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Original Article

Eupomatia laurina R. Br. fruit solvent extractions inhibit the growth of a panel of pathogenic bacteria

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ABSTRACT

Introduction: *Eupomatia laurina* are trees native to Australia and Papua New Guinea. The fruit of this species are consumed as a bushfood. Infusions and decoctions produced from leaves and bark have reputed antiseptic properties and were used traditionally to treat a variety of bacterial diseases. Despite this, *E. laurina* fruit solvent extractions have not been rigorously examined for antibacterial properties against many pathogens. **Methods:** The antimicrobial activity of *E. laurina* fruit solvent extractions was investigated by disc diffusion and growth time course assays against a panel of pathogenic bacteria. The growth inhibitory activity was quantified by MIC determination. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** Methanolic and aqueous *E. laurina* fruit extracts inhibited the growth of a wide range of bacterial species. Growth of both gram positive and gram negative bacteria was inhibited by the *E. laurina* fruit extracts to approximately the same extent. With some noteable exceptions, the methanolic extracts were generally more potent than the aqueous extracts. The methanolic and aqueous *E. laurina* fruit extracts were particularly potent inhibitors of *P. mirabilis* growth, with MIC values as low as 83 µg/mL (aqueous extract against the *P. mirabilis* clinical isolate). The antibacterial activity of the methanolic *E. laurina* fruit extract was further investigated by growth time course assays which showed significant growth inhibition in cultures of *K. pneumoniae* and *P. mirabilis* within 1 h of exposure. All extracts were determined to be nontoxic in the *Artemia franciscana* nauplii bioassay, indicating their safety for internal use as well as for topical uses. **Conclusions:** The lack of toxicity of the *E. laurina* fruit extracts and their growth inhibitory bioactivity against a panel of pathogenic bacteria partially validate the traditional usage of these species to treat bacterial diseases and indicate their potential in the development of antiseptic agents.

Key words: *Eupomatia laurina*, Eupomatiaceae, Native guava, Copper laurel, Australian plants, Antibacterial activity, Medicinal plants.

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INTRODUCTION

Plants produce a wide variety of compounds, which in addition to giving them characteristic pigment, odour and flavour characteristics, may also have antimicrobial properties.[1] For thousands of years, traditional plant derived medicines have been used in most parts of the world and their use in fighting microbial disease is becoming the focus of intense study.[2,3] Whilst much of the research into traditional medicinal plant use has focused on Asian,[4] African[5] and South American[6] plants, the therapeutic potential of the flora of Australia has been recognised for many thousands of years. The first Australians had well developed ethnopharmacological systems and understood the therapeutic properties of a wide variety of aromatic Australian plants.[7] Despite this, relatively few studies have rigorously examined the antibacterial activity of Australian native plants, although recently there has been increased study in this field.

Eupomatia laurina R. Br. (family Eupomatiaceae; commonly known as Bolwarra, native guava, copper laurel) is a small-medium tree which is native to the Australian continent and Papua New Guinea. The trees grow 3-5 m tall and have elliptical, glossy green leaves (Figure 1a) which can be dried and used as an herb and food flavouring agent. White to cream flowers (approximately 20 mm diameter; Figure 1b) develop into globose berries (Figure 1c) which were used by Australian Aborigines as a traditional bushfood. Interestingly, we were unable to find confirmed reports of Aboriginal medicinal use of any part of *E. laurina*. In contrast, Papua New Guinea tribes have been reported to boil the leaves and stem bark and drink the infusion to treat diarrhoea and dysentery.[8] Previous studies have demonstrated the antibacterial activity of the leaves, stem bark[8] and root bark[9] of this species. In contrast, antibacterial studies of *E. laurina* fruit are lacking.

Several interesting phytochemical components have been identified in *E. laurina* bark extracts. In particular, several alkaloid components have been isolated from *E. laurina* root bark and identified as sampangine (Figure 1d), eupomatidine-1 (Figure 1e), liriodenine (Figure 1f) and lanuginosine (Figure 1g).[9] Interestingly, that study also reported broad spectrum antibacterial and anti-protozoal activity for the bark extract and all of the isolated compounds. Indeed, the extract and compounds inhibited the growth of all bacteria and protozoa screened. Broad spectrum antifungal activity was also reported, albeit at doses which would indicate only moderate to low growth inhibitory activity. Despite these promising earlier studies using other parts of this species, examination of the antibacterial properties and phytochemistry of the fruit from *E. laurina* is lacking. The current report was undertaken to screen *E. laurina* fruit for growth inhibitory properties against a panel of pathogenic bacteria.

