Pharmacogn. Commn. 2019;9(4):124-129
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcogcommn.org

Original Article

Preliminary Evaluations of the Antibacterial Activity of *Tasmannia lanceolata* against *Bacillus anthracis*: Natural Resource Probing to Prevent Anthrax

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ABSTRACT

Introduction: *Bacillus anthracis* is bacterial cause of the highly fatal, zoonotic disease anthrax. Tasmanian pepper (*Tasmannia lanceolata*) has been previously documented for its antiseptic properties against other pathogenic bacteria. This study sought to investigate the effectiveness of *T. lanceolata* as an inhibitory agent against *B. anthracis*. **Methods:** *Tasmannia lanceolata* berry and leaf extracts were prepared with either water or methanol as the extraction solvent. Growth inhibition was assessed against *B. anthracis* strain PMO through disc diffusion as assaying. The relative MIC values of each extract was quantified to evaluate efficacy as a sterilant. The degree of toxicity of each extract was achieved using the widely used *Artemia franciscana* nauplii bioassay. **Results:** *T. lanceolata* leaf extracts inhibited the growth of *B. anthracis* in the disc diffusion assay, with MIC values of 2333 and 1873 µg/mL respectively. In contrast, the *T. lanceolata* berry extracts were completely devoid of growth inhibitory activity. All *T. lanceolata* extracts (both berry and leaf) were non-toxic (LC_{50} values substantially >1000 µg/mL) as determined via the *Artemia franciscana* bioassay. **Conclusion:** *T. lanceolata* berry and leaf extracts are not only non-toxicity, but also had moderate growth inhibitory bioactivity against *B. anthracis*, highlighting their potential in the treatment of anthrax.

Key words: *Bacillus anthracis*, Anthrax, Antioxidant, Zoonotic, *Tasmannia lanceolata*, Tasmanian pepper.

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DOI: 10.5530/pc.2019.4.26

INTRODUCTION

Bacillus anthracis is a gram-positive, endospore-forming bacterium responsible for the disease anthrax. Whilst anthrax became notorious as a result of its use in bioterrorism in the early 2000s, livestock infection via endospore inhalation is a far more common manifestation of the disease.[1] From a cultural standpoint, the most notable reporting of weaponised anthrax to date occurred in 2001, where *B. anthracis* spores were mailed to several locations in the US. This resulted in the infection of twenty people and this, among other incidents, resulted in widespread knowledge of weaponised anthrax.[2] However, this practice is not new and in fact, anthrax has been weaponised and used in warfare, at least since medieval times. Looking past its associations with life-threatening infections and bioterrorism, *B. anthracis* is more importantly, one of many microorganisms naturally present in soil flora. In fact, inadvertent mass infections can be traced as far back as Ancient Egypt and it is theorised that the plagues described in ancient literature may have been mass infections of anthrax.[3] Human anthrax is relatively uncommon compared to the prevalence in other vertebrates. Indeed, outbreaks in both wildlife and livestock are a significant health and economic issue in many parts of the world.

Anthrax infection in humans occurs when *B. anthracis* endospores enter the body through inhalation, ingestion or through abrasions in the skin.[4] Members of the genus *Bacillus* can produce endospores in response to adverse environmental conditions. These endospore structures protect the bacterial genetic material until conditions are once again favourable to support growth. Endospores are metabolically dormant, non-dividing cells that are highly resistant to drying, heat and ultraviolet radiation and are the causative agent of disease initiation, rather than the vegetative cells.[5] Once internalised, the body elicits an immune response, although the encapsulating endospore coating provides protection for the cells and can contribute to germination (a process known as macrophage-enhanced germination).[6] The germinating bacterium can then resume normal metabolic functions and toxins are subsequently produced. Inhalation anthrax is the most dangerous of the three forms of the disease and infection, often resulting in death unless rapid treatment is administered.[7]

