

## Research Article

# The Potential of Bunya Nut Extracts as Antibacterial Functional Food Agents

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**ABSTRACT: Introduction:** *A. bidwillii* is an endemic Australian plant traditionally used by Australian Aborigines as a food. Its nuts are known to keep well, raising the possibility that they may contain antimicrobial compounds and therefore may have value as a functional food to retard spoilage and prevent food poisoning, as well as potential medicinal antibiotic uses. **Methods:** *A. bidwillii* solvent extracts were tested for antimicrobial activity against a panel of bacteria (*Aeromonas hydrophila*, *Alcaligenes faecalis*, *Bacillus cereus*, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Salmonella newport*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes*) and toxicity *in vitro*. **Results:** All extracts displayed antibacterial activity in the disc diffusion assay. The methanol extract proved to have the broadest specificity, inhibiting the growth of 8 of the 14 bacteria tested (57.1 %). The water, ethyl acetate, chloroform and hexane extracts inhibited the growth of 5 (35.7 %), 6 (42.9 %), 7 (50.0 %) and 6 (42.9 %) of the 14 bacteria tested respectively. All extracts were more effective at inhibiting the growth of Gram-negative bacteria than Gram-positive bacteria. Indeed, only the methanol extract was capable of inhibiting the growth of any of the Gram-positive bacteria, inhibiting the growth of only 1 (*B. cereus*) of the 4 Gram-positive bacteria tested (25%). All *A. bidwillii* extracts were non-toxic in the *Artemia franciscana* bioassay with no significant increase in mortality above that of the negative control induced by any extract at 24, 48 and 72 h. **Conclusions:** The lack of toxicity of the *A. bidwillii* extracts and their antibacterial bioactivity against a broad range of bacteria demonstrate their potential as food additives to inhibit bacterial spoilage and food borne illnesses without the need for chemical preservative additives. Furthermore, *A. bidwillii* extracts also have promise as antimicrobial agents for medicinal purposes.

**KEYWORDS:** *Araucaria bidwillii*, Bunya nut, Australian plants, functional food, antimicrobial activity

## INTRODUCTION

Antimicrobial food spoilage and microbial induced food poisoning are major concerns to the food production industry. Incidences of food-borne illnesses were estimated at 76 million cases annually in the USA alone in a 1999 study, with at least 5000 deaths annually directly attributed to food poisoning.<sup>[1]</sup> Currently, the major method of controlling food borne microbes and thereby reducing spoilage and food toxin production is by the addition of chemical preservatives during the food production process.

Commonly used chemical food preservatives include butylhydroxyanisol (BHA), butylated hydroxytoluene (BHT), calcium propionate, nitrates, nitrites, sulphur dioxide (SO<sub>2</sub>) and sulfites (SO<sub>3</sub>).<sup>[2]</sup> The effectiveness of these chemical preservatives is dependent on the type of microbial flora and the physical and chemical characteristics of the food.<sup>[2, 3]</sup> Of concern, the safety of many of the chemical preservatives used in food has yet to be determined and in some cases these preservatives have been linked with serious health problems. Studies have indicated that chemical preservatives may cause respiratory problems,<sup>[4]</sup> aggravate attention deficit hyperactivity disorder (ADHD)<sup>[5]</sup> and cause anaphylactic shock in susceptible individuals.<sup>[4]</sup>

Due to greater consumer awareness and the negative perceptions of artificial preservatives, consumers are increasingly avoiding foods containing preservatives of

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chemical origin. Natural antimicrobial alternatives are increasingly being sought to increase the shelf life and safety of processed foods.<sup>[6]</sup> Plant extracts and oils are candidates for antimicrobial agents/bio-preservatives that would be more acceptable to consumers due to their natural origin and consumer perception of safety. In addition, many plants have well established antimicrobial activity.<sup>[7]</sup>

