

# *Heritiera actinophyllum* (F.M.Bailey) Kosterm. Leaf Extracts Lack Antibacterial Activity and are non-toxic *in vitro*

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## ABSTRACT

**Introduction:** The development of bacterial strains that are resistant to multiple antibiotics has made the discovery of new antibiotics a priority for medical research. Examination of plants for new antimicrobial agents is an attractive prospect and numerous recent studies have screened plants for antibacterial activity. Despite this, Australian native plants have been relatively neglected. *Heritiera actinophyllum* (F.M.Bailey) Kosterm. is a rainforest tree that is native to eastern coastal regions of Australia. Material from this species has not yet been examined for antibacterial properties against human pathogens. **Materials and Methods:** The ability of *H. actinophyllum* leaf extracts to inhibit the growth of a panel of bacterial pathogens was investigated by disc diffusion assay. Toxicity was examined using the *Artemia franciscana* nauplii bioassay. **Results:** *H. actinophyllum* methanolic and aqueous extracts were ineffective at inhibiting the growth of gram-positive and gram-negative panels of bacteria. The extracts were non-toxic or of low toxicity following 24 h exposure. **Conclusion:** The *H. actinophyllum* leaf extracts lacked growth inhibitory bioactivity against

a panel of pathogenic bacteria and were nontoxic in the *Artemia nauplii* assay. However, these extracts may have other therapeutic properties and testing against protozoa, fungi, and virus and tumour cells is required.

**Key words:** Malvaceae, Black booyong, Blackjack, Blush tulip oak, Traditional medicine, Medicinal plants, Toxicity.

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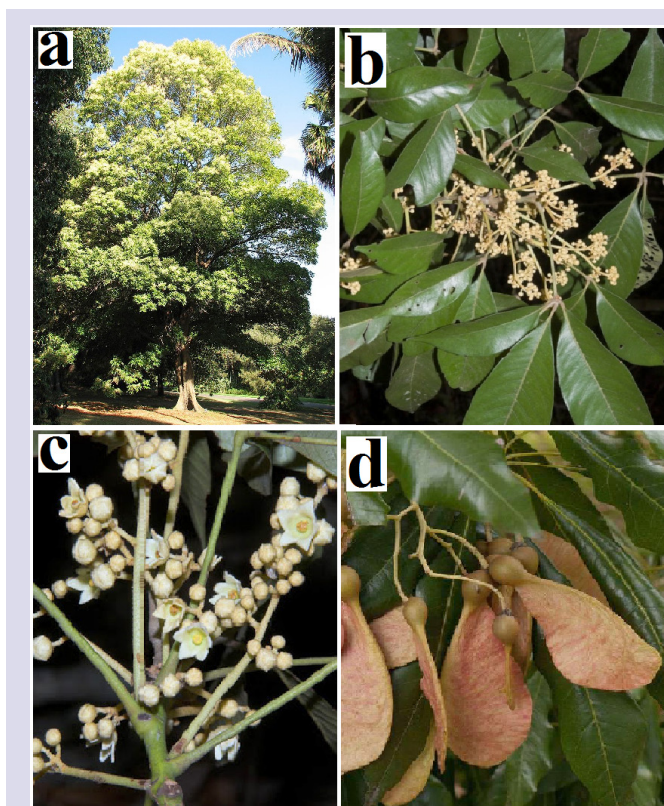
## INTRODUCTION

