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*Original Article*

# Australian *Acacia* spp. extracts as natural food preservatives: Growth inhibition of food spoilage and food poisoning bacteria

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## ABSTRACT

**Introduction:** *A. auriculiformis*, *A. disparrima* and *A. leptoloba* are native Australian *Acacia* spp. which were used as both foods and medicines by the first Australians. 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, Australian *Acacia* spp. solvent extractions have not been rigorously examined for antibacterial properties against food spoilage and food poisoning bacteria. **Methods:** The antimicrobial activity of *A. auriculiformis*, *A. disparrima* and *A. leptoloba* leaf extracts extractions was investigated by disc diffusion and growth time course assays against a panel of food spoilage and food poisoning bacteria. The growth inhibitory activity was quantified by MIC determination. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** *A. auriculiformis*, *A. disparrima* and *A. leptoloba* leaf extracts inhibited the growth of a wide range of bacterial species which cause food spoilage and food poisoning. *A. auriculiformis* extracts were generally more potent growth inhibitors than extracts prepared from the other species, although *A. disparrima* extracts were also potent inhibitors of bacterial growth. With few exceptions, the methanolic extracts were more potent growth inhibitors than the other solvent extractions. The methanolic *A. auriculiformis* leaf extract was a particularly potent inhibitor of *K. pneumoniae* and *P. mirabilis*, *B. cereus* and *S. aureus* growth, with MIC values of 97, 132, 178 and 109 µg/mL respectively. This extract was also a good inhibitor of *A. faecalis*, *A. hydrophilia* and *S. newport* growth (MIC's <1000 µg/mL range). The *A. disparrima* extracts had a similar, albeit slightly less potent activity profiles. In contrast, the *A. leptoloba* leaf extracts were substantially less potent. All extracts were determined to be nontoxic in the *Artemia franciscana* nauplii bioassay, indicating their safety for use as natural food preservatives. **Conclusions:** The lack of toxicity of the *A. auriculiformis*, *A. disparrima* and *A. leptoloba* leaf extracts and their growth inhibitory bioactivity against a panel of food spoilage and food poisoning bacteria indicate their potential in the development of natural food preservatives.

**Key words:** *Acacia auriculiformis*, *Acacia disparrima*, *Acacia leptoloba*, *Fabaceae*, Natural food preservatives, Australian plants, Antibacterial activity, Medicinal plants.

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## INTRODUCTION

Food loss through spoilage is a major global problem. Spoilage can render food unpalatable and/or increase the risks of diseases and food poisoning and can be caused by a variety of physiochemical causes and biological agents. Of perhaps most concern to the food production industry is microbial induced food spoilage and food poisoning. 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.[1] This is an area of concern to the food industry and there is considerable effort to develop improved preservation strategies. Methods aimed at inhibiting microbial growth in food must control initial populations as well as regrowth of post-processing microbial survivors and contaminant induced populations. This may be achieved by several methodologies including alteration of temperature (heating, chilling), pH, water activity (fermentation or dehydration) or oxygen availability (canning, shrink wrap, reduced oxygen packaging, high pressures), irradiation or by chemical preservation.[2]

A major method of controlling food-borne microbes and thereby reducing spoilage and food toxin production is through the use of chemical preservatives. Commonly used chemical food preservatives include butylhydroxyanisol (BHA), butylated hydroxytoluene (BHT), calcium propionate, nitrates, nitrites, sulphur dioxide ($SO_2$) and sulfites ($SO_3$).[2] The effectiveness of these chemical preservatives is dependent on the type of microbial flora and the physical and chemical characteristics of the food.[2-4] However, the safety of many of the chemical food preservatives used in food has yet to be determined and in some cases these preservatives have been linked with serious health problems. Indeed, chemical preservatives may cause respiratory problems,[4] aggravate attention deficit hyperactivity disorder (ADHD)[5] and cause anaphylactic shock in susceptible individuals.[4]

Consumers are increasingly avoiding foods containing synthetic preservatives due to greater consumer awareness and the negative perceptions of artificial preservatives. Instead, natural antimicrobial alternatives are increasingly being sought to increase the shelf life and safety of processed foods.[6] Plant extracts and oils are candidates for antimicrobial agents 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 and several plant species have already been identified for their potential as natural preservatives.[7-12]

The genus *Acacia* (family *Fabaceae*, subfamily *Mimosaceae*) consists of over 1200 species of which more than 700 are indigenous to Australia[13] Other species are spread throughout tropical to warm temperate regions of Africa, India and the Americas. *Acacias* have also been introduced globally for ornamental and economic purposes. Most *Acacia* species produce quality wood and some are also valuable sources of proteins, tannins, gum, perfumes, paint, ink and flavouring agents.[14,15] Furthermore, *Acacia* seed formed an important part of the diet for Australian Aborigines as an easily obtainable, high energy food.[16,17] *Acacia* seed can easily be ground to a flour which is then mixed with water and eaten

either raw or cooked to produce an unleavened bread. Powdered *Acacia* seed flour inhibits the growth of several species of food spoilage bacteria and thus has potential as natural food preservatives.[18] Other parts of some *Acacia* species are also eaten. Several species exude a sugary gum from wounds to the stem and branches[14,17] whilst others are hosts for edible grubs often referred to as witchetty grubs by non-Aboriginal Australians.[19]

Australian *Acacia* species were also used as traditional bush medicines by the first Australians. Several species were used to prepare antimicrobial washes and lotions.[20,21] Unfortunately most of our understanding of the antimicrobial potential of Australian *Acacia* species is anecdotal with few species being rigorously studied. These anecdotal accounts demonstrate that the first Australians knew of the antibacterial properties of the Australian *Acacia* spp. and used them for an array of therapeutic purposes to treat many diseases (Table 1).

