

Gas chromatography-mass spectroscopy analysis of bioactive *Petalostigma* extracts: Toxicity, antibacterial and antiviral activities

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ABSTRACT

Background: *Petalostigma pubescens* and *Petalostigma triloculare* were common components of pharmacopeia's of multiple Australian Aboriginal tribal groupings which traditionally inhabited the areas in which they grow. Among these groups, they had a myriad of medicinal uses in treating a wide variety of bacterial, fungal and viral infections. This study was undertaken to test *P. pubescens* and *P. triloculare* leaf and fruit extracts for the ability to inhibit bacterial and viral growth and thus validate Australian Aboriginal usage of these plants in treating bacterial and fungal diseases. **Materials and Methods:** *P. pubescens*, and *P. triloculare* leaves and fruit were extracted and tested for antimicrobial, antiviral activity and toxicity. The bioactive extracts were further examined by RP-HPLC and GC-MS to identify the component compounds. **Results:** The methanol, water and ethyl acetate leaf and fruit extracts displayed potent antibacterial activity. The methanol and ethyl acetate extracts displayed the broadest specificity, inhibiting the growth of 10 of the 14 bacteria tested (71%) for the leaf extract and 9 of the 14 bacteria tested (64%) for the fruit extracts. The water extracts also had broad spectrum antibacterial activity, inhibiting the growth of 8 (57%) and 7 (50%) of the 14 bacteria tested, respectively. All antibacterial extracts were approximately equally effective against Gram-positive and Gram-negative bacteria, inhibiting the growth of 50-75% of the bacteria tested. The methanol, water and ethyl acetate extracts also displayed antiviral activity in the MS2 plaque reduction assay. The methanol and water extracts inhibited 26.6-49.0% and 85.4-97.2% of MS2 plaque formation, respectively, with the fruit extracts being more potent inhibitors. All ethyl acetate extracts inhibited 100% of MS2 plaque formation. All extracts were also non-toxic or of low toxicity. Analysis of these extracts by RP-HPLC showed that the *P. triloculare* ethyl acetate fruit extract was the least complex of the bioactive extracts. Subsequent analysis of this extract by GC-MS revealed that it contained 9 main compounds: acetic acid; 2,2-dimethoxybutane; 4-methyl-1,3-dioxane; decane; undecane; 2-furanmethanol; 1,2-benzenediol; 1,2,3-benzenetriol; and benzoic acid. **Conclusion:** These studies validate Australian Aboriginal therapeutic usage of *Petalostigma* species and indicate their medicinal potential.

Key words: Antibacterial, antiviral, Australian medicinal plants, euphorbiaceae, *Petalostigma pubescens*, *Petalostigma triloculare*, quinine bush

INTRODUCTION

Plants have been used for thousands of years as medicines for treating a variety of different diseases and medical complaints by most, if not all civilisations. Phytotherapy in Asia is particularly widespread and documented. Asian

plant-based medications have been used in the treatment of numerous disorders including eczema, malaria and respiratory disorders.^[1] Similarly, plant based medicinal systems continue to be the primary therapeutic system in many parts of Africa. For example, *Phytolacca dodecandra* is used as a molluscicide in the control of schistosomiasis.^[2] Some African plant derived medicines have also found a place in modern Western medicinal systems. The antitumour agents vinblastine and vincristine (derived from *Catharanthus roseus*) are currently used in the treatment of a variety of tumours.^[3,4] Studies have also examined

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the myriad of medicinal plant uses by indigenous North, Central^[5] and South Americans.^[6] Approximately 1500 plant species are currently used in Europe to treat a wide variety of medical conditions.^[1]

While much of the research into traditional medicinal plant use has focused on Asian, African and South American plants, recent studies have begun to examine the therapeutic potential of Australian medicinal plants. In particular, the antibacterial nature of Australian plants is receiving attention due to the development of super-resistant bacterial strains and the need to find new antibacterial agents. In recent studies in this laboratory, we have examined the antimicrobial activity^[7,8] and toxicity^[9] of various Australian native plants, many from the Toohey Forest area of Brisbane, Australia. Several plants were identified as having promising antimicrobial activities.

Petalostigma, an Australian genus of the Euphorbiaceae family, consists of seven species. Two of these species which occur in the Toohey Forest region (*Petalostigma pubescens* and *Petalostigma trilobulare*) are collectively known as ‘quinine tree’. The two species differ slightly in terms of leaf and fruit shape and size.^[10] Both trees grow to between 2 to 10 metres in height, with bright orange fruit. They are distinguished by the longer leaves of *P. trilobulare* and the slightly larger fruit of *P. pubescens* [Figure 1]. Ethnobotanical evidence exists that an infusion of *Petalostigma* spp. bark or fruit in water is effective in relieving sore eyes, and as an antiseptic. Indeed, *Petalostigma* spp. were used extensively by Australian Aborigines to treat a myriad of bacterial, fungal and viral diseases.^[11] Fruit was also held in the mouth by Australian Aborigines to relieve toothache.^[11] Despite this range of traditional medicinal uses, the phytochemistry and therapeutic potential of *Petalostigma* spp. has not been extensively studied. Although the common name suggests quinine is present within the fruit or leaves, there is no scientific evidence to support this.

While the therapeutic potential and phytochemistry of *Petalostigma* spp. has yet to be extensively examined, several other species of family Euphorbiaceae are known to produce diterpenes with cytotoxic, antitumor/tumor promoting and antimicrobial activities.^[12,13] Furthermore, a recent investigation into the chemical composition of *P. pubescens* heartwood identified five polycyclic diterpenes with antitumor activity: 5,9-syn-rosane petalostigmone A and B and erythroxylyne petalostigmone C, pubescenone, and sonderianol [Figure 1].^[14] The current study was undertaken to determine the toxicity and antibacterial and antiviral activities of *P. pubescens* and *P. trilobulare* leaf and fruit extracts. GC-MS analysis was used to examine the phytochemistry of a bioactive extract.

MATERIALS AND METHODS

Plant collection and extraction

P. pubescens and *P. trilobulare* leaves and fruit were collected from Toohey Forest, Australia and were identified with reference to a taxonomic key to Toohey Forest plants.^[15] Voucher specimens (PPL090224, PPF090224, PTL090226 and PTF090226) were stored in Biomolecular and Physical Science, Griffith University, Australia. Samples were dried in a Sunbeam food dehydrator and then ground to a coarse powder. 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 and were AR grade. Leaf and fruit 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 deionized water.