*Araucaria bidwillii* (family Araucariaceae) (commonly known as Bunya Pine) is large woody perennial tree that grows in limited locations in South-east Queensland, Australia, especially in the Bunya Mountains National Park.<sup>[8]</sup> *A. bidwillii* produces its reproductive structures in large cones which reach to 30 cm in length. The large nuts within these cones were highly prized as a food source by Australian Aborigines due to their flavour and nutritional value. Aborigines valued the nuts so much that they travelled long distances to collect them, even suspending hostilities for the duration of the Bunya nut season.<sup>[9]</sup> Nutritionally Bunya nuts are similar to chestnuts, consisting largely of starches and proteins rather than oils. The nuts were traditionally eaten raw or were roasted, but occasionally were buried in the mud for several months to ferment and improve their flavour.<sup>[9]</sup> *A. bidwillii* nuts are known to keep well, raising the possibility that the nuts may contain antimicrobial compounds and therefore may have value to the food industry to retard spoilage and prevent food poisoning.

The current study was undertaken to test *A. bidwillii* nut extracts for the ability to inhibit microbial growth/contamination against a variety of bacteria involved in food spoilage and/or food poisoning. Through examining the antibacterial capability of *A. bidwillii* nut extracts, we aim to assess their potential as additives to foods to retard spoilage and to potentially reduce food poisoning in processed foods.

## MATERIALS AND METHODS

### Plant collection and extraction

Fresh *A. bidwillii* nuts were provided by Aboriginal elder Beverly Hand, member of the Barung Landcare council and manager of Hand E Seeds, Queensland. The nuts were cut up, weighed and dried in a Sunbeam food dehydrator until no further loss of mass was recorded with subsequent drying time. The resultant dried nuts were subsequently coarsely ground and stored at -30 °C until use.

1 g of plant material was weighed into each of five tubes and five different extracts were prepared by adding 50 mL of methanol, water, ethyl acetate, chloroform, or hexane respectively. All solvents were obtained from Ajax Finechem, Australia and were analytical (AR) grade. The ground *A. bidwillii* nut material was extracted in each solvent for 24

hours at 4 °C with gentle shaking. 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 dry extract was weighed and redissolved in 10 mL deionised water.

### Antimicrobial screening

#### Test microorganisms

All bacterial strains were obtained from Michelle Mendell and Tarita Morais, Griffith University, Australia. Stock cultures of *Aeromonas hydrophila*, *Alcaligenes faecalis*, *Bacillus cereus*, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Salmonella newport*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were subcultured and maintained in nutrient broth at 4 °C.

#### Evaluation of antibacterial activity

Antimicrobial activity of all plant extracts was determined using a modified Kirby-Bauer disc diffusion method.<sup>[10]</sup> Briefly, 100 µL of the test bacteria were grown in 10 mL of fresh broth until they reached a count of approximately 10<sup>8</sup> cells/mL (as determined by direct microscopic determination). One hundred microliters of bacterial suspension was spread onto agar plates corresponding to the broth in which they were maintained.

The extracts were tested using 5 mm sterilised filter paper discs. Discs were impregnated with 10 µL of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4 °C for 2 hours before incubation with the test bacterial agents. Plates inoculated with *Alcaligenes faecalis*, *Aeromonas hydrophila*, *Bacillus cereus*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas fluorescens*, and *Serratia marcescens*, were incubated at 30 °C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Escherichia coli*, *Salmonella newport*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were incubated at 37 °C for 24 hours, then the diameters of the inhibition zones were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values are reported in this study. Standard discs of ampicillin (2 µg) and chloramphenicol (10 µg) were obtained from Oxoid Ltd. and served as positive controls for antibacterial activity. Filter discs impregnated with 10 µL of distilled water were used as negative controls.

#### Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the *A. bidwillii* extracts were determined by the disc diffusion method across a range of doses. The plant extracts were diluted in deionised water across a concentration range of 5 mg/mL

to 0.1 mg/mL. Discs were impregnated with 10  $\mu$ L of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values. All  $R^2$  values were  $\geq 0.95$ .

