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# Centella asiatica (L.) Urban Leaf 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 *Centella asiatica* (Gotu Kola) 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  $\Sigma$ FIC values obtained from these assays were used to determine the class of combinational effects and isobologram analysis was used to determine the ideal synergistic ratio(s). Toxicity was evaluated by *Artemia nauplii* mortality assays. **Results:** The methanolic extracts showed good inhibitory activity against several microbial triggers of autoimmune inflammatory diseases, whilst the chloroform and hexane extracts were also potent inhibitors of *K. pneumoniae* growth. Combinations of the *C. asiatica* extracts with conventional antibiotics were often substantially more effective in inhibiting bacterial growth. One synergistic and 10 additive interactions were noted. Notably, the methanolic extract restored significant growth inhibitory activity to chloramphenicol and tetracycline when tested in combination against *K. pneumoniae*. In contrast, two antagonistic

interactions were noted for combinations containing gentamycin (against *A. baylyi* and *S. pyogenes*), indicating that those combinations should be avoided when treating infections caused by those bacteria. **Conclusion:** *C. asiatica* extracts have potential as inhibitors of bacterial triggers of selected autoimmune inflammatory diseases. Furthermore, extract components may also potentiate the activity of two antibiotics that are relatively ineffective alone. Isolation of these agents may be beneficial in drug design against several bacteria, including the microbial triggers of rheumatoid arthritis, ankylosing spondylitis 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.<sup>1</sup> Additionally, the discovery of new antimicrobial agents has decreased dramatically in recent years, making many bacterial infections difficult to manage using current therapeutic strategies.<sup>2</sup> The development of alternative antibacterial treatment modalities is considered by the World Health Organisation (WHO) to be perhaps the biggest challenge currently facing medical science.<sup>3</sup> For a number of reasons reviewed elsewhere,<sup>2</sup> 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).<sup>4</sup> 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, MS and RV are induced in genetically 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.<sup>4</sup> 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.<sup>1</sup> Furthermore, the prophylactic use of pure antibiotics over prolonged periods would certainly induce resistance, thereby rendering the bacteria refractory to their actions. A better approach may be to use combinations of antibacterial components.<sup>2</sup>

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.<sup>5</sup> 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 antibiotic resistant strains.<sup>6,7</sup> 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.<sup>2</sup> Combinations of plant extracts/isolated compounds with conventional antibiotics may also prove to have economic advantages.<sup>5</sup> 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.<sup>5</sup> 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.<sup>6</sup>

*Centella asiatica* (L.) Urban (commonly known as Gotu Kola and Indian pennywort; Family Apiaceae) is a medicinal plant that is native to wetland regions of Asia. It is particularly prevalent in India, Madagascar and Sri Lanka, but also occurs in southern Africa, Australia, China, Indonesia and throughout the South Pacific region. In India, *C. asiatica* is used to treat skin conditions associated with eczema, lupus and psoriasis and for varicose ulcers.<sup>8</sup> It is also particularly beneficial in female conditions such as amenorrhea and diseases of the urogenital tract. It is also used in traditional Chinese medicine (TCM) for treating fevers, dysentery and urinary tract infections, as well as infectious hepatitis and jaundice.<sup>9</sup> *C. asiatica* decoctions have also been reported to be effective as an antidote for poisoning by arsenic, toxic mushrooms and *Gelsemium elegans* (Gardner and Chapm.) Benth.,. A poultice is also applied externally to treat snakebites, scabies, herpes, fractures, contusions and sprains.<sup>9</sup> However, despite its well documented traditional uses, there has been relatively little research into the therapeutic properties of *C. asiatica*.

Several studies have reported growth inhibitory effects of *C. asiatica* essential oils against a panel of bacterial pathogens including *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Shigella sonnei*.<sup>10</sup> The oil is a particularly good inhibitor of gram negative bacteria, with MIC values as low as 39µg/mL recorded against *E. coli*. It also inhibited the growth of gram positive bacteria, although the MIC values obtained indicated more moderate growth inhibition. Similarly, leaf extracts are good inhibitors of a panel of bacteria, including several of the same species.<sup>11</sup> However, that study tested a single high concentration of each extract using an agar diffusion technique. MIC values were not determined, making comparisons with other studies impossible. Chemical profiling of *C. asiatica* essential oil has identified high levels of the sesquiterpenoid germacrene. This compound has strong antimicrobial activity and is likely to contribute to the antibacterial effects of *C. asiatica*. Despite these earlier studies, *C. asiatica* preparations are yet to be tested against the bacterial triggers of rheumatoid arthritis (*Proteus mirabilis*), ankylosing spondylitis (*Klebsiella pneumoniae*), multiple sclerosis (*Acinetobacter baylyi*, *Pseudomonas aeruginosa*) and rheumatic fever (*Streptococcus pyogenes*).<sup>4</sup> Furthermore, we were unable to find any studies testing the antibacterial activity of *C. asiatica* extracts in combination with conventional antibiotics. Therefore, this study was undertaken to investigate the antimicrobial effects of *C. asiatica* 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

Certified *C. asiatica* (L.) Urban leaf powder was obtained from Noodles Herbal Emporium, Australia and a voucher specimen (GU2017aGC) was deposited in the School of Natural Sciences, 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 24h 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 *C. asiatica* extracts for the presence of alkaloids, cardiac glycosides, flavonoids, phenolic compounds, phytosterols, saponins, tannins and triterpenoids was achieved as previously described.<sup>12,13</sup>

### Antibacterial screening Conventional Antibiotics

Penicillin-G (1440-1680µg/mg), chloramphenicol (≥98% purity), erythromycin (≥850µg/mg), gentamycin (600µg/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.<sup>4</sup> 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. A clinical isolate strain of *Streptococcus pyogenes* was obtained from the School of Environment and Science 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 37°C for 24h and were subcultured and maintained in nutrient broth at 4°C until use.

