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Original Article

Tasmannia lanceolata (Poir.) A.C. Sm. and *Tasmannia insipida* R.Br. ex. DC. Extracts Inhibit the Growth of Axillary and Foot Odour Producing Bacteria

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ABSTRACT

Introduction: *Tasmannia* spp. extracts inhibit the growth of many 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. **Methods:** *T. lanceolata* and *T. insipida* fruit and leaf solvent extracts were investigated by disc diffusion and liquid dilution MIC assays against the most significant bacterial contributors to axillary and plantar malodour formation. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. Non-targeted HPLC separation of the *T. lanceolata* methanolic berry extracts, coupled to high resolution time-of-flight (TOF) mass spectroscopy was used for the identification and characterisation of individual components in the extract. **Results:** The methanolic and aqueous *T. lanceolata* and *T. insipida* fruit and leaf extracts displayed noteworthy bacterial growth inhibitory activity against all of the malodour forming bacteria tested. The *T. lanceolata* methanolic fruit extract was particularly potent, with low MIC values recorded against *C. jeikeium* (480µg/mL) and *S. epidermidis* (513µg/mL), as well as moderate activity against *P. acnes* (1750µg/mL) and *B. linens* (1250µg/mL). Similar MIC values were noted for the aqueous *T. lanceolata* fruit extract against *C. jeikeium* and *S. epidermidis,* although this extract was ineffective against the other bacteria tested. Similar, albeit less potent inhibitory profiles were noted for the *T. lanceolata* and *T. insipida* leaf extracts against *C. jeikeium* and *S. epidermidis*. All *Tasmannia* spp. extracts were nontoxic in the *Artemia fransiscana* bioassay. Non-biased phytochemical analysis of the methanolic leaf extract highlighted several notable compounds, including polygodial, capsidiol, salutarisolide gallic acid and combretastatin A1 in relative abundance. **Conclusion:** The lack of toxicity of the *T. lanceolata* and *T. insipida* fruit and leaf extracts and their potent growth inhibition of axillary and plantar malodour producing bacteria indicate their potential as deodorant components.

Key words: Mountain pepper berry, Tasmanian pepper, Safety testing, Body odour, Deodorant, *Corynebacterium*.

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INTRODUCTION

A range of synthetic antimicrobial compounds are currently included in deodorants and antiperspirants to inhibit the growth of skin bacteria and their subsequent odour formation. These additives include propylene glycol, triclosan, benzalkonium chloride and metal (e.g. Al) salts.[1] However, the safety of many of these chemical additives has yet to be determined. Of concern, these additives have been linked with some serious health problems. Aluminium salts, which are common additives to anti-perspirants, have been linked with a wide range of negative health 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. Triclosan has been reported to cause a variety of health problems including dermal irritation and allergies, although the authors of that study report relatively high levels of exposure are required for these effects to become evident.[4] Natural alternatives that inhibit the growth of odour forming bacteria are desirable and may be more acceptable to consumers due to their natural origin and consumer perception of safety. There has been a recent trend to search natural resources for safe and effective plant preparations to replace these synthetic additives and several promising plan species have been identified.[5,6]

The genus *Tasmannia* (family Winteraceae) grow exclusively in the southern hemisphere and many species are native to Australia.[7] The species *Tasmannia lanceolata* (Poir.) A.C.Sm. (commonly known as Tasmanian pepper or mountain pepper) has attracted particular interest due to its high antioxidant capacity and ethnobotanical uses.[7,8] Recent studies have also reported that *T. lanceolata* berry and leaf extracts have strong antibacterial,[9-11] antifungal[12] and antiprotozoal[13,14] activities against an extensive panel human pathogens. In several of those studies, varying polarity *T. lanceolata* solvent extracts were screened against gram-positive and gram-negative bacterial species. Polar methanolic and aqueous extracts were the most effective antibacterial agents against a broad range of bacteria, indicating that the antibacterial components were polar. Indeed, the polar extracts inhibited the growth of nearly every bacterial pathogen tested. Both gram-positive and gram-negative bacterial species were susceptible, indicating that the inhibitory compounds may readily cross the gram-negative cell wall. Similarly, the related species *Tasmannia stipitata* (Vickery) A.C.Sm.[15] and *Tasmannia insipida*[16] have also been reported to have noteworthy growth inhibitory activity against several human bacterial pathogens. Further screening of these species against other bacteria is warranted. Due to their ethnobotanical usage and the previously reported bacterial growth inhibitory activity of several *Tasmannia* spp. extracts against a broad panel of bacteria, it is likely that they may also inhibit the growth of bacteria directly involved in axillary and foot malodour formation. This study aimed to test *Tasmannia* spp. leaf and fruit extracts against the axillary and foot odour forming bacteria *Corynebacterium jeikeium, Staphylococcus epidermidis, Propionibacterium acnes* and *Brevibacterium linens,* with the aim of identifying safe and effective deodorant components.

MATERIALS AND METHODS

Plant source and extraction

Tasmannia lanceolata semi-dry fruit/berry (without seed) and dried leaf material was purchased from GoWild Harvest, Australia. *Tasmannia insipida* leaves were supplied by the Queensland Bushfoods Association, Australia and were dried using a Sunbeam food dehydrator. The dried material was stored at -30°C until use. The plant materials were thawed and freshly ground to a coarse powder prior to extraction. Individual 1g quantities of the ground plant materials were prepared by weighing each powdered plant part into each of three tubes and adding 50mL of methanol, water, ethyl acetate, chloroform or hexane respectively. All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The fruit and leaf material was 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 by rotary evaporation in an Eppendorf concentrator 5301. The resultant dry extracts were weighed and redissolved in 10mL of deionised water (containing 0.5 % DMSO), and passed through a 0.22µm filter (Sarstedt) and stored at 4°C until use.

