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Antimicrobial Activity of Extracts from Native Plants of Temperate Australia

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ABSTRACT

Introduction: Significant effort has been invested in looking at the antimicrobial activity of plant extracts from tropical regions of Australia, with less interest in those from more temperate environments. We sought to redress this imbalance by examining antimicrobial activities of extracts from native plants of Victoria. **Methods:** Sixteen plant samples were obtained around the Ballarat region of Victoria. Plant material was desiccated, ground and extracted with methanol at room temperature. Methanol extracts were subsequently dissolved in water, filtered and freeze dried. Extracts were dissolved in water and their activity determined against eight bacterial species. Plant extracts that showed appreciable antibacterial activity in the initial antimicrobial screen were examined further with both their MICs and MBCs determined. **Results:** Ten of the sixteen plant extracts showed antimicrobial activity. Extracts of *Eucalyptus*, *Melaleuca*, *Prostanthera* and *Westringia* were particularly active with MICs as low as 0.25 mg/ml against organisms including *P. aeruginosa* and

S. aureus. **Conclusion:** The current study demonstrates the antimicrobial activity of plant extracts from temperate Australia. These may serve as precursors for future chemotherapy agents.

Key words: Antibacterial Activity, *Melaleuca*, *Prostanthera*, *Westringia*, *P. aeruginosa*.

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INTRODUCTION

The development of antibiotic resistance in pathogenic bacteria is a major public health concern.¹ The search for alternative antimicrobial compounds is an urgent area of biomedical research and extracts derived from plants have long held interest as potential sources of new therapeutic agents.² Australian plants have been shown to be a promising source of potent antimicrobial agents.³⁻⁷

Aboriginal populations have used Australia's native flora for medicinal purposes for thousands of years. However, written records of medical applications pertaining to plants are few and where they do exist, focus mostly on northern and western indigenous populations,⁸ with less work done on the southern and eastern populations. It is feared that much of this tribal knowledge may have been lost from more temperate parts of Australia.^{9,10} The aim of the current research was to demonstrate whether plant extracts from temperate regions of Australia showed antibacterial activity. The plants selected were:

- *Acacia* species are prevalent throughout Australia and are very important in Aboriginal medicine.¹¹ They have been described as having antiseptic properties⁸ and have been used to treat infected eyes.¹⁰ They are believed to have been important medicinal plants in southern Australia.¹²
- *Eucalyptus* species have been used as antiseptics.⁸ Roots and leaves have been boiled and drunk as a cure for colds and have been documented as being used in western Victoria.¹² Aqueous preparations of *Eucalyptus* kinos have been used to treat wounds and eye infections.¹³
- *Hakea* species have been shown to have antiseptic properties⁸ and *H. eyreana* mixed with animal fat has been used as emollient to treat burns.¹⁴
- *Melaleuca* species produce potent antibacterial essential oils¹⁵ and are used as antiseptics by Aborigines.⁸

- *Prostanthera* species have been documented as being used as antiseptics in northern Australia.⁸
- *Solanum laciniatum* is prolific in the southern temperate regions of Australia and New Zealand. Maori, indigenous people of New Zealand, used the leaf of *S. laciniatum* to form poultices to treat ulcers.¹⁶ It is an important source of solasodine¹⁷ and it is very plausible it would have been investigated for medicinal purposes by Aborigines in this region.

In addition to these plants with known traditional medicinal applications, we also prepared extracts from the following: *Duboisia hopwoodii*, *Hymenosporum flavum*, *Philotheca myoporoides* and *Westringia fruticosa*. With the exception of *Duboisia*, there is very little documented evidence for medicinal application of these plants. They were included due to their widespread distribution and accessibility to the native population. *Duboisia* is well known as an important plant among pre-contact Aborigines, it was chewed to release nicotine.¹⁸ It was included in the study due to its importance to indigenous people, rather than documented antiseptic use.

