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Original Article

GC-MS Headspace Analysis of *Terminalia ferdinandiana* fruit and Leaf Extracts which inhibit *Bacillus anthracis* Growth

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ABSTRACT

Background: *Terminalia ferdinandiana* (Kakadu plum) is an endemic Australian plant with an extremely high antioxidant capacity. The fruit has long been used by the first Australians as a nutritional food and as a medicine and recent studies have reported its potent growth inhibitory activity against a broad panel of bacteria. Despite this, *T. ferdinandiana* extracts are yet to be tested for the ability to inhibit the growth of *Bacillus anthracis.* **Materials and Methods:** Solvent extracts were prepared using both the fruit and leaf of Kakadu plum.The ability to inhibit the growth of *B. anthracis* was investigated using a disc diffusion assay. 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 investigated using non-targeted GC-MS head space analysis (with screening against a compound database) for the identification and characterisation of individual components in the crude plant extracts. **Results:** Solvent extractions of *T. ferdinandiana* fruit and leaf displayed good growth inhibitory activity in the disc diffusion assay against *B. anthracis*. Fruit ethyl acetate and methanolic leaf extracts were particularly potent growth inhibitors, with MIC values of 451 and 377μg/mL respectively. The fruit methanolic and chloroform extracts, as well as the aqueous leaf extracts also were good inhibitors of *B. anthracis* growth, albeit with lower efficacy (MIC values of 1800 and 1414 μg/mL respectively).The aqueous fruit extract and leaf chloroform extracts had only low inhibitory activity. All other extracts were completely devoid of growth inhibitory activity. Furthermore, all of the extracts with growth inhibitory activity were nontoxic in the *Artemia fransiscana* bioassay, with LC_{50} values >1000 μg/mL. Non-biased GC-MS phytochemical analysis of the most active extracts (fruit ethyl acetate and methanolic leaf) putatively identified and highlighted several compounds that may contribute to the ability of these extracts to inhibit the growth of *B. anthracis*. **Conclusions:** The low toxicity of the *T. ferdinandiana* fruit ethyl acetate and methanolic leaf extracts, as well as their potent growth inhibitory bioactivity against *B. anthracis,* indicates their potential as medicinal agents in the treatment and prevention of anthrax.

Key words: *Bacillus anthracis*, Anthrax, Kakadu plum, Zoonotic, Tannin, Combretastatin, stilbene, Metabolomics.

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INTRODUCTION

Zoonotic infections are diseases that can be transmitted indirectly or directly between humans and animals and are a significant burden from both health and economic standpoints.[1] These diseases can be spread to humans from both domesticated and wild animals and can be transferred through direct contact, the contamination of drinking water by animal secretions, or the consumption of contaminated meat products.[2] These diseases pose an exceptional set of problems in the control and treatment of infections, as the traditionally effective strategies of herd immunity and isolation of infected individuals are not feasible. Furthermore, unlike humans who can verbalise otherwise indistinguishable symptoms, infected animals may go unnoticed and further contribute to the spread of disease. From 1940 to 2004, approximately 60% of all emerging infectious diseases were of a zoonotic nature with the majority originating in wildlife.[3] Therefore, the development of cross species treatments plays a key role in the effective control and eradication of zoonotic diseases.

Bacillus anthracis, the etiological agent of anthrax, is a sporulating gram-positive bacterium found predominately in soils.[4] Similar to other organisms within the *Bacillus* genus, *B. anthracis* is capable of producing endospores that can remain dormant for several years until conditions are again favourable for growth. These spores are metabolically inactive and are capable of surviving environmental conditions that would kill vegetative cells, including

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temperature, desiccation and enzymatic destruction.[5] Although the vegetative *B. anthracis* cells produce the toxins associated with the disease, infection is generally initiated when spores are introduced into a host through inhalation, ingestion or via direct contact with open wounds. Once internalised, the spores revert to viable cells, proliferate and begin producing the deadly anthrax toxins.[6] The disease has been controlled to varying degrees internationally through careful monitoring and strong eradication measures. However, anthrax is endemic worldwide and is often fatal if infection occurs.[7]

