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Original Article

Arctium lappa L. Root Extracts Inhibit the Growth of Bacterial Triggers of Selected Autoimmune Inflammatory Diseases and Potentiate the Activity of Conventional Antibiotics

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ABSTRACT

Introduction: An increase in antibiotic resistance and a corresponding decrease in antimicrobial discovery have directed researchers towards alternative therapies, including plant based medicines. However, synergistic combinations of plant extracts with conventional antibiotics may be a far more effective approach in overcoming resistance and potentiating the activity of antibiotics that are otherwise ineffective against resistant bacterial strains. **Methods:** The antibacterial activity of *Arctium lappa* L. root extracts was investigated by disc diffusion and quantified by liquid dilution and solid phase MIC assays. The extracts were also combined with a range of conventional antibiotics and tested against various microbial triggers of autoimmune diseases. The ΣFIC values obtained from these assays were used to determine the class of combinational effects. Toxicity was evaluated by *Artemia* nauplii mortality and HDF cytotoxicity assays. **Results:** Methanolic and ethyl acetate *A. lappa* root extracts showed good inhibitory activity against several microbial triggers of autoimmune inflammatory diseases, including *P. mirabilis, P. vulgaris* and *A. baylyi*. The aqueous extract was also a noteworthy inhibitor of *A. baylyi* growth. Of further interest, some combinations of the *A. lappa* root extracts and conventional antibiotics potentiated bacterial growth inhibition compared to the individual components alone. One synergistic and six additive interactions were noted. Notably, no antagonistic interactions were evident, indicating that all combinations could be used without decreasing the antibacterial activity of the components. All extracts were nontoxic in the ALA and HDF assays. **Conclusion:** *Arctium lappa* L. root extracts have potential as inhibitors of bacterial triggers of selected autoimmune inflammatory diseases. Furthermore, extract components may also potentiate the activity of three antibiotics that are relatively ineffective alone. Isolation and identification of these compounds may be beneficial in drug design against several bacteria, including the microbial triggers of rheumatoid arthritis and multiple sclerosis.

Key words: Synergy, Conventional antimicrobials, Interaction, Medicinal plants, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Drug combinations.

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INTRODUCTION

Despite their initial efficacy, the overuse of antibiotics has resulted in a wide range of bacterial pathogens developing resistance towards multiple antibiotics.[1] Additionally, the discovery of new antimicrobial agents has decreased dramatically in recent years making many bacterial infections difficult to manage using current therapeutic strategies.[2] The development of alternative antibacterial treatment modalities is considered by the World Health Organisation (WHO) to be one of the biggest challenge currently facing medical science.[3] For a number of reasons reviewed elsewhere,[2] it is unlikely that the current methods of antibiotic discovery/development will be as successful in the future. This is particularly true for the treatment of autoimmune inflammatory diseases. These are a group of debilitating diseases including rheumatoid arthritis (RA), ankylosing spondylitis (AS), lupus, Lyme disease, multiple sclerosis (MS), celiac disease, and rheumatic fever (RV).[4-6] All of these diseases result from an abnormal immune response to self-tissue as a consequence of antigen challenge, often by bacterial pathogens. There is currently no cure for any of these diseases and the current treatment strategy is to alleviate the symptoms with analgesics and anti-inflammatory therapies. However, as RA, AS and MS are induced in genetically susceptible people by bacterial pathogens, a more effective preventative treatment may be to target the growth of the specific trigger bacteria, thereby blocking the disease etiological events.[4-6] Whilst antibiotics are already available for the treatment of all of these bacteria, the development of resistant strains in recent years have decreased their efficacy.[1] Furthermore, the prophylactic use of pure antibiotics over prolonged periods would certainly induce further antibiotic resistance, thereby rendering the bacteria refractory to their actions. A better approach may be to use combinations of antibacterial components.[2]

Traditional medicines have great potential for antimicrobial drug development. 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 compounds often have much lower activity than the crude extract.[7] 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 against antibiotic resistant strains.[8,9] Combinational therapies are already preferred over mono-therapy to treat multiple life-threatening infectious diseases such as malaria, tuberculosis and HIV/AIDS due to their ability to target multiple facets of a disease and to curb resistance.[2] Combinations of plant extracts/isolated compounds with conventional antibiotics may also prove to have economic advantages.[7] 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 antimicrobial agents.[7] Furthermore, synergistic combinations may have increased efficiency, reduced side effects, increased stability and bioavailability, and require lower doses in comparison to synthetic alternatives to achieve therapeutic outcomes.[8]