Recent studies[20,21] have demonstrated the antibacterial activity of methanolic extracts of several species of Australian *Acacia* against a limited panel of bacteria. However, the therapeutic properties of many other Australian *Acacia* spp. are yet to be investigated. The current study was undertaken to assess the growth inhibitory properties of 3 species of Australian *Acacia* spp. (*Acacia auriculiformis* A.Cunn. Ex Benth., *Acacia disparrima* M.W.McDonald & Maslin and *Acacia leptoloba*) with documented antiseptic uses against of a panel of food spoilage and food poisoning bacteria. Furthermore, the toxicity of the extracts was evaluated to further assess their suitability as natural food preservatives.

## MATERIALS AND METHODS

### Plant collection and extraction

*Acacia auriculiformis* A.Cunn. Ex Benth., *Acacia disparrima* M.W.McDonald & Maslin and *Acacia leptoloba* Pedley leaves were obtained from, and identified by, Philip Cameron, senior botanic officer, Mt Cootha Botanical Gardens, Brisbane, Australia. The leaf samples were dried in a Sunbeam food dehydrator and stored at -30ºC. Prior to use, the dried leaves were 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 hr at 4ºC with gentle shaking. All solvents were obtained from Ajax, Australia and were AR grade. The extracts was 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 4ºC until use.

### Qualitative phytochemical studies

Phytochemical analysis of the *A. auriculiformis*, *A. disparrima* and *A. leptoloba* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[22-24]

### Antibacterial screening

### Test microorganisms

All media was supplied by Oxoid Ltd., Australia. Clinical isolate microbial strains of *Aeromonas hydrophilia*, *Alcaligenes feacalis*, *Bacillus cereus*, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus 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 4ºC.

### Evaluation of antimicrobial activity

Antimicrobial activity of all *Acacia* leaf extracts was determined using a modified disc diffusion assay.[25-27] 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.[28,29] Briefly, the *A. auriculiformis*, *A. disparrima* and *A. leptoloba* leaf 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.[30] Briefly, 3 mL of the *K. pneumoniae* and *P. mirabilis* cultures 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 30ºC 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.[31-33] Briefly, 400 µL of seawater containing approximately 52 (mean 52.4, n=75, SD 15.2) *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 ± 1ºC 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

**Table 1:** The ethnobotanical usage, synonyms and common names of the *Acacia* species tested in this study.

| Plant Species | Synonym | Common Name | Part Used Medicinally | Part Used in This Study | Medicinal Use | References |
|---|---|---|---|---|---|---|
| *Acacia auriculiformis* A.Cunn. Ex Benth. | *Acacia moniliformis* Griseb., *Racosperma auriculiforme* (Benth.) Pedley | earleaf acacia, earpod wattle, northern black wattle. Papuan wattle, tan wattle | The leaves and and seed pods are used as extracts, decoctions or infusions. | leaves | A decoction of the leaves was applied to cuts and wounds. A leaf decoction and a lather prepared from crushed ripe seed pods was used to treat itchy skin and several skin disorders and rashes. An infusion prepared from leaves and seed pods was used as an analgesic | 20, 21 |
| *Acacia disparrima* M.W.McDonald & Maslin | *Acacia aulacocarpa* Morrison & Davies, *Racosperma disparrimum* (M.W.McDonald & Maslin) Pedley | Hickory wattle, Southern Salwood | The leaves, seed pods and bark are used as extracts, decoctions or infusions. | leaves | A decoction of the leaves was applied to cuts and wounds. A leaf decoction and a lather prepared from crushed ripe seed pods was used to treat itchy skin and several skin disorders and rashes. An infusion prepared from leaves and seed pods was used as an analgesic | 20, 21 |
| *Acacia leptoloba* Pedley | *Racosperma leptolobum* Pedley | Irvinebank wattle | leaves | leaves | Seed pods are mashed and a decoction is prepared. This is applied to infected eyes. The decoction is also used to treat skin disorders and as a wound antiseptic. | 20, 21 |

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 *A. auriculiformis*, *A. disparrima* and *A. leptoloba* leaves with solvents of varying polarity yielded dried extracts ranging from 25 mg (*A. auriculiformis* hexane extract) to 150 mg (aqueous *A. leptoloba* extract) (Table 2). The methanolic and aqueous extracts of all *Acacia* spp. produced high yields (generally >100 mg). Cloroform also extracted relatively high masses of extracted material (80-100 mg). In contrast, ethyl acetate and hexane extracted only low masses for all *Acacia* spp. (25-35 mg). The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 2.

Qualitative phytochemical studies showed that the higher polarity methanol and water solvents generally extracted the greatest diversity and highest levels of phytochemicals. These extracts of all *Acacia* spp. contained high levels of polyphenolics (particularly water soluble phenolics), saponins, flavonoids and tannins. They also contained low levels of phytosterols and alkaloids. All ethyl acetate extracts contained similar phytochemical classes, albeit generally at lower levels. Interestingly, despite extracting relatively large amounts of material, the chloroform and hexane extracts were generally 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 *A. auriculiformis*, *A. disparrima* and *A. leptoloba* leaf extracts against the panel of pathogenic bacteria, aliquots (10 µL) of each extract were screened in the disc diffusion assay. The methanolic, aqueous and ethyl acetate extracts of *A. auriculiformis* inhibited a broad spectrum of gram negative (Figure 1a) and gram positive bacterial species (Figure 1b). Indeed, the methanolic, aqueous and ethyl acetate extracts inhibited 9 (90%), 8 (80%) and 8 (80%) of the gram negative bacteria screened. Furthermore, the methanolic, aqueous and ethyl acetate extracts all inhibited 100 % of the gram positive bacteria tested. The methanolic extract was the most potent growth inhibitor (as assessed by the sizes of the zones of inhibition). It was a particularly potent inhibitor of *K. pneumoniae*, *P. mirabilis* and *S. aureus* growth, with zones of inhibition of 10.6 ± 0.6 mm, 9.6 ± 0.3 mm and 12.5 ± 1.0 mm respectively. This inhibition was particularly noteworthy compared to the inhibition by the ampicillin control (10 µg: inhibition zones of approximately 8 mm for each of these bacteria). The chloroform extracts also inhibited the growth of a range of bacteria (4 gram negative bacteria (40%) and 2 gram positive bacteria (50%), albeit generally with substantially smaller inhibition zones than were recorded for methanolic, aqueous and ethyl acetate extracts. The hexane extract was devoid of growth inhibitory activity.