### Evaluation of antibacterial activity

Antibacterial activity screening of the *C. asiatica* extracts was assessed using a modified disc diffusion assay.<sup>12,13</sup> 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.<sup>14</sup> 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.<sup>15</sup> All plates were incubated at 37°C for 24h. 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 6h 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.<sup>12,13</sup> 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 *C. asiatica* extracts and the conventional antibiotics were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC) for each combination.<sup>15</sup> 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).<sup>15</sup>

Varied ratio combination studies (isobolograms)

For each combination producing synergistic interactions, nine different ratios spanning the range 10:90 (extract:antibiotic) to 90:10 (extract:antibiotic) were tested. All combinations were tested in duplicate in two independent experiments, providing four replicates for each combination ratio. The data is presented as the mean of four replicates. Data points for each ratio examined were plotted on a isobologram and this was used to determine optimal combination ratios to obtain synergy. Data points on or below the 0.5:0.5 line indicate synergy; those above the 0.5:0.5 line, up to and including the 1.0:1.0 line indicate an additive interaction; data points above the 1.0:1.0 line indicate indifferent interaction.

## 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 MTT cellular proliferation assay was used to determine a cellular evaluation of toxicity.

## *Artemia franciscana* Kellogg nauplii toxicity screening

Potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) (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 *C. asiatica* extracts, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.<sup>16,17</sup> The LC<sub>50</sub> with 95% confidence limits for each treatment was calculated using probit analysis.

## Cellular viability assay

All extracts were also screened individually using a normal human primary dermal fibroblast (HDF) standard assay.<sup>18</sup> Briefly, the HDF cells were obtained from American Type Culture Collection (ATCC PCS-201-012) and were cultured and maintained in Dulbecco's modified eagle medium (DMEM; ThermoFisher Scientific, Australia), supplemented with 10% foetal calf serum (Life Technologies, Australia), 50µg/mL streptomycin (Sigma-Aldrich, Australia) and 50 IU/mL penicillin (Sigm-Aldrich, Australia) at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere. Individual 70µL volumes of culture media (containing approximately 5000 cells) were added to wells of a 96 well plate and 30µL

of the test extracts or cell media (for the negative control) was added to each well. The plates were incubated at 37°C, 5% CO<sub>2</sub> for 24h in a humidified atmosphere. All extracts were screened at 200µg/mL. The cells were then washed in PBS (pH 7.2) to remove interference due to sample colour. A 20µL volume of Cell Titre 96 Aqueous One solution (Promega) was added to each well and the plates were incubated for a further 3h. 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 in at least triplicate and 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* value < 0.01 considered to be statistically significant.

## RESULTS

Liquid extraction yields ranged from 124mg (*C. asiatica* hexane extract) to 238mg (methanolic *C. asiatica* extract) (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*)

*P. mirabilis* growth was inhibited by the mid to high polarity *C. asiatica* water, methanol 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 18.2±0.4 mm. A volume of 10µL of this extract was infused into the disc, which equates to approximately 240µg of extract infused into the disc. The ZOI for this extract is substantially larger than that of the chloramphenicol controls (12.5±0.5 mm). Notably, the chloramphenicol control antibiotic was pure and was tested and 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 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. Interestingly, this bacterium was resistant to the ampicillin control. The aqueous and ethyl acetate extracts had substantially smaller ZOIs than the methanolic extract, (9.2 and 7.2 mm respectively), although this inhibition was still noteworthy. 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 slightly smaller ZOIs were measured. As for *P. mirabilis*, the methanolic extract was the strongest inhibitor of *P. vulgaris* growth (ZOI 14.8±0.4 mm). The ZOIs measured for the methanolic extract were similar to those recorded for the pure chloramphenicol control and substantially bigger than those noted for the ampicillin control (7.6mm). Whilst also displaying inhibitory activity, the aqueous and ethyl acetate extracts had substantially lower efficacy than the methanolic extract, with ZOIs of 8.5 and 6.6 mm respectively. All other extracts were ineffective at inhibiting *P. vulgaris* growth.

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

Most of the *C. asiatica* extracts inhibited the growth of *K. pneumoniae*, albeit with much smaller ZOIs than measured for the *Proteus* spp. (Figure 3). The methanolic and chloroform extracts were the strongest growth inhibitors (ZOIs of 8.3 and 8.6mm respectively). These ZOIs were comparable to that of the ampicillin control (8.4mm), but substantially smaller than those measured for chloramphenicol (12.6mm). Only the ethyl acetate extract was completely ineffective against *K. pneumoniae*, although it is noteworthy that this extract was tested at a substantially lower concentration (~5mg/mL) than the other *C. asiatica* extracts, which may account for its lack of apparent inhibitory activity against this bacterium. As *K. pneumoniae* can induce ankylosing spondylitis in genetically susceptible individuals,<sup>4</sup> the *C. asiatica* extracts (particularly the methanolic and chloroform extracts) may be beneficial in the

prevention and treatment of that disease.

