, sweet annie, sweet sagewort; Chinese 黄花蒿;, pinyin huánghuāhāo) is a small annual herbaceous plant of the family Asteraceae. It is native to temperate regions of Asia, although it has now been widely naturalised in many regions of the world. Preparations prepared from the leaves of this plant have numerous uses in traditional Chinese medicine (TCM), although its use for the treatment of fever is particularly well documented.[9-11] Based on its traditional uses in TCM, a Chinese study examined *A. annua* extracts for the ability to treat malaria and identified the sesquiterpene lactone artemisinin as a potent inhibitor of *Plasmodium falciparum*, the main parasitic that causes of malaria in humans.[12] However, whilst *A. annua* is nowadays best known for its anti-plasmoidal properties, numerous studies have also screened extracts and essential oils prepared from its leaves against bacterial pathogens and have reported noteworthy activities.[13-15] However, *A. annua* extracts are yet to be tested against the bacterial triggers of rheumatoid arthritis (*Proteus mirabilis*), ankylosing spondylitis (*Klebsiella pneumoniae*) and multiple sclerosis (*Acinetobacter baylyi*, *Pseudomonas aeruginosa*).[1,2,16] This study investigates the antimicrobial effects of *A. annua* leaf extracts and their ability to potentiate the growth inhibitory properties of conventional antibiotics against the bacterial triggers of some autoimmune inflammatory diseases.

MATERIALS AND METHODS

Sourcing and preparation of plant samples

The dried *Artemisia annua* L. 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 AALI-AA1-2016-1). Individual quantities (1g) of the plant material were weighed into separate tubes and 50mL of methanol, deionised water, ethyl acetate or hexane were added. All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The seeds were extracted in each solvent for 24 hr at 4°C with gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum. The solvent extracts were air dried at room temperature in the shade. The aqueous extracts were lyophilised by freeze drying at -50°C. The resultant dried extracts were weighed to determine the extraction yield and were dissolved in 10mL of deionised water (containing 1% DMSO).

Qualitative phytochemical analysis

Phytochemical analysis of the *A. annua* leaf extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved using standard assays.[17,18]

Antibacterial Analysis

Conventional Antibiotics

Penicillin-G (potency of 1440-1680µg/mg), chloramphenicol (≥98% purity by HPLC), erythromycin (potency ≥850µg/mg), and tetracycline (≥95% purity by HPLC) were purchased from Sigma-aldrich, Australia and used for the microplate liquid dilution assay. All antibiotics were prepared in sterile deionised water at stock concentrations of 0.01mg/mL and stored at 4°C until use. For the disc diffusion studies, ampicillin (2µg), tetracycline (10µg) and chloramphenicol (10µg) standard discs were obtained from Oxoid Ltd., Australia and used as positive controls.

Bacterial cultures

All bacterial strains were selected based on their ability to trigger autoimmune inflammatory diseases in genetically susceptible individuals.[19-21] Reference strains of *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC21719) *Klebsiella pneumoniae* (ATCC31488), *Acinetobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Type Culture Collection, USA. All bacteria were cultured in nutrient broth (Oxoid Ltd., Australia). Streak nutrient agar (Oxoid Ltd., Australia) plates were tested in parallel to ensure the purity of all bacterial cultures and for sub-culturing. All bacterial cultures were incubated at 37°C for 24hr and were subcultured and maintained in nutrient broth at 4°C until use.

Evaluation of bacterial susceptibility to growth inhibition

The susceptibility of the bacteria to the *A. annua* leaf extracts and the conventional antibiotics was initially assessed using a modified disc diffusion assay.[22,23] Ampicillin (2 µg), tetracycline (10µg) and chloramphenicol discs (10µg) were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using two methods. A liquid dilution MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay.[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 plant samples and conventional antimicrobials independently and in combinations. Briefly, 100µL of sterilized distilled water was dispensed into each well of 96 well micro-titre plate. The plant samples and conventional antibiotics (100µL) were then added into separate wells of the first row of the plate. The *A. annua* extracts were introduced at a starting concentration of 32mg/mL whilst the conventional antibiotics were introduced at a starting concentration of 0.01mg/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 (100µL) was then added to all wells of the plate except the sterile control wells. Each inoculum contained approximately $1x10^6$ colony forming units (CFU)/mL. All plates were subsequently incubated at 37°C. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, Australia and dissolved in sterile deionised water to prepare a 0.2mg/mL INT solution. A 40µL volume of this solution was added into all wells and the plates were incubated for a further 6 hr at 30°C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