Qualitative phytochemical studies

Phytochemical analysis of the *T. lanceolata* and *T. insipida* fruit and leaf extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved as previously described.[17-19]

Antioxidant capacity

The antioxidant capacity of each sample was assessed using a modified DPPH free radical scavenging method.[20,21] Ascorbic acid (0-25 µg per well) was used as a reference and the absorbances were measured and recorded at 515 nm. All tests were completed alongside controls on each plate and all tests were performed in triplicate. 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), *Corynebacterium 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 24hr. Cultures of *C. jeikeium* were grown and maintained in nutrient broth supplemented with 300µL Tween 80/L of broth at 37°C for 24hr. *Propionibacterium acnes* was cultured using a thioglycollate liquid media under induced anaerobic conditions through the use of anaerobic jars and AnaeroGen™ 3.5L atmospheric generation systems (Thermo Scientific). Incubation was at 37°C for 72hr. All stock cultures were subcultured and maintained in liquid media at 4°C.

Evaluation of antibacterial activity

Antibacterial activity screening of the *T. lanceolata* and *T. insipida* fruit and leaf extracts was assessed using a modified disc diffusion assay.[22-24] Briefly, 100µL of each individual bacteria was grown separately in 20mL of the appropriate broth until an approximate count of 10^8 cells/mL was reached. A volume of 100µL of each bacterial suspension was spread onto agar plates and the extracts were tested for antibacterial activity using 5mm 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 300µL Tween 80/L of agar. Discs were infused with 10µL of the individual extracts, allowed to dry and placed onto the inoculated plates. The plates were left to stand at 4°C for 2hr before incubation. Plates inoculated with *B. linens*, *S. epidermidis* or *C. jeikeium* cultures were incubated aerobically at 37°C for 24hr. Plates spread with *P. acnes* cultures were incubated under induced anaerobic conditions at 37°C for 72hr. The diameters of the inhibition zones were measured to the closest whole millimetre. Each assay was completed in at least triplicate. Mean values (± SEM) are reported in this study. Ampicillin (10µg) and vancomycin (5µg) discs were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10µL of distilled water were used as a negative control.

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.[25] Furthermore, as microplate liquid dilution 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 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.[26,27] 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 100µL sterile broth was added to all wells of a 96 well plate. Test extracts or control antibiotics (100µL) 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 100µL 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 100µL 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 24hr. Plates inoculated with *P. acnes* cultures were incubated under induced anaerobic conditions at 37°C for 72hr. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma, Australia and dissolved in sterile deionised water to prepare a 0.2mg/mL INT solution. A 40µL 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.[28,29] Briefly, the *T. lanceolata* and *T. insipida* fruit and leaf extracts were diluted in deionised water (containing 0.5% DMSO) and tested across a range of concentrations. Discs were infused with 10µL 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.

Toxicity Screening

Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4mg/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.[30,31] Briefly, 400µL of seawater containing ~43 (mean 42.7, *n* = 125, SD 14.7) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used in the bioassay. A volume of 400µL 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 400µL 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 24hr, 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.

Non-targeted HPLC-MS QTOF analysis

Chromatographic separations were performed as previously described.[32] Briefly, 2µL of sample was injected onto an Agilent 1290 HPLC system fitted with a Zorbax Eclipse plus C_{18} column (2.1 x 100 mm, 1.8µm particle size). The mobile phases consisted of (A) ultrapure water and (B) 95:5 acetonitrile/water at a flow rate of 0.7mL/min. Both mobile phases were modified with 0.1% (v/v) glacial acetic acid for mass spectrometry analysis in positive mode and with 5mM 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 positive and negative mode.

Data was analysed using the Masshunter Qualitative analysis software package (Agilent Technologies). Blanks using each of the solvent extraction systems were analysed 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.

Statistical Analysis

Data is expressed as the mean ± SEM of at least three independent experiments.

RESULTS

Liquid Extraction Yields and Qualitative Phytochemical Screening

Tasmannia lanceolata and *T. insipida* fruit and leaf extractions (1 g) with various solvents yielded dried plant extracts ranging from 27 mg to 263mg (Table 1). Aqueous and methanolic extracts provided significantly greater yields of extracted material relative to the chloroform, ethyl acetate and hexane counterparts, which gave low to moderate yields. The dried extracts were resuspended in 10 mL of deionised water (containing 1 % DMSO), resulting in the concentrations presented in Table 1.

Antioxidant Content

Antioxidant capacity for the plant extracts (Table 1) ranged from 0.3mg (hexane leaf extract) to a high of 286mg ascorbic acid equivalence per gram of dried plant material extracted (aqueous fruit extract). The aqueous and methanolic extracts had substantially higher antioxidant capacities than the corresponding chloroform, hexane and ethyl acetate extracts for both the leaf and fruit extracts.