Current strategies in the treatment of anthrax typically involve a combination of antibiotic therapies to fight infection, as well as supportive care to manage associated symptoms.[8] The administration of intravenous or oral antibiotics are generally effective in the management of anthrax, however there is always an inherent risk that the bacteria may develop drug resistance. As such, the discovery of new drugs is of significant importance, either through the design and synthesis of new compounds, or through the investigation of antimicrobials within pre-existing natural assets.[5,9] The antimicrobial effects of medicinal plants have long been recognised by many cultures and phytochemical analysis to identify the active compounds offers promise in the development of new anti-*B. anthracis* agents.Thus the investigation of natural assets provides great potential in the discovery of compounds effective in managing anthrax.

Terminalia ferdinandiana, commonly referred to as Kakadu plum, is a small flowering tree endemic to the tropical northern regions of Australia. It has been used as a source of food by the first Australians for thousands of years.[10] The fruit has a remarkably high antioxidant capacity and has been defined as one of the best sources of vitamin C of any plant in the world.[10,11] The medicinal benefits of the fruit were well known by the first Australians who considered the plum both a medicine and a food source. It has been proposed that the health benefits may stem from one or many antimicrobial compounds.[12] These include flavonols, flavonones, as well as benzoic acid, gallic acid and ellagic acid derivatives, all of which have been previously associated with microbial inhibition.[13] Indeed, the antiseptic potential of both the fruit and leaves is well documented in the prevention of several disease-causing microorganisms[14-17] and it is likely that Kakadu plum may have inhibitory compounds that aid in the prevention of *B. anthracis* growth. The current study investigates both the fruit and leaf components of *T. ferdinandiana* for the ability to inhibit the proliferation of vegetative *B. anthracis* cells as a natural alternative in the treatment of anthrax.

MATERIALS AND METHODS

Plant source and extraction

T. ferdinandiana fruit pulp and leaves were supplied and verified by David Boehme of Wild Harvest, Northern Territory (Australia). The pulp was frozen for transport and kept at -10°C until processed. A voucher specimen of the pulp (KP2014GD) is stored at Griffith University. The leaves were thoroughly dehydrated in a Sunbeam food dehydrator and the dried material was subsequently stored at -30°C. A voucher specimen (KP2015LA) is stored at the School of Natural Sciences, Griffith University. Prior to use, the plant materials were thoroughly dried and ground into a coarse powder. A mass of 1g of ground powder was extracted extensively in 50 mL of either methanol, deionised water, ethyl acetate, chloroform or hexane for 24 h at 4°C with gentle shaking. All solvents were supplied by Ajax (AR grade). The extracts were filtered through filter paper (Whatman No. 54) and air dried at room temperature. The aqueous extract was lyophilised by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellets were dissolved in10 mL deionised water (containing 0.5 % DMSO). The extract was passed through a 0.22 µm filter (Sarstedt) and stored at 4°C.

Qualitative phytochemical studies

Phytochemical analysis of the extracts for the presence of alkaloids, anthraquinones, cardiac glycosides, flavonoids, phenolic compounds, phytosteroids, saponins, tannins and triterpenoids were conducted by previously described assays.[18-20]

Antioxidant capacity

The antioxidant capacity of each sample was assessed using the DPPH free radical scavenging method with modifications.[21-23] Ascorbic acid (0-25 µg per well) was used as a reference and the absorbances were recorded at 515 nm. All tests were completed alongside controls on each plate and all 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

Environmental *Bacillus anthracis* screening

An environmental strain of *Bacillus anthracis* was isolated as previously described[5]. All growth studies were performed 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, 2.4 g/L HEPES buffer (pH 7.5) and 16g/L bacteriological agar when required. 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. 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.[24-26] Briefly, 100 µL of the test bacterium was grown in 10 mL of fresh nutrient broth media until they reached a count of ~10^8 cells/mL. A volume of 100 µL of the bacterial suspension was spread onto nutrient agar plates and 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 the 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 to the closest whole millimetre. Each assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of penicillin (2 µg) and ampicillin (10 µg) were obtained from Oxoid Ltd. and used 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 concentrations (MIC) of the extracts was determined as previously described[27]. Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were impregnated with 10 µL 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. MIC values were determined using linear regression.