Disc diffusion MIC assay

The minimum inhibitory concentrations (MIC) of the extracts was also evaluated by disc diffusion assay as previously described.[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 the combinations of plant samples and conventional antimicrobials were further classified using the sum of the fractional inhibitory concentration (ΣFIC). The FIC was calculated using the following equation, where (a) represents the plant sample and (b) the conventional antimicrobial sample.[24-26]

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

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

The ΣFIC was then calculated using the equation: $\Sigma FIC = FIC^{(i)} + FIC^{(ii)}$. The interactions were classified as being synergistic for ΣFIC 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 *A. annua* 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 (4mg/mL) and serially diluted in artificial seawater as a reference toxin. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Statistical analysis

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

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extractions of the dried *A. annua* leaf material (1g) with solvents of varying polarity yielded dried plant extracts ranging from 28mg (hexane extract) to 156mg (aqueous extract) (Table 1). Qualitative phytochemical screening (Table 1) showed that the higher polarity solvents (methanol and water) extracted the greatest amount and widest diversity of phytochemical classes.

Bacterial growth inhibition screening

Proteus mirabilis growth was susceptible to the high to mid-polarity methanolic, aqueous and ethyl acetate *A. annua* extracts (Figure 1a). The methanolic, aqueous and ethyl acetate extracts (ZOIs of 7.4, 7.8 and 8mm respectively) produced comparable zones of inhibition (ZOIs) to the ampicillin control (7.4mm), although the chloramphenicol control was a substantially stronger inhibitor of *P. mirabilis* growth (13.8mm). In contrast, the chloroform and hexane extracts were completely devoid of *P. mirabilis* growth inhibitory activity. Similar trends and susceptibilities were noted for the inhibition of *P. vulgaris* growth by the *A. annua* extracts, with inhibition recorded for only the methanolic, aqueous and ethyl acetate extracts (7.2, 7.8 and 7mm respectively) (Figure 1b).

Interestingly, a different susceptibility profile was evident for *K. pneumoniae* (Figure 1c). Whilst, the methanolic and ethyl acetate extracts inhibited bacterial growth, the aqueous extract was completely devoid of inhibitory activity (as judged by ZOIs). Furthermore, the lower polarity chloroform and hexane extracts had noteworthy growth inhibitory activity. Thus, as the low and mid-polarity extracts inhibited *K. pneumoniae* growth yet the polar aqueous extract did not, it is likely that the antibacterial component(s) are relatively low polarity. It is also likely that the compounds responsible for inhibiting the growth of *A. baylyi* may also be relatively nonpolar as only the methanolic and

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *A. annua* 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	102	10.2	+++	+++	++	-	-	+	-	-	-	++	+	++	-	-
Water	156	15.6	+++	+++	+	-	-	+	-	-	-	+++	++	++	-	-
Ethyl Acetate	54	5.4	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Chloroform	66	6.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	28	2.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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