### Toxicity screening by *Artemia franciscana* nauplii bioassay

#### Reference toxins for the *Artemia franciscana* nauplii bioassay

Potassium dichromate ( $K_2Cr_2O_7$ ) (AR grade, Chem-Supply, Australia) was prepared as a 2 mg/mL solution in distilled water and was serially diluted in synthetic seawater for use in the *A. franciscana* nauplii bioassay. Mevinphos (2-methoxycarbonyl-1-methylvinyl dimethyl phosphate) was obtained from Sigma-Aldrich as a mixture of cis (76.6%) and trans (23.0%) isomers and prepared as a 4 mg/mL stock in distilled water. The stock was diluted in artificial seawater for use in the bioassay.

#### *Artemia franciscana* nauplii Bioassay

Toxicity was tested using the *A. franciscana* nauplii lethality assay developed by Meyer et al.<sup>[11]</sup> for the screening of active plant constituents with the following modifications. *A. franciscana* cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Synthetic seawater was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/l distilled water were prepared prior to use. 2 g of *A. franciscana* cysts were incubated in 1 l synthetic seawater under artificial light at 25°C, 2000 Lux with continuous aeration. Hatching commenced within 16-18 h of incubation. Newly hatched *A. franciscana* (nauplii) were used within 10 h of hatching. Nauplii were separated from the shells and remaining cysts and were concentrated to a suitable density by placing an artificial light at one end of their incubation vessel and the nauplii rich water closest to the light was removed for biological assays. 400  $\mu$ L of seawater containing approximately 42 (mean 41.6,  $n = 150$ , SD 17.8) nauplii were added to wells of a 48 well plate and immediately used for bioassay. The plant extracts were diluted to 4 mg/mL in seawater for toxicity testing, resulting in a 2 mg/mL concentration in the bioassay. 400  $\mu$ L of diluted plant extract and the reference toxins were transferred to the wells and incubated at  $25 \pm 1^\circ\text{C}$  under artificial light (1000 Lux). A negative control (400  $\mu$ L seawater) was run in at least 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 observed within 10 sec. After 72 h all nauplii were sacrificed and counted to determine the total number per well. The  $LC_{50}$  with 95% confidence limits for each treatment was calculated using Probit analysis.<sup>[12]</sup>

### Statistical analysis

Data are expressed as the mean  $\pm$  SD of at least three independent experiments. The Paired *T*-Test was used to calculate statistical significance between control and treated groups with a *P* value  $< 0.05$  considered to statistically significant.

## RESULTS

### Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried plant material with the various solvents yielded dried plant extracts ranging from 32.5 mg to 264.8 mg (Table 1). Deionised water and chloroform both gave relatively high yields of dried extracted material (147.7 and 264.8 mg respectively) whilst ethyl acetate and hexane extracted the lowest masses (35.6 and 32.5 mg respectively). The dried extracts were resuspended in 10 mL of deionised water resulting in the extract concentrations shown in Table 1.

### Antibacterial activity

10  $\mu$ L of each extract was tested in the disc diffusion assay against 14 bacteria (Table 2). All extracts displayed broad spectrum antibacterial activity, being capable of inhibiting the growth of between 5 and 8 of the 14 bacteria tested. The methanolic extract displayed the broadest antibiotic specificity, inhibiting the growth of 8 of the 14 bacteria tested (57.1 %). The methanolic extract was particularly potent against *B. cereus* as determined from the zone of inhibition ( $10.2 \pm 0.2$  mm). *A. bidwillii* chloroform extract also displayed good antibacterial specificity, inhibiting the growth of 7 of the 14 bacteria tested (50 %). The ethyl acetate and hexane extracts both inhibited the growth of 6 of the 14 bacteria tested (42.9 %). The water extract had the narrowest antibacterial specificity, inhibiting the growth of 5 of the 14 bacteria tested (35.7 %).