Toxicity screening

Reference toxin for toxicity screening

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

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *A. franciscana* nauplii lethality assay.[28-30] 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 in the bioassay. A volume of 400 µL of reference toxin or the diluted plant extracts 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 LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Non-targeted GC-MS head space analysis

Separation and quantification were performed using a Shimadzu GC-2010 plus (USA) linked to a Shimadzu MS TQ8040 (USA) mass selective detector system as previously described.[15] Briefly, the system was equipped with a Shimadzu auto-sampler AOC-5000 plus (USA) fitted with a solid phase micro-extraction fibre (SPME) handling system utilising a Supelco (USA) divinyl benzene/carbowax/polydimethylsiloxane (DVB/CAR/PDMS). Chromatographic separation was accomplished using a 5% phenyl, 95% dimethylpolysiloxane (30 m x0.25 mm id x 0.25 um) capillary column (Restek USA). Helium (99.999%) was employed as a carrier gas at a flow rate of 0.79 ml/min. The injector temperature was set at 230°C. Sampling utilised a SPME cycle which consisted of an agitation phase at 500 rpm for a period of 5 sec. The fibre was exposed to the sample for 10 min to allow for absorption and then desorbed in the injection port for 1 min at 250°C. The initial column temperature was held at 30°C for 2 min, increased to 140°C for 5 min, then increased to 270°C over a period of 3 mins and held at that temperature for the duration of the analysis. The GC-MS interface was maintained at 200°C with no signal acquired for a min after injection in split-less mode. The mass spectrometer was operated in the electron ionisation mode at 70 eV. The analytes were then recorded in total ion count (TIC) mode. The TIC was acquired after a min and for duration of 45 mins utilising a mass range of 45 - 450 m/z.

Statistical analysis

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

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extractions of the various dried Kakadu plum plant materials (1 g) with various solvents yielded dried plant extracts ranging from 18 mg (hexane fruit extract) to 483 mg (aqueous fruit extract) (Table 1). Methanolic and aqueous extracts gave significantly higher yields of dried extracted material compared to the chloroform, hexane and ethyl acetate counterparts, which gave low to moderate yields. The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 1.

Antimicrobial activity

To determine the ability of the crude plant extracts to inhibit the growth of *B. anthracis*, aliquots (10 µL) of each extract were screened using a disc diffusion assay. The bacterial growth was strongly inhibited by 7 of the 10 extracts screened (70%) (Figure 1). The methanolic leaf extract