Arctium lappa L. (family Asteraceae; commonly known as burdock, greater burdock, lappa, thorny burr, beggar's buttons) is a medicinal

plant that is native to temperate regions of Europe and Asia, although it has been widely naturalised and is now common in disturbed regions globally. *Arctium lappa* roots have been used for hundreds of years as traditional medicines by multiple European, Asian and North American cultures[10] for a variety of purposes including to improve the immune system and enhance metabolism,[11] as well as for its anti-inflammatory,[12-15] anticancer,[16,17] and antibiabetic properties,[18,19] Many of these illnesses are caused by bacterial pathogens and several studies have reported that *A. lappa* leaf extracts inhibit the growth of *Bacillus subtilus*, *Lactobacillus acidophilus* and *Pseudomonas aeruginosa*.[20] However, MIC values were not determined in that study so it is not possible to compare the activity to other extracts in other studies. Notably, whilst the roots are generally used medicinally, they have been relatively neglected in antibacterial studies. Instead the leaf is most frequently examined, despite the root being the part most frequently used medicinally. Additionally, chlorogenic acid isolated from *A. lappa* leaves has bacteriostatic effects against *Escherichia coli* and *Staphylococcus aureus,* although MIC values were also not reported in that study.[21] Despite these earlier studies and its traditional uses, *A. lappa* root 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*).[4-6] Furthermore, we were unable to find any studies testing the antibacterial activity of *A. lappa* root extracts in combination with conventional antibiotics. Therefore, this study was undertaken to investigate the antimicrobial effects of *A. lappa* root 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

Plant source and extraction

Arctium lappa L. root material was obtained from Noodles Herbal Emporium, Australia and a voucher specimen (GU2017aALR1) was deposited in the School of Environment and Science, Griffith University, Australia. Individual 1g masses of the ground plant material were weighed into separate 50mL Falcon tubes and 50mL of methanol, deionised water, ethyl acetate, chloroform or hexane were individually added. All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The ground plant materials were extracted in each solvent for 24 hr at 4°C with gentle shaking. The extracts were filtered through Whatman No. 54 filter paper under vacuum and 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 then dissolved in 10mL deionised water (containing 1% DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the *A. lappa* extracts for the presence of alkaloids, cardiac glycosides, flavonoids, phenolic compounds, phytosterols, saponins, tannins and triterpenoids was achieved as previously described.[22,23]

Antibacterial screening
Conventional Antibiotics

Penicillin-G (1440-1680µg/mg), chloramphenicol (≥98% purity), erythromycin (≥850µg/mg), and tetracycline (≥95% purity) 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 (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.[24-26] 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 antibacterial activity

Antibacterial activity screening of the *A. lappa* root extracts was assessed using a modified disc diffusion assay.[27,28] Ampicillin (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 (containing 1% DMSO) 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.[29] Furthermore, as microplate liquid dilution MIC assays are perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method facilitates comparisons with other studies. A solid phase agar disc diffusion assay was also used in this study for comparison as it more accurately represents the growth patterns of the bacteria on solid surfaces.

Microplate liquid dilution MIC assay

The MICs of the extracts were evaluated by standard methods.[30-32] All plates were incubated at 37°C for 24hr. 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 6hr at 37°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.[33,34] Graphs of the zone of inhibition versus Ln concentration were plotted and MIC values were achieved using linear regression.

Sum of fractional inhibitory concentration (ΣFIC) assessment

Interactions between the *A. lappa* root extracts and the conventional antibiotics were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC) for each combination.[29] The FIC values for each component (a and b) were calculated using the following equations where a represents the plant extract sample and b represents the conventional antibiotic:

$$FIC(a) = \left(\frac{MIC\,[a \text{ in combination with } b]}{MIC\,[a \text{ independently}]} \right)$$

$$FIC(b) = \left(\frac{MIC\,[b \text{ in combination with a}]}{MIC\,[b \text{ independently}]} \right)$$

The ΣFIC was then calculated using the formula ΣFIC = FIC(a) + FIC(b). The interactions were classified as synergistic (ΣFIC ≤0.5), additive (ΣFIC >0.5-1.0), indifferent (ΣFIC >1.0-4.0) or antagonistic (ΣFIC >4.0).[29]

Toxicity screening

Two assays were used to assess the toxicity of the individual samples. The *Artemia* nauplii lethality assay (ALA) was utilised for rapid preliminary toxicity screening, whereas the MTS cellular proliferation assay was used to determine a cellular evaluation of toxicity.

Artemia franciscana Kellogg nauplii toxicity screening

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. Toxicity of the *A. lappa* extracts, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.[35,36] The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Cellular viability assay

All extracts and conventional antibiotics were screened for toxicity towards normal human primary dermal fibroblasts (HDF; ATCC PCS -201-012). The HDF cells were cultured and screened in Dulbecco's modified eagle medium (DMEM; ThermoFisher Scientific, Australia), supplemented with 10% foetal calf serum (Life Technologies), 50µg/mL streptomycin (Sigma-Aldrich, Australia) and 50 IU/mL penicillin (Sigm-Aldricha, Australia). All extracts were screened at 200µg/mL with incubation at 37°C and 5% CO_2 in a humidified atmosphere following standard protocols.[25] Following the incubation, 20µL of Cell Titre 96 Aqueous One solution (Promega) was added to each well and the plates were incubated for a further 3 hr. Absorbances were recorded at a test wavelength of 540nm and a blank wavelength of 690nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed three time, each with internal triplicates (n = 9). Triplicate controls were included on each plate. The % cellular viability of each test was calculated using the following formula:

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

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

Statistical analysis

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

RESULTS

Liquid extraction yields ranged from 23mg (*A. lappa* ethyl acetate root extract) to 469mg (aqueous *A. lappa* root extracts) (Table 1). Qualitative phytochemical screening (Table 1) showed that the higher polarity solvents (methanol and water) extracted the greatest mass and widest diversity of phytochemical classes.

