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Original Article

Growth Inhibitory Properties of *Kunzea pomifera* F.Muell. and *Podocarpus elatus* R.Br. Fruit Extracts against Axillary and Foot Odour Producing Bacteria

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ABSTRACT

Introduction: Many high antioxidant fruit extracts can inhibit the growth of multiple bacterial pathogens. They may also inhibit the growth of malodour producing bacteria and thus be useful deodorant components, although this is yet to be tested for many Australian native fruits. **Materials and Methods :** Methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts were investigated by disc diffusion and liquid dilution MIC assays against the most significant bacterial contributors to axillary and plantar malodour. Toxicity was determined using *Artemia franciscana* nauplii bioassays and unbiased HPLC-MS QTOF analysis was used to identify interesting consituents of the most active extract. **Results:** Methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts displayed noteworthy bacterial growth inhibitory activity against all of the malodour forming bacteria tested. The methanolic *K. pomifera* extract had particularly good antibacterial effects, strongly inhibiting the growth of all bacteria, with MIC values substantially less than 1000 µg/mL. Indeed, liquid dilution (LD) MIC values of 610, 894, 663 and 625 µg/mL were recorded against C. *jeikeium, P. acnes, B. linens* and *S. epidermidis* respectively. Similar, albeit slightly higher LD MIC values were noted for the aqueous *K. pomifera* fruit extract, and for the methanolic and aqueous *P. elatus* fruit extracts against these bacteria. All *K. pomifera* and *P. elatus* fruit extracts were non-toxic in the *Artemia fransiscana* bioassay. Several interesting phytochemicals, including several tannins, were identified in the methanolic *K. pomifera* fruit extract. **Conclusion:** The lack of toxicity of the methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts and their noteworthy growth inhibition of axillary and plantar malodour producing bacteria indicate their potential as deodorant components. Further studies are warranted to isolate and identify the active components and to determine the antibacterial mechanism.

Keywords: Muntries, Illawarra plum, Body odour, Deodorant, *Corneybacterium jeikeium*, *Propionobacter acnes, Brevibacter linens, Staphylococcus epidermidis.*

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INTRODUCTION

Axillary and plantar malodours have social consequences and may lead to social exclusion and mental health issues. To address these odours, sales of commercial preparations (antiperspirants and deodarants) account for one of the largest sectors of the cosmetic industry globally. Multiple synthetic compounds (including propylene glycol, triclosan, benzalkonium chloride and metal (e.g. Al) salts) are currently included in deodorants and antiperspirants to decrease sweating, as well inhibiting the growth of skin bacteria, with the aim of decreasing odour formation.[1] Notably, the safety of many of these additives has yet to be definitively verified. Indeed, the use of several of these additives is considered to be hazardous to human health. Aluminium salts have been linked with a wide range of negative side-effects, including degenerative neurological conditions (e.g. Alzheimers disease, encephalopathy)[2] and cancer.[3] Aluminium salt additives may also cause respiratory problems and induce anaphylactic shock in susceptible individuals.[2] There are also concerns about chronic exposure to triclosan (a common bacterial growth inhibitor in many deodorants) due to reports of dermal irritation and allergies.[4] Natural alternatives that inhibit the growth of odour forming bacteria may be less concern for human health, and may also be more acceptable to consumers due to their natural origin. Indeed, there has been a recent trend to search natural resources for safe and effective plant preparations to replace these synthetic additives and several promising plant species have been identified.[5-8]

Recent studies have highlighted the antibacterial properties of multiple fruits with high antioxidant contents. Extracts prepared using *Terminalia ferdinandiana* Exell. fruit, which contain the highest ascorbic acid levels of any plant in the world (up to 6 % of wet weight), have been reported to have potent inhibitory activity against multiple human pathogens.[9-15] Similarly, *Tasmannia lanceolata* (Poir.) Sm.,[10,16] and multiple *Syzygium* spp.[17-19] are also good inhibitors of a broad-spectrum of bacterial pathogens. Interestingly, extracts produced from all of these plant species have also been reported to be potent inhibitory of *Corynebacterium* spp., *Staphylococcus* spp., *Propionibacterium* spp. and *Staphylococcus* spp. growth, which are major contributors to axillary and plantar odour formation.[5-8] Numerous other Australian plants with high antioxidant capacities have also been identified and have been screened against limited panels of bacterial pathogens relevant to human health,[20,21] although these plants are yet to be screened against malodour forming bacteria.

In particular, the fruit of *Kunzea pomifera* (commonly called muntries) and *Podocarpus elatus* (commonly known as Illawarra plum and brown plum) have been highlighted for their therapeutic potential as they have similar antioxidant capacities to blueberries, which are themselves considered to have a high antioxidant capacity.[22] The inhibitory activity of extracts prepared from the fruits of these plants has been documented against limited bacterial panels,[20] and screening against other bacterial species is warranted. This study aimed to test solvent extracts produced from *K. pomifera* and *P. elatus* fruit against the axillary and foot odour forming bacteria *Corneybacterium jeikeium, Propionibacterium acnes, Brevibacterium linens* and *Staphylococcus epidermidis,* with the aim of identifying potential safe and effective deodorant additives`.

MATERIALS AND METHODS

Plant Source and Extraction

The *Kunzea pomifera* F. Meull. (muntries) and *Podocarpus elatus* R.Br. (Illawarra plum) fruit used in this study were purchased from Taste of Australia Bush Food, Australia. The fruit were washed in deionised water and thoroughly dehydrated using a Sunbeam food dehydrator. The dried material was stored at -30°C until use. The dried fruit were freshly ground to a coarse powder prior to extraction. Individual 1 g quantities of the ground fruit were weighed separately into tubes and 50 mL of methanol (AR grade, Ajax Fine Chemicals, Australia) or sterile deionised water were added. The fruits were extracted in each solvent for 24 hr at 4°C with gentle shaking. The extracts were filtered through filter paper (Whatman No. 54) under vacuum, followed by drying in a vacuum oven at 50°C. The resultant dry extracts were weighed and redissolved in 10 mL of deionised water (containing 0.5 % DMSO), and passed through a 0.22 µm filter (Sarstedt) to remove particulates. The extracts were then stored at 4°C until use.