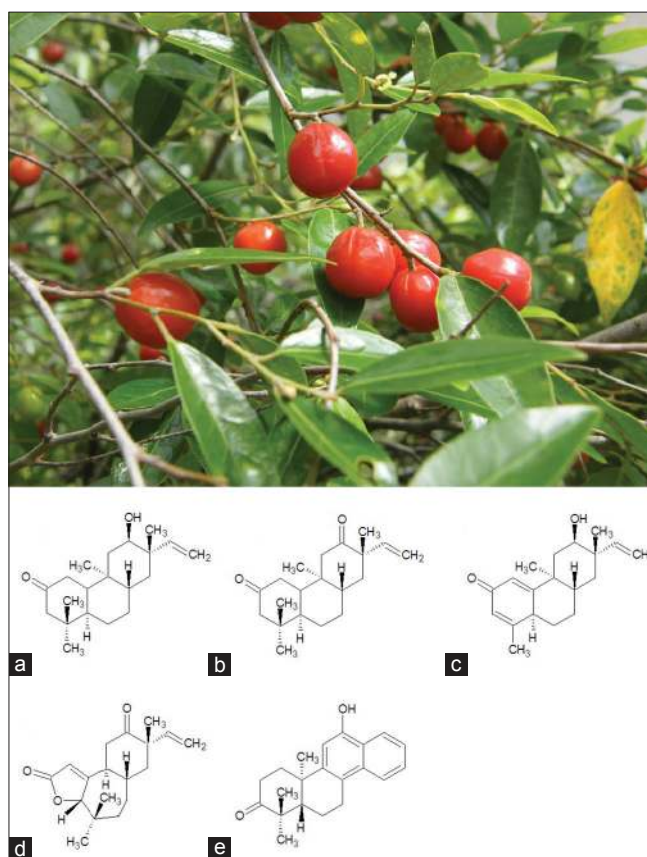


Figure 1: *Petalostigma pubescens* leaves and fruit (photograph was taken in January 2011 in Toohey Forrest, Australia by Dr Ian Cock) and five tricyclic diterpenes present within *P. pubescens* heartwood: (a) 5,9-syn-rosane petalostigmone A, (b) 5,9-syn-rosane petalostigmone B, (c) erythroxylyne petalostigmone C, (d) pubescenone, (e) sonderianol

Qualitative phytochemical studies

Phytochemical analysis of *Petalostigma* spp. extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.^[16,17]

Antibacterial screening

Test microorganisms

All microbial 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 antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion method.^[18-20] Briefly, 100 µl of the test bacteria were grown in 10 ml of fresh nutrient broth until they reached a count of approximately 10⁸ cells/ml as determined by direct microscopic determination. One hundred microliters of microbial suspension was spread onto nutrient agar plates.

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 microbial agents. Plates inoculated with *Alcaligenes faecalis*, *Aeromonas hydrophila*, *Bacillus cereus*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Serratia marcescens*, were incubated at 30°C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *E. coli*, *S. newport*, *S. sonnei*, *S. aureus*, *S. 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 and mean values were determined. Standard discs of ampicillin (2 µg) and chloramphenicol (10 µg) were obtained from Oxoid Ltd. and served as positive controls for 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 *Petalostigma* spp. 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 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. All stock cultures were subcultured and maintained in nutrient broth at 4°C.

Production of MS2 virus

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 hours at 37°C and 160 rpm. Once bacterial cells had reached log phase, 1 ml of stock MS2 virus (containing approximately 10⁸ 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 CaCl₂ solution and 1% w/v MgSO₄ 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.^[21] Briefly, 490 µl of crude plant extract was inoculated with 10 µl of MS2 virus (containing approximately 10¹⁰ plaque forming units/ml) and incubated overnight at 4°C. The solution was added to 500 µl F + Amp + *E. coli* and incubated at 37°C for 20 minutes. The bacteria/virus/extract mixture was then added to 3 ml soft agar overlay and immediately poured over pre-made 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 *C. sinensis* water extract, and UV irradiation (microwave of 10 µl virus only for 4 × 30 seconds) were used as positive controls.

Toxicity screening

Reference toxins for biological screening

Potassium dichromate (K₂Cr₂O₇) (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.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *A. franciscana* nauplii lethality assay.^[9,22] Briefly, *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. Four hundred microliters of seawater containing approximately 41 (mean 40.8, *n* = 180, SD 16.4) 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. Four hundred microliters of 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 sec. After 72 h all nauplii were sacrificed and counted to determine the total number per well. The LC₅₀ with 95% confidence limits for each treatment was calculated using probit analysis.

Reverse phase high performance liquid chromatography

Gradient RP-HPLC was performed at a flow rate of 1 mg/ml using dual LC-10 AT VP pumps (Shimadzu

HPLC class VP series), a variable wavelength programmable photodiode array detector SPD MIOA VP (Shimadzu), a CTO-IOAS VP column oven (Shimadzu), an SCL-10A VP system controller (Shimadzu) and a Bio-Rad reverse phase C₁₈ column (150 mm × 4.6 mm). The HPLC system was equipped with software class VP series version 6.12 (Shimadzu). The gradient was performed as follows: 5 min 0% methanol, followed by a 10 min gradient to 5% methanol. This was followed by a 10 min gradient to 20% methanol; 5 min gradient to 100% methanol; 100% methanol for 5 min; 5 min gradient to 0% methanol. The column temperature was maintained at 24°C throughout. A 20-µl sample was injected using the autoinjector (Shimadzu). Twenty microliter of deionized water was used as a negative control to produce the baseline curve and zero the machine prior to each experiment.