Bacterial growth inhibition screening
Inhibition of bacterial triggers of rheumatoid arthritis (*P. mirabilis* and *P. vulgaris*)

Proteus mirabilis growth was inhibited by the mid to high polarity *A. lappa* root methanol, aqueous and ethyl acetate extracts (Figure 1). The methanolic extract was the strongest inhibitor of *P. mirabilis* growth (as judged by ZOI), with a ZOI of 16.7mm. A volume of 10µL of this extract was infused into the disc, which equates to approximately 270µg of extract infused into the disc. The ZOI for this extract is substantially larger than that of the ampicillin and chloramphenicol controls (7.5 and 13.8mm respectively). Notably, the ampicillin and chloramphenicol control antibiotics were pure and were tested at relatively high dose (10µg/disc). In contrast, the extracts were crude mixtures and the antimicrobial compounds would be expected to account for a small % of

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

Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
M	267	26.7	+++	+++	+++	-	+	-	-	-	-	+++	++	-	-
W	469	46.9	+++	+++	++	-	+	-	-	-	-	+++	++	-	-
E	23	2.3	+	+	+	-	-	-	-	-	-	+	-	-	-
C	125	12.5	-	-	+	-	-	-	-	-	-	-	-	-	-
H	32	3.2	-	-	+	-	-	-	-	-	-	-	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. W = aqueous extract; M = methanolic extract; C = chloroform extract; H = hexane extract; E = ethyl acetate extract.

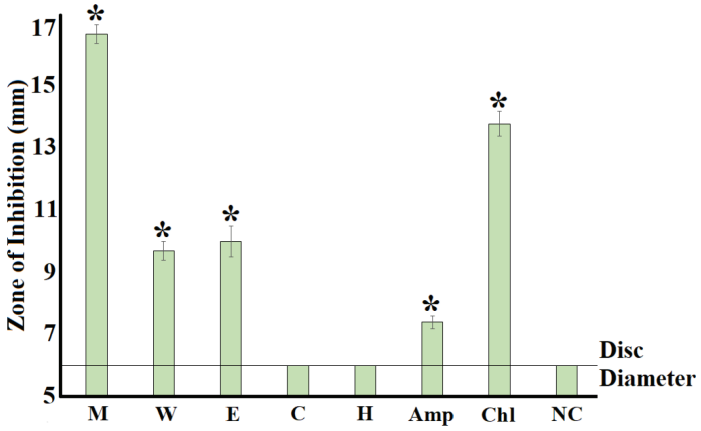

Figure 1: Antibacterial activity of *A lappa* root extracts against *P. mirabilis* (ATCC21721) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates (n=6) ± SEM. * indicates results that are significantly different to the negative control (P<0.01).

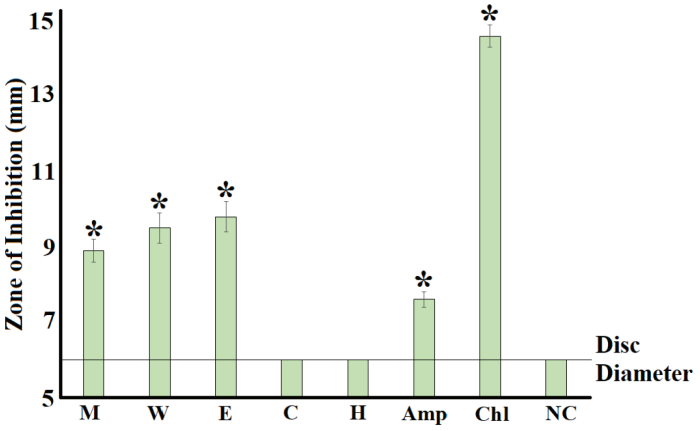

Figure 2: Antibacterial activity of *A. lappa* root extracts against *P. vulgaris* (ATCC21719) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates (n=6) ± SEM. * indicates results that are significantly different to the negative control (P<0.01).

