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Original Article

Bidens biternata (Lour.) Merr. and Sherff Whole Plant Extract Fractions Inhibit the Growth of a Panel of Pathogenic Bacteria

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ABSTRACT

Introduction: *Bidens biternata* (Lour.) Merr. and Sherff is used in several medicinal systems (including traditional Chinese medicine) for multiple purposes, including to treat bacterial infections. Despite this, *B. biternata* extracts, fractions and isolated compounds have not been rigorously examined for antibacterial properties against many pathogens. **Materials and Methods:** The antimicrobial activity of *B. biternata* whole plant extracts was investigated by disc diffusion assays and the MIC values were determined using liquid dilution assays against a panel of pathogenic bacteria. Toxicity was determined using the *Artemia franciscana* nauplii and HDF proliferation toxicity bioassays. **Results:** Fractions prepared from an ethanolic *B. biternata* whole plant extract inhibited the growth of a panel of bacterial species. Growth of both gram positive and gram negative bacteria was inhibited by the fractions, although the gram negative bacteria were substantially more susceptible to the extracts than the gram positive bacteria were. The 100% methanolic fraction prepared from the ethanolic *B. biternata* whole plant extract was a substantially better antibacterial agent compared to the other fractions. The 100% methanolic fraction was a strong inhibitor *of P. aeruginosa* and *S. newport*, with MIC values substantially <1000 µg/mL. This fraction also was a noteworthy inhibitor of *A, faecalis, E. coli* and *S. pyogenes* growth. Furthermore, all of the *B. biternata* fractions were nontoxic in the *Artemia* nauplii and human dermal fibroblast toxicity assays, indicating their safety for therapeutic use. **Conclusion:** The lack of toxicity of the *B. biternata* whole plant extracts and their growth inhibitory bioactivity against a panel of pathogenic bacteria partially validate the traditional usage of this species to treat bacterial diseases and indicates their potential in the development of antiseptic agents.

Keywords: Antibacterial Activity, Antibiotic-Resistant Bacteria, Asteraceae, Medicinal Plants, Spanish needles, Toxicity.

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INTRODUCTION

The rapid development of bacterial strains expressing resistance genes in recent years has resulted in increasing numbers of *Extremely* (XDR) or totally drug-resistant pathogens (TDR).[1] Often, these pathogens are resistant to multiple classes of antibiotics, and there is now few therapeutic options remaining to treat diseases caused by infection of these pathogens. Of greater concern, the spread of resistance genes is rapid and this situation is likely to worsen as more bacterial strains become multi-drug resistant (MDR). New antibiotic chemotherapies are urgently required to combat these infections, and improved antibiotic stewardship is required to maintain the efficacy of these therapies. Indeed, the World Health Organisation (WHO) considers the development of alternative antibiotic chemotherapies to be one of the most serious challenges facing medical science.[2] Whilst, medical researchers had previously been able to maintain a steady antibiotic pipeline by bioprospecting bacteria and fungi for novel antibiotic compounds, it is unlikely that these previous methods of antibiotic discovery/development will provide adequate new antibiotics in the future and new therapeutic options are urgently required.

A re-examination of traditional plant-derived medicines is an attractive option for the development of new antibiotic therapies as many plants have been used therapeutically for hundereds (or even thousands) of years and their uses have often been documented.[3] Indeed, the ability of plant extracts to block the growth of pathogenic bacteria has become the focus of substantial recent study.[4-9] Much of the research into traditional medicinal plant use has focused on Asian,[10,11] African[12-15] and South American[16,17] plants. Similarly, the medicinal properties of Australia flora has also received recent attention. Australian

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Aborigines understood the therapeutic properties of a wide variety of Australian plants and how to use them effectively.[18-22]

Bidens (family Asteraceae: Heliantheae) is a complicated genus of flowering plants that consists of approximately 230 species, which are widely disturbed globally.[23] Several species of this genus have well documented therapeutic uses. The therapeutic properties of *Bidens pilosa* L. have been particularly well documented. Whilst this species is native to the Americas, it has also been introduced into Asia, Africa, Australia and the Oceania region. Due to its wide distribution, this species is used in several traditional healing systems for the treatment of inflammation,[24] fever,[25] viral[26] and bacterial respiratory diseases,[27] as well as oral[28] and ophthalmic infections.[29] Similarly, *Bidens biternata* (Lour.) Merr. and Sherff (Figures 1a-c) is used in traditional chinese medicine (TCM) to treat diabetes, inflammation, enteritis, bacillary dysentery and pharyngitis.[30] Additionally, this species is used in Brazil to treat pain, fever, angina, diabetes, edema, infections and inflammation.[31] Biological studies have demonstrated that it has anti-hyperglycemic,[32,33] antihypertensive,[34] antiulcerogenic, antiulcerogenicm,[35] hepatoprotective,[36] antipyretic,[37] immunosuppressive and anti-inflammatory,[38-40] anti-leukemia,[41,42] anti-malarial,[43] anti-bacterial,[44] and antitumor[45] effects.

