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# An examination of the medicinal potential of *Scaevola spinescens*: Toxicity, antibacterial, and antiviral activities

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## ABSTRACT

**Background:** *Scaevola spinescens* is an endemic Australian native plant with a history of use as a medicinal agent by indigenous Australians. Yet the medicinal bioactivities of this plant are poorly studied. **Materials and Methods:** *S. spinescens* solvent extracts were tested for antimicrobial activity, antiviral activity 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 7 of the 14 bacteria tested (50%). The water, ethyl acetate, chloroform, and hexane extracts inhibited the growth of 6 (42.9%), 5 (35.7%), 5 (35.7%), and 4 (28.6%) of the 14 bacteria tested, respectively. *S. spinescens* methanolic extracts were equally effective against Gram-positive (50%) and Gram-negative bacteria (50%). All other extracts were more effective at inhibiting the growth of Gram-negative bacteria. All extracts also displayed antiviral activity in the MS2 plaque reduction assay with the methanol, water, ethyl acetate, chloroform, and hexane extracts inhibiting 95.2 ± 1.8%, 72.3 ± 6.3%, 82.6 ± 4.5%, 100 ± 0% and 47.7 ± 12.9% of plaque formation, respectively. All *S. spinescens* extracts were nontoxic in the *Artemia fransiscana* bioassay with no significant increase in mortality induced by any extract at 24 and 48 h. The only increase in mortality was seen for the water extract at 72 h, although even this extract displayed low toxicity, inducing only 41.7 ± 23.3% mortality. **Conclusions:** The lack of toxicity of the *S. spinescens* extracts and their inhibitory bioactivity against bacteria and viruses validate the Australian Aboriginal usage of *S. spinescens* and indicates its medicinal potential.

**Key words:** Antiviral, Australian medicinal plants, *maroon bush*, medicinal plants, MS2 bacteriophage, *Scaevola spinescens*

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## INTRODUCTION

*Scaevola spinescens* (commonly known as currant bush, *maroon bush*, and fanflower; family *Goodeniaceae*) is a rigid, scrubby bush that grows to approximately 1 m height and is distributed throughout the drier areas of the Australian continent. It has short hair covered branchlets which in many plants are converted to short sharp spines. In the summer, the shrub develops a profusion of cream to yellow colored flowers which develop to small purple fruits, each containing a single seed. Australian Aborigines used *S. spinescens* as a medical plant to treat a variety of conditions.[1-3] An infusion of the roots was used to treat stomach pain and urinary disorders. A decoction of the crushed stem was used to treat boils, rashes, and skin disorders. The whole plant was burnt and the fumes were inhaled to treat colds. Leaves and twigs were steamed and sores treated by exposure to this steam.

Despite its range of traditional medicinal uses, the phytochemistry and therapeutic potential of *S. spinescens* has not been extensively studied. A study by Semple *et al.* examined 40 different Australian plants for antiviral bioactivities.[4] The study found that *S. spinescens* leaf extracts were capable of inhibiting greater than 25% of human cytomegalovirus (CMV) late antigen production. More recently, preliminary studies in this laboratory have detected an antiviral bioactivity of the *S. spinescens* methanolic extract against MS2 bacteriophage.[5] These studies demonstrate the antiviral potential of *S. spinescens* and provide support for the traditional Aboriginal use of *S. spinescens* infusions to treat viral diseases.

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*S. spinescens* also had uses in the treatment of various cancers. This ethnopharmacological knowledge was traditionally passed on by word of mouth instead of written record, and unfortunately, much of our understanding of Aboriginal medicine has been lost as the Aboriginal society has merged into the mainstream Australian society. Accounts exist of aqueous extracts of the *S. spinescens* root bark being used to cure cancer,[2] although their efficacy has yet to be verified in controlled laboratory studies. Anecdotal accounts have also credited *S. spinescens* with an anticancer activity[6] although these also have yet to be verified by rigorous scientific examination.

While individual bioactive compounds are yet to be identified, *S. spinescens* has been reported to contain high yields of a number of taraxerene type pentacyclic triterpenoids.[7] In particular, high levels of 14-taraxerene-3,28-diol (1; myricadiol) were isolated from *S. spinescens* in this study. Similar pentacyclic triterpenoids isolated from *Alchornea latifolia* have been linked with the cytotoxic activity toward Hep-G2 and A-431 human cancer cell lines and are potent inhibitors of topoisomerase II.[8] Taraxerene triterpenoids from *Laggera pterodonta* have also been shown to have an antiviral activity against herpes viruses.[9] Studies have also demonstrated the antibacterial activity of pentacyclic triterpenoids from a variety of plants.[10-12]

Surprisingly, the antiseptic and antiviral properties of *S. spinescens* remain largely unstudied. The antibacterial, antifungal, and antiviral properties of other Australian plants are well known. *Eucalypts*,[13-16] *Callistemons*,[17] *Leptospermums*,[8,18-20] *Melaleucas*,[21,22] and *Syzygiums*[18,21-24] are all known to have antimicrobial activities. The current study reports on the antibacterial and antiviral properties of *S. spinescens* extracts as well as their toxicity to determine their potential as antibiotic agents.