The use of natural plant therapeutics is as old as human civilisation and in many regions of the world is still the primary modality of health care. Ayurvedic medicine in India for example is still commonly practiced, with approximately 85 % of Indians using crude plant preparations for the treatment of various diseases and ailments.<sup>1</sup> Even in Western civilisations, plants play an important role in medicine. At least 25 % of pharmaceuticals prescribed worldwide are directly obtained from plants and many more drugs are semi-synthetic derivatives of natural plant precursors.<sup>2-4</sup> Examples of medicinally important plant derived compounds include the anti-malarial drug quinine and its derivatives (from *Cinchona* spp.), the antitumour drugs vincristine and vinblastine (from *Catharanthus roseus*) along with the semi-synthetic analogue vindesine, the analgesics morphine and codeine (from *Papaver somniferum*), the anticholinergic drug atropine derived from plants of the family Solanaceae (*Atropa belladonna*, *Datura stramonium* and *Mandragora officinarum*), the anticancer drug taxol (derived from *Taxus brevifolia*) and the cardiac glycoside digoxin (from *Digitalis purpurea*).<sup>5</sup>

Despite the potential of plants to provide us with useful pharmaceutical agents, the field is still 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.<sup>5</sup> 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 closely 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 discovery.<sup>2</sup> Plants species may also be selected for study based on their phytochemical contents, and are often also selected randomly.<sup>6</sup>

In recent years, the development of bacterial pathogens that are either extremely (XDR) or totally drug resistant (TDR) to common clinically used antibiotics<sup>7</sup> has resulted in the need to develop new 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.<sup>8</sup> For a number of reasons reviewed elsewhere,<sup>7</sup> 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.<sup>9-20</sup>

*Heritiera actinophyllum* (F.M.Bailey) Kosterm. (synonyms *Argyrodendron actinophyllum* (F.M.Bailey) Edlin, *Tarrietia actinophyllum* F.M.Bailey; commonly known as black booyong, blackjack, blush tulip oak, crow's foot elm and Mackay tulip oak) is a large rainforest tree that is native to eastern coastal regions of Australia (Figure 1a). *H. actinophyllum* has glossy green lanceolate to oblanceolate leaves (10-18 cm long by 2-5cm wide) (Figure 1b). Small white coloured flowers (Figure 1c) form on panicles and develop into ovoid seed pods (Figure 1d). The seed-bearing portion of the pod is small (5-7mm in diameter) and smooth with a 4-6 cm long wing. We were unable to find records of usage by the first Australians for medicinal purposes. Nor were any studies screening this plant species for any medicinal properties available. Similarly, there is a lack of information on the phytochemical composition of this species. This study was undertaken to screen *H. actinophyllum* leaf extracts for the ability to inhibit the growth of a panel of gram-positive and gram-negative bacterial pathogens.



**Figure 1:** *H. actinophyllum* (a) whole tree, (b) leaves, (c) flowers and (d) seed pods.

## MATERIALS AND METHODS

### Collection of Plant Material and Extraction

*Heritiera actinophyllum* (F.M.Bailey) Kosterm leaves were obtained from and identified by Philip Cameron, senior botanic officer, Mt Cootha Botanical Gardens, Brisbane, Australia. The leaves were washed in deionised water and dried in a Sunbeam food dehydrator dried within 4 hours of collection. The dried leaves were subsequently was ground to a coarse powder using a coffee grinder. Individual 1g masses of the dried plant material was extracted extensively in 50 mL methanol (Ajax, AR grade) or deionised water for 24h 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 (containing 1% DMSO) and passed through 0.22µm filter (Sarstedt) and stored at 4°C.

### Qualitative Phytochemical Studies

Phytochemical analysis of the *H. actinophyllum* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted using standard assays.<sup>21-23</sup>

### Antibacterial Screening

#### Test Micro-organisms

All media was purchased from Oxoid Ltd., Australia. The reference strains of *E. 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 faecalis*, *Bacillus cereus*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Salmonella newport*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus* and *Staphylococcus epidermidis* strains were obtained from Ms Michelle Mendell and Ms Jane Gifkins, Griffith University. All stock cultures were subcultured and maintained in nutrient broth at 4°C.