Due to its myriad of therapeutic uses, the phytochemistry of *B. biternata* has been reasonably well studied and a number of compounds have been highlighted. In particular, *B. biternata* is a rich source of flavonoids, including okanin (Figure 1d), cymaroside (Figure 1e), luteolin (Figure 1f), apienin (Figure 1g), and apigenin 7-o-glucoside (Figure 1h). The pentacyclic triterpenoids friedelin (Figure 1i) and friedelanol (Figure 1j) have also been identified in *B. biternata*.[46] Despite the earlier studies, *B. biternata* extracts are yet to be screened against many bacterial pathogens. This study screened a fractionated *B. biternata* whole plant extract against a panel of bacterial pathogens of importance to human health, and tested them for toxicity to evaluate their safety for therapeutic use.

MATERIALS AND METHODS

Plant collection and extraction

A 5 g mass of dried *Bidens biternata* (Lour.) Merr. and Sherff whole plant extract was kindly provided by Jiayu Zhang of Griffith University. The extract had been prepared using ethanol maceration and was dried by lyophilisation. 1 g masses of the extract were individually resuspended in a minimal volume of either 100%, 75%, 50% or 25% methanol in water. All solvents were AR grade and were supplied by Ajax Fine Chemicals, Australia. The extracts was absorbed individually onto C_{18} bonded silica gel, dried by evaporation at room temperature, and fractionated by C_{18} silica flash chromatography, eluting with 100% methanol (containing 0.1% TFA). Each fraction was lyophilised and resuspended in sterile deionised water (containing 1% DMSO).

Antibacterial screening

Conventional antibiotics

Penicillin-G (potency of 1440-1680 µg/mg), chloramphenicol (≥98% purity by HPLC, erythromycin (potency ≥850 µg/mg), ciprofloxacin (potency=600 µg/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 0.01 mg/mL and stored at 4°C until use. For the disc diffusion studies, ampicillin (10 µg) chloramphenicol discs (10 µg/disc) and tetracycline (10 µg/disc) standard discs were obtained from Oxoid Ltd., Australia and used as positive controls.

Test microorganisms

All media was supplied by Oxoid Ltd., Australia. Reference strains of *Acinetobacter baylyi* (ATCC33304), *Bacillus cereus* (ATCC 14579), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721), *Pseudomonas aeruginosa* (ATCC39324), *Shigella sonneii* (ATCC 25931), and *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pyogenes* (ATCC 12384) were purchased from American Type Culture Collection, USA. Clinical isolate microbial strains of *Alcaligenes feacalis, Enterobacter aerogenes*, and *Salmonella newport* were obtained from the School of Environment and Science technical laboratory, Griffith University, Australia. All bacteria were cultured in Meuller-Hinton broth (Oxoid Ltd., Australia). Streak Meuller-Hinton 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 24 hr and were subcultured and maintained in nutrient broth at 4°C until use.

Evaluation of antimicrobial activity

Antibacterial activity screening of the *B. biternata* extracts was assessed using a modified disc diffusion assay.[47,48] Ampicillin (10 µg/disc), chloramphenicol discs (10 µg/disc) and tetracycline discs (10 µg/disc) were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water (containing 1% DMSO) were used as a negative control.

Minimum Inhibitory Concentration (MIC) determination

The MICs of the *B. biternata* extracts and the antibiotic standards were evaluated by standard methods.[14] All plates were incubated at 37°C for 24 hr. *p*-Iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, Australia and dissolved in sterile deionised water to prepare a 0.2 mg/mL INT solution. A 40 µL volume of this solution was added into all wells and the plates were incubated for a further 6 hr at 37°C. Following incubation,

the MIC was visually determined as the lowest dose at which colour development was inhibited.