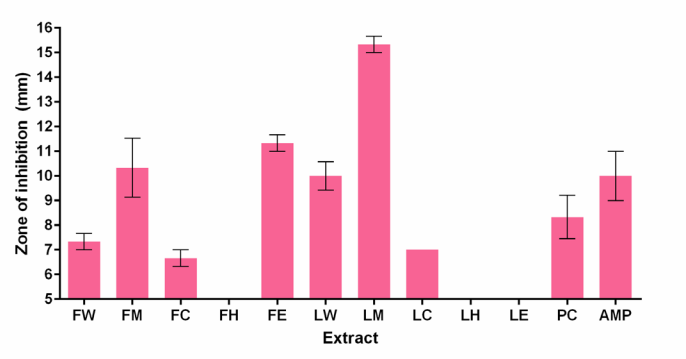

Figure 1: Growth inhibitory activity of Kakadu plum plant extracts against the *B. Anthracis* environmental isolate measured as zones of inhibition (mm). FW = aqueous Kakadu plum fruit extract; FM = methanolic Kakadu plum fruit extract; FC = chloroform Kakadu plum fruit extract; FH = hexane Kakadu plum fruit extract; FE = ethyl acetate Kakadu plum fruit extract; LW = aqueous Kakadu plum leaf extract; LM = methanolic Kakadu plum leaf extract; LC = chloroform Kakadu plum leaf extract; LH = hexane Kakadu plum leaf extract; LE = ethyl acetate Kakadu plum leaf extract; PC = penicillin (2 µg); AMP = ampicillin (10 µg). Results are expressed as mean zones of inhibition ± SEM.

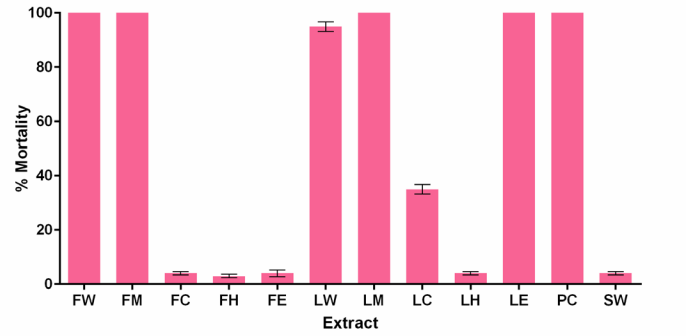

Figure 2: The lethality of the Australian plant extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *Artemia franciscana* nauplii after 24 h exposure. FW = aqueous Kakadu plum fruit extract; FM = methanolic Kakadu plum fruit extract; FC = chloroform Kakadu plum fruit extract; FH = hexane Kakadu plum fruit extract; FE = ethyl acetate Kakadu plum fruit extract; LW = aqueous Kakadu plum leaf extract; LM = methanolic Kakadu plum leaf extract; LC = chloroform Kakadu plum leaf extract; LH = hexane Kakadu plum leaf extract; LE = ethyl acetate Kakadu plum leaf extract; PC = potassium dichromate control; SW = seawater control. Results are expressed as mean % mortality ± SEM.

was the most potent inhibitor of *B. anthracis* growth (as judged by zone of inhibition), with inhibition zones of 15.3 ± 0.6 mm. This compares favourably with the penicillin (2 µg) and ampicillin controls (10 µg), with zones of inhibition of 8.3 ± 0.6 and 10.0 ± 0.7 respectively. The methanolic fruit extract as well as the ethyl acetate and aqueous leaf extracts also displayed good inhibition of *B. anthracis* growth, with ≥ 8 mm zones of inhibition. In general, the leaf extracts were more potent inhibitors of *B. anthracis* growth than were their fruit extract counterparts.

The antimicrobial efficacy was further quantified through the determination of MIC values against the Kakadu plum extracts (Table 2). Most of the extracts were effective at inhibiting *B. anthracis* growth, with MIC values <1000 µg/ml for several extracts (<10 µg impregnated in the disc). The ethyl acetate fruit extract and the methanolic leaf extract were particularly potent, with MIC values of 451 µg/mL (approximately 4.5 µg infused into the disc) and 377µg/mL (approximately 3.8 µg infused into

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant capacities of the Kakadu plum 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	Polysteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	0Combined Anthraquinones
FW	483	48.3	264	+++	+++	+++	–	+	–	–	–	–	+++	++	–	–
FM	359	35.9	660	+++	+++	+++	–	++	+	–	+	+	+++	++	–	–
FC	62	6.2	7	+	–	–	–	–	–	–	–	–	–	–	–	–
FH	18	1.8	1	–	–	–	–	–	–	–	–	–	–	–	–	–
FE	30	3	39	++	++	+	–	+	++	–	–	–	++	–	–	–
LW	471	47.1	340	+++	+++	+++	++	+++	++	–	–	–	++	+++	+	+
LM	331	33.1	150	+++	+++	+++	+++	++	+	–	+	+	++	+++	+	+
LC	59	5.9	5	+	–	–	–	–	–	–	–	–	–	–	–	–
LH	58	5.8	0.4	+	–	–	–	–	–	–	–	–	++	+	–	–
LE	59	5.9	22	+++	+++	+++	–	–	–	–	–	–	++	++	–	–

