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*Original Article*

# Antibacterial Activity and Toxicity Profiles of *Apium graveolens* L. Extracts and Conventional Antibiotics against Bacterial Triggers of some Autoimmune Diseases

**Jason Batten[1], Ian E Cock[1,2,*]**
[1]School of Environment and Science, Griffith University, Nathan, Queensland, AUSTRALIA.
[2]Centre for Planetary Health and Food Security, Nathan Campus, Griffith University, Nathan, Queensland, AUSTRALIA.

## ABSTRACT

**Background:** *Apium graveolens* L. (commonly known as celery) seeds have been used traditionally to treat a variety of conditions including bacterial infections and inflammation. There is also considerable recent interest in its use as a complementary medicine. However, they are yet to be tested for the ability to inhibit the growth of bacterial triggers of autoimmune diseases. **Methods:** Antimicrobial activity was assessed using disc diffusion and liquid dilution minimum inhibitory concentration (MIC) assays against a panel of bacterial triggers of some autoimmune diseases. Interactions between the *A. graveolens* extracts and conventional antibiotics were studied and classified using the sum of the fractional inhibitory concentration (ΣFIC). Notable synergistic interactions were further examined across a range of ratios using isobologram analysis. The toxicity of the individual samples and the combinations was assessed using the *Artemia* lethality assay (ALA) assay. **Results:** *Apium graveolens* seed extracts displayed notable antibacterial activity against the bacterial trigger of rheumatoid arthritis (*P. mirabilis*), but were ineffective against *K. pneumoniae*, *A. baylyi, P. aeruginosa* and *S. pyogenes*. The ethyl acetate extract was a particularly good inhibitor of *P. mirabilis* growth, with an MIC of 64µg/mL recorded. The hexane (MIC=256µg/mL) and methanolic extracts (MIC=750µg/mL) also displayed noteworthy inhibitory activity towards *P. mirabilis*. Furthermore, combining the extracts with conventional antibiotics resulted in significant potentiation of the inhibitory activity for some combinations. Interestingly, all combinations containing the ethyl acetate extract produced either synergistic or additive effects against *P. mirabilis*. None of the individual components (nor the combinations) were toxic in the ALA assay. **Conclusion:** The *A. graveolens* ethyl acetate extract displayed clinically relevant antibacterial activity against *P. mirabilis* when tested alone, and potentiated the activity of chloramphenicol, gentamicin and ciprofloxacin in combination. Furthermore, the lack of toxicity of the extract and combinations indicates that *A. graveolens* ethyl acetate extract and antibiotic combinations may provide leads in the development of new therapies to prevent and treat rheumatoid arthritis.

**Key words:** Medicinal plants, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Conventional antimicrobials, Synergy, Drug interaction, Toxicity.

**Correspondence:**
**Dr. I E Cock**
[1]School of Environment and Science, Griffith University, 170 Kessels Rd, Nathan, Queensland, 4111, AUSTRALIA.
[2]Centre for Planetary Health and Food Security, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Queensland, 4111, AUSTRALIA.
E-mail: i.cock@griffith.edu.au
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## INTRODUCTION

Medicinal plants have formed the basis of traditional medicine since before written record and are now used as complementary therapies globally. Even in regions where allopathic medicine has supplanted traditional and herbal medicine, the importance of therapeutic plants is still evident. Indeed, numerous conventional allopathic drugs currently used in clinical practice have been derived from plant sources, or are semi-synthetic analogues of plant compounds. Examples include the antimalarial drugs quinine and artemisinin, the cardiac drug atropine, and the opioid based analgesic drugs. Furthermore, cancer chemotherapeutics are dominated by modified versions of plant derived drugs including paclitaxel, vincristine, vinblastine etc.

Recent increases in bacterial resistance to clinical antibiotics and a corresponding decrease in antibiotic discovery has made the development of new antibiotic therapies a high priority.[1] As a result, interest in medicinal plant research is increasing, with the aim of identifying alternative antibiotic therapies.[2] Unfortunately, whilst the antibiotic potential of some plant species is promising, plant-derived antimicrobials usually possess lower potency than conventional antimicrobials.[3] Combinational approaches may be more effective in overcoming resistance and potentiating the activity of conventional antibiotics, even in bacteria that are otherwise recalcitrant to their effects. Several studies investigating natural product and conventional antibiotic combinations have already reported promising results.[3,4-6] Most of these studies focussed on antibiotic combinations with common herbal formulations, such as *Rosmarinus officinalis* Spenn., *Origanum vulgare* L., *Thymus vulgaris* L., *Mentha piperita* L. and *Melaleuca alternifolia* (Maiden and Betche) Cheel. More recently, studies have also begun to report on the interactive effects of antibiotics in combination with African,[7,8] Asian[9,10] and Australian traditional medicinal plants.[11] These studies have identified several plant species as synergistic enhancers of conventional antimicrobials, even when the plants do not possess noteworthy antimicrobial in isolation.[2,3,8,11] Other studies have focused on isolated phytochemicals such as phenols, tannins and flavonoids, in combination with conventional antibiotics and many promising synergistic formulations have been identified.[12-15] These studies highlight the potential of medicinal plants and their individual components for potentiating the activity of conventional antimicrobials, or even restoring the antibiotic activity, even against bacteria otherwise resistant to their effects. Not only is it important to investigate these combinations to identify possible alternatives to overcome resistance, but combinational studies may also provide valuable information for use in the clinical settings where complementary therapy-allopathic drug interactions may occur.