the total extract mass. Therefore, the methanolic extract was considered to be a particularly effective inhibitor of *P. mirabilis* growth and may be effective in the prevention and treatment of rheumatoid arthritis. The aqueous and ethyl acetate extracts were also good inhibitors of *P. mirabilis* growth, albeit with substantially smaller ZOIs than the methanolic extract (9.7 and 10mm respectively). In contrast, the chloroform and hexane extracts were completely ineffective against *P. mirabilis* growth. Similar inhibitory trends were noted for *P. vulgaris* growth (Figure 2), although smaller ZOIs were measured. Unlike the trends noted for *P. mirabilis* inhibition, the aqueous and ethyl acetate *A. lappa* root extracts were the strongest inhibitors of *P. vulgaris* growth (ZOI = 9.5 and 9.8mm respectivelty). The methanolic extract, whilst also a good inhibitor of *P. vulgaris* growth, induced slightly smaller ZOIs (8.9mm). Notably, the ZOIs measured for the *A. lappa* root extracts methanolic, aqueous and ethyl acetate extracts were substantially larger than those recorded for the ampicillin control, although chloroamphenicol (ZOI = 14.6mm) was a substantially better inhibitor of *P. vulgaris* growth. All other extracts were ineffective at inhibiting *P. vulgaris* growth.

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

The methanolic, aqueous and ethyl acetate *A. lappa* root extracts also inhibited the growth of *K. pneumonia,* albeit with much smaller ZOIs than measured for the *Proteus* spp. (ZOIs = 6.7, 6.4 and 6.6mm respectively; Figure 3). These ZOIs were comparable to that of the ampicillin and chloramphenicol control (7.3 and 8.4mm respectively). In contrast, the chloroform and hexane *A. lappa* root extracts were completely ineffective against *K. pneumonia.* As *K. pneumoniae* can induce ankylosing spondylitis in genetically susceptible individuals,[4,5] the methanolic, aqueous and ethyl acetate *A. lappa* root extracts may be beneficial in the prevention and treatment of that disease.

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

The methanolic, aqueous and ethyl acetate *A. lappa* root extracts also inhibited *A. baylyi* growth, with ZOIs of 10.6, 12.7 and 9.8mm respectively (Figure 4). This *A. baylyi* strain was resistant to ampicillin (ZOI = 7.2mm),

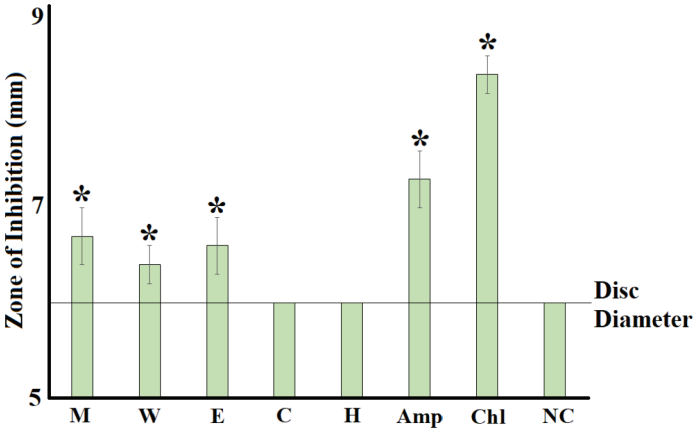

Figure 3: Antibacterial activity of *A. lappa* root extracts against extracts against *K. pneumoniae* (ATCC31488) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates (n=6) ± SEM. * indicates results that are significantly different to the negative control (P<0.01).

but was highly susceptible to chloramphenicol (ZOI = 11.4mm). In contrast to the trends noted for the *Proteus* spp. and *K. pneumoniae*, the lower polarity chloroform and hexane extracts also inhibited *A. baylyi* growth, with ZOIs of 8 and 7.8mm respectively. Surprisingly, all of the *A. lappa* root extracts also strongly inhibited the growth of the *P. aeruginosa* strain tested in this study (Figure 5). This was noteworthy as previous studies have reported that this is a particularly antibiotic-resistant strain.[37-39] Furthermore, our study confirmed that this *P. aeruginosa* strain is ampicillin resistant, although it was relatively sensitive to chloramphenicol (ZOI = 9.8mm). Therefore, due to their noteworthy growth inhibitory activity against *A. baylyi* and *P. aeruginosa*, the *A. lappa* root extracts may be useful in preventing and treating multiple sclerosis in genetically susceptible people.[4-6]

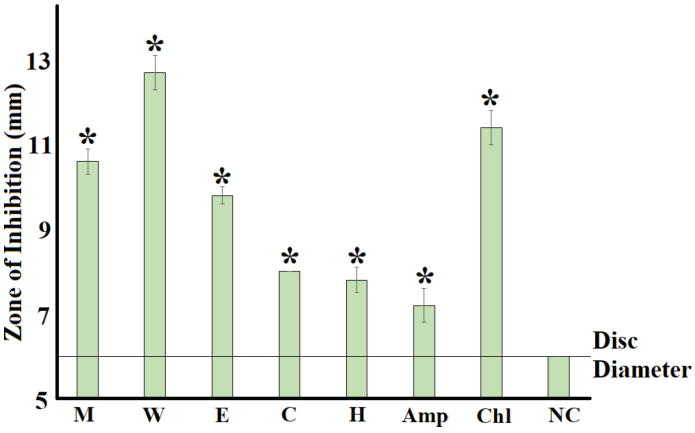

Figure 4: Antibacterial activity of *A. lappa* root extracts against *A. baylyi* (ATCC33304) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates (n=6) ± SEM. * indicates results that are significantly different to the negative control (P<0.01).