The methanol extract was the only extracts capable of inhibiting the growth of any Gram-positive bacteria, inhibiting 1 (*B. cereus*) of the 4 bacteria tested (25 %). All *A. bidwillii* extracts inhibited the growth of  $\geq 5$  of the 10 Gram-negative bacteria tested ( $\geq 50$  %). The most

**Table 1: The mass of dried material extracted with the various solvents and the concentration after resuspension in deionised water.**

Solvent	Mass (mg)	Concentration (mg/mL)
Methanol	82.1	8.2
Deionised Water	147.7	14.8
Ethyl Acetate	35.6	3.6
Chloroform	264.8	26.5
Hexane	32.5	3.3

**Table 2: Antibacterial activity of *A. bidwillii* solvent extracts measured as zones of inhibition (mm).**

	<i>Methanol extract</i>	<i>Water extract</i>	<i>Ethyl acetate extract</i>	<i>Chloroform extract</i>	<i>Hexane extract</i>	<i>Ampicillin</i>	<i>Chloramphenicol</i>
<b>Gram negative rods</b>							
<i>A. faecalis</i>	-	-	-	-	-	9.8 ± 0.8	6.3 ± 0.6
<i>A. hydrophilia</i>	-	-	-	6.7 ± 0.2	-	9.5 ± 1.4	28.7 ± 1.6
<i>C. freundii</i>	9.3 ± 0.5	8.0 ± 0	9.5 ± 0.2	9.7 ± 0.2	9.2 ± 0.2	9.8 ± 0.8	13.2 ± 1.2
<i>E. coli</i>	7.8 ± 0.2	8.0 ± 0.4	8.3 ± 0.2	9.3 ± 0.5	8.2 ± 0.2	13.7 ± 1.6	20.0 ± 1.8
<i>K.pneumoniae</i>	8.7 ± 0.2	7.8 ± 0.2	7.7 ± 0.2	7.7 ± 0.2	8.5 ± 0.5	9.3 ± 0.6	21.3 ± 1.5
<i>P. mirabilis</i>	7.5 ± 0.5	7.8 ± 0.2	8.2 ± 0.2	8.2 ± 0.2	8.0 ± 0.4	14.6 ± 0.7	8.7 ± 0.6
<i>P. fluorescens</i>	9.0 ± 0.4	-	8.7 ± 0.2	8.5 ± 0.5	7.8 ± 0.2	8.2 ± 0.5	12.2 ± 1.2
<i>S. newport</i>	-	-	-	-	-	9.5 ± 1.4	10.3 ± 1.8
<i>S. marcescens</i>	7.8 ± 0.6	7.3 ± 1.0	6.0 ± 0	8.0 ± 0	6.7 ± 0.2	-	11.7 ± 1.8
<i>S. sonnei</i>	7.8 ± 0.2	-	-	-	-	14.0 ± 0	14.3 ± 0.6
<b>Gram positive rods</b>							
<i>B. cereus</i>	10.2 ± 0.2	-	-	-	-	16.7 ± 2.0	11.3 ± 1.2
<b>Gram positive cocci</b>							
<i>S. aureus</i>	-	-	-	-	-	11.7 ± 2.1	18.6 ± 0.6
<i>S. epidermidis</i>	-	-	-	-	-	26.3 ± 1.5	12.3 ± 0.6
<i>S. pyogenes</i>	-	-	-	-	-	7.0 ± 1.0	10.3 ± 0.7

Numbers indicate the mean diameters (mm) of inhibition of at least triplicate experiments ( $\pm$  standard deviation). - indicates no growth inhibition. Ampicillin (2  $\mu$ g) and Chloramphenicol (10  $\mu$ g) were used as the positive controls. Deionised water was included as a negative control.

susceptible bacteria to the *A. bidwillii* extracts was *C. freundii* (as seen from the zone of inhibition). Indeed, with exception of the water extract, all extracts showed > 9 mm zones of inhibition for *C. freundii* growth. The water extract was the least effective antibacterial agent (as determined by the number of bacteria inhibited), inhibiting the growth of 5 of the 10 Gram-negative bacteria tested (50 %).