Inhibition of Bacterial Growth

To determine the ability of the *Tasmannia lanceolata* and *T. insipida* fruit and leaf extracts to inhibit the growth of some bacterial species associated with the production of body and foot malodour, 10µL of each extract was screened using a disc diffusion assay. *Corynebacterium jeikeium* growth was inhibited by all of the *Tasmannia* spp. fruit and leaf extracts screened (Figure 1). Whilst both the fruit and leaf extracts were good inhibitors of *C. jeikeium* growth, the *T. lanceolata* leaf extracts were generally more potent inhibitors (as judged by zone of inhibition). The methanolic and aqueous *T. lanceolata* leaf extracts were particularly potent inhibitors of *C. jeikeium* growth, with inhibition zones of 10.4±0.4mm and 9.6±0.3mm respectively. Similarly, the methanolic (7.6±0.3mm) and aqueous extracts (8.2±0.4mm) also displayed the greatest growth inhibitory activity of the *T. lanceolata* fruit extracts. The *T. insipida* leaf extracts followed a similar trend, with the methanolic and aqueous extracts (7.8±0.3mm and 8.2±0.4mm respectively) also producing substantially larger zones of inhibition than the other extracts. Indeed, whilst the ethyl acetate, chloroform and hexane extracts of all *Tasmannia* spp. fruit and leaf extracts also inhibited *C. jeikeium* growth, the zones of inhibition that they induced were generally substantially smaller, indicating that the most potent and/or most abundant bacterial growth inhibitory compounds in the *Tasmannia* spp. extracts are polar. Whilst the ampicillin (10µg) and vancomycin (5µg) controls produced greater zones of inhibition (10.3±0.3mm and 11.6±0.6mm respectively), they 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 noteworthy. As *C. jeikeium* is responsible for the formation of strongest axillary malodour and also contributes to foot odour formation, the aqueous and methanolic *Tasmannia* spp. extracts have potential for use in odour mitigating personal hygiene products.

When *C. jeikeium* growth becomes inhibited, there would be less competition for other bacteria and the growth of other malodour producing bacteria may 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 to axillary odour formation, and *Propionibacterium* spp. and *Brevibacterium* spp. produce malodorous volatile compounds from foot sweat, an effective deodorant would also need to inhibit the growth of these bacteria. Notably, the growth

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant capacities of the *T. lanceolata* and *T. insipida* fruit and leaf extracts.

Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Antioxidant Capacity (mg Ascorbic Acid Equivalency)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
TLF M	171	17.1	153	+++	+++	+++	–	++	+	–	–	–	+++	+	–	–
TLF W	111	11.1	286	+++	+++	+++	–	-	–	–	–	–	+++	+	–	–
TLF E	57	5.7	18	+	+	++	–	+	++	–	–	–	++	–	–	–
TLF C	83	8.3	7	+	–	–	–	–	–	–	–	–	–	–	–	–
TLF H	46	4.6	0.3	–	–	–	–	–	–	–	–	–	–	–	–	–
TLL M	144	14.4	67	+++	+++	+++	–	+	+	–	–	–	+++	++	–	–
TLL W	136	13.6	92	+++	+++	+++	–	++	–	–	–	–	+++	+	–	–
TLL E	27	2.7	4	+	+	++	–	+	+	–	–	–	++	–	–	–
TLL C	43	4.3	BDT	+	–	–	–	–	–	–	–	–	–	–	–	–
TLL H	35	3.5	BDT	–	–	–	–	–	–	–	–	–	–	–	–	–
TIL M	232	23.2	48	+++	+++	++	–	+++	+	–	–	–	+++	+	–	–
TIL W	263	26.3	63	+++	+++	+++	–	++	+	–	–	–	+++	+	–	–
TIL E	68	6.8	2.2	+	+++	+	–	–	+	–	–	–	++	–	–	–
TIL C	59	5.9	BDT	–	–	+	–	–	–	–	–	–	–	–	–	–
TIL H	38	3.8	BDT	–	–	–	–	–	–	–	–	–	–	–	–	–

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. TLF = *T. lanceolata fruit*; TLL = *T. lanceolata leaf*; TIL = *T. insipida* leaf; M= methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; BDT = below detection threshold. Antioxidant capacity was determined by DPPH reduction and is expressed as mg ascorbic acid equivalence per g plant material extracted.

of *S. epidermidis* was also highly susceptible to some *Tasmannia* spp. fruit and leaf extracts (Figure 2). Consistent with the trend noted for *C. jeikeium* growth inhibition, the higher polarity methanolic and aqueous extracts were again the best growth inhibitors, indicating that the antimicrobial components of the extracts were polar. As reported for *C. jeikeium* growth inhibition, the *T. lanceolata* methanolic and aqueous leaf extracts were particularly potent inhibitor of *S. epidermidis* growth, with inhibition zones of 9.2±0.6mm and 8.3±0.5mm respectively. The inhibition of *S. epidermidis* growth was particularly noteworthy compared to the 7.8±0.4mm growth inhibition of the ampicillin (10 µg) control, although the inhibition by the vancomycin control (9±0.5mm) was more pronounced. However, it is noteworthy that the controls tested high doses of pure antibiotics. The aqueous and methanolic extracts of *T. lanceolata* fruit and *T. insipida* leaf were also noteworthy inhibitors of *S. epidermidis* growth, although the mid to low polarity extracts were generally ineffective *S. epidermidis* growth inhibitors.

The growth of *P. acnes* was also inhibited by all of the *Tasmannia* spp. fruit and leaf extracts, albeit with zones of inhibition that indicate lower antibacterial potency (Figure 3). Indeed, only the mid to low polarity *T. lanceolata* leaf extracts, as well as the methanolic and chloroform *T. insipida* leaf extracts produced zones of inhibition greater than 8mm (compared to approximately 12 and 12.6mm for the ampicillin and vancomycin controls respectively). Whilst all other *Tasmannia* spp. fruit and leaf extracts also inhibited *P. acnes* growth, they generally produced substantially smaller inhibition zones.