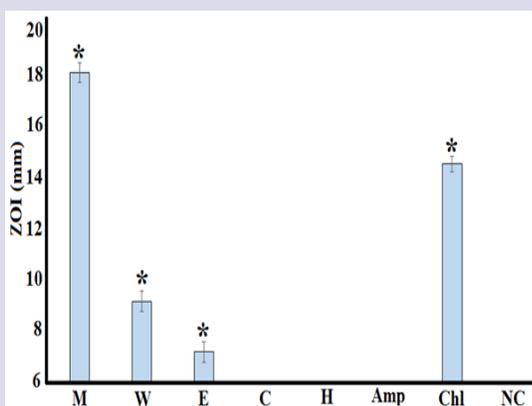
## Inhibition of bacterial triggers of multiple sclerosis (*A. baylyi* and *P. aeruginosa*)

The methanolic, *R. canina* extract also inhibited *A. baylyi* growth, with a ZOI of 9.3±0.6mm (Figure 4). This *A. baylyi* strain was highly susceptible to ampicillin and chloramphenicol, ZOIs of 11.6 and 14.3mm respectively. The aqueous and ethyl acetate extracts also inhibited *A. baylyi* growth, although the small ZOIs (7.3 and 6.8mm respectively) that indicate only low inhibitory activity. In contrast, the *P. aeruginosa* strain tested in this study was completely resistant to all of the *C. asiatica* extracts (Figure 5). However, it is noteworthy that this was a particularly resistant bacterium. It was also completely resistant to the ampicillin control and displayed only low sensitivity towards chloramphenicol, with a ZOI of ~7.3mm. Despite the lack of activity against *P. aeruginosa*, the

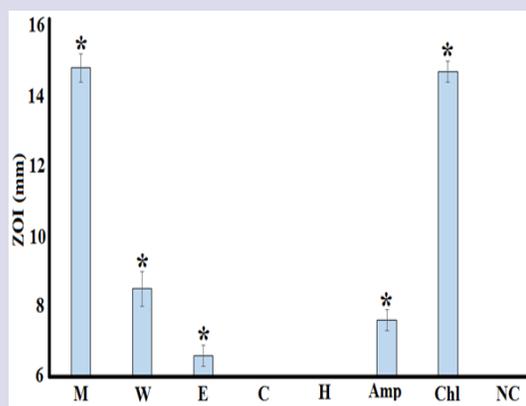
**Table 1:** The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *C. asiatica* extracts.

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	238	23.8	+++	++	+++	-	-	-	-	-	-	+++	+	-	-
W	260	26.0	+++	+++	++	-	+	+	-	-	-	+++	+	-	-
E	49	4.9	+	+	-	-	-	-	-	-	-	+	-	-	-
C	199	19.9	-	-	+	-	-	-	-	-	-	-	-	-	-
H	124	12.4	-	-	+	-	-	-	-	-	-	-	-	-	-

+++ 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 *C. asiatica* 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 *C. asiatica* 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 ( $P<0.01$ ).

*C. asiatica* extracts may still be useful in preventing the onset of multiple sclerosis as *A. baylyi* and *P. aeruginosa* can induce multiple sclerosis in genetically susceptible people.<sup>4</sup>

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

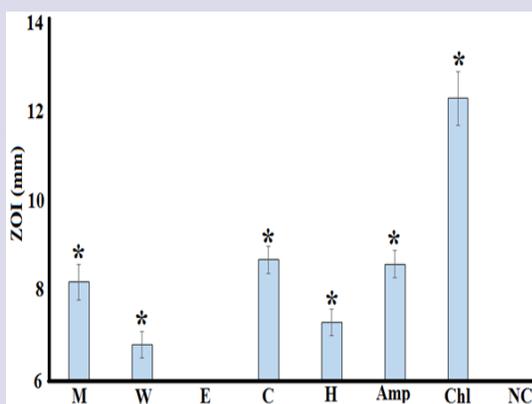
*S. pyogenes* growth was inhibited by the methanolic, aqueous and ethyl acetate *C. asiatica* extracts (Figure 6). The chloroform and hexane extracts were completely ineffective at inhibiting the growth of this bacterium. The methanolic extract was the strongest growth inhibitor, albeit with relatively small ZOIs (ZOI ~7.8mm). Notably, this inhibition was comparable to that of chloramphenicol (ZOI ~8.2mm). This is noteworthy as the chloramphenicol control was pure and was tested at relatively high doses (10µg/disc). In contrast, *S. pyogenes* was substantially more susceptible to ampicillin (~12.4mm) The water and ethyl acetate extracts also inhibited *S. pyogenes* growth, albeit with substantially smaller ZOIs (7.3 and 6.6mm respectively), indicating low to moderate growth inhibitory activity. As *S. pyogenes* can trigger rheumatic fever in genetically susceptible people,<sup>4</sup> the *C. asiatica* methanolic extract (and to a lesser extent, the aqueous and ethyl acetate extracts) may be effective in the prevention and treatment of this disease (and other diseases caused by this bacterium).