## MATERIALS AND METHODS

### Plant collection and extraction

The *S. spinescens* plant material was provided by Jeannie Cargo of Outback Books Australia (an commercial supplier of *S. spinescens* tea) as a predried and coarse-milled whole plant material. The material was stored at −30°C until use.

One gram of the 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, and hexane, respectively. All solvents were obtained from Ajax and were of AR grade. The leaf material was extracted in each solvent for 24 h at 4°C with gentle shaking. The extracts were filtered through a filter paper (Whatman no. 54) under vacuum followed by drying by rotary evaporation in Eppendorf concentrator 5301. The resultant dry extract was weighed and redissolved in 10 ml deionized water.

### Qualitative phytochemical studies

Phytochemical analysis of *S. spinescens* extracts was conducted by modified versions of previously described assays.[25-27] The modified assays are briefly outlined below.

### Saponins

One milliliter of the pure extract was added to 1 ml deionized water and shaken vigorously for 30s. The tubes were allowed to stand for 15 min and the presence or absence of persistent frothing was noted. Persistent frothing indicated the presence of saponins.

### Phenolic compounds

Phenolic compounds were detected using a modified version of the Folin–Ciocalteu procedure.[27] A total of 200 µl of the crude extract was added to 2 ml of 3% aqueous sodium carbonate, followed by the addition of 200 µl Folin–Ciocalteu reagent. The mixture was allowed to stand for 30 min at room temperature. The formation of blue/gray color indicated the presence of phenolic groups.

### Water-soluble phenol test

Two drops of 1% ferric chloride were added to 500 µl of each extract. A red color change indicated the presence of water-soluble phenols.

### Water insoluble phenol test

A total of 500 µl of dichloromethane, 3 drops of 1% ferric chloride, and 1 drop of pyridine were added to 500 µl of each extract and mixed. The presence of insoluble phenols was indicated by a color change.

### Flavonoids

Flavonoids were detected using a modified Kumar test.[26] A total of 100 µl of aqueous sodium hydroxide was added to 1 ml of each extract. The development of an intense yellow color indicated the presence of flavonoids. Also, 100 µl of concentrated HCl was added to the solution. Reversion to the original color confirmed the presence of flavonoids.

### Polysteroids

Polysteroids were detected using a modified version of the Leiberman–Buchard test.[26] Three drops of acetic anhydride were added to 500 µl of the crude extract followed by the addition of a few drops of concentrated sulphuric acid. The solution was allowed to stand at room temperature for 5 min. The formation of a blue/green color indicated the presence of polysteroids.

### Triterpenoids

Triterpenoids were detected using a modified version of the

Salkowski test.[25] One milliliter of the extract was slowly added to 400 µl chloroform, followed by careful addition of 400 µl concentrated sulphuric acid. The formation of a red/brown/purple color at the interface indicated the presence of triterpenoids.

### Cardiac glycosides

Cardiac glycosides were detected using a modified version of the Keller–Kiliani test.[27] A total of 500 µl of the extract was added to 500 µl glacial acetic acid. A few drops of 1% aqueous iron chloride and concentrated sulphuric acid were then carefully added. The presence of a red/brown ring on the interface or the formation of a green/blue colour throughout the solution indicated the presence of cardiac glycosides.

### Anthraquinones

Anthraquinones were detected using modified versions of the Kumar and Ajaiyeoba tests.[26,27] The modified Kumar test involved the addition of a few drops of concentrated sulphuric acid to 500 µl pure extract, followed by the careful addition of 500 µl of ammonia. A rose pink color indicated the presence of free anthraquinones. For the Ajaiyeoba test, 450 µl of the crude extract was added to 50 µl concentrated HCl and allowed to stand at room temperature for several minutes. A total of 500 µl chloroform was then carefully added. The formation of a rose pink colour indicated the presence of combined anthraquinones.

### Tannins

Tannins were detected using a modified version of the ferric chloride test.[26] Two drops of the 1% aqueous ferric chloride reagent were added to 500 µl of the crude extract. The mixture was observed for the formation of blue, blue-black, green, or green-black coloration which indicated the presence of tannins.

### Alkaloids

Two methods were used to test for the presence of alkaloids.

#### *Mayer's reagent test*

A total of 200 µl of the pure extract was treated with a few drops of the aqueous solution of hydrochloric acid and 500 µl Mayer's reagent. The formation of a white precipitate was taken to indicate the presence of alkaloids.