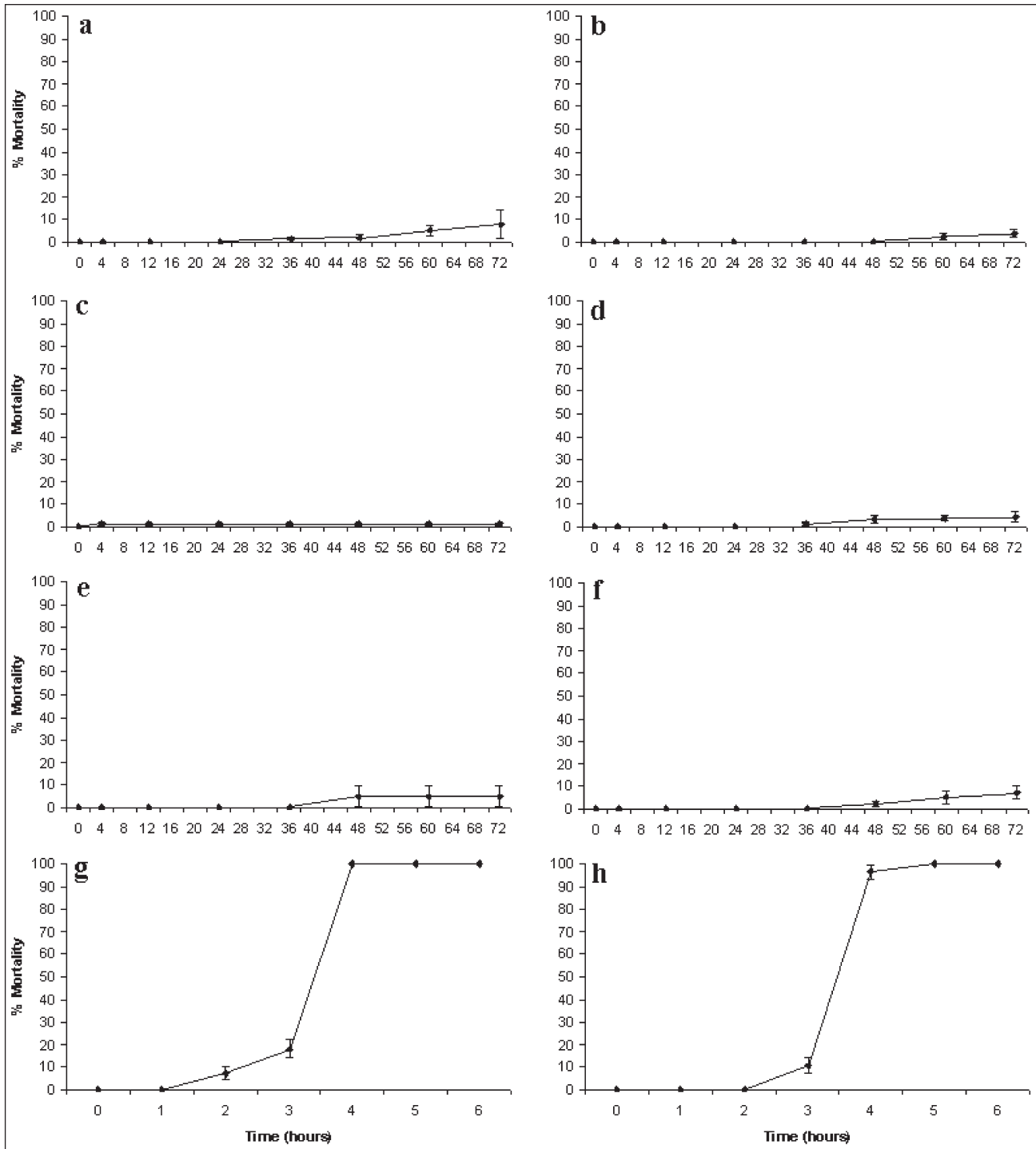
The relative level of antibacterial activity was further evaluated by determining the MIC values for each extract against the bacterial species which were shown to be susceptible by disc diffusion assays. MIC's were evaluated in the current studies by disc diffusion across a range of concentrations. This has previously been determined to be a valid method of MIC determination as MIC values determined by disc diffusion correlate well with those determined by broth dilution assays.<sup>[13]</sup>