Tasmannia lanceolata (Poir.) A.C.Sm (commonly known as Tasmanian pepper or mountain pepperberry) is a shrub indigenous to the woodlands and cool temperate rainforests of Tasmania and south-eastern coastal regions of the mainland Australia, as far north as Sydney.[8] It is a medium-large shrub of approximately 2-5 m in height. The leaves, berries and bark of this plant are traditionally used medicinally as well as a food source and flavouring agent. When dried, the berry forms a small, solid peppercorn suitable for crushing/milling and provides a spicy flavour and sharp aroma. Historically, the leaves have been used as herbs whilst its berries were used as a spice. Australian Aborigines used *T. lanceolata* as a therapeutic agent to treat stomach disorders.[8,9] *T. lanceolata* has also been used traditionally for the treatment and cure of colic, venereal diseases, skin disorders and stomach aches.[10] European colonists also documented the therapeutic properties of *T. lanceolata* and the bark was commonly used as a substitute for other herbal remedies and to treat scurvy (due to its high anti-antioxidant content).[10] Despite its extensive ethnobotanical usage, limited scientific studies into the therapeutic properties of

T. lanceolata have been published. It has been theorised that the plants high antioxidant capacity may provide therapeutic benefits. Indeed, investigations of *T. lanceolata* within our laboratory have highlighted the inhibition of bacterial growth by *T. lanceolata* berries, leaves and peppercorns against various pathogenic bacteria,[11] as well as protozoal parasites.[12] Despite the documented ability of *T. lanceolata* to inhibit the growth of many bacterial species, to the best of our knowledge there have been no studies focusing on *T. lanceolata* against *B. anthracis* growth. For this work, *T. lanceolata* berry and leaf extracts were prepared and their antibacterial potential was assessed against this pathogen to determine their potential in preventing and treating anthrax.

MATERIALS AND METHODS

Plant collection and extraction

T. lanceolata semi-dry berry (without seed) and dried leaf material was purchased from GoWild Harvest, Australia. Voucher specimens (TP-GU-GWH-2016B and TP-GU-GWH-2016L for the berry and leaf respectively) are stored in the School of Environment and Science, Griffith University. The berries were further dried in a Sunbeam food dehydrator until constant mass was acquired upon repeated measurements. All plant materials were stored at -30°C until use. Extract preparation was performed as previously described.[13,14] The plant materials were thawed and freshly ground to a coarse powder prior to extraction. Individual 1g quantities of the ground plant materials were weighing into each of two tubes and 50mL of methanol or water were added respectively. Methanol (AR grade) was obtained from Ajax Fine Chemicals, Australia. The berry and leaf material were extracted in each solvent for 24 hr at 4°C with gentle shaking. The extracts were subsequently filtered under vacuum through Whatman No. 54 filter paper, followed by drying in an Eppendorf concentrator 5301. The resultant dry extract was weighed and redissolved in 10mL deionised water (containing 1% DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the extracts for the presence of glycosides, flavonoids, phenolic compounds, alkaloids, anthraquinones, cardiac, polysteroids, saponins, tannins and triterpenoids were conducted using established assays.[15,16]

Antioxidant capacity

The antioxidant capacity of each sample was assessed using the DPPH free radical scavenging method with modifications.[17,18] Briefly, ascorbic acid (0-25µg per well) was used as a reference and the absorbances recorded at 515nm. All were performed alongside controls on each plate and all tests performed in triplicate, each with three technical replicates (n=9). 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.

Bacillus anthracis isolation and screening

Bacillus anthracis was originally obtained from a water sample taken from Paralana hot springs (30°17'49"S, 139°44'15"E), South Australia. Isolation was achieved through successive culturing steps using a modified Peptone/Yeast Extract (PYE) agar as previously described.[19,20] Genomic DNA was extracted using phenol/chloroform separation as previously described.[21] PCR amplification of the 16S rRNA gene was performed as previously described[22] and sequence analyses of the isolate generated a contig of 1428bp. The bacterium was revealed to be 99.92% similar to *B. anthracis* by EzTaxon and designated as *Bacillus anthracis* strain PMO. The GenBank accession number for the 16S rRNA gene sequence for the isolate is KR003287.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay.[23,24] Briefly, 100µL of the test bacteria were grown in 10mL of fresh nutrient broth media until they reached a count of ~10^8 cells/mL. A 100µL volume of the bacterial suspension was spread onto nutrient agar plates and extracts were tested for antibacterial activity using 5mm sterilised filter paper discs. Discs were infused with 10µL of individual test samples, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4°C for 2h before incubation at 30°C for 24h. The diameters of the zones of inhibition (ZOIs) were measured to the closest whole millimetre. Each assay was performed three times, each with technical triplicates (n=9). Mean values (± SD) are reported in this study. Standard discs of penicillin (2µg) and ampicillin (10µg) were obtained from Oxoid Ltd., Australia and used as positive controls for antibacterial activity. Filter discs impregnated with 10µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The Minimum Inhibitory Concentrations (MIC) of the extracts was determined as previously described.[25,26] Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were impregnated with 10µL of the extract dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted. In linear regression was used to calculate the MIC values.