A similar trend was noted for the *A. disparrima* leaf extracts, although less bacterial species were inhibited (Figure 2a and 2b). The methanolic and aqueous extracts were again the most potent growth inhibitors. However, the *A. disparrima* leaf extracts were substantially less potent growth inhibitors than the corresponding *A. auriculiformis* extracts, as judged by the zones of inhibition. The *A. leptoloba* leaf extracts had even lower potency (Figure 3a and 3b). Indeed, the methanolic *A. leptoloba* leaf extract inhibited only 6 of the 14 bacterial species screened (43%), and generally with only small zones of inhibition (<7 mm).

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, aqueous and ethyl acetate *A. auriculiformis* and *A. disparrima* leaf extracts were potent growth inhibitors of several bacterial species (as judged by MIC; Table 3). *K. pneumoniae* and *P. mirabilis, B. cereus* and *S. aureus* were the most susceptible bacteria to the *A. auriculiformis* and *A. disparrima* leaf extracts, with MIC values generally <500 µg/mL (<5 µg infused into the disc) recorded for the aqueous and methanolic extracts against these bacteria. The potency of the methanolic *A. auriculiformis* extract was

**Table 2:** The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the methanolic *A. auriculiformis*, *A. disparrima* and *A. leptoloba* extracts.

| Plant Species | Extract | Mass of Dried Extract (mg) | Resuspended Extract (mg/mL) | Phenols | | | Cardiac Glycosides | Saponins | | Triterpenes | Phytosteroids | Alkaloids | | | | Flavanoids | | Tannins | | Anthraquinones | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Total Phenolics | Water Soluble | Water Insoluble | Keller-Kiliani Test | Froth Persistence | Emulsion test | Salkowski Test | Acetic Anhydride Test | Meyers Test | Wagners Test | Draggendoffs Test | Shinoda Test | Kumar test | Ferric Chloride Test | Lead Acetate Test | Free | Combined |
| *A. auriculiformis* | M | 97 | 9.7 | +++ | +++ | ++ | - | +++ | + | - | + | + | + | + | +++ | ++ | +++ | ++ | - | - |
| | W | 112 | 11.2 | +++ | +++ | + | - | +++ | ++ | - | + | ++ | + | + | ++ | + | +++ | + | - | - |
| | E | 28 | 2.8 | ++ | ++ | - | - | - | - | - | - | - | - | - | + | + | + | + | - | - |
| | C | 87 | 8.7 | + | + | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| | H | 25 | 2.5 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| *A. disparrima* | M | 117 | 11.7 | +++ | +++ | ++ | - | + | + | + | + | - | - | - | +++ | ++ | +++ | +++ | - | - |
| | W | 147 | 14.7 | +++ | +++ | + | - | + | + | - | + | + | - | - | ++ | + | +++ | ++ | - | - |
| | E | 33 | 3.3 | + | + | - | - | - | - | - | - | - | - | - | + | + | ++ | + | - | - |
| | C | 83 | 8.3 | + | + | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | H | 30 | 3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *A. leptoloba* | M | 130 | 13 | +++ | +++ | + | - | ++ | + | - | + | + | - | - | +++ | ++ | +++ | ++ | - | - |
| | W | 150 | 15 | +++ | +++ | + | - | ++ | + | - | + | + | - | - | +++ | + | +++ | ++ | - | - |
| | E | 35 | 3.5 | ++ | + | + | - | - | - | - | - | - | - | - | ++ | + | + | + | - | - |
| | C | 103 | 10.3 | + | + | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | H | 35 | 3.5 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |

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

**Table 3:** Minimum bacterial growth inhibitory concentration (μg/mL) of the *A. auriculformis*, *A. disparrima* and *A. leptoloba* extracts.

| Bacteria | *A. auriculiformis* | | | | | *A. disparrima* | | | | | *A. leptoloba* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | W | E | C | H | M | W | E | C | H | M | W | E | C | H |
| **Gram negative bacteria** | | | | | | | | | | | | | | | |
| *A. faecalis* | 523 | 627 | 880 | 1282 | - | 799 | 922 | 1200 | - | - | - | - | - | - | - |
| *A. hydrophilia* | 771 | 920 | 875 | 1693 | - | 1275 | 1186 | 1573 | - | - | 1086 | 1459 | 1472 | - | - |
| *C. freundi* | 895 | 1018 | 1147 | - | - | - | - | - | - | - | - | - | - | - | - |
| *E. coli* | 1266 | 1472 | 1386 | - | - | 2287 | 2639 | - | - | - | - | - | - | - | - |
| *K.pneumoniae* | 97 | 154 | 227 | 1263 | - | 783 | 1017 | 993 | - | - | 1324 | 2488 | 1653 | - | - |
| *P. mirabilis* | 132 | 188 | 203 | 697 | - | 965 | 1342 | 1728 | - | - | 1880 | 3879 | 2564 | - | - |
| *P. fluroscens* | 803 | 1220 | 976 | - | - | - | - | - | - | - | - | - | - | - | - |
| *S. newport* | 528 | 575 | 820 | - | - | 911 | 1235 | 1194 | - | - | 2237 | - | - | - | - |
| *S. marcenscens* | 2405 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *S. sonnei* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Gram positive bacteria** | | | | | | | | | | | | | | | |
| *B. cereus* | 178 | 226 | 495 | 1147 | - | 226 | 419 | 283 | 1427 | - | 311 | 723 | - | - | - |
| *S. aureus* | 109 | 240 | 408 | 992 | - | 476 | 589 | 770 | 2318 | - | 825 | 1425 | - | - | |
| *S. epidermidis* | 1140 | 1825 | 2207 | - | - | 2682 | 2404 | 1665 | - | - | - | - | - | - | - |
| *S. pyogenes* | 2085 | 2865 | 3244 | - | - | 3380 | 3674 | - | - | - | - | - | - | - | - |

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.

particularly noteworthy (MICs of 97, 132, 178 and 109 μg/mL against each of these bacteria respectively). The chloroform *A. auriculiformis* and *A. disparrima* leaf extracts were also moderate inhibitors of these bacteria, although the MIC values recorded were generally >1000 μg/mL (>10 μg infused into the disc).

