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Original Article

Growth inhibition of the zoonotic bacteria *Bacillus anthracis* by high antioxidant Australian plants: New leads for the prevention and treatment of anthrax

Mitchell Henry Wright[1], Ben Matthews[2], Anthony Carlson Greene[1] and Ian Edwin Cock,[1,3*]

[1]*School of Natural Sciences, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, Australia.*
[2]*Smart Waters Research Centre, Griffith University, Gold Coast, Australia.*
[3]*Environmental Futures Research Institute, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, Australia.*

ABSTRACT

Introduction: Anthrax is severe acute disease caused by *Bacillus anthracis* infections. If untreated, it often results in mortality. High antioxidant plant extracts have documented therapeutic properties as general antiseptics, inhibiting the growth of a wide variety of bacterial species. This study examines the ability of selected high antioxidant Australian plant extracts to inhibit *B. anthracis* growth. **Methods:** Solvent extracts were prepared using various high antioxidant Australian fruits and herbs and investigated by disc diffusion assay for the ability to inhibit the growth of an environmental strain of *B. anthracis*. Their MIC values were determined to quantify and compare their efficacies. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. The most potent extracts were analysed by non-targeted HPLC-QTOF mass spectroscopy (with screening against 3 compound databases) for the identification and characterisation of individual components in crude plant extracts. **Results:** Methanolic and aqueous extracts of several high antioxidant plant extract sdisplayed potent antibacterial activity in the disc diffusion assay against *B. anthracis*. The aqueous and methanolic extracts of lemon aspen, as well as the methanolic extracts of muntries, Illawarra plum and native tamarind were particularly potent growth inhibitors with MIC values < 1000 μg/mL. Furthermore, all of these extracts were nontoxic in the *Artemia fransiscana* bioassay, with LC50 values substantially > 1000 μg/mL. Non-biased phytochemical analysis of the lemon aspen aqueous and methanolic extracts putatively identified 85 compounds and highlighted several that may contribute to the ability of these extracts to inhibit the growth of *B. anthracis*. **Conclusion:** The low toxicity of several high antioxidant plant extracts and their potent inhibitory bioactivity against *B. anthracis* indicates their potential as medicinal agents in the treatment and prevention of anthrax. Lemon aspen is particularly worthy of further study.

Key words: Anthrax, Antioxidant, *Bacillus anthracis*, lemon aspen, Muntries, Metabolomic profiling, Syzygium, Zoonotic.

INTRODUCTION

The prevention and treatment of zoonotic diseases poses a unique set of difficulties not encountered for other diseases. Many pathogens may be controlled by isolating the infected individual so that they cannot infect others and allowing the disease to run its course. Alternatively, targeted immunization programs may be useful to establish herd immunity, allowing the disease to dissipate.[1] Indeed, such approaches have allowed the eradication of the infectious disease smallpox.[2] However, zoonotic pathogens may persist in a non-human reservoir until they are able to infect new hosts. The recent outbreaks of Ebola in West Africa, Hendra virus in Australia and swine influenza globally, demonstrate how new infectious

Corresponding Address
Dr. Ian Edwin Cock
School of Natural Sciences and Environmental Futures Research Institute Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, Australia.
E-mail: I.Cock@griffith.edu.au
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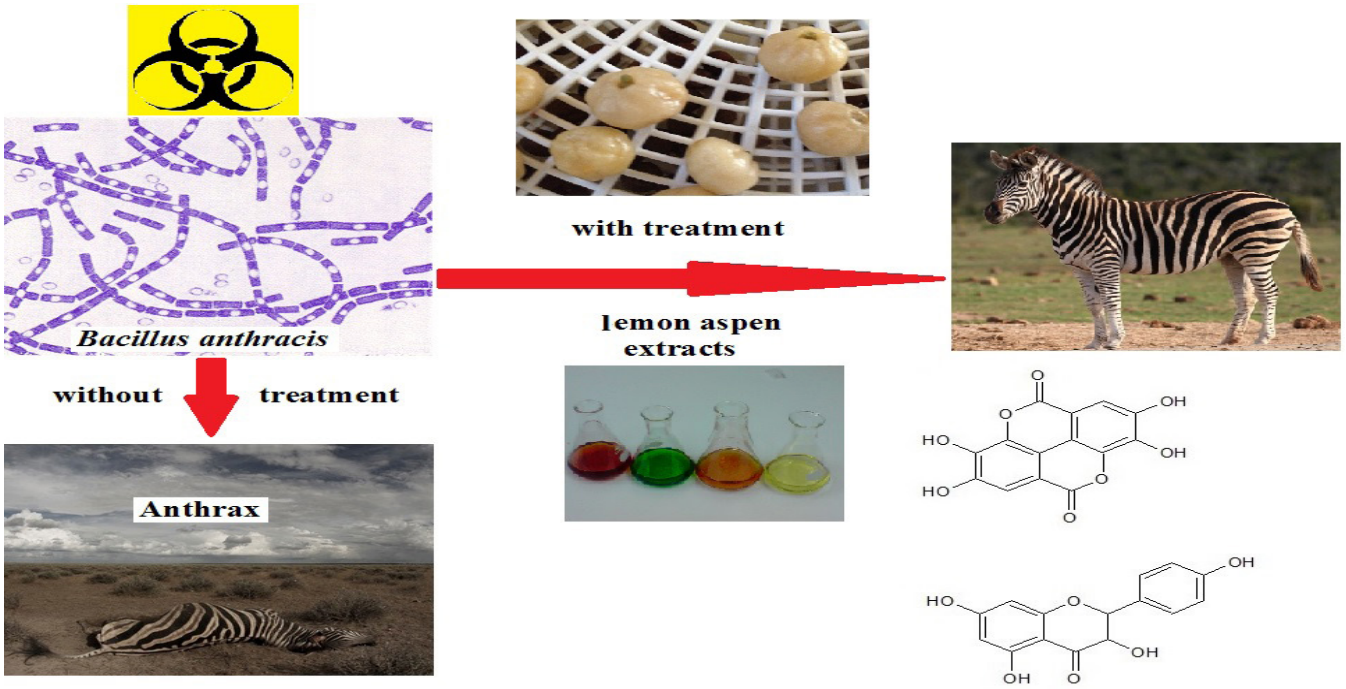

Graphical Abstract

agents may develop in other species and spread to human populations with dramatic results. However, it is not only new zoonotic diseases that are of concern. International outbreaks of ancient zoonotic diseases occur relatively frequently and highlight the need to monitor and treat livestock and wildlife, as well as the human population.

Anthrax is an ancient acute disease which is caused by *Bacillus anthracis*. The disease is not contagious through contact and is usually contracted in 3 main ways:

- Cutaneous anthrax is perhaps the most common *B. anthracis* infection in humans.[3] It may occur when spores enter via skin cuts and abrasions. It is most commonly associated with individuals that are in contact with contaminated animals or animal products (eg. leather from contaminated livestock). Alternatively, as the bacterial spores may be present in soil, it may be contracted from environmental sources through broken skin. This form of anthrax is rarely fatal if treated.
- Gastrointestinal anthrax is a much more serious form of the disease and is generally contracted in humans by consuming *B. anthracis* infected meat.[4] After invading the gastrointestinal tract (GIT), the bacteria spreads through the body via the bloodstream, producing toxins. Whilst still treatable, this form of the disease has a much higher mortality rate.
- Inhalation/pulmonary anthrax is rare in humans, but relatively common in herbivorous livestock and wild animal populations, which may inhale the bacterial spores whilst grazing.[5] This form of the disease generally has the highest mortality rate (over 85% historically), although modern medical advances have reduced this rate substantially. As for cutaneous anthrax, individuals working with contaminated animal products (eg. wool, animal skins etc) are most prone to pulmonary anthrax.