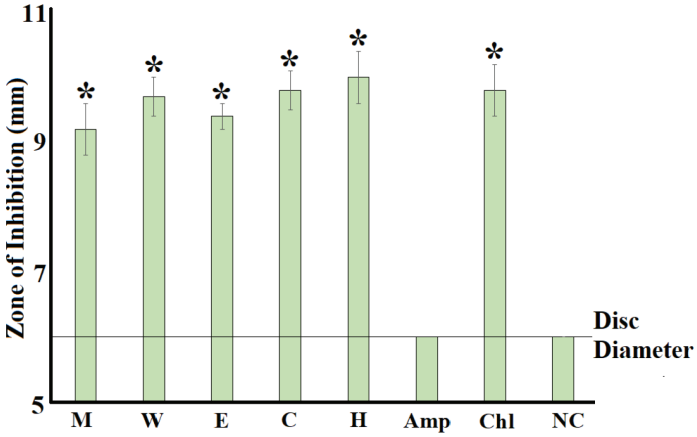

Figure 5: Antibacterial activity of *A. lappa* root extracts against *P. aeruginosa* (ATCC39324) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. The positive controls were Amp (ampicillin 10µg) and Chl (chloramphenicol 10µg). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates (n=6) ± SEM. * indicates results that are significantly different to the negative control (P<0.01).

Table 2: Disc diffusion (DD) and liquid dilution (LD) MIC values (µg/mL) for the *A. lappa* root 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	798	436	688	420	>5000	>5000	1295	926	1675	1284
W	>5000	>5000	>5000	>5000	>5000	>5000	670	558	2216	1570
E	747	385	397	254	1675	1186	728	655	1884	1466
C	-	-	-	-	-	-	>5000	>5000	3650	2287
H	-	-	-	-	-	-	2462	1963	1163	1080
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.

Quantification of minimum inhibitory concentration (MIC)

The relative antimicrobial strength of the extracts was further evaluated by determining the MIC values using two methods: the liquid dilution MIC assay and the disc diffusion MIC assay (Table 2). Consistent with the antibacterial screening assays, the mid to higher polarity methanol, aqueous and ethyl acetate *A. lappa* root extracts were the most effective at inhibiting the growth of the bacterial triggers of the selected autoimmune diseases. The MIC values of the conventional antibiotic controls were only determined for the liquid dilution assay. Commercially manufactured discs with set amounts of antibiotics loaded were used for the disc diffusion assay and thus the zones of only single doses were recorded. Chloramphenicol was the most versatile antibiotic as it inhibited all bacteria tested except *P. aeruginosa*. Notably, the *P. aeruginosa* strain used in these studies was completely resistant to all other antibiotics. Notably, all bacteria except *A. baylyi* were completely resistant to erythromycin. Furthermore, with the exception of *P. mirabilis* and *P. vulgaris*, all of the other bacterial strains were completely resistant to penicillin. Both *Proteus* spp. were completely resistant to tetracycline. As MIC values >1µg/mL for pure antibiotics in this assay indicates resistance,[39-42] all of these bacteria were considered resistant to all of the conventional antibiotics tested.

The MIC values determined for the *A. lappa* root extracts compare relatively well between the disc diffusion and liquid dilution assays. All bacterial species were susceptible to the methanolic and ethyl acetate extracts, although the inhibition was only noteworthy (<1000µg/mL) against the *Proteus* spp. and *A. baylyi*. The ethyl acetate extract was a particularly good growth inhibitor (MIC values of 385, 254 and 655µg/mL against *P. mirabilis, P. vulgaris* and *A. baylyi* respectively). The methanolic extract also displayed noteworthy activity (MIC values of 436, 420 and

926µg/mL against *P. mirabilis, P. vulgaris* and *A. baylyi* respectively), whilst noteworthy activity was only evident for the aqueous extract when tested against *A. baylyi* (MIC = 558µg/mL). Therefore, the *A. lappa* root extracts (particularly the ethyl acetate extract) may be useful in the prevention and treatment of rheumatoid arthritis and multiple sclerosis. In contrast, only relative low potency was noted for the extracts against *K. pneumoniae* and *P. aeruginosa*. Therefore, the *A. lappa* root extracts may be of limited use against infections of those bacteria. However, as the *K. pneumoniae* and *P. aeruginosa* strains tested in our study were resistant against all control antibiotics, the extracts may still be useful against these bacteria and testing against other strains of these bacteria is required.