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 ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4 mg/mL) and serially diluted in artificial seawater as a reference toxin. Toxicity of the *B. biternata* fractions, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.[49,50] The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Cellular viability assay

All *B. biternata* fractions were also screened individually using a normal human primary dermal fibroblast (HDF) standard assay.[15] 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), 50 µg/mL streptomycin (Sigma-Aldrich, Australia) and 50 IU/mL penicillin (Sigm-Aldricha, Australia) at 37°C, 5% CO_2 in a humidified atmosphere. Individual 70 µL volumes of culture media (containing approximately 5000 cells) were added to wells of a 96 well plate and 30 µL 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_2 for 24 hr in a humidified atmosphere. All extracts were screened at 200 µg/mL. The cells were then washed in PBS (pH 7.2) to remove interference due to sample colour. A 20 µL volume of Cell Titre 96 Aqueous One solution (Promega) was added to each well and the plates were incubated for a further 3 hr. Absorbances were recorded at a test wavelength of 540 nm and a blank wavelength of 690 nm 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:

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

Table 1: Minimum Inhibitory Conncentrations (MIC) against the tested bacterial species expressed in µg/mL.

	B. biternata Whole Plant Extract Fractions				Antibiotic Controls			Toxicity Control	
	100% Methanol	75% Methanol	50% Methanol	25% Methanol	Amp	Chlor	Tet	PD	Quin
Gram Negative Bacteria									
A. baylyi	-	-	-	-	>2.5	0.32	0.63	NT	NT
A. faecalis	1250	>5000	-	-	0.32	-	>2.5	NT	NT
E. aerogenes	>5000	>5000	-	-	1.25	-	1.25	NT	NT
E. coli	1250	1250	-	-	0.32	0.16	0.63	NT	NT
K. pneumoniae	-	-	-	-	>2.5	1.25	0.63	NT	NT
P. aeruginosa	625	1250	>5000	-	-	>2.5	-	NT	NT
P. mirabilis	2500	>5000	-	-	0.63	0.32	>2.5	NT	NT
S. newport	625	625	>5000	-	0.95	0.63	0.16	NT	NT
S. sonneii	2500	>5000	-	-	0.63	1.25	0.32	NT	NT
Gram Positive Bacteria									
B. cereus	-	-	-	-	0.32	0.16	0.64	NT	NT
S. aureus	-	-	-	-	0.64	1.25	0.32	NT	NT
S. pyogenes	1250	>5000	>5000	-	1.25	-	0.63	NT	NT
Toxicity testing									
ALA	NT	NT	N T	NT	NT	NT	NT	CND	CND
HDF	NT	NT	NT	NT	NT	NT	NT	CND	CND

Numbers indicate the mean MIC values of triplicate determinations. - indicates that the test was ineffective against that bacterium at all concentrations tested. Amp= Ampicillin; Chlor= Chloramphenicol; Tet= Tetracycline; ALA= *Artemia* lethaliry assay; HDF= Human dermal fibroblasat assay; PD= Potassium dichromate control; Quin= Quinine control; NT= Not tested: CND= could not be determined as mortality or proliferation inhibition did not exceed 50% at any concentration tested. MIC values >1 µg/mL for pure antibiotics indicates resistance to that antibiotic.