MATERIALS AND METHODS

Plant collection and extraction

Eupomatia laurina R. Br. Fruit were obtained from and identified by Mr John King of the Queensland Bushfoods Association, Australia. Fruit samples were dried in a Sunbeam food dehydrator and stored at -30°C. Prior to use, the dried fruit was freshly ground to a coarse powder and 1 g quantities were weighed into separate tubes. A volume of 50 mL methanol, sterile deionised water, ethyl acetate, chloroform or hexane was added to individual tubes and extracted for 24 hours at 4°C with gentle shaking. All solvents were obtained from Ajax, Australia and were

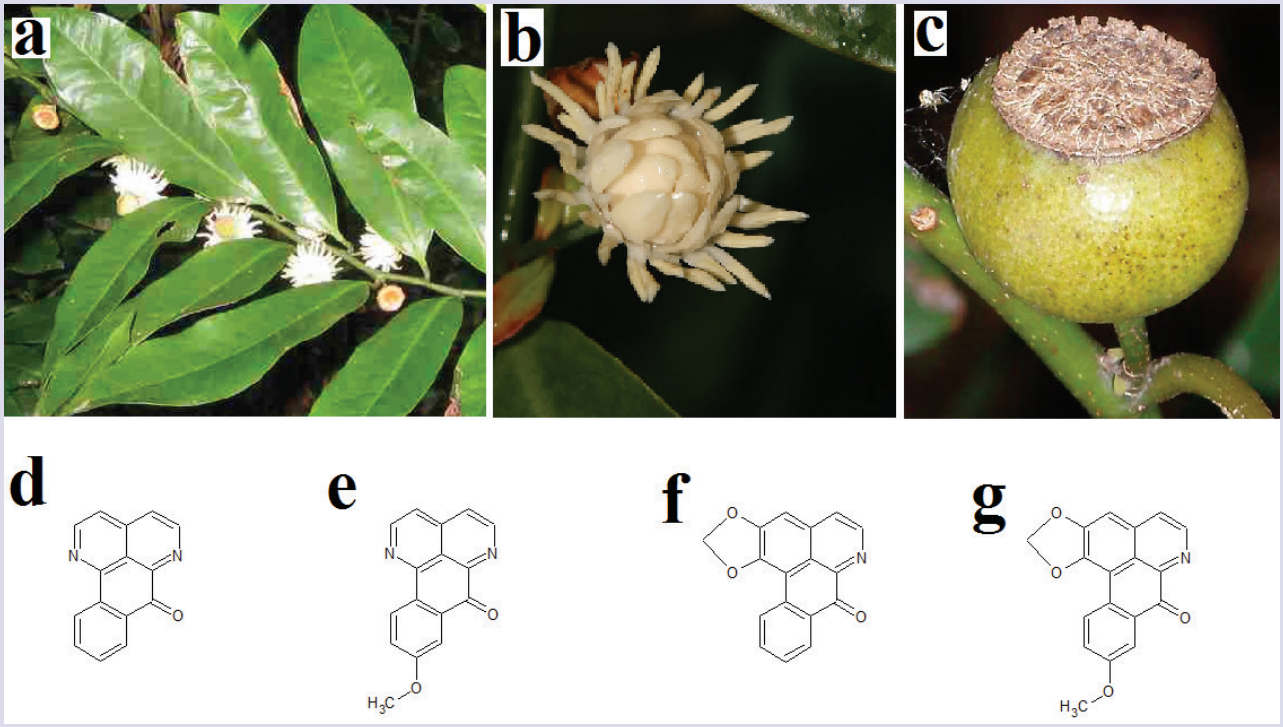

Figure 1: *E. laurina* (a) leaves, (b) flower and (c) fruit, as well as the chemical structures of alkaloid components (d) sampangine, (e) eupomatidine-1, (f) liriodenine, and (g) lanuginosine.

AR grade. The extracts were filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellets were dissolved in 10 mL sterile deionised water (containing 1% DMSO). The extracts were passed through 0.22 µm filter (Sarstedt) and stored at 4°C until use.

Qualitative phytochemical studies

Phytochemical analysis of the *E. laurina* fruit extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[10-12]

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd., Australia. Reference strains of *Klebsiella pneumoniae* (ATCC31488), *Escherichia coli* (ATCC 25922) and *Proteus mirabilis* (ATCC21721) were purchased from American Tissue Culture Collection, USA. Clinical isolate microbial strains of *Aeromonas hydrophilia*, *Alcaligenes feacalis*, *Bacillus cereus*, *Citrobacter freundii*, *E. coli*, *K. pneumoniae*, *P. mirabilis*, *Pseudomonas fluorescens*, *Salmonella newport*, *Serratia marcescens*, *Shigella sonneii*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyonenes* 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 all plant extracts was determined using a modified disc diffusion assay.[13-15] Briefly, 100 µL of each bacterial culture was grown in 10 mL of fresh nutrient broth until they reached a count of ~10^8 cells/mL. A volume of 100 µL of the bacterial suspension was spread onto nutrient agar plates and extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were infused with 10 µL of the plant extracts, allowed to dry and placed onto the inoculated plates. The plates were allowed to stand at 4°C for 2 h before incubation at 30°C for 24 h. The diameters of the inhibition zones were measured to the closest whole millimetre. Each assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (10 µg) were obtained from Oxoid, Australia and were used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of each extract against susceptible bacteria was determined as previously described.[16,17] Briefly, the *E. laurina* extracts were diluted in deionised water and tested across a range of concentrations. Discs were infused with 10 µL of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was completed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to determine the MIC values of each extract.

Bacterial growth time course assay

Bacterial growth time course studies were performed as previously described.[18] Briefly, 3 mL of the susceptible ATCC reference bacterial cultures (*Klebsiella pneumoniae* (ATCC31488) and *Proteus mirabilis* (ATCC21721)) in nutrient broth were added to 27 mL nutrient broth containing 3 mL of 10 mg/mL methanolic plant extract to give a final concentration of 1000 µg/mL in the assay. The tubes were incubated at

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

Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
M	257	25.7	++	++	+	+	++	-	-	+	+	+	+++	++	++
W	242	24.2	+	+	-	++	+	-	-	-	-	++	+++	++	++
E	133	13.3	+	+	-	+	+	-	-	-	-	++	++	-	-
C	269	26.9	-	-	-	-	-	-	-	-	-	-	-	-	-
H	102	10.2	-	-	-	-	-	-	-	-	-	-	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; H = hexane extract.

