Ficus racemosa L. Leaf Extracts Lack Antibacterial activity and are 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 genus are known to possess therapeutic properties. The genus *Ficus* has a long history of medicinal usage in many areas of the world. Many species are known to have therapeutic properties, several species of which have well established antibacterial bioactivities. **Methods:** The ability of *F. racemosa* 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:** *F. racemosa* methanolic and aqueous extracts were completely ineffective at inhibiting the growth of gram-positive and gram-negative panels of bacteria. The extracts were nontoxic or of low toxicity following 24 h exposure. **Conclusion:** Despite the close taxonomic relationship with several bioactive Ficus spp. and its therapeutic uses in traditional medicine, *F. racemosa* leaf extracts were completely ineffective

bacterial growth inhibitors. However, these extracts may have other therapeutic properties and testing against protozoa, fungi, virus and tumour cells is required.
Key words: *Myrtaceae*, Rose gum, Antibacterial activity, Australian plant, Traditional medicine, Medicinal plants, Toxicity.

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. Ayuverdic 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 with many more drugs being semi-synthetic derivatives of natural plant precursors.[2-4] 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 anticholinogenic drug atropine derived from plants of the family *Solinaceae* (*Atropa belladonna*, *Datura stramonium* and *Mandragora officinarum*), the anticancer drug taxol (derived from *Taxus brevifolia*) and the cardiac glycoside digoxin (from *Digitalis purpurea*).[5]

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 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 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.[2]

In recent years, the development of bacterial pathogens that are either extremely (XDR) or totally drug resistant (TDR) to common clinically used antibiotics[6] 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.[7] For a number of reasons reviewed elsewhere,[6] 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.[8,9]

The genus *Ficus* (family *Moraceae*) consists of approximately 850 species of trees, shrubs, vines and epiphytes. Most species are native to the tropics, although the distribution of some species extends into warm temperate zones. Whilst all species produce edible fruit, only *Ficus carica* L. is widely cultivated for its fruit (commonly known as figs). The fruit of all other species is also eaten as a bushfood and is only of importance to the areas in which they grow naturally. Several *Ficus* spp. also have medicinal uses in the treatment of a wide variety of medical disorders and conditions.[10] *Ficus racemosa* L. (commonly known as cluster fig, gular or Indian fig) is a large tree (Figure 1a) that is native to Australia, Indo-China and the Indian subcontinent. It produces clusters of globular edible fruit (35 x 40mm) that are borne on specialised shoots that protrude directly from the trunk and major branches (Figure 1b). *F. racemosa* has light green ovate leaves (up to 20cm by 9cm) (Figure 1c).

F. racemosa has a history of usage by the first Australians to treat rheumatism, inflammation, sexually transmitted diseases and numerous infections diseases.[11] In traditional Indian medicine, the leaves, bark, roots and sap have similar uses.[10] The phytochemical composition of the bark of this species has been relatively well studied and a number of compounds have been identified including bergenin (Figure 1d),

racemosic acid (Figure 1e), lupeol (Figure 1f), α-amyrin (Figure 1g), β-amyrin (Figure 1h), β-sitosterol (Figure 1i), stigmasterol (Figure 1j), coumarin (Figure 1k), psoralen (Figure 1l), kaempherol (Figure 1m), gallic acid (Figure 1n), ellagic acid (Figure 1o) and rutin (Figure 1p).[10] In comparison, the phytochemistry of the fruit and leaves has been relatively poorly studied. This study was undertaken to screen of *F. racemosa* leaf extracts for the ability to inhibit the growth of a panel of gram-positive and gram-negative bacterial pathogens.

MATERIALS AND METHODS

Plant Material
Collection of Plant Material and Extraction
Ficus racemosa L. leaves were were obtained from and identified by Philip Cameron, senior botanic officer, Mt Cootha Botanical Gardens, Brisbane, Australia. 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 50 ml methanol (Ajax, AR grade) or deionised water for 24 hrs at 4ºC 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 5 ml deionised water. The extract was passed through 0.22 μm filter (Sarstedt) and stored at 4ºC.

Qualitative Phytochemical Studies
Phytochemical analysis of the *F. racemosa* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by standard assays.[12-14]

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, Alcaligenes feacalis, Bacillus cereus, Citrobacter freundii, Pseudomonas fluorescens, 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 stock cultures were subcultured and maintained in nutrient broth at 4ºC.