Many practitioners of complementary and alternative medicine use traditional and conventional medications concurrently, without an understanding of the interactions and side effects that may occur. This lack of understanding of drug interactions may pose serious risks to patient safety.[16,17] Indeed, the practice of combining complementary therapies with conventional medicine is prevalent, with some reports

estimating that 20% of the population in Western countries use herbal drugs concurrently with prescription drugs.[18,19] A Canadian study estimated that approximately 23% of the population used herbal drugs, with more than 5% confirming that the used them together with prescription drugs.[19] Similarly, a US survey reported that 72% of patients using herbal remedies were also using prescription drugs.[20] The same survey reported that 84% of patients used over-the-counter medications in combination with natural therapies and many patients combined these two forms of healthcare with the belief that there would be an enhanced effect. There have been many instances where the co-use of natural products and conventional medicines have resulted in severe reactions.[21,22] Much more work is required to test the safety of allopathic and complementary drug combinations.

There is a common misconception amongst consumers that all natural products are safe. However, like synthetic drugs, natural products may induce severe interactions and are not devoid of toxicity.[23,24] Natural product combinational studies generally focus on the efficacy of the drug combination, and studies examining the safety of these combinations have been relatively neglected, despite extensive studies reporting interactions between herbal medicines and natural products when used with conventional antimicrobials.[21] There are limited reports of interactions between traditional medicinal plants and conventional antimicrobials. Such interactions may have considerable effects on the efficacy of conventional treatments, as many patients do not report their concurrent usage of traditional medicines to their healthcare providers. Hence, a comprehensive investigation of these interactions is warranted for any herbal medicine.

*Apium graveolens* L. (family Apiaceae) (commonly known as celery) is widely used as a vegetable for human consumption. Depending on the cultivar and culinary use, either the stalks and leaves are usually consumed, although *A. graveolens* seeds may also be used as a spice and flavouring agent. The seeds are also widely used in several Asian herbal medicine systems including Ayuverda,[25] as well as Middle Eastern[26] and African traditional medicine systems[27] to treat a wide variety of illnesses and conditions, including stomach diseases and spasms,[26] as a heart tonic, and to lower blood pressure.[27] It is also used as a laxative, a sedative,[26] as an aphrodisiac,[28,29] and to increase milk secretion.[30] However, it is perhaps best known for its anti-inflammatory properties and as an alternative treatment for arthritis, rheumatism and gout.[25] Indeed, the anti-inflammatory activity of *A. graveolens* seed extracts has been previously reported in several *in vivo* models.[31,32] However, those studies examined the downstream effects of inflammation and did not evaluate the extracts against the initiating events of inflammation. Notably, several autoimmune inflammatory diseases are caused by specific bacterial pathogens in genetically susceptible individuals. In particular, *Proteus mirabilis* has been highlighted as a trigger of rheumatoid arthritis, *Klebsiella pneumoniae* triggers ankylosing spondylitis, *Acinetobacter baylyi* and *Pseudomonas aeruginosa* can trigger multiple sclerosis, and *Streptococcus pyogenes* can trigger rheumatic heart disease in genetically susceptible people.[33-35] Whilst *A. graveolens* seed extracts have previously been reported to have antibacterial activity,[36,37] the inhibitory activity against the bacterial triggers of the autoimmune inflammatory diseases is yet to be tested. Our study aimed to evaluate the growth inhibitory properties of *A. graveolens* seed extracts against some bacterial triggers of selected autoimmune inflammatory diseases. Furthermore, the interactive antimicrobial and toxicity profiles of combinations of the *A. graveolens* seed extracts and six conventional antibiotic drugs was examined.

# MATERIALS AND METHODS

## Sourcing and preparation of plant samples

The *Apium graveolens* L. seeds used in this study were purchased from Noodles Emporium, Australia. Voucher specimens are deposited in the School of Natural Sciences, Griffith University, Australia (voucher number AGS-A1-2018-1A). Individual quantities (1g) of the dried seeds were weighed into separate tubes and 50mL of methanol, deionised water, ethyl acetate or hexane were added. All solvents were obtained from Ajax FineChemicals, Australia and were AR grade. The seeds were extracted in each solvent for 24 hr at 4°C with gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum. The solvent extracts were air dried at room temperature in the shade. The aqueous extracts were lyophilised by freeze drying at -50°C. The resultant dried extracts were weighed and dissolved in 10mL of deionised water (containing 1% DMSO).

## Qualitative phytochemical analysis

Phytochemical analysis of the *A. graveolens* extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved using standard assays.[38,39]

## Antibacterial analysis

### *Conventional antibiotics*

Penicillin-G (potency of 1440-1680µg/mg), chloramphenicol (≥98% purity by HPLC), ciprofloxacin (≥98%), erythromycin (potency ≥850µg/mg), gentamicin (potency of 600µg/mg), and tetracycline (≥95% purity by HPLC) were purchased from Sigma-Aldrich, Australia and used for the microplate liquid dilution assay. All antibiotics were prepared in sterile deionised water at stock concentrations of 0.01mg/mL and stored at 4°C until use. For the disc diffusion studies, ampicillin (2µg), tetracycline (10µg/mL) and chloramphenicol discs (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.[33-35] Reference strains of *Proteus mirabilis* (ATCC21721), *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 Natural Sciences teaching laboratory, Griffith University, Australia. All bacteria were cultured in nutrient broth (Oxoid Ltd., Australia). Streak nutrient agar (Oxoid Ltd., Australia) plates were tested in parallel to ensure the purity of all bacterial cultures and for sub-culturing. All bacterial cultures were incubated at 37°C for 24hr and were subcultured and maintained in nutrient broth at 4°C until use.