30°C with gentle shaking. The optical density was measured hourly at 550 nm for a 6 h incubation period. Control tubes were incubated under the same conditions but without the extract. All assays were performed in triplicate.

Toxicity screening

Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 4 mg/mL solution in distilled water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using an adapted *Artemia franciscana* nauplii lethality assay.[19-21] Briefly, 400 µL of seawater containing approximately 48 (mean 47.8, n = 125, SD 10.3) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400 µL of diluted plant extracts or the reference toxin were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A 400 µL seawater negative control was run in triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was detected within 10 seconds. After 24 h, all nauplii were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits for each treatment was determined using probit analysis.

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

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried and powdered *E. laurina* fruit with solvents of varying polarity yielded dried extracts ranging from 102mg (hexane extract) to 269 mg (chloroform extract) (Table 1). The aqueous (242 mg) and methanolic extracts (257 mg) also yielded relatively high levels of extracted materials. The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 1.

Qualitative phytochemical studies showed that the higher polarity methanol and water solvents extracted the greatest diversity and highest levels of phytochemicals. Both contained high levels of tannins as well as moderate levels of phenolics, saponins, flavonoids and anthraquinones. The ethyl acetate extract contained similar phytochemical classes, albeit generally at lower levels. Interestingly, despite extracting relatively large amounts of material, the chloroform and hexane extracts were devoid of all classes of phytochemicals screened. Due to their nonpolar nature, these extracts would be expected to contain high levels of lipids, hydrocarbons etc. As our qualitative phytochemical studies did not screen for these compounds, they were not detected and other techniques are required to further examine the nature of these nonpolar components.

Antimicrobial activity

To determine the growth inhibitory activity of the *E. laurina* fruit extracts against the panel of pathogenic bacteria, aliquots (10 µL) of each extract were screened in the disc diffusion assay. The aqueous and methanolic *E. laurina* fruit extracts inhibited a broad spectrum of the bacterial species screened (Figure 1). The methanolic *B. myrtifolia* extract was a more potent growth inhibitor than the aqueous extract against most bacterial species (as assessed by the sizes of the zones of inhibition). Noteably, the opposite trend was noted for *P. mirabilis* growth inhibition, with the aqueous extracts inhibiting the reference and clinical strains by 11.3 ± 0.6 and 12.0 ± 1.0 mm respectively. This inhibition was particularly noteworthy compared to the inhibition by the ampicillin control (10 µg: inhibition zones of approximately 10 mm). In contrast, the methanolic *E. laurina* fruit extract inhibited *P. mirabilis* growth by 9.3 ± 0.6 mm (reference strain) and 10.0 ± 0 mm (clinical isolate strain). The ethyl acetate and chloroform extracts also inhibited the growth of a range of bacteria (6 (43%) and 5 (36%) of the 14 bacterial species tested respectively), albeit generally with substantially smaller inhibition zones than were recorded for methanolic and aqueous extracts. The hexane extract was generally devoid of growth inhibitory activity.

Both gram positive and gram negative bacteria were approximately equally affected by the *E. laurina* fruit extracts. Of the 10 gram negative bacterial strains tested, 6 (60%) were inhibited by both the methanolic and aqueous *E. laurina* fruit extracts. In contrast, the methanolic and aqueous *E. laurina* fruit extracts each inhibited 3 of the 4 (75%) of the gram positive bacterial species tested. In contrast, gram negative bacteria were significantly more susceptible to the ethyl acetate and chloroform extracts than were the gram positive bacterial species. Indeed, 5 of the 10 gram negative bacteria (50%) and 1 of the 4 gram positive bacteria (25%) were susceptible to the ethyl acetate extract. This trend was even more evident for the chloroform extract, with the growth of 4 of the gram negative bacteria (40%) and none of the gram positive bacteria inhibited.

The antimicrobial efficacy was further quantified by determining the MIC values for each extract against the microbial species which were determined to be susceptible. The methanolic and aqueous *E. laurina* fruit extracts were potent growth inhibitors of several bacterial species (as judged by MIC; Table 2). *P. mirabilis* was the most susceptible bacteria to the *E. laurina* fruit extracts, with MIC values <200 μg/mL (<2 μg infused into the disc) recorded for the aqueous and methanolic extracts against both strains of the bacteria. The ethyl acetate extract was also a moderate *P. mirabilis* growth inhibitor, with MIC values of 1885 (reference *P. mirabilis* strain) and 1603 μg/mL (clinical isolate *P. mirabilis* strain). As *P. mirabilis* infection is a common cause of urinary tract infections and has also been identified as a trigger of rheumatoid arthritis,[22,23] the aqueous and methanolic *E. laurina* fruit extracts have potential for the prevention of these diseases in genetically susceptible individuals.