Gas chromatography-mass spectroscopy (GC-MS)

GC-MS analysis of bioactive *Petalostigma* extracts were performed as previously described.^[23] Briefly, the extracts were diluted to 1 mg/ml and 0.5 µl was separated on a nonpolar DB5-HT capillary column (20 m × 0.18 mm) with a 0.1-µm film (J and W Scientific) fitted to an AutoSystem XL GC-MS. The injector temperature was 270°C and the oven temperature was programmed at an initial temperature of 50°C for 1 min, rising at 25°C per minute to 160°C and maintained at that temperature for 1 min. The temperature was subsequently increased by 10°C per min to 300°C and maintained at that temperature for a further 4.6 min. The carrier gas was helium at a constant pressure of 5 kPa. The GC was directly interfaced with an AutoSystem XL quadrupole mass spectrometer with an interface temperature of 270°C. Sample ionization was by 70 eV electron impact and was analysed in positive mode. Structural determination was by comparison of mass spectral patterns to ChemSpider data bases.

Statistical analysis

Data are expressed as the mean ± SEM of at least three independent experiments. The paired *t*-test was used to calculate statistical significance between control and treated groups with a *P* < 0.05 considered to be statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried plant material with various solvents yielded dried plant extracts ranging from 10 mg to 275 mg [Table 1]. *P. trilocilare* extracts generally had higher yields of extracted material than *P. pubescens* extracts and fruit extracts had higher extraction yields than the leaf extracts.

Table 1: The mass of dried extracted material, the concentration of extracts after resuspension in deionised water and qualitative phytochemical screenings of solvent extractions

Extract	Mass of dried extract (mg)	Resuspended extract concentration (mg/ml)	Total phenolics	Water soluble	Water insoluble	Cardiac glycosides	Saponins	Triterpenes	Polysteroids	Alkaloids (Meyer test)	Alkaloids (Wagners test)	Flavanoids	Tannins	Free anthraquinones	Combined anthraquinones
<i>P. pubescens</i> (L)															
Methanol	90.0±4.8	9.0±0.48	+++	+++	+	++	+++	-	-	-	-	++	+++	++	+
Water	87.3±19.9	8.7±2.0	+	+	-	+	++	-	-	-	-	++	+++	++	+
Ethyl acetate	23.1±5.1	2.3±0.5	-	-	-	+	+	-	-	-	-	-	-	+	+
Chloroform	46.5±11.9	4.7±1.2	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	20.3±4.6	2.0±0.5	+	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. pubescens</i> (F)															
Methanol	154.4±36.0	15.4±3.6	++	++	+	+	++	-	-	+	+	+	+++	++	++
Water	64.8±6.9	6.5±0.7	+	+	-	+++	+	-	-	-	-	++	+++	++	++
Ethyl acetate	24.3±5.3	2.4±0.5	+	+	-	+	+	-	-	-	-	++	++	-	-
Chloroform	79.7±13.7	8.0±1.4	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	10.6±1.7	1.1±0.2	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. triloculare</i> (L)															
Methanol	123.2±14.8	12.3±1.5	+++	+++	+	++	+++	-	-	-	-	++	+++	++	+
Water	111.6±24.1	11.2±2.4	+	+	-	+	++	-	-	-	-	++	+++	++	+
Ethyl acetate	40.3±4.2	4.0±0.4	-	-	-	+	+	-	-	-	-	-	-	+	+
Chloroform	92.3±18.8	9.2±1.9	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	36.1±7.7	3.6±0.8	+	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. triloculare</i> (F)															
Methanol	272.7±31.8	27.3±3.2	++	++	+	+	++	-	-	+	+	+	+++	++	++
Water	86.6±16.4	8.7±1.6	+	+	-	+++	+	-	-	-	-	++	+++	++	++
Ethyl acetate	47.7±6.0	4.8±0.6	+	+	-	+	+	-	-	-	-	++	++	-	-
Chloroform	105.7±18.3	10.6±1.8	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	22.3±5.2	2.2±0.5	-	-	-	-	-	-	-	-	-	-	-	-	-

(L) indicates leaf extract, (F) indicates fruit extract, +++ indicates a large response, ++ indicates a moderate response, + indicates a minor response, - indicates no response in the assay

Methanol, deionised water and chloroform gave relatively high yields of dried extracted material (up to approximately 275 mg for *P. triloculare* fruit methanolic extract) while hexane extracted the lowest mass (as low as approximately 10 mg for *P. pubescens* fruit hexane extract). The dried extracts were resuspended in 10 ml of deionised water resulting in the extract concentrations shown in Table 1.

Phytochemical studies [Table 1] show that methanol extracted the widest range and largest amount of phytochemicals in this study. The methanol extracts of both leaves and fruit for both plants showed moderate to high levels of water-soluble phenolics, saponins and tannins, with lower levels of flavonoids and cardiac glycosides present. Similar classes of phytochemicals were detected in the water and ethyl acetate extracts, although generally at lower levels. Alkaloids were only detected in the methanol extract of the fruits of both plants, and only at low levels. No alkaloids were detected in extracts using any other solvent, or in the leaf extracts. The chloroform and hexane

extracts showed only low levels of any class of extracted phytochemicals tested.

Antibacterial activity

Aliquots (10 µl) of dilutions of each extract were tested in the disc diffusion assay against 14 bacteria and the MIC's were determined [Table 2]. The methanol, water and ethyl acetate extracts all displayed broad-spectrum antibacterial activity, inhibiting the growth of ≥ 7 of the 14 bacteria tested. The number of bacterial species inhibited was higher for the methanol and ethyl acetate leaf extracts (10 out of 14 bacteria tested; 71%) than in the methanol and ethyl acetate fruit extracts (9 out of 14 bacteria tested; 64%). Similarly, the water leaf extracts inhibited the growth of 8 of the 14 bacteria tested (57%) compared to 7 bacteria inhibited (50%) for the water fruit extracts. No inhibitory activity was evident for any of the chloroform or hexane extracts at the doses tested. The methanol, water and ethyl acetate extracts were all potent inhibitors of antibacterial growth, with

Table 2: Minimum inhibitory concentrations ($\mu\text{g/ml}$) of *P. pubescens* and *P. triculore* fruit and leaf extracts against susceptible bacteria