## Evaluation of bacterial susceptibility to growth inhibition

The susceptibility of the bacteria to the *A. graveolens* extracts and the conventional antibiotics was initially assessed using a modified disc diffusion assay.[40] Ampicillin (2 µg), tetracycline (10µg) and chloramphenicol discs (10µg) were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10µL of distilled water were used as a negative control.

## Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using two methods. A liquid dilution MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay.[7] Furthermore, as microplate liquid dilution MIC assays are perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method allows for comparisons with other studies. A solid phase agar disc diffusion assay was also used in this study for comparison.

## Microplate liquid dilution MIC assay

A standard liquid dilution MIC assay[7] was used to evaluate the antimicrobial activity of the plant samples and the conventional antimicrobials independently and in combinations. Briefly, 100µL of sterilized distilled water was dispensed into each well of 96 well micro-titre plate. The plant samples and conventional antibiotics (100µL) were then added into separate wells of the first row of the plate. Plant samples were introduced at a starting concentration of 32mg/mL whilst the conventional antibiotics were introduced at a starting concentration of 0.01mg/mL. A negative control (nutrient broth), a sterile control (without bacteria) and a sample-free culture control (to ensure the media was capable of supporting microbial growth) were included on all plates. After addition of the test samples to the plate, each was serially diluted by doubling dilution. The relevant bacterial culture inoculum (100µL) was then added to all wells of the plate except the sterile control wells. Each inoculum contained approximately $1x10^6$ colony forming units (CFU)/mL. All plates were subsequently incubated at 37°C. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, Australia and dissolved in sterile deionised water to prepare a 0.2mg/mL INT solution. A 40µL volume of this solution was added into all wells and the plates were incubated for a further 6 hr at 30°C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

## Disc diffusion MIC assay

The minimum inhibitory concentrations (MIC) of the extracts was also evaluated by disc diffusion assay as previously described.[39,40] Graphs of the zone of inhibition (ZOI) versus Ln of the concentration were plotted and MIC values were achieved using linear regression.

## Fractional inhibitory concentration (FIC) assessment

Interactions between the combinations of plant samples and conventional antimicrobials were further classified using the sum of the fractional inhibitory concentration (ΣFIC). The FIC was calculated using the following equation, where (a) represents the plant sample and (b) the conventional antimicrobial sample:[3]

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

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

The ΣFIC was then calculated using the equation: $\Sigma FIC = FIC^{(i)} + FIC^{(ii)}$. The interactions were classified as being synergistic for ΣFIC values of ≤ 0.5, additive (> 0.5 – 1.0), indifferent (> 1.0 – ≤ 4.0) or antagonistic (> 4.0).[3]

## Varied ratio combination studies (isobolograms)

For the identified synergistic interactions, nine different ratios of the combination were prepared and the MIC values determined. The samples were combined at fixed concentrations of 0.01mg/mL for antibiotic and 32mg/mL for the plant extract, at various volume ratios (antimicrobial: plant), resulting in varied concentrations for each ratio (Table 1). Data points for each ratio were plotted on an isobologram using the GraphPad Prism® software (Version 5). The construction of isobolograms allowed for the identification of the agent (plant or antimicrobial sample) most responsible for the synergistic effects within the combination. Data points falling below the 0.5:0.5 line indicated synergy, while those above the 0.5:0.5 line, but below the 1.0:1.0 line indicated an additive interaction. Data points above the 1.0:1.0 line, but below the 4.0:4.0 line indicated a non-interactive or indifferent interaction, and data points falling above the 4.0:4.0 line indicated antagonism.[3]

## *Artemia franciscana* lethality assay (ALA)

Toxicity of the *A. graveolens* extracts, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* Kellogg nauplii lethality assay (ALA).[41,42] Artificial salt water was prepared by dissolving 16g of Sigma sea salts in 500mL distilled water and decanted into a bottomless, inverted plastic bottle. Dessicated *A. franciscana* cysts were obtained from Ocean Nutrition™ and a mass of 0.5g was added to the salt water. An aquarium pump was used to aerate the water and disperse the eggs to achieve maximise hatch rate. The eggs were exposed to a concentrated source of light and warmth from a halogen lamp (220–240V) and incubated under these conditions for 18–24hr before use. A volume of 400µL artificial seawater containing 40–60 live *A. franciscana* nauplii, was added to each well of a 48 well micro-titre plate. A volume of 400µL of the test samples (plant extracts, antimicrobials or combinations were diluted in distilled water and added to triplicate wells. All samples were initially screened for toxicity at a concentration of 1mg/mL as extract concentrations >1mg/mL as extracts with $LC_{50}$ values <1000µg/ml towards *Artemia* nauplii have previously been defined as non-toxic.[42] The $LC_{50}$ values were determined in the *Artemia* nauplii bioassay following 24hr exposure. A negative control (32g/L) artificial salt water and a positive control (1mg/mL potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was included in triplicate on all plates. The plates were observed using a light microscope (Olympus) (40X magnification) immediately after sample addition (at time 0) to record any dead brine-shrimp, which are then excluded from percentage mortality calculations. The moribund *A. franciscana* were then counted after 24hr and 48hr. A lethal dose of 50µL of glacial acetic acid (100% v/v; Ajax FineChemicals, Australia) was added to each well