Outbreaks of anthrax are relatively common globally amongst wild animal populations.[6] Africa is particularly prone, with outbreaks reported every year amongst the wildlife populations.[7] As *B. anthracis* can infect a wide variety of species, a large reservoir of the bacterium is constantly available to cross species and infect farm animals and humans. Indeed, the same study also reported recent outbreaks in livestock and in humans in Namibia, Zimbabwe and Lesotho in 2013 and 2014. Whilst perhaps less prevalent, anthrax outbreaks are also reasonably common in other regions internationally. Sizeable recent outbreaks have been reported in China in 2012,[8] in India in 2011[9] and 2014,[10] in Canada in 2006[11] and in the United States in 2005.[12] There have also been reports of anthrax amongst European heroin users, with reported cases in Denmark, England, France, Germany, Norway and Scotland.[13] The threat of anthrax outbreaks via bioterrorism has also received much recent attention following multiple cases of inhalational anthrax in the United States resulting from intentional release of *B. anthracis* spores.[14]

Current strategies for the prevention and treatment of anthrax generally rely on either vaccination or antibiotic

administration. Effective vaccines have been available since the 19th century. However, severe adverse reactions may occur in approximately 1% of the population.[15] Furthermore, *B. anthracis* vaccines are most useful as a preventative measure and are generally of little use once the disease is contracted. Instead, large doses of intraveneous and/or oral antibiotics (eg. fluoroquinolones, penicillin, erythromycin, vancomycin) are used and are generally effective for treating anthrax infections.[16] However, due to the potential development of super-resistant strains, it is important to search for new antibiotics with high efficacy.[17] The search is ongoing by (a) the design and synthesis of new agents, and (b) re-searching the repertoire of natural resources for as yet unrecognised or poorly characterised antimicrobial agents. Furthermore, the development of new anti-*B.anthracis* products to disinfect contaminated sites, soils etc. would decrease the spread of anthrax and thus decrease the impact of the disease.

The antiseptic qualities of medicinal plants have been long recognised by many cultures. Recently there has been a revival of interest in herbal medications due to perceptions that there is often a lower incidence of adverse reactions to natural phytochemicals compared to synthetic pharmaceuticals. Antimicrobial plant extracts with high antioxidant contents are particularly attractive as they may treat the symptoms of anthrax as well as blocking growth of the pathogen and thus have pleuripotent effects. Recent studies have demonstrated the potent inhibitory activity of several Australian plants with high antioxidant capacities against a wide panel of medicinally important bacteria.[18-21] Furthermore, potent growth inhibition of the bacterial triggers of autoimmune inflammatory diseases has also been reported for high antioxidant Australian fruits[22-24] and culinary herbs.[25] Despite this, many high antioxidant Australian plants are yet to be rigorously tested for the ability to inhibit the growth of pathogenic bacteria. The current study examines the growth inhibitory activity of extracts of selected high antioxidant Australian plants against *B. anthracis* with the aim of determining new leads for the prevention and treatment of anthrax.

MATERIALS AND METHODS

Plant source and extraction

The *Syzygium australe* (brush cherry), *Syzygium leuhmannii* (riberry), *Davidsonia pruriens* (Davidson's plum) and *Elaeoparpus angustifolius* (blue quandong) plant materials used in this study were supplied and verified by the Queensland Bush foods Association, Australia. *Kunzea pomifera* (muntries), *Podocarpus elatus* (Illawarra plum), *Diploglottis australis* (native tamarind), *Acronychia acidula* (lemon aspen), *Citrus glauca* (desert lime), *Solanum aviculare* (bush tomato), *Acacia vivtoriae* (wattle seed), *Prostanthera rotundifolia* (native thyme), *Prostanthera incise* (native sage), *Ocimum tenuiflorum* (native basil) and *Mentha australis* (river mint) were obtained from Taste of Australia Bush Food, Australia. Voucher samples have been stored in the School of Natural Sciences, Griffith University.The plant materials were thoroughly dried in a Sunbeam food dehydrator and the dried plant materials were stored at -30°C. Prior to use, the plant materials were thawed and freshly ground to a coarse powder. Individual 1 g quantities of the ground plant material were weighed into separate tubes and 50 mL of methanol or water were added. All solvents were obtained from Ajax and were AR grade. The ground plant materials were individually extracted in each solvent for 24 h at 4°C with gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant dry extract was weighed and redissolved in 10 mL deionised water (containing 1% DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[22-24]

Antioxidant capacity

The antioxidant capacity of each sample was assessed using the DPPH free radical scavenging method[26] with modifications. Briefly, a DPPH solution 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 amounts of 5, 10, 25, 50, 75 μL in triplicate. Methanol was added to each well to give a volume of 225 μL. A volume of 75 μL of the fresh DPPH solution was added to each well for a total reaction volume of 300 μL. A blank of each extract concentration, methanol solvent, and DPPH was also performed in triplicate. Ascorbic acid was prepared fresh and examined across the range 0-25 μg per well as a

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

Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (µg/mL)	Antioxidant Capacity (µg Ascorbic Acid Equivalency)	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
1	350	35	2.9	+++	+++	+++	-	+++	++	-	-	-	+++	++	-	-
2	524	52.4	6.9	+++	+++	-	-	+++	++	-	-	-	+++	++	-	-
3	195	19.5	2.7	+++	++	+++	-	+++	++	-	-	-	++	++	++	++
4	314	31.4	6.8	+++	+++	+++	-	+++	++	-	-	-	++	++	+	++
5	52	5.2	8.4	++	-	+++	-	-	-	-	-	-	+	-	++	-
6	107	10.7	9.2	+++	-	+++	-	++	-	-	-	-	+	-	-	-
7	162	16.2	7.2	+++	-	-	+	+++	++	-	-	-	+++	-	-	-
8	360	36	15.9	+++	-	-	+	-	++	-	-	-	+++	-	-	-
9	182	18.2	6.3	+++	++	++	+	-	++	-	-	++	+++	-	-	-
10	247	24.7	11.7	+	-	-	+	++	++	-	-	-	+++	-	-	-
11	79	7.9	5.6	+++	++	+++	-	+++	++	-	-	++	+++	++	-	-
12	313	31.3	9.1	+++	-	+++	-	-	++	-	-	++	++	-	-	-
13	360	36	55	+++	+++	+++	-	-	+	-	-	-	+++	+	-	-
14	240	24	41	+++	+++	++	-	+	-	-	-	-	+++	+	-	-
15	180	18	25	+++	+++	++	-	+	+	-	-	-	++	+	-	-
16	360	36	40	+++	+++	+++	-	+	+	-	-	-	+++	+	-	-
17	110	11	2.6	++	-	++	-	-	-	-	-	-	+	-	-	-
18	120	12	59	+++	+++	++	-	+	+	-	-	-	+++	+	-	-
19	560	56	95	+++	+++	+++	-	+	+	-	-	-	+++	+	-	-
20	130	13	1.5	++	-	++	-	-	-	-	-	-	-	+	-	-
21	88	8.8	45	+++	+++	++	-	+	+	-	-	-	++	+	-	-
22	190	19	43	+++	+++	+++	-	+	+	-	-	-	++	+	-	-
23	62	6.2	5.5	++	-	++	-	-	-	-	-	-	++	-	-	-
24	220	22	16	++	+++	++	-	+	+	-	-	-	+++	+	-	-
25	530	53	23	++	+++	++	-	+	+	-	-	-	++	+	-	-
26	36	3.6	39	++	++	+	-	+	-	-	-	-	+++	+	-	-
27	230	23	35	++	++	++	-	+	-	-	-	-	+++	+	-	-
28	140	14	21	+++	++	++	-	-	+	-	-	-	++	-	-	-
29	490	49	23	+++	++	++	-	+	+	-	-	-	+++	-	-	-
30	120	12	0.6	++	++	+	++	-	++	-	-	-	+++	-	-	-
31	88	8.8	3.9	++	+++	++	-	++	++	-	-	++	+++	-	-	-
32	52	5.2	5.1	+++	+++	+++	++	+++	++	-	-	++	+++	++	++	-
33	171	17.1	11.1	+++	+++	++	++	+++	++	-	-	++	+++	++	-	-
34	25	2.5	6.5		+++	++	++	+++	++	-	-	-	+++	++	-	-
35	109	10.9	12.3	+++	+++	+++	++	+++	++	-	-	+	++	++	-	-
36	108	10.8	6.8	+++	+	++	++	+++	++	-	-	+	++	+	-	-
37	192	19.2	11.7	+++	+++	+++	-	+++	++	-	-	++	+++	++	-	-

