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*Original Article*

# *Westringa fruticosa* (Willd.) Druce Leaf and Flower Extracts Lack Antibacterial Activity and are Non-toxic *in vitro*

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## ABSTRACT

**Introduction:** Closely related plant species often share similar secondary metabolites and bioactivities and are therefore good targets for bioactivity testing when one or more species within a family are known to possess therapeutic properties. The family Laminaceae has a long history of medicinal usage globally. Many species are known to have therapeutic properties, several species of which have well established antibacterial bioactivities. **Methods:** The ability of *Westringa fruticosa* leaf and flower extracts to inhibit the growth of a panel of bacterial and fungal pathogens was investigated by disc diffusion assay. Toxicity was examined using the *Artemia franciscana* nauplii bioassay. **Results:** *W. fruticosa* leaf methanolic and aqueous extracts were both completely ineffective at inhibiting the growth of gram-positive and gram-negative panels of bacteria, as well as fungi. The extracts were non-toxic or of low toxicity in the Artemia bioassay following 24 h exposure. **Conclusion:** Despite the taxonomic relationship to several bioactive Laminaceae spp., *W. fruticosa* leaf and flower extracts were completely ineffective bacterial and fungal growth inhibitors. However, these extracts may have other therapeutic properties and testing against protozoa, virus and tumour cells is required.

**Key words:** Lamiaceae, Coastal rosemary, Antibacterial activity, Australian plant, Traditional medicine, Medicinal plants, Toxicity.

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## INTRODUCTION

Traditional plant derived medicines have been used in most parts of the world for a variety of therapeutic purposes, including fighting microbial disease. Indeed, the ability of plant extracts to block the growth of pathogenic bacteria has become a focus of substantial recent study.[1-5] Much of the research into traditional medicinal plant use has focused on Asian,[6-8] African,[9-11] Middle Eastern[12-14] and South American[15] plants. However, despite the potential of plants to provide us with useful pharmaceutical agents, the field is still relatively poorly studied. Only an estimated 5-10 % of the approximately 300,000-500,000 plant species worldwide have been screened for one or more bioactivities.[9] With so many plant species yet to be tested, it is essential that plant selection processes narrow the field. The main selection criteria currently used is to select plants on the basis of ethnobotanical usage as traditional medicines. Another important selection method is to examine plants related to plants for which medicinal potential is well established. Many plant secondary metabolites are regarded as family, genus or species specific and investigation of species closely related to those used as traditional medicines may lead to natural therapeutic discoveries.[16]

The development of new antibiotic therapies is particularly urgent. The recent establishment of bacterial pathogens that are either extremely (XDR) or totally resistant (TDR) to common clinically used antibiotics[17] has resulted in the need to develop new and effective antibiotic chemotherapies. There are now limited therapeutic options for many diseases caused by bacterial pathogens and the situation is expected to worsen in the future as bacteria exchange resistance genes. Indeed, the development of alternative antibacterial treatment modalities has become crucial and is considered by the World Health Organisation (WHO) to be one of the most serious challenges facing medical science.[18] For a number of reasons reviewed elsewhere,[17] it is unlikely that the previous methods of antibiotic discovery/development will be as successful in the future and new treatment modalities are urgently required. Traditional medicines and herbal remedies have great potential for antimicrobial drug development and there has recently been a substantial increase in interest in this field.[19,20]

The first Australians had well-developed medicinal systems and understood the therapeutic properties of a wide variety of Australian plants and how to use them effectively.[16] Despite this, relatively few studies have rigorously examined the antibacterial activity of Australian native plants, although there has recently been a substantial increase in interest in this field.[21-24] The genus *Westringa* (family Lamiaceae) consists of more than 30 species of Australian shrubs.[25] *Westringa fruticosa* (Willd.) Druce (commonly known as coastal rosemary) is an endemic Australian shrub (Figure 1a) that grows in coastal regions of Eastern Australia. Its growth pattern resembles *Rosmarinus officinalis* L. (rosemary) and both species are in the same family. *W. fruticosia* flowers in spring and summer in temperate southern climates but can flower continuously in warmer climates. The flowers are small (2-1mm long) and are white in colour with purple to brown dots (Figure 1b).