Qualitative Phytochemical Studies

Phytochemical analysis of the *K. pomifera* and *P. elatus* fruit extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved as previously described.[23-25]

Antioxidant Capacity

The antioxidant capacity of each sample was evaluated using the DPPH free radical scavenging method[26] with modifications. Briefly, DPPH was prepared fresh as a 400 µM solution by dissolving DPPH (Sigma) in AR grade methanol (Ajax, Australia). The initial absorbance of the DPPH solution was measured at 515 nm using a Molecular Devices, Spectra Max M3 plate reader and did not change significantly throughout the assay period. A 2 mL aliquot of each extract was dried by evaporation and the residue resuspended in 2 mL of methanol. Each extract was added to a 96-well plate in triplicate as of 5, 10, 25, 50, 75 µL volumes. Methanol was added to each well to give a volume of 225 µL. A volume of 75 µL of the fresh DPPH solution was then added to each well to give a total reaction volume of 300 µL. A blank of each extract concentration, methanol solvent, and DPPH was also performed in triplicate. Ascorbic acid (Sigma, Australia) was prepared fresh each day and examined across the range 0-25 µg per well as a reference. The absorbances were recorded at 515 nm. All tests were performed in triplicate (n = 3) and triplicate controls were included on each plate. The antioxidant capacity based on DPPH free radical scavenging ability was determined for each extract and expressed as µg ascorbic acid equivalents per gram of original plant material extracted.

Antibacterial Screening

Test bacterial strains

All media and components was supplied by Oxoid Ltd., Australia. Reference strains of *Brevibacterium linens* (ATCC9172), *Corneybacterium jeikeium* (ATCC43734) and *Propionibacterium acnes* (ATCC6919) were purchased from American Type Culture Collection, USA. The clinical isolate strain of *Staphylococcus epidermidis* used in this study was supplied by Ms. Jane Gifkins of the School of Environment and Science, Griffith University, Australia. To culture the bacteria, *B. linens* and *S. epidermidis* were inoculated into separate flasks of nutrient broth and grown aerobically at 37°C for 24 hr. Cultures of *C. jeikeium* were grown and maintained in nutrient broth supplemented with 300 µL Tween 80/L of broth at 37°C for 24 hr. *Propionibacterium acnes* was cultured using a thioglycollate liquid media under induced anaerobic conditions through the use of anaerobic jars and AnaeroGen™ 3.5 L atmospheric generation systems (Thermo Scientific). Incubation was at 37°C for 72 hr. All stock cultures were maintained and subcultured in liquid media at 4°C.

Evaluation of Antibacterial Activity

Antibacterial activity screening of the *K. pomifera* and *P. elatus* fruit extracts was achieved using modified disc diffusion assays.[27-29] Briefly, 100 µL of each individual bacteria was grown separately in 20 mL of the appropriate broth until an approximate count of 10^8 cells/mL was reached. A volume of 100 µL of each bacterial suspension was spread onto agar plates and the extracts were tested for antibacterial activity using 6 mm sterilised filter paper discs. *Brevibacterium linens, S. epidermidis* and *P. acnes* cultures were spread onto nutrient agar plates. *Corneybacterium jeikeium* cultures were spread onto nutrient agar plates supplemented with 300 µL Tween 80/L of agar. Discs were infused with 10 µL of the individual extracts, allowed to dry, and placed onto the inoculated plates. The plates were left to stand at 4°C for 2 hr before incubation. Plates inoculated with *B. linens*, *S. epidermidis* or *C. jeikeium* cultures were incubated aerobically at 37°C for 24 hr. Plates spread with *P. acnes* cultures were incubated under induced anaerobic conditions at 37°C for 72 hr. The diameters of the inhibition zones (ZOIs) were measured to the closest whole millimetre. Each assay was completed three times, each with internal triplicates (n = 9). Mean values (± SEM) are reported in this study. Ampicillin (10 µg) and vancomycin (5 µg) discs were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water (containing 0.5 % DMSO) were used as negative controls.

Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration for each extract was determined using two methods. A liquid dilution MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay.[30] Furthermore, as microplate liquid dilution (LD) MIC assays are perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method allows for comparisons with other studies. A solid phase agar disc diffusion (DD) assay was also used in this study as this bioassay was deemed to provide a closer representation of the environment and conditions relevant to solid axillary and foot skin systems.

Microplate Liquid Dilution MIC Assay

The MICs of the extracts were evaluated by standard methods.[30,31] Briefly, overnight bacterial cultures were added dropwise to fresh liquid broth and the turbidity was visually adjusted to produce a McFarlands number 1 standard culture. This was subsequently diluted 1 in 50 with fresh broth, resulting in the MIC assay inoculum culture. A volume of 100 µL sterile broth was added to all wells of a 96 well plate. Test extracts or control antibiotics (100 µL) were then added to the top row of each plate and 1 in 2 serial dilutions were prepared in each column of wells by transferring 100 µL from the top well to the next well in each column, etc. A growth control (without extract) and a sterile control (without inoculum) were included on each plate. A volume of 100 µL of bacterial culture inoculum was added to all wells except the sterile control wells. Plates inoculated with *B. linens*, *S. epidermidis* or *C. jeikeium* cultures were incubated aerobically at 37°C for 24 hr. Plates inoculated with *P. acnes* cultures were incubated under induced anaerobic conditions at 37°C for 72 hr. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma, Australia and dissolved in sterile deionised water to prepare a 0.2 mg/mL INT solution. A 40 µL volume of this solution was added into all wells and the plates were incubated for a further 6 hr at 30°C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

Disc Diffusion MIC Assay

The minimum inhibitory concentrations (MIC) of the extracts was also evaluated by disc diffusion assay as previously described.[32,33] Briefly, the *K. pomifera* and *P. elatus* fruit extracts were diluted in deionised water (containing 0.5 % DMSO) and tested across a range of concentrations. Discs were infused with 10 µL of the extract dilutions, allowed to dry and placed onto inoculated plates. The assay was achieved as outlined above and graphs of the zone of inhibition versus Ln concentration were plotted. Determination of MIC values were achieved using linear regression.