Evaluation of Antimicrobial Activity
Antimicrobial activity of the *F. racemosa* leaf extracts was determined using a modified disc diffusion assay.[15-17] 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.[18-20] Briefly, *A. franciscana* nauplii were incubated in the pres-

ence of the extracts, reference toxin (1mg/mL potassium dichromate) or artificial seawater (negative control) at 25±1ºC under artificial light. All treatments were performed three times in triplicate (*n*=9). The number of dead were counted in each well at 24h. At the completion of the 24h 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 *F. racemosa* leaves with methanol and water yielded 338 and 250mg of extracted material respectively (Table 1). The extracts were resuspended in 10mL of deionised water (containing 1% DMSO), resulting in an extract concentration shown in Table 1. Qualitative phytochemical studies showed that both extracts had similar phytochemical profiles. Both contained moderate to high levels of phenolic compounds, flavonoids and tannins. Lower levels of saponins, phytosterols and alkaloids were also detected. Cardiac glycosides, triterpenoids and anthraquinones were generally absent or below the detection thresholds for these assays.

Antibacterial activity
To determine the growth inhibitory activity of the *F. racemosa* leaf extracts, aliquots (10μL) of each extract were screened in the disc diffusion assay. The *F. racemosa* leaf extracts were ineffective at inhib-
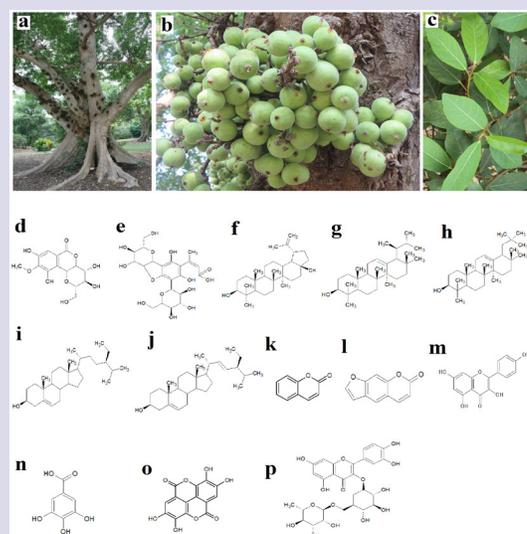

Figure 1: *F. racemosa* (a) tree, (b) fruit, (c) leaves and the structures of some compounds identified in ths species (d) bergenin, (e) racemosic acid, (f) lupeol, (g) α-amyrin, (h) β-amyrin, (i) β-sitosterol, (j) stigmasterol, (k) coumarin, (l) psoralen, (m) kaempherol, (n) gallic acid, (o) ellagic acid and (p) rutin.[10]

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

		Methanolic extract	Aqueous extract
Mass of extracted material (mg)		338	33.8
Concentration of resuspended extract (mg/mL)		250	25
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	-	-

(Left vertical label: Qualitative Phytochemical Tests)

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.
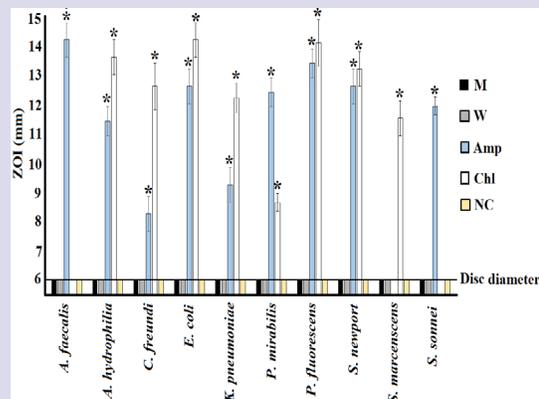
Figure 2: Growth inhibitory activity of *F. racemosa* leaf extracts and reference antibiotics against gram-negative bacterial species measured as ZOIs (mm) ± SEM. Ampicillin (Amp) and chloramphenicol (Chl) standard discs (10μg) were used as positive controls. M = methanolic extract; W = aqueous extract; 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.
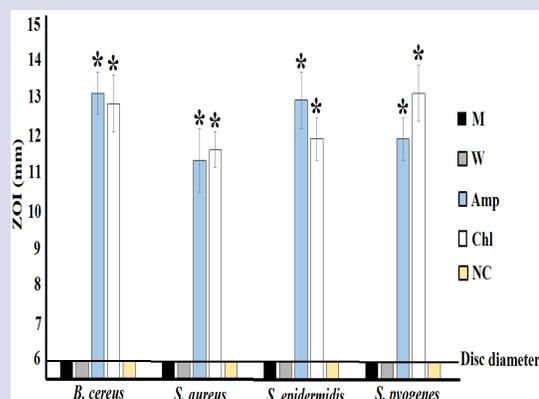

Figure 3: Growth inhibitory activity of *F. racemosa* leaf extracts and reference antibiotics against gram-positive bacterial species measured as ZOIs (mm) ± SEM. Ampicillin (Amp) and chloramphenicol (Chl) standard discs (10μg) were used as positive controls. M = methanolic extract; W = aqueous extract; 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.

iting 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 determe the MIC values for any extract as they were completely ineffective at all concentrations tested.