### Evaluation of Antimicrobial Activity

Antimicrobial activity of the *H. actinophyllum* leaf extracts was determined using a modified disc diffusion assay.<sup>24-27</sup> Briefly, 100µL of the each bacterial suspension in log phase was spread onto individual nutrient agar plates and the extracts were tested for antibacterial 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 2h before incubation at 37°C for 24h. 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) and chloramphenicol (10µg) were obtained from Oxoid, Australia and were used as positive controls to compare antibacterial 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.<sup>28-30</sup> 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, each with internal triplicates (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<sub>50</sub> 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 *H. actinophyllum* leaves with methanol and water yielded 243 and 168mg of extracted material respectively (Table 1). The extracts were resuspended in 10mL of deionised water (containing 1% DMSO), resulting in an extract concentrations shown in Table 1. Qualitative phytochemical screening studies showed that both extracts had similar phytochemical profiles. Both contained high levels of phenolic compounds and flavonoids. Moderate levels of saponins and tannins were also detected in each extract. Lower levels of saponins, triterpenoids and tannins were also detected. Cardiac glycosides, phytosterols, alkaloids and anthraquinones were completely absent or below the detection thresholds for these assays.

### Antibacterial activity

To determine the growth inhibitory activity of the *H. actinophyllum* leaf extracts, aliquots (10µL) of each extract were screened in the disc

diffusion assay. The *H. actinophyllum* leaf 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 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.

### Quantification of Toxicity

The toxicity of the *H. actinophyllum* leaf extracts was initially tested at 2mg/mL in the *A. franciscana* nauplii bioassay (Figure 4). The mortality in the presence of both extracts was not significantly different to that of the untreated control at 24h and thus both extracts were deemed to be non-toxic. Extracts with 24h LC<sub>50</sub> values >1000µg/mL have previously been defined as non-toxic.<sup>28</sup> 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 *H. actinophyllum* 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

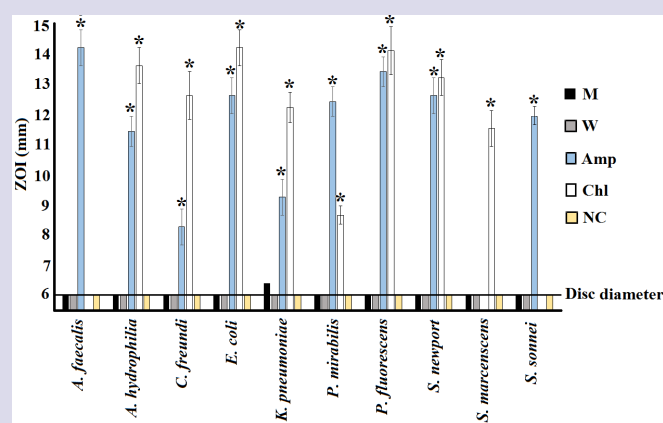
**Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *H. actinophyllum* leaf extracts.**

|                                 |  | Methanolic extract | Aqueous extract |
|---------------------------------|--|--------------------|-----------------|
| Qualitative Phytochemical Tests | Mass of extracted material (mg)              | 243                | 168             |
|                                 | Concentration of resuspended extract (mg/mL) | 24.3               | 16.8            |
|                                 | Total phenols                                | +++                | +++             |
|                                 | Phenols                                      |                    |                 |
|                                 | Water soluble phenols                        | +++                | +++             |
|                                 | Insoluble phenols                            | +++                | ++              |
|                                 | Saponins                                     |                    |                 |
|                                 | Froth persistence                            | +                  | ++              |
|                                 | Emulsion test                                | -                  | +               |
|                                 | Cardiac glycosides                           |                    |                 |
|                                 | Keller-Kiliani Test                          | -                  | -               |
|                                 | Triterpenoids                                |                    |                 |
|                                 | Salkowski Test                               | ++                 | +               |
|                                 | Phytosterols                                 |                    |                 |
|                                 | Acetic Anhydride Test                        | -                  | -               |
|                                 | Meyer's Test                                 | -                  | -               |
|                                 | Alkaloids                                    |                    |                 |
|                                 | Wagner's Test                                | -                  | -               |
|                                 | Draggendorff's Test                          | -                  | -               |
|                                 | Flavonoids                                   |                    |                 |
|                                 | Kumar Test                                   | +++                | +++             |
| Tannins                         | Ferric Chloride Test                         | ++                 | ++              |
|                                 | Lead Acetate Test                            | ++                 | ++              |
|                                 | Free   | -                  | -               |
|                                 | Combined                                     | -                  | -               |
| Anthraquinones                  |  |                    |                 |