For Mayer's reagent, mercuric chloride (1.358 g) was dissolved in 60 ml deionized water. Potassium iodide (5.0 g) was dissolved in 10 ml deionized water. The mercuric chloride and potassium iodide solutions were mixed and made up to 100 ml with deionized water.

#### *Wagner's reagent test*

A total of 200 µl of each extract was treated with a few drops of an aqueous solution of hydrochloric acid and 500 µl Wagner's reagent. A reddish-brown flocculent precipitate indicated the presence of alkaloids.

For Wagner's reagent, 1.27 g iodine and 2 g potassium iodide were dissolved in 5 ml deionized water and made up to a final volume of 100 ml with deionized water.

### Antibacterial screening

#### *Test microorganisms*

All microbial strains were obtained from Michelle Mendell and Tarita Morais, Griffith University, Australia. Stock cultures of *Aeromonas hydrophila*, *Alcaligenes feacalis*, *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 the antimicrobial activity

The antimicrobial activity of all plant extracts was determined using a modified Kirby–Bauer disc diffusion method.[28] Briefly, 100 µl of the test bacteria was grown in 10 ml of the fresh nutrient broth until the bacteria reached a count of approximately $10^8$ cells/ml as determined by direct microscopic determination. One hundred microliters of the microbial suspension was spread onto nutrient agar plates.

The extracts were tested using 5-mm sterilized 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 h before incubation with the test microbial agents. Plates inoculated with *A. feacalis*, *A. hydrophila*, *B. cereus*, *C. freundii*, *K. pneumoniae*, *P. mirabilis, P. fluorescens*, and *S. marcescens* were incubated at 30°C for 24 h; then the diameters of the inhibition zones were measured in millimeters. Plates inoculated with *E. coli*, *S. newport*, *S. sonnei*, *S. aureus*, *S. Epidermidis*, and *S. pyogenes* were incubated at 37°C for 24 h; then the diameters of the inhibition zones were measured. All measurements were to the closest whole millimeter. 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 the antimicrobial activity. Filter discs impregnated with 10 µl of distilled water or 10 µl of 10% methanol were used as negative controls.

### Minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) of the *S. spinescens* extracts were determined by the disc diffusion

method across a range of doses. The plant extracts were diluted in deionized water across a concentration range from 5 mg/ml to 0.1 mg/ml. Discs were impregnated with 10 µl 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.

## Screen for antiviral bioactivity

### *Viral and bacterial stocks*

MS2 bacteriophage, and F+ Amp+ *E. coli* used in this study were supplied by Dr. Jatinder Sidhu and Dr. Simon Toze of CSIRO, St. Lucia Qld, Australia. *S. aureus* were obtained from Michelle Mendell, Griffith University. All stock cultures were subcultured and maintained in nutrient broth at 4°C.

### *Production of MS2 virus*

A total of 100 ml of nutrient broth (25 g/l) containing ampicillin (100 µg/ml) was inoculated with 1 ml F + Amp+ *E. coli* culture and incubated overnight at 37°C. The following day, flasks containing 30 ml of nutrient broth (containing 100 µg/ml ampicillin) were inoculated with 1 ml of the F+ Amp+ *E. coli* overnight culture and incubated for 2 h at 37°C and 160 rpm. Once bacterial cells had reached log phase, 1 ml of stock MS2 virus (containing approximately $10^8$ plaque forming units) was added and incubated overnight at 35°C. The solution was centrifuged at 4000 rpm for 10 min and the supernatant was collected and passed through a 22 µm Sarstedt filter. All stock and working solutions were stored at 4°C until use.

### *Soft agar overlay*

A soft agar overlay was prepared to a final concentration of 0.7% w/v agar, 1% w/v glucose, 1% w/v $CaCl_2$ solution, and 1% w/v $MgSO_4$ and autoclaved at 120°C for 20 min. The soft agar overlay was allowed to cool to 65°C; then nalidixic acid was added to a final concentration of 0.4% w/v. The overlay was used immediately for the MS2 plaque inhibition assay described below.

### *MS2 plaque inhibition assay*

The MS2 plaque assay was performed as previously described.[5] Briefly, 490 µl of the crude plant extract was inoculated with 10 µl of MS2 virus (containing approximately $10^{10}$ plaque forming units/ml) and incubated overnight at 4°C. The solution was added to 500 µl *S. aureus* and incubated at 37°C for 20 min. The bacteria/virus/extract mixture was then added to 3 ml soft agar overlay and immediately poured over premade agar plates (2.8% w/v agar). Plates were allowed to set for 15 min at room temp, inverted, and incubated overnight at 37°C. The following morning plaques were counted and a percentage inhibition recorded. Serial dilution was used to determine the antiviral strength of samples where necessary. Nutrient broth and deionized water were used as negative controls, while the *C. sinensis* water extract, and UV irradiation (microwave of 10 µl virus only for 4 × 30 s) were used as positive controls.