Furthermore, the methanolic, aqueous and ethyl acetate extracts were also good *A. faecalis, A. hydrophilia* and *S. newport* growth inhibitors, with MIC values generally <1000 μg/mL range. The chloroform *A. auriculiformis* and *A. disparrima* leaf extracts were generally only moderate growth inhibitors of most bacteria (MIC >100 μg/mL), although the chloroform *A. auriculiformis* extract was a potent inhibitor of *P. mirabilis* (MIC 697 μg/mL). 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 methanolic *A. auriculformis* and *A. disparrima* extracts was further investigated against the most susceptible bacterial species (*K. pneumoniae* and *P. mirabilis*) by bacterial growth time course assays in the presence and absence of the extract. The growth inhibitory properties of the methanolic *A. leptoloba* extract were not further evaluated due to it substantially lower potency (as assessed by MIC determination). Furthermore, only the effect of the methanolic extracts was evaluated as these extracts were the most potent bacterial growth inhibitors. The starting concentration of the extract used in these assays was 1000 μg/mL. The methanolic *A. auriculformis* and *A. disparrima* extracts both significantly inhibited *K. pneumoniae* (Figure 4a) and *P. mirabilis* (Figure 4b) growth within 1 h, indicating a rapid anti-microbial action. The growth of both *K. pneumoniae* and *P. mirabilis* were inhibited for at least the first 5 h of the time course. However, both bacteria were generally able to overcome the inhibition by *A. disparrima* within 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 *A. disparrima* extract at the concentrations tested. In contrast, inhibition of by the methanolic *A. auriculformis* extract was substantially more profound, with growth still significantly inhibited by the end of the 6 h time course study. This may indicate that the methanolic *A. auriculformis* extract may have bactericidal activity 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 *A. auriculformis*, *A. disparrima* and *A. leptoloba* leaf extracts were initially tested in the *Artemia franciscana* nauplii bioassay at a concentration of 2000 μg/mL (Figure 5). All extracts induced low levels of mortality at 24 h, similar to the % mortality seen for the seawater control. By 48 h, the mortality induced by the aqueous and methanolic extracts had increased although it was still not significantly higher than that in the untreated control. As all of the *A. auriculformis*, *A. disparrima* and *A. leptoloba* leaf extracts induced <50 % toxicity at 48 h, all were deemed to be nontoxic. Extracts with an $LC_{50}$ of greater than 1000 μg/mL towards *Artemia* nauplii following 24 h exposure have previously been defined as being nontoxic.[33] In contrast, the potassium dichromate positive control induced mortality within 4 h (results not shown), with 100 % mortality induction seen by 24 h.

# DISCUSSION

There is increasing consumer demand to find alternatives for chemical based artificial preservatives as consumers become more aware of the