**Table 1:** The concentration ratios used for antimicrobial and plant sample combination studies.

| Volume ratio of extract: antibiotic (µL) | Concentration of plant extract in combination (mg/mL) | Concentration of antibiotic[a] in combination (µg/mL) |
|---|---|---|
| 90:10 | 90.00 | 3.20 |
| 80:20 | 80.00 | 6.40 |
| 70:30 | 70.00 | 9.60 |
| 60:40 | 60.00 | 12.80 |
| 50:50 | 50.00 | 16.00 |
| 40:60 | 40.00 | 19.20 |
| 30:70 | 30.00 | 22.40 |
| 20:80 | 20.00 | 25.60 |
| 10:90 | 10.00 | 28.80 |

[a] = penicillin G / chloramphenicol / ciprofloxacin / erythromycin / gentamicin / tetracycline.

and the total dead brine-shrimp count recorded after 30 min exposure. The percentage mortality was then calculated.[41,42] Samples providing a percentage mortality greater than 50% were considered toxic.[41,42] These samples were serially diluted and tested across the concentration range 1- 0.032mg/mL to obtain a log-sigmoid dose response curve, generated with GraphPad Prism® software (Version 5), from which the $LC_{50}$ values were determined. The $LC_{50}$ value represented the concentration of a test substance necessary to have a lethal effect on 50% of the *A. franciscana* nauplii.

### Statistical analysis

Data is expressed as the mean ± S.D. 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 and qualitative phytochemical screening

Extractions of the *A. graveolens* seeds (1g) with solvents of varying polarity yielded dried plant extracts ranging from 2.8mg (*A. graveolens* seed ethyl acetate extract) to 159mg (*A. graveolens* seed aqueous extract) (Table 1). Qualitative phytochemical screening (Table 2) showed that the higher polarity solvents (methanol and water) extracted the greatest amount and widest diversity of phytochemical classes.

### Bacterial growth inhibition screening

Only *P. mirabilis* growth was susceptible to the *A. graveolens* extracts in the disc diffusion screening assays (Figure 1a). The methanol extract was a particularly potent inhibitor of *P. mirabilis* growth, with a ZOI of approximately 9.6mm. This compares favourably to the antibiotic controls included in this assay. Indeed, this extract produced considerably stronger inhibition of this bacterium than either ampicillin or tetracycline (on the basis of the size of the ZOI), which produced ZOIs of ~8mm and 8.2mm respectively. The chloramphenicol control was substantially more effective (ZOI~14.3mm) than the extracts or other antibiotic controls. The aqueous and ethyl acetate extracts were also noteworthy inhibitors of the growth of this bacterium, with ZOIs of 8.2 and 8.7mm respectively. Notably, the extracts tested in this screening assay were initially tested at the concentration at which they were prepared to provide an approximation of the concentration at which the *A. graveolens* seed extracts would be used traditionally. However, the methanolic and aqueous extracts were tested at approximately 26 and 57 fold higher concentrations than the ethyl acetate extract. Similarly, whilst the hexane extract produced substantially smaller ZOIs than the other extracts, it was also tested at a much lower concentration than the methanolic and aqueous extracts. Therefore, it is likely that the ethyl acetate and hexane extracts are much stronger inhibitors of *P. mirabilis* growth than is initially evident in this screening study. In contrast to the *P. mirabilis* susceptibility results, all *A. graveolens* extracts were completely ineffective inhibitors of *K. pneumoniae* (Figure 1b), *A. baylyi* (Figure 1c), *P. aeruginosa* (Figure 1d) and *S. pyogenes* growth (Figure 1e).

### Quantification of minimum inhibitory concentration (MIC)

The relative level of antimicrobial activity was further evaluated by determining the MIC values using two methods: The liquid dilution MIC assay and the disc diffusion MIC assay (Table 3). Consistent with the antibacterial disc screening assays, only *P. mirabilis* was susceptible to the *A. graveolens* extracts, whilst all of the other bacteria tested were unaffected. The mid polarity ethyl acetate extract was a particularly good inhibitor of *P. mirabilis* growth, with an $LC_{50}$ of 64µg/mL recorded. Therefore,

**Table 2: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *A. graveloens* seed extracts.**

| Extract | Mass of Dried Extracted Material (mg) | Conventration of ectract (mg/mL) | Phenols | | | Cardiac Glycosides | Saponins | Triterpenes | Phytosterols | Alkaloids | | Flavanoids | | Tannins | Anthraquinones | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total Phenolics | Water Soluble | Water Insoluble | Keller-Kiliani Test | Froth Persistence | Salkowski Test | Acetic Anhydride Test | Meyers Test | Wagners Test | Shinoda Test | Kumar test | Ferric Chloride Test | Free | Combined |
| Methanol | 74 | 7.4 | +++ | + | ++ | - | +++ | + | + | - | - | ++ | +++ | - | - | - |
| Water | 159 | 15.9 | +++ | + | +++ | - | +++ | + | + | - | - | +++ | +++ | - | - | - |
| Ethyl Acetate | 2.8 | 0.28 | - | - | - | - | + | - | - | - | - | + | + | - | - | - |
| Hexane | 7.8 | 0.78 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

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

**Table 3: Disc diffusion (DD) and liquid dilution (LD) MIC values (µg/mL) for *A. graveolens* seed extracts against *P. mirabilis*.**

| | Test | DD MIC | LD MIC |
|---|---|---|---|
| Extracts | M | 852 | 750 |
| | W | 1521 | 1521 |
| | E | 100 | 64 |
| | H | 920 | 256 |
| Controls | Penicillin | ND | - |
| | Chloramphenicol | ND | 1.25 |
| | Erythromycin | ND | - |
| | Tetracycline | ND | - |
| | Gentamicin | ND | 0.63 |
| | Ciprofloxacin | ND | 0.63 |

M = methanol extract; W = water extract; E = ethyl acetate extract; H = hexane; DD = disc diffusion; LD = liquid dilution; ND indicates that the MIC was not determined in the DD assay as only a single dose was tested; - indicates no inhibition was noted at any concentration. Bacteria with MIC values >1µg/mL for pure antibiotics are considered resistant to that antibiotic.