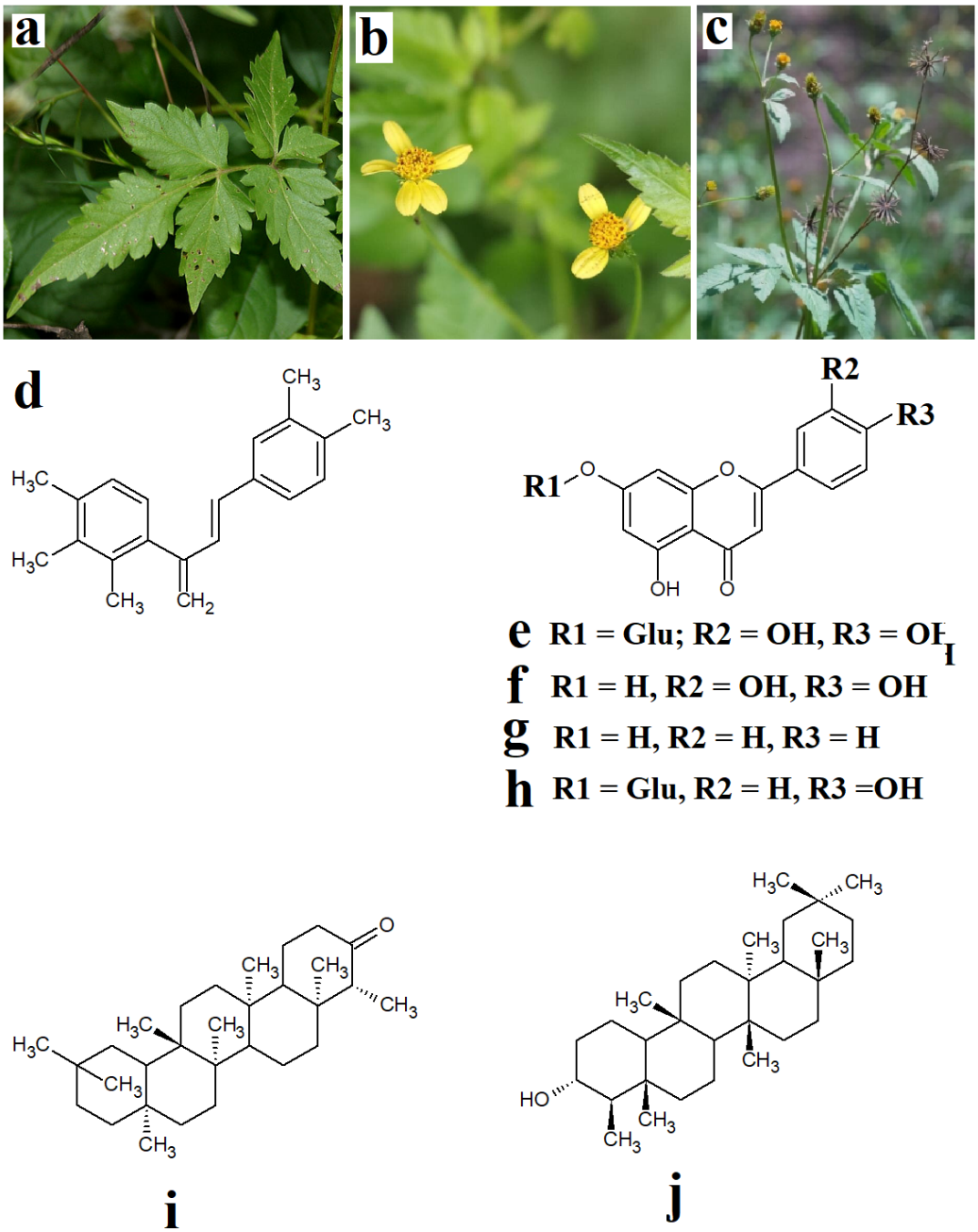

Figure 1: *Bidens biternata* (a) leaves, (b) flowers, (c) fruit, as well as the chemical structures of (d) okanin, (e) cymaroside, (f) luteolin, (g) apienin, (h) apigenin 7-o-glucoside, (i) friedelin, (j) friedelanol.

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

RESULTS

Antimicrobial activity

To determine the growth inhibitory activity of the *B. biternata* whole plant extract fractions against the panel of pathogenic bacteria, aliquots (10 µL) of each fraction were initially screened against a panel of gram negative (Figure 2) and gram positive bacteria (Figure 3) in the disc diffusion assay. Of the 9 gram negative bacterial strains tested, seven (~78%) were inhibited by at least two of the *B. biternata* extract fractions. However, all fractions were completely ineffective against *A. baylyi* (Figure 2a) and *K. pneumoniae* (Figure 2e). Against the other gram negative

bacteria, the 75 and 100% methanolic extracts were substantially more effective inhibitors of bacterial growth than the lower methanol % fractions. Indeed, the 25 and 50% methanol extracts completely lacked growth inhibitory activity against *A. faecalis* (Figure 2b), *E. aerogenes* (Figure 2c), *E. coli* (Figure 2d), *P. mirabilis* (Figure 2g) and *S. sonnei* (Figure 2i). Whilst the 25% methanol extract was also ineffective against *P. aeruginosa* (Figure 2f) and *S. newport* (Figure 2h), the 50% methanol inhibited bacterial growth, albeit with zones of inhibition (ZOIs) that indicate only low inhibitory activity. In contrast, all of the tested gram negative bacteria were inhibited by at least one of the conventional antibiotics tested, but not by the negative control, indicating that the assay was functioning correctly.