Characterisation of the antimicrobial activity of some of these plants has been investigated previously.^{15,19} However, the focus tends to be on the essential oils and organic extracts. Essential oils are rich in terpenes, which are relatively insoluble in water. The focus of the current manuscript was to attempt to replicate Aboriginal plant preparations which were typically made by infusion, decoction or maceration: Aborigines did not use distillates or alcoholic extracts.¹⁴ To this effect we used a two stage extraction consisting of a methanol extraction followed by an aqueous extraction allowing recovery of only the aqueous polar components from each plant. Ultimately it is hoped this research initiative will yield new compounds to help combat the rise of antibiotic resistant bacteria.

MATERIALS AND METHODS

Plant collection

Sixteen samples of plant material representing ten Australian plant genera were investigated. In two instances two variants (based on difference in foliage) of the same plant were sampled (*Melaleuca alternifolia* and *Prostanthera ovalifolia*). Sample types included were: leaves (n=12), fruit (n=2), flowers (n=1), and flower buds (n=1). Plant material was collected from the Ballarat region of Victoria, Australia, with the exception of the *Duboisia hopwoodii* sample, which was obtained from Nanya Station in south-west New South Wales.

Plant extraction

Plant material was dried in a food dehydrator and ground to obtain a coarse powder. Five gram portions of dried plant material were exhaustively extracted with 100 mL of analytical methanol (Sigma-Aldrich) using a mechanical shaker for 15 h. The methanolic extract was filtered using a Whatman filter paper (Whatman No. 4) before concentrating it to dryness using a rotary evaporator (water bath temperature 27°C). The dried methanol extract was reconstituted with 20–30 mL of water and filtered using a Whatman filter paper (Whatman No. 4). The resulting aqueous filtrate was frozen at -80°C prior to freeze drying (Christ Alpha 2-4 LD Plus). The freeze dried residue was kept in a desiccator at 0–4°C until required for bioassays.

Microorganisms

Eight microorganisms were assayed to represent both gram positive and negative species: *Bacillus subtilis* (NCTC 10400), *Listeria monocytogenes* (ATCC 7644), *Micrococcus luteus* (University of Melbourne culture collection), *Staphylococcus aureus* (NCTC 6571), *Escherichia coli* (ATCC 11775), *Klebsiella pneumoniae* (HAC8), *Pseudomonas aeruginosa* (NCTC 10662) and *Salmonella enterica* serovar Typhimurium (UQ 723). Routine cultivation of these organisms was performed on nutrient agar incubated at 37°C under ambient oxygen.

Antibacterial assays

Aqueous extracts were tested for antimicrobial efficacy by broth dilution using the EUCAST method.²⁰ Extracts were filter sterilized (0.22 µm) and approximately 4 mg/ml of each extract was used for an initial screen. Dilutions were performed in Mueller-Hinton (Oxoid, Basingstoke, UK) broth and incubated in ambient air at 37°C for 20 h. Antimicrobial activity was assessed by visual examination and semi-quantitated by measuring absorbance at 600 nm relative to a broth culture with no extract. The extracts showing the greatest activity had their MIC/MBC determined using a doubling dilution from 0.12–4 mg/ml, again using the EUCAST method. Bactericidal activity was determined by applying 10 µl of each broth culture to nutrient agar and incubated at 37°C for 48 h.

RESULTS

An initial screen of extract activity was performed on the sixteen aqueous extracts. Assays were performed by broth dilution, rather than plate-hole or disc diffusion methods commonly employed. The rationale for this was to provide a more sensitive assay and one that was aligned with clinical methods of antimicrobial susceptibility testing. The extracts were assayed for activity at 4 mg/ml against eight different bacteria (Table 1). Of the 16 extracts six (38%) showed no appreciable antimicrobial effect, and were not investigated further.

In general, the extracts had greater activity against Gram positive bacteria, particularly *M. luteus* and *S. aureus*, which is consistent with the findings of other researchers.^{3,5} The activity against Gram-negative bacteria was minimal: none of the extracts showed activity against the three Enterobacteriaceae species, while three extracts showed some

activity against *P. aeruginosa*. Interestingly, there was little difference between plant variants of the same species: both *M. alternifolia* variant extracts were active against *M. luteus*, *S. aureus*, and *P. aeruginosa*, while both *P. ovalifolia* variant extracts were active against all the Gram-positive pathogens. The most potent antimicrobial activities generally came from leaf material. A notable exception being *S. laciniatum* where extracts from fruit (particularly ripe fruit) were the most active.