Non-targeted HPLC-MS QTOF Analysis

Chromatographic separations were performed using previously optimised parameters.[34] Briefly, 2 µL of sample was injected onto an Agilent 1290 HPLC system fitted with a Zorbax Eclipse plus C_{18} column (2.1 × 100 mm, 1.8 µm particle size). The mobile phases consisted of (A) ultrapure water and (B) 95:5 acetonitrile/water at a flow rate of 0.7 mL/min. Both mobile phases were modified with 0.1% (v/v) glacial acetic acid for mass spectrometry analysis in positive mode and with 5 mM ammonium acetate for analysis in negative mode. The chromatographic conditions utilised for the study consisted of the first 5 min run isocratically at 5% B, a gradient of (B) from 5% to 100% was applied from 5 min to 30 min, followed by 3 min isocratically at 100%. Mass spectrometry analysis was performed on an Agilent 6530 quadrapole time-of-flight spectrometer fitted with a Jetstream electrospray ionisation source in both negative and positive mode.

Data was analysed using the Masshunter Qualitative analysis software package (Agilent Technologies). Blanks using each of the solvent extraction systems were assessed using the Find by Molecular Feature algorithm in the software package to generate a compound list of molecules with abundances greater than 10,000 counts. This was then used as an exclusion list to eliminate background contaminant compounds from the analysis of the extracts. Each extract was then analysed using the same parameters using the Find by Molecular Feature function to generate a putative list of compounds in the extracts. Compound lists were then screened against three accurate mass databases; a database of known plant compounds of therapeutic importance generated specifically for this study (800 compounds); the Metlin metabolomics database (24,768 compounds); and the Forensic Toxicology Database by Agilent Technologies (7,509 compounds). Empirical formula for unidentified compounds was determined using the Find Formula function in the software package.

Toxicity Screening

Reference Toxin for Toxicity Screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4 mg/mL) and serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay.

Artemia franciscana Nauplii Toxicity Screening

Toxicity was assessed using a modified *Artemia franciscana* nauplii lethality assay.[35,36] Briefly, 400 µL of seawater containing ~42 (mean 41.8, n = 125, SD 15.2) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used in the bioassay. A volume of 400 µL of the reference toxin or the diluted plant extracts were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). For each plate, a 400 µL seawater negative control was run in triplicate. The wells were assessed at regular intervals and the number of dead counted. The nauplii were deemed dead if no movement of the appendages was observed within 10 sec. After 24 hr, all nauplii were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95 % confidence limits for each treatment was calculated using probit analysis.

Statistical Analysis

Data is expressed as the mean ± SEM of at least three independent experiments, each with internal triplicates (n=9).

RESULTS

Liquid Extraction Yields and Qualitative Phytochemical Screening

Dried *K. pomifera* and *P. elatus* fruit powder (1 g) were extracted with methanol and water resulting in yields ranging from 195 mg (*P. elatus* aqueous extract) to 524 mg (*K. pomifera* methanolic extract) (Table 1). In general, higher extraction yields were noted for *K. pomifera* fruit than the corresponding *P. elatus* extracts, and methanol was a better extractant (based on extraction yields) than water. The dried extracts were resuspended in 10 mL of deionised water (containing 1 % DMSO), resulting in the concentrations presented in Table 1. Qualitative phytochemical studies showed that the *K. pomifera* and *P. elatus* extracts had similar phytochemical profiles. All contained moderate to high levels of phenolic compounds, saponins and flavonoids, as well as moderate levels of tannins and low levels of triterpenoids.

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water (0.5 % DMSO) and qualitative phytochemical screenings of the *K. pomifera* and *P. elatus* fruit extracts.

			K. pomifera		*P. elatus*	
			Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract
Mass of extracted material (mg)			524	350	314	195
Concentration of resuspended extract (mg/mL)			52	35	31	20
Antioxidant capacity (µg ascorbic acid equivalency)			6.9	2.9	6.8	2.7
Qualitative Phytochemical Tests	**Phenols**	Total phenols	+++	+++	+++	+++
		Water soluble phenols	+++	+++	+++	+++
		Insoluble phenols	+	+++	+++	+++
	Saponins	Froth persistence	++	+++	++	+++
		Emulsion test	++	+++	++	+++
	Cardiac glycosides	Keller-Kiliani Test	-	-	-	-
	Tri-terpenoids	Salkowski Test	++	+	+	+
	Phyto-sterols	Acetic Anhydride Test	-	-	-	-
	Alkaloids	Meyer's Test	-	-	-	-
		Wagner's Test	-	-	-	-
		Draggendoff's Test	-	-	-	-
	Flavo-noids	Kumar Test	+++	+++	++	++
	Tannins	Ferric Chloride Test	++	++	++	++
		Lead Acetate Test	++	++	++	++
	Anthra-quinones	Free	-	-	+	++
		Combined	-	-	++	++

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

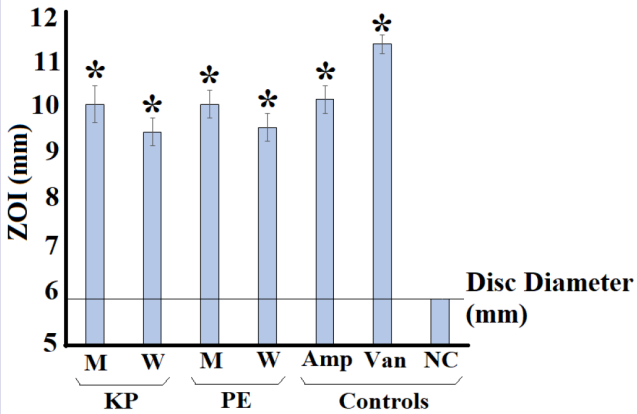

Figure 1: Growth inhibitory activity of the *K. pomifera* and *P. elatus* fruit extracts against *Corneybacterium jeikeium* (ATCC 43734) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; KP = *K. pomifera*; PE = *P. elatus*; Amp = ampicillin (10 µg); Van = vancomycin (5 µg); NC = negative control (0.5 % DMSO). Results are expressed as mean zones of inhibition ± SEM. * indicates results that are significantly different to the negative control (p<0.05).