The gram positive bacteria appeared to be substantially more resistant to the *B. biternata* whole plant fractions. Indeed, only one (*S. pyogenes*; Figure 3c) of the 3 g positive bacteria tested (~33%) was inhibited by the *B. biternata* 50-100% methanol extract fractions. In contrast, *B. cereus* (Figure 3a) and *S. aureus* (Figure 3b) were unaffected by all of the fractions. However, it is uncertain whether the lower susceptibility of the gram positive bacteria (compared to the gram negative bacteria) is genuine, or is due to the substantially fewer gram positive tested. Future studies screening the fractions against a larger panel of gram positive bacteria are required to confirm whether this trend is genuine. Notably, all of the gram positive bacteria were inhibited by ≥2 of the antibiotic controls, but not by the negative control, confirming that the assay was functioning correctly.

The antimicrobial efficacy was further quantified by determining the MIC values for each extract against the microbial species that were determined to be susceptible. For this study, fractions with MICs <1000 µg/mL were deemed to be strong; MICs 1000-3000 µg/mL were deemed to be moderate; and MIC values >3000 µg/mL were deemed to be weak. The *B. biternata* fractions were found to be good growth inhibitors of several bacterial species (as judged by MIC; Table 1). In general, the 100% methanolic fraction was the most effective antibacterial agent. Indeed,