	Gram-negative bacteria										Gram-positive bacteria			
	Rods										Rods	Cocci		
	<i>A. faecalis</i>	<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. newport</i>	<i>S. marcescens</i>	<i>S. sonnei</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>S. pyogenes</i>
<i>P. pubescens</i> (leaf)														
Methanol	0.7	1.2	0.6	-	1	0.3	0.5	-	1.4	-	0.4	1.2	0.3	-
Water	4.5	1.9	-	-	1.2	4.2	10.2	-	11	-	3.8	3.1	-	-
Ethyl acetate	0.4	0.4	0.2	-	0.3	0.2	0.4	-	0.8	-	0.4	0.8	0.2	-
Chloroform	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. pubescens</i> (fruit)														
Methanol	-	2.4	1.1	-	1	1.3	0.8	-	1.9	-	2.3	1.6	0.8	-
Water	-	4.2	-	-	9.5	1.7	4.1	-	11.1	-	11.3	11.2	-	-
Ethyl acetate	-	0.4	0.4	-	0.1	0.6	0.4	-	10.4	-	1.1	0.9	0.4	-
Chloroform	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. triculare</i> (leaf)														
Methanol	0.6	1.1	0.6	-	1	0.3	0.5	-	1.5	-	0.4	1.3	0.3	-
Water	4.7	1.8	-	-	1.1	3.7	9.8	-	10.3	-	3.4	3.3	-	-
Ethyl acetate	0.5	0.4	0.2	-	0.3	0.2	0.4	-	0.8	-	0.4	0.8	0.2	-
Chloroform	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. triculare</i> (fruit)														
Methanol	-	2.1	1	-	1	0.7	0.8	-	2.1	-	0.7	1.4	1.2	-
Water	-	2.5	-	-	9.4	0.5	3.8	-	10.4	-	4.8	8.5	-	-
Ethyl acetate	-	0.2	0.2	-	0.1	0.2	0.3	-	0.7	-	0.4	0.7	0.1	-
Chloroform	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Numbers indicate the mean MIC values of at least triplicate determinations. - indicates no growth inhibition

MIC values generally $<10 \mu\text{g/ml}$. Ethyl acetate extracts were particularly potent, achieving MIC values less than $1 \mu\text{g/ml}$ for nearly every bacterial species for which they displayed antibacterial activity.

The methanol and ethyl acetate extracts inhibited the growth of Gram-positive and Gram-negative approximately equally. Methanolic and ethyl acetate leaf extracts of both species inhibited the growth of 7 of the Gram-negative bacteria tested (70%) compared with 3 of the 4 Gram-positive bacteria tested (75%). Similarly, *P. pubescens* and *P. trilobulare* methanol and ethyl acetate fruit extracts inhibited the growth of 6 of the Gram-negative bacteria tested (60%) and 3 Gram-positive bacteria (75%). The *P. pubescens* and *P. trilobulare* water leaf and fruit extracts inhibited the growth of 6 (60%) and 5 (50%) of the Gram-negative bacteria tested respectively. Similarly, they inhibited 2 (50%) of the Gram-positive bacteria tested.

Antiviral activity

Figure 2 shows the formation of MS2 bacteriophage plaques in the presence of the *Petalostigma* extracts ($1000 \mu\text{g/ml}$) or the positive and negative controls as a % of control plaque formation. The methanol, water and ethyl acetate extracts of the leaf and fruit of both *Petalostigma* species displayed antiviral activity when tested in the MS2 plaque bioassay. The water and ethyl acetate extracts were particularly effective at inhibiting MS2 plaque formation, inhibiting approximately 85-97% (for the water extracts) and $100 \pm 0\%$ (for ethyl acetate extracts) of the plaque formation, respectively. The methanol extracts were also effective, inhibiting between 27% (PTLM) and 49% (PPFM) of plaque formation. In contrast, the *C. sinensis* control inhibited approximately 91% of bacteriophage plaque formation.

The *P. pubescens* and *P. trilobulare* 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 3]. The minimum dosage of the positive control (*C. sinensis* leaf extract) capable of totally blocking 100% MS2 plaque formation (PI_{100}) was 2960 $\mu\text{g/ml}$ while the minimum concentration of *C. sinensis* leaf extract capable of blocking 50% of MS2 plaque formation (PI_{50}) was 590 $\mu\text{g/ml}$. All of the *Petalostigma* water and ethyl acetate extracts were more effective antiviral agents than the *C. sinensis* control as determined by their lower PI_{50} values. The water extracts of the fruit proved the most effective, with a PI_{50} values of 220 $\mu\text{g/ml}$ and 180 $\mu\text{g/ml}$ for the *P. pubescens* and *P. trilobulare* ethyl acetate extracts respectively. The order of effectiveness of MS2 plaque reduction was *P. trilobulare* fruit water extract > *P. pubescens* fruit water extract > *P. trilobulare* fruit ethyl acetate extract > *P. pubescens* fruit ethyl acetate extract > *P. pubescens* leaf ethyl acetate extract > *P. pubescens* leaf water extract > *P. trilobulare* leaf ethyl acetate extract > *P. trilobulare* leaf water extract > *C. sinensis* extract > *P. pubescens* fruit methanol extract > *P. trilobulare* fruit methanol extract > *P. pubescens* leaf methanol extract > *P. trilobulare* leaf methanol extract.