38	30	3	5	++	++	++	++	-	++	-	-	-	+++	-	-	-
39	120	12	10.6	+++	+++	+++	++	+++	++	-	-	++	+++	++	+	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. 1 = aqueous muntries extract; 2 = methanolic muntries extract; 3 = aqueous Illawarra plum extract; 4 = methanolic Illawarra plum extract;5 = aqueous native tamarind extract; 6 = methanolic native tamarind extract; 7 = aqueous lemon aspen extract; 8 = methanolic lemon aspen extract; 9 = aqueous desert lime extract; 10 = methanolic desert lime extract; 11 = aqueous bush tomato extract; 12 = methanolic bush tomato extract; 13 = methanolic *S. australe* fruit extract; 14 = aqueous *S. australe* fruit extract; 15 =aqueous *S. australe* leaf extract; 16 = methanolic *S. australe* leaf extract;17 = *S. australe* fruit ethyl acetate extract; 18 = aqueous *S. leuhmannii* fruit extract; 19 = methanolic *S. leuhmannii* fruit extract; 20 =*S. leuhmannii* fruit ethyl acetate extract; 21 = aqueous *S. leuhmannii* leaf extract; 22 = methanolic *S. leuhmannii* leaf extract; 23 = *S. leuhmannii* leaf ethyl extract acetate; 24 = aqueous Davidson's plum fruit extract; 25 = methanolic Davidson's plum fruit extract; 26 = aqueous Davidson's plum leaf extract; 27 = methanolic Davidson's plum leaf extract; 28 = aqueous quandong extract; 29 = methanolic quandong extract; 30 = aqueous wattle seed extract; 31 = methanolic wattle seed extract; 32 = aqueous native thyme extract; 33 = methanolic native thyme extract; 34 = aqueous native sage extract; 35 = methanolic native sage extract; 36 = aqueous native basil extract; 37 = methanolic native basil extract; 38 = aqueous river mint extract; 39 = methanolic river mint extract.

reference and the absorbances were recorded at 515 nm. All tests were performed in triplicate 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

Environmental *Bacillus anthracis* strain

An environmental strainof *Bacillus anthracis* was isolated and used in these studies. The bacterium was originally isolated 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: 1 g/L peptone, 1.5 g/L yeast extract, 7.5 g/L NaCl, 1 g/L ammonium persulfate and 2.4 g/L HEPES buffer(pH 7.5). Incubation was at 30°C and the stock culture was subcultured and maintained in PYE media at 4°C. The media nutrient components were supplied by Oxoid Ltd. Sequence analysis of the environmental isolate generated a contig of 1428bp which was revealed to be 99.92% similar to *B. anthracis* by EzTaxon and designated as *Bacillus anthracis* strain PMO. The Gen Bank 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.[22-24] Briefly, 100 μL of the test bacteria were grown in 10 mL of fresh nutrient broth media until they reached a count of approximately 10^8 cells/mL. An amount of 100 μL of bacterial suspension was spread onto nutrient agar plates. The extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were impregnated with 10 μL of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4°C for 2 h before incubation at 30°C for 24 h. The diameters of the inhibition zones were measured in millimetres. All measurements were to the closest whole millimetre. Each assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (10 μg) and penicillin (2 μg) were obtained from Oxide Ltd. and served as positive controls for antibacterial activity. Filter discs impregnated with 10 μL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the extracts was determined as previously described.[22-24] Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were impregnated with 10 μL of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

Toxicity screening

Reference toxin for toxicity screening

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

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.[22-24] 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 400 μL 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 (400 μL 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 seconds. After 24 h all nauplii were sacrificed and counted to determine the total % mortality per well. The LC50 with 95% confidence limits for each treatment was calculated using probit analysis.

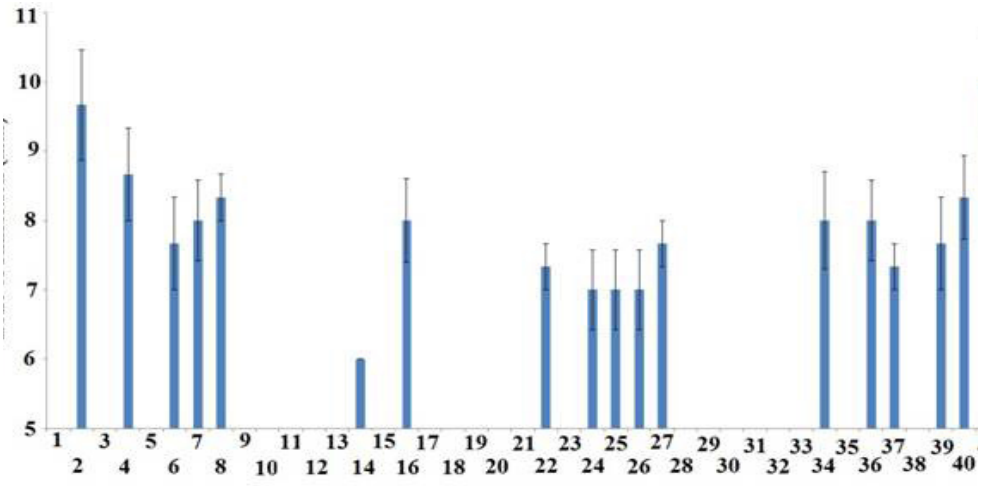

Figure 1: Growth inhibitory activity of high antioxidant Australian plant extracts against the *B. anthracis* environmental isolate measured as zones of inhibition (mm). 1=aqueous muntries extract; 2=methanolic muntries extract; 3=aqueous Illawarra plum extract; 4=methanolic Illawarra plum extract; 5=aqueous native tamarind extract; 6=methanolic native tamarind extract; 7=aqueous lemon aspen extract; 8=methanolic lemon aspen extract; 9=aqueous desert lime extract; 10=methanolic desert lime extract; 11=aqueous bush tomato extract; 12=methanolic bush tomato extract; 13=methanolic *S. australe* fruit extract; 14=aqueous *S. australe* fruit extract; 15=aqueous *S. australe* leaf extract; 16=methanolic *S. australe* leaf extract; 17=*S. australe* fruit ethyl acetate extract; 18=aqueous *S. leuhmannii* fruit extract; 19=methanolic *S. leuhmannii* fruit extract; 20=*S. leuhmannii* fruit ethyl acetate extract; 21 = aqueous S. *leuhmannii* leaf extract; 22=methanolic *S. leuhmannii* leaf extract; 23=*S. leuhmannii* leaf ethyl extract acetate; 24=aqueous Davidson's plum fruit extract; 25=methanolic Davidson's plum fruit extract; 26=aqueous Davidson's plum leaf extract; 27=methanolic Davidson's plum leaf extract; 28=aqueous quandong extract; 29=methanolic quandong extract; 30=aqueous wattle seed extract; 31 = methanolic wattle seed extract; 32=aqueous native thyme extract; 33=methanolic native thyme extract; 34=aqueous native sage extract; 35=methanolic native sage extract; 36=aqueous native basil extract; 37=methanolic native basil extract; 38=aqueous river mint extract; 39=methanolic river mint extract; 40=penicillin (2 μg); 41=ampicillin (10 μg). Results are expressed as mean zones of inhibition ± SEM

Non-targeted HPLC-MS QTOF analysis

Chromatographic separations were performed as previously described.[23,26] Briefly, 2 μL of sample was injected onto an Agilent 1290 HPLC system fitted with a Zorbax Eclipse plus C18 column (2.1x100 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 positive and negative mode.

Data was analysed using the Mass hunter 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.