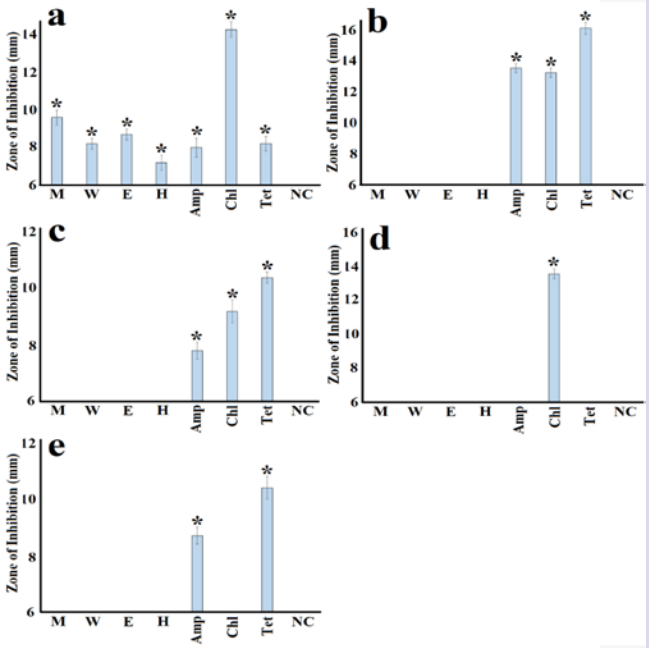


**Figure 1:** Antibacterial activity of *A. graveolens* seed extracts against (a) *P. mirabilis* (ATCC21721); (b) *K. pneumoniae* (ATCC31488); (c) *A. baylyi* (ATCC33304); (d) *P. aeruginosa* (ATCC: 39324); and (e) *S. pyogenes* clinical strain, measured as zones of inhibition (mm). M = methanolic extract;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

the ethyl acetate extract would be useful for preventing and treating rheumatoid arthritis in genetically susceptible people. Interestingly, the hexane and methanolic extracts also displayed noteworthy inhibitory activity towards *P. mirabilis* was (256 and 750µg/mL respectively), whilst the aqueous extract had substantially lower inhibitory activity (1521µg/mL). Notably, each of the methanol, ethyl acetate and hexane extracts would contain lower polarity extracts, indicating that lower polarity compounds may be responsible (at least in part) for the inhibitory activity against *P. mirabilis*. The inhibitory activity of the *A. graveolens* extracts against this bacterium were particularly interesting as the *P. mirabilis* strain tested in our study was resistant to multiple antibiotics. Indeed, it was completely resistant to penicillin-G, erythromycin and tetracycline as these antibiotics did not inhibit bacterial growth at any concentration tested. Similarly, an MIC value of 1.25µg/mL was measured for chloramphenicol against *P. mirabilis*. As MIC values >1µg/mL for pure antibiotics indicates antibiotic resistance in this assay,[7] it was determined that this bacterium was only considered susceptible to gentamicin and ciprofloxacin of the antibiotic controls tested. The good activity of the *A. gravelens* extracts against the bacterium indicates that it may therefore be particularly useful in preventing and treating rheumatoid arthritis.

In contrast, all of the other bacteria tested were unaffected by the *A. graveolens* extracts (Figures 1b-e). Notably, several other studies have screened these same bacterial strains previously and have reported all of them to have multi-drug antibiotic resistance.[43-46] The *P. aeruginosa* strain has been reported to be particularly resistant to most conventional antibiotics. Notably, some of the extracts could only be tested at low concentrations. The ethyl acetate and hexane extracts were extracted at low concentrations (280 and 780µg/mL respectively). Therefore, testing of these extracts was only possible using maximum concentrations of 70 and 195µg/mL respectively in the assay. As MIC values <1000µg/mL are considered noteworthy in this assay, it is possible that these extracts may be useful growth inhibitors of the other bacterial species if tested at higher concentrations. Further studies are planned to test this hypothesis.

## Fractional inhibitory concentration (FIC) assessment

Three combinations (25%) of the *A. graveolens* seed leaf extracts and conventional antibiotic combinations that inhibited *P. mirabilis* growth produced synergistic effects when tested together (Table 4). This is particularly noteworthy as these combinations have substantially higher growth inhibitory activity than either component separately (>4 times higher activity). Therefore, these combinations would be particularly useful in preventing and treating rheumatoid arthritis (and other illnesses caused by *P. mirabilis* infections). Additionally, four combinations (33%) produced additive effects against *P. mirabilis*. As these combinations produce effects greater than either the individual extract or conventional antibiotic components, these combinations would also be beneficial in the prevention and treatment of rheumatoid arthritis. All of the other combinations were non-interactive. Whilst these combinations provide no added benefit over that of the individual components alone, the components do not antagonise each other's effects and are therefore safe to use concurrently without risk of lessening the efficacy of either component.