Toxicity screening

Reference toxin for toxicity screening

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

Artemia franciscana nauplii toxicity screening

Toxicity was evaluated using a modified *Artemia franciscana* nauplii lethality assay.[27] Briefly, 400 µL of seawater containing approximately 43 (mean 43.2, n=155, SD 14.5) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400µL of diluted plant extracts or the reference toxin were transferred to the wells and incubated at 25±1°C under artificial light (1000 Lux). A negative control (400µL seawater) was run in triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was observed within 10 sec. After 24h, 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 ± SD of three independent experiments, each with technical triplicates (n=9).

RESULTS

Extraction of 1g of the *T. lanceolata* plant materials with the solvents yielded dried plant extracts ranging from 111mg (*T. lanceolata* aqueous berry) to 144mg (methanolic *T. lanceolata* leaf extract; Table 1). The dried extracts were resuspended in 10mL of deionised water (containing 1% DMSO) resulting in the extract concentrations shown in Table 1.

Qualitative phytochemical studies showed little difference between the aqueous and methanolic extracts. Similarly, few differences were noted between the phytochemical classes detected between the berry and

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

Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Polysteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
ML	144	14.4	+++	+++	+++	–	+++	+	–	–	–	+++	–	–	–
AL	134	13.4	+++	+++	+++	–	++	–	–	–	–	+++	–	–	–
MF	171	17.1	+++	+++	+++	–	++	+	–	–	–	+++	–	–	–
AF	111	11.1	+++	+++	+++	–	–	–	–	–	–	+++	–	–	–

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. ML = methanolic *T. lanceolata* leaf extract; AL = aqueous *T. lanceolata* leaf extract; MF = methanolic *T. lanceolata* berry extract; AF = aqueous *T. lanceolata* berry extract.

corresponding fruit extracts. High levels of polyphenolic compounds (both water soluble and insoluble) were extracted in all the *T. lanceolata* extracts. Similarly, high levels of flavonoids and moderate levels of saponins were detected in the aqueous and methanolic berry and leaf extracts.

To assess the inhibitory activity of the crude *T. lanceolata* plant extracts against *B. anthracis*, 10µL aliquots of each were screened using standard disc diffusion assays. Antibacterial activity against *B. anthracis* was observed in all 4 extracts tested (Figure 1). The methanolic and aqueous leaf extract inhibited *B. anthracis* growth, with ZOIs of 6.7 ± 0.58mm and 7.0 ± 0.0mm respectively. In contrast, the berry extracts were completely devoid of growth inhibitory activity. The ampicillin control had inhibitory zones of 10.0 ± 1.7 mm, indicating that the assay was functioning correctly.

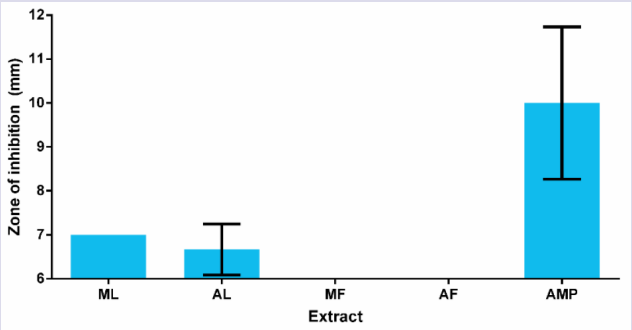

Figure 1: Growth inhibitory activity of *T. lanceolata* leaf and berry extracts against the *B. anthracis* environmental isolate ML = methanolic *T. lanceolata* leaf extract; AL = aqueous *T. lanceolata* leaf extract; MF = methanolic *T. lanceolata* berry extract; AF = aqueous *T. lanceolata* berry extract. AMP = Ampicillin (10µg). Measured as zones of inhibition (mm) and expressed as mean zones of inhibition ± SD.