Table 2: Minimum inhibitory concentration (µg/mL) of the plant extracts and LC50 values (µg/mL) in the Artemia nauplii bioassay

Extract	MIC	LC50
aqueous muntries extract	-	-
methanolic muntries extract	361	1965
aqueous Illawarra plum extract	-	1956
methanolic Illawarra plum extract	883	1664
aqueous native tamarind extract	-	1862
methanolic native tamarind extract	461	1595
aqueous lemon aspen extract	397	1872
methanolic lemon aspen extract	306	1500
aqueous desert lime extract	-	3875
methanolic desert lime extract	-	-
aqueous bush tomato extract	-	5372
methanolic bush tomato extract	-	3467
methanolic *S. australe* fruit extract	-	3310
aqueous *S. australe* fruit extract	>10,000	1879
aqueous *S. australe* leaf extract	-	244
methanolic *S. australe* leaf extract	>10,000	294
S. australe fruit ethyl acetate extract	-	-
aqueous *S. leuhmannii* fruit extract	-	478
methanolic *S. leuhmannii* fruit extract	-	414
S. leuhmannii fruit ethyl acetate extract	-	-
aqueous *S. leuhmannii* leaf extract	-	813
methanolic *S. leuhmannii* leaf extract	1428	450
S. leuhmannii leaf ethyl extract acetate	8,800	-
aqueous Davidson's plum fruit extract	>10,000	2883
methanolic Davidson's plum fruit extract	>10,000	6443
aqueous Davidson's plum leaf extract	3,600	-
methanolic Davidson's plum leaf extract	>10,000	-
aqueous quandong extract	-	3762
methanolic quandong extract	-	5418
aqueous wattle seed extract	-	6254
methanolic wattle seed extract	-	5763
aqueous native thyme extract	-	-
methanolic native thyme extract	-	3358
aqueous native sage extract	2,500	1831
methanolic native sage extract	-	4015
aqueous native basil extract	535	2480
methanolic native basil extract	>10,000	7185
aqueous river mint extract	-	-
methanolic river mint extract	>10,000	2658
potassium dichromate	-	186
seawater control	-	-

Numbers indicate the mean MIC and LC_{50} values of triplicate determinations. - indicates no inhibition.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of the various dried Australian plant materials with the solvents yielded dried plant extracts ranging from 25 mg (native sage aqueous extract) to 524 mg (muntries methanolic extract) (Table 1). Methanolic extracts generally gave relatively high yields of dried extracted material whilst the aqueous extracts had moderate to high yields for most species. The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO) resulting in the extract concentrations shown in Table 1.

Qualitative phytochemical studies showed that methanol generally extracted the widest range of phytochemicals (Table 1). It extracted high levels of phenolics (both water soluble and insoluble phenolics), flavonoids and saponins, as well as moderate levels of triterpenoids. Muntries and Illawarra plum also showed moderate levels of tannins. The aqueous extracts generally extracted similar but slightly lower phytochemical profiles. Low to moderate levels of alkaloids were also noted for the methanolic and aqueous extracts of desert lime and bush tomato. Cardiac glycosides and alkaloids were detected in low to moderate levels in the methanolic and aqueous extracts of most of the culinary herbs.

Antioxidant content

Antioxidant capacity for the plant extracts (Table 1) ranged from 0.6 mg (aqueous wattle seed extract) to a high of 15.9 mg ascorbic acid equivalence per gram of dried plant material extracted (lemon aspen fruit methanolic extract). The methanol extracts generally had higher antioxidant capacities than the corresponding water extracts.

Antimicrobial activity

To determine the ability of the crude plant extracts to inhibit the growth of *B. antharcis*, aliquots (10 µL) of each extract were screened using a disc diffusion assay. The bacterial growth was strongly inhibited by 16 of the 39 extracts screened (41%) (Figure 1). The muntries methanolic extract appeared to be the most potent inhibitor of *B. antharcis* growth (as judged by zone of inhibition), with inhibition zones of 9.7 ± 0.8 mm. This compares favourably with the penicillin and ampicillin controls, with zones of inhibition of 8.3 ± 0.6 and 10.0 ± 0.7 respectively. Illawarra plum, lemon aspen, *S. australe* leaf, native sage and native basil all displayed good inhibition of *B. antharcis* growth, with ≥8 mm zones of inhibition. In general,

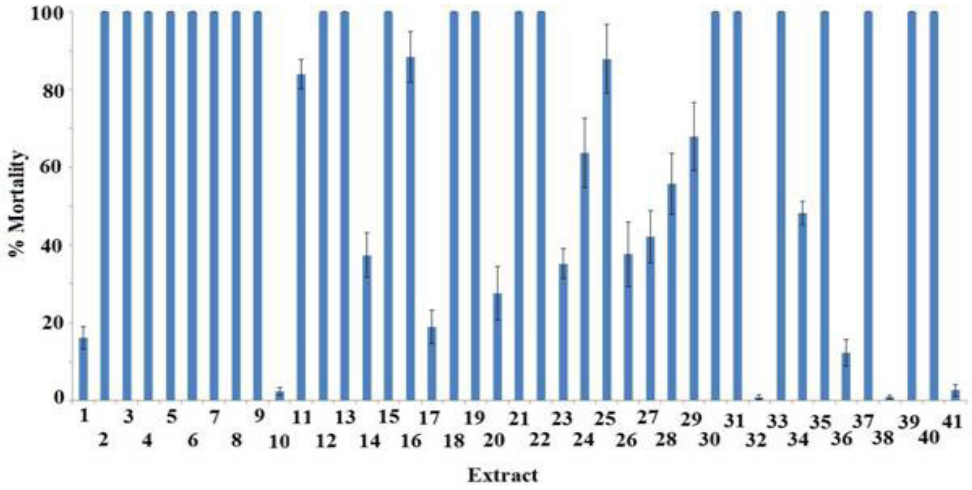

Figure 2: The lethality of the Australian plant extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *Artemia franciscana* nauplii after 24 h exposure. 1=aqueous muntries extract; 2=methanolic muntries extract; 3=aqueous Illawarra plum extract; 4=methanolic Illawarra plum extract;5=aqueous native tamarind extract; 6=methanolic native tamarind extract; 7=aqueous lemon aspen extract; 8=methanolic lemon aspen extract; 9=aqueous desert lime extract; 10=methanolic desert lime extract; 11=aqueous bush tomato extract; 12=methanolic bush tomato extract; 13=methanolic *S. australe* fruit extract; 14=aqueous *S. australe* fruitextract; 15=aqueous *S. australe* leaf extract; 16=methanolic *S. australe* leaf extract; 17=*S. australe* fruit ethyl acetate extract; 18=aqueous *S. leuhmannii* fruit extract; 19=methanolic *S. leuhmannii* fruit extract; 20=*S. leuhmannii* fruit ethyl acetate extract; 21=aqueous *S. leuhmannii* leaf extract; 22=methanolic *S. leuhmannii* leaf extract; 23=*S. leuhmannii* leaf ethyl extract acetate; 24=aqueous Davidson's plum fruit extract; 25=methanolic Davidson's plum fruit extract; 26=aqueous Davidson's plum leaf extract; 27=methanolic Davidson's plum leaf extract; 28=aqueous quandong extract; 29=methanolic quandong extract; 30=aqueous wattle seed extract; 31=methanolic wattle seed extract; 32=aqueous native thyme extract; 33=methanolic native thyme extract; 34=aqueous native sage extract; 35=methanolic native sage extract; 36=aqueous native basil extract; 37=methanolic native basil extract; 38=aqueous river mint extract; 39=methanolic river mint extract; 40=potassium dichromate control; 41=seawater control. Results are expressed as mean % mortality ± SEM

the methanolic extracts were more potent inhibitors of *B. antharcis* growth than were their aqueous counterparts. Indeed, the muntries, Illawarra plum, native tamarind, *S. australe* leaf and *S.leuhmannii* leaf methanolic extracts were all good growth inhibitors, whilst the corresponding aqueous extracts did not inhibit *B. antharcis* growth at all.

The antimicrobial efficacy was further quantified by determining the MIC values (Table 2). Several of the extracts were effective at inhibiting microbial growth, with MIC values against *B. anthracis* <1000 µg/mL (<10 µg impregnated in the disc). The lemon aspen extracts were particularly potent, with MIC values for both the aqueous and methanolic extracts 300-400 µg/mL (3-4 µg impregnated in the disc). The muntries, Illawarra plum and native tamarind methanolic extracts, as well as the aqueous native basil extracts were similarly effective growth inhibitors. Although less potent, the aqueous *S. Leuhmannii* leaf, Davidson's plum leaf and native sage aqueous extracts were also good anti-*B. anthracis* agents (MIC values 1500-3500 µg/mL). Several other extracts also inhibited *B. anthracis* growth when tested undiluted (Figure 1), although with MIC values >5000 µg/mL, these were deemed to be of only low potency against *B. anthracis*.