## Varied ratio combination studies (isobolograms)

Three synergistic combinations were detected against *P. mirabilis*. One of these interactions was in a combination containing chloramphenicol (with the ethyl acetate extract), whereas two combinations contained ciprofloxacin and either the aqueous or ethyl acetate extracts. These combinations were further examined using isobologram analysis across a range of extract: antibiotic ratios to identify the ideal ratios to obtain synergy (Figure 2). Interestingly, similar trends were noted for both of the combinations containing the *A. graveolens* ethyl acetate extract, with synergy generally evident for ratios of ≥40% extract, up to ~80% extract (Figures 2a and 2c). Ratios outside this range produced additive effects and would thus also be beneficial for inhibiting *P. mirabilis* growth.

**Table 4: ΣFIC values for the *A. graveolens* seed extract and conventional antibiotic combinations against *P. mirabilis*.**

| | M | W | E | H |
|---|---|---|---|---|
| **Chloramphenicol** | *0.67* | 1.28 | **0.38** | *0.85* |
| | *(ADD)* | (IND) | **(SYN)** | *(ADD)* |
| **Gentamicin** | 1.5 | 1.32 | *0.94* | 1.15 |
| | (IND) | (IND) | *(ADD)* | (IND) |
| **Ciprofloxacin** | 1.25 | **0.09** | **0.42** | *0.66* |
| | (IND) | **(SYN)** | **(SYN)** | *(ADD)* |

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

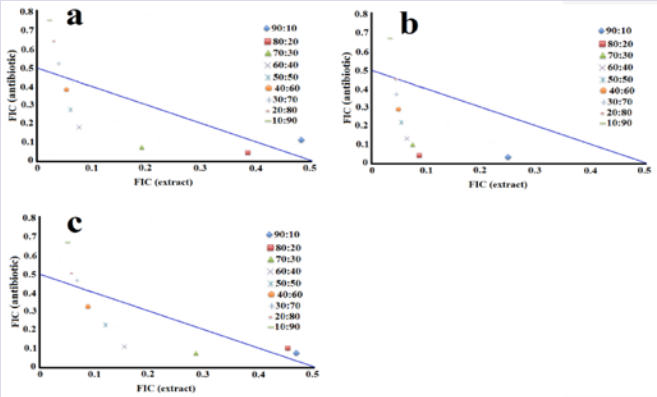


**Figure 2:** Isobolograms of extract and antibiotic combinations with *A. graveolens* seed extract: (a) ethyl acetate extract and chloramphenicol, (b) aqueous extract and ciprofloxacin and (c) ethyl acetate extract and ciprofloxacin, tested at various ratios against the *P. mirabilis*. Results represent 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 (Σ FIC ≤ 0.5). Any points between the 0.5:0.5 and 1.0:1.0 lines are deemed additive (Σ FIC > 0.5-1.0).

These results indicate that extract component(s) may be competing with the antibiotic component for interaction with the bacterial resistance mechanism (e.g. an efflux pump or antibiotic inactivating enzyme). Therefore, as the ratio of extract increases across the combinations, the extract component(s) outcompete the antibiotic for interaction with the resistance mechanism, effectively blocking its ability to inhibit the antibiotics effects. Thus, the ethyl acetate extracts may contain competitive inhibitors of the bacterial resistance protein(s), although the specific mechanism is yet to be determined. A different trend was evident for the combination of the aqueous extract and ciprofloxacin (Figure 2b). That combination produced synergy across a wider range of ratios, even at very low levels of the extract. Indeed, all ratios except the 10% extract ratio produced synergy against *P. mirabilis* growth. The ability to counteract the effects of the bacterial resistance mechanism, even when only low extract % is present, indicates that the aqueous extract components may function via irreversible mechanisms. A similar trend has been reported for the combination of amoxycillin and clavulanic acid in the clinical antibiotic therapy Augmentin®.[1] Indeed, the profile of the synergistic ratios of the Augmentin® components is the basis of the 15% clavulanic acid: 85% amoxicillin composition of that chemotherapeutic. As all combinations of the aqueous extract and ciprofloxacin >10% extract produced synergy, all of these combination ratios would be beneficial to enhance *P. mirabilis* growth inhibition. However, bacteria would be less likely to develop resistance when combinations are used in ratios that minimise the amount of conventional antibiotic used. Thus, for long term prophylactic treatment (as would be required to prevent and treat rheumatoid arthritis), the ideal extract: antibiotic ratio may be 90:10. However, when used for the treatment of acute infections (e.g. urinary tract infections), the ratio which maximises the efficacy of the treatment (i.e. the 20:80 ratio) may be the preferred option.

## Toxicity evaluation

All plant extracts and antibiotics were individually screened at 1000µg/mL in the assay. The extracts were only considered toxic if they induced percentage mortalities greater than 50% ($LC_{50}$) following 24 hr of exposure to the *Artemia* nauplii.[42] When tested individually, the antimicrobials demonstrated no toxicity in the ALA (Table 5). Similarly, none of the *A. graveolens* extracts produced mortality significantly different to that of the negative control. When tested together in the ALA, none of the extract-antibiotic combinations produced mortality significantly different to the negative controls, and no single component nor combination induced >50% mortality. Therefore, all combinations and individual components were deemed nontoxic. In contrast, the positive control potassium dichromate induced 100% mortality in the ALA.