## Toxicity screening

### *Reference toxins for biological screening*

Potassium dichromate ($K_2Cr_2O_7$ AR grade; Chem-Supply, Australia) was prepared as a 1.6 mg/ml solution in distilled water and was serially diluted in synthetic seawater for use in the *Artemia 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 serially diluted in artificial seawater for use in the bioassay.

### *A. franciscana nauplii toxicity screening*

Toxicity was tested using the *A. franciscana* nauplii lethality assay developed by Meyer *et al.*[29] 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, UT, USA). Synthetic seawater was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/l distilled water were prepared prior to use. Two grams of *A. franciscana* cysts was 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. A total of 400 µl of seawater containing approximately 42 (mean 41.6, $n$ = 150, SD 17.8) nauplii was 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. A total of 400 µl of the diluted plant extract and the reference toxins were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A negative control (400 µl 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 moribund if no movement of the appendages was observed within 10 s. 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.[30]

## Statistical analysis

Data are expressed as the mean ± 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

The extraction of 1 g of the dried plant material with various solvents yielded dried plant extracts ranging from 44 mg to 232 mg [Table 1]. Deionized water and chloroform both gave high yields of the dried extracted material (210 and 232 mg, respectively) while hexane extracted the lowest mass (44 mg). The dried extracts were resuspended in 10 ml of deionized water resulting in the extract concentrations shown in Table 1.

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

| Solvent | Mass of the dried extract (mg) | Resuspended extract concentration (mg/ml) |
|---|---|---|
| Methanol | 116 | 11.6 |
| Deionized water | 210 | 21 |
| Ethyl acetate | 160 | 16 |
| Chloroform | 232 | 23.2 |
| Hexane | 44 | 4.4 |

Phytochemical studies [Table 2] showed that methanol and water extracted the widest range of phytochemicals. Both showed moderate to high levels of phenolics (water-soluble phenolics only), flavonoids, and tannins with lower levels of saponins present in both extracts. The only difference detected between the methanol and water extracts was the possible presence of low levels of alkaloids in the methanol extract. However, alkaloids were only detected in the methanol extract by the Meyer test. In contrast, the Wagner test was negative for the presence of alkaloids, indicating that if present, the level of alkaloids in the methanol extract was low. No alkaloids were detected in the water extract. The ethyl acetate, chloroform, and hexane extracts all only had detectable levels of water insoluble phenols and only a low response was seen for each solvent.

### Antibacterial activity

A total of 10 µl of each extract was tested in the disc diffusion assay against 14 bacteria [Table 3]. All extracts displayed an antibacterial activity, being capable of inhibiting the growth of between 4 and 7 of the bacteria tested. The methanolic extract displayed the broadest antibiotic specificity, inhibiting the growth of 7 of the 14 bacteria tested (50%). The methanolic extract was particularly potent against *S. marcenscens* as determined from the zone of inhibition (10.3 ± 0.6 mm). The *S. spinescens* water extract also had a broad-range antibacterial specificity inhibiting the growth of 6 of the 14 bacteria tested (42.9%). The ethyl acetate and chloroform extracts each inhibited 5 of the 14 bacteria tested (35.7%) while the hexane extract inhibited 4 of the 14 bacteria tested (28.6%).

**Table 2: Qualitative phytochemical screenings of solvent extractions**

| | Methanol | Deionized water | Ethyl acetate | Chloroform | Hexane |
|---|---|---|---|---|---|
| Phenolics | | | | | |
| Total phenolics | +++ | +++ | – | – | – |
| Water soluble | ++ | ++ | – | – | – |
| Water insoluble | – | – | + | + | + |
| Cardiac glycosides | | | | | |
| Keller–Kiliani test | – | – | – | – | – |
| Saponins | | | | | |
| Froth persistence | + | + | – | – | |
| Triterpenes | | | | | |
| Salkowski test | – | - | – | – | – |
| Phytosteroids | | | | | |
| Acetic anhydride test | – | – | – | – | – |
| Alkaloids | | | | | |
| Meyer's test | + | – | – | – | – |
| Wagner's test | – | – | – | – | – |
| Flavonoids | | | | | |
| Shinoda test | ++ | ++ | – | – | – |
| Kumar test | ++ | ++ | – | – | – |
| Tannins | | | | | |
| Ferric chloride test | ++ | +++ | – | – | – |
| Anthraquinones | | | | | |
| Free | – | – | – | – | – |
| Combined | – | – | – | – | – |

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

The methanol extract was the most effective at inhibiting Gram-positive bacterial growth, inhibiting 2 of the 4 bacteria tested (50%). In contrast, all other extracts inhibited only a single bacteria (*S. epidermidis*) of the 4 Gram-positive bacteria tested (25%). The methanol extract was also the most efficient at inhibiting Gram-negative growth, inhibiting the growth of 5 of the 10 bacterial tested (50%). In comparison, the growth of 5 Gram-negative bacteria was inhibited by the water extract (50%) and 4 Gram-negative bacteria were inhibited by each of the ethyl acetate, chloroform, and hexane extracts (40%).