The extracts from four plant species; *Eucalyptus* spp., *M. alternifolia*, *P. ovalifolia* and *W. fruticosa* were particularly potent against certain bacteria and in an attempt to quantify this activity, MIC and MBC assays were performed against a selected group of particularly susceptible bacteria (Table 2). It should be noted that the extraction process employed is not specific and crude plant extracts are generally a mixture of active and non-active compounds. Crude mixtures will have markedly higher MICs than single active compounds and for this reason, an MIC of less than 1 mg/ml is interpreted in this study as showing strong antibacterial potential. The extracts showed remarkably high potency in this assay. An especially striking finding is the activity *M. alternifolia* and *Eucalyptus* spp. extracts against *P. aeruginosa* with MICs of 0.25–0.5 mg/ml.

DISCUSSION

Recent decades have seen an increased interest in examining plants for potential antimicrobial agents²¹ driven by the concomitant rise of antibiotic resistant bacteria. The ten plants examined herein showed a range of antimicrobial activity against the panel of eight bacteria. Unsurprisingly, for most of the plants with limited documented indigenous usage, *in vitro* antimicrobial activity was minimal. Conversely, however, *Westringia fruticosa*, a plant with no described medicinal usage was potent in *in vitro* antimicrobial assays.

The extract of the white sallow wattle (*Acacia floribunda*) was effective against *L. monocytogenes*, but had little activity against other bacteria. This builds on previous studies that found little antimicrobial activity from methanolic extracts of eight *Acacia* species in a disc diffusion assay.²² However, this study was only performed against two bacterial pathogens: *S. aureus* and *S. pyogenes* and therefore overlooked the inhibitory effect against *Listeria*. Conversely, Pennacchio *et al* found methanolic extracts of two different *Acacia* species were active against both *S. aureus* and *S. pyogenes*.⁷

There is little published on the antimicrobial activity of *Solanum laciniatum* extracts. Some anti-fungal activity in extracts of *S. laciniatum* leaves against *C. albicans* has been demonstrated, but no antibacterial activity.²³ This supports our finding—there was little activity in the leaves, but there was activity in the fruit, particularly as they ripened.

Extracts from four plant genera were particularly active in the initial antimicrobial screen: *Eucalyptus* spp., *M. alternifolia*, *P. ovalifolia*, *W. fruticosa*. To quantify the activity of these extracts, MICs and MBCs were determined against selected bacteria. *Eucalyptus* extracts showed potent activity against gram-positive bacteria (although not *B. subtilis*) and also the gram-negative bacteria *P. aeruginosa*. This matches findings of other researchers: aqueous extracts of kinos from 13 *Eucalyptus* species had activity against the gram-positive bacteria *S. aureus* and *B. subtilis*,¹³ whilst aqueous extracts of *E. olida* and *E. staigerana* also showed activity against *S. aureus* (MIC 15.6 µg/ml and 125 µg/ml, respectively) although no activity against six other bacteria.³

The essential oils of *M. alternifolia* have well documented antibacterial activity against a broad spectrum of bacteria^{15,19,24,25} while the activity of aqueous *Melaleuca* extracts are less well documented. The *M. alternifolia* extracts in the current study were notable for their activity against *P. aeruginosa* (MIC 0.25 mg/ml). Previous studies have shown *M. alternifolia* essential oils have MICs of 1–8% (vol/vol) against *P. aeruginosa*.¹⁵

Table 1: Absorbance of bacterial cultures was measured at 600 nm. The level of extract activity was compared to positive (no extract) control: "+++" <10% control absorbance (very inhibitory); "++" 10-49% control absorbance (inhibitory); "+/-" 50-99% control absorbance (neutral); "-" 100% control absorbance (enhanced growth)