Brevibacterium spp. are also major contributors to foot malodour.[1] Thus, the ability of the *Tasmannia* spp. fruit and leaf extracts to inhibit *B. linens* growth was also tested in this study (Figure 4). The *B. linens* strain tested

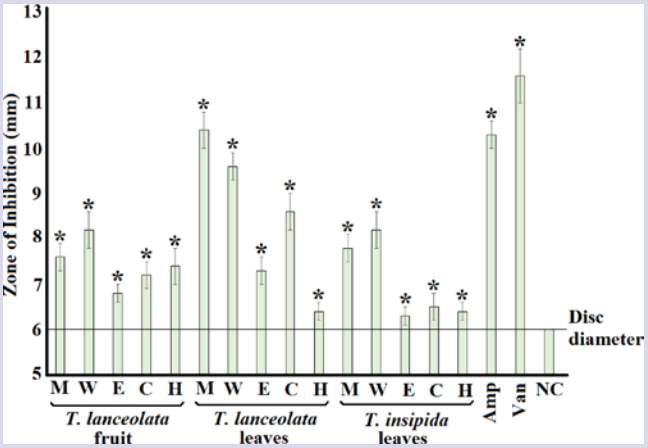

Figure 1: Growth inhibitory activity of the *T. lanceolata* and *T. insipida* fruit and leaf extracts against *Corneybacterium jeikeium* (ATCC 43734) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10µg); Van = vancomycin (5µg). * indicates results that are significantly different to the negative control (p<0.05). Results are expressed as mean zones of inhibition ± SEM.

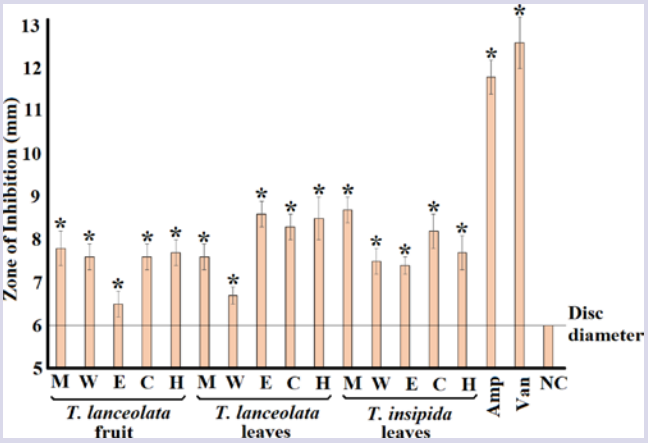

Figure 3: Growth inhibitory activity of the *T. lanceolata* and *T. insipida* fruit and leaf extracts against *Propionibacterium acnes* (ATCC 6919) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10µg); Van = vancomycin (5µg). * indicates results that are significantly different to the negative control (p<0.05). Results are expressed as mean zones of inhibition ± SEM.

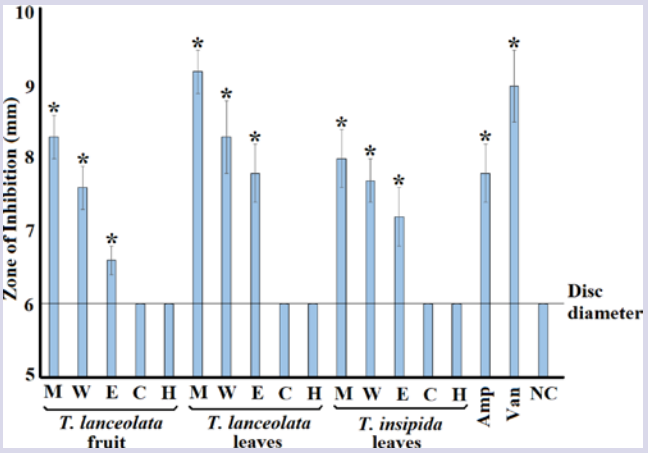

Figure 2: of the *T. lanceolata* and *T. insipida* fruit and leaf extracts against a *Staphylococcus epidermidis* clinical isolate measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10µg); Van = vancomycin (5µg). * indicates results that are significantly different to the negative control (p<0.05). Results are expressed as mean zones of inhibition ± SEM.

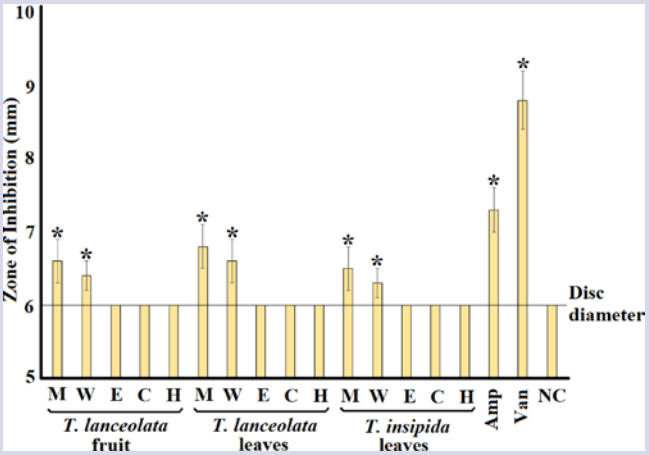

Figure 4: Growth inhibitory activity of the *T. lanceolata* and *T. insipida* fruit and leaf extracts against *Brevibacterium linens* (ATCC9172) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10µg); Van = vancomycin (5µg). * indicates results that are significantly different to the negative control (p<0.05). Results are expressed as mean zones of inhibition ± SEM.

was relatively resistant to all of the *Tasmannia* spp. Indeed, only the methanolic and aqueous extracts inhibited the growth of this bacterium, albeit with zones of inhibition indicative of low strength (<7mm). Notably, this bacterial strain was also resistant to the ampicillin control, with a zone of inhibition of 7.3±0.3mm. In contrast, the vancomycin control was a potent inhibitor of *B. linens* growth, with inhibition zones of 8.8±0.4mm. The mid to lower polarity extracts were all completely ineffective against this bacterium, indicating that the inhibitory compounds are polar.