The relative level of the 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 [Table 4]. MICs 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.[31]

The water extract was effective at low concentrations against several bacteria. Indeed, the growth of *A. faecalis*, *K. pneumoniae*, and *P. mirabilis* was inhibited at concentrations below 100 μg/ml. With the exception of *C. freundi* (MIC 2501 μg/ml), the water extract displayed MIC values less than 500 μg/ml for all the bacterial species whose growth it inhibited. Similarly, the ethyl acetate extract also inhibited *P. mirabilis* at low concentrations (MIC 23.1 μg/ml). It was less effective at inhibiting the growth of the four other bacterial species, although in all cases the MIC was less than 500 μg/ml. While the methanol extract had the broadest specificity of the extracts [Table 3], it was not as effective against any of these bacteria as the water or ethyl acetate extract, with MIC values ranging from 151.9 to 531.7 μg/ml. Also noteworthy was the low MIC value of the chloroform extract against *S. newport*.

## Antiviral activity

Figure 1 shows the formation of MS2 bacteriophage plaques in the presence of the extracts or the positive and negative controls as a percentage of control plaque formation. All extracts displayed an antiviral activity when

**Table 3: Antibacterial activity of *S. spinescens* solvent extracts measured as zones of inhibition**

| | Methanol extract | Water extract | Ethyl acetate extract | Chloroform extract | Hexane extract | Ampicillin | Chloramphenicol | Negative control (water) |
|---|---|---|---|---|---|---|---|---|
| Gram-negative rods | | | | | | | | |
| *A. faecalis* | – | 6.3 ± 0.6 | – | – | – | 15.2 ± 1.2 | 6.3 ± 0.6 | – |
| *A. hydrophilia* | – | – | – | – | – | 12.0 ± 1.0 | 28.7 ± 1.6 | – |
| *C. freundi* | 6.0 ± 0 | 6.7 ± 1.5 | – | 7.7 ± 0.6 | 7.7 ± 0.6 | 8.3 ± 0.6 | 15.7 ± 1.2 | – |
| *E. coli* | – | – | – | – | – | 14.7 ± 0.6 | 17.3 ± 0.6 | – |
| *K. pneumoniae* | 7.7 ± 1.2 | 7.7 ± 0.6 | 8.0 ± 0 | 7.7 ± 1.2 | 9.0 ± 0 | 10.3 ± 0.6 | 21.3 ± 1.5 | – |
| *P. mirabilis* | 6.0 ± 1.0 | 9.3 ± 0.6 | 9.7 ± 1.2 | – | 9.0 ± 1.0 | 17.3 ± 0.6 | 8.7 ± 0.6 | – |
| *P. fluroscens* | – | – | – | – | – | 18.2 ± 0.5 | 21.2 ± 1.2 | – |
| *S. newport* | 8.0 ± 1.0 | – | 7.7 ± 0.6 | 8.7 ± 0.6 | – | 18.7 ± 0.6 | 20.3 ± 0.6 | – |
| *S. marcenscens* | 10.3 ± 0.6 | 6.7 ± 1.2 | 6.0 ± 0 | 6.0 ± 0 | 6.3 ± 0.6 | – | 14.7 ± 0.6 | – |
| *S. sonnei* | – | – | – | – | – | 14.0 ± 0 | 14.3 ± 0.6 | – |
| Gram-positive rods | | | | | | | | |
| *B. cereus* | 7.0 ± 0 | – | – | – | – | 26.7 ± 0.6 | 13.3 ± 1.2 | – |
| Gram-positive cocci | | | | | | | | |
| *S. aureus* | – | – | – | – | – | 11.7 ± 2.1 | 16.0 ± 1.0 | – |
| *S. epidermidis* | 6.3 ± 0.6 | 6.3 ± 0.6 | 6.3 ± 0.6 | 5.7 ± 0.6 | – | 26.3 ± 1.5 | 12.3 ± 0.6 | – |
| *S. pyogenes* | – | – | – | – | – | 17.0 ± 1.0 | 24.0 ± 1.0 | – |

Numbers indicate the mean diameters (mm) of the inhibition of at least triplicate experiments ± standard deviation. Ampicillin (2 μg) and chloramphenicol (10 μg) were used as the positive controls. Artificial seawater was included as a negative control; – = Indicates no growth inhibition.