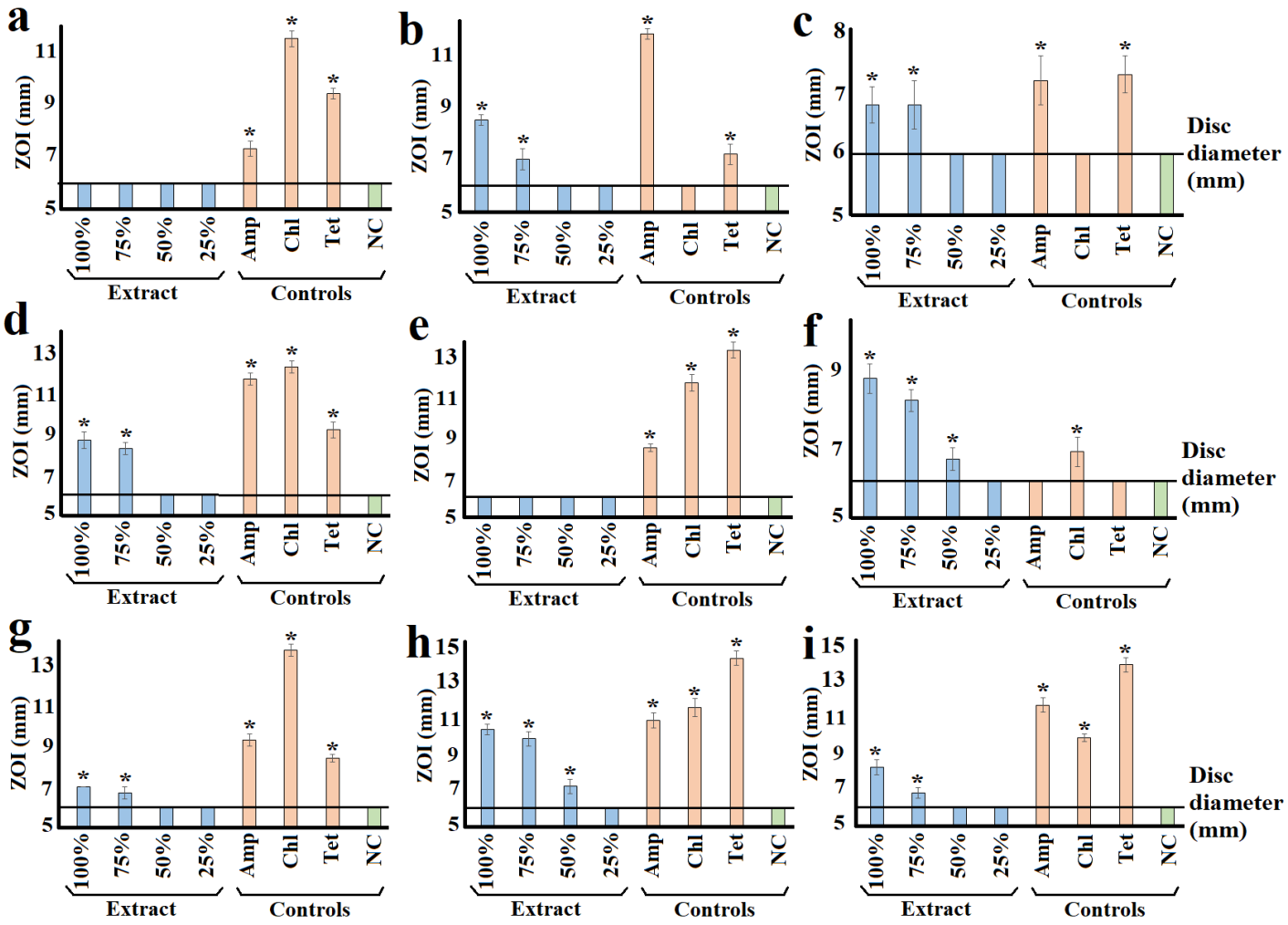

Figure 2: Antibacterial activity of B. biternata whole plant extracts measured as zones of inhibition (ZOIs; mm) against gram negative bacteria (a) *A. baylyi,* (b) *A. faecalis,* (c) *E. aerogenes*, (d) *E. coli*, (e) *K. pneumoniae*, (f) *P. aeruginosa*, (g) *P. mirabilis*, (h) *S. newport*, (i) *S. sonnei*. The % extracts refer to the % methanol that was used for fractionation. Amp = ampicillin (10 µg/disc); Chl = chloramphenicol (10 µg/disc); Tet = tetracycline (10 µg/disc). Results are expressed as mean±SEM of three determinations, each in triplicate (n=9). * indicates results that are significantly different to the untreated control (p<0.01).