Quantification of toxicity

All extracts were initially screened at 2000 µg/mL in the assay (Figure 2). For comparison, the reference toxin potassium dichromate (1000 µg/mL) was also tested in the bioassay. The potassium dichromate reference toxin was rapid in its onset of mortality, inducing nauplii death within the first 3 h of exposure and 100% mortality was evident following 4-5 h (results not shown). Most of the extracts displayed >50% mortality rates at 24h.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in artificial seawater to test across a range of concentrations in the Artemia nauplii bioassay. Table 2 shows the LC50 values of the extracts towards *A. franciscana*. No LC50 values are reported for the muntries aqueous extracts, desert lime methanolic extract, *S. australe* leaf and *S. leuhmannii* fruit and leaf ethyl acetate extracts, the Davidson's plum

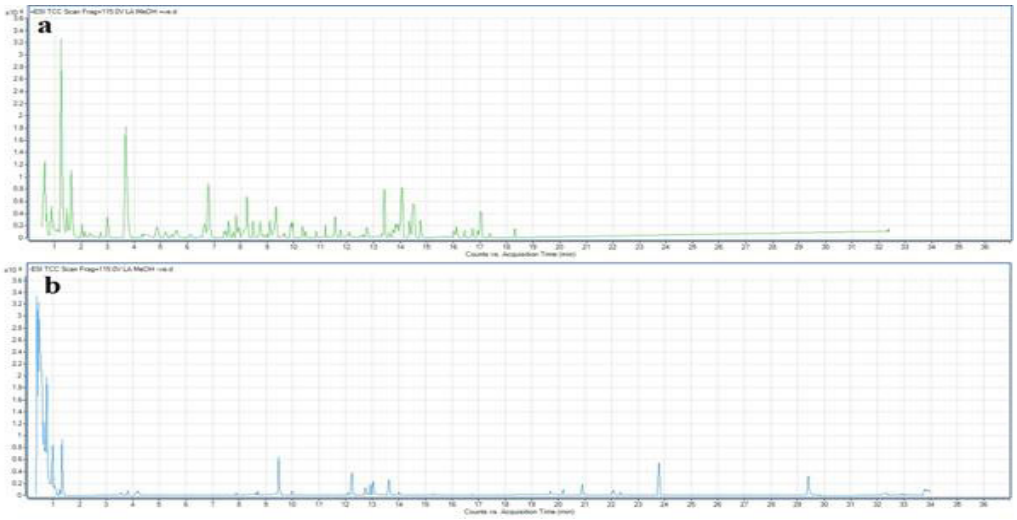

Figure 3: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µL injections of lemon aspen methanolic extract

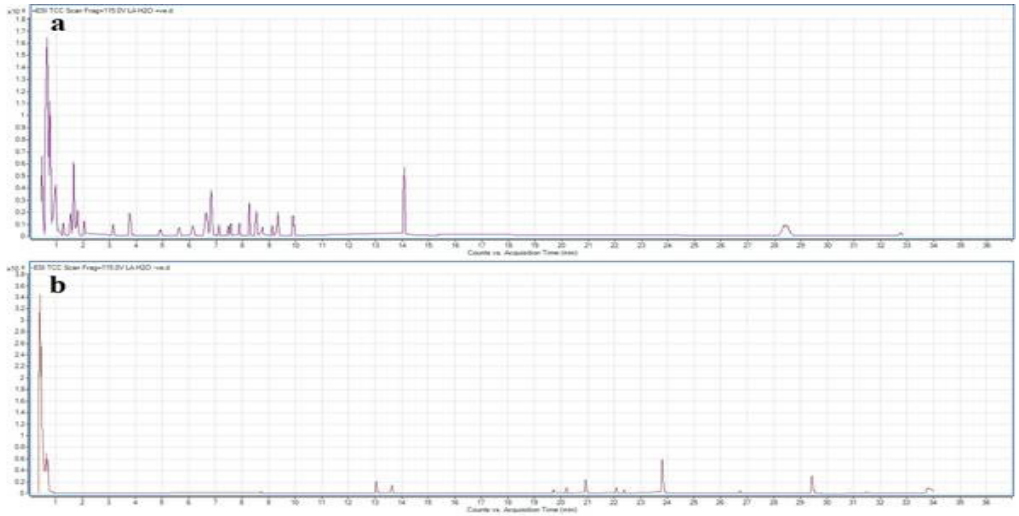

Figure 4: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µL injections of lemon aspen aqueous extract

leaf methanolic and aqueous extracts, the native thyme aqueous extract, as well as the river mint aqueous extract as <50% mortality was seen for all concentrations tested. Significant toxicity was noted for several of the Syzygium spp. extracts, with LC50 values substantially <1000 µg/mL. All other extracts were determined to be nontoxic, with LC50 values substantially greater than 1000 µg/mL following 24 h exposure. Extracts with an LC50 of greater than 1000 µg/mL towards Artemia nauplii have been defined as being nontoxic.[27]

HPLC-MS QTOF analysis

As the lemon aspen extracts displayed the greatest potency in the *B. anthracis* growth inhibition assay, the methanolic and aqueous extracts were deemed the most promising extracts for further phytochemical analysis. Optimised HPLC-MS parameters were developed and used to profile and compare the compound profiles from the aqueous and methanolic extractions of lemon aspen fruit. The resultant total compound chromatograms for the positive ion and negative ion chromatograms of the methanolic extract are presented in Figure 3a and Figure 3b respectively. The positive ion chromatogram had a significantly greater number of mass signal peaks detected. However, the negative ion chromatogram had a higher base peak signal to noise ratio in the total ion chromatograms which may have hidden some peaks in the negative ionisation mode.

Both the positive and negative ion lemon aspen fruit chromatograms of the methanolic extract revealed

Table 3: Qualitative HPLC-MS QTOF analysis of the lemon aspen fruit methanolic and aqueous extracts, elucidation of empirical formulas and putative identification (where possible) of the compounds

Putative Identification	Empirical Formula	Molecular Mass	Retention Time	Methanol Extract	Aqueous Extract
	$C_4H_6O_3$	102.0328	0.478	-	
Quinone	$C_6H_4O_2$	108.0217	1.603	+	+
3-furanoic acid	$C_5H_4O_3$	112.0161	0.387		-
Purine	$C_5H_4N_4$	120.0436	32.377	+	
phloroglucinol	$C_6H_6O_3$	126.0322	1.603	+	+
2-Desoxy-D-ribose	$C_5H_{10}O_4$	134.0584	0.45		-
m-nitrotoluene	$C_7H_7NO_2$	137.047	0.521	+	
(1S,5R)-4-hydroxy-6,7-dioxabicyclo[3.2.1]oct-2-en-8-one (isomer 1)	$C_6H_6O_4$	142.0269	3.647	+	
(1S,5R)-4-hydroxy-6,7-dioxabicyclo[3.2.1]oct-2-en-8-one (isomer 2)	$C_6H_6O_4$	142.027	1.231	+	
(E)-2-Methylglutaconic acid	$C_6H_8O_4$	144.0418	0.714	-	
Xylitol	$C_5H_{12}O_5$	152.0699	0.44		-
	$C_4H_4N_4O_3$	156.0286	0.617	+	
Oxanamide	$C_8H_{15}NO_2$	157.1109	0.614	+	+
2-Oxoadipic acid	$C_6H_8O_5$	160.0376	1.229	+	
ethyl (ethylperoxy) (oxo) acetate	$C_6H_{10}O_5$	162.0528	2.116	+/-	-
	$C_8H_4O_4$	164.0111	3.106		+
	$C_9H_{11}NO_2$	165.0768	1.451	+	
Ethyl 4-hydroxybenzoate	$C_9H_{10}O_3$	166.0635	0.956	-	
(1S,5R)-4-Oxo-6,8-dioxabicyclo[3.2.1]oct-2-ene-2-carboxylic acid	$C_7H_6O_5$	170.0219	1.216	+	
dehydroascorbic acid (oxidised vitamin C)	$C_6H_6O_6$	174.0164	0.384		-
shikimic acid	$C_7H_{10}O_5$	174.0532	3.646	+	
2-Hydroxyethyl salicylate	$C_9H_{10}O_4$	182.0578	0.742	-	
	$C_7H_8O_6$	188.0326	1.219	+	
citric acid	$C_6H_8O_7$	192.0268	0.749	-	+/-
Hydroxy-7-methyl-4H,5H-pyrano[4,3-b]pyran-dione	$C_9H_6O_5$	194.0221	2.001	+	+
ferulic acid	$C_{10}H_{10}O_4$	194.0582	1.222	-	
Feroxidin	$C_{11}H_{14}O_3$	194.0948	11.548	+	
Cantharidin	$C_{10}H_{12}O_4$	196.074	1.309	-	
	$C_8H_{10}O_6$	202.0478	3.645	+	
calamenene (isomer 1)	$C_{15}H_{22}$	202.1722	13.379	+	
calamenene (isomer 2)	$C_{15}H_{22}$	202.1724	16.403	+	
2,3-O-(Oxomethylene) hexopyranose	$C_7H_{10}O_7$	206.0432	1.22	+/-	+
Cyclazodone	$C_{12}H_{12}N_2O_2$	216.0901	5.567	+	+
5-hydroxycalamenene	$C_{15}H_{22}O$	218.1669	12.582	+	
	$C_8H_{12}O_7$	220.0589	3.645	+/-	
	$C_9H_8N_4O_3$	220.059	2.971	+	
spathulenol (isomer 1)	$C_{15}H_{24}O$	220.1828	13.575	+	
spathulenol (isomer 2)	$C_{15}H_{24}O$	220.1831	15.998	+	