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. A similar trend was noted as seen for the screening assays i.e. the methanolic and aqueous *Tasmannia* spp. fruit and leaf extracts were generally better inhibitors of the growth of all bacterial species than were the corresponding ethyl acetate, chloroform or

Table 2: Disc diffusion and liquid dilution MICs against *C. jeikeium*, *S. epidermidis*, *P. acnes* and *B. linens* growth (µg/mL) and *Artemia* nauplii bioassay LC_{50} values (µg/mL) of *the* T. lanceolata *and T. insipida* fruit and leaf extracts.

Extract	MIC (µg/mL)								*Artemia* nauplii bioassay
	C. jeikeium		*S. epidermidis*		*P. acnes*		*B. linens*		LC_{50} (µg/mL)
	DD	LD	DD	LD	DD	LD	DD	LD	
TLF M	685	480	644	513	2858	1750	>5,000	1250	1437
TLF W	876	655	890	587	>5,000	>5,000	>5,000	1500	1563
TLF E	843	729	826	625	942	723	-	-	-
TLF C	770	-	-	-	2150	-	-	-	-
TLF H	928	-	-	-	1587	-	-	-	-
TLL M	860	573	789	667	>5,000	>5,000	>5,000	2500	1265
TLL W	1268	705	1075	1000	>5,000	>5,000	>5,000	3000	1580
TLL E	953	614	953	875	1806	1400	-	-	1655
TLL C	1450	-	-	-	3485	-	-	-	-
TLL H	1863	-	-	-	2769	-	-	-	-
TIL M	1146	876	927	843	>5,000	>5,000	>5,000	2500	1528
TIL W	1352	1263	1297	986	>5,000	>5,000	>5,000	3000	1685
TIL E	1063	1157	1143	1054	1309	1000	-	-	-
TIL C	4285	-	-	-	4351	-	-	-	-
TIL H	3529	-	-	-	3210	-	-	-	-

DD disc diffusion; LD liquid dilution; TLF = *T. lanceolata fruit*; TLL = *T. lanceolata leaf*; TIL = *T. insipida* leaf; M= methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Numbers indicate the mean DD MIC, LD MIC and LC_{50} values of triplicate determinations. - indicates no inhibition at any concentration tested.

hexane extracts. Indeed, all *Tasmannia* spp. fruit and leaf methanolic and aqueous extracts yielded MIC values substantially <1000µg/mL. A further trend was also evident: the methanolic extracts were generally the most potent inhibitors of bacterial growth, as judged by MIC values. *Corynebacterium jeikeium* was the most susceptible bacteria to all of the the extracts, with liquid dilution (LD) MIC values of 480, 573 and 876µg/mL for the *T. lanceolata* fruit, *T. lanceolata* leaf and *T. insipida* leaf methanolic extracts respectively. The methanolic extracts was also potent inhibitors of *S. epidermidis* growth, with LD MIC values of 513, 667 and 843µg/mL respectively. Whilst still displaying noteworthy inhibitory activity, the methanolic *T. lanceolata* fruit extract was a less potent inhibitors of *P. acnes* (LD MIC values of 1750µg/mL), whilst the other methanolic extracts were essentially ineffective against this bacterium (LD MIC values >5000µg/mL). Notably the *Tasmannia* spp. fruit and leaf ethyl acetate extracts displayed stronger activity against *P. acnes* growth than the corresponding methanolic and aqueous extracts, with LD MICs 723-1400µg/mL. Similarly, only moderate to low inhibitory activity (LD MICs 1250-3000µg/mL) was recorded for the methanolic and aqueous *Tasmannia* spp. fruit and leaf extracts against *B. linens*, whilst all other extracts were completely ineffective against that bacterium. Therefore, all of the *Tasmannia* spp. methanolic extracts have potential to ameliorate axillary malodour via the inhibition of *C. jeikeium* and *S. epidermidis* growth, but were substantially less effective against the foot odour producing bacteria (*P. acnes* and *B. linens*).

Quantification of Toxicity

All extracts were initially screened in the *Artemia* nauplii assay at 2000µg/mL (Figure 5). Additionally, potassium dichromate was also