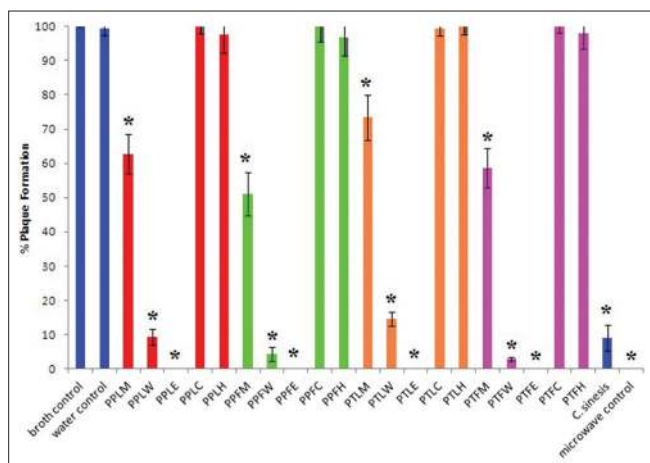


Figure 2: MS2 plaque formation in a *E. coli* lawn expressed as % of control plaque formation following incubation of the MS2 with nutrient broth (negative control); deionized water (negative control); (PPLM) *P. pubescens* leaf methanol extract; (PPLW) *P. pubescens* leaf water extract; (PPLE) *P. pubescens* leaf ethyl acetate extract; (PPLC) *P. pubescens* leaf chloroform extract; (PPLH) *P. pubescens* leaf hexane extract; (PPFM) *P. pubescens* fruit methanol extract; (PPFW) *P. pubescens* fruit water extract; (PPFE) *P. pubescens* fruit ethyl acetate extract; (PPFC) *P. pubescens* fruit chloroform extract; (PPFH) *P. pubescens* fruit hexane extract; (PTLM) *P. trilobulare* leaf methanol extract; (PTLW) *P. trilobulare* leaf water extract; (PTLE) *P. trilobulare* leaf ethyl acetate extract; (PTLC) *P. trilobulare* leaf chloroform extract; (PTLH) *P. trilobulare* leaf hexane extract; (PTFM) *P. trilobulare* fruit methanol extract; (PTFW) *P. trilobulare* fruit water extract; (PTFE) *P. trilobulare* fruit ethyl acetate extract; (PTFC) *P. trilobulare* fruit chloroform extract; (PTFH) *P. trilobulare* fruit hexane extract; *C. sinensis* leaf water extract (1000 $\mu\text{g/ml}$); nutrient agar and microwave irradiated MS2 phage (positive control). All *Petalostigma* extracts were tested at 1000 $\mu\text{g/ml}$. Results are reported as the mean of triplicate assays \pm SEM. * indicates a statistically significant difference to the negative control ($P < 0.05$)

Quantification of toxicity

P. pubescens and *P. trilobulare* leaf and fruit extracts were serially diluted in artificial seawater for toxicity testing in the *Artemia* nauplii lethality bioassay. For comparison, the reference toxins potassium dichromate and Mevinphos were also tested in the bioassay. Table 4 shows the extract or control toxin concentrations required to achieve 50% mortality (LC_{50}) at various times. Potassium dichromate and Mevinphos (reference toxins) were rapid in their onset of mortality. Both reference toxins induced mortality within the first 3 hours of exposure while greater than 12 hours was required for mortality induction by the *Petalostigma* extracts (results not shown). As toxicity of crude plant extracts has previously been defined as 24 or 48 h LC_{50} values < 1000 $\mu\text{g/ml}$,¹²⁴ the measured LC_{50} values indicate that all *Petalostigma* extracts were non-toxic or of low toxicity. Only the *P. trilobulare* leaf and fruit ethyl acetate extracts displayed LC_{50} values below 1000 $\mu\text{g/ml}$ at 24 and/or 48 h and even these LC_{50} values indicate low toxicity. All *Petalostigma* methanolic extracts also induced mortality above that of the seawater control, although their

Table 3: Extract and control concentrations ($\mu\text{g/ml}$) required to achieve 100% (PI_{100}) and 50% (PI_{50}) MS2 plaque reduction

Extract	PI_{100}	PI_{50}
<i>P. pubescens</i> (leaf)		
Methanol	2160	1230
Water	1560	390
Ethyl acetate	760	350
Chloroform	-	-
Hexane	-	-
<i>P. pubescens</i> (fruit)		
Methanol	1610	1040
Water	1480	220
Ethyl acetate	740	340
Chloroform	-	-
Hexane	-	-
<i>P. trilobulare</i> (leaf)		
Methanol	2510	1630
Water	1780	570
Ethyl acetate	840	470
Chloroform	-	-
Hexane	-	-
<i>P. trilobulare</i> (fruit)		
Methanol	1720	1070
Water	1250	180
Ethyl acetate	680	290
Chloroform	-	-
Hexane	-	-
Controls		
Broth control	-	-
Water control	-	-
<i>C. sinensis</i>	2960	490

Numbers indicate the means of at least triplicate determinations. - indicates no plaque reduction

Table 4: LC₅₀ (95% confidence interval) for brine shrimp nauplii exposed to *P. pubescens* and *P. trilobulare* fruit and leaf extracts, the reference toxins potassium dichromate and Mevinphos, and a seawater control

Extract	LC ₅₀ (µg/ml)		
	24 hours	48 hours	72 hours
<i>P. pubescens</i> (leaf)			
Methanol	1622	1278	375
Water	-	-	-
Ethyl acetate	1568	1240	385
Chloroform	-	-	-
Hexane	-	-	-
<i>P. pubescens</i> (fruit)			
Methanol	-	-	1500
Water	-	-	-
Ethyl acetate	1523	1281	373
Chloroform	-	-	-
Hexane	-	-	-
<i>P. trilobulare</i> (leaf)			
Methanol	-	1245	608
Water	-	-	-
Ethyl acetate	814	685	312
Chloroform	-	-	-
Hexane	-	-	-
<i>P. trilobulare</i> (fruit)			
Methanol	-	1636	654
Water	-	-	-
Ethyl acetate	1650	834	188
Chloroform	-	-	-
Hexane	-	-	-
Controls			
Mevinphos	1346	505	104
Potassium dichromate	264	36	14
Seawater control	-	-	-

- denotes values that were not obtained as $\geq 50\%$ mortality was not obtained at this time point

LC₅₀ values indicate that they are nontoxic. The water, chloroform and hexane extracts not induce mortality above that of the seawater controls at any time point measured and are therefore considered nontoxic.

RP-HPLC analysis of extracts displaying antimicrobial activity

As the *P. trilobulare* extracts displayed the greatest antibacterial activity, the methanol and ethyl acetate leaf and fruit extracts of this plant were further analysed by RP-HPLC. The *P. trilobulare* fruit methanol extract chromatogram [Figure 3b] revealed numerous overlapping peaks, particularly in the very early stages of the chromatogram corresponding to the elution of polar compounds. The *P. trilobulare* leaf methanol extract [Figure 3a] also had large amounts of polar material eluting early in the chromatogram. However, a large peak later in the chromatogram indicates the broad spread of

polarities of the compounds in the leaf methanol extract. Similarly, the *P. trilobulare* ethyl acetate leaf extract [Figure 3c] and *P. trilobulare* ethyl acetate fruit extract [Figure 3d] also revealed a broad spread of compound polarities. As the ethyl acetate extracts contained much less extracted material yet still retained the same degree of bioactivity, the *P. trilobulare* ethyl acetate extracts were deemed suitable for further analysis by GC-MS. Furthermore, the ethyl acetate extracts also displayed much greater antiviral activity, making them of interest for further study.