Furthermore, the aqueous and methanolic extracts were also potent *S. aureus* and *S. epidermidis* growth inhibitors, with MIC values generally in the 500-900 μg/mL range. The ethyl acetate extract was also a potent *A. hydrophilia* growth inhibitor (MIC <700 μg/mL). The aqueous and methanolic *E. laurina* fruit extracts were also moderate inhibitors of *A. hydrophilia*, albeit with MIC values >1000 μg/mL, indicating moderate growth inhibition. Moderate to low growth inhibition (or no inhibition) was noted for all other extract/bacterium combinations.

Bacterial growth time course assay

The antibacterial activity of the *E. laurina* extracts was further investigated in the reference bacterial strains by bacterial growth time course assays in the presence and absence of the extract. Only the effect of the methanolic extract on the bacterial growth time course were evaluated as this extract was generally as potent or more potent than the other *E. laurina* extracts. Furthermore, the time course studies only examined the effect of the extracts on the reference strains which were inhibited in the screening studies (*K. pneumoniae* and *P. mirabilis*). The starting concentration of the extract used in these assays was 1000 μg/mL. The methanolic *E. laurina* extract significantly inhibited *K. pneumoniae* (Figure 3a) and *P. mirabilis* (Figure 3b) growth within 1 h, indicating a rapid antimicrobial action. Whilst *K. pneumoniae* growth was inhibited for at least the first 4 h of the time course, the bacteria were generally able to overcome this inhibition by 6 h, with the recorded turbidity not significantly different to that of the untreated control. This indicates that the growth inhibition of these bacteria was bacteriostatic for the methanolic *E. laurina* extract at the concentrations tested. In contrast, inhibition of *P. mirabilis* by the methanolic *E. laurina* extract was substantially more profound, with growth still significantly inhibited by the end of the 6 h time course study. This may indicate that these extracts have bactericidal activity against *P. mirabilis* at the dose tested. Indeed, the turbidity at 6 h was not greatly increased from the starting turbidity.

Quantification of toxicity

The toxicity of the *E. laurina* extracts was initially tested in the *Artemia franciscana* nauplii bioassay at a concentration of 2000 μg/mL (Figure 4). All extracts induced low levels of mortality at 24 h, similar to the % mortality seen for the seawater control. By 48 h, the aqueous and methanolic extracts has begun to induce mortality significantly higher than that in the untreated control. As only the methanolic extract induced > 50% toxicity at 48 h, all other extracts were deemed to be nontoxic. In contrast, the potassium dichromate positive control induced mortality within 4 h (results not shown), with 100% mortality induction seen by 24 h.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay (Table 3). For comparison, serial dilutions of potassium dichromate were also tested. All extracts were determined to be nontoxic, with LC_{50} values substantially greater than 1000 μg/mL following 24 h exposure. Extracts with an LC_{50} of greater than 1000 μg/mL towards *Artemia* nauplii have previously been defined as being nontoxic.[21]

DISCUSSION

Plant derived remedies are becoming increasingly sought after in the treatment of a myriad of diseases and disorders due both to their perception of greater safety than synthetic drugs, and the failure of current drug regimens to effectively treat many diseases. Our study reports on the growth inhibitory properties of *E. laurina* fruit extracts against a panel of pathogenic bacteria, and on their toxicity. The gram positive and gram negative bacteria tested in this study demonstrated similar susceptibilities towards the *E. laurina* fruit extracts. In contrast, many previous studies with other plant species report a greater susceptibility of many bacterial species towards solvent extracts for South American,[24] African[25] and Australian plant extracts.[26,27]

Our study examined the ability of *E. laurina* fruit to inhibit the growth of a panel of medicinally important bacterial pathogens. The aqueous and methanolic extracts were identified as being particularly potent inhibitors of *P. mirabilis*. MIC values against the clinical *P. mirabilis* isolate strain of 125 and 83 μg/mL (aqueous extract) and 213 and 137 μg/mL (methanolic extract) were determined for the extracts against the reference and clinical strains respectively. As *P. mirabilis* can trigger rheumatoid arthritis in genetically susceptible individuals,[22,23] these extracts have potential for the development of rheumatoid arthritis inhibitory therapies. The methanolic extract also displayed moderate *K. pneumoniae* growth inhibitory properties, with MIC values 1774 and 2018 μg/mL for both the reference and clinical strains respectively. As *K. pneumoniae* can trigger ankylosing spondylitis in genetically susceptible individuals,[28,29] this extract may also be useful in the prevention of ankylosing spondylitis.

The aqueous and methanolic *E. laurina* fruit extracts also were moderate to good inhibitors of several other bacterial pathogens. Both the aqueous and methanolic *E. laurina* extracts were also good inhibitors of *S. aureus* and *S. epidermidis* growth (MICs 500-900 μg/mL). As both of these bacteria are skin disease pathogens, the aqueous and methanolic *E. laurina* fruit extracts also may have applications as topical treatments of these diseases.

Whilst a detailed investigation of the phytochemistry of the *E. laurina* fruit extracts was beyond the scope of our study, qualitative screening studies were used to determine the classes of compounds present. Several commanalities were noted: the most potent aqueous, methanolic and ethyl acetate extracts all contained relatively high levels of tannins and flavonoids. Many studies have reported potent growth inhibitory activities for a number of tannin compounds. Gallotannins have been

Table 2: Minimum bacterial growth inhibitory concentration (µg/mL) of the *E. laurina* extracts.