Plant	Common name	Sample type	<i>B. subtilis</i>	<i>L. monocytogenes</i>	<i>M. luteus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. Typhimurium</i>
<i>Acacia floribunda</i>	White sallow wattle	Leaf	-	+++	++	+/-	+/-	+/-	+/-	-
<i>Duboisia hopwoodii</i>	Pituri	Leaf	-	-	+/-	+/-	+/-	-	-	-
<i>Eucalyptus</i> spp.	Eucalyptus	Leaf	+/-	++	+++ [#]	+++ [#]	+/-	+/-	++	+/-
<i>Hakea</i> spp.	Hakea	Leaf	-	++	+/-	+++	+/-	+/-	+/-	-
<i>Hymenosporum flavum</i>	Native Frangipani	Flower	-	-	++	+/-	+/-	-	+/-	-
<i>Hymenosporum flavum</i>	Native Frangipani	Leaf	-	-	+/-	+/-	+/-	-	-	-
<i>Melaleuca alternifolia</i> var. 1	Tea tree	Leaf	-	+/-	+++ [#]	+++ [#]	-	-	++	-
<i>Melaleuca alternifolia</i> var. 2	Tea tree	Leaf	+/-	++	++	+++	+/-	+/-	++	+/-
<i>Philotheca myoporoides</i>	Long-leaf waxflower	Leaf	-	-	+/-	+/-	+/-	+/-	-	-
<i>Philotheca myoporoides</i>	Long-leaf waxflower	Flower bud	-	+/-	+/-	+/-	+/-	+/-	-	-
<i>Prostanthera ovalifolia</i> var. 1	Native Mint	Leaf	+++	+++	+++ [#]	+++ [#]	+/-	+/-	+/-	-
<i>Prostanthera ovalifolia</i> var. 2	Native Mint	Leaf	++	+++	+++	+++	+/-	-	+/-	+/-
<i>Solanum laciniatum</i>	Kangaroo apple	Fruit (ripe)	+++	-	+++	+/-	-	-	-	-
<i>Solanum laciniatum</i>	Kangaroo apple	Fruit (unripe)	++	-	++	+/-	+/-	+/-	+/-	+/-
<i>Solanum laciniatum</i>	Kangaroo apple	Leaf	-	-	+/-	+/-	-	-	-	-
<i>Westringia fruticosa</i>	Coast Rosemary	Leaf	+/-	+++	+++ [#]	+++ [#]	+/-	+/-	+/-	-

[#]Bactericidal assay showed complete killing of the bacteria; [#] Bactericidal assay showed >99% reduction of the bacterial numbers.

Table 2: MIC (MBC) determination of selected plant extracts shown in mg/ml

Plant	Sample type	<i>B. subtilis</i>	<i>L. monocytogenes</i>	<i>M. luteus</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
<i>Eucalyptus spp.</i>	Leaf	-	1 (>2)	0.25 (1.0)	0.5 (1.0)	0.5 (>2)
<i>Melaleuca alternifolia var. 1</i>	Leaf	-	-	-	0.25 (1.0)	0.25 (1.0)
<i>Melaleuca alternifolia var. 2</i>	Leaf	-	0.5 (>2)	0.06 (0.5)	0.5 (1.0)	0.25 (1.0)
<i>Prostanthera ovalifolia var. 1</i>	Leaf	0.5 (>2)	0.5 (1.0)	0.25 (1.0)	0.5 (1.0)	-
<i>Prostanthera ovalifolia var. 2</i>	Leaf	1.0 (>2)	1.0 (1.0)	0.5 (2.0)	0.25 (2.0)	-
<i>Westringia fruticosa</i>	Leaf	-	1.0 (>2)	1.0 (2)	0.5 (1.0)	-

Prostanthera species, like many other Australian plants, have been shown to have essential oils with potent antimicrobial activity. Essential oils from the desert species *P. centralis* have been shown to be effective against gram-positive bacteria with MICs against *B. subtilis* and *S. aureus* of approximately 0.1 mg/ml²⁶ only two double-dilutions lower than the activity of the aqueous extracts in the current report (0.5 mg/ml).