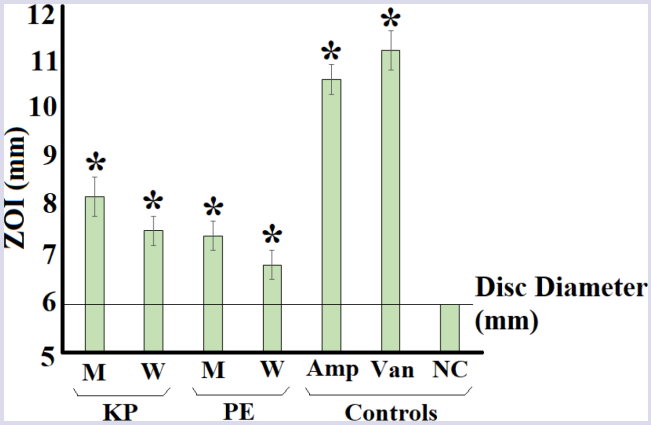

Figure 2: Growth inhibitory activity of the *K. pomifera* and *P. elatus* fruit extracts against *Propionibacterium acnes* (ATCC 6919) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; KP = *K. pomifera*; PE = *P. elatus*; Amp = ampicillin (10 µg); Van = vancomycin (5 µg); NC = negative control (0.5 % DMSO). Results are expressed as mean zones of inhibition ± SEM. * indicates results that are significantly different to the negative control (p<0.05).

Antioxidant ContentAntioxidant capacities for the plant extracts (Table 1) ranged from 2.7 mg (aqueous *P. elatus* extract) to 6.9 mg (*K. pomifera* methanolic extract) ascorbic acid equivalents per gram of dried plant material. Methanolic extracts typically had higher antioxidant capacities than the corresponding water extracts.

Inhibition of Bacterial Growth

To determine the ability of the *K. pomifera* and *P. elatus* fruit extracts to inhibit the growth of some bacterial species associated with the production of body and foot malodour, 10 µL of each extract was screened using a disc diffusion assay. *Corneybacterium jeikeium* growth was inhibited by both the methanolic and aqueous of both *K. pomifera* and *P. elatus* fruit (Figure 1). Whilst all extracts were good inhibitors of *C. jeikeium* growth, the methanolic extracts were better growth inhibitors than the corresponding aqueous extracts (as judged by zone of inhibition (ZOI), with inhibition zones of 10.2 mm for the methanolic extracts, compared to ~9.7 mm for the aqueous extracts of both species. Notably, the inhibition by the extracts was similar to that of the ampicillin control (10 µg). In contrast, the vancomycin (5 µg) control produced slightly larger zones of inhibition (11.5 mm) than the extracts against *C. jeikeium*, However, it is noteworthy that the antibiotic controls consisted of relatively high doses of pure antibiotics. In contrast, the extracts are crude mixtures, which would contain many individual compounds. Thus, it is likely that the growth inhibitory activity of individual bioactive extract component(s) is/are particularly promising. As *C. jeikeium* is responsible for the formation of strongest axillary malodour (and also contributes to foot odour formation), the methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts have potential for use in odour mitigating personal hygiene products and further study is warranted.

Decreased *C. jeikeium* growth through inhibition may subsequently result in less competition for other bacteria and the growth of other malodour producing bacteria may therefore increase. Thus, it would be beneficial for personal hygiene products aimed at reducing body odour to also inhibit the growth of the other odour forming bacteria. As *Staphylococcus* spp. and *Propionibacterium* spp. contribute significantly to axillary odour formation, and *Propionibacterium* spp.

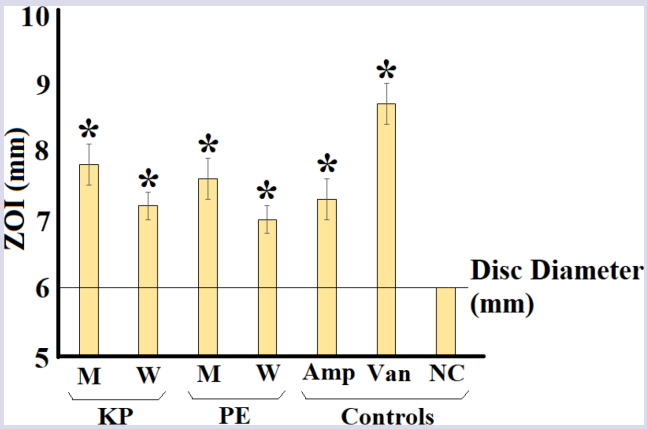

Figure 3: Growth inhibitory activity of the *K. pomifera* and *P. elatus* fruit extracts against measured *Brevibacterium linens* (ATCC9172) as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; KP = *K. pomifera*; PE = *P. elatus*; Amp = ampicillin (10 µg); Van = vancomycin (5 µg); NC = negative control (0.5 % DMSO). Results are expressed as mean zones of inhibition ± SEM. * indicates results that are significantly different to the negative control (p<0.05).

and *Brevibacterium* spp. produce malodorous volatile compounds from foot sweat, an effective deodorant would also need to inhibit the growth of those bacteria. Interestingly, the methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts also significantly inhibited the growth of *P. acnes,* albeit with substantially smaller ZOIs than measured against *C. jeikeium* (Figure 2). Indeed, the methanolic (8.2 and 7.4 mm for the methanolic *K. pomifera* and *P. elatus* fruit extracts respectively) and aqueous extracts (7.5 and 6.8 mm respectively) significantly inhibited *P. acnes* growth. Whilst these ZOIs are substantially smaller than those of the ampicillin (10.6 mm) and vancomycin controls (11.2 mm), they still indicate that these extracts are promising inhibitors of this bacterium.