GC-MS analysis of the *P. trilobulare* ethyl acetate extracts

Gas chromatography of the ethyl acetate extract [Figure 4] resulted in the separation of 11 main peaks for the *P. trilobulare* ethyl acetate leaf extract and 9 main peaks for the *P. trilobulare* ethyl acetate fruit extract. As fewer peaks were evident for the fruit extract gas chromatogram yet this extract had stronger antibacterial and antiviral bioactivities, the *P. trilobulare* ethyl acetate fruit extract peaks were further analysed by MS [Figure 5]. Structural identification was achieved for all 9 of the main compound peaks detected in the *P. trilobulare* ethyl acetate fruit extract. The major peak from the gas chromatogram had a retention time of 3.22 min. MS analysis [Figure 5d] determined the mass of this compound to be 142 and the empirical formula to be C₁₀H₂₂. A comparison with MS data bank ionization patterns showed similarity with the 10 carbon hydrocarbon decane. Another major peak with a retention time of 5.43 min [Figure 5h] was determined to have a mass of 126 and the empirical formula C₆H₆O₃. A comparison with MS data bank ionisation patterns showed similarity with 1, 2, 3-benzenetriol (pyrogallol). Other compounds in the extract were determined to be acetic acid (retention time 1.14; Figure 5a), 2,2-dimethoxybutane (retention time 1.57 min; Figure 5b), 4-methyl-1,3-dioxane (retention time 2.17 min; Figure 4c) and the straight chain hydrocarbon unadecane (retention time 3.88 min; Figure 5e). 2-furanmethanol (retention time 3.97; Figure 4f), 1,2-benzenediol (catechol) (retention time 4.43; Figure 5g) and benzoic acid (p-salicylic acid) (retention time 6.11; Figure 5i) were also determined to be present in the *P. trilobulare* ethyl acetate fruit extract.

DISCUSSION

The current study reports on the antimicrobial and antiviral activities of various *Petalostigma* leaf and fruit extracts, and on their toxicity. The ability of *P. pubescens* and *P. trilobulare* 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

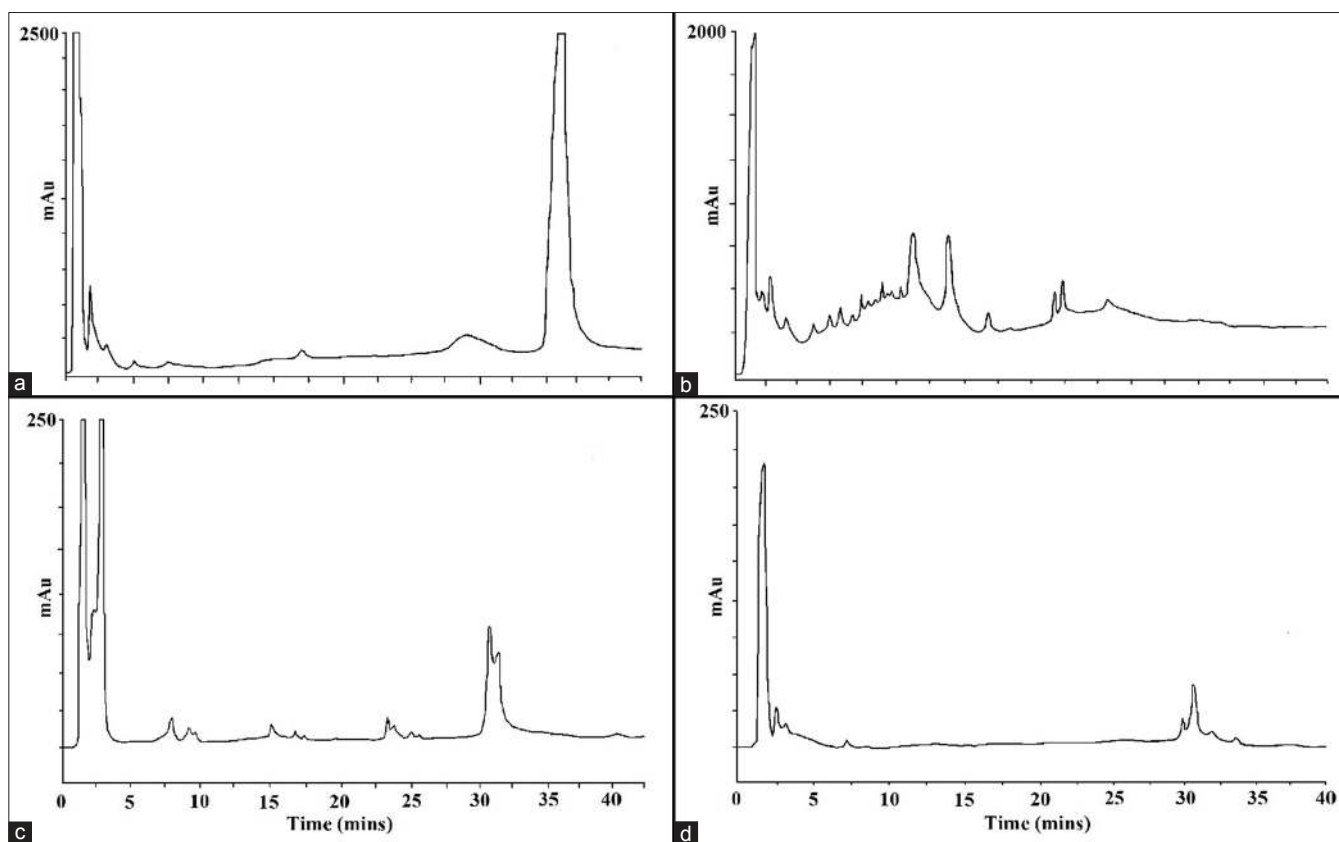


Figure 3: RP-HPLC chromatogram of 20 μ l injections of (a) *P. triliculore* methanol leaf extract, (b) *P. triliculore* methanol fruit extract, (c) *P. triliculore* ethyl acetate leaf extract, (d) *P. triliculore* ethyl acetate fruit extract. Extracts were dried and resuspended in deionized water. Absorbance was recorded at 210 nm and chromatography conditions were as described in the methods section