The hexane extract proved to be most effective bacterial growth inhibitor at low concentrations, with MIC values

against all 6 bacteria that it inhibited at < 350  $\mu$ g/mL (< 3.5  $\mu$ g impregnated in the disc). The ethyl acetate extract also displayed low MIC's for all bacteria except *S. marcescens*, having MIC values below 500  $\mu$ g/mL (< 5  $\mu$ g impregnated in the disc). Indeed, the lowest MIC of the tested extracts was seen for the ethyl acetate extract against *P. fluorescens* (217.8  $\mu$ g/mL). The methanol extract also displayed low MIC values (< 900  $\mu$ g/mL (< 9  $\mu$ g impregnated in the disc)) for all of the bacteria that it inhibited. In contrast, both the water and chloroform extracts showed higher MIC values (> 1000  $\mu$ g/mL (> 10  $\mu$ g impregnated in the disc)) for all of the bacteria that they inhibited.

### Quantification of toxicity

*A. bidwillii* extracts were diluted to a concentration of 4000  $\mu$ g/mL in artificial seawater for toxicity testing, resulting in 2000  $\mu$ g/mL concentrations in the *Artemia nauplii* lethality bioassay. For comparison, the reference toxins potassium dichromate (1000  $\mu$ g/mL) and Mevinphos (2000  $\mu$ g/mL) were also tested in the bioassay. Figure 1



**Figure 1:** Brine shrimp lethality of (a) *A. bidwillii* methanol extract (2000 µg/mL), (b) *A. bidwillii* water extract (2000 µg/mL), (c) *A. bidwillii* ethyl acetate extract (2000 µg/mL), (d) *A. bidwillii* chloroform extract (2000 µg/mL), (e) *A. bidwillii* hexane extract (2000 µg/mL), (f) artificial seawater negative control, (g) potassium dichromate (1000 µg/mL), (h) Mevinphos (2000 µg/mL). All bioassays were performed in at least triplicate and are expressed as mean ± standard deviation.

shows the % mortality induced by each extract and by the controls at various times. The potassium dichromate (Figure 2G) and Mevinphos (Figure 2H) reference toxins were rapid in their onset of mortality. Both reference toxins induced mortality within the first 3 hours of exposure and 100 % mortality was evident following 4-5 hours. In contrast,

all of the *A. bidwillii* extracts (Figures 2A-E) displayed mortality rates similar to those of the artificial seawater negative control (Figure 2F) at 24, 48 and 72 h. It was not possible to accurately determine an LC<sub>50</sub> for any extract as the mortality never exceeded 50 % for any extract at any time tested.

**Table 3: Minimum inhibitory concentrations ( $\mu\text{g/mL}$ ) of *A. bidwillii* extracts against susceptible bacteria.**

	MIC ( $\mu\text{g/mL}$ )								
	<i>A. hydrophilia</i>	<i>C. freundii</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>P. fluorescens</i>	<i>S. marcescens</i>	<i>S. sonnei</i>	<i>B. cereus</i>
Methanol extract	-	722.2	631.2	715.4	569.4	849.5	863	891.4	863
Water extract	-	1163.1	1092.7	1163.1	1591.4	-	1688.3	-	-
Ethyl acetate extract	-	335.9	297	338	401.1	217.8	645.5	-	-
Chloroform extract	1783.1	2535.4	1818.3	1976.9	2363.9	2388.7	3011.2	-	-
Hexane extract	-	306.6	285.9	255.9	341	271.2	341.6	-	-

Numbers indicate MIC values determined by linear regression from plotting the mean of at least triplicate determinations. - indicates no growth inhibition at any concentration tested.

## DISCUSSION

There is increasing consumer demand to find alternatives for chemical based artificial preservatives as consumers become more aware of the potential for chemical induced health problems. Edible plants could potentially provide a source of inhibitory substances for food borne pathogens and bacteria associated with food spoilage. The current study reports on the antibacterial activities of various *A. bidwillii* extracts and on their toxicity. The ability of *A. bidwillii* extracts to inhibit the growth of Gram-negative, and to a lesser extent, Gram-positive bacteria, is in agreement with previous reports of the antibacterial activity of other Australian native plants that have a history of usage by Australian Aborigines. The antiseptic properties of the *Eucalypts*,<sup>[14-17]</sup> *Leptospermum*,<sup>[18-21]</sup> and *Melaleucas*,<sup>[22, 23]</sup> have been extensively studied and shown to inhibit the growth of a wide variety of both Gram-positive and Gram-negative bacteria.