### Quantification of minimum inhibitory concentration (MIC)

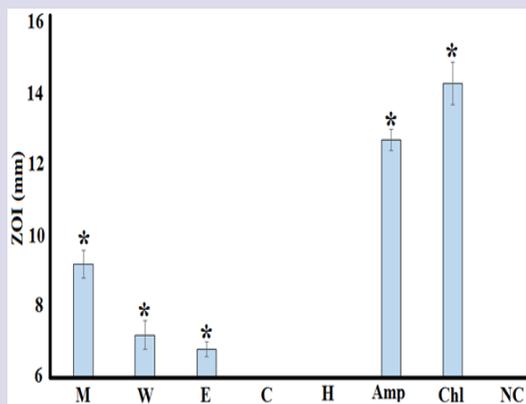
The relative antimicrobial strength of the extracts was further evaluated by determining the MIC values using two methods: the liquid dilution MIC assay and the disc diffusion MIC assay (Table 2). Consistent with the antibacterial screening assays, the higher polarity methanol and water *C. asiatica* extracts were generally most effective at inhibiting the growth of the bacterial triggers of the selected autoimmune diseases. The complete lack of inhibition of *P. aeruginosa* by all *C. asiatica* extracts was the exception to this trend. 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. Gentamycin was generally the most potent antibiotic (as judged by its MIC) and was the only antibiotic to inhibit the growth of all of the bacterial species tested. Notably, the *P. aeruginosa* strain used in these studies was resistant to all of antibiotics except gentamycin. Furthermore, with the exception of *P. mirabilis* and *P. vulgaris*, all of the other bacterial strains were completely resistant to penicillin.

The MIC values determined for the *C. asiatica* extracts compare relatively well between the disc diffusion and liquid dilution assays with some notable exceptions. All bacterial species except *P. aeruginosa* were most susceptible to the methanolic extract, although the ethyl acetate extract had similar efficacy towards the *Proteus* spp. and *S. pyogenes* (based on MIC values). The growth of *P. mirabilis* was inhibited by methanolic (DD MIC 800µg/mL; LD MIC 672µg/mL) and ethyl acetate extracts (DD MIC 1200µg/mL; LD MIC 850µg/mL) with MIC values that indicate noteworthy growth inhibitory activity. The aqueous extract was also a moderate inhibitor of this bacterium (DD MIC 1742µg/mL; LD MIC 1570µg/mL). Similar, albeit slightly higher MIC values were also determined for these extracts against *P. vulgaris*. Therefore, these extracts may be useful in the prevention and treatment of rheumatoid arthritis. The methanolic extract was also a strong inhibitor of *K. pneumoniae* growth (DD MIC 950µg/mL; LD MIC 672µg/mL). Notably, the chloroform and hexane extracts were potent inhibitors of *K. pneumoniae* growth in the liquid dilution assay (LD MIC 95 and 50 µg/mL respectively) and thus may also be useful in the prevention and treatment of ankylosing spondylitis. Whilst the methanolic, aqueous and ethyl acetate extracts also inhibited the growth of *A. baylyi* and *S. pyogenes*, the MIC values were >1000µg/mL against those bacteria, indicating only low to moderate potency. However, as these bacteria were also resistant against all control antibiotics except gentamycin, the *C. asiatica* extracts may still be useful in the prevention of multiple sclerosis and rheumatic fever.



**Figure 3:** Antibacterial activity of *C. asiatica* 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 ( $n=6$ )  $\pm$  SEM. \* indicates results that are significantly different to the negative control ( $P<0.01$ ).



**Figure 4:** Antibacterial activity of *C. asiatica* 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 ( $n=6$ )  $\pm$  SEM. \* indicates results that are significantly different to the negative control ( $P<0.01$ ).

## Fractional inhibitory concentration (FIC) assessment

### Combinational effects on a bacterial trigger of rheumatoid arthritis (*Proteus spp.*)

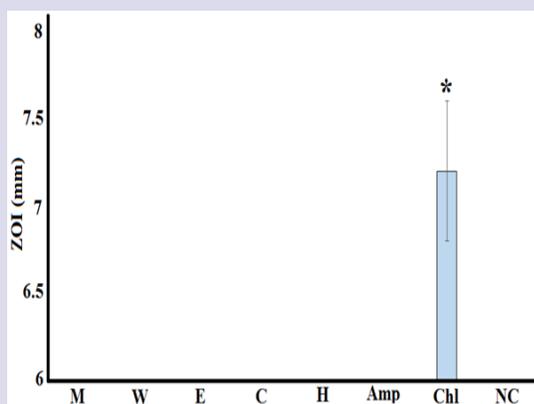
Combinations of the *C. asiatica* extracts with conventional antibiotics were tested against *P. mirabilis* and *P. vulgaris* to determine the classes of interactions for these combinations (Table 3).  $\Sigma$ FIC values could not be determined for many of the combinations as one or both of the components in the combination were ineffective against the tested bacterium when tested alone. Of the effective combinations, the

majority of were non-interactive (approximately 89% of the inhibitory combinations). Whilst these combinations have no additional benefit over the individual monotherapies alone, 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. Three combinations also produced additive effects (methanol extract and chloramphenicol against *P. mirabilis*; methanol and ethyl acetate extracts in combination with tetracycline against *P. vulgaris*), with  $\Sigma$ FIC values of 0.83, 0.87 and 0.98