**Table 4: Minimum inhibitory concentrations (μg/ml) of *S. spinescens* extracts against susceptible bacteria**

| | MIC (μg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *A. faecalis* | *C. freundi* | *K. pneumoniae* | *P. mirabilis* | *S. newport* | *S. marcenscens* | *B. cereus* | *S. epidermidis* |
| Methanol extract | – | 353.6 | 581.5 | 151.9 | 197.9 | 374.6 | 353.5 | 531.7 |
| Water extract | 85.3 | 2501.1 | 66.8 | 25.4 | – | 350.3 | – | 489.3 |
| Ethyl acetate extract | – | – | 336.1 | 23.1 | 335.2 | 441.1 | – | 292.7 |
| Chloroform extract | – | 789.2 | 171.2 | – | 75.8 | 495 | – | 409.9 |
| Hexane extract | – | 540.2 | 342 | 239.1 | – | 239.1 | – | – |

Numbers indicate the mean MIC values of at least triplicate determinations; – = Indicates no growth inhibition.

tested in the MS2 plaque bioassay. The methanol and chloroform extracts were particularly effective at inhibiting MS2 plaque formation, inhibiting 95.2 ± 1.8% and 100 ± 0% of the plaque formation, respectively. Water and ethyl acetate extracts were also effective, in both cases inhibiting more than 50% of plaque formation (72.3 ± 6.3% and 82.6 ± 4.5%, respectively). While still capable of inhibiting the formation of MS2 plaque formation, the hexane extract was a less effective antiviral agent than the other extracts tested as determined by % plaque inhibition (47.7 ± 12.9%).

The *S. spinescens* extracts were further tested over a range of concentrations to determine the minimum concentration capable of inhibiting 100% of plaque formation ($PI_{100}$) and the minimum concentration capable of inhibiting 50% of plaque formation ($PI_{50}$; Table 5). The minimum dosage of the positive control (*C. sinensis* leaf extract) capable of totally blocking 100% MS2 plaque formation ($PI_{100}$) was 2540.0 ± 812.5 µg/ml while the minimum concentration of the *C. sinensis* leaf extract capable of blocking 50% of MS2 plaque formation ($PI_{50}$) was 612.5 ± 198.7 µg/ml. All of the *S. spinescens* extracts were more effective antiviral agents than the *C. sinensis* control as determined by their lower $PI_{50}$ values. The chloroform extract proved the most effective, with a $PI_{50}$ value of 366.9 ± 167.2 µg/ml. The order of effectiveness of MS2 plaque reduction was as follows: chloroform extract > methanol extract > ethyl acetate extract > water extract > hexane extract > *C. sinensis* control. $PI_{100}$ values were only obtained for the *S. spinescens* chloroform extract and for the *C. sinensis* extract control at the concentrations tested in the bioassay. Again, the chloroform extract was the most effective inhibitor of MS2 plaque formation, blocking 100% of plaque formation at approximately half the concentration of the *C. sinensis* extract control (1321.2 ± 602.4 µg/ml compared with 2450.0 ± 812.5 µg/ml).

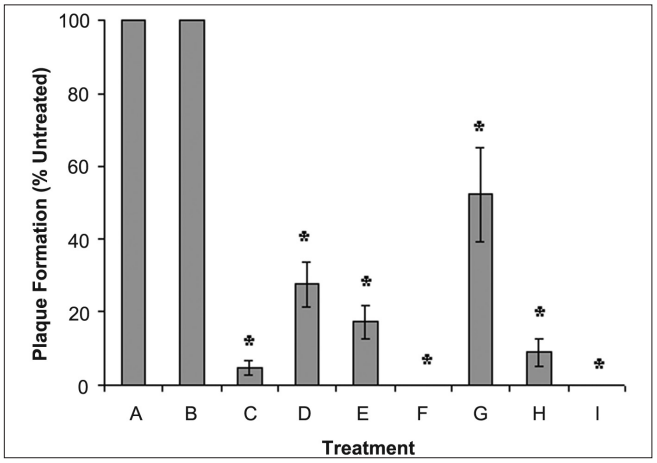


**Figure 1:** MS2 plaque formation in a *S. aureus* lawn expressed as the percentage of control plaque formation following the incubation of the MS2 with (A) nutrient broth (negative control), (B) deionized water (negative control), (C) methanol extract (1500 µg/ml), (D) water extract (2600 µg/ml), (E) ethyl acetate extract (2000 µg/ml), (F) chloroform extract (2900 mg/ml), (G) hexane extract (600 µg/ml), (H) *C. sinensis* leaf water extract (1000 µg/ml), and (I) nutrient agar and microwave irradiation (positive control). All Results are reported as the mean of triplicate assays ± standard deviation. *indicates statistically significant results