spathulenol (isomer 3)	$C_{15}H_{24}O$	220.1832	10.321	+	
Gladiolic acid	$C_{11}H_{10}O_5$	222.0532	9.873	+	+
	$C_7H_{13}NO_7$	223.0696	1.228	+	
	$C_{13}H_{10}N_2O_2$	226.0746	8.511		+
Heptylheptanoate	$C_{14}H_{28}O_2$	228.2098	20.927		-
(2R,3S)-3-(3-Carboxylatopropanoyl)-5-oxotetrahydro-2-furancarboxylate	$C_9H_8O_7$	228.0251	1.23	+	
Ozagrel	$C_{13}H_{12}N_2O_2$	228.09	7.84	+	+
Heptylheptanoate	$C_{14}H_{28}O_2$	228.2093	20.899	-	
Metomidate	$C_{13}H_{14}N_2O_2$	230.1059	7.704	+	+
	$C_9H_{14}O_7$	234.0746	7.91	+	
drimenin	$C_{15}H_{22}O_2$	234.1624	16.105	+	
capsidiol (isomer 1)	$C_{15}H_{24}O_2$	236.1779	11.496	+	
capsidiol (isomer 2)	$C_{15}H_{24}O_2$	236.178	17.371	+	
capsidiol (isomer 3)	$C_{15}H_{24}O_2$	236.1781	14.516	+	
	$C_{12}H_{19}N_3O_2$	237.1479	7.076		+
Kessyl alcohol	$C_{15}H_{26}O_2$	238.1936	14.479	+	
	$C_{12}H_5N_3O_3$	239.0323	2.97	+	
Isopentadecylic acid	$C_{15}H_30O_2$	242.2247	22.342	-	-
	$C_7H_4N_2O_8$	243.999	1.227	+	
LeuLeu	$C_{12}H_{24}N_2O_3$	244.1794	7.424	+	+
nodakenetin	$C_{14}H_{14}O_4$	246.0897	12.068	+	
Acetyltryptophane	$C_{13}H_{14}N_2O_3$	246.1007	5.157	+	
Nitrefazole	$C_{10}H8N_4O_4$	248.0538	0.737		+
	$C_{15}H_{26}O_3$	254.1881	16.722	+	
7-palmitoleic acid	$C_{16}H_{30}O_2$	254.225	22.059	-	-
11-keto pentadecanoic acid	$C_{15}H_{28}O_3$	256.2042	13.987	-	
palmitic acid	$C_{16}H_{32}O_2$	256.241	23.786	-	-
	$C_{10}H_{16}O_8$	264.085	0.64		-
Diftalone	$C_{16}H_{12}N_2O_2$	264.0913	10.429	+	
Propacetamol	$C_{14}H_{20}N_2O_3$	264.1482	3.692	+	+
	$C_9H_{14}O_9$	266.0642	0.737		+/-
Lauroylsarcosine	$C_{15}H_{29}NO_3$	271.2155	13.978	+	
Lauroylsarcosine	$C_{15\ H29}NO_3$	271.2155	17.008	+	
	$C_{12}H_{11}N_5O_3$	273.0857	0.686	+	
macowine	$C_{16}H_{19}NO_3$	273.1372	8.452	+	+
2,15-dihydroxy-pentadecylic acid	$C_{15}H_{30}O_4$	274.2148	9.96	-	
	$C_9H_{13}N^{11}$	275.1351	0.864	+	
Menthyl salicylate	$C_{17}H_{24}O_3$	276.1709	17.046	+	
Queuine	$C_{12}H_{15}N_5O_3$	277.1171	0.556		+
7-hydroxy-10E,16-heptadecadien-8-ynoic acid	$C_{17}H_{26}O_3$	278.1861	14.479	+	
Dihydroartemisinin	$C_{15}H_{24}O_5$	284.1631	12.734	+/-	
Hexyl dodecanoate	$C_{18}H_{36}O_2$	284.2723	26.727		-
N,N-Didemethylchlorpromazine	$C_{15}H_{15}ClN_2S$	290.0648	0.655		+
	$C_9H_{15}N_{11}O$	293.146	0.863	+	

gingerol	$C_{17} H_{26} O_4$	294.184	13.005	-	-
Sulazepam	$C_{16} H_{13} Cl N_2 S$	300.0483	6.089	+	+/-
TEGASEROD (isomer 1)	$C_{16} H_{23} N_5 O$	301.1893	2.708	+	
TEGASEROD (isomer 2)	$C_{16} H_{23} N_5 O$	301.1895	12.731	+	
	$C_{14} H_8 O_8$	304.0206	0.735		+
	$C_{12} H_{18} O_9$	306.0954	0.636		-
Pyrethrosin	$C_{17} H_{22} O_5$	306.1469	15.328		+
Propentofylline	$C_{15} H_{22} N_4 O_3$	306.1705	8.22	+	+
2,2'-[Methylenebis(oxy)] bis(ethylmalonic acid	$C_{11} H_{16} O_{10}$	308.0752	0.647		+
Dantrolene	$C_{14} H_{10} N_4 O_5$	314.0643	8.988	+	
	$C_{16} H_{29} N O_5$	315.2053	7.365	+	
9,13-dihydroxy-11-octadecenoic acid	$C_{18} H_{34} O_4$	314.2464	20.177	-	-
Butalamine	$C_{18} H_{28} N_4 O$	316.2259	12.903	-	
Denbufylline	$C_{16} H_{24} N_4 O_3$	320.1855	7.567	+	
	$C_{15} H_{24} N_4 O_4$	324.1801	1.669	+	
	$C_{13} H_{10} N_8 O_3$	326.0883	0.408		-
	$C_{11} H_{22} N_{10} O_2$	326.1922	19.696	-	-
	$C_{15} H_{21} N O_7$	327.1323	1.521	+	
	$C_{13} H_{12} N_4 O_7$	336.0704	0.614		+
	$C_{16} H_{18} O_8$	338.1006	3.52	-	
Clorotepine	$C_{19} H_{21} Cl N_2 S$	344.1114	4.138	-	
Granisetron metabolite 1	$C_{18} H_{24} N_4 O_3$	344.1846	12.218	-	
	$C_{13} H_{14} N_4 O_8$	354.0813	0.401	-	-
Oxipurinol-7-ribonucleotide	$C_{10} H_{13} N_4 O_9 P$	364.0418	0.623		+
	$C_{14} H_{16} N_4 O_8$	368.0972	0.529	-	
9-(5-O-Benzoylpentofuranosyl)-3,9-dihydro-6H-purin-6-one	$C_{17} H_{16} N_4 O_6$	372.1058	3.787	-	
	$C_{20} H_{17} F_2 N_3 O_3$	385.1218	0.996	+	
	$C_{15} H_{38} N_{10} O_2$	390.3181	29.397	-	-
	$C_{13} H_8 N_6 O_9$	392.0366	0.57	+/-	+
	$C_{24} H_{10} N_2 O_5$	406.0575	0.538	-	
	$C_{12} H_{20} O_{16}$	420.0728	0.637	-	
	$C_{16} H_{14} N_4 O_{10}$	422.0703	1.499		+
Ketorolac glucuronide	$C_{21} H_{21} N O_9$	431.1205	0.601		+
Propranolol glucuronide	$C_{22} H_{29} N O_8$	435.1906	7.811	+	
	$C_{17} H_21 N_5 O_9$	439.1341	0.685		+
	$C_{13} H_{20} O_{17}$	448.0678	0.601		-
	$C_{15} H_{23} N O_{15}$	457.1074	1.766		+
trans-1,4-bis(2-Chlorobenzaminomethyl) cyclohexane dihydrochloride	$C_{22} H_{30} Cl_4 N_2$	462.117	12.726	-	
	$C_{17} H_{20} O_{15}$	464.0807	0.926		+
	$C_{19} H_6 N_4 O_{11}$	466.005	1.241	+	
	$C_{22} H_{28} N_8 O_4$	468.223	4.834	+	
	$C_{22} H_{28} N_8 O_4$	468.2235	7.519	+	+
Chlortetracycline	$C_{22} H_{23} Cl N_2 O_8$	478.1121	12.068	-	
	$C_{17} H_{22} O_{16}$	482.091	0.914		+