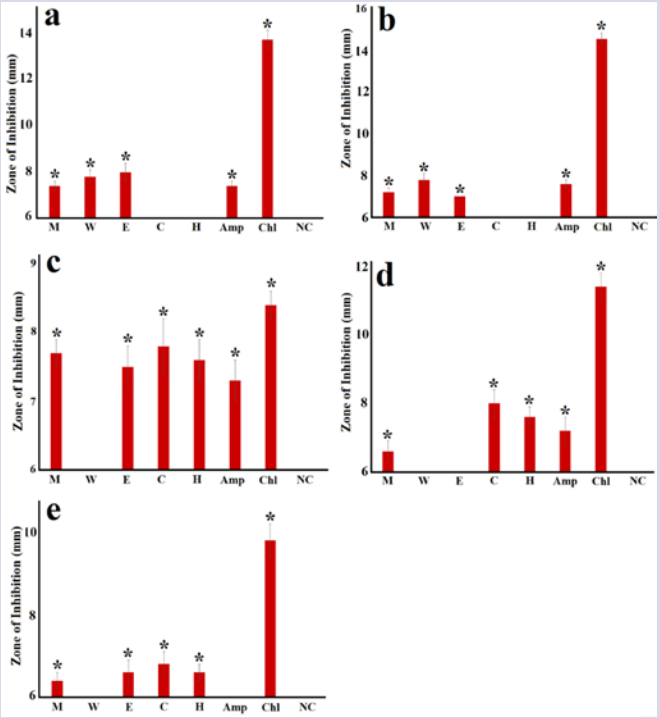

Figure 1: Antibacterial activity of A. annua leaf extracts against (a) *P. mirabilis* (ATCC21721); (b) *P. vulgaris* (ATCC21719); (c) *K. pneumoniae* (ATCC31488); (d) *A. baylyi* (ATCC33304); and (e) *P. aeruginosa* (ATCC: 39324), measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; H = hexane extract; Amp = ampicillin (2µg); Chl = chloramphenicol (10µg); Tet = tetracycline (10µg); NC = negative control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates ± SEM * indicates results that are significantly different to the negative control (P<0.01).

hexane extracts inhibited the growth of that bacterium, with ZOIs of 6.6 and 7.6mm respectively (Figure 1d). All of the *A. annua* extracts except the aqueous extract inhibited *P. aeruginosa* growth. However, the small ZOIs (<7mm for all extracts) indicates that these extracts have relatively weak activity against that bacterium. It is noteworthy that the *P. aeruginosa* strain tested in this study was relatively resistant to antibiotic exposure. 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]

Quantification of minimum inhibitory concentration (MIC)

The relative level of antimicrobial activity was further evaluated by determining the MIC values using two methods: The liquid dilution MIC assay and the disc diffusion MIC assay (Table 2). Consistent with the antibacterial disc screening assays, all bacterial strains tested were susceptible to the *A. annua* extracts, although, only weak inhibitory activity was recorded against *P. aeruginosa*. However, as previously noted, this bacterial strain was particularly resistant to the antibiotic controls. Indeed, *P. aeruginosa* growth was completely unaffected by penicillin-G, erythromycin and chloramphenicol in the liquid dilution assay. The growth of this bacteria was only inhibited by tetracycline, with an MIC of 2.5µg/mL. However, this MIC value indicates that this bacterium is also resistant to tetracycline as MIC values >1µg/mL in this assay are indicative of resistance.[24,25] Similarly, the *A. annua* extracts and the antibiotic controls were relatively weak inhibitors of *A. baylyi* growth. Indeed, whilst all of the antibiotics except penicillin-G inhibited the growth of this bacterium, all had MIC values substantially >1µg/mL. Similarly, only the methanolic extract inhibited the growth of this bacterium, although the high MIC (>5000µg/mL) indicates only weak activity. As both *A. baylyi* and *P. aeruginosa* can trigger multiple sclerosis in genetically susceptible people,[1,2] it is unlikely that these extracts would be useful for the prevention and treatment of this disease, due to the low inhibitory activity.

The *Proteus* spp. were substantially more susceptible to the *A. annua* extracts compared to *A. baylyi* and *P. aeruginiosa*. The ethyl acetate extracts were particularly noteworthy, with MIC values of 686 and 875µg/mL against *P. mirabilis* and *P. vulgaris* respectively. The methanolic extract also had noteworthy activity against the *Proteus* spp., with MIC values of 1108 and 1256µg/mL against *P. mirabilis* and *P. vulgaris* respectively. Only weak activity was seen for the aqueous extract against these bacteria, whilst the chloroform and hexane extracts were completely devoid of activity. As *Proteus* spp. can trigger rheumatoid arthritis in genetically susceptible people.[1,2] the *A. annua* extracts (particularly the ethyl acetate extract) may be useful for the prevention of this disease, as well as other diseases caused by these bacteria.