Fractional inhibitory concentration (FIC) assessment

Combinations of the *A. lappa* root extracts with conventional antibiotics against the bacterial pathogens were tested to determine the classes of interactions for these combinations (Table 3). ΣFIC values could not be determined for many of the combinations as one or both of the components in the combination were ineffective against the tested bacterium when tested alone. Of the effective combinations, the majority of were non-interactive (approximately 82% of the inhibitory combinations). Whilst these combinations have no additional benefit over the individual monotherapies, the lack of antagonism indicates that taking these therapies in combination would not have detrimental effects. This is important information as allopathic and complementary therapies are often taken concurrently. One synergistic combination (methanolic extract and chloramphenicol against *P. mirabilis*) was noted. Furthermore, three combinations also produced additive effects against the *Proteus* spp. (ethyl acetate extract and chloramphenicol against *P. mirabilis*; methanol and ethyl acetate extracts in combination with chloramphenicol against *P. vulgaris*). As these combinations have enhanced effects compared to either component alone, they would be beneficial for the treatment and prevention of rheumatoid arthritis (and other diseases causes by *Proteus* spp.). Notably, none of the combinations produced antagonistic effects. Therefore, all combinations are safe to use without decreasing the activity of either component.

No synergistic or additive interactions were noted for combinations of the *A. lappa* root extracts and conventional antibiotics against the growth of *K. pneumoniae* (Table 3). All combinations were either non-interactive or ineffective. Similarly, no synergistic interactions were recorded against *A. baylyi* or *P. aeruginosa*. However, three additive interactions were noted (methanolic or ethyl acetate extracts in combination with chloramphenicol against *A. baylyi*; methanolic extract in combination with tetracycline against *P. aeruginosa*). Thus, these combinations may be beneficial in treating infections of these bacteria due to their increased growth inhibitory efficacies. As *A. baylyi* and *P. aeruginosa* are bacterial triggers of multiple sclerosis,[4-6] these combinations may therefore be beneficial in the prevention and treatment of that disease. All of the other combinations were non-interactive. Whilst there is no added benefit in combining these therapies, their concurrent use would not decrease the activity of either component and therefore they may be safely used in combination without decreasing the efficacy of the treatment.

Quantification of toxicity

No LC_{50} values were determined for the ethyl acetate, chloroform or hexane extracts as <50 % mortality was seen in all tested concentrations (Table 4). In contrast, LC_{50} values of 1656 and 1458µg/ml were determined for the methanolic and aqueous extracts respectively. As extracts with LC_{50} values <1000 µg/ml towards *Artemia* nauplii have previously been defined as being toxic in this assay,[35,36] all extracts were deemed to be nontoxic. Furthermore, all plant extracts demonstrated a lack of toxicity

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

Bacteria	Extract	Penicillin-G	Chloramphenicol	Erythromycin	Tetracycline
P. mirabilis	M	1.75 (IND)	0.48 (SYN)	-	-
	W	2.23 (IND)	1.27 (IND)	-	-
	E	1.13 (IND)	*0.58 (ADD)*	-	-
P. vulgaris	M	1.65 (IND)	*0.72 (IND)*	-	-
	W	2.2 (IND)	1.46 (IND)	-	-
	E	1.42 (IND)	*0.83 (ADD)*	-	-
K. pneumoniae	M	-	2.52 (IND)	-	1.62 (IND)
	W	-	2.66 (IND)	-	1.84 (IND)
	E	-	1.78 (IND)	-	1.45 (IND)
A. baylyi	M	-	1.5 (IND)	*0.58 (ADD)*	1.13 (IND)
	W	-	1.78 (IND)	1.08 (IND)	1.35 (IND)
	E	-	1.33 (IND)	*0.84 (ADD)*	1.25 (IND)
	C	-	2.66 (IND)	2.33 (IND)	2.19 (IND)
	H	-	3.2 (IND)	2.74 (IND)	2.43 (IND)
P. aeruginosa	M	-	-	-	0.92 (ADD)
	W	-	-	-	1.17 (IND)
	E	-	-	-	1.46 (IND)
	C	-	-	-	2.75 (IND)
	H	-	-	-	1.86 (IND)

M = methanolic extract; W = aqueous extract ; E = ethyl acetate extract; H = hexane extract; SYN = synergistic interaction; *ADD = additive interaction*; IND = indifferent interaction; - = a ΣFIC could not be determined as at least one component of the combination was inactive.

Table 4: LC_{50} values determined for *A. lappa* root extracts in the *Artemia* nauplii and HDF bioassays following 24 hr exposure.

Extract	LC_{50} value (µg/mL)	
	ALA	HDF assay
M	1656	-
W	1458	-
E	-	-
C	-	-
H	-	-
PC	56	NT

- indicates that less than 50% mortality was induced by the extract at all concentrations tested. ALA = *Artemia* nauplii toxicity assay; HDF = human dermal fibroblast toxicity assay; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NT = Not tested.

towards normal human primary dermal fibroblasts, with cellular viability for all tests substantially >50% of the untreated control. All extracts were therefore deemed to be nontoxic.