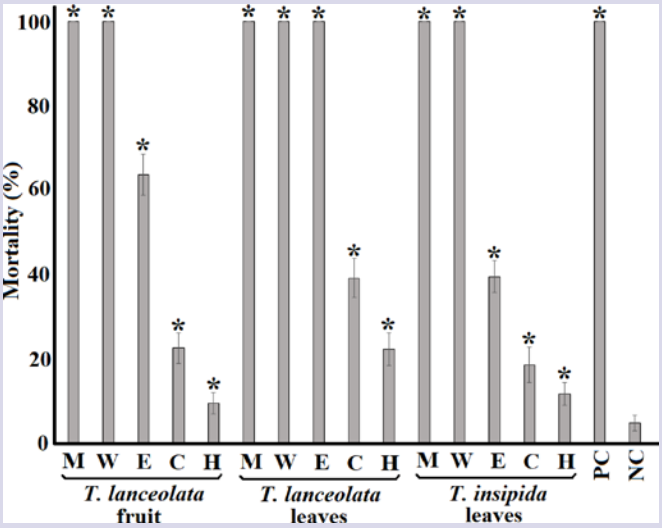

Figure 5: The lethality of the *T. lanceolata* and *T. insipida* fruit and leaf extracts (2000µg/mL) and the potassium dichromate (1000µg/mL) and seawater controls towards *Artemia franciscana* nauplii after 24 hr exposure. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; 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.

tested in the bioassay as a reference toxin. The reference toxin was rapid in its onset of mortality, promoting nauplii death within the first 3hr of exposure, with 100% mortality evident within 5 hr (unpublished results). All of the methanolic and aqueous extracts also induced 100% mortality following 24hr exposure. Similarly, the *T. lanceolata* ethyl acetate leaf extract also induced 100% mortality at 24hr exposure, whilst the fruit extract induced approximately 64% mortality. All other extracts induced <50% mortality and were therefore deemed to be nontoxic.

To further quantify the effects of toxin concentration on the initiation of mortality, the extracts were serially diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay. The 24hr LC_{50} values of the *T. lanceolata* and *T. insipida* fruit and leaf extracts towards *A. franciscana* are displayed in Table 2. No LC_{50} are reported for any chloroform or hexane extracts, nor for the *T. insipida* leaf ethyl acetate extract as <50% mortality was seen in all tested concentrations. LC_{50} values substantially >1000µg/mL were determined for all of the other extracts. As extract with LC_{50} values >1000µg/mL towards *Artemia* nauplii have been defined as being nontoxic in this assay,[30,31] all of the *Tasmannia* spp. fruit and leaf extracts were deemed to be non-toxic.

HPLC-MS QTOF Analysis

As the *T. lanceolata* fruit extracts displayed the best antibacterial activity against most of the tested bacteria, the *T. lanceolata* methanolic, aqueous and ethyl acetate fruit extracts were further examined by high accuracy HPLC-MS QTOF. The *T. lanceolata* berry methanolic (Figure 6a) and aqueous extract (Figure 6b) positive ion base peak chromatograms revealed multiple overlapping peaks, particularly in the early stages of each chromatogram, corresponding to the elution of polar compounds. Most of the extracted compounds had eluted in the first 10 min, corresponding to 5-25% acetonitrile. Indeed, many of the peaks eluted in the first 5 min during the isocratic stage of the chromatogram (5% acetonitrile). However, the presence of several prominent peaks between 10 and 16.5min indicates the broad spread of polarities of the compounds in this extract. Substantially fewer peaks were evident in the ethyl acetate extract (Figure 6c) than in the methanolic and aqueous extracts, and a shift towards a great percentage of the compounds eluting at higher acetonitrile %'s.

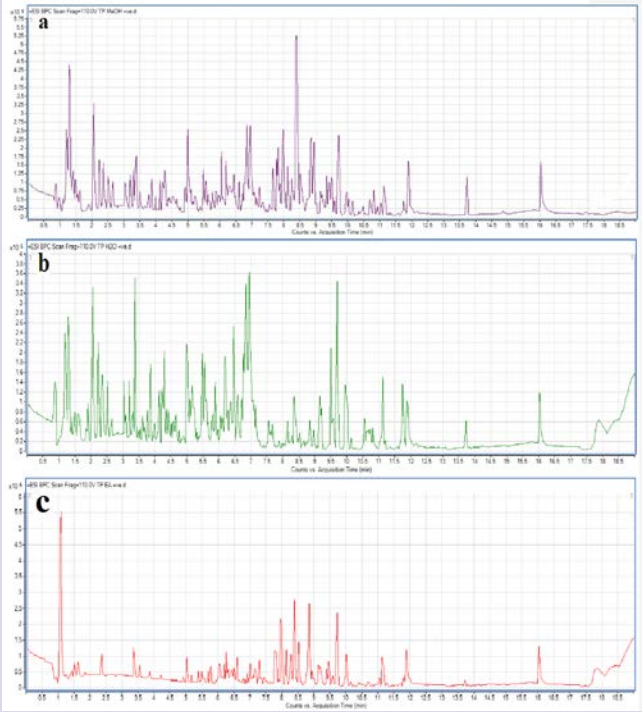

Figure 6: Positive ion RP-HPLC base peak chromatograms (BPC) of 2µl injections of *T. lanceolata* fruit (a) methanolic, (b) aqueous and (c) ethyl acetate extracts.

Qualitative mass spectral analysis of methanolic *T. lanceolata* fruit extract

In total, 998 unique mass signals were detected across the *T. lanceolata* fruit extracts (results not shown). Of these, 22 mass signals were present in each of the ethyl acetate, chloroform and hexane fruit extracts and putative empirical formulas were achieved for all of these compounds (Table 3). Each of the unique molecular mass signals detected across these extracts were putatively identified by comparison to the Metlin metabolomics, forensic toxicology (Agilent) and phytochemicals (developed in this laboratory) databases (Table 3).