A substantially different trend was noted for *K. pneumoniae*. In contrast to the other bacteria tested, the low polarity chloroform and hexane extracts produced the greatest growth inhibition (MICs of 256 and 894µg/mL respectively). The mid-polarity ethyl acetate extract also produced noteworthy inhibition (927µg/mL). The methanolic extract also had moderate inhibitory activity against *K. pneumoniae* (MIC=1460µg/mL), whilst the aqueous extract was devoid of inhibitory activity. Therefore, as this bacterium can trigger ankylosing spondylitis in genetically susceptible people,[1,2] the low to mid-polarity *A. annua* extracts may be useful in the prevention and treatment of this disease, as well as other diseases caused by this bacterium.

Fractional inhibitory concentration (FIC) assessment

None of the combinations of the *A. annua* extracts and conventional antibiotic combinations produced synergistic effects when tested together against any of the bacteria tested (Table 3). However, four combinations (two each against *P. mirabilis* and *A. baylyi*) had additive effects in the assay. Whilst these combinations would not be as effective as synergistic combinations, they are still an improvement on using either the antibiotic or the extract alone. It would therefore be beneficial to use these combinations in the prevention and treatment of rheumatoid arthritis and multiple sclerosis. Interestingly, the majority of additive combinations contained chloramphenicol as the antibiotic component. The exception was the additive combination of tetracycline and the hexane extract against *A. baylyi*. No obvious trend was apparent regarding the extract component of the combination, with two additive combinations containing the hexane extract and one each containing the methanolic and ethyl acetate extracts. All of the other inhibitory combinations were non-interactive. Whilst these combinations provide no added benefit over that of the individual components alone, the components do not antagonise each other's effects and are therefore safe to use concurrently without risk of lessening the efficacy of either component.

Toxicity evaluation

All plant extracts and antibiotics were individually screened at 1000µg/mL in the ALA assay (Table 4). The extracts were only considered toxic if they induced percentage mortalities greater than 50% (LC_{50}) following 24 hr of exposure to the *Artemia* nauplii.[30] When tested individually, the antimicrobials demonstrated no toxicity in the ALA. Similarly, none

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

Extract	*P. mirabilis*		*P. vulgaris*		*K. pneumoniae*		*A. baylyi*		*P. aeruginosa*	
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC
M	2540	1108	2540	1256	2540	1460	>5000	>5000	>5000	>5000
W	>5000	>5000	3888	2500	-	-	-	-	-	-
E	1340	686	2360	875	1340	927	-	-	>5000	>5000
C	-	-	-	-	819	256	3275	2856	>5000	>5000
H	-	-	-	-	1390	894	1390	1042	>2800	>2800
					Controls					
Penicillin-G	ND	2.5	ND	1.25	ND	-	ND	-	ND	-
Erythromycin	ND	-	ND	-	ND	-	ND	2.5	ND	-
Tetracycline	ND	-	ND	-	ND	1.25	ND	1.25	ND	2.5
Chloramphenicol	ND	1.25	ND	1.25	ND	2.5	ND	2.5	ND	-

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

Table 3: ∑FIC values for the *A. annua* leaf extract and conventional antibiotic combinations against susceptible bacteria.

Bacteria	Extract	Penicillin-G	Chloramphenicol	Erythromycin	Tetracycline
P. mirabilis	**M**	1.29 (IND)	*0.88 (ADD)*	-	-
	W	3.28 (IND)	3.56 (IND)	-	-
	E	1.14 (ADD)	*0.73 (ADD)*	-	-
	C	-	-	-	-
	H	-	-	-	-
P. vulgaris	**M**	1.98 (ADD)	1.03 (IND)	-	-
	W	2.32 (IND)	2.85 (IND)	-	-
	E	1.36 (IND)	1.05 (IND)	-	-
	C	-	-	-	-
	H	-	-	-	-
K. pneumoniae	**M**	-	2.25 (IND)	-	3.44 (IND)
	W	-	-	-	-
	E	-	1.87 (IND)	-	2.72 (IND)
	C	-	1.54 (IND)	-	1.85 (IND)
	H	-	2.06 (IND)	-	2.16 (IND)
A. baylyi	**M**	-	1.48 (IND)	3.36 (IND)	2.2 (IND)
	W	-	-	-	-
	E	-	-	-	-
	C	-	1.58 (IND)	2.88 (IND)	2.55 (IND)
	H	-	*0.88 (ADD)*	1.37 (IND)	*0.97 (ADD)*

M = methanolic extract; W = aqueous extract ; E = ethyl acetate extract; H = hexane extract; *ADD* = *additive interaction*; IND = indifferent interaction.