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. FW = aqueous Kakadu plum fruit extract; FM = methanolic Kakadu plum fruit extract; FC = chloroform Kakadu plum fruit extract; FH = hexane Kakadu plum fruit extract; FE = ethyl acetate Kakadu plum fruit extract; LW = aqueous Kakadu plum leaf extract; LM = methanolic Kakadu plum l eaf extract; LC = chloroform Kakadu plum leaf extract; LH = hexane Kakadu plum leaf extract; LE = ethyl acetate Kakadu plum leaf extract. Antioxidant capacity was determined by DPPH reduction and is expressed as mg ascorbic acid equivalence per g plant material extracted.

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

Extract	MIC	LC_{50}
aqueous fruit extract	>10,000	2,080
methanolic fruit extract	877	2,115
chloroform fruit extract	1800	-
hexane fruit extract	-	-
ethyl acetate fruit extract	451	-
aqueous leaf extract	1414	1,330
methanolic leaf extract	377	1,133
chloroform leaf extract	5,000	-
hexane leaf extract	-	-
ethyl acetate leaf extract	-	767

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

the disc) respectively. These results compare well with the growth inhibitory activity of the penicillin and ampicillin controls which were tested at 2 µg and 10 µgrespectively. The methanolic fruit extract was also a potent *B. anthracis* growth inhibitor (MIC value of 877 µg/ml). Whilst less potent, the fruit chloroform and aqueous leaf extracts also had good growth inhibitory activity (MIC values of 1800 and 1414 µg/ml respectively). In contrast, the aqueous fruit and hexane extracts, as well as the leaf chloroform hexane and ethyl acetate extracts, were not active, or were of only low efficacy in the assay.

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). All methanolic and aqueous extracts showed>90 % mortality rates at 24h, as did the ethyl acetate leaf extract. The remainder of the extracts showed < 10% mortality rates at 24 h, with the exception of the chloroform leaf extract.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted inartificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay at 24 hours. Table 2 shows the LC_{50} values of the Kakadu plum extracts towards *A. franciscana*. No LC_{50} values are reported for either of the chloroform and hexane extracts, nor for the ethyl acetate fruit extract, as less than 50% mortality was seen for all concentrations tested. Extracts with an LC_{50} greater than 1000 µg/ml towards *Artemia* nauplii have been defined as being nontoxic in this assay.[31]As only the ethyl acetate fruit extract had a LC_{50} <1000 µg/ml, all other extracts were considered nontoxic. Whilst the LC_{50} value for leaf ethyl acetate extract is below 1000 µg/ml, the value of 767 µg/ml indicates low to moderate toxicity.

Non-targeted GC-MS headspace analysis of Kakadu plum extracts

As the fruit ethyl acetate and methanolic leaf extracts had the greatest growth inhibitory efficacy against *B. anthracis* (as determined by MIC; Table 2), they were deemed the most promising extracts for further phytochemical analysis. Optimised GC-MS parameters were developed and used to examine the phytochemical composition of these extracts. The resultant gas chromatograms for the fruit ethyl acetate and methanolic leaf extracts are presented in Figures 3 and 4 respectively. Several major peaks were noted in the fruit ethyl acetate extract at approximately 15.1 (3,3-dimethyl-hexane, 7.1% relative abundance), 19.7 (2-methyl-2-phenyl-oxirane, 14.6% relative abundance), 20.9 (m-di-tert-butylbenzene,22% relative abundance) and 28.9 min (3,5-bis(1,1-dimethylethyl)-phenol, 19.4% relative abundance). Numerous overlapping peaks were also evident in the middle stages of the chromatogram from 10-25 min. In total, 42 unique mass signals were noted for the *T. ferdinandiana* fruit ethyl acetate extract (Table 3). Putative empirical formulas and identifications were achieved for all of these compounds.