There have been few investigation into the antibacterial activity of *Westringia* species. Cinnamate esters of catechol isolated from *W. fruticosa* were antifungal against *Cladosporium*,²⁷ but methanolic extracts of *W. fruticosa* (leaves and flowers) were shown not to exhibit an antibacterial effect on four bacterial species (including *B. subtilis*).²⁸ We also found activity against *B. subtilis* was minimal, but the extract was very active against the three other gram-positive bacteria examined. This is a novel observation: while work has been performed on other rosemary genera²⁹ there is no previous evidence of antibacterial activity from *W. fruticosa* extracts.

Many researchers have investigated the antimicrobial properties of Australian plants, but such studies are often focussed on the essential oils^{15,19} and to a lesser extent methanol extracts.^{30,31} Studies on aqueous extracts have typically shown poor antimicrobial activity compared to other solvents.³ While some Aboriginal preparations are made by mixing plant material with animal fat¹⁴ which could contain non-polar components in essential oils, many Aboriginal plant preparations are aqueous, involving simple infusion, decoction or maceration procedures. The pharmacologically active components in these preparations must therefore be water soluble.¹⁴ It was decided therefore, that aqueous extracts represented the most accurate ethnomedical approach.

The MICs described in the current manuscript are higher than those of their corresponding essential oils.^{15,19,26} However, the aqueous extracts would consist of a mixture of non-active components including carbohydrates, organic acids, proteins and minerals. It is tempting to speculate that active compounds may be present in the extracts bound to carbohydrates in the form of glycosides: highly polar compounds containing one or more sugar units. Bacterial glycosidases may hydrolyse these compounds to yield free sugar and an aglycone component, the latter of which, may have enhanced antimicrobial activity. Extracts of bergamot peel, for example, were showed increased activity after enzymatic deglycosylation to yield the flavenoid agylcones.³²

Combating the global increase in antibiotic resistance will inevitably require the development of new antimicrobial agents. This study highlights the antimicrobial potential of several Australian plants: the traditional medicinal knowledge of Australian indigenous people may provide options for future antimicrobial therapy.

CONCLUSION

In an era of reduced therapeutic options to treat multidrug resistant infections, the current study demonstrates the antimicrobial activity of

plant extracts from temperate Australia. Plants from southern Australia are often overlooked in favour of those from the warmer northern and western parts of the country about which there is more indigenous medicinal knowledge. However, the extracts tested herein showed potent activity against a number of pathogens including *P. aeruginosa*. These extracts may serve as precursors for future chemotherapy agents, either alone or as a combination therapy.

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CONFLICT OF INTEREST

No conflict of interest declared.

ABBREVIATION USED

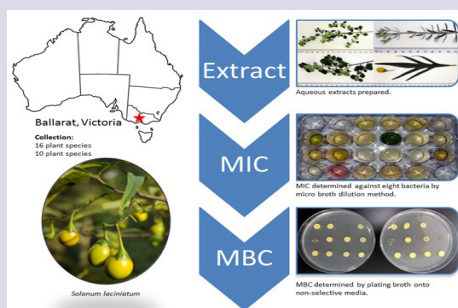
ATCC: American Type Culture Collection; **EUCAST:** European Committee on Antimicrobial Susceptibility Testing; **MIC:** Minimum Inhibitory Concentration; **MBC:** Minimum Bactericidal Concentration; **NCTC:** National Collection of Type Cultures; **UK:** United Kingdom.

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PICTORIAL ABSTRACT



SUMMARY

- Compounds derived from plant sources have great potential for use as antimicrobial agents.
- Ten of sixteen plant extracts showed antimicrobial activity in a broth dilution assay.
- Gram positive bacteria were especially susceptible to the extracts.
- Extracts of *Eucalyptus*, *Melaleuca*, *Prostanthera* and *Westringia* were particularly active with MICs as low as 0.25 mg/ml against organisms including *P. aeruginosa* and *S. aureus*.

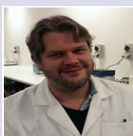
ABOUT AUTHORS



Sarah Wigmore: Sarah completed her Bachelor of Biomedical Science at Federation University Australia in 2014. She has subsequently been involved in researching the antimicrobial activity of Australian plant extracts. Other research interests include the epidemiology of veterinary pathogens in companion animals and antibiotic resistance in bacterial pathogens.



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