In contrast with many other members of family Lamiaceae, there is little evidence that *W. fruticosa* was used medicinally by the first Australians. However, a recent study reported potent growth inhibitory activity for *W. fruticosa* leaf extracts against *Listeria monocytogenes*, *Micrococcus luteus* and *Staphylococcus aureus*.[26] The same study reported lower activity or no activity at all against against *Bacillus subtilis, Escherichia coli, Klebsiellia pneumonia Pseudomonas aeruginosa* and *Salmonella typhimurium*. In contrast, a different study reported that *W. fruticosa* leaf and flower extracts were completely ineffective against *Aeromonas hydrophilia, Pseudomonas fluorescens, Bacillus cereus* and *Bacillus subtilis*.[27] There is also a lack of information on the phytochemical composition of this species. However, the phytochemistry of other Laminacaeae species has been reported[28] and is characterised by relatively high levels of luteolin-7- rutinoside (Figure 1c), luteolin-7-glucuronide (Figure 1d), lithospermic acid (Figure 1e), rosmarnic acid (Figure 1f) and methyl rosmarinate (Figure 1g). Many of these compounds have been reported to have potent antibacterial activity[29] and may therefore contribute to the antibacterial properties of *W. fruticosa*. This study was

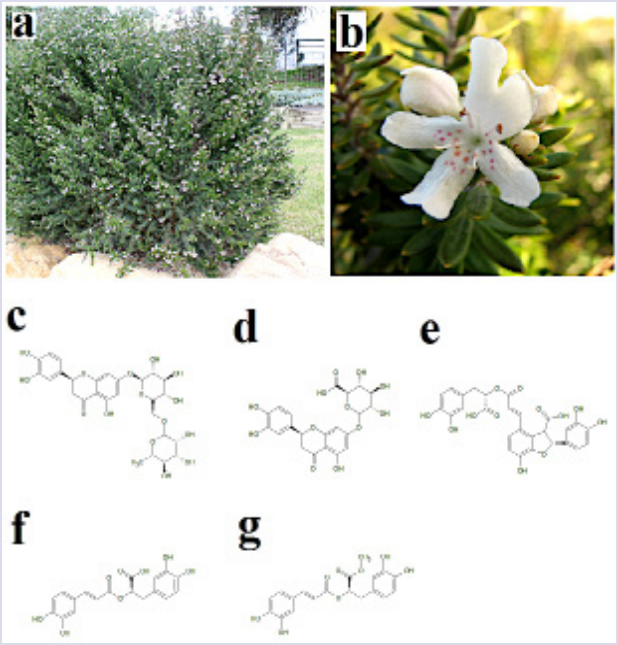


**Figure 1:** *W. fruticosa* (a) whole plant and (b) flower and the structures of some common Lamiacaeae compounds: (c) luteolin-7- rutinoside, (d) luteolin-7-glucuronide, (e) lithospermic acid, (f) rosmarnic acid and (g) methyl rosmarinate.

undertaken to screen *W. fruticosa* leaf and flower extracts for the ability to inhibit the growth of a panel of gram-positive and gram-negative bacterial pathogens and three fungi.

## MATERIALS AND METHODS

### Plant material

### Collection of plant material and extraction

*Westringa fruticosa* (Willd.) Druce leaves and flowers were harvested from a confirmed suburban plant in the southern suburbs of Brisbane, Australia. The plant was monitored for 3 months prior to harvesting to ensure that it received no pesticides or fertilisers and none were used in a 3-metre radius of the plant. The harvested leaves and flowers were washed in deionised water and processed within 4 h of collection. The leaves were dried in a Sunbeam food dehydrator and the dried material was ground to a coarse powder. Individual 1g masses of the dried plant material was extracted extensively in 50mL methanol (Ajax, AR grade) or deionised water for 24 h at 4°C with gentle shaking. The extract was filtered through filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation. The resultant pellet was dissolved in 5mL deionised water. The extract was passed through 0.22µm filter (Sarstedt) and stored at 4°C.

### Qualitative phytochemical studies

Phytochemical analysis of the *W. fruticosa* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by standard assays.[30-32]

### Antibacterial screening

### Test microorganisms

All media was purchased from Oxoid Ltd., Australia. The reference strains of *Escherichia coli* (ATCC157293), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721) and *Streptococcus pyogenes* (ATCC19615) were purchased from American Tissue Culture Collection (ATCC), USA. Clinical isolate microbial strains of *Aeromonas hydrophilia*, *Alcaligenes feacalis*, *Aspergillus niger*, *Bacillus cereus, Candida albicans, Citrobacter freundii*, *Pseudomonas fluorescens*, *Saccromyces cerviseae*, *Salmonella newport, Serratia marcescens, Shigella sonneii, Staphylococcus aureus* and *Staphylococcus epidermidis* strains were obtained from Ms Michelle Mendell and Ms Jane Gifkins, Griffith University. All bacterial stock cultures were subcultured and maintained in nutrient broth at 4°C. The fungal strains were cultured in Sabourand broth (Oxoid, Australia).