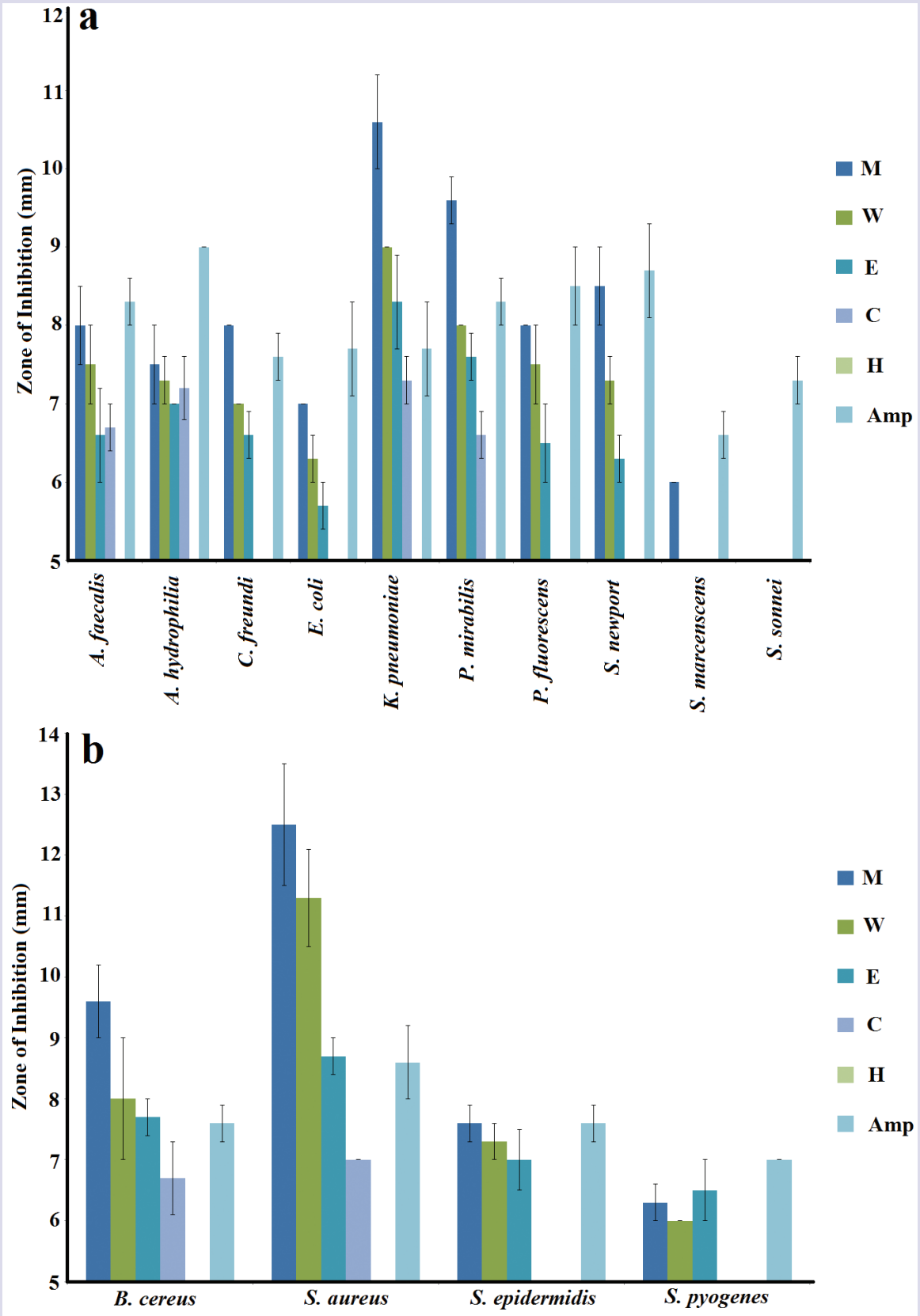


**Figure 1:** Growth inhibitory activity of the *A. auriculiformis* leaf extracts against (a) gram negative and (b) gram positive bacterial species and an ampicillin (10 µg) control. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg) control. All determinations were performed in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

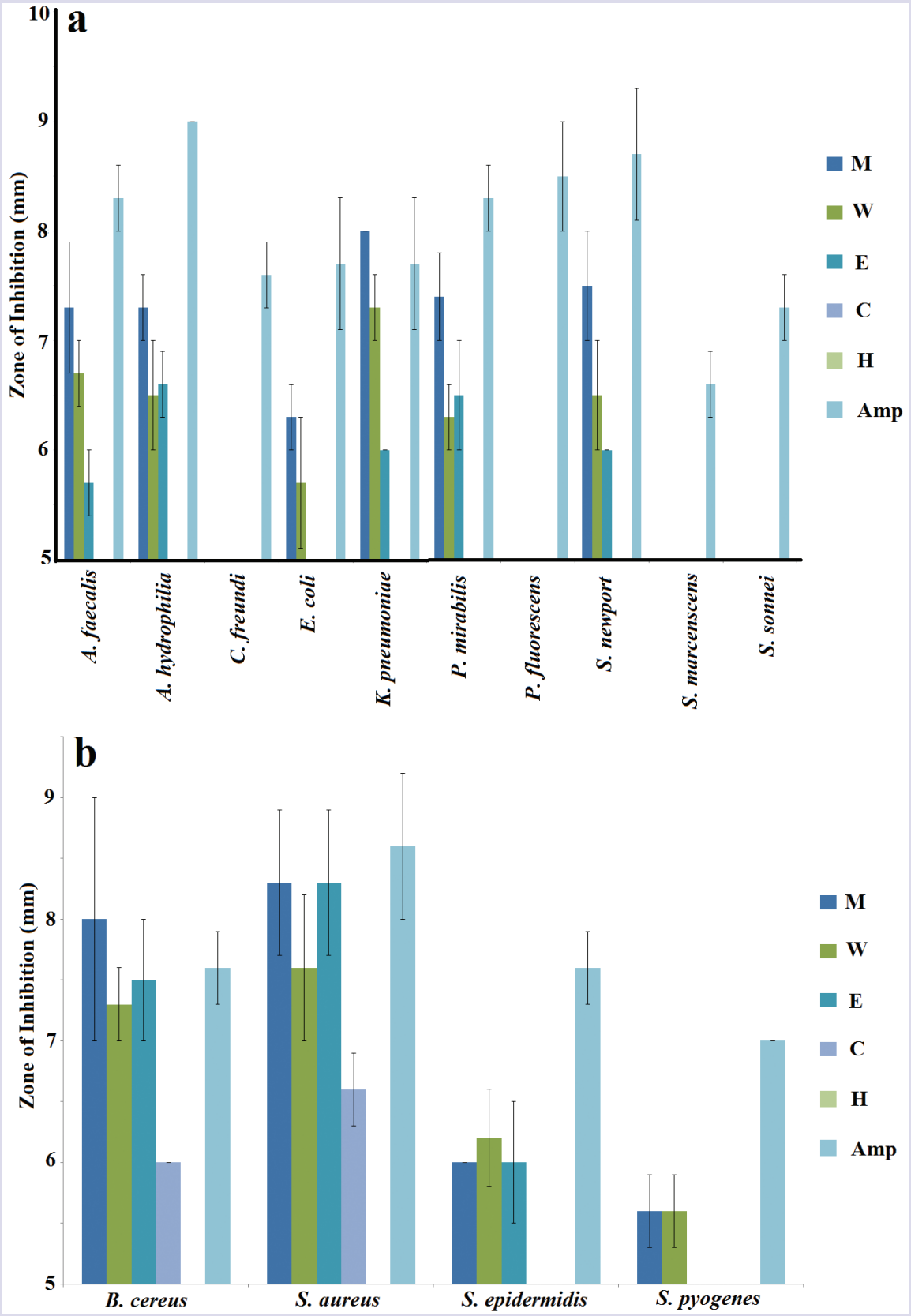


**Figure 2:** Growth inhibitory activity of the *A. disparrima* leaf extracts against (a) gram negative and (b) gram positive bacterial species and an ampicillin (10 µg) control. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg) control. All determinations were performed in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