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

0	Sample	Mortality ± SEM (%)	
		After 24 hr	After 48 hr
Antimicrobials	Penicillin G	1.8 ± 1.4	4.3 ± 2.4
	Chloranphenicol	2.7 ± 1.3	5.6 ± 3.3
	Erythromycin	1.2 ± 0.6	5.8 ± 2.3
	Tetracycline	2.4 ± 1.5	5.1 ± 2.8
Extracts	M	17.3 ± 3.5	44.6 ± 3.2
	W	9.6 ± 2.9	23.4 ± 3.6
	E	12.5 ± 2.5	28.9 ± 4.2
	C	9.3 ± 3.3	21.4 ± 3.1
	H	10.9 ± 4.8	25.2 ± 3.4
Combinations	M + Pen	28.6 ± 3.7	63.9 ± 3.5
	W + Pen	18.3 ± 2.9	35.5 ± 4.2
	E + Pen	22.6 ± 3.8	52.4 ± 3.5
	C + Pen	20.5 ± 3.6	47.9 ± 4.4
	H + Pen	19.8 ± 3.7	42.9 ± 3.6
	M + Chl	30.8 ± 4.6	68.7 ± 3.6
	W + Chl	21.4 ± 2.8	39.3 ± 3.5
	E + Chl	23.2 ± 3.4	45.8 ± 4.4
	C + Chl	17.8 ± 3.8	33 ± 2.9
	H + Chl	14.5 ± 3.3	32.7 ± 3.2
	M + Eryth	17.4 ± 4.6	37.5 ± 3
	W + Eryth	9.5 ± 3.3	28.8 ± 2.7
	E + Eryth	15.7 ± 3.7	29 ± 3.2
	C + Eryth	11.4 ± 4.6	33.6 ± 3.8
	H + Eryth	9.6 ± 3.7	24.7 ± 3.6
	M + Tet	16.3 ± 3.9	32.5 ± 3.8
	W + Tet	6.6 ± 3.5	19.4 ± 4
	E + Tet	14.8 ± 3.6	28.7 ± 3.5
	C + Tet	10.6 ± 4.2	21.5 ± 3.4
	H + Tet	7.3 ± 3.8	18.4 ± 3.2
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; E = ethyl acetate extract; H = hexane extract; Chl = chloramphenicol; Eryth = erythromycin; Tet = tetracycline; SEM = standard error of the mean. Results represent means ± SEM of 3 independent experiments, each preformed in triplicate (n = 9).

of the *A. annua* extracts produced mortality above 50% following 24hr exposure. Additionally, when the extract-antibiotic combinations were tested in the ALA, none of them produced mortality >50% mortality. Therefore, all combinations and individual components were deemed nontoxic. In contrast, the positive control potassium dichromate induced 100% mortality in the ALA.

DISCUSSION

This study investigated the ability of *A. annua* extracts to inhibit the growth of some bacterial triggers of auto-immune inflammatory diseases, both alone and in combination with conventional antibiotics. *Artemisia annua* was selected for this study as it is traditionally used to treat multiple pathogenic illnesses, including several diseases caused by bacterial pathogens.[1,2] Furthermore, previous studies have reported antibacterial properties for *A. annua* extracts against multiple bacteria.[13-15] However, to the best of our knowledge, none of these previous studies has tested *A. annua* extracts for the ability to inhibit the growth of the bacterial triggers of autoimmune inflammatory diseases. Several extracts were identified as effective growth inhibitors against *P. mirabilis*, *P. vulgaris, K. pneumoniae* and *A. baylyi* growth, with clinically relevant potency. The ethyl acetate extract had the strongest inhibitory activity against all bacteria, indicating that it may be particularly useful in preventing and treating rheumatoid arthritis, ankylosing spondylitis, and (to a lesser extent) multiple sclerosis, as well as other infections caused by these bacteria, when used by alone.