Bacterial Species	Strain	M	W	E	C	H
A. hydrophilia	Clinical isolate	1331	1580	677	4387	-
A. feacalis	Clinical isolate	-	-	-	-	-
B. cereus	Clinical isolate	3762	>10,000	5855	-	-
C. freundii	Clinical isolate	-	-	-	-	-
E. coli	ATCC:25922	-	-	-	-	-
E. coli	Clinical isolate	-	-	-	-	-
K. pneumoniae	ATCC:31488	1774	6560	>10,000	-	-
K. pneumoniae	Clinical isolate	2018	4839	>10,000	-	-
P. mirabilis	ATCC:21721	213	125	1885	>10,000	-
P. mirabilis	Clinical isolate	137	83	1603	>10,000	-
P. fluorescens	Clinical isolate	-	-	-	-	-
S. newport	Clinical isolate	-	-	-	-	-
S. marcescens	Clinical isolate	2954	3277	-	1405	873
S. sonneii	Clinical isolate	-	-	-	-	-
S. aureus	Clinical isolate	493	873	-	-	-
S. epidermidis	Clinical isolate	845	1455	-	-	-
S. pyonenes	Clinical isolate	-	-	-	-	-

Numbers indicate the mean MIC and LC_{50} values of triplicate determinations. - indicates no inhibition. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract.

Table 3: LC_{50} (95% confidence interval) for *Artemia* nauplii exposed to the *E. laurina* fruit extracts and the reference toxin potassium dichromate.

Extract	LC_{50} (µg/mL)	
	24 h	48 h
Methanol	3766 ± 183	1735 ± 85
Water	4653 ± 172	2480 ± 107
Ethyl acetate	-	-
Chloroform	5583 ± 206	2285 ± 183
Hexane	-	-
Potassium dichromate	88 ± 5	82 ± 4

Results represent the mean ± SEM of triplicate determinations.

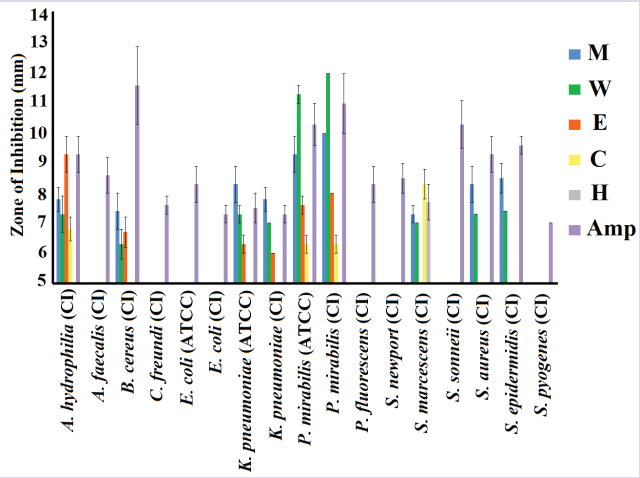

Figure 2: Growth inhibitory activity of *E. laurina* fruit extracts against the clinical isolate (CI) and reference (ATCC) bacterial strains measured as zones of inhibition (mm) ± SEM. Blue bars represent inhibition by the methanolic extract; green bars represent inhibition by the aqueous extract; orange bars represent inhibition by the ethyl acetate extract; yellow bars represent inhibition by the chloroform extract; grey bars represent inhibition by the hexane extract; purple bars represent the inhibition by the ampicillin control. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg) control. All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

reported to inhibit the growth of a broad spectrum of bacterial species[30] through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins,[31,32] and by inhibiting glucosyltransferase enzymes.[33] Elligitannins are also highly potent inhibitors of bacterial growth, with MIC values as low as 62.5 µg/mL.[30,32] Ellagitannins have also been reported to function via several antibiotic mechanisms including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls.[30,32] Thus, it is likely that *E. laurina* fruit tannins may contribute to the inhibition of bacterial growth reported in our study.

It is likely that other phytochemical classes also contribute to the growth inhibitory properties of these extracts. Our qualitative phytochemical screening studies indicate that polyphenolics, flavonoids, saponins and cardiac glycosides were present in the *E. laurina* fruit extracts. Many