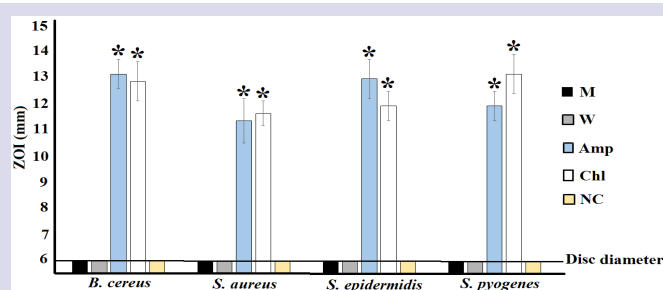
+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

for medical science.<sup>7,8</sup> A concurrent decrease in the discovery of new antibiotic medicines by conventional strategies has increased interest in evaluating plants for new antibiotic chemotherapies.<sup>31</sup> As *H. actinophyllum* has not previously been tested for any therapeutic activity, it was deemed a viable target for antibacterial screening. Interestingly, the *H. actinophyllum* methanolic and aqueous extracts were completely inactive against all gram-positive and gram-negative bacteria tested. However, it is noteworthy that a single assay technique was used to screen for antibacterial activity in this study. We chose to use the disc diffusion assay as it is rapid and it has previously been widely utilised in other studies. Therefore, comparisons between studies are relatively simple.

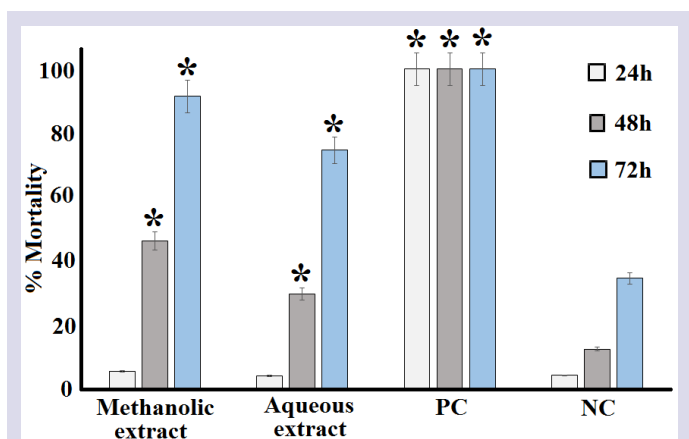
As the disc diffusion method is reliant on the diffusion of a molecule through the aqueous environment of an agar gel, this assay may be affected by the solubility of the extract compounds in the aqueous environment. Polar compounds that are highly soluble in water would be expected to diffuse easily in the gel, whereas less soluble compounds would not diffuse as readily and thus will be concentrated around the disc. For this reason, whilst this is a handy assay for screening aqueous



**Figure 2:** Growth inhibitory activity of *H. actinophyllum* leaf extracts and reference antibiotics against gram-negative bacterial species measured as ZOIs (mm) ± SEM. Ampicillin (Amp) and chloramphenicol (Chl) standard discs (10µg) were used as positive controls. M = methanolic extract; W = aqueous extract; 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. 8 = results that are significantly different to the negative control ( $p<0.05$ )