The antimicrobial efficacy was further quantified by determining the MIC values (Table 2). Only the leaf extracts were growth inhibitory. Therefore, no MIC values are reported for the berry extracts. Both the methanolic and aqueous leaf extracts had similar MIC values (~1900-2300µg/mL) and are indicative of moderate growth inhibitory activity.

All extracts were initially screened undiluted in the *Artemia* nauplii assay as a measure of toxicity (Figure 2). For comparison, the reference toxin potassium dichromate (1000µg/mL) was also tested in the bioassay. The potassium dichromate reference toxin was rapid in its onset, promoting nauplii death within the first 3h of exposure with 100% mortality evident following 4-5hr (unpublished results). Similarly, all the *T. lanceolata* extracts displayed significant mortality rates following 24h exposure (>50%). The extracts were therefore tested across a range of dilutions to determine the LC_{50} values. Notably, all extracts had LC_{50} values substantially >1000µg/mL. As LC_{50} values >1000µg/mL have

Table 2: Minimum inhibitory concentration (µg/mL) of the plant extracts and LC_{50} values (µg/mL) in the *Artemia nauplii* bioassay.

Extract / Control	MIC	LC_{50}
ML	2333	2096
AL	1873	2665
MF	-	2573
AF	-	2376
PC	-	186
SW		

Numbers indicate the mean MIC and LC_{50} values of triplicate determinations. - indicates no inhibition. ML = methanolic *T. lanceolata* leaf extract; AL = aqueous *T. lanceolata* leaf extract; MF = methanolic *T. lanceolata* berry extract; AF = aqueous *T. lanceolata* berry extract; PC = Potassium dichromate; SW = artificial seawater.

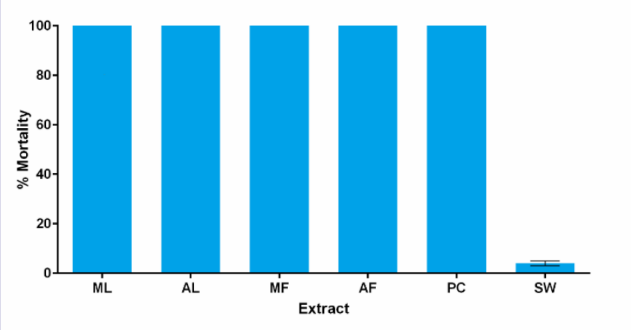

Figure 2: The lethality of the *T. lanceolata* extracts (2000µg/mL) alongside the potassium dichromate control (1000µg/mL) towards *Artemia franciscana* nauplii after 24hr exposure. ML = methanolic *T. lanceolata* leaf extract; AL = aqueous *T. lanceolata* leaf extract; MF = methanolic *T. lanceolata* berry extract; AF = aqueous *T. lanceolata* berry extract; PC = potassium dichromate; SW = artificial seawater (negative control). Results are expressed as mean % mortality ± SD.

previously been defined as non-toxic,[27] all *T. lanceolata* extracts were determined to be non-toxic.