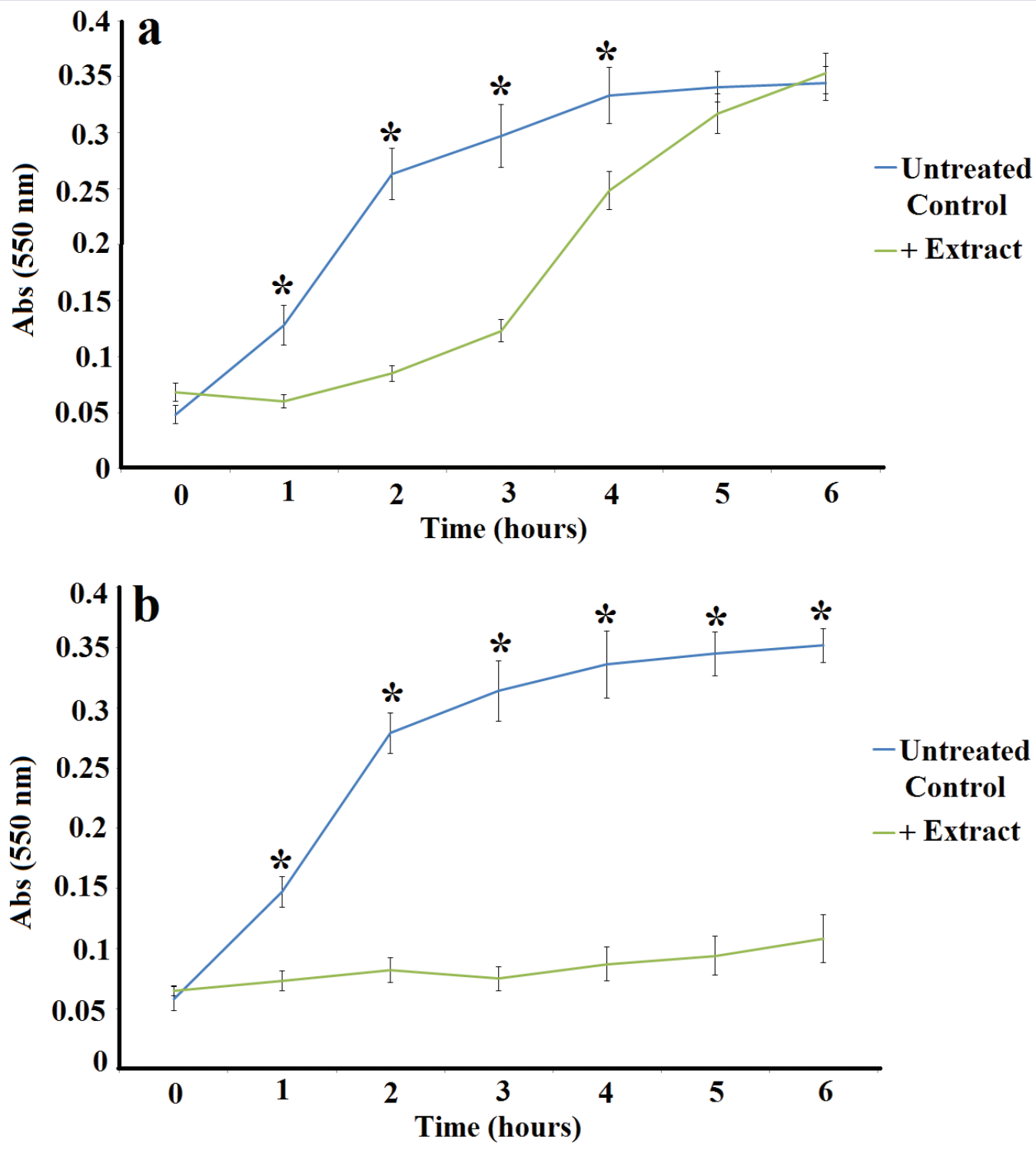

Figure 3: Bacterial growth curves for the methanolic *E. laurina* fruit extract against (a) *K. pneumoniae* (ATCC31488) and (b) *P. mirabilis* (ATCC21721). All bioassays were performed in at least triplicate and are expressed as mean ± SEM. * = results that are significantly different between the treated and the untreated control growth ($p<0.01$).

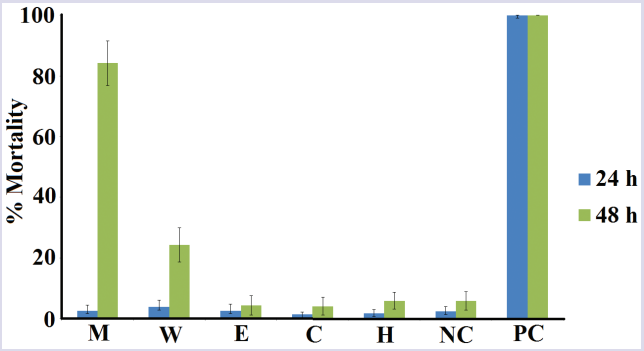

Figure 4: The lethality of the *E. laurina* leaf extracts (2000 µg/mL), potassium dichromate (1000 µg/mL) and a seawater control. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = negative (seawater) control; PC = positive control (1000 µg/mL potassium dichromate). All bioassays were performed in at least triplicate and are expressed as mean ± SEM.

studies have reported potent antibacterial activities for a wide variety of polyphenolic compounds, including many flavonoids.[39] Further phytochemical evaluation studies and bioactivity driven isolation of active components is required to further evaluate the mechanism of bacterial growth inhibition.

The findings reported here also demonstrate that all of the *E. laurina* fruit extracts were nontoxic towards *Artemia franciscana* nauplii, with LC_{50} values substantially > 1000 µg/mL. Extracts with LC_{50} values > 1000 µg/mL towards *Artemia* nauplii are defined as being nontoxic.[21] Whilst our preliminary toxicity studies indicate that these extracts may be safe for therapeutic use, studies using human cell lines are required to further evaluate the safety of these extracts. Furthermore, whilst these studies have demonstrated the potential of the *E. laurina* fruit extracts in the development of future antibiotic chemotherapeutics for the prevention and treatment of urinary tract infections, autoimmune diseases (particularly rheumatoid arthritis and ankylosing spondylitis) and some skin diseases, 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 the *E. laurina* fruit as inhibitors of pathogenic bacteria growth. Furthermore, their lack of toxicity indicates than they are safe for internal as well as topical treatment. Further studies aimed at the purification and identification of bioactive components are needed to examine the mechanisms of action of these agents.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS

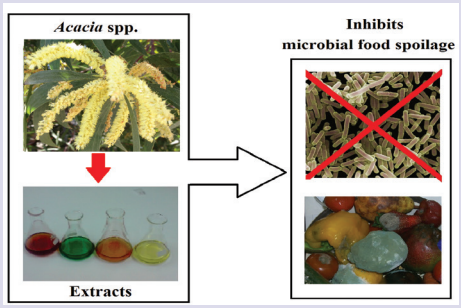
DMSO: Dimethyl sulfoxide; **LC_{50}:** The concentration required to achieve 50% mortality; **MIC:** minimum inhibitory concentration.