The gas chromatogram for the methanolic leaf extract (Figure 4) had substantially fewer peaks evident than the fruit ethyl acetate extract (Figure 3). In total, 19 unique mass signals were noted in themethanolic leaf extract chromatogram. Several major peaks were present at approximately 11.3 (methoxy-phenyl-oxime, 22.7% relative abundance), 13.7 (1-octen-3-ol, 2.4 % relative abundance),14.4 (2-(1,1-dimethylethoxy)-ethanol, 27.7% relative abundance), 19.5 (2-methyl-2-phenyl-oxirane, 11.4% relative abundance) and 21.5 min (3,5-dimethyl-benzaldehyde, 15.6% relative abundance). Several small peaks were also evident throughout the chromatogram. Of the 19 unique mass signals, putative empirical formulas and identifications were achieved for 16 of these compounds.

DISCUSSION

Many *Terminalia* spp. have a history of therapeutic usage to treat microbial infections and numerous recent investigations have reported on their antibacterial properties.[32] The Australian species *T. ferdinandiana* has proven to be particularly effective, with growth inhibitory activity reported against a broad panel of bacterial pathogens,[17] as well as against some bacterial triggers of rheumatoid arthritis[14,16] and multiple sclerosis.[15] Furthermore, *T. ferdinandiana* has also recently been reported to inhibit the proliferation of the gastrointestinal protozoan parasite *Giardia duodenalis*[13] indicating its therapeutic potential against both prokaryotic and eukaryotic pathogens. Interestingly, whilst inhibition of *B. anthracis* growth was not evaluated in any of the previous studies, one recent study reported potent growth inhibition of the related bacterial species *B.cereus*, with MIC values as low as approximately 100 µg/mL.[17] *B. cereus* is very closely related to *B. anthracis* with >99% 16S RNA gene sequence homology.[33] Indeed, some bacterial 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.[34-36] Therefore, it is perhaps not surprising that the *T. ferdinandiana* extracts screened in our study displayed potent growth inhibitory activity towards *B. anthracis*.

Qualitative GC-MS headspace analysis of the most potent *B. anthracis* growth inhibitory *T. ferdinandiana* extracts (fruit ethyl acetate and methanolic leaf extracts) identified a number of interesting compounds which may contribute to this activity. The presence of the furan compounds 1-(2-furanyl)-ethanone (Figure 5a) and ethyl 2-(5-methyl-5-vinyl tetra hydrofuran-2-yl) carbonate (Figure 5b) are particularly note worthy as many furan derivatives are potent inhibitors of bacterial growth. The nitro furans have particularly well studied antimicrobial mechanisms, acting via the inhibition of nucleic acid synthesis.[37] Similarly, another study reported synthetic furan derivatives (modified by the addition of a rhodanine moiety) to be potent inhibitors of the growth of a panel of

Table 3: Qualitative GC-MS analysis of the *T. ferdinandiana* fruit ethyl acetate extract, elucidation of empirical formulas and putative identification of each compound