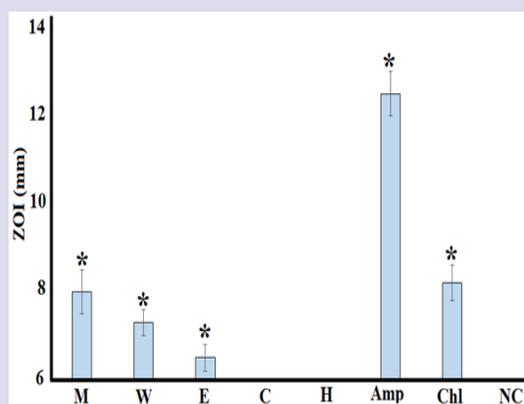
**Table 2:** Disc diffusion and liquid dilution MIC values for the *C. asiatica* extracts against *P. mirabilis*, *P. vulgaris*, *K. pneumoniae*, *A. baylyi*, *P. aeruginosa* and *S. pyogenes* growth ( $\mu\text{g/mL}$ ).

EXTRACT	<i>P. mirabilis</i> (ATCC33304)		<i>P. vulgaris</i> (ATCC21719)		<i>K. pneumoniae</i> (ATCC31488)		<i>A. baylyi</i> (ATCC21721)		<i>P. aeruginosa</i> (ATCC39324)		<i>S. pyogenes</i>	
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC
M	800	672	1405	968	950	672	2486	1635	-	>5000	>5000	1890
W	1742	1570	>5000	4822	>5000	>5000	>5000	>5000	-	-	>5000	2685
E	1200	850	1460	1158	-	-	ND	>5000	-	-	ND	1688
C	-	-	-	-	ND	95	-	-	-	-	-	-
H	-	-	-	-	ND	50	-	-	-	-	-	-
Positive controls												
Penicillin	ND	2.5	ND	1.25	ND	-	ND	-	ND	-	ND	-
Chloramphenicol	ND	2.5	ND	2.5	ND	1.25	ND	2.5	ND	-	ND	-
Gentamycin	ND	1.25	ND	1.25	ND	0.31	ND	0.31	ND	0.63	ND	0.63
Erythromycin	ND	2.5	ND	2.5	ND	-	ND	2.5	ND	-	ND	-
Tetracycline	ND	-	ND	2.5	ND	1.25	ND	1.25	ND	-	ND	2.5
Negative control	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-

M = methanol; W = water; E = ethyl acetate; C = chloroform; H = hexane. DD = disc diffusion; LD = liquid dilution. - indicates no inhibition at any dose tested. Numbers indicate the mean DD MIC and LD MIC values of triplicate determinations, expressed in  $\mu\text{g/mL}$ . ND = MIC could not be determined as only a single dose was tested.



**Figure 5:** Antibacterial activity of *C. asiatica* 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 10 $\mu\text{g}$ ) and Chl (chloramphenicol 10 $\mu\text{g}$ ). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates ( $n=6$ )  $\pm$  SEM. \* indicates results that are significantly different to the negative control ( $P<0.01$ ).



**Figure 6:** Antibacterial activity of *C. asiatica* extracts against a clinical isolate of *S. pyogenes* 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 10 $\mu\text{g}$ ) and Chl (chloramphenicol 10 $\mu\text{g}$ ). Negative control (NC) = water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates ( $n=6$ )  $\pm$  SEM. \* indicates results that are significantly different to the negative control ( $P<0.01$ ).

respectively. 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 caused by *Proteus* spp.). None of the combinations produced synergistic effects.

### Combinational effects on a bacterial trigger of ankylosing spondylitis (*K. pneumoniae*)

Two synergistic interactions were noted for combinations of the *C. asiatica* extracts and conventional antibiotics against the growth of *K. pneumoniae* (Table 3). Interestingly, both of these combinations contained the methanolic extract with either chloramphenicol or tetracycline ( $\Sigma$ FIC 0.43 and 0.37 respectively). These combinations may therefore be effective in the prevention and treatment of ankylosing spondylitis (and other diseases caused by *K. pneumoniae*). Of further note, combinations of chloramphenicol with chloroform or hexane extracts and the hexane extract with erythromycin or tetracycline, produced additive effects. This

indicates that these combinations may also be beneficial in the treatment of those diseases due to their increased growth inhibitory efficacies compared to the individual components. All other combinations were either non-interactive or ineffective.

### Combinational effects on bacterial triggers of multiple sclerosis (*A. baylyi* and *P. aeruginosa*)

A range of interactions were observed for combinations of the *C. asiatica* extracts and conventional antibiotics against *A. baylyi* (Table 3). Combinations of the methanolic extract with erythromycin or tetracycline resulted in additive interactions. Similarly, the ethyl acetate-tetracycline combination also produced additive effects. Thus, these combinations may be beneficial due to their increased growth inhibitory efficacies. As *A. baylyi* is one of the bacterial triggers of multiple sclerosis,<sup>4</sup> these combinations may be beneficial in the prevention and treatment of that disease. The majority of the other combinations were non-interactive.