# DISCUSSION

This study investigated the ability of *A. graveolens* seed extracts to inhibit the growth of some bacterial triggers of auto-immune inflammatory diseases, both alone and in combination with conventional antibiotics. Several extracts were identified as effective growth inhibitors against *P. mirabilis*, although, all extracts were completely ineffective against all of the other bacterial strains tested. Thus, the *A. graveolens* seed extracts tested in our study only have clinically relevant potency against *P. mirabilis* if used alone. The ethyl acetate extract had the strongest inhibitory activity, with an MIC of 64µg/mL. This indicates that this extract would be particularly useful in preventing and treating rheumatoid arthritis and other infections of this bacterium when used by itself. However, the combinational studies combining the *A. graveolens* seed extracts with conventional antibiotics were of greater interest. Several combinations displayed substantially enhanced potential as therapeutic agents against *P. mirabilis* than either the extracts or antibiotics alone. Indeed, three synergistic combinations were noted, with two of these containing the ethyl acetate extract (in combination with both chloramphenicol and ciprofloxacin). The implications of these synergistic interactions include enhanced efficacy, the requirement for lower dose administration and a reduction in side effects, as well as possibly reduced antimicrobial resistance.[1] The use of synergistic combinations may ultimately enhance or repurpose ineffective drugs with greater efficacy.

Bacteria have developed numerous resistance mechanisms to block or inhibit the effects of antibiotics. A common method is through the use of multi-drug resistant (MDR) efflux pumps which are encoded chromosomally and utilized 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 antibiotics. 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 MDR pump inhibitors in order to enhance the activity of their own natural antimicrobial compounds. Such MDR pump inhibitors become useful tools in combination with some previously ineffective/resistance prone antibiotic compounds.[48] Isoflavones isolated from *Lupinus argenteus* Pursh potentiate the activity of the natural plant antibiotic berberine as well as the synthetic fluoroquinoline antibiotic,

**Table 5: Mortality (%) assessment for extracts and conventional antibiotics tested individually and as combinations in the *Artemia* lethality assay.**

| | Sample | Mortality ± SD (%) | |
|---|---|---|---|
| | | After 24 hr: | After 48 hr: |
| Antimicrobials | Penicillin G | 1.8 ± 1.4 | 4.3 ± 2.4 |
| | Chloranphenicol | 2.7 ± 1.3 | 5.6 ± 3.3 |
| | Erythromycin | 1.2 ± 0.6 | 5.8 ± 2.3 |
| | Tetracycline | 2.4 ± 1.5 | 5.1 ± 2.8 |
| | Gentamicin | 3.1 ± 1.8 | 6.7 ± 2.6 |
| | Ciproflaxacin | 5.5 ± 2.0 | 8.3 ± 2.1 |
| Extracts | M | 8.4 ± 2.8 | 27.3 ± 3.2 |
| | W | 7.1 ± 2.3 | 22.5 ± 3.2 |
| | E | 4.2 ± 1.6 | 10.5 ± 1.9 |
| | H | 3.7 ± 1.6 | 8.8 ± 1.4 |
| Combinations | M + Chl | 7.7 ± 3.6 | 32.4 ± 2.8 |
| | M + Gent | 8.7 ± 2.3 | 27.6 ± 4.4 |
| | M + Cip | 10.4 ± 3 | 33.8 ± 2.9 |
| | W + Chl | 5.3 ± 2.4 | 18.3 ± 2.7 |
| | W + Gent | 5.4 ± 3.6 | 15.7 ± 2.6 |
| | W + Cip | 8.9 ± 3.6 | 24.7 ± 4.1 |
| | E + Chl | 4.7 ± 2.6 | 19.3 ± 3.4 |
| | E + Gent | 7.6 ± 3.5 | 23.1 ± 4.2 |
| | E + Cip | 10.4 ± 2.8 | 29.6 ± 4.2 |
| | H + Chl | 5.3 ± 2.2 | 17.4 ± 4.1 |
| | H + Gent | 9.4 ± 3.5 | 21.5 ± 3.8 |
| | H + Cip | 10.9 ± 2.8 | 37.6 ± 3.5 |
| Controls | Deionised water | 2.7 ± 1.7 | 3.6 ± 2.5 |
| | Potassium dichromate | 100.00 ± 0.00 [a] | |

Potassium dichromate was tested at a concentration of 1000µg/mL; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; H = hexane extract; Chl = chloramphenicol; Gent = gentamicin; Cip = ciprofloxacin; SD = standard deviation. Results represent means ± SD of 3 independent experiments, each preformed in triplicate ($n$ = 9).

norfloxacin as inhibitors of *S. aureus* growth.[48] That study reported that the isoflavone allows a greater concentration of berberine to accumulate in the bacteria by inhibiting the efflux mechanism (MDR pump). Similarly, *Mezoneuron benthamianum* (Roxb.) Benth. and *Securinega virosa* Leandri extracts act as efflux pump inhibitors for fluoroquinolone, tetracycline and erythromycin in resistant strains of *S. aureus* (MRSA).[47] 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 approximately 4.