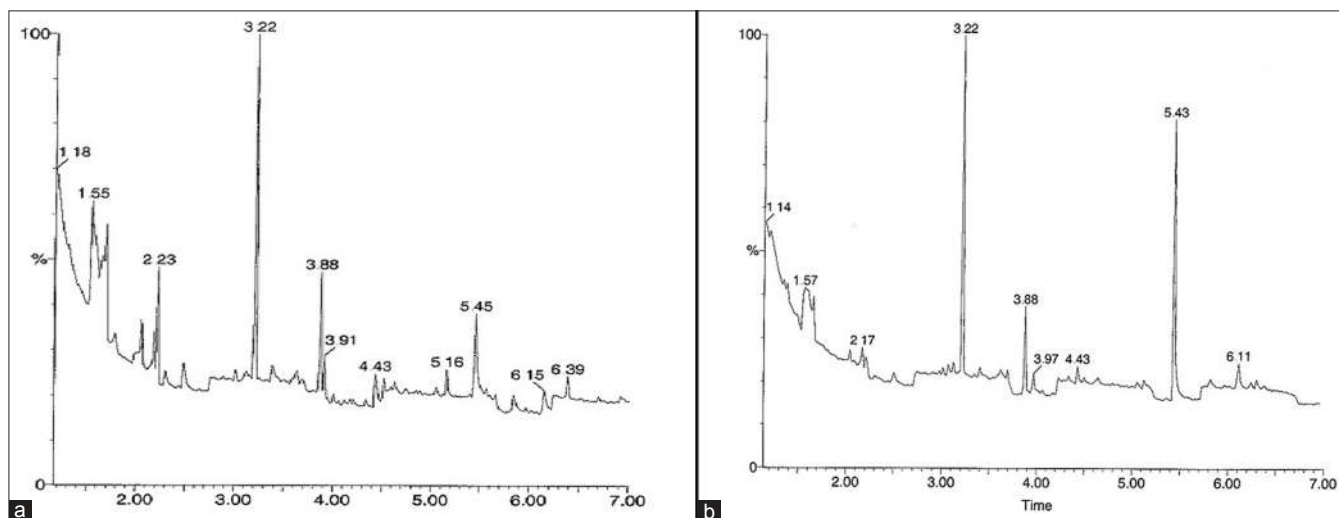


Figure 4: Gas chromatogram of 0.5 μ l injections of (a) *P. triliculore* ethyl acetate leaf extract and (b) *P. triliculore* ethyl acetate fruit extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section

Australian native plants that have a history of medicinal usage by Australian Aborigines. The antiseptic properties of the Eucalypts,^[25] Leptospermums^[8,26] and Melaleucas^[27] have been extensively studied and shown to inhibit the growth of a wide variety of bacteria. The current study shows Gram-positive and Gram-negative bacteria to

be equally susceptible to *P. pubescens* and *P. triliculore* extracts. This is in contrast to previous studies which have reported a greater susceptibility of Gram-positive bacteria toward solvent extracts for South American,^[28] African^[29] and Australian^[30] plant extracts. Results within this laboratory^[8] have also confirmed the greater susceptibility