### Evaluation of antimicrobial activity

Antimicrobial activity of the *W. fruticosa* leaf extracts was determined using a modified disc diffusion assay.[33-35] Briefly, 100µL of the each microbial suspension in log phase was spread onto individual nutrient agar plates (or Sabourand agar for the fungal strains) and the extracts were tested for antimicrobial activity using 6mm sterilised filter paper discs. The discs were each infused with 10µL of the individual plant extract, allowed to dry and placed onto the inoculated plates. The plates were allowed to stand at 4°C for 2 h before incubation at 37°C for 24 h. The diameters of the zones of inhibition (ZOIs) were measured to the closest whole millimetre. Each assay was performed three times in triplicate ($n$=9). Mean values (± SEM) are reported in this study. Standard discs of ampicillin (10µg), chloramphenicol (10µg) and nystatin (100µg) were obtained from Oxoid, Australia and were used as positive controls to compare antibacterial and antifungal activity. Filter discs infused with 10µL of distilled water were used as a negative control.

### *Artemia franciscana* nauplii toxicity screening

Toxicity was tested using an adapted *Artemia franciscana* nauplii lethality assay.[36-38] Briefly, *A. franciscana* nauplii were incubated in the presence of the extracts, reference toxin (1mg/mL potassium dichromate) or artificial seawater (negative control) at 25±1°C under artificial light. All treatments were performed three times in triplicate ($n$=9). The number of dead were counted in each well at 24h, 48h and 72h. At the completion of the 72h exposure period, the remaining live nauplii were sacrificed and the total number of nauplii in each well were counted and used to calculate the % mortality per well. $LC_{50}$ values were calculated for each treatment using probit analysis.

### Statistical analysis

Data are expressed as the mean ± SEM of three independent experiments with internal triplicates ($n$=9). 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

### Liquid extraction yields and qualitative phytochemical screening

Extraction of 1g of dried and powdered *W. fruticosa* leaves with methanol and water yielded 279 and 224mg of extracted material respectively (Table 1). The extracts were resuspended in 10mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 1. Qualitative phytochemical studies showed that both leaf extracts had similar phytochemical profiles. Both contained high levels

**Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *W. fruticosa* leaf.**

| | | | Leaf | | Flower | |
|---|---|---|---|---|---|---|
| | | | Methanolic extract | Aqueous extract | Methanolic extract | Aqueous extract |
| Mass of extracted material (mg) | | | 279 | 224 | 283 | 216 |
| Concentration of resuspended extract (mg/mL) | | | 27.9 | 22.4 | 28.3 | 21.6 |
| Qualitative Phytochemical Tests | Phenols | Total phenols | +++ | +++ | +++ | +++ |
| | | Water soluble phenols | +++ | +++ | +++ | +++ |
| | | Insoluble phenols | ++ | ++ | ++ | ++ |
| | Saponins | Froth persistence | - | - | + | + |
| | | Emulsion test | - | - | + | + |
| | Cardiac glycosides | Keller-Kiliani Test | - | - | - | - |
| | Triterpenoids | Salkowski Test | + | - | - | - |
| | Phytosterols | Acetic Anhydride Test | - | - | - | - |
| | Alkaloids | Meyer's Test | - | - | - | - |
| | | Wagner's Test | - | - | - | - |
| | | Draggendoff's Test | - | - | - | - |
| | Flavonoids | Kumar Test | +++ | +++ | +++ | +++ |
| | Tannins | Ferric Chloride Test | ++ | ++ | + | + |
| | | Lead Acetate Test | + | - | + | + |
| | Anthraquinones | Free | - | - | - | - |
| | | Combined | - | - | - | - |