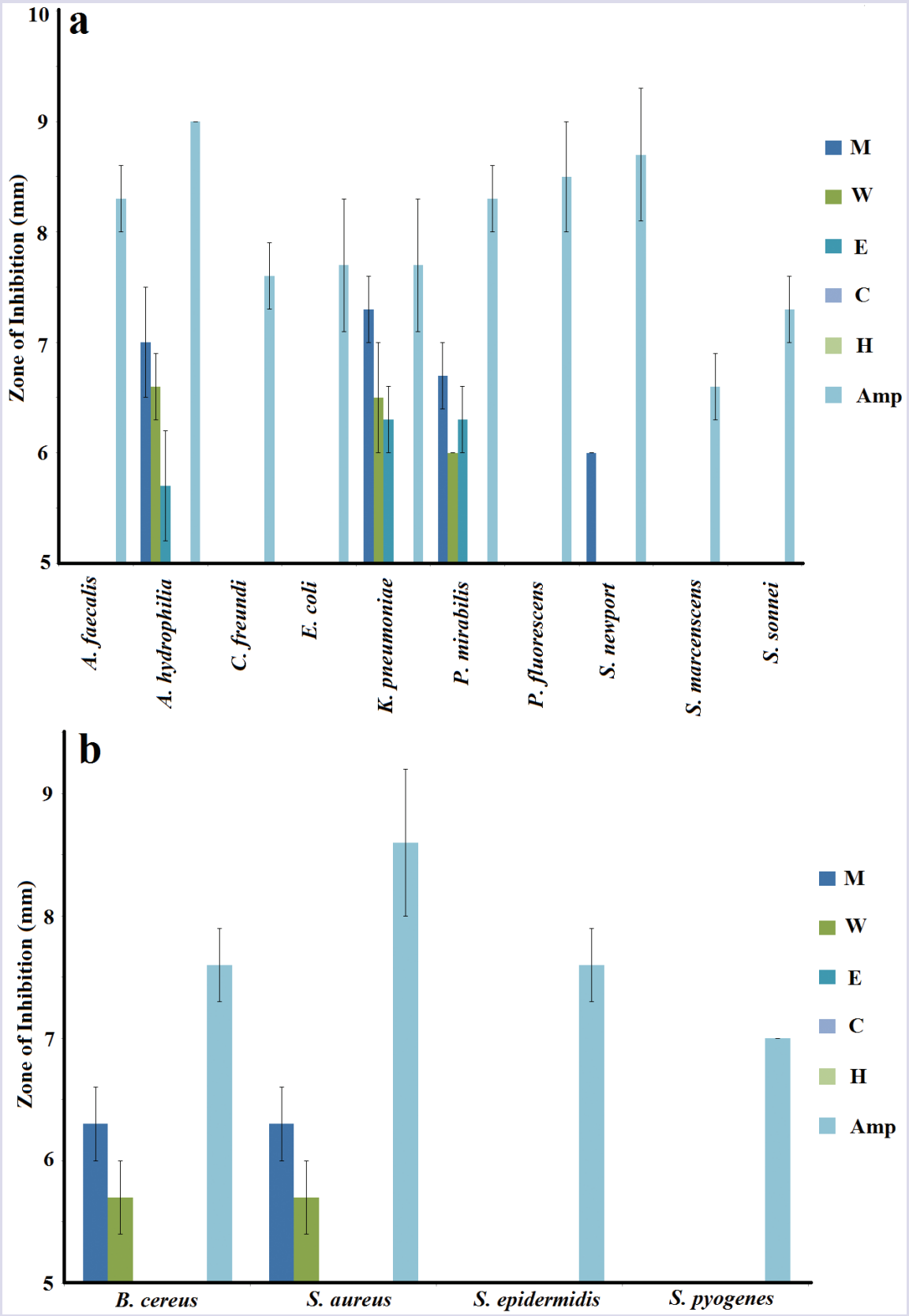


**Figure 3:** Growth inhibitory activity of the *A. leptoloba* leaf extracts against (a) gram negative and (b) gram positive bacterial species and an ampicillin (10 µg) control. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg) control. All determinations were performed in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