DISCUSSION

Body malodour (encompassing both axillary and foot odour) has social consequences related to embarrassment and social exclusion. The production of excessive axillary and foot odours may also have economic consequences as individuals that produce strong odours seek to replace damaged/stained clothing and footware. The nature and extent of these malodours is directly related to the production and content of an individual's sweat, which in turn is controlled genetically and influenced by environmental factors (e.g. diet, medicinal intake etc). Sweating provides fats and nutrients which provide ideal growth conditions for many bacteria. *Corynebacterium* spp. possess enzymes to degrade protein and lipid sweat components to produce strongly malodorous volatile components.[33] *Staphylococcus* spp. possess similar (albeit less active) enzymes and are therefore capable of producing similar volatile compounds and thus also contribute to axillary malodour.[33] *Propionbacterium* spp. and *Brevibacterium* spp. (and to a lesser extent, *Corynebacterium* spp. and *Staphylococcus* spp.) are the main bacteria responsible for foot malodour production.[1]

Current deodorant compositions seek to control axillary and plantar malodour in several ways:

- Antiperspirants reduce sweating, usually by mechanical obstruction of eccrine sweat ducts.[35] Aluminium salts are the most commonly used compound in antiperspirant formulations. However, there are concerns about chronic exposure to these chemicals. Acute exposure to aluminium 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 antiperspirant usage, can also result in structural and functional degradation of eccrine sweat ducts and the loss of secretory functionality.[34]
- Inhibition/reduction of the bacteria which cause axillary and/or plantar malodours. Propylene glycol, triclosan, benzalkonium chloride and metal (e.g. Al) salts are common additives in commercial deodorant formulations.[1] There are also concerns about chronic exposure to these compounds. The deleterious effects of chronic exposure to metal salts (particularly aluminium) have already been discussed. There are similar concerns regarding chronic exposure to several other deodorant additives. Triclosan is a very common bacterial growth inhibitor in commercial deodorants. Despite this, chronic triclosan exposure has been linked to a myriad of serious

health problems.[4] Furthermore, triclosan persists for a relatively short period and is rapidly inactivated.[35] Therefore, there is a need to develop more effective antimicrobial deodorant components.

- Masking the malodour with other, more palatable aromas.

Due to the health concerns associated with some of the current antiperspirant/deodorant components, the development of safer formulations is required. These products are used at least daily by large section of the population and thus the potential for chronic and additive effects needs to be taken into account. Products with greater functionality would allow for lower doses and thus lower health risks. Of concern, bioactive deodorant components may be added to deodorant compositions in relatively high levels. The antibacterial compound triclosan is present in some products at up to 1% of the formulation (although 0.1-0.15 % is more common).[36] Triclosan is readily absorbed through the skin and conjugated forms have been reported to persist in the plasma for several days.[37] Indeed, the time required to clear 50% of a single triclosan dose in humans has been reported to be 96hr.[38] As deodorants are applied frequently, and often as repeated applications, the implications and possibility for systemic accumulation and chronic toxicity are obvious.

Table 3: Putative identification of the major compounds detected in all of the *T. lanceolata* berry methanolic, aqueous and ethyl acetate extracts.

Putative Identification	Empirical Formula	Molecular Mass	Retention Time (mins)
7-Hydroxy-5-heptynoic acid	$C_7 H_{10} O_3$	142.0619	0.842
Glucose	$C_6 H_{12} O_6$	180.0633	1.713
Quinic acid	$C_7 H_{12} O_6$	192.0629	1.775
Butibufen	$C_{14} H_{20} O_2$	220.1462	10.933
Cyclohexyl (1-hydroxycyclopentyl) acetic acid	$C_{13} H_{22} O_3$	226.1566	10.074
Polygodial	$C_{15} H_{22} O_2$	234.1628	7.768
Capsidiol	$C_{15} H_{24} O_2$	236.1779	5.949
3,3'-(2-Oxo-1,1-cyclohexanediyl) dipropanoic acid	$C_{12} H_{18} O_5$	242.1136	1.917
Salutarisolide	$C_{15} H_{20} O_3$	248.1416	7.969
3-(1-adamantyl)-2-oxopropyl acetate	$C_{15} H_{22} O_3$	250.1534	9.061
Punctaporin B	$C_{15} H_{24} O_3$	252.1719	8.524
Abscisic acid	$C_{15} H_{20} O_4$	264.1357	8.958
5-(4-Hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-pentanoic acid	$C_{15} H_{22} O_4$	266.1497	9.192
Lauroylsarcosine	$C_{15} H_{29} N O_3$	271.2151	7.396
Gallic acid, 2-ethylhexyl ester isomer 2	$C_{15} H_{22} O_5$	282.1461	7.459
Gallic acid, 2-ethylhexyl ester isomer 3	$C_{15} H_{22} O_5$	282.1462	5.305
Dihydroartemisinin	$C_{15} H_{24} O_5$	284.1605	8.31
Vomitoxin	$C_{15} H_{20} O_6$	296.1259	11.018
Benzeneacetic acid, 4-phenyl-, 4-methylphenyl ester	$C_{21} H_{18} O_2$	302.1283	9.661
Matricin	$C_{17} H_{22} O_5$	306.1463	7.97
9S,10S,11R-trihydroxy-12Z-octadecenoic acid	$C_{18} H_{34} O_5$	330.2407	8.811
Combretastastin A-1	$C_{18} H_{20} O_6$	332.1319	11.166

Only compounds detected in all berry extracts by high accuracy QTOF LC-MS are shown.