Quantification of Toxicity

The toxicity of the *F. racemosa* leaf extracts was initially tested at 1mg/mL in the *A. franciscana* nauplii bioassay (Figure 4). All extracts induced 100% mortality at 24h and were therefore deemed to be toxic. Extracts with 24h LC_{50} values >1000μg/mL have previously been defined as non-toxic.[20] The potassium dichromate positive control also induced
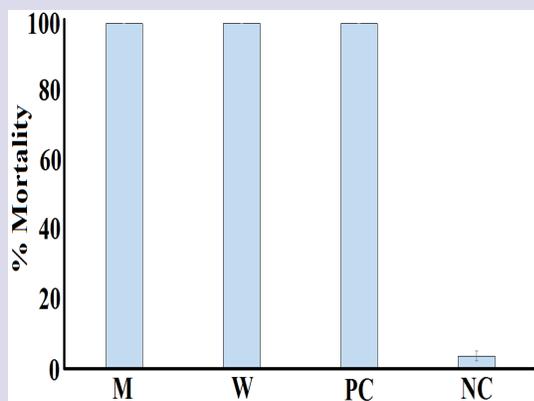
Figure 4: The lethality of the *F. racemosa* leaf extracts (1000μg/mL), potassium dichromate control (1000μg/mL) and seawater (negative control) following 24, hrs of exposure. All bioassays were performed three times in triplicate (*n*=9) and are expressed as mean ± SEM. * indicates results that are significantly different to the untreated (seawater) control at the equivalent exposure time (*P*<0.01).

substantial mortality within 4h (results not shown), with 100% mortality induction seen by 24h, whilst the seawater (negative control) induced <5% mortality at 24 hrs.

DISCUSSION

Due to recent increases in bacterial resistance to many antibiotics, the development of new antibiotic chemotherapies is a high priority for medical science.[6,7] A parallel decrease in the discovery of new antibiotic medicines by conventional strategies has increased interest in re-evaluating medicinal plants for new antibiotic chemotherapies.[21] *F. racemosa* was used by the first Australians to treat a number of diseases,[11] some of which are caused by bacterial pathogens. Similarly, traditional Indian healers also used the bark of this species to treat numerous pathogens.[10] Interestingly, the *F. racemosa* extracts were completely inactive against all gram-positive and gram-negative bacteria tested. 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 a rapid methodology 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. 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 oil and their components). Liquid dilution studies may have been better suited to screen the *F. racemosa* for activity and future studies will use these techniques to re-examine the extracts for antibacterial activity.

Diffusion of molecules within aa 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 distorted view

of its inhibitory potential. The findings reported here indicate that the extracts examined were toxic (24 hr 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.[20] However, further studies are required to determine whether this is also true for the *F. racemosa* leaf extracts examined in these studies.

CONCLUSION

Methanolic and aqueous *F. racemosa* leaf extracts displayed no antibacterial activity in the disc diffusion assay against a panel of human pathogenic bacteria, despite their traditiojnal medicinal uses and their close taxonomic relationship with *Ficus* spp. with known antibacterial properties. The extracts were toxic towards *Artemia* nauplii.

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
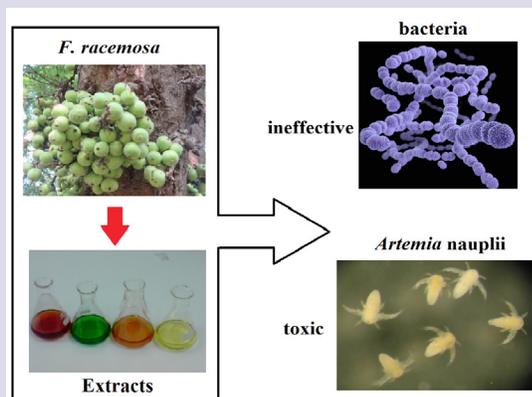
SUMMARY

- *F. racemosa* 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 *F. racemosa* extracts was determined using the Artemia nauplii toxicity bioassay.

- Both the methanolic and aqueous extracts were nontoxic.

ABOUT AUTHORS

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.

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.