Brevibacterium spp. are major contributors to foot malodour.[1] Thus, the ability of the *K. pomifera* and *P. elatus* fruit extracts to inhibit *B. linens* growth was also tested in this study (Figure 3). A similar growth

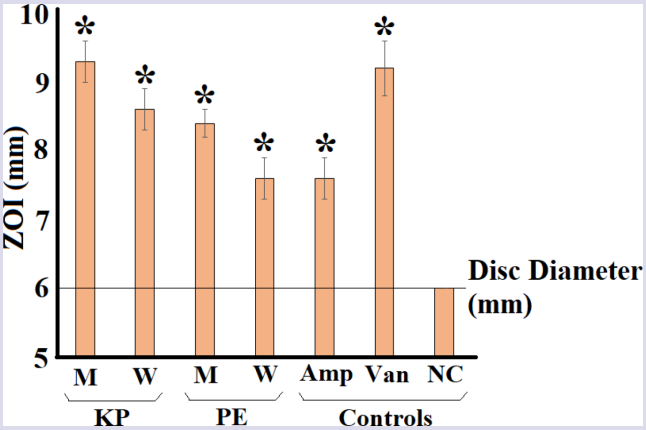

Figure 4: Growth inhibitory activity of the *K. pomifera* and *P. elatus* fruit extracts against *Staphylococcus epidermidis* (clinical isolate) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; KP = *K. pomifera*; PE = *P. elatus*; Amp = ampicillin (10 µg); Van = vancomycin (5 µg); NC = negative control (0.5 % DMSO). Results are expressed as mean zones of inhibition ± SEM. * indicates results that are significantly different to the negative control (p<0.05).

inhibitory profile was seen to the other bacteria, with ZOIs ~7.7 mm and ~7.1 mm measured for the methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts respectively. Notably, these ZOIs were comparable to the ZOIs measured for the pure ampicillin and vancomycin controls, indicating that these extracts may also be useful for inhibiting the growth of this bacterium.

The growth of *S. epidermidis* was also highly susceptible to the methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts (Figure 4). Consistent with the trend noted for the growth inhibition of the other bacteria screened, the methanolic extracts (ZOI = 9.3 and 8.4 mm for *K. pomifera* and *P. elatus* respectively) were substantially better inhibitors of *S. epidermidis* growth than the aqueous extracts were (ZOI = 8.6 and 7.6 mm for *K. pomifera* and *P. elatus* respectively). The inhibition of *S. epidermidis* growth by the extracts was particularly noteworthy compared to the 7.6 mm growth inhibition of the ampicillin (10 µg) control, and 9.2 mm ZOIs recorded for the vancomycin control (ZOI = 9.2 mm).

Quantification of Minimum Inhibitory Concentration (MIC)

The relative level of antimicrobial activity was further evaluated by determining the MIC values (Table 2) for each extract against the bacteria which were shown to be susceptible in the disc diffusion screening assays. Notably, substantial differences were evident between the results obtained in the disc diffusion (DD) and liquid dilution (LD) MIC screening assays, with substantially lower MIC values generally recorded for the liquid dilution assay, compared to the solid phase assay, and these findings may reflect the classes of molecules in the extracts. Larger and/or lower polarity molecules do not readily diffuse through agar and therefore solid phase assays may provide erroneous results for these compounds. In contrast, larger and lower polarity molecules are more suitable for testing in liquid phase assays and these assays may provide a better understanding of their antibacterial activity. Given the substantially higher MIC values measured in the solid phase assays, it is likely that the bioactive compounds are relatively large and/or nonpolar, although this remains to be verified. Alternatively, potentiating compounds (with different physiochemical properties to the inhibitory compounds) may separate as they diffuse through the agar gel, whereas

Table 2: Disc diffusion and liquid dilution MICs against *C. jeikeium*, *P. acnes*, *B. linens* and *S. epidermidis* (µg/mL) of the *K. pomifera* and *P. elatus* extracts.

Extract	MIC (µg/mL)							
	C. jeikeium		*P. acnes*		*B. linens*		*S. epidermidis*	
	DD	LD	DD	LD	DD	LD	DD	LD
KP M	1150	610	1386	915	3042	1830	896	407
KP W	1382	894	1505	1125	3950	2250	1143	563
PE M	923	663	1420	1326	3255	2652	1013	663
PE W	1250	625	1753	938	3856	2500	987	469

DD disc diffusion; LD liquid dilution; KP = *K. pomifera*; PE = *P. elatus*; M= methanolic extract; W = aqueous extract; Numbers indicate the mean DD MIC and LD MIC values of triplicate determinations. - indicates no inhibition at any concentration tested.

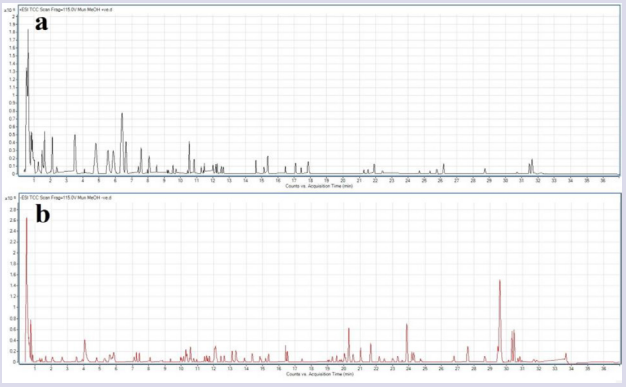

Figure 5: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µL injections of muntries fruit methanolic extract.

they will remain together in the liquid media assays, possibly accounting for these differences.

Noteworthy antibacterial activity (LD MIC values substantially <1000 µg/mL) were calculated for all of the methanolic *K. pomifera* fruit extract against all of the bacteria screened in this study. Additionally, the aqueous *K. pomifera* and the methanolic and aqueous *P. elatus* fruit extracts were also good inhibitors of against *C. jeikeium* and *S. epidermidis*. Whilst the aqueous *K. pomifera* extract, and the methanolic and aqueous *P. elatus* fruit extracts also inhibited *P. acnes* and *B. linens* growth, the higher LD MIC values (generally substantially >1000 µg/mL) indicated that these extracts had only moderate to low growth inhibitory activity. Therefore, the methanolic *K. pomifera* fruit extract was considered particularly promising for decreasing axillary malodour via the inhibition of *C. jeikeium, P. acnes, B. linens* and *S. epidermidis* growth.

HPLC-MS QTOF

As the muntries methanolic extract displayed the lowest MIC values against all of the bacteria tested in this study, it was selected for further examination by high-accuracy HPLC-mass spectroscopy (MS) quadrupole time-of-flight (QTOF) analysis. The muntries methanolic fruit extract total compound chromatograms for the positive ion and negative ion chromatograms are presented in Figure 5a and Figure 5b respectively. The negative ion chromatogram yielded significantly higher

Table 3: Qualitative HPLC-MS QTOF analysis of the muntries methanolic fruit extract, elucidation of empirical formulas and putative identification of the compound.