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

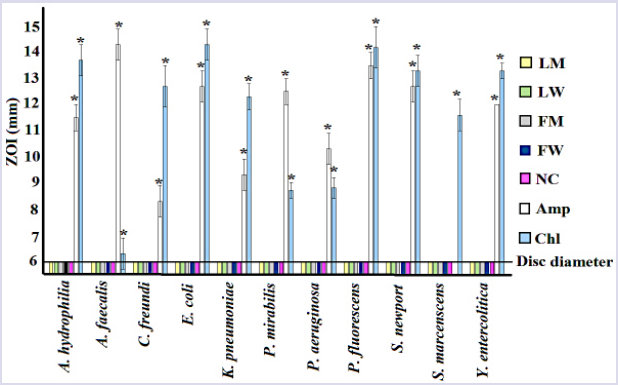


**Figure 2:** Growth inhibitory activity of *W. fruticosa* leaf and flower extracts and reference antibiotics against gram-negative bacterial species measured as ZOIs (mm) ± SEM. L = leaf; F = fruit; M = methanolic extract; W = aqueous extract; Amp = ampicillin (10µg); Chl = chloramphenicol (10µg); NC = negative control. All assays were completed three times, each with internal triplicates (n=9) and the results are expressed as mean zones of inhibition (mm) ± SEM.

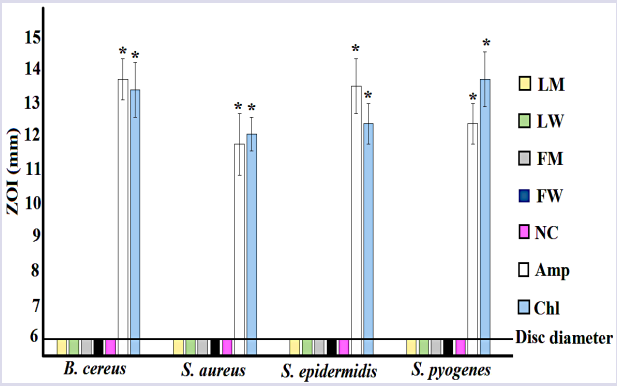


**Figure 3:** Growth inhibitory activity of *W. fruticosa* leaf extracts and reference antibiotics against gram-positive bacterial species measured as ZOIs (mm) ± SEM. L = leaf; F = fruit; M = methanolic extract; W = aqueous extract; Amp = ampicillin (10µg); Chl = chloramphenicol (10µg); NC = negative control. All assays were completed three times, each with internal triplicates (n=9) and the results are expressed as mean zones of inhibition (mm) ± SEM.

of phenolic compounds and flavonoids, as well as moderate levels of tannins. Similar yields of dried extracted material were obtained for the *W. fruticosa* flower extracts. The flower extracts also had similar phytochemical profiles to the leaf extracts, albeit generally with lower relative abundance.

## Antimicrobial activity

To determine the growth inhibitory activity of the *W. fruticosa* leaf and flower extracts, aliquots (10µL) of each extract were screened in the disc diffusion assay. The *W. fruticosa* leaf and flower extracts were ineffective at inhibiting the growth of all gram-negative (Figure 2) and gram positive (Figure 3) bacterial species tested. In contrast, both positive control antibiotics (ampicillin and chloramphenicol) were effective

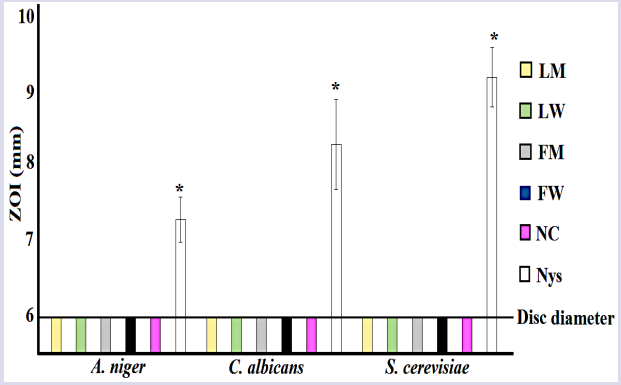


**Figure 4:** Growth inhibitory activity of *W. fruticosa* leaf extracts and reference antibiotics against fungal species measured as ZOIs (mm) ± SEM. L = leaf; F = fruit; M = methanolic extract; W = aqueous extract; Nys = Nystatin (100µg); NC = negative control. All assays were completed three times, each with internal triplicates (n=9) and the results are expressed as mean zones of inhibition (mm) ± SEM.