DISCUSSION

This study investigated the ability of *A. lappa* root extracts to inhibit the growth of some bacterial triggers of autoimmune inflammatory diseases, both alone and in combination with conventional antibiotics. Several *A. lappa* root extracts were identified as effective bacterial growth inhibitors. The ethyl acetate extract was a particularly strong inhibitor of *P. mirabilis, P. vulgaris* and *A. baylyi* growth, with MIC values as low as 254µg/mL. Noteworthy activity was also noted for the methanolic extract against those bacteria. Whilst the *A. lappa* root extracts extracts also inhibited the growth of *K. pneumoniae* and *P. aeruginosa,* the MIC values were generally substantially >1000µg/mL and are thus indicative of only low to moderate inhibitory activity. Whilst a detailed investigation of the phytochemistry of the *A. lappa* root extracts was beyond the scope of this study, the qualitative phytochemical studies highlighted several phytochemical classes that may contribute to the bacterial growth inhibitory activity. Interestingly, the methanolic and aqueous *A. lappa* root extracts had relatively high abundances of polyphenolics and flavonoids, as well as moderate levels of tannins. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.[43-45] This has been attributed to a variety of mechanisms, including their ability to complex with extracellular and soluble proteins, as well as bacterial cell walls.[45] Similarly, multiple tannins have broad-spectrum antibacterial activity via a variety of intra- and extra-cellular mechanisms, including the precipitation of microbial proteins.[46] It is likely that other phytochemical classes may also contribute to the growth inhibitory properties of these extracts. Therefore, phytochemical evaluation studies and bioactivity driven isolation of the active components are required to evaluate the mechanism of the *A. lappa* root extracts growth inhibitory activity.

The combinational studies combining the *A. lappa* root extracts with conventional antibiotics also yielded interesting results. Several combinations displayed enhanced potential as therapeutic agents (particularly against *Proteus* spp.) compared with the inhibitory activity of either the extract or antibiotic components alone. Indeed, one synergistic and one additive interactions were noted against *P. mirabilis*, whilst two additive interactions were noted against *P. vulgaris*. Notably, all of these potentiating combinations contained chloramphenicol as the antibiotic component, in combination with either the methanolic or ethyl acetate *A. lappa* root extract. Three additional additive combinations were also noted (erythromycin and either the methanolic or ethyl acetate *A. lappa* root extract against *A. baylyi*; or tetracycline and the methanolic extract against *P. aeruginosa*). The implications of these potentiating combinations include enhanced efficacy, the requirement for lower dose administration and a reduction in side effects, as well as possibly reduced antimicrobial resistance.[2,29] Importantly, none of the combinations produced antagonistic effects. This is an important finding as it indicates that it is safe to use the *A. lappa* root extracts and conventional antibiotics in combination without decreasing the efficacy of either component.

A further trend was evident in our study: the extract-antibiotic combinations that did not produce additive effects, generally did not greatly affect the efficacy of the antibiotic i.e. they appear to not counter-indicate with the antibiotics tested in this study. This is important as many users of herbal and traditional medicines self-diagnose/treat, often with multiple therapies concurrently. Thus, an understanding of drug herbal medicine interactions is important. This is an important finding as it indicates that all of the combinations tested are safe to use without decreasing the efficacy of either component.

Microbes have developed numerous resistance mechanisms to avoid the effects of antibiotics. One main method is through the use of multi-drug resistant (MDR) efflux pumps that are encoded chromosomally and are used to rapidly remove antibiotics that have entered the bacterial cells, thus rendering them resistant to the effects of the antibiotic.[47,48] A single pump may allow the bacteria to escape several types of antimicrobials. When these efflux pumps are inhibited, the intracellular concentration of antibiotic will increase, allowing the treatment to once again be effective. Interestingly, many plants possess multi-drug resistance (MDR) pump inhibitors in order to enhance the activity of their own natural antimicrobial compounds. Such MDR pump inhibitors become effective tools when used in combination with some previously ineffective/resistance prone antibiotic compounds and several examples have previously been reported.[48] Isoflavones isolated from *Lupinus argenteus* Pursh potentiate the activity of the natural plant antibiotic berberine as well as the synthetic fluoroquinoline antibiotic, norfloxacin as inhibitors of *S. aureus* growth.[48] That study reported that the isoflavone allows a greater concentration of berberine to occur inside the bacteria by inhibiting the efflux mechanism (MDR pump). Similarly, *Mezoneuron benthamianum* Baill. and *Securinega virosa* (Roxb. Ex Willd) Baill. extracts act as efflux pump inhibitors for fluoroquinolone, tetracycline and erythromycin in resistant strains of *S. aureus* (MRSA).[49] As a consequence, the *M. benthamianum* ethanol extract and chloroform extract of *S. virosa* reduce the MIC (minimum inhibitory concentration) of norfloxacin against *S. aureus* by a factor of 4.