Putative Identification	Empirical Formula	Molecular Mass	Retention Time	Methanol Extract
Rutinose	$C_7 H_{10} O_7$	206.0429	1.233	+
Metomidate	$C_{13} H_{14} N_2 O_2$	230.1059	6.647	+
Buthiopurine	$C_{10} H_{12} N_4 O_2 S$	252.0672	4.678	+/-
Palmitic acid	$C_{16} H_{32} O_2$	256.2413	23.877	-
Doxofylline	$C_{11} H_{14} N_4 O_4$	266.1004	0.978	+/-
Hexyl dodecanoate	$C_{18} H_{36} O_2$	284.2723	26.782	-
Gingerol	$C_{17} H_{26} O_4$	294.1839	13.12	-
Ellagic acid	$C_{14} H_6 O_8$	302.0082	9.699	-
Gallocatechin	$C_{15} H_{14} O_7$	306.0734	0.609	+/-
Phloionolic acid	$C_{18} H_{36} O_5$	332.2568	14.629	+/-
Ttrimethyl ellagic acid	$C_{17} H_{12} O_8$	344.0538	15.125	+/-
9-(5-O-Benzoylpento furanosyl)-3,9-dihydro-6H-purin-6-one	$C_{17} H_{16} N_4 O_6$	372.1064	3.889	-
Nerolidyl diphosphate	$C_{15} H_{28} O_7 P_2$	382.1276	10.523	+/-
19-hydroxy-17-oxoandrost-5-en-3-beta-yl sulfate	$C_{19} H_{28} O_6 S$	384.1637	1.081	-
Lys Cys His	$C_{15} H_{26} N_6 O_4 S$	386.1732	17.077	+/-
1-α-25-Dihydroxy-3-deoxy-19-nor-22-oxavitamin D3	$C_{25} H_{42} O_3$	390.3172	29.815	-
Xanthiol	$C_{23} H_{29} Cl N_2 O S$	416.1686	8.826	-
Buclizine	$C_{28} H_{33} Cl N_2$	432.2361	10.483	-
Tyr Val Tyr	$C_{23} H_{29} N_3 O_6$	443.2019	7.556	+
Asp Glu Trp	$C_{20} H_{24} N_4 O_8$	448.157	7.56	+/-

+ and - indicates the mass spectral mode in which the molecule was detected. Only compounds detected in both the methanolic and aqueous extracts by high accuracy QTOF LC-MS are shown.

signals than those observed for the positive ion chromatograms due to a significantly higher base peak signal to noise ratio. The positive ion chromatogram (Figure 5a) revealed numerous overlapping peaks, particularly in the early and middle stages of the chromatogram corresponding to the elution of polar compounds. The majority of the methanol extract peaks in positive ion mode had eluted by 10 min (corresponding to approximately 25 % acetonitrile). Indeed, a large number of peaks eluted in the first 5 min with 5 % acetonitrile. However, a number of peaks were also seen later in the positive ion chromatogram (at approximately 31.5 min) indicating the broad spread of polarities of the compounds in this extract. In contrast, more peaks were evident in the muntries methanol fruit extract negative ion chromatogram (Figure 5b) at elution times corresponding to the mid and low polarity compounds.

Qualitative Mass Spectral Analysis of the Muntries Fruit Extracts

In total, 182 unique mass signals were noted for the muntries fruit extracts (results not shown). Of these 29 unique molecular mass signals,

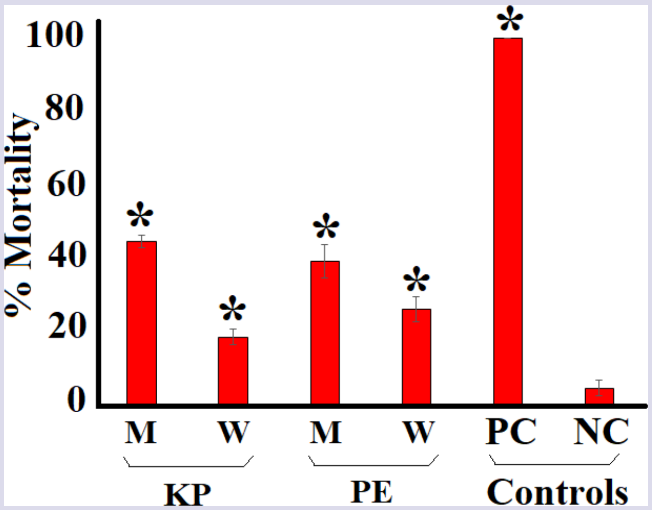

Figure 6: The lethality of the *K. pomifera* and *P. elatus* fruit extracts (2000 µg/mL) and the potassium dichromate (1000 µg/mL) and seawater controls towards *Artemia franciscana* nauplii after 24 hr exposure. M = methanolic extract; W = aqueous extract; KP = *K. pomifera*; PE = *P. elatus*; PC = potassium dichromate control; NC = negative (seawater) control. * indicates results that are significantly different to the negative control (p<0.05). Results are expressed as mean % mortality ± SEM.

20 compounds (Table 3) were putatively identified by comparison against three accurate mass databases; a database of known plant compounds of therapeutic importance generated specifically for this study (650 compounds); the Metlin metabolomics database (24,768 compounds); and the Forensic Toxicology Database by Agilent Technologies (7,509 compounds).

Quantification of Toxicity

All extracts were initially screened in the *Artemia* nauplii assay at 2000 µg/mL (Figure 6). Additionally, potassium dichromate was also tested in the bioassay as a reference toxin. Potassium dichromate was rapid in its onset of mortality, promoting nauplii death within the first 3h of exposure, with 100% mortality evident within 5 hr (unpublished results). In contrast, the methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts both induced substantially less than 50 % moratlity following 24 hr exposure. As 24 h LC_{50} values >1000 µg/mL have previously been defined as non-toxic in this assay,[35,36] the methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts were deemed to be non-toxic and their LC_{50} values were not determined.