There is a need to develop safe effective new deodorant compounds that address these concerns. Plant formulations are ideal candidates for potential deodorant compounds. Many plant extracts and oils have been used traditionally to inhibit bacterial growth, and in some case their efficacy has been verified by rigorous scientific examination. Furthermore, natural alternatives that inhibit the growth of odour forming bacteria are desirable as they may be more acceptable to consumers due to their natural origin and consumer perception of safety. This study examined the growth inhibitory properties of *Tasmannia* spp. extracts against several bacterial species associated with axillary and plantar malodour formation. *Tasmannia* spp. extracts was selected for study as previous studies have reported good activity for plants of this genus against several human pathogens.[9-16]

Our study confirmed the potential of *Tasmannia* spp. fruit and leaf extracts for inhibiting the growth of axillary and foot malodour producing bacteria. The methanolic leaf extract was the most promising growth inhibitor of all bacterial species. As *Corynebacterium* spp. have been reported to produce the most unpleasant and strongest malodours,[39] the strong inhibition of *C. jeikeium* growth (LD MIC = 480µg/mL) by the *T. lanceolata* methanolic fruit extract 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 3130ppm (equivalent to 3130µg/mL).[40] In contrast, an MIC of 513µg/mL was determined against *S. epidermidis* for the *T. lanceolata* methanolic fruit extract screened in our studies. This represents an approximately six-fold increased potency compared to the *Caesalpinia minosoides* study. Similarly, *Cassia alata,*[41] *Barleria lupulina* and *Psidium guajava*[42] were 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 625µg/mL.[42] 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 methanolic *T. lanceolata* fruit extract reported in our study had marginally better potency compared to the *H. sabdariffa* formulation. Thus, the addition of methanolic *T. lanceolata* fruit extract to deodorant preparations has commercial potential.

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 *Corynebacterium xerosis* growth.[44] However, that study is of limited value as a single, high dose of the extract was screened (approximately 500µg/disc). Furthermore, MIC values were not determined, making a comparison with other studies impossible. Similarly, *Anethum graveolens* essential oils inhibited *Corynebacterium* spp. Growth.[45] However, MIC values were also not reported, making a comparison difficult. Furthermore, that study utilised a disc diffusion assay to test the growth inhibitory activity of the oil. Whilst disc diffusion

assays are suited to the 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. Another study reported potent inhibition of *C. xerosis* and *S. epidermidis* growth by a supercritical *Humulus lupulus* (commonly known as hops) extract.[46] The authors reported 15mm zones of inhibition for a 2mg/mL *H. lupulus* extract using a disc diffusion assay. It is difficult to compare the MIC quantification results reported in that study to those in our study due to the use of differing methods. However, a direct comparison of the disc diffusion studies is possible. Our study screened substantially lower doses in the disc diffusion assay compared to the *H. lupulus* study, yet recorded similar inhibition zones. Thus, it is likely that the potency of the *Tasmannia* spp. extracts compare favourably with the *H. lupulus* extract.

Notably, all of the *Tasmannia* spp. extracts were nontoxic towards *Artemia* nauplii and are thus likely to be safe to use as 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 (and multiple previous studies on *Tasmannia* spp. extracts)[9-16] have all reported a general lack of toxicity, it is noteworthy that all of these studies have examined acute toxicity. Pharmacodynamics and pharmacokinetics 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 polar *Tasmannia* spp. extracts as a natural antibacterial components for deodorant formulation. These extracts were potent growth inhibitors of all of the malodour forming bacteria tested. Furthermore, the lack of toxicity of the extract indicates its suitability for topical use.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ALA: Brine-shrimp lethality assay; **DMSO:** Dimethyl sulfoxide; **FIC:** Fractional Inhibitory Concentration; **INT:** ρ-iodonitrotetrazolium chloride; **LC_{50}:** Dose of sample necessary to have a lethal effect on 50% of test organisms or cells; **MIC:** Minimum Inhibitory Concentration.

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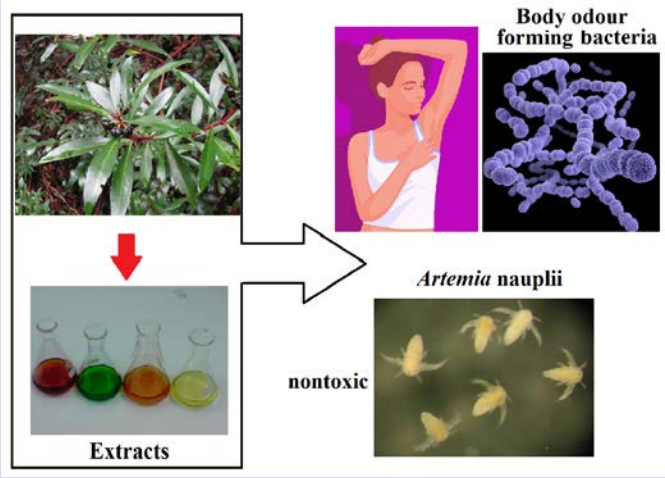
PICTORIAL ABSTRACT



SUMMARY

- *Tasmannia* spp. extracts were screened for the ability to block the growth of malodour producing bacteria .
- The extracts were also tested for toxicity in the Artemia nauplii bioassay.
- The phytochemical composition of most active *T. lanceolata* extracts were evaluated by LC-MS analysis.

About Authors

Dr. Ian Cock leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin including *Aloe vera*, South Asian and South American tropical fruits, as well as Australia plants including *Scaevola spinescens, Pittosporum phylliraeoides, Terminalia ferdinandiana* (Kakadu plum), Australian Acacias, Syzygiums, Petalostigmas and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in nearly 200 scientific publications in a variety of peer reviewed journals.