The combinational studies combining the *A. annua* extracts with conventional antibiotics also yielded interesting results. Several combinations displayed enhanced potential as therapeutic agents against *P. mirabilis, P. vulgaris* and *A. baylyi* than either the extracts or antibiotics alone. Indeed, four additive combinations were noted, with three of these containing chloramphenicol (in combination with either the methanolic, ethyl acetate or hexane). The implications of this potentiation include enhanced efficacy, the requirement for lower dose administration and a reduction in side effects, as well as possibly reduced antimicrobial resistance.[3] Importantly, none of the combinations produced antagonistic effects. This is an important finding as it indicates that it is safe to use any of the *A. annua* extracts and conventional antibiotics in combination without decreasing the efficacy of either component.

None of the *A. annua* 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 *A. annua* extract may also be unsurprising as they have long been used in several traditional medicine systems to treat pathogenic diseases.[12-15] The lack of toxicity of the combinations in our study also confirms their potential for therapeutic usage. However, further *in vitro* studies using human cell lines are required to verify their safety. Furthermore, *in vivo* testing is also required to confirm that the extracts and combinations retain efficacy and remain non-toxic in complex biological systems.

CONCLUSION

Whilst the findings reported herein support the therapeutic properties of the *A. annua* extracts as preventative and therapeutic options against rheumatoid arthritis and ankylosing spondylitis, 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 *A. annua* extracts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ALA: brine-shrimp lethality assay; **DMSO**; dimethyl sulfoxide; **FIC**; fractional inhibitory concentration; **INT**; ρ-iodonitrotetrazolium chloride; **LC_{50}**; dose of sample necessary to have a lethal effect on 50% of test organisms or cells; **MIC**; minimum inhibitory concentration; **ΣFIC**; the sum of the fractional inhibitory concentration.

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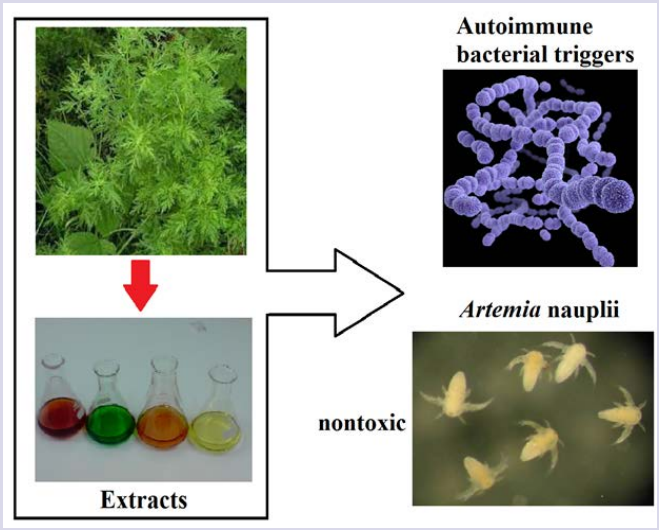
PICTORIAL ABSTRACT



SUMMARY

- *Artemisia annua* L. leaf extracts were screened for the ability to block the growth of a panel of bacterial triggers of autoimmune diseases.
- The extracts displayed inhibitory activity against all of the bacterial species tested
- Toxicity of the *A. annua* extracts was determined using the *Artemia* nauplii toxicity bioassay.
- Both the methanolic and aqueous extracts were non-toxic.

About Authors

Dr Ian Cock leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin, including Aloe vera, South Asian and South American tropical fruits, as well as Australia plants including *Scaevola spinescens, Pittosporum phylliraeoides, Terminalia ferdinandiana* (Kakadu plum), Australian *Acacias, Syzygiums, Petalostigmas* and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in nearly 200 publications in a variety of peer reviewed journals.