DISCUSSION

This study was performed to investigate the antibacterial potential *T. lanceolata* to inhibit the growth of *B. anthracis*, thereby blocking the induction of anthrax treating the disease once it is established. Despite the efficacy of commonly used antibiotics, recent increases in bacterial resistances have made the development of new antibiotic therapies a high priority.[28] Furthermore, parallel decreases in the introduction novel antibiotic therapies in recent years has further compounded this problem. Because of this, interests in the potential for medicinal plants for new antibiotic chemotherapies has drastically increased in recent years.[29] Numerous investigations into high antioxidant plants have reported on their antibacterial properties against a broad panel of bacterial pathogens, including those capable of inhibiting *B. anthracis* growth.[24,25] With respect to *T. lanceolata*, in addition to inhibiting bacterial pathogens,[12-16] extracts prepared from the berries and leaves have recently been reported to inhibit the proliferation of the gastrointestinal protozoan parasite *Giardia duodenalis*.[30] Demonstrating its therapeutic potential against both prokaryotic and eukaryotic pathogens. However, despite the relative wealth of information into the therapeutic potential of *T. lanceolata*, it is yet to be comprehensively studied for antibacterial activity against an endospore forming bacterial pathogen. Herein, we report moderate growth inhibitory activity for *T. lanceolata* berry and leaf extracts against the bacterial cause of the disease anthrax, a potentially fatal infection acquired through inhalation, ingestion or through skin abrasions in contact with *B. anthracis*.

B. anthracis growth inhibitory activity was evident in the *T. lanceolata* fruit and leaf extracts. Therefore, these extracts have potential for the prevention and treatment of anthrax and are potential targets for drug discovery. Detailed analyses into the phytochemistry of the *T. lanceolata* fruit and leaf extracts was beyond the scope of this study. However, several notable compounds have been previously reported in *T. lanceolata* extracts and essential oils.[8] These include multiple monoterpenoids (e.g. 1, 8-cineole, terpinen-4-ol, α-pinene and β-pinene) and sesquiterpenoids (particularly polygodial), flavonoids (including quercetin and rutin), other phenolics (including coumaric acid and caffeic acid) and hydrocarbons. Many of these compounds have also been isolated from other plant species and have been shown to have potent antimicrobial activity.[8] Therefore, these components may also contribute to the *B. anthracis* growth inhibitory properties of the extracts tested in this study. Of particular note, high abundances of the sesquiterpenoid polygodial have been reported in *T. lanceolata* berry and leaf extracts, although higher relative levels have been reported in the berry extracts. Indeed, polygodial can account for approximately 40% of commercial *T. lanceolata* essential oil components.[8,31] Interestingly, several studies have reported the therapeutic properties of this compound, including its antibacterial,[31] antifungal,[32] anti-hyperalgesia,[33] anti-inflammatory, antiallergic and vasorelaxation activities.[34] Other structurally similar sesquiterpenoids have also been reported in *T. lanceolata* extracts prepared in a similar way to our study.[30] Of further interest, salidroside has previously been reported in *T. lanceolata* berry and leaf methanolic and aqueous extracts. Salidroside has been linked with antibacterial and anti-parasitic activity in multiple plants. Plants of the genus *Warburgia* are known to have significant levels of s salidroside[35] and several *Warburgia* spp. have been reported to have strong antimicrobial and antiparasitic activities.[36,37]

T. lanceolata berry and leaf extracts have also been reported to contain an abundance of gallotannin components.[30] Gallotannins have been reported to inhibit the growth of a broad spectrum of microbial species[38] via binding cell surface lipoteichoic acid and proline-rich cell surface proteins[39,40] and by inhibiting glucosyltransferase enzymes.[41] The stilbene combretastatin A1 has also been putatively identified in all *T. lanceolata* berry extracts,[30] albeit, in low abundance. Combretastatin's are well known for their potent ability to block cancer cell progression and induce apoptosis by binding intracellular tubulin, thereby disrupting microtubule formation.[42] Combretastatin's act in a similar fashion to that of colchicine (N-[(7S)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptlen-7-yl] acetamide) by binding the colchicine's binding site in tubulin.[43] Thus, the *T. lanceolata* combretastatin's may block bacterial replication events.

The findings reported here also indicate that the *T. lanceolata* berry and leaf extracts were nontoxic, with LC_{50} values substantially >2000 µg/mL. Toxicity was assessed in this study using the test organism *A. franciscana*. The lack of toxicity of the *T. lanceolata* extracts in our study indicates that the extracts are safe for medicinal usage. This is hardly surprising as *T. lanceolata* is highly nutritious and has long been used as a spice (berries) and herb (leaves). However, whilst the extracts examined in this report have potential as *B. anthracis* growth inhibitory agents, caution is required before these compounds can be applied to medicinal purposes. Toxicity towards *A. franciscana* has previously been shown to correlate well with toxicity towards human cells for many toxins.[30] However, further studies are required to determine whether this is also true for the *T. lanceolata* extracts examined in these studies. The results of this study indicate that the *T. lanceolata* extracts examined warrant further study due to their *B. anthracis* growth inhibitory activity. Purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents.