DISCUSSION

Eccrine gland secretions (sweating) produces fats and nutrients, which provide ideal energy sources for many bacteria. *Corneybacterium* spp. enzymes degrade protein and lipid components in the sweat to produce malodorous volatile components.[37] *Staphylococcus* spp. also use similar (albeit less active) enzymes to degrade these molecules, producing similar volatile compounds, thereby also contributing to axillary malodour production.[37] Similarly, *Propionbacterium* spp. and *Brevibacterium* spp. produce volatile foot malodour molecules via similar processes.[1] Body malodour formation may be controlled either by:

- Using chemical antiperspirants to reduce sweating, thereby depriving odour producing bacteria of the fuels to produce volatile malodorous compounds. Aluminium salts are commonly used in antiperspirant formulations to block eccrine sweat glands, despite

being associated with numerous health issues.

- Direct inhibition/reduction of the bacteria that cause axillary and/or plantar malodours through the use of formulations, which most frequently contain propylene glycol, triclosan, benzalkonium chloride or metal (e.g. Al) salts. There are also serious concerns with the safety of several of these additives for long-term exposure.
- The use of masking agents (e.g. perfumes) to mask the malodour.

There is concern about the safety of many of the additives to antiperspirant/deodorants. Exposure to aluminium salts has been linked with a wide range of negative health effects including degenerative neurological conditions (e.g. Alzheimers disease, encephalopathy)[6] and cancer.[3] Chronic exposure, as is the case with long term daily use in body odour management, may also result in structural and functional degradation of eccrine sweat ducts and the loss of secretory functionality.[38] Similarly, there is concern about including triclosan (and other deodorant compounds) in commercial deodorants as they have also been linked to a myriad of serious health problems.[4] There is a need to develop safer and more effective antimicrobial deodorant components to address these concerns. Plant formulations are ideal candidates for the development of new deodorant compounds. Many plant extracts and oils have been used traditionally for hundreds (or even thousands) of years to inhibit bacterial growth, and in some case their efficacy has been verified by rigorous scientific examination. Additionally, the use of natural alternatives to inhibit the growth of malodour forming bacteria may be more acceptable to consumers due to their natural origin and consumer perception of safety. This study examined the growth inhibitory properties of methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts against several bacterial species associated with axillary and plantar malodour formation. These species were selected for this study as they have high antioxidant contents[22] and previous studies have reported noteworthy antibacterial activity for these plants against several human pathogens.[20] Additionally, *K. pomifera* and *P. elatus* fruit have been used by the Frist Australian for thousands of years as a nutritious food and are considered to be safe.

Our study confirmed the potential of *K. pomifera* and *P. elatus* fruit extracts for inhibiting the growth of axillary and foot malodour producing bacteria. The methanolic *K. pomifera* fruit extract was the most promising growth inhibitor of all bacterial species, with MIC values substantially less than 1000 µg/mL against all of the tested bacteria. As *Corneybacterium* spp. and *S. epidermidis* are responsible for producing the strongest and most unpleasant malodours,[39] the strong inhibition of *C. jeikeium* and *S. epidermidis* growth by the methanolic *K. pomifera* fruit extract (LD MIC = 610 and 407 µg/mL respectively) was particularly encouraging. Studies using extracts from other plants have reported comparable or considerably higher MIC values as signifying potent inhibitory activity. Inhibitory activity against *S. epidermidis* has been most extensively reported. Extracts produced from the Asian medicinal and edible plant *Caesalpinia minosoides* were reported to be "potent inhibitors" of *S. epidermidis* growth with an MIC value of 3130 ppm (equivalent to 3130 µg/mL).[40] In contrast, an LD MIC of 407 µg/mL was determined for the methanolic *K. pomifera* fruit extract against *S. epidermidis* in our study. This represents a more than seven-fold increase in potency compared to the *Caesalpinia minosoides* study. Similarly, *Cassia alata,*[41] as well as *Barleria lupulina* and *Psidium guajava,*[42] were reported to be moderate inhibitors of *S. epidermidis* growth, with MIC values equivalent to 2500-5000µg/mL. *Hibiscus sabdariffa* and *Eupatorium odoratum* were reported to be potent *S. epidermidis* growth inhibitors, each with MICs equivalent to 625 µg/mL.[42] Indeed, on the basis of its *S. epidermidis* growth inhibitory properties, a *H. sabdariffa* deodorant formulation was the basis of for a US patent application.[43] The MIC value of the *K. pomifera* fruit extract reported in our study indicated that this extract was more potent than the *H. sabdariffa* formulation. Thus, the addition of methanolic *K. pomifera* fruit extract to deodorant preparations has commercial potential and should be explored further.

Examination of the growth inhibition of other malodour producing bacteria by herbal formulations has been less extensively reported. *Rubia tinctorium* (commonly known as madder) crude extracts were reported to be strong inhibitors of *Corneybacterium xerosis* growth.[44] However, that study is of limited value as a single, high dose of the extract was screened (approximately 500 µg/disc). Furthermore, MIC values were not determined, making a comparison with other studies impossible. Similarly, *Anethum graveolens* essential oils inhibited *Corneybacterium* spp. Growth.[35] However, MIC values were also not reported in that study, making a comparison difficult. Furthermore, that study utilised disc diffusion assays to test the growth inhibitory activity of the oil. Whilst disc diffusion assays are generally suitable for study of extracts, they are not recommended when testing oils for antibacterial activity due to the insolubility of the oils in the aqueous gel matrix. More recently, several studies have screened other plant extracts against *C. jeikeium* and have reported noteworthy growth inhibitory activity. Particularly good inhibitory activity was reported for *Terminalia ferdinandiana* Exell. Extracts,[5] as well as other Australian, Indian and African *Terminalia* spp.,[8] with MIC values of approximately 200 µg/mL against *C. jeikeium*. Similarly, low MIC values (200-1000 µg/mL) were reported for several Australian *Syzygium* spp.,[5] whilst higher (but still noteworthy) MIC values (~1500 µg/mL) were reported for *Acronychia acidula* F. Muell. fruit extracts.[45]