	$C_{18} H_{20} N_4 O_{13}$	500.1031	0.341		-
	$C_{17} H_{27} N O_{17}$	517.1281	0.931		+
	$C_{19} H_{14} N_{10} O9$	526.0945	0.557		+
	$C_{23} H_{50} N_{10} O_4$	530.4014	subscript.412		+
	$C_{23} H_{22} O_{16}$	554.0902	0.544	+	
	$C_{29} H_{39} N_9 O_3$	561.3177	11.174	+	
Apiin	$C_{26} H_{28} O_{14}$	564.1485	9.609	+/-	+/-
	$C_{26} H_{16} N_8 O_8$	568.1099	0.61	-	
	$C_{32} H_{35} N_9 O_2$	577.2921	14.062	+/-	+/-
Glucofrangulin	$C_{27} H_{30} O_{14}$	578.1651	9.915	+	+
	$C_{25} H_{18} N_{14} O_5$	594.1582	8.718	+	+
	$C_{28} H_{26} N_4 O_{11}$	594.1603	9.097	+/-	
8'-Hydroxydihydroergotamine	$C_{33} H_{37} N_5 O_6$	599.2729	14.067	+	+
rutin	$C27 H_{30} O_{16}$	610.154	8.777	+	+
	$C_{29} H_{28} N_{18} O$	644.27	10.823	+	
	$C_{28} H_{20} N_2 O_{17}$	656.0766	1.225	+	
	$C_{34} H_{44} N_6 O_8$	664.322	13.165	+/-	
	$C_{53} H_{54} O_2$	722.4123	15.728	+/-	
	$C_{31} H_{26} N_{24}$	734.2773	11.548	+	

+ and - indicates the mass spectral mode in which that the molecule was detected.

numerous peaks, particularly in the early and middle stages of the chromatogram corresponding to the elution of polar compounds. Nearly all of the methanol extract compounds had eluted by 17 minutes (corresponding to approximately 50% acetonitrile). Indeed, multiple over lapping peaks eluted in the first 2 minutes with 5% acetonitrile. However, multiple peaks eluting later in the chromatogram (particularly evident in the negative ionisation mode (Figure 3b) indicate the broad spread of polarities of the compounds in this extract.

The lemon aspen fruit aqueous extract in negative ionisation mode (Figure 4a) also had large amounts of polar material eluting early in the chromatogram at similar elution volumes to many of the compounds in the methanol extract, although the aqueous extract in positive ion mode had a lesser amount and size of peaks corresponding to the mid polarity compounds in the middle of the chromatogram (10-20 min) at approximately 25-60% acetonitrile. Much fewer peaks were evident in the negative ion chromatograms (Figure 4b). The aqueous extract negative ion chromatograms showed several major peaks, particularly at very early elution times. Many of these elution times correspond to peaks at similar elution times in the positive ion chromatograms (Figure 4a), indicating the corresponding compounds eluting at these times may have functional groups that are capable of both gaining and losing electrons.

Qualitative mass spectral analysis of the lemon aspen fruitextracts

In total, 145 unique mass signals were noted for the lemon aspen fruit extracts (Table 3). Putative empirical formulas were achieved for all of these compounds. Of the 145 unique molecular mass signals detected, 85 compounds were putatively identified by comparison 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).

DISCUSSION

Previous studies have reported potent growth inhibitory activity for several of the high antioxidant plant species screened in our study against different pathogenic bacterial species. The fruit[19] and leaves[28-30] of several Australian Syzygium species, including *S. australe* and *S. leuhmannii*, have been reported to inhibit the growth a broad range of pathogenic bacteria. Interestingly, whilst *B. anthracis* was not tested in any of the previous studies, Australian Syzygium spp. strongly inhibit the growth of the related bacterial species *Bacillus cereus* and *Bacillus subtilis*.[19,30] *B. cereus* is very closely related to *B. anthracis* with >99% 16S rRNA gene sequence homology.[31] Indeed, some bacte-

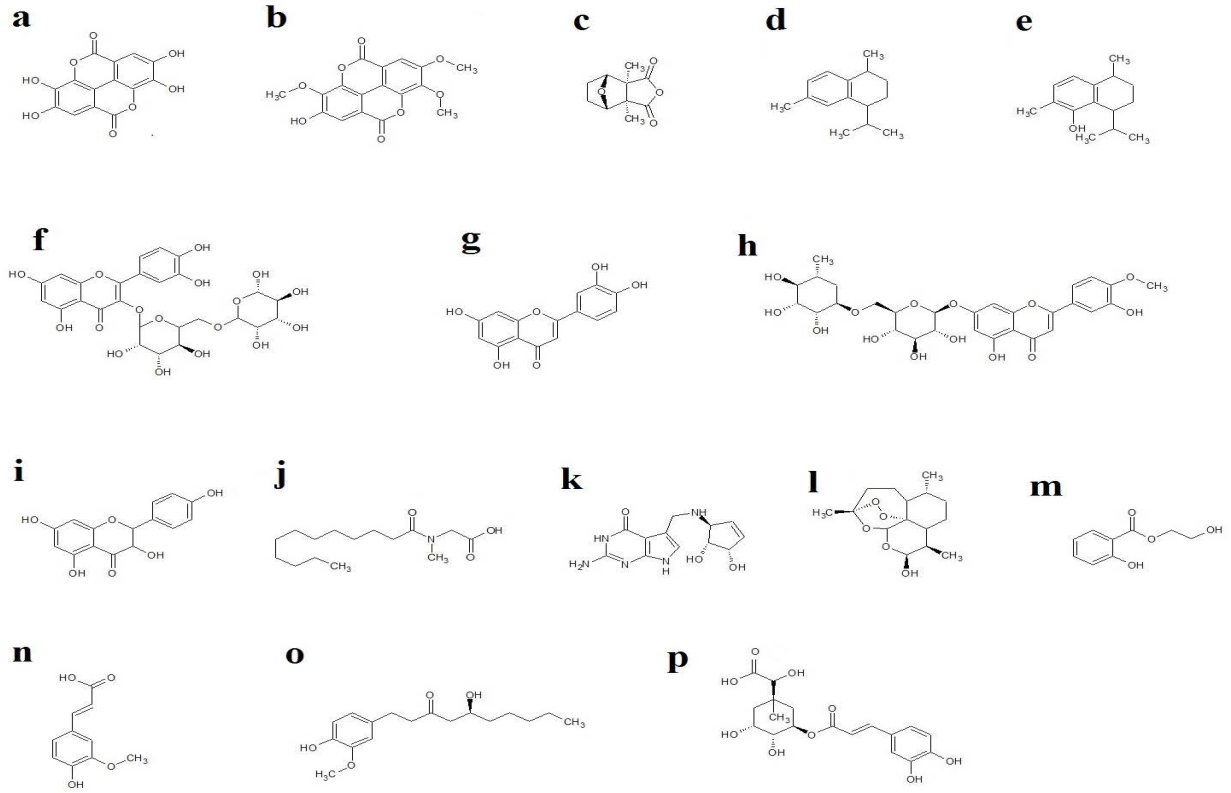

Figure 5: Chemical structures of lemon aspen fruit compounds detected in the methanolic and aqueous extracts: (a) ellagic acid; (b) trimethylellagic acid; (c) cantharidin; (d) calamanene; (e) hydroxycalamanene; (f) rutin; (g) luteolin; (h) diosmin; (i) dihydrokaempferol; (j) lauryl sarcosinate; (k) queuine; (l) dihydroxyarteminisin; (m) hydroxyethyl salicylate; (n) ferulic acid; (o) gingerol; (p) chlorogenic acid

