Mirbelia oxylobioides F. Muell. 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. Mirbelia oxylobioides F. Muell. is a native Australian shrub of the family Fabaceae. Very few studies have yet examined species for antibacterial properties against human pathogens. Methods: The ability of M. oxylobioides 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: M. oxylobioides 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 M. oxylobioides leaf extracts lacked growth inhibitory bioactivity against a panel of pathogenic bacteria and were non-toxic in the Artemia nauplii assay. However, these extracts may have other therapeutic properties and testing against protozoa, fungi, virus and tumour cells is required.

Key words: Fabaceae, Mountain mirbelia, Sandstone bushpea, Australian plants, 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.1 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.2,3 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 anticolinergic 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).4 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 1 or more bioactivities.5 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 or 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.6 Plants species may also be selected for study based on their phytochemical contents, and are often also selected randomly.6

In recent years, the development of bacterial pathogens that are either extremely (XDR) or totally drug resistant (TDR) to common clinically used antibiotics7 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.8 For a number of reasons reviewed elsewhere,9 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.9,10

Mirbelia oxylobioides F.Muell. (commonly known as sandstone bushpea, mountain Mirbelia; Figure 1) is an erect shrub to 1.5m high that grows in dry sclerophyll forests (particularly at high altitudes) on the east coastal regions of southeastern and eastern Australia. It has ovate to elliptical leaves 2-10mm long by 1-4mm wide that are generally opposite or whorled on the stems. M. oxylobioides produces small orange/yellow flowers (up to 10mm) with red markings between October and January. We were unable to find records of usage by the first Australians for medicinal purposes. Similarly, few studies have screened this plant species for therapeutic properties, although some recent studies have screened this species against limited panels of bacteria.11 Similarly, there is a lack of information on the phytochemical composition of this species. This study was undertaken to screen M. oxylobioides leaf extracts for the ability to inhibit the growth of a panel of gram-positive and gram-negative bacterial pathogens of importance to human health.
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The mortality increased, leaf 50% plates. The plates were allowed to stand at 4°C for 2h before incubation individual plant plates and the extracts were tested for antibacterial activity using 6mm bacterial suspension in log phase was spread onto individual nutrient agar using a modified disc diffusion assay.

**Antimicrobial activity of the**

Evaluation of Antimicrobial Activity

**Phytochemical analysis of the**

Qualitative Phytochemical Studies

**Collection of Plant Material and Extraction**

*Mirbelia oxylobioides* F.Muell. leaves were obtained from and identified by Philip Cameron, senior botanic officer, Mt Coota Botanical Gardens, Brisbane, Australia. The leaves were washed in deionised water and dried in a Sunbeam food dehydrator dried within 4 h 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 50mL 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.

**Liquid extraction yields and qualitative phytochemical screening**

Extraction of 1g of dried and powdered *M. oxylobioides* leaves with methanol and water yielded 314 and 306mg 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 triterpenoids were also detected. Cardiac glycosides, phytosterols, alkaloids and anthraquinones were completely absent or below the detection thresholds for these assays.

**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**

**Antibacterial Screening**

**Test Microorganisms**

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, Alkaligenes faecalis, Bacillus cereus, Citrobacter freundii, Pseudomonas fluorescens, Salmonella newport, Serratia marcescens, Shigella sonneti, 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 *M. oxylobioides* leaf extracts was determined using a modified disc diffusion assay. Briefly, 100µL of 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.

**Artemia franciscana Nauplii Toxicity Screening**

Toxicity was tested using an adapted *Artemia franciscana* nauplii lethality assay. 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. LC50 values were calculated for each treatment using probit analysis.

**Antibacterial activity**

To determine the growth inhibitory activity of the *M. oxylobioides* leaf extracts, aliquots (10µL) of each extract were screened in the disc diffusion assay. The *M. oxylobioides* 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 *M. oxylobioides* 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 LC50 values >1000µg/mL have previously been defined as non-toxic. 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...
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**DISCUSSION**

Due to recent increases in bacterial resistance to many antibiotics, the development of new antibiotic chemotherapies is a high priority for medical science. A concurrent decrease in the discovery of new antibiotic medicines by conventional strategies has increased interest in evaluating plants for new antibiotic chemotherapies. As *M. oxylobioides* has not been rigorously tested for any therapeutic activity, it was further increased following 72h exposure.

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

<table>
<thead>
<tr>
<th></th>
<th>Methanolic extract</th>
<th>Aqueous extract</th>
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<tbody>
<tr>
<td>Mass of extracted material (mg)</td>
<td>314</td>
<td>306</td>
</tr>
<tr>
<td>Concentration of resuspended extract (mg/mL)</td>
<td>314</td>
<td>30.6</td>
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<tr>
<td>Total phenols</td>
<td>+++</td>
<td>+++</td>
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<td>Water soluble phenols</td>
<td>+++</td>
<td>+++</td>
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<td>Insoluble phenols</td>
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<td>+</td>
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<tr>
<td>Froth persistence</td>
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<td>Emulsion test</td>
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<td>Keller-Kiliani Test</td>
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<td>Salkowski Test</td>
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<td>Free</td>
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<tr>
<td>Combined</td>
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</tbody>
</table>

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

following exposure to the *M. oxylobioides* leaf extracts at 48h and was further increased following 72h exposure.
deemed a viable target for antibacterial screening. Interestingly, the *M. oxyloboides* 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 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 are preferred. Interestingly, the phytochemical screening studies presented herein reports the presence of saponins (which are relatively nonpolar) within the mentholic 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 *M. oxyloboides* extracts may have been significantly underestimated using this assay. Liquid dilution studies may have been better suited to screen the *M. oxyloboides* 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 hr LC₅₀ >1000μg/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. However, further studies are required to determine whether this is also true for the *M. oxyloboides* leaf extracts examined in these studies.

**CONCLUSION**

Methanolic and aqueous *M. oxyloboides* 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 authors report no conflicts of interest.

**ABBREVIATIONS**

DMSO: Dimethyl sulfoxide; LC₅₀: The concentration required to achieve 50 % mortality; MIC: minimum inhibitory concentration; ZOI: zone of inhibition.

**REFERENCES**


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M. oxyloboides 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 M. oxyloboides extracts was determined using the Artemia nauplii toxicity bioassay. Both the methanolic and aqueous extracts were non-toxic.