CONCLUSION

The lack of toxicity and the moderate *B. anthraicis* growth inhibitory activity of the *T. lanceolata* leaf extracts demonstrate their potential as therapeutic agents for the prevention and treatment of anthrax.

ACKNOWLEDGEMENT

Financial support for this work was provided by the Environmental Futures Research Institute and the School of Environment and Science, Griffith University, Australia.

CONFLICT OF INTEREST

The authors declare no conflict of interest

ABBREVIATIONS

DMSO: Dimethyl sulfoxide; **LC_{50}:** The concentration required to achieve 50% mortality; **MIC:** Minimum inhibitory concentration.

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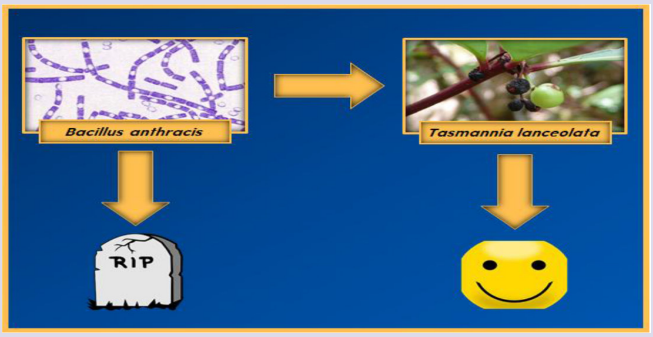
PICTORIAL ABSTRACT



SUMMARY

- *T. lanceolata* berry and leaf extracts were tested for growth inhibitory activity against *B. anthracis*.
- The leaf extracts were moderate bacterial growth inhibitors.
- The berry extracts were completely devoid of growth inhibitory activity.
- All extracts were nontoxic in the Artemia nauplii assay.

ABOUT AUTHORS

Mr. Cameron Lee completed his Bachelor of Science (BSc) in 2015 and is currently undertaking his Ph.D with Dr. Anthony Carlson Greene. His research involves the investigation of halophilic aerobes and anaerobes capable of manganese oxidation and perchlorate reduction. He has extensive experience in aerobic and anaerobic cultivation/isolation and in numerous analytical techniques associated with heavy metal analysis.

Dr. Mitchell Henry Wright is a Geomicrobiologist who received his Ph.D in 2014 for his work investigating the manganese reduction/oxidation characteristics of environmental bacteria. From 2016 to 2018 he undertook a postdoctoral researcher role under the mentorship of Prof. Bradley Tebo, where he explored the bacterial oxidative formation and removal of complexed Mn (III) and the implications of these processes on the global ocean. Upon returning to Australia, Dr. Mitchell H. Wright was recruited by First Choice College and to date, oversees their Department of Research and Development. Additionally, he has returned to his former lab (lead by Dr. Ian Cock) to continue his research into the antimicrobial potential of native plants.

Dr. Anthony Carlson Greene is a senior lecturer and researcher at Griffith University, Brisbane, Australia. He obtained his Ph.D in Microbiology from the University of New South Wales and focuses on extreme environments, bioremediation and geomicrobiology. His specific interests include the microbial ecology of thermophilic, saline and alkaliphilic environments and the mechanisms and industrial potential of extremophilic bacteria contained therein.

Ms. Huda Aldosary is undertaking her Ph.D at Griffith University (Brisbane, Australia) under the supervision of Dr. Anthony Greene. Her primary interests involve the investigation of bacterial hydrocarbon degradation and the potential for these processes in the remediation of contaminated sites. She has extensive experience with anaerobic organisms and specialises in extremophiles.

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 over 150 publications across a variety of peer reviewed journals.