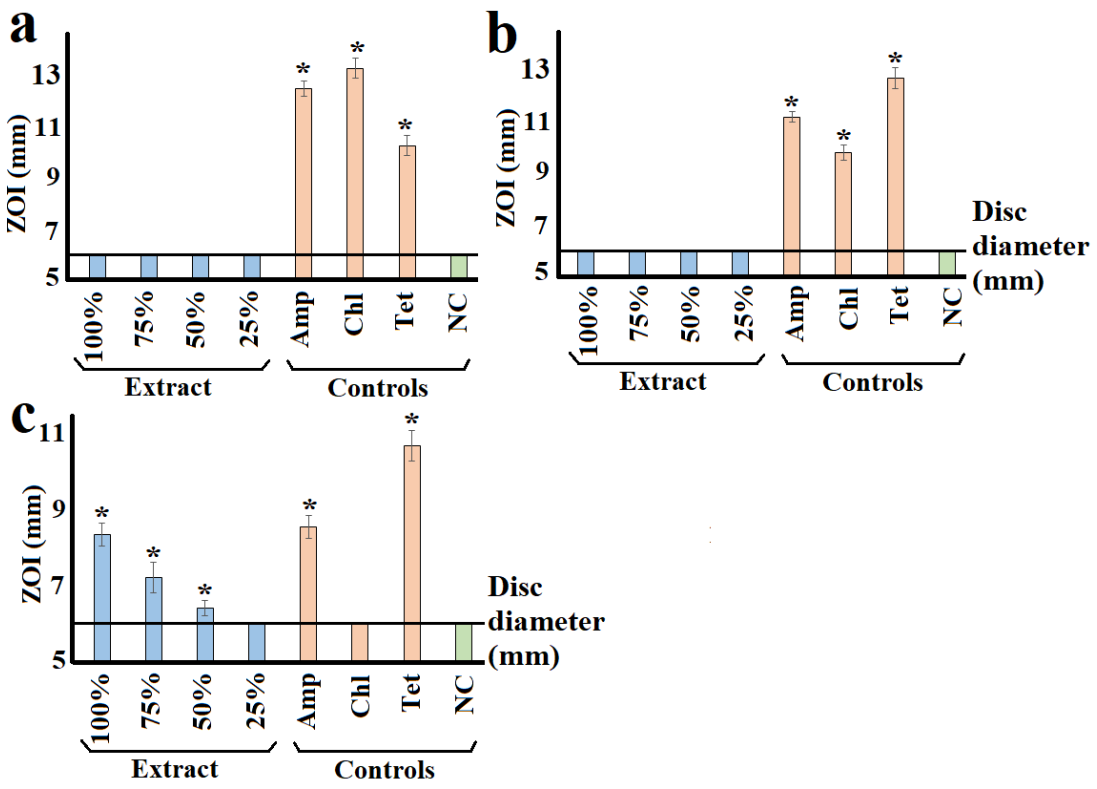

Figure 3: Antibacterial activity of *B. biternata* whole plant extracts measured as zones of inhibition (ZOIs; mm) against gram positive bacteria (a) *B. cereus*, (b) *S. aureus*, (c) *S. pyogenes*. The % extracts refer to the % methanol that was used for fractionation. Amp = ampicillin (10 µg/disc); Chl = chloramphenicol (10 µg/disc); Tet = tetracycline (10 µg/disc). Results are expressed as mean±SEM of three determinations, each in triplicate (n=9). * indicates results that are significantly different to the untreated control (p<0.01).

strong MIC values of 625 µg/mL were recorded for the 100% methanolic fraction against *P. aeruginosa* and *S. newport*, whilst noteworthy inhibition was noted for this fraction against *A. faecalis, E. coli, P. mirabilis, S. sonneii* and *S. pyogenes*. This is perhaps not surprising as this fraction would be expected to be similar to the original ethanolic extract as both ethanol and methanol have similar extractabilities. Therefore, it is likely that this fraction would contain the maximum amount and greatest diversity of *B. biternata* compounds. The 75% methanol fraction also displayed good antibacterial activity against *S. newport*, as well as noteworthy activity against *E. coli* and *P. aeruginosa*. These results are particularly interesting as the MIC values for the pure antibiotics indicated that several of these bacterial strains were antibiotic-resistant, with MIC values >1 µg/mL. Indeed, *E. aeruginosa* and *P. aeruginosa* were resistant to all of the control antibiotics tested, and *A. faecalis* was only susceptible to ampicillin. Similarly, *K. pneumonuiae* and *S. pyogenes* were only susceptible to tetracycline.

In contrast, the lower % methanol fractions (with corresponding higher water concentrations) were substantially less effective bacterial growth inhibitors that the corresponding higher % methanol fractions. Notably, lower amounts of mid to lower polarity compounds as the methanol concentration decreases, and therefore it is likely that these fractions contain fewer classes of compounds. Notably, with the exception of *E. coli, P. aeruginosa* and *S. newport*, the 75% methanol fraction was generally ineffective, whilst the 25 and 50% methanol fractions were ineffective against all of the bacterial species tested.

Quantification of toxicity

The toxicity of the *B. biternata* whole plant extracts was tested in both an *Artemia franciscana* nauplii lethality assay (ALA), and in a human dermal fibroblast (HDF) viability assay at a concentration of 1000 µg/mL (Figure 4). Both fractions induced substantially <50% mortality in the ALA, and inhibited HDF viability by substantially >50% and were therefore deemed to be non-toxic. As the *Artemia* mortality or decreased cellular viability did not exceed 50% at any concentration tested, it was not possible to determine LC_{50} values for the fractions. In contrast, potassium dichromate (positive control for ALA screening) and quinine (positive control for the HDF viability assay) induced approximately 100% mortality or 100% reduced HDF viability respectively following 24 hr exposure, indicating that both assays were functioning correctly.

DISCUSSION

Recent increases in bacterial resistance has made the development of new antibiotic therapies a high priority.[1,2] A concurrent decrease in the development of new antibiotic therapies has further increased this problem and new antibiotic therapies are