The current study shows Gram-negative bacteria to be much more susceptible to *A. bidwillii* extracts than Gram-positive bacteria. Indeed, only the methanolic extract was capable of inhibiting any Gram-positive bacteria, albeit inhibiting the growth of only one (*B. cereus*) of the 4 Gram-positive bacteria tested (25%). The greater susceptibility of Gram-negative bacteria observed in this study is in contrast to previous studies which have reported a greater susceptibility of Gram-positive bacteria towards solvent extracts for South American,<sup>[24]</sup> African<sup>[25, 26]</sup> and Australian<sup>[27]</sup> plant extracts. Results within this laboratory<sup>[14]</sup> have also confirmed the greater susceptibility of Gram-positive bacteria towards many other Australian plant extracts, although examples of Australian plants having a greater effect on Gram-negative bacteria have also been reported.<sup>[28, 29]</sup>

The bacteria examined in this study were chosen because they are all important bacteria in food spoilage and/or food

poisoning/intoxication. Staphylococcus spp. (especially *S. aureus*) is one of the most common sources of food borne diseases worldwide.<sup>[2]</sup> *S. pyogenes* contamination (especially of dairy products and salads) can cause pharyngitis as well as gastroenteritis and diarrhoea.<sup>[30]</sup> *B. cereus*,<sup>[31]</sup> *E. coli*,<sup>[32]</sup> *C. freundii*,<sup>[33]</sup> *K. pneumoniae*<sup>[33]</sup> and *S. sonnei*<sup>[34]</sup> all produce toxins and other proteins that induce gastroenteritis and diarrheal diseases. Many of these toxins are heat stable and are not destroyed by heat treatments/pasteurisation. Therefore, control of these bacteria in food is particularly important. Similarly, *P. mirabilis* releases factors that stimulate histamine production resulting in gastrointestinal, neurological (palpitations, headaches, itching), cutaneous (hives, rash) and hypertension symptoms.<sup>[35]</sup> Whilst storage of food at refrigerated temperatures inhibits the growth of many of these pathogenic bacteria, the inclusion of antibacterial food components would further enhance food safety.

Of the pathogenic/toxic bacteria tested in this study, the Staphylococcus spp. and *S. pyogenes* were unaffected by any of the *A. bidwillii* extracts. This is likely related to the fact that these are Gram-positive coccus bacteria. As previously noted, Gram-positive bacteria were less susceptible to the *A. bidwillii* extracts tested in this study. The only susceptible Gram-positive bacteria examined (*B. cereus*) was only susceptible to the methanolic extract. As *B. cereus* is a Gram-positive rod, it would be of interest to test the susceptibility of other Gram-positive rod bacteria in future studies. Of the Gram-negative bacteria tested, only *S. newport* was resistant to all of the *A. bidwillii* extracts. Whether *S. newport* is representative of other Salmonella species in unclear and the testing of further species in this genus is warranted. All other bacteria were inhibited by at least 1 of the extracts. Indeed, *C. freundii*, *E. coli*, *K. pneumoniae*, *P. mirabilis* and *S. sonnei* were inhibited by all of the extracts tested, demonstrating the potential of *A. bidwillii* inclusion in processed foods for controlling food borne diseases.

Also particularly interesting was the ability of the *A. bidwillii* extracts to inhibit the growth of psychrotropic bacteria. Many foods are stored below 5 °C in refrigerators to retard bacterial growth. These foods are expected to have long shelf lives, in some cases up to 50 days or more. Between processing and consumption, foods may become temperature abused to 10 °C or higher allowing psychrotropic bacteria (eg. *A. faecalis*, *A. hydrophilia*, *B. cereus* and *P. fluorescens*) to cause spoilage. Some pathogenic bacteria are also psychrotropic (eg. *B. cereus* and some strains of *C. freundii*, *E. coli* and *K. pneumoniae*).<sup>[31-33]</sup> Therefore, food based antibacterial agents with inhibitory activity against psychrotropic bacteria are especially useful. With the exception of *A. faecalis* which was not inhibited by any extract in this study, all of the psychrotropic bacteria tested were inhibited by at least one *A. bidwillii* extract. Indeed, of the psychrotropic bacteria associated with spoilage, *P. fluorescens* growth was inhibited by all extracts except the water extract. The low observed MIC values of the ethyl acetate and hexane extracts for *P. fluorescens* (217.8, 217.2 µg/mL respectively) indicate that they may be especially useful. Of the bacteria associated with food poisoning, *C. freundii*, *E. coli* and *K. pneumoniae* were susceptible to all of the *A. bidwillii* extracts.