In our study, all bacterial species were resistant to penicillin-G, chloramphenicol, erythromycin and tetracycline, with only low susceptibility or complete resistance to each antibiotic. All of these antibiotics are susceptible to resistance due to efflux pumps.[49,50] A single pump can provide bacteria with resistance to a wide array of chemically and structurally diverse antibiotics and it is not uncommon for an organism to code for more than one efflux pump.[49,50] It is therefore imperative to identify agents that can block the efflux mechanism (efflux pump inhibitors - EPIs) or alter the process of efflux, and in so doing, extend the life of existing antibacterial drugs. Plants produce various secondary metabolites that are used as defense mechanisms against pathogenic invaders. Some plants produce antimicrobials which, along with other compounds, inhibit the efflux of those antimicrobials from a bacterial cell. There are currently no EPI/antimicrobial drug combinations on the market, although research into identifying potential EPIs is ongoing.[49] The synergistic and additive interactions noted in our study suggest the possibility of a common EPI in the *A. lappa* root extracts that could be inhibiting a MDR efflux pump in these bacteria.

Alternatively (or in addition to MDR efflux pumps), the bacteria screened in our study may have acquired genes encoding for reduced-affinity penicillin-binding protein 2a (PBP2a) (rendering β-lactam

antibiotics ineffective).[51] As penicillin binding proteins are a group of protein enzymes, these phytochemicals may form nonspecific interactions and affect the bacterial cell wall biosynthesis. The *A. lappa* root extracts may also contain a β-lactamase inhibitor. β-lactamases are the major defense of gram-negative bacteria against β-lactam antibiotics.[52] Clavulanic acid is an irreversible β-lactamase inhibitor, which in combination with β-lactam antibiotics, can block the bacterial antimicrobial resistance mechanism.[2] Further studies are required to identify whether extract compounds mirror the chemical and biological characteristics of clavulanic acid (i.e. the presence of a β-lactam ring).

None of the *A. lappa* root extracts or conventional antibiotics were toxic, indicating their potential for therapeutic use. The non-toxicity of the *A. lappa* root extracts is unsurprising as this species has long been used in several traditional medicine systems to treat a wide variety of diseases.[10-20] However, *in vitro* studies using further 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

The results of this study demonstrate the potential of the *A. lappa* root extracts in inhibiting the growth of some bacterial triggers of autoimmune inflammatory diseases. Extract components may also potentiate the activity of antibiotics that are relatively ineffective alone. Therefore, a combinational approach not only increases the effectiveness of these antibiotics, but may also potentially reduce the side effects and reduce the development of drug resistant pathogens. Isolation of the bioactive and potentiating compounds may be beneficial in drug design against several bacteria including the microbial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ABBREVIATIONS

ALA: *Artemia* lethality assay; **DMSO:** Dimethyl sulfoxide **EPI:** Efflux pump inhibitor; **FIC:** Fractional inhibitory concentration; **HDF:** Human dermal fibroblasts; **LC_{50}:** The concentration required to achieve 50 % mortality; **MIC:** Minimum inhibitory concentration; **MDR:** Multi-drug resistant; **ZOI:** Zone of inhibition.

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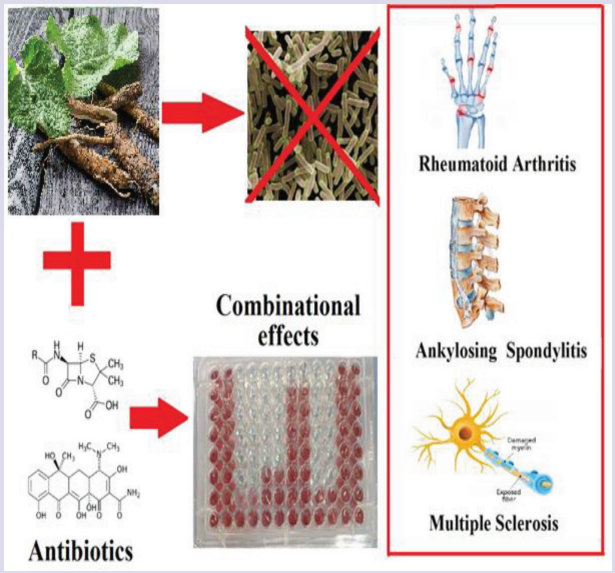
PICTORIAL ABSTRACT



SUMMARY

- *Arctium lappa* root extracts were screened for the ability to block the growth of a panel of bacterial triggers of autoimmune inflammatory diseases.
- The antibacterial activity was quantified by determining the MIC values of each extract.
- The extracts were also tested in combination with conventional antibiotics and the class of interaction was determined
- Toxicity of *A. lappa* root extracts was determined using the *Artemia* nauplii and HDF cell viability toxicity bioassays.

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 250 publications in a variety of peer reviewed journals.

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