**Table 3:**  $\Sigma$ FIC values of *C. asiatica* extracts in combination with conventional antibiotics against *P. mirabilis*, *P. vulgaris*, *K. pneumoniae*, *A. baylyi* and *S. pyogenes*.

		Penicillin	Chloramphenicol	Gentamycin	Erythromycin	Tetracycline
<i>P. mirabilis</i>	M	1.75	0.83	2.2	1.05	-
	W	1.3	1.3	1.8	1.8	-
	E	2.2	1.1	2.3	1.27	-
	C	-	-	-	-	-
	H	-	-	-	-	-
<i>P. vulgaris</i>	M	1.63	1.45	2.2	1.2	0.87
	W	1.27	1.6	2.4	1.83	1.17
	E	1.45	1.53	1.9	1.47	0.98
	C	-	-	-	-	-
	H	-	-	-	-	-
<i>K. pneumoniae</i>	M	-	0.43	2.9	-	0.37
	W	-	1.46	2.7	1.45	2.3
	E	-	-	-	-	-
	C	-	0.92	2.9	-	-
	H	-	0.58	3.2	0.66	0.85
<i>A. baylyi</i>	M	-	1.5	4.2	0.7	0.65
	W	-	2	3.5	1.5	1.25
	E	-	1.75	2.6	1.2	0.8
	C	-	-	-	-	-
	H	-	-	-	-	-
<i>S. pyogenes</i>	M	-	-	4.2	-	1.5
	W	-	-	3.0	-	1.5
	E	-	-	-	-	-
	C	-	-	-	-	-
	H	-	-	-	-	-

M = methanol; W = water; E = ethyl acetate; C = chloroform; H = hexane. - indicates that the  $\Sigma$ FIC could not be determined. M = methanol; W = water; E = ethyl acetate; C = chloroform; H = hexane; - indicates that the  $\Sigma$ FIC could not be determined; Synergy (bold highlighting) =  $\leq$  0.5; Additive (*italics highlighting*) =  $>$  0.5-1.0; Indifferent (no highlighting) =  $>$  1.0 -  $\leq$  4; Antagonistic (*underlined highlighting*) =  $>$  4.0. Numbers indicate the mean  $\Sigma$ FIC values of 4 determinations.

**Table 4:** LC<sub>50</sub> values determined for *C. asiatica* extracts in the *Artemia* nauplii and HDF bioassays following 24 hr exposure.

Extract	LC <sub>50</sub> value (µg/mL)	
	ALA	HDF assay
M	1765	-
W	2468	-
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.

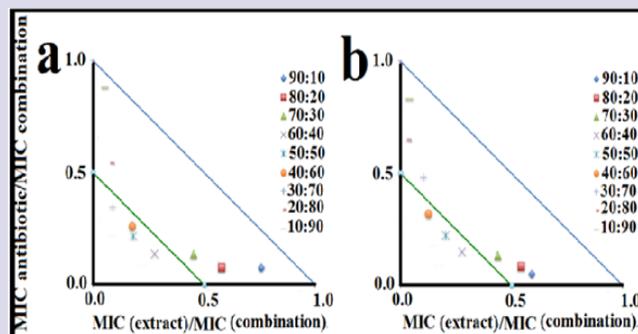
Whilst there is no added benefit in combining these therapies, their concurrent use would not decrease the activity of either component and therefore they may be safely used in combination without decreasing the efficacy of the treatment. However, one antagonistic interaction was detected (methanolic extract with gentamycin). This combination should therefore be avoided for the prevention and treatment of multiple sclerosis (and other diseases caused by *A. baylyi*). No interactive effects could be determined against *P. aeruginosa* as all *C. asiatica* extracts and most of the conventional antibiotics were completely ineffective against this bacterium. It was therefore not possible to calculate  $\Sigma$ FIC values for combinations containing those components.

### Combinational effects on a bacterial trigger of rheumatic fever (*S. pyogenes*)

The combinational antimicrobial effects of the *C. asiatica* extracts with various conventional antibiotics against *S. pyogenes* are summarised in Table 3. The majority of the combinations produced non-interactive effects. These combinations therefore have no therapeutic advantage over the use of either monotherapy alone, although the use of the combination would also not decrease the effects of either component. No synergistic or additive effects were detected for any combination. It is noteworthy that this bacterial strain displayed substantial resistance to most of the conventional antibiotics. Indeed, only gentamycin and tetracycline inhibited the growth of this bacterium, although the relatively high MIC for tetracycline (2.5µg/mL) indicates only low efficacy. Perhaps of greater interest, the combination of the methanolic extract and gentamycin was antagonistic. This combination should therefore be avoided as a chemotherapeutic option to treat *S. pyogenes* infections.