Notably, all of the synergistic combinations noted in our study contained either chloramphenicol or ciprofloxacin. Both of these antibiotics are susceptible to resistance due to efflux pumps[1] and therefore it is possible that the *A. graveolens* seed extracts may contain one or more efflux pump inhibitors (EFIs). 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.[1] It is therefore imperative to identify agents that can block the efflux mechanism or alter the process of efflux to extend the life of multiple antibacterial drugs. Plants produce various secondary metabolites that are used as defense compounds against pathogenic invaders. Some plants produce antimicrobials which, along with other compounds, inhibit the efflux of those antimicrobials from a bacterial cell. *Berberis* spp. are known for their production of the antimicrobial alkaloid berberine.[49] However, they also produce an inhibitor of a *Staphylococcus aureus* efflux pump, identified as 5-methoxyhydnocarpin (5-MHC). 5-MHC induces a significantly decreased MIC for berberine against *S. aureus*, thereby greatly potentiating its efficacy. Recently, many other natural plant based phytoconstituents have been identified as potential efflux pump inhibitors.[49] Similarly, methanolic extracts of *Punica granatum* L. have synergistic activity with chloramphenicol, gentamicin, ampicillin, tetracycline and oxacillin against strains of MRSA and MSSA.[50] The same extracts also either inhibit the MDR efflux pump NorA or enhance the influx of the antibiotics. Therefore, methanolic *P. granatum* extracts may dramatically enhance the activity of various different antibiotics including chloramphenicol, potentially, extending the lifespan of the antibiotic.[1] Furthermore, baicalein extracted from the leaves of *Scutellaria baicalensis* Georgi also has the potential to inhibit NorA efflux pump and thus potentiate the activity of gentamicin.[51] Carnosic acid from *Rosmarinus officinalis* L. has been shown to successfully potentiate the activity of erythromycin by the inhibition of MDR pumps.[52] Similar MDR efflux pump inhibitors may also be present in the *A. graveolens* extracts. There are currently no EPI/antimicrobial drug combinations on the market, although research into identifying potential EPIs is ongoing.[1,49]

Interestingly, one of the synergistic interactions detected in our study (aqueous extract in combination with ciprofloxacin) occurred with ratios as low as 20% extract to 80% antibiotic, emphasising the efficacy of these extracts. Such a trend is consistent with irreversible mechanisms such as that of clavulanic acid/β-lactam antibiotic combinations[1,53] and future studies will aim at testing the synergistic mechanism of these combinations. In contrast, other synergistic extract: antibiotic combinations produced a wider range of interactions, including synergistic and additive. This is more consistent with reversible competition between the extract component(s) and the conventional antibiotic for binding to an effector.[1] Of the combinations which were not synergistic, approximately 33% were additive and would therefore provide additional benefit compared to using either therapy alone. A further 41% of the tested combinations were indifferent. Although these combinations did not provide any significant benefit by enhancing the efficacy of the antibiotics, they also did not counter-indicate with the antibiotic. Thus, co-administration of the extracts with the conventional antibiotics in these combinations will not lessen the efficacy of the conventional therapies. This information is important as many individuals self-medicate with herbal and traditional medicines and it is therefore important to understand how these medicines interact. Notably, none of the combinations were antagonistic to the action of the conventional antibiotics.

None of the *A. graveolens* seed extract or conventional antibiotics demonstrated toxicity in the ALA assay when tested independently. Similarly, all combinations were nontoxic, indicating their potential for therapeutic use. The non-toxicity of the conventional antibiotics is hardly surprising as these drugs have a long history of therapeutic use and their lack of toxicity has previously been verified in clinical trials. The lack of toxicity determined for the *A. graveolens* seed extracts may perhaps be unsurprising as they have long been used in several traditional medicine systems to treat inflammation and rheumatic conditions.[25] However, to the best of our knowledge, there is a lack of rigorous toxicity studies for *A. graveolens* seed extracts. Indeed, whilst several studies have reported on the side effects of *A. graveolens* seeds,[30] or on its modulation of the toxicity of other compounds,[29,54] we were unable to find studies

quantifying the concentrations of *A. graveolens* seed extracts required to induce mortality. The lack of toxicity of the combinations also indicate their potential for therapeutic usage. However, further *in vitro* studies using human cell lines are required to verify their safety. Furthermore, *in vivo* testing is also required to confirm that the extracts and combinations retain efficacy and remain nontoxic in complex biological systems.

## CONCLUSION

Whilst the findings reported here indicate the potential of *A. graveolens* seed extracts (particularly in combination with ciprofloxacin or chloramphenicol) as preventative and therapeutic options against *P. mirabilis*, further *in vivo* investigations are required to support these *in vitro* findings. Furthermore, studies to determine the possible mechanism of action resulting in the observed interaction are warranted, and bioactivity driven compound isolation and/or metabolomics studies are also required to determine the active compound(s), as well as those responsible for the antibiotic potentiation, within the *A. graveolens* seeds.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

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

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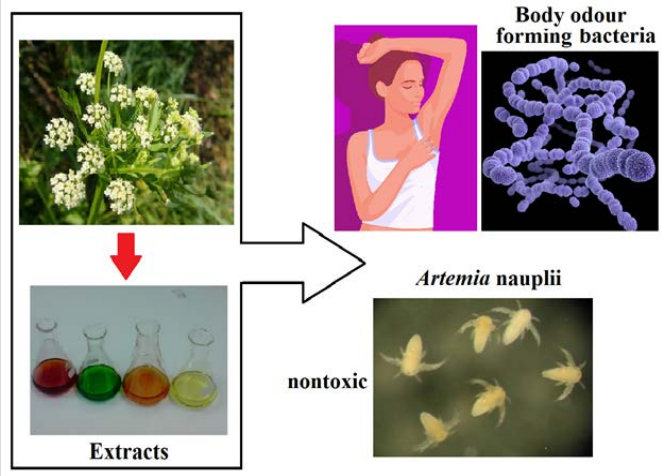
## PICTORIAL ABSTRACT




## SUMMARY

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

## 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.