Also noteworthy was the ability of the *A. bidwillii* methanol extract to limit the growth of spore forming bacteria. Heat treatment/pasteurisation is commonly used as a method of destroying food bacteria prior to processing and storage. However, when a bacterium produces heat resistant spores (as *B. cereus* does) heat treatment may kill the bacteria present, only to have further *B. cereus* growth occurring from spores. As *B. cereus* is also psychrotropic, it is especially difficult to control. As the *A. bidwillii* methanolic extract demonstrated good inhibitory activity against *B. cereus* (as seen from the zone of inhibition) its incorporation into prepared/processed foods may be a valuable method of controlling *B. cereus* induced food spoilage and food poisoning. The current study focussed on the effect of *A. bidwillii* extracts on aerobic bacteria. However, the anaerobic spore forming bacteria *Clostridium botulinum* is of greater concern to the food industry due to its degree of incidence and the severity of the symptoms seen with botulism poisoning.<sup>[1]</sup> Future studies into the effects of *A. bidwillii* extracts into anaerobes, including *C. botulinum* are warranted to further evaluate their usefulness as a food preservative.

Individual *A. bidwillii* extract components responsible for the antibacterial potential of the solvent extracts were not identified in the current study. Previous reports have identified various bioactive components of other Australian medicinal plants (*Eucalypts*,<sup>[36]</sup> *Leptospermum*,<sup>[37]</sup> *Melaleucas*<sup>[38]</sup>). These plants all contain terpenes including 1, 8-cineole, terpinen-4-ol,  $\alpha$ -pinene and  $\beta$ -pinene. Both 1, 8-cineole and terpinen-4-ol have antimicrobial activity.<sup>[39, 40]</sup> Recent

studies have also reported on the antibacterial activities of the *Callistemons*<sup>[41]</sup> and *Syzygiums*.<sup>[42-45]</sup> (It has been postulated that terpene components may also be responsible for the antiseptic properties of these plants.<sup>[46]</sup> *A. bidwillii* resins have been previously been shown to contain a number of oxygenated di- and tricyclic diterpenoids including clerodanes),<sup>[47]</sup> whilst the leaves contain sesquiterpenoid enantiomers, diterpenes<sup>[48]</sup> and biflavones.<sup>[49]</sup> The phytochemistry of the *A. bidwillii* extracts investigated in the current study was not examined. Further studies are required to identify which phytochemical(s) is/are responsible for the recorded bioactivities of these extracts.

The findings reported here also demonstrate that none of the *A. bidwillii* extracts displayed significant toxicity towards *Artemia franciscana*. Indeed, none of the extracts tested induced mortality above that of the negative control at the dose tested (2000 µg/mL). Previously, compounds with an LC<sub>50</sub> of greater than 1000 µg/mL towards *Artemia nauplii* have been defined as being non-toxic.<sup>[11]</sup> It was therefore determined that all *A. bidwillii* extracts were non-toxic towards *Artemia nauplii*.

In conclusion, the results of this study demonstrate the antibacterial potential of *A. bidwillii* in food preparation and indicate that *A. bidwillii* nut extracts are worthy of further study. Further evaluation of the antibacterial properties of these extracts against a more extensive panel of microbial agents is warranted. Likewise, purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents. Whilst the extracts examined in this report are promising as antibacterial agents, caution is needed before these compounds can be applied to medicinal purposes. In particular, further toxicity studies using human cell lines are needed to verify the suitability of these extracts for these purposes.

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