REFERENCES

1. Cowan MM. Plant products as antibacterial agents. Clinical Microbiology Reviews. 1999;12:564-82. PMid:10515903 PMCid:PMC88925
2. Bhavnani SM, Ballow CH. New agents for Gram-positive bacteria. Current Opinion in Microbiology. 2000;3:528-534. http://dx.doi.org/10.1016/S1369-5274(00)00134-X
3. Chiariandy CM, Seaforth CE, Phelps RH, Pollard GV, Khambay BP. Screening of medicinal plants from Trinidad and Tobago for antimicrobial and insecticidal properties. Journal of Ethnopharmacology. 1999;64:265-270. http://dx.doi.org/10.1016/S0378-8741(98)00130-5
4. Patwardhan B, Warude D, Pushpangadan P, Bhatt N. Ayurveda and traditional Chinese medicine: a comparative overview. Evidence-based Complimentary and Alternative Medicine. 2005;2:465-73. http://dx.doi.org/10.1093/ecam/neh140 PMid:16322803 PMCid:PMC1297513
5. Hostettmann K, Marston A, Ndjoko K, Wolfender J. The potential of African plants as a source of drugs. Current Organic Chemistry. 2000;4:973-1010. http://dx.doi.org/10.2174/1385272003375923
6. Paz EA, Cerdeiras MP, Fernandez J, Ferreira F, Moyna P, Soubes M, et al. Screening of Uruguayan medicinal plants for antimicrobial activity. Journal of Ethnopharmacology. 1995;45:67-70. http://dx.doi.org/10.1016/0378-8741(94)01192-3
7. Cock IE. Medicinal and aromatic plants – Australia. In Ethnopharmacology, Encyclopedia of Life Support Systems (EOLSS), 2011. Developed under the auspices of UNESCO. Oxford, UK: EOLSS Publishers; 2011. Available from: http://www.eolss.net. Accessed 1 April 2013.
8. Khan MR, Kihara M, Omoloso AD. Antimicrobial activity of *Eupomatia laurina*. Pharmaceutical Biology. 2001;39(4):297-9. http://dx.doi.org/10.1076/phbi.39.4.297.5907
9. Khan MR, Kihara M, Omoloso AD. Antimicrobial activity of the alkaloidal constituents of the root bark of *Eupomatia laurina*. Pharmaceutical Biology. 2003;41(4):277-80. http://dx.doi.org/10.1076/phbi.41.4.277.15671
10. Arkhipov A, Sirdaarta J, Rayan P, et al. An examination of the antibacterial, antifungal, anti-Giardial and anticancer properties of *Kigelia africana* fruit extracts. Pharmacognosy Communications. 2014;4(3):62-76. DOI: 10.5530/pc.2014.3.7 http://dx.doi.org/10.5530/pc.2014.3.7
11. Kalt FR, Cock IE. Gas chromatography-mass spectroscopy analysis of bioactive Petalostigma extracts: Toxicity, antibacterial and antiviral activities. Pharmacognosy Magazine. 2014;10(Suppl 1):S37-49. DOI: 10.4103/0973-1296.127338 http://dx.doi.org/10.4103/0973-1296.127338
12. Vesoul J, Cock IE. The potential of Bunya Nut as an antibacterial food agent. Pharmacognosy Communications. 2012;2(1):72-9. DOI: 10.5530/pc.2012.1.13 http://dx.doi.org/10.5530/pc.2012.1.13
13. Courtney R, Sirdaarta J, Matthews B, et al. Tannin components and inhibitory activity of Kakadu plum leaf extracts against microbial triggers of autoimmune inflammatory diseases. Pharmacognosy Journal. 2015;7(1):18-31. http://dx.doi.org/10.5530/pj.2015.7.2
14. Cock IE. Antimicrobial activity of *Acacia aulacocarpa* and *Acacia complanta* methanolic extracts. Pharmacognosy Communications. 2012;2(1):66-71. DOI: 10.5530/pc.2012.1.12 http://dx.doi.org/10.5530/pc.2012.1.12
15. Wright MH, Matthews B, Arnold MSJ, et al. The prevention of fish spoilage by high antioxidant Australian culinary plants: *Shewanella putrefaciens* growth inhibition. International Journal of Food Science and Technology. 2016; DOI: 10.1111/ijfs.13026 http://dx.doi.org/10.1111/ijfs.13026
16. Sautron C, Cock IE. Antimicrobial activity and toxicity of *Syzygium australe* and *Syzygium leuhmanii* fruit extracts. Pharmacognosy Communications. 2014;4(1):53-60. DOI: 10.5530/pc.2014.1.8 http://dx.doi.org/10.5530/pc.2014.1.8
17. Hart C, Ilanko P, Sirdaarta J, *et al. Tasmannia stipitata* as a functional food/natural preservative: Antimicrobial activity and toxicity. Pharmacognosy Communications. 2014;4(4):33-47. DOI: 10.5530/pc.2014.4.4
18. Cock IE. Antimicrobial activity of *Acacia aulacocarpa* and *Acacia complanta* methanolic extracts. Pharmacognosy Communications. 2012;2(1):66-71. DOI: 10.5530/pc.2012.1.12 http://dx.doi.org/10.5530/pc.2012.1.12
19. Cock IE. Assessment of the toxicity of selected Australian native plant extracts using the *Artemia franciscana* nauplii bioassay. Internet Journal of Toxicology. 2008;5:2.
20. Ruebhart DR, Wikramasinghe WA, Cock IE. Protective efficacy of the antioxidants vitamin E and Trolox against Microcystis aeruginosa and microcystin-LR in *Artemia franciscana* nauplii. Journal of Toxicology and Environmental Health Part A. 2009;72(24):1567-75. http://dx.doi.org/10.1080/15287390903232459; PMid:20077231
21. Cock IE, Ruebhart DR. Comparison of the brine shrimp nauplii bioassay and the ToxScreen-II test for the detection of toxicity associated with Aloe vera (*Aloe barbadensis Miller*) leaf extract. Pharmacognosy Research. 2009;1(2):98-101.