Varied ratio combination studies (isobolograms)

The combination of the methanolic *C. asiatica* extract with chloramphenicol (Figure 7a) or tetracycline (Figure 7b) induced synergistic interactions against *K. pneumoniae* and therefore these combinations were further investigated by isobologram analysis across a range of ratios to determine the ideal combination compositions to obtain synergy. All combination ratios containing 30-60% of the methanolic extract produced synergistic interactions in combination with chloramphenicol against *K. pneumoniae* (Figure 7a). Thus these combination ratios would be beneficial to enhance *K. pneumoniae* growth inhibition. However, bacteria would be less likely to develop resistance when combinations are used in ratios which minimise the amount of conventional antibiotic used. Thus, for long term prophylactic treatment



**Figure 7:** Isobologram for combinations of the methanolic *C. asiatica* extract with (a) chloramphenicol or (b) tetracycline against *K. pneumoniae*. Results represent mean FIC values of four replicates. Ratio = % extract:% antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic ( $\Sigma$ FIC  $\leq$ 0.5). Any points between the 0.5:0.5 and 1.0:1.0 lines are deemed additive ( $\Sigma$ FIC  $>$ 0.5-1.0). Chl = chloramphenicol.

(as would be required to prevent and treat ankylosing spondylitis), the ideal extract:chloramphenicol ratio may be 60:40. However, when used for the treatment of acute infections (e.g. lung infections), the ratio which maximises the efficacy of the treatment (i.e. the 30:70 ratio) may be the preferred option.

Isobologram analysis for the combination of methanolic *C. asiatica* extract and tetracycline showed synergistic interactions against *K. pneumoniae* across a wide range of ratios (Figure 7b). All combinations containing between 40-60% methanolic *C. asiatica* extract produced synergistic effects. All other combination ratios were additive. Therefore, the combination containing 40% methanolic *R. canina* extract and 60% tetracycline was deemed to be the best combination ratio for prophylactic treatment to prevent ankylosing spondylitis, as well as decreasing the possibility of further increasing bacterial resistance to chloramphenicol.

### Quantification of toxicity

No LC<sub>50</sub> 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<sub>50</sub> values of 1765 and 2468µg/ml were determined for the methanolic and aqueous extracts respectively. As extracts with LC<sub>50</sub> values  $<$ 1000 µg/ml towards *Artemia* nauplii have previously been defined as being toxic in this assay [16], all extracts were deemed to be nontoxic. Furthermore, all plant extracts demonstrated a lack of toxicity 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 *C. asiatica* extracts to inhibit the growth of some bacterial triggers of autoimmune inflammatory diseases, both alone and in combination with conventional antibiotics. Several *C. asiatica* extracts were identified as effective bacterial growth inhibitors. The methanolic extract was particularly strong inhibitors of *P. mirabilis*, *P. vulgaris* and *K. pneumoniae* growth, with MIC values as low as 672µg/mL. Whilst these extracts also inhibited the growth of *A. baylyi*, *P. aeruginosa* and *S. pyogenes*, the MIC values were generally substantially  $>$ 1000µg/mL and are thus indicative of only low to moderate inhibitory activity. Interestingly, the chloroform and hexane extracts were particularly good inhibitors of *K. pneumoniae* growth, with MIC values of 95 and 50µg/mL respectively, indicating the inhibitory compounds may be nonpolar in nature. Whilst a detailed investigation of the phytochemistry of the

*C. asiatica* 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 *C. asiatica* extracts had relatively high abundances of polyphenolics and flavonoids, as well as lower levels of tannins, triterpenoids and saponins. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.<sup>19-21</sup> 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.<sup>22</sup> Similarly, multiple tannins have broad spectrum antibacterial activity via a variety of intra- and extra-cellular mechanisms, including the precipitation of microbial proteins.<sup>23</sup> 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 *C. asiatica* extracts growth inhibitory activity.

The studies combining the extracts with conventional antibiotics also highlighted other therapeutic options. In particular, combinations containing methanolic *C. asiatica* extract and either chloramphenicol or tetracycline produced synergistic effects against *K. pneumoniae*, despite this bacterium being relatively resistant against both of these antibiotics. It appears that the extract functioned as a resistance-modifying agent, inhibiting bacterial resistance mechanisms and allowing the antibiotic to function with improved efficacy. This is particularly interesting and highlights an effective treatment modality for use against resistant *K. pneumoniae* infections. As several multi-antibiotic strains of this bacterium (and one strain that was resistant to all commonly used clinical antibiotics) have recently been reported,<sup>4</sup> this is a particularly interesting and relevant finding. Furthermore, several additive combinations were also detected. Whilst the extract does not increase the activity of the antibiotics to as great an extent in these combinations, the potency of the treatment is still substantially increased compared to either monotherapy when used separately. Thus, these combinations would also be beneficial when used as a preventative therapy for ankylosing spondylitis, or for other diseases caused by *K. pneumoniae*.

Several combinations also produced additive effects against other bacterial triggers of autoimmune diseases. The methanolic extract (in combination with chloramphenicol) against *P. mirabilis*, the methanolic and ethyl acetate extracts (in combination with tetracycline) against *P. vulgaris* and the methanolic and ethyl acetate extracts (in combination with erythromycin or chloramphenicol) against *A. baylyi* all produced enhanced efficacy. Therefore, the use of these combinations would be beneficial in the treatment of rheumatoid arthritis and multiple sclerosis, as well, as other diseases caused by these bacteria.