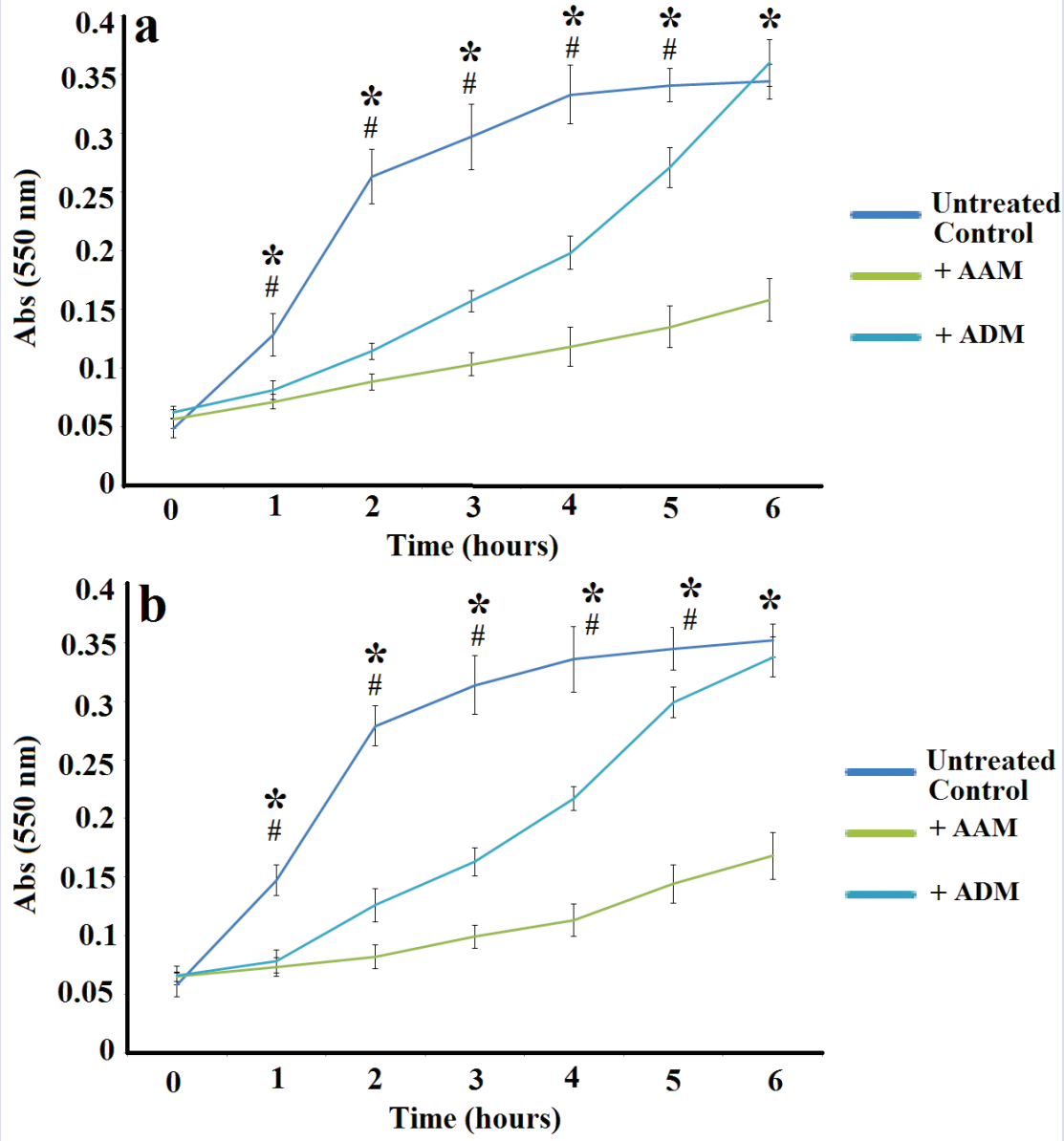


**Figure 4:** Bacterial growth curves for the methanolic *A. auriculformis* and *A. disparrima* leaf extracts against (a) *K. pneumoniae* and (b) *P. mirabilis*. All bioassays were performed in at least triplicate and are expressed as mean ± SEM. AAM = *A. auriculformis* methanolic extract; ADM = *A. disparrima* methanolic extract; * = results that are significantly different between the treated and the untreated control growth ($p<0.01$).

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. This study reports on the antimicrobial activities of *A. auriculformis*, *A. disparrima* and *A. leptoloba* leaf extracts, and on their toxicity. The gram positive and gram negative bacteria tested in this study demonstrated similar susceptibilities towards the *Acacia* spp. extracts. Previous studies with other plant species generally report a greater susceptibility of gram positive bacteria towards solvent extracts for South American,[34] African[35] and Australian plant extracts[36,37] although examples of plants having a greater effect on gram negative bacteria have also been reported.[24,38]

The bacteria examined in this study were chosen because they are all important 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.[1] *B. cereus* and *B. subtilise,*[39] *E. coli,*[40] *C. freundii*[41] and *K. pneumoniae*[41] 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.[42] 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, *Staphylococcus* spp. are generally considered to be the most common source of food poisoning worldwide.[1] *S. aureus* and *S. epidermidis* were each inhibited by 10 (67 %)

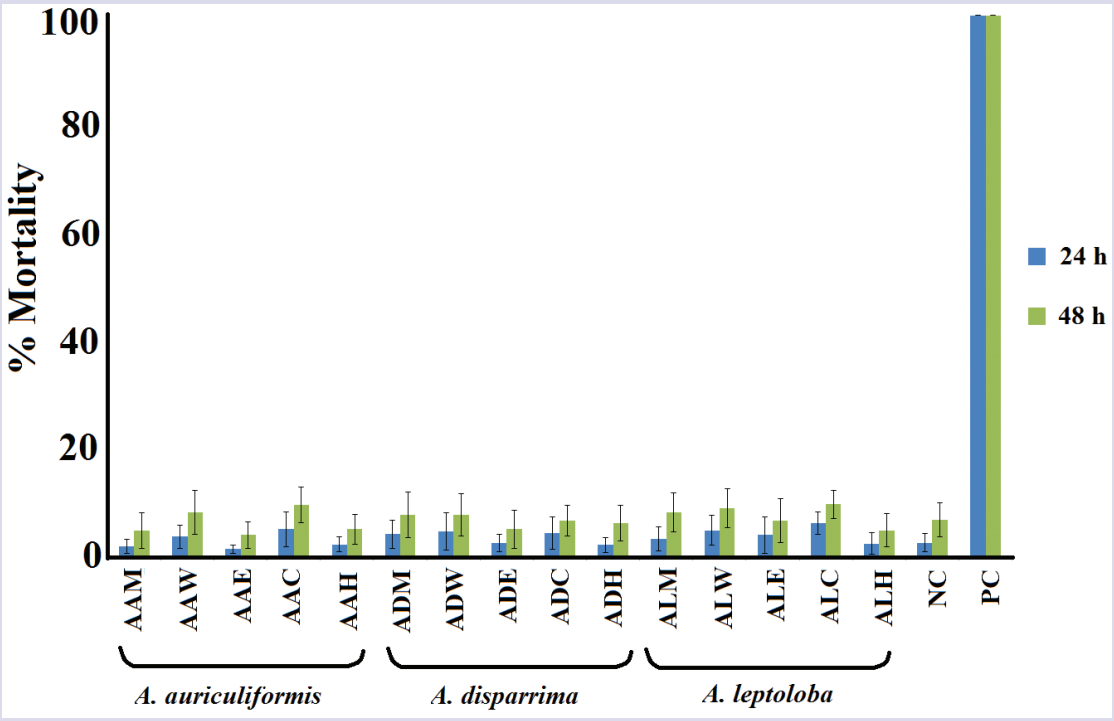


**Figure 5:** The lethality of the *A. auriculformis*, *A. disparrima* and *A. leptoloba* leaf extracts (2000 µg/mL), potassium dichromate (1000 µg/mL) and a seawater control. AAM = *A. auriculformis* methanolic extract; AAW = *A. auriculformis* aqueous extract; AAE = *A. auriculformis* ethyl acetate extract; AAC = *A. auriculformis* chloroform extract; AAH = *A. auriculformis* hexane extract; ADM = *A. disparrima* methanolic extract; ADW = *A. disparrima* aqueous extract; ADE = *A. disparrima* ethyl acetate extract; ADC = *A. disparrima* chloroform extract; ADH = *A. disparrima* hexane extract; ALM = *A. leptoloba* methanolic extract; ALW = *A. leptoloba* aqueous extract; ALE = *A. leptoloba* ethyl acetate extract; ALC = *A. leptoloba* chloroform extract; ALH = *A. leptoloba* 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.