22. Cock IE, van Vuuren SF. Anti-Proteus activity of some South African plants: Their potential for the prevention of rheumatoid arthritis. Inflammopharmacology. 2014;22:23-36. DOI: 10.1007/s10787-013-0179-3 http://dx.doi.org/10.1007/s10787-013-0179-3
23. Ebringer A, Rashid T. Rheumatoid arthritis is an autoimmune disease triggered by Proteus urinary tract infection. Clinical and Developmental Immunology. 2006;13(1):41-8. http://dx.doi.org/10.1080/17402520600576578; PMid:16603443 PMCid:PMC2270745
24. Mohanty S, Cock IE. Evaluation of the antibacterial activity of *Mycaria caulifloria* methanolic leaf and fruit extracts. The Internet Journal of Microbiology. 2009;7(2).
25. Cock IE, van Vuuren SF. South African food and medicinal plant extracts as potential antimicrobial food agents. Journal of Food Science and Technology. 2015;52(11):687999. DOI: 10.1007/s13197-015-1806-3 http://dx.doi.org/10.1007/s13197-015-1806-3
26. Kukkonen L, Cock IE. An examination of the medicinal potential of *Scaevola spinescens*: Toxicity, antibacterial, and antiviral activities. Pharmacognosy Research 2011;3(2):85-94. DOI: 10.4103/0974-8490.81955 http://dx.doi.org/10.4103/0974-8490.81955
27. Vesoul J, Cock IE. An examination of the medicinal potential of *Pittosporum phylliraeoides*: Toxicity, antibacterial, and antifungal activities. Pharmacognosy Communications. 2011;1(2):8-17. DOI: 10.5530/pc.2011.2.3 http://dx.doi.org/10.5530/pc.2011.2.3
28. Cock IE, van Vuuren SF. The potential of selected South African plants with anti-Klebsiella activity for the treatment and prevention of ankylosing spondylitis. Inflammopharmacology 2015;23:21-35. DOI: 10.1007/s10787-014-0222-z http://dx.doi.org/10.1007/s10787-014-0222-z
29. Ebringer A, Rashid T, Wilson C, et al. Ankylosing spondylitis as an auto-immune disease linked to intestinal Klebsiella infection: prospects for a new therapeutic approach. Current Rheumatology Reviews 2006;2:55-68. http://dx.doi.org/10.2174/157339706775697044
30. Buzzini P, Arapitsas P, Goretti M, et al. Antimicrobial activity of hydrolysable tannins. Mini-Reviews in Medicinal Chemistry 2008;8:1179-87. http://dx.doi.org/10.2174/138955708786140990 ; PMid:18855732
31. Wolinsky LE, Sote EO. Isolation of natural plaque-inhibiting substances from 'Nigerian chewing sticks'. Caries Research 1984;18:216-25. http://dx.doi.org/10.1159/000260768
32. Hogg SD, Embery G. Blood-group-reactive glycoprotein from human saliva interacts with lipoteichoic acid on the surface of Streptococcus sanguis cells. Archives in Oral Biology 1982;27:261-8. http://dx.doi.org/10.1016/0003-9969(82)90060-7
33. Wu-Yuan CD, Chen CY, Wu RT. Gallotannins inhibit growth, water-soluble glucan synthesis, and aggregation of Streptococci mutans. Journal of Dental Research 1988;67:51-55. http://dx.doi.org/10.1177/00220345880670011001; PMid:11039045

PICTORIAL ABSTRACT



SUMMARY

- *E. laurina* fruit extracts inhibited the growth of a wide range of bacterial species.
- Growth of both gram positive and gram negative bacteria was inhibited to approximately the same extent.
- *E. laurina* fruit extracts were particularly potent inhibitors of *P. mirabilis* growth.
- All *E. laurina* fruit extracts were nontoxic in the *Artemia franciscana* nauplii bioassay.

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.