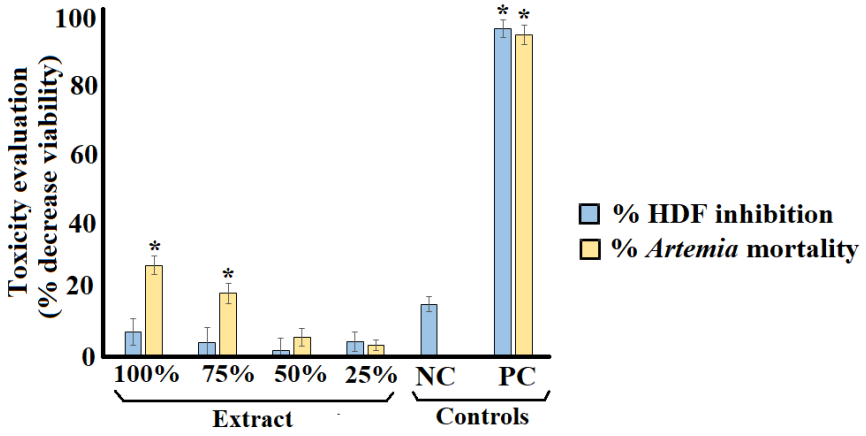

Figure 4: The lethality of the *B. biternata* whole plant extracts (1000 µg/mL), negative controls (artificial seawater for *Artemia* assay; cell growth media for HDF assay) and positive controls (1000 µg/mL potassium dichromate for *Artemia* toxicity assay; 200 µg/mL quinine for HDF assay). Blue bars represent the % decreased cell viability following 24 hr exposure of HDF cells to the extract/toxin; yellow bars represent the % *Artemia* nauplii mortality following 24 hr exposure to the extract/toxin; NC=negative (untreated) control; PC = positive control. All bioassays were performed three times, each with internal triplicates (n=9) and are expressed as Mean±SEM. * indicates results that are significantly different to the negative controls (p<0.01).

urgently needed. Interest in re-evaluating medicinal plants for new antibiotic chemotherapies has increased substantially in recent years. Notably, several *Bidens* spp. (including *B. biternata*) are used in multiple traditional therapeutic systems for a variety of medical conditions, including for treating bacterial infections. Despite this, limited scientific studies have rigorously evaluated the antibacterial properties of *B. biternata*.

Our study examined the ability of fractions prepared from a *B. biternata* whole plant extract to inhibit the growth of a panel of medicinally important bacterial pathogens. The 100% methanol fraction was a noteworthy growth inhibitor of *A. faecalis, E. coli, P. aeruginosa, S. newport* and *S. pyogenes* (as determined by their MIC values). Several of these bacteria are associated with food poisoning and diarrhoea. Of further interest, some of these bacteria were also resistant to one or more conventional antibiotics. Therefore, the *B. biternata* whole plant fractions (particularly the 100% methanol fraction) have potential for the treatment of diarrhoea and gastrointestinal disease, although further testing against other human-infective bacterial pathogens is required to fully evaluate the antibacterial potential of this fraction. An investigation of the phytochemistry of the *B. biternata* whole plant extract fractions was beyond the scope of our study and future studies are required to identify the active component(s) in these fractions.

The ability of the *B. biternata* fractions to inhibit the growth of both gram-positive and gram-negative bacteria is in agreement with previous reports of the antibacterial activity of other plant species.[52] However, in this study, the gram-negative bacteria were more susceptible to the *B. biternata* fractions. In contrast, multiple previous studies have reported substantially greater susceptibility for gram-positive bacteria to South American,[53] African[54,55] and Australian[52] plant extracts. Results within our laboratory have also confirmed the greater susceptibility of gram-positive bacteria towards many other plant extracts.[56,57] The gram-negative bacterial cell wall outer membrane is thought to act as a barrier to many substances including several antibiotics.[58] In contrast, other studies have demonstrated that gram-negative bacteria are often as susceptible (or more susceptible) to plant extracts from other plant species.[59]

The findings of this study also demonstrate that the *B. biternata* whole plant extract fractions were nontoxic towards *Artemia franciscana* nauplii and HDF cells, with little difference noted to the untreated controls in both assays. Whilst our preliminary toxicity studies indicate that these fractions may be safe for therapeutic use, studies using further human cell lines are required to rigorously evaluate the safety of these fractions. Furthermore, whilst these studies have demonstrated the potential of the *B. biternata* whole plant extract fractions in the development of future antibiotic chemotherapeutics, more work is required to isolate the inhibitory components and determine the mechanism of inhibition.

CONCLUSION

The results of this study demonstrate the potential of *B. biternata* whole plant extracts as inhibitors of pathogenic bacteria growth. Furthermore, their lack of toxicity indicates that they are safe for therapeutic uses. Further studies aimed at the purification and identification of bioactive components are required to examine the mechanisms of action of these agents.

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ABBREVIATIONS

DMSO: Dimethyl sulfoxide; **LC_{50}:** The concentration required to achieve 50% mortality; **MIC:** Minimum inhibitory concentration.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

- *Bidens biternata* whole plant extracts were screened for the ability to block the growth of a panel of bacterial pathogens.
- The growth inhibition of both gram-positive and gram-negative bacteria was tested.
- The antibacterial activity was quantified by determining the MIC values of each extract.
- Toxicity of the *B. biternata* extracts was determined using the *Artemia* nauplii and HDF toxicity bioassays.

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