A further trend was evident in our study: most of the extract-antibiotic combinations which did not produce synergistic or 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. Only two combinations tested in this study produced antagonistic interactions with the conventional antibiotics (methanolic extract in conjunction with gentamycin against *A. baylyi* or *S. pyogenes*). This is an important finding and highlights that this combination should be avoided when treating *A. baylyi* and *S. pyogenes* infections. Interestingly, previous studies indicate that antagonistic combinations of plant extracts with gentamycin are not uncommon.<sup>24</sup>

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 which 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.<sup>25,26</sup> 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.<sup>27</sup> Isoflavones isolated from *Lupinus argenteus* Pursh potentiate the activity of the natural plant antibiotic berberine as well as the synthetic fluoroquinolone antibiotic, norfloxacin as inhibitors of *S. aureus* growth.<sup>27</sup> 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).<sup>28</sup> 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.<sup>27,29</sup> 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.<sup>27,29</sup> 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.<sup>27</sup> The synergistic interactions in our study suggests the possibility of a common EPI in the *C. asiatica* 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  $\beta$ -lactam antibiotics ineffective).<sup>28</sup> As penicillin binding proteins are a group of protein enzymes, these phytochemicals may form nonspecific interactions and affect the bacterial cell wall biosynthesis. The *C. asiatica* extracts may also contain a  $\beta$ -lactamase inhibitor.  $\beta$ -lactamases are the major defense of gram-negative bacteria against  $\beta$ -lactam antibiotics.<sup>30</sup> Clavulanic acid is an irreversible  $\beta$ -lactamase inhibitor, which in combination with  $\beta$ -lactam antibiotics can block the bacterial antimicrobial resistance mechanism.<sup>31</sup> Further studies are required to identify whether extract compounds mirror the chemical and biological characteristics of clavulanic acid (i.e. the presence of a  $\beta$ -lactam ring).

Ultimately, the preparation of combinations of *C. asiatica* extracts (or purified compounds) with conventional antibiotic will depend on the nature of the pathogen and of the disease treated. In general, combinations of antibiotic with pure *C. asiatica* derived compounds would be preferred for acute infections as they are much less complex, easier to standardize and have lower chances of unwanted side effects. The use of crude extracts in these preparations is also effective and may still be acceptable to treat some diseases. However, when treating chronic

illness, or using a combinational approach to prevent illness (as would be required in preventing autoimmune inflammatory diseases), the use of a pure potentiator compound in combination with the antibiotic may not be preferred. Continuous exposure of bacteria to a pure antibiotic (or to a combination of a single antibiotic and single potentiator) is likely to induce resistance to one or both of the compounds in the bacteria. Indeed, some *E. coli* strains are now resistant to amoxicillin-clavulanic acid combinations.<sup>32</sup> However, crude plant extracts often contain numerous antibacterial compounds which may affect multiple bacterial targets. Thus, using a plant extract (rather than pure plant compounds) in combination with an antibiotic is less likely to result in resistant bacteria. Indeed, we were unable to find reports of any bacteria developing resistance to a crude plant extract. For this reason, when recommending preferred combination ratios throughout this study, we have recommended two different ratios for acute and chronic conditions. The lowest extract:highest antibiotic ratio which produced synergy has been deemed as the ideal ratio for treating acute bacterial infections, whilst we deemed the highest extract:lowest antibiotic ratio which produced synergy to be preferred for preventing and treating chronic disease

## CONCLUSION

The results of this study demonstrate the potential of the *C. asiatica* 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 also 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.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

## ABBREVIATIONS

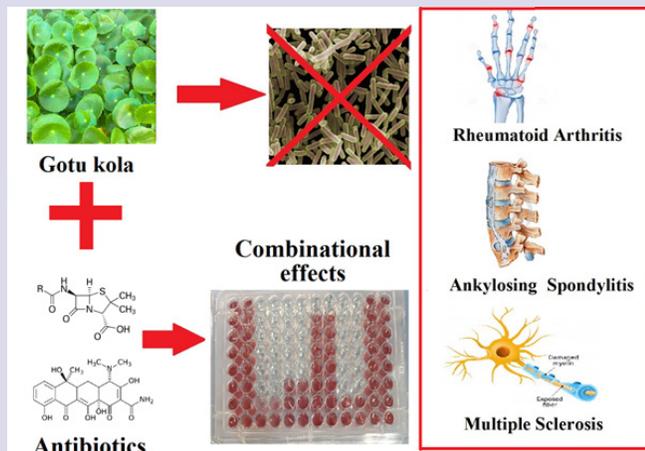
**ALA:** *Artemia* lethality assay; **DMSO:** Dimethyl sulfoxide; **EPI:** Efflux pump inhibitor; **FIC:** Fractional inhibitory concentration; **HDF:** Human dermal fibroblasts; **LC<sub>50</sub>:** The concentration required to achieve 50 % mortality; **MIC:** Minimum inhibitory concentration; **MDR:**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

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## PICTORIAL ABSTRACT



## 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.

## SUMMARY

- *C. asiatica* 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
- Synergistic combinations were screened at various ratios to determine the ideal ratios to provide synergy.
- Toxicity of *C. asiatica* extracts was determined using the *Artemia nauplii* and HDF cell viability toxicity bioassays.