of the 15 plant extracts tested. Most of the extracts capable of inhibiting *S. aureus* growth displayed potent activity, with MIC values generally < 1000 µg/mL and as low as 109 µg/mL (*A. auriculformis* methanolic extract). With the exception of *S. sonnei*, all pathogenic bacteria were inhibited by at least 1 of the extracts. Of the bacteria associated with food poisoning, *A. faecalis*, *K. pneumoniae* and *P. mirabilis* were particularly susceptible. The potent anti-*Proteus* activity has further therapeutic implications as *Proteus mirabilis* has been shown to be a trigger of rheumatoid arthritis (RA) and several plant species have already been highlighted as inhibitors of RA via *Proteus mirabilis* inhibition.[43]

Also particularly interesting was the ability of the extracts to inhibit the growth of psychrotrophic 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 psychrotrophic bacteria (e.g. *A. faecalis*, *A. hydrophilia*, *B. cereus* and *P. fluorescens*) to cause spoilage. Some pathogenic bacteria are also psychrotrophic (e.g. *B. cereus* and some strains of *C. freundii*, *E. coli* and *K. pneumoniae*).[39-42] Therefore, food based antibacterial agents with inhibitory activity against psychrotrophic bacteria are especially useful. All of the psychrotrophic bacteria tested were inhibited by at least 1 *Acacia* spp. extracts. The *A. auriculformis* leaf methanolic extract was the strongest and most versatile inhibitor of the psychrotrophic bacteria associated with spoilage, based on the number of MIC's and the number of psychrotrophic bacteria inhibited. Indeed, that extract blocked the growth of every psychrotrophic bacterial species tested. Furthermore, this extract generally displayed low MIC values, indicating that it may be especially useful.

Also noteworthy was the ability of many of the extracts 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 the spores. As *B. cereus* is also psychrotrophic, it is especially difficult to control. All methanolic and aqueous *A. auriculformis*, *A. disparrima* and *A. leptoloba* leaf extracts demonstrated good inhibitory activity against *B. cereus* (all with MIC values substantially <1000 µg/mL). Therefore their 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. auriculformis*, *A. disparrima* and *A. leptoloba* leaf extracts on aerobic bacteria. However, the anaerobic spore forming bacteria *Clostridium botulinium* is of greater concern to the food industry due to its incidence and the severity of the symptoms seen with botulism poisoning.[44] Future studies into the effects of *A. auriculformis*, *A. disparrima* and *A. leptoloba* leaf extracts on anaerobes, including *C. botulinium* are warranted to further evaluate their usefulness as food preservatives.

Individual extract components responsible for the antimicrobial potential of the plant extracts were not identified in the current study. However, qualitative screening studies were used to determine the classes of compounds present. Several commonalities 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 reported to inhibit the growth of a broad spectrum of bacterial species[45] through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins,[46,47] and by inhibiting glucosyltransferase enzymes.[48] Elligitannins are also highly potent inhibitors of bacterial growth, with MIC values as low as 62.5 µg/mL.[45,47] Ellagitannins have also been reported to function via several antibiotic mechanisms including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls.[45,47] Thus, it is likely that *Acacia* spp. leaf tannins may contribute to the inhibition of bacterial growth reported in our study. It is also likely that other phytochemical classes may contribute to the growth inhibitory properties of these extracts. Further phytochemical evaluation studies and bioactivity driven isolation of active *A. auriculformis*, *A. disparrima* and *A. leptoloba* leaf extract components is required to further evaluate the mechanism of bacterial growth inhibition.

The findings reported here also demonstrate that none of the *A. auriculformis*, *A. disparrima* and *A. leptoloba* leaf extracts displayed significant toxicity towards *Artemia franciscana* nauplii. Previously, compounds with an $LC_{50}$ >1000 µg/mL towards *Artemia* nauplii have been defined as being nontoxic.[33] None of the extracts tested in this study displayed $LC_{50}$ values < 1000 µg/mL. It was therefore determined that all *A. auriculformis*, *A. disparrima* and *A. leptoloba* leaf extracts examined in this study were nontoxic.

## CONCLUSIONS

The results of this study demonstrate the potential of *A. auriculformis*, *A. disparrima* and *A. leptoloba* leaf extracts to block bacterial food spoilage and food poisoning. Furthermore, the broad spectrum antimicrobial activity and the low MICs indicate the potential of the *Acacia* spp. extracts as natural food preservatives. 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.

## ACKNOWLEDGEMENTS

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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.

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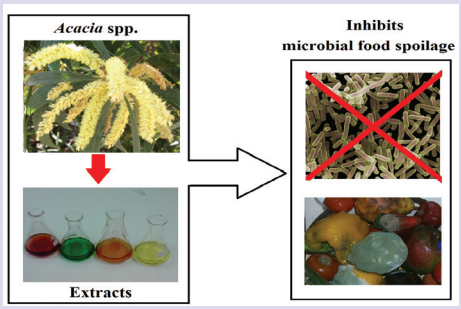
## PICTORIAL ABSTRACT




## SUMMARY


- *A. auriculiformis* and *A. disparrima* leaf extracts display broad spectrum antibacterial activity against gram positive and gram negative bacteria.
- Methanolic *A. auriculiformis* extract was a particularly potent inhibitor of *K. pneumoniae*, *P. mirabilis, B. cereus* and *S. aureus* growth (MICs of 97, 132, 178 and 109 µg/mL respectively).
- Methanolic *A. auriculiformis* extract was also a good inhibitor of *A. faecalis, A. hydrophilia* and *S. newport* growth (MIC's <1000 µg/mL range).
- The *A. disparrima* extracts were similarly potent inhibitors of bacterial growth.
- All *A. auriculiformis*, *A. disparrima* and *A. leptoloba* leaf extracts were nontoxic.


## 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.