### Quantification of toxicity

*S. spinescens* extracts were diluted to a concentration of 4000 µg/ml in artificial seawater for toxicity testing, resulting in 2000 µg/ml concentrations in the *Artemia* nauplii lethality bioassay. For comparison, the reference toxins potassium dichromate (1000 µg/ml) and Mevinphos (2000 µg/ml) were also tested in the bioassay. Figure 2 shows the percent 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 h of exposure and 100% mortality was evident following 4–5 h. In contrast, all of the *S. spinescens* extracts displayed mortality rates similar to those of the artificial seawater negative control [Figure 2f] at 24 and 48 h. The water extract did show some increase in mortality by 72 h although the mortality was still below 50% so is considered to be of low toxicity. It was not possible to accurately determine an $LC_{50}$ for any extract as the mortality never exceeded 50% for any extract at any time tested.

## DISCUSSION

The current study reports on the antimicrobial and antiviral activities of various *S. spinescens* extracts and on their toxicity. The ability of *S. spinescens* extracts to inhibit the growth of both Gram-positive and Gram-negative bacteria is in agreement with previous reports of the antibacterial activity of other Australian native plants that have a history of medicinal usage by Australian Aborigines. The antiseptic properties of the *Eucalypts*,[13-16] *Leptospermums*,[8,18-20] and *Melaleucas*[21,22] have been extensively studied and shown to

**Table 5: Minimum concentrations capable of inhibiting 100% ($PI_{100}$) and 50% ($PI_{50}$) of MS2 plaque formation of anti-viral plant extracts**

| Phage treatment | $PI_{100}$ | $PI_{50}$ |
|---|---|---|
| *S. spinescens* methanol extract | NPI | 405.6 ± 189.9 |
| *S. spinescens* water extract | NPI | 519.0 ± 198.7 |
| *S. spinescens* ethyl acetate extract | NPI | 435.1 ± 224.6 |
| *S. spinescens* chloroform extract | 1321.2 ± 602.4 | 366.9 ± 167.2 |
| *S. spinescens* hexane extract | NPI | 557.8 ± 162.2 |
| *C. sinesis* water extract | 2450.0 ± 812.5 | 612.5 ± 198.7 |

All $PI_{100}$ and $PI_{50}$ values are expressed as µg/ml ± standard deviation. NPI denotes that $PI_{100}$ was not achieved. All values are the mean of at least triplicate determinations.

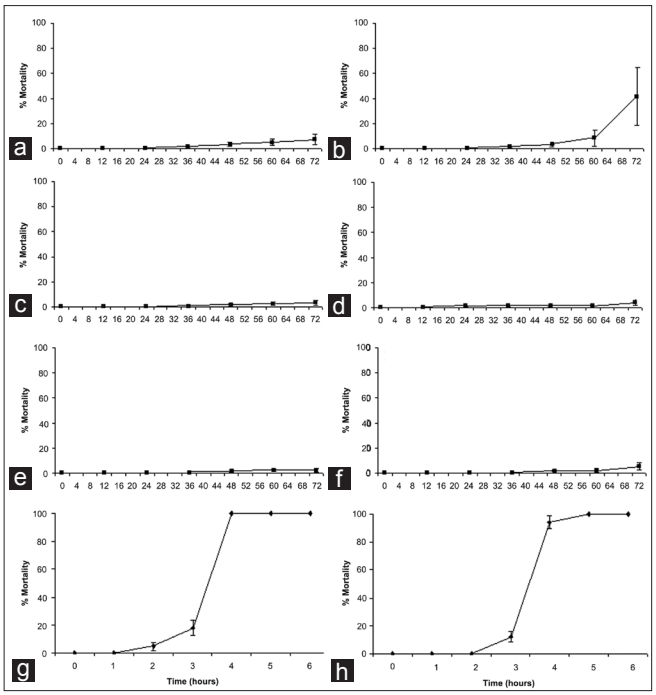


**Figure 2:** Brine shrimp lethality of the (a) *S. spinescens* methanol extract (2000 µg/ml), (b) S. spinescens water extract (2000 µg/ml), (c) *S. spinescens* ethyl acetate extract (2000 µg/ml), (d) *S. spinescens* chloroform extract (2000 µg/ml), (e) *S. spinescens* hexane extract (2000 µg/ml), (f) artificial seawater negative control, (g) potassium dichromate (1000 µg/ml), and (h) Mevinphos (2000 µg/ml). All bioassays were performed in at least triplicate and are expressed as mean ± standard deviation

inhibit the growth of a wide variety of both Gram-positive and Gram-negative bacteria.