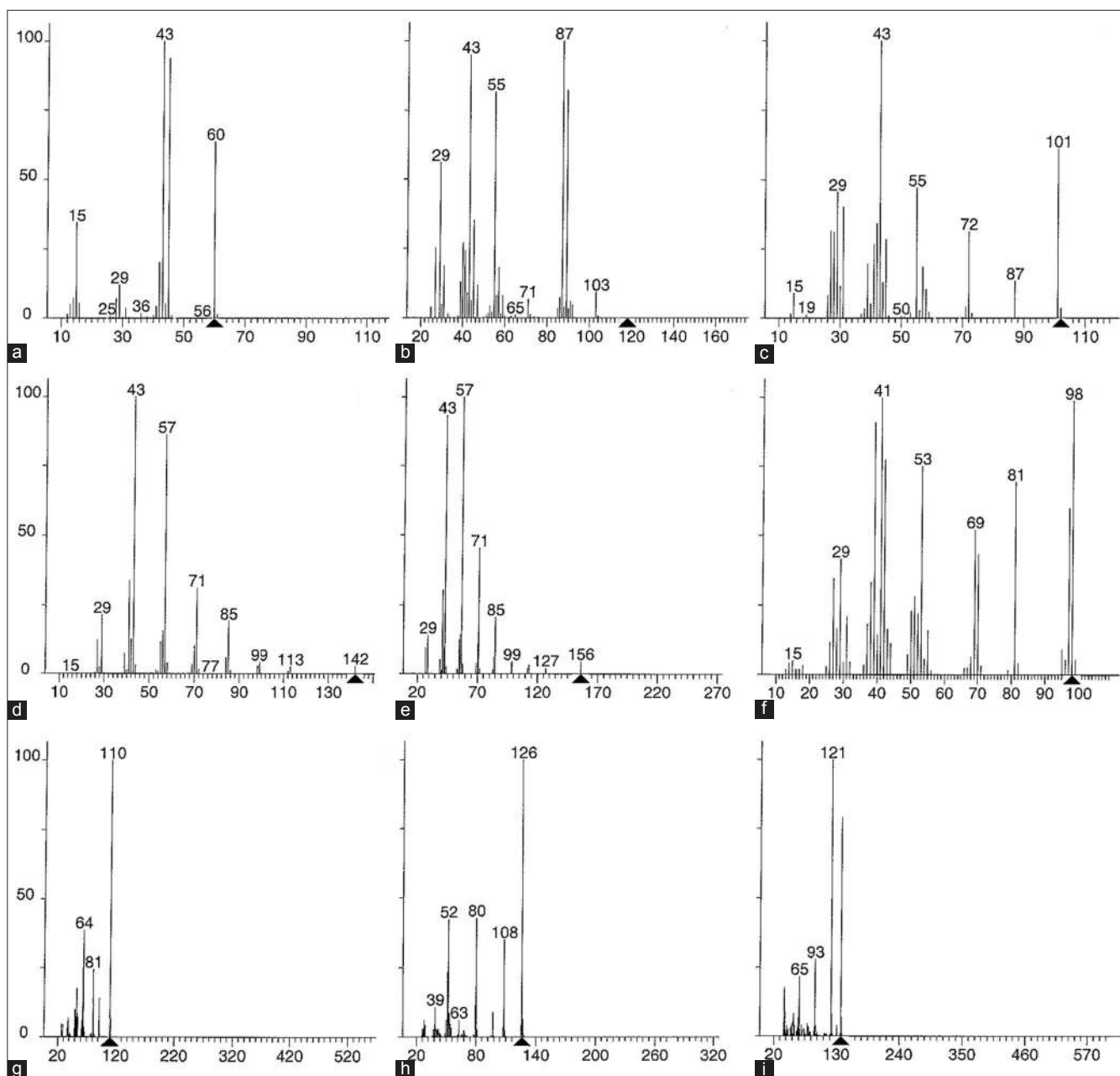


Figure 5: Mass spectra of *P. triloculare* ethyl acetate fruit extract peaks from GC separation. The spectra correspond to peaks from the GC chromatogram with retention times of (a) 1.14 min, (b) 1.57 min, (c) 2.17 min, (d) 3.22 min, (e) 3.88 min, (f) 3.97 min, (g) 4.43 min, (h) 5.43 min, (i) 6.11 min

of Gram-positive bacteria toward other Australian plant extracts.

The methanol, water and ethyl acetate solvent extracts also displayed antiviral activity toward the RNA virus MS2 bacteriophage. While no previous reports of antiviral activity of *Petalostigma* extracts were found, previous studies have reported antiviral activity of extracts of another Australian species of Euphorbiaceae (*Euphorbia australis*) toward the DNA virus cyclomeglovirus (CMV).^[31] Interestingly, no antiviral activity toward the RNA viruses

tested in the earlier study (Ross River virus and polio virus type 1) was reported. Neither the current study nor the earlier study examined the mechanism of 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, reverse transcription of viral RNA (in the case of RNA viruses), viral nucleic acid or protein synthesis, viral assembly and release). Further studies are required to determine the antiviral mechanism of the *Petalostigma* extracts. Furthermore, while the current studies have detected

antiviral activity toward MS2 bacteriophage and the earlier study^[31] has shown anti-CMV activity, the antiviral specificity is yet to be determined toward other viruses of medicinal importance. Future studies need to rigorously test *Petalostigma* extracts against a wider panel of viruses to determine their medicinal potential.

The findings reported here also demonstrate that none of the *P. pubescens* and *P. triliculore* extracts displayed significant toxicity toward *A. franciscana*. Indeed, only the *P. triliculore* leaf and fruit ethyl acetate extracts displayed LC₅₀ values below 1000 µg/ml at 24 and/or 48 h, and even these LC₅₀ values indicate low toxicity. While still classified as lowly toxic, it is evident that the *P. triliculore* ethyl acetate leaf extract did induce mortality above that of the seawater control at 24 h. Therefore, further toxicity studies against human cell lines are warranted to determine the suitability of these *P. triliculore* extracts for medicinal purposes as antimicrobial agents.

Of the compounds detected by GC-MS in the bioactive *P. triliculore* ethyl acetate fruit extract, several have been reported to have medicinally important bioactivities. Benzoic acid and its derivatives have previously been used in the treatment of fungal disorders including tinea, ringworm and athlete's foot.^[32,33] They were also used as expectorants, analgesics and antiseptics until the early part of the 20th century.^[32,34] Furthermore, sodium benzoate (a soluble benzoic acid salt) is used as a common antimicrobial preservative in foods, although its usage is limited to ≤ 0.1% due to its toxicity.^[32,35] Similarly, the benzene alcohols detected in the *P. triliculore* ethyl acetate fruit extract (1,2-benzenediol (catechol) and 1,2,3-benzenetriol (pyrogallol) also have documented antiseptic properties.^[32] They are also powerful reducing agents and therefore have potential applications in protection against oxidative stress. Furthermore, catechol is also a component of some pesticides and a precursor for several pharmaceuticals.

Several other compounds detected in the *P. triliculore* ethyl acetate fruit extract are toxic and may therefore contribute to the limited toxicity reported in this extract. The unbranched (decane, undecane) paraffins detected in this study have previously been reported to be toxic.^[36] The branched ether 2,2-dimethoxybutane has also been shown to be toxic. Similarly, acetic acid has previously been shown in our laboratory to induce mortality in *A. franciscana* nauplii and is routinely used to induce mortality in the assay prior to total nauplii counts.^[37]

Also of interest is the 2-furanmethanol detected in the *P. triliculore* ethyl acetate fruit extract. Many furans have been shown to have strong bacterial growth inhibitory activity. Furfural (an oxidised form of 2-furanmethanol) has potent antibacterial activity, inhibiting the growth of

some microorganisms at concentrations as low as 1mM.^[38] 2-furanmethanol also has demonstrated antibacterial activity against bacteria,^[38] albeit at slightly higher concentrations than those reported for furfural. Various other furan derivatives were also been shown to be good antibacterial agents.^[39] No reports were found of antiviral activity of furan compounds, except as components of substituted nucleosides to block reverse transcription of RNA viruses.^[40] This study also reported that the presence of the ribo-sugar moiety was of absolute importance for the antiviral activity of the substituted nucleosides, indicating that the antiviral activity of the *P. triliculore* ethyl acetate fruit is likely to be due to components other than 2-furanmethanol.

The phytochemical 4-methyl-1,3-dioxane detected in *P. triliculore* ethyl acetate fruit extract may also contribute to the antibacterial and antiviral activity reported in this study. A number of dioxane^[41] and dioxolane^[42] compounds have previously been shown to have good antibacterial and antifungal activities. Furthermore, several dioxane compounds have been reported to have potent antiviral activities, inhibiting Sindbis virus at concentrations as low as 40 µM.^[43] It is believed that the dioxane-based antiviral agents tested against Sindbis virus blocked virus production by targeting the hydrophobic-binding pocket of the capsid protein. While no reports were found of antiviral activity of 4-methyl-1,3-dioxane, it is possible that it may act in a similar manner in the MS2 phage assay, blocking virus production, although this is yet to be tested.

CONCLUSIONS

The results of this study partially validate the traditional Australian Aboriginal usage of *Petalostigma* species to treat bacterial and viral diseases and indicate that *P. pubescens* and *P. triliculore* are worthy of further study. Further evaluation of the antibacterial and antiviral properties of these extracts against a more extensive panel of microbial agents is warranted. Likewise, purification and examination of the mechanisms of action of these agents is required. 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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