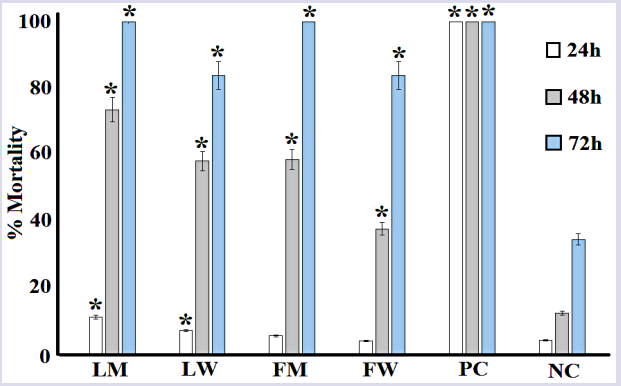


**Figure 5:** The lethality of the *W. fruticosa* leaf and flower extracts, potassium dichromate control (PC; 1000µg/mL) and seawater negative control (NC) following 24, 48 and 72 h of exposure. L = leaf; F = flower; M = methamolic extract; W = aqueous extract. All bioassays were performed three times in triplicate (n=9) and are expressed as mean ± SEM. * indicates results that are significantly different to the untreated (seawater) control at the equivalent exposure time (P<0.01).

growth inhibitors, with ZOI's of up to 14.3mm (chloramphenicol against *E. coli*). We were therefore unable to determine the MIC values for any extract as they were completely ineffective at all concentrations tested. Similarly, all of the extracts were ineffective at inhibiting the growth of the three fungal species screened in this study (Figure 4).

## Quantification of Toxicity

The toxicity of the *W. fruticosa* leaf and flower extracts was initially tested at 2mg/mL in the *A. franciscana* nauplii bioassay (Figure 5). The mortality in the presence of all extracts was not significantly different to that of the untreated control at 24h and thus were deemed to be non-toxic. Extracts with 24h $LC_{50}$ values >1000µg/mL have previously been defined as non-toxic.[38] In contrast, the potassium dichromate positive control induced substantial mortality within 4h (results not shown), with 100% mortality induction seen by 24h. The mortality increased following exposure to the *W. fruticosa* leaf extracts at 48h and was further increased following 72h exposure.

# DISCUSSION

Due to recent increases in bacterial resistance to many antibiotics, the development of new antibiotic chemotherapies is a high priority for medical science.[17,18] A concurrent decrease in the discovery of new antibiotic medicines by conventional strategies has increased interest in re-evaluating medicinal plants for new antibiotic chemotherapies.[39] Whilst we were unable to find reports of the traditional use of *W. fruticosa* medicinally, it is taxonomically related to other Lamiaceae species with extensive therapeutic uses. For example, *Mentha piperita* L. (peppermint), *Melissa officinalis* L. (Melissa leaf, lemon balm leaf) and *Salvia officinalis* L. (sage) have widespread uses as herbal teas and essential oils. *M. piperita* is used topically for muscle and nerve pain and for the relief of itching. Oral administration is useful for relieving gastrointestinal complaints.[40] *M. officinalis* and *Salvia officinalis* are used for disorders of the gastrointestinal tract, nervous system, as well as liver and bile disorders.[28] Many of these diseases are caused by bacterial infections and numerous studies have reported antibacterial properties for species of this genus.[41-43] However, many of these studies either do not quantify the activity, or report low to moderate activity for the extracts and oils of these plants.

The antibacterial activity of Australian Lamiaceae species is even less well examined. Indeed, we were only able to find two studies examining the antibacterial activity of *W. fruticosa*. One study reported that a *W. fruticosa* leaf extract was a potent inhibitor of *L. monocytogenes*, *M. luteus* and *S. aureus* growth in a disc diffusion assay.[26] The same extract was inactive or had only low activity against *B. subtilis, E. coli, K, pneumonia P. aeruginosa* and *S. typhimurium*. This contrasts with the results of our current study, which determined that *W. fruticosa* leaf and flower extracts were completely ineffective against an extended panel of bacteria. These results more closely align with a previous study from our group that screened *W. fruticosa* leaf and flower extracts against *A. hydrophilia, P. fluorescens, B. cereus* and *B. subtilis.* The *W. fruticosa* extracts were completely ineffective at inhibiting the growth of those bacteria.[27]