**Figure 3:** Growth inhibitory activity of *H. actinophyllum* leaf extracts and reference antibiotics against gram-positive bacterial species measured as ZOIs (mm) ± SEM. Ampicillin (Amp) and chloramphenicol (Chl) standard discs (10µg) were used as positive controls. M = methanolic extract; W = aqueous extract; 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. 8 = results that are significantly different to the negative control ( $p<0.05$ )



**Figure 4:** The lethality of the *H. actinophyllum* leaf extracts, potassium dichromate control (1000µg/mL) and seawater (negative control) following 24, 48 and 72 hours of exposure. All bioassays were performed three times in triplicate ( $n=9$ ) and are expressed as mean  $\pm$  SEM. \* indicates results that are significantly different to the untreated (seawater) control at the equivalent exposure time ( $P<0.01$ )

extracts, this technique may not be ideal for non-polar compounds (e.g. when screening essential oils and their components). For examining non-polar mixtures, other techniques such as liquid dilution assays are preferred. Interestingly, the phytochemical screening studies presented herein reports the presence of triterpenoids and saponins (both of which are relatively non-polar) within the methanolic and aqueous extracts. It is therefore possible that these compounds may not contribute significantly to the potential antibacterial activity of these extracts as they remain at or near the discs and are unable to diffuse through the solid agar media. Thus, the growth inhibitory activity of the *H. actinophyllum* extracts may have been significantly underestimated using this assay. Liquid dilution studies may have been better suited to screen the *H. actinophyllum* extracts for activity and future studies will use these techniques to re-examine the extracts for antibacterial activity.

Diffusion of molecules within an agar gel is also affected by the size of the molecules. The movement of large, complex phytochemicals (e.g. 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 distorted view of its inhibitory potential.

The findings reported here also indicate that the extracts examined were non-toxic (24 hour  $LC_{50} > 1000 \mu\text{g/mL}$ ) 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.<sup>28</sup> However, further studies are required to determine whether this is also true for the *H. actinophyllum* leaf extracts examined in these studies.

## CONCLUSION

Methanolic and aqueous *H. actinophyllum* leaf extracts displayed low or no antibacterial activity in the disc diffusion assay against a panel of human pathogenic bacteria. The extracts were non-toxic towards *Artemia nauplii*.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The author Declare no conflict of interest.

## ABBREVIATIONS

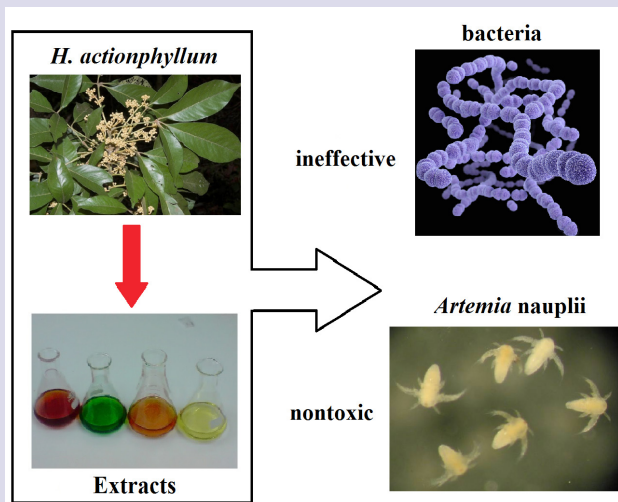
**DMSO:** Dimethyl sulfoxide;  **$LC_{50}$ :** The concentration required to achieve 50 % mortality; **MIC:** Minimum inhibitory concentration; **ZOI:** Zone of inhibition.

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### PICTORIAL ABSTRACT



### SUMMARY

- *H. actinophyllum* leaf extracts were screened for the ability to block the growth of a panel of human bacterial pathogens.
- Low or no inhibitory activity was evident against the bacterial species tested
- Toxicity of the *H. actinophyllum* extracts was determined using the *Artemia nauplii* toxicity bioassay.
- Both the methanolic and aqueous extracts were non-toxic.

### 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.