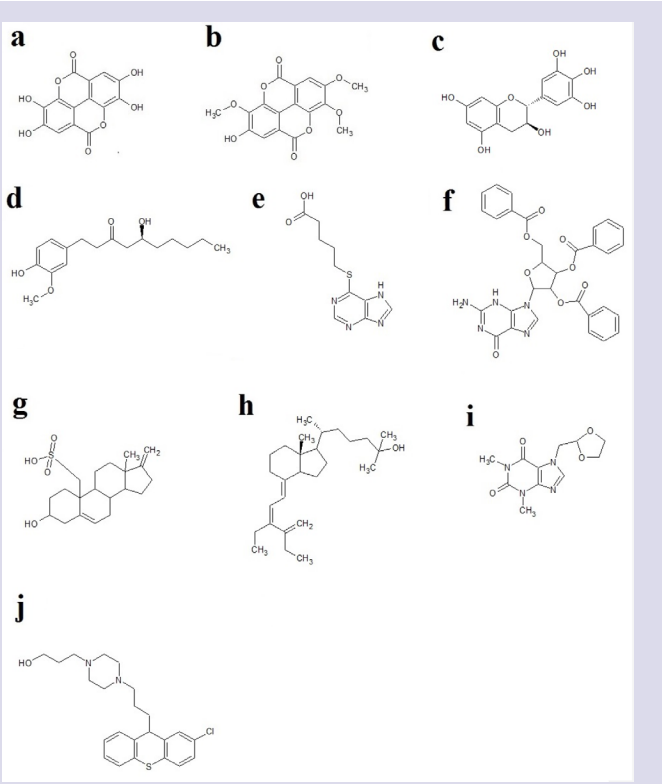

Figure 7: Chemical structures of notable muntries fruit compounds detected in the methanolic *K. pomifera* fruit extract: (a) ellagic acid; (b) trimethyl ellagic acid; (c) gallocatechin, (d) gingerol; (e) buthiopurine; (f) 9-(5-O-benzoylpentofuranosyl)-3,9-dihydro-6H-purin-6-one; (g) 19-hydroxy-17-oxoandrost-5-en-3-beta-yl sulfate; (h) 1-α,25-dihydroxy-3-deoxy-19-nor-22-oxavitamin D3; (i) doxofylline; (j) xanthiol.

Whilst the antibacterial components were not identified in this study, a number of compounds were putatively identified by HPLC-MS QTOF analysis. In particular, ellagic acid (Figure 7a), trimethyl ellagic acid (Figure 7b), gallocatechin (Figure 7c), gingerol (Figure 7d), buthiopurine (Figure 7e), 9-(5-O-benzoylpentofuranosyl)-3,9-dihydro-6H-purin-6-one (Figure 7f), 19-hydroxy-17-oxoandrost-5-en-3-beta-yl sulfate (Figure 7g), 1-α,25-dihydroxy-3-deoxy-19-nor-22-oxavitamin D3 (Figure 7h), doxofylline (Figure 7i), and xanthiol (Figure 7j) were highlighted in our study. Several of these compounds have strong antibacterial activities. The antibacterial properties of tannins (including ellagic acid and its derivatives, and gallocatechin have been particularly well studied (as reviewed in).[9] Similarly, gingerol (and its analogues) inhibit the growth of several bacterial pathogens, including methicillin-resistant *S. aureus* (MRSA) strains.[46] It is likely that these compounds may contribute to the antibacterial activity reported herein, although it is possible that other methanolic *K. pomifera* fruit extract components may also contribute to this activity. Further studies to elucidate the phytochemicals in this extract (and their potential therapeutic mechanisms) are warranted.

Notably, the methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts tested herein were both non-toxic towards *Artemia* nauplii and are thus likely to be safe for topical application as deodorants. However, further toxicity studies using human cell lines (and subsequent *in vivo* studies) are required to confirm the safety of these extracts before they are accepted as natural deodorant alternatives. Furthermore, whilst our study reported the *K. pomifera* and *P. elatus* fruit extracts to be non-toxic, it is noteworthy that all of these studies have examined acute toxicity. Pharmacodynamic and pharmacokinetic studies are required to determine the ability of the extract components to cross the skin barrier, their duration in the blood stream prior to clearance, and the urinary excretory products. As deodorants are applied frequently, such studies are required for any formulation to ensure that their components do not accumulate and cause chronic toxicity.

CONCLUSION

The results of this study demonstrate the potential of the *K. pomifera* and *P. elatus* fruit extracts as natural antibacterial components for deodorant formulation. In particular, the methanolic *K. pomifera* extract was a potent inhibitor of all of the malodour forming bacteria tested. Furthermore, the lack of toxicity of the extract indicates the suitability of that extract for topical use.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide; **LC_{50}:** The concentration required to achieve 50 % mortality; **MIC:** minimum inhibitory concentration; **ZOI:** zone of inhibition.

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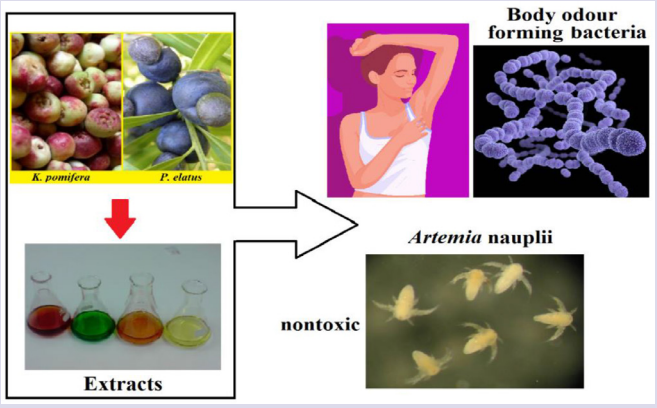
PICTORIAL ABSTRACT



SUMMARY

- Methanolic and aqueous *K. pomifera* and *P. elatus* fruit extracts were screened for the ability to block the growth of a panel of axillary and plantar odour producing bacteria.
- The methanolic *K. pomifera* fruit extract was a particularly good inhibitor of bacterial growth.
- LD MIC values of 610, 894, 663 and 625 µg/mL were measured for the methanolic *K. pomifera* extract against C. *jeikeium, P. acnes, B. linens* and *S. epidermidis* respectively.
- The aqueous *K. pomifera* extract, as well as both *P. elatus* fruit extracts also had good antibacterial activity, albeit with higher MIC values.
- The notoxicity of the *K. pomifera* and *P. elatus* fruit extracts was verified using the *Artemia* nauplii toxicity bioassay.
- Non-biased HPLC-MS QTOF analysis was used to identify and highlight noteworthy constituents of the methanolic *K. pomifera* extract.

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 more than 250 scientific publications in a variety of peer reviewed journals.

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