A single assay technique was used to screen for antibacterial activity in this study (as well as in the previous studies examining the antibacterial activity of this species). We chose to use the disc diffusion assay as it is a rapid method and it has previously been widely utilised in other studies. Therefore, comparisons between studies are relatively simple. However, as the disc diffusion method is reliant on the diffusion of a molecule through the aqueous environment of an agar gel, this assay may be affected by the solubility of the extract compounds in the aqueous environment. Polar compounds that are highly soluble in water would be expected to diffuse easily in the gel, whereas less soluble compounds would not diffuse as readily and thus be concentrated around the disc. Diffusion of molecules within an agar gel is also affected by the size of the molecules. The movement of large, complex phytochemicals (eg. complex tannins) through agar gels by diffusion would also be retarded and may provide a false idea of the efficacy of an extract. As many tannins have well described antibiotic properties, screening for growth inhibition using agar diffusion techniques may give a fallacious view of its inhibitory potential. For this reason, whilst this is a handy assay for screening aqueous extracts, this technique may not be ideal for nonpolar compounds (e.g. when screening essential oils and their components). For examining nonpolar mixtures, other techniques such as liquid dilution assays may be preferred. As Lamiaceae spp. are known to contain nonpolar terpenoid components,[44-46] their activity may have been significantly underestimated. Liquid dilution studies may

have been better suited to screen *W. fruticosa* extracts for activity and future studies will use these techniques to re-examine the extracts for antibacterial activity.

The findings reported here also indicate that the extracts examined were non-toxic (24 h $LC_{50}$ >1000 µg/mL) in the *Artemia* nauplii bioassay. Whilst toxicity was assessed in this study with the test organism *A. franciscana*, toxicity towards *A. franciscana* has previously been shown to correlate well with toxicity towards human cells for many toxins.[38] However, further studies are required to determine whether this is also true for the *W. fruticosa* leaf extracts examined in these studies.

## CONCLUSION

Methanolic and aqueous *W. fruticosa* leaf and flower extracts displayed no antibacterial activity in the disc diffusion assay against panels of human pathogenic bacteria and fungi, despite their close taxonomic relationship with other Laminaceae spp. with well-known antibacterial properties. The extracts were nontoxic towards *Artemia* nauplii.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors report no conflicts of interest.

## ABBREVIATIONS

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

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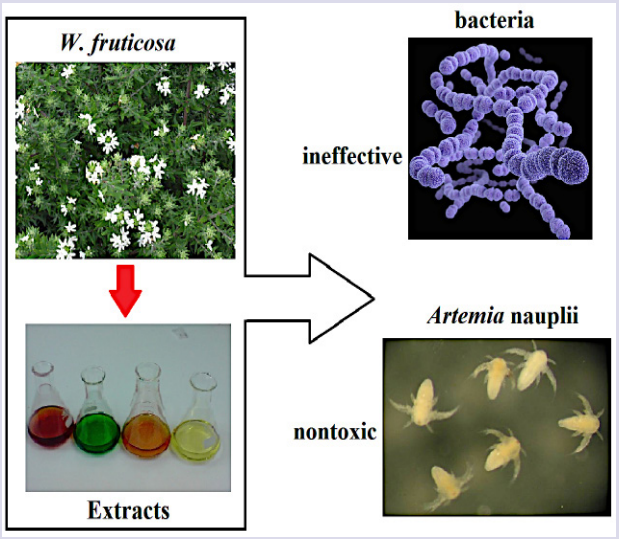
## PICTORIAL ABSTRACT




## SUMMARY

- *W. fruticosa* leaf and flower extracts leaf extracts were screened for the ability to block the growth of a panel of human bacterial pathogens.
- No inhibitory activity was evident against any of the bacterial species tested.
- Toxicity of the *W. fruticosa* extracts was determined using the *Artemia* nauplii toxicity bioassay.
- Both the methanolic and aqueous extracts were nontoxic.

## ABOUT AUTHORS

**Ms Getmore Chikowe** completed at BSc at Griffith University in life sciences. Following graduation, she undertook a research project in Dr Ian Cock's laboratory in the School of Natural Sciences at Griffith University. The project examined the growth inhibitory properties of a variety of Australian native plants against an extensive panel of bacterial pathogens.

**Ms Lindiwe Mpala** completed at BSc at Griffith University in life sciences. Following graduation, she undertook a research project in Dr Ian Cock's laboratory in the School of Natural Sciences at Griffith University. The project examined the growth inhibitory properties of a variety of Australian native plants against an extensive panel of bacterial pathogens.


**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.