rial taxonomonists believe that *B. anthracis, B. cereus, B. thuringiensis*, *B. mycoides*, *B. pseudomycoides* and *B. weinstephanensis* should be classified as a single species under current standards (>97% 16S rRNA sequence homology) and are only classified as separate species as a result of the different diseases that they cause.[32-34] Whilst *B. subtilise* has less similarity(>94% homology with *B. anthracis* 16S rRNA), it is still considered a closely related species.[31] It is therefore perhaps not surprising that the Syzygium spp. extracts screened in our study displayed growth inhibitory activity towards *B. anthracis*.

Recently, we also reported growth inhibitory activity of several high antioxidant fruits[22-24] and culinary herb extracts[25] against some microbial triggers of selected autoimmune inflammatory diseases. The Illawarra plum, lemon aspen, desert lime, wattle seed, native thyme and rivermint were particularly potent inhibitors of the microbial triggers of rheumatoid arthritis and ankylosing spondylitis.[24,25] All of the bacterial species associated with the onset of the autoimmune diseases screened in the previous studies are Gram negative and thus substantially different to the Gram positive *B. anthracis* screened in this study. However, the previously reported inhibitory activity does indicate the presence of antibacterial components in these extracts and is consistent with the inhibitory activity reported in this study.

The same panel of high antioxidant plant extracts was also previously reported to inhibit the proliferation of $CaCo_2$ and HeLa cancer cells.[35,36] Although those studies examined growth inhibition ineukaryotic cells, they are interesting as they report the phytochemical compositions of the bioactive extracts. A commonality between many of the inhibitory extracts was the relatively high levels of a number of tannin components including exifone (4-galloylpyrogallol), ellagic acid dehydrate, trimethylellagic acid, chebulic acid, corilagen, castalagin and chebulagic acid. Gallotannins have been reported to inhibit the growth of a broad spectrum of bacterial species[37] through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins,[38,39] and by inhibiting glucosyltransferase enzymes.[40] Elligitannins are also highly potent inhibitors of bacterial growth, with MIC values as low as 62.5 μg/ml.[37,39,41] Ellagitannins have also been reported

to function via several antibiotic mechanisms including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls.[37,39]

The lemon aspen fruit extracts displayed the most potent growth inhibitory activity against *B. anthracis* of all the extracts screened in our study, with MIC values <400 µg/mL. Our study employed a metabolomics profiling approach to putatively identify as many of the phytochemical components as possible in the lemon aspen aqueous and methanolic extracts. We putatively identified several of the same tannins in the lemon aspen extracts as previously reported for other high antioxidant extracts. In particular, ellagic acid (Figure 5a) and trimethylellagic acid (Figure 5b) were detected in the lemon aspen extracts and are likely to contribute to their inhibition of *B. anthracis* growth. However, it is likely that other phytochemical classes also contribute to this bioactivity. Alkaloids, anthraquionones, flavonoids, polyphenolics, phytosterols, saponins, stilbenes and terpenes have also been linked with antibacterial activity in different plant species and thus may be responsible (at least in part) for the bacterial growth inhibitory activities reported here.

Terpenoids and naphthalenes including cantharidin (Figure 5c), calamanene (Figure 5d) and hydroxycalamanene (Figure 5e) were also identified in the lemon aspen extracts. Terpenoids and naphthalenes have been previously reported to have potent broad spectrum antibacterial activity[42] and therefore may contribute to the inhibitory activity against *B. anthracis*. Interestingly, cantharidin, calamanene and hydroxycalamanene have also been shown to inhibit the proliferation of pancreatic cancer cells via oxidative stress-independent cell cycle arrest and the induction of apoptosis.[43,44] Furthermore, compounds structurally similar to calamanene and hydroxycalamanene have also been associated with the induction of apoptosis in human lung cancer cell lines by upregulating DR4 and DR5 cell death receptors and enhancing the activation of caspases 3, 7, 8 and 9,[43,44] possibly accounting for the anticancer activity previously reported for lemon aspen extracts.[45]

Flavonoids were also identified in the lemon aspen extracts. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.[46] Several flavonoids including rutin (Figure 5 f), luteolin (Figure 5 g), diosmin (Figure 5 h) and dihydrokaempferol (Figure 5i) were putatively identified in our study. These flavonoids have been reported to be good inhibitors of bacterial growth, particularly of Gram positive bacteria.[46] Whilst we were unable to find any reports of *B. anthracis* growth inhibitory activity of these flavonoids, they have been reported to inhibit growth of the closely related species *B. Cereus*.[47] Furthermore, a number of other phytochemicals including lauryl sarcosinate (Figure 5j), queuine (Figure 5k), dihydroxyarteminisin (Figure 5l), hydroxyethyl salicylate (Figure 5m), ferulic acid (Figure 5n), gingerol (Figure 5o), and chlorogenic acid (Figure 5p) were putatively identified in the lemon aspen extracts. Several recent studies have demonstrated the potent antimicrobial activity of plant extracts with high levels of these components.[18-24] In particular, *Tasmannia lanceolata,*[20] *Terminalia ferdinandiana*[21] and several Syzygium species[19] have demonstrated potent antimicrobial activity against a wide panel of bacteria. Thus, it is likely that multiple compounds within the lemon aspen extracts are contributing to the growth inhibition of *B. anthracis*.

An important consideration of any metabolomic technique is that it will not detect all compounds in a complex mixture, but instead will only detect a portion of them. This is not necessarily a problem when a directed/biased study is undertaken to detect a particular compound or class of compounds and the separation and detection conditions can be optimised for the study. However, when the aim of the study is metabolomic profiling rather than metabolomic finger printing, the technique conditions must be chosen and optimised to separate and detect the largest amount of compounds, with the broadest possible physical and chemical characteristics. Generally, HPLC-MS is a good choice for such metabolomic profiling studies as it generally detects a larger amount of compounds of varying polarities than the other commonly used techniques. However, this method is limited to studies of the mid-highly polar compounds and is not as useful for studies aimed at highly nonpolar compounds. Thus, many nonpolar phytosterols, saponins, stilbenes and terpenes which may contribute to the inhibitory activity of the lemon aspen extracts may escape detection by HPLC-MS. For this reason, future studies should also utilise GC-MS analysis to detect and identify the less polar compounds. Furthermore, mass spectral techniques are generally not capable on their own of differentiating between structural isomers. Further studies using a wider variety of techniques are required to confirm the identity of the compounds putatively identified here.

CONCLUSION

The results of this study demonstrate the potential of high antioxidant Australian plant extractsto block the growth of *B. anthracis*. Lemon aspen was particularly potent and thushas potential in the prevention and treat-

ment of anthrax. Further studies aimed at the purification of the bioactive components are needed to examine the mechanisms of action of these agents.

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CONFLICT OF INTEREST

All authors declare no conflicts of interest.

Highlights of Paper

- High antioxidant Australian plantextracts were potent inhibitors of *Bacillus anthracis* (the pathogenic agent of anthrax).
- The muntries, Illawarra plum and native tamarind extracts were particularly potent growth inhibitors with MIC values substantially < 1000 μg/mL.
- With the exception of *Syzygium australe* and *Syzygium leuhmannii* extracts, all inhibitory extracts were nontoxic.
- LC-MS analysis of the most potent lemon aspen extracts identified 85 compounds and highlighted several that may contribute to the ability of these extracts to inhibit the growth of *B. anthracis*.

Author Profile

- 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 approximately 150 scientific publications in a variety of peer reviewed journals.

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