The current study shows Gram-positive and Gram-negative bacteria to be equally susceptible to the *S. spinescens* methanolic extract. All other *S. spinescens* extracts showed a greater specificity for Gram-negative bacteria, although this may be due to the small sample of Gram-positive bacteria tested. 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 toward solvent extracts of South American,[32] African,[33,34] and Australian[35] plant extracts. Results within this laboratory have also confirmed the greater susceptibility of Gram-positive bacteria toward other Australian plant extracts.[18]

Individual *S. spinescens* 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*,[36] *Leptospermum*,[37] *Melaleucas*[38]). These plants all contain terpenes including 1,8-cineole, terpinen-4-ol, α-pinene, and β-pinene. Both 1,8-cineole and terpinen-4-ol have an antimicrobial activity.[39,40] Recent studies have also reported on the antibacterial activities of the *Callistemons*[17] and *Syzygiums*.[21,22,24,41] It has been postulated that terpene components may also be responsible for the antiseptic properties of these plants.[42] *S. spinescens* has been reported to contain high yields of a number of taraxerene-type pentacyclic triterpenoids, particularly 14-taraxerene-3,28-diol (1; myricadiol).[7] While the phytochemistry of the *S. spinescens* extracts was not extensively examined in the current study, phenolic compounds, saponins, flavonoids, and tannins were detected by qualitative assays. Interestingly, no triterpenes were detected in any extract. The presence of other terpenoids was not tested for in the current studies so it is possible that other non-triterpene terpenoids may still be responsible for the antibacterial activity of the *S. spinescens* extracts.

Similarly, the antiviral components were not identified in the current study. All solvent extracts tested displayed an antiviral activity toward MS2 bacteriophage. This is in agreement with a previous study which reported an antiviral activity of *S. spinescens* extracts toward cyclomeglovirus (CMV).[35] Neither study examined the mechanism of the antiviral activity. The extract may be acting by direct viral inactivation, or by disrupting one or more stages of viral replication (cellular attachment and penetration, viral uncoating, and reverse transcription of viral RNA (in the case of RNA viruses, viral nucleic acid synthesis, viral assembly and release). Further studies are required to determine the antiviral mechanism of the *S. spinescens* extracts. Furthermore, while the current studies have detected an antiviral activity toward MS2 bacteriophage and a previous study has shown an anti-CMV activity,[35] the antiviral specificity is yet to be determined toward other viruses. Future studies need to rigorously test *S. spinescens* extracts against a wider panel of viruses to determine their medicinal potential.

Noteworthy was the high antiviral activity in the chloroform extract in the current studies. Of the compounds assayed for, only water insoluble phenols were detected in the chloroform extract, and then only in low levels. It is possible that other classes of compounds not tested for may be responsible for the observed antiviral activity. Fatty acids and other lipids would be expected to be extracted into the chloroform extract. Previous reports have shown various fatty acids and fatty acid derivatives to display an antiviral activity. Lauric acid and monolaurin are effective at inactivating HIV, measles, herpes simplex virus, vesicular somatitis, visna virus, and CMV.[43] Similarly, 9*S*, 12*S*, 13*S*-trihydroxy-10*E*-octadecenoic acid has a potent antiviral activity against H1N1 and H3N2 subtypes of influenza A and B viruses.[44] It is possible that fatty acids extracted into the chloroform extract may be responsible

for its potent antiviral activity toward MS2. Similarly, fatty acids would also be expected to be extracted with ethyl acetate, hexane, and methanol solvents and therefore may also be responsible for the antiviral activity of these extracts.

The findings reported here also demonstrate that none of the *S. spinescens* extracts displayed significant toxicity toward *A. franciscana*. Indeed, of the extracts tested, only the water extract was found to induce mortality above that of the negative control, and then only following 72 h of exposure. As most toxicity studies using *Artemia* nauplii usually report 24-h and/or 48-h $LC_{50}$s, the reporting of mortality at 72 h may be excessive. Furthermore, even at the 72-h time, the mortality induced by the water extract was not high and we were unable to determine an $LC_{50}$. The extracts were all tested in the current studies at concentrations of 2000 μg/ml in the bioassay. Previously, compounds with an $LC_{50}$ of greater than 1000 μg/ml toward *Artemia* nauplii have been defined as being nontoxic.[29] We report the mortality induction of the extracts at 2000 μg/ml in this study as no increase in mortality was seen for any extract at 1000 μg/ml at any time (unreported results). It was therefore determined that all *S. spinescens* extracts were nontoxic toward *Artemia* nauplii.

In conclusion, the results of this study partially validate the traditional Australian Aboriginal usage of *S. spinescens* to treat bacterial and viral diseases and indicate that *S. spinescens* is worthy of a further study. Further evaluation of the antibacterial and antiviral properties of these extracts against a more extensive panel of microbial agents is warranted. Likewise, the purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents. While the extracts examined in this report are promising as antibacterial and antiviral 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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