Putative Identification	Molecular Mass	Empirical Formula	Retention Time (min)	Relative Abundance (% Area)
Ethanone, 1-(2-furanyl)-	110	$C_6H_6O_2$	10.202	0.9
2-Heptanone, 4-methyl-	128	$C_8H_{16}O$	11.184	3.8
Propanoic acid, 2-acetylhydrazono-	144	$C_5H_8N_2O_3$	11.8	1
Tridecane	184	$C_{13}H_{28}$	12.087	0.6
Acetic acid, heptyl ester	158	$C_9H_{18}O_2$	12.263	0.2
3-Pentanol, 2,2-dimethyl-	116	$C_7H_{16}O$	12.525	2.8
2-Heptanone, 4,6-dimethyl-	142	$C_9H_{18}O$	12.866	3.2
3-Heptanol, 2-methyl-	130	$C_8H_{18}O$	13.063	0.2
1-Octanol, 2-butyl-	186	$C_{12}H_{26}O$	13.233	0.8
Heptane, 3,3,5-trimethyl-	142	$C_{10}H_{22}$	13.531	0.3
Undecane	156	$C_{11}H_{24}$	13.69	1.5
Octane, 3,3-dimethyl-	142	$C_{10}H_{22}$	13.96	2.5
1-Hexanol, 2-ethyl-	130	$C_8H_{18}O$	14.105	3.8
1,3-Propanediamine, N,N-dimethyl-	102	$C_5H_{14}N_2$	14.3	0.3
Hexane, 3,3-dimethyl-	114	C_8H_{18}	15.079	7.1
1-Octanol	130	$C_8H_{18}O$	15.422	0.3
Propanoic acid, anhydride	130	$C_6H_{10}O_3$	15.625	0.2
1,3-Benzenediol, 4-ethyl-	138	$C_8H_{10}O_2$	16.034	0.4
1-Undecene, 4-methyl-	168	$C_{12}H_{24}$	16.461	1.9
Carbonic acid, nonyl prop-1-en-2-yl ester	228	$C_{13}H_{24}O_3$	17.241	0.2
Octanoic acid	144	$C_8H_{16}O_2$	18.481	0.8
Oxalic acid, 6-ethyloct-3-yl isohexyl ester	314	$C_{18}H_{34}O_4$	18.665	0.4
2-Octanol, 2,6-dimethyl-	158	$C_{10}H_{22}O$	18.914	0.1
Undecane, 4,7-dimethyl-	184	$C_{13}H_{28}$	19.387	0.8
Oxirane, 2-methyl-2-phenyl-	134	$C_9H_{10}O$	19.699	14.6
Nonane, 2,6-dimethyl-	156	$C_{11}H_{24}$	19.803	0.6
Dodecane, 4-methyl-	184	$C_{13}H_{28}$	20.035	0.3
Undecane, 2,4-dimethyl-	184	$C_{13}H_{28}$	20.678	0.3
4-tert-Butylcyclohexyl methyl ethylphosphonate	262	$C_{13}H_{27}O_3P$	20.777	0.3
m-Di-tert-butylbenzene	190	$C_{14}H_{22}$	20.931	22
Hexane, 3,3-dimethyl-	114	C_8H_{18}	21.08	0.2
Nonadecane	268	$C_{19}H_{40}$	21.703	1
Butyl 2-butoxyacetate	188	$C_{10}H_{20}O_3$	21.841	0.7
Octane, 2,3,6,7-tetramethyl-	170	$C_{12}H_{26}$	22.087	0.1
Hexane, 3,3-dimethyl-	114	C_8H_{18}	22.959	0.2
1,1,6,6-Tetramethylspiro[4.4]nonane	180	$C_{13}H_{24}$	23.348	0.6
2,2,4-Trimethyl-1,3-pentanediol diisobuty	286	$C_{16}H_{30}O_4$	23.611	0.4
n-Decanoic acid	172	$C_{10}H_{20}O_2$	23.862	0.1
Propanoic acid, 2-methyl-, 3-hydroxy-2,2	216	$C_{12}H_{24}O_3$	24.169	0.6
1-Dodecanol	186	$C_{12}H_{26}O$	27.352	0.1
Phenol, 3,5-bis(1,1-dimethylethyl)-	206	$C_{14}H_{22}O$	28.856	19.4
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	286	$C_{16}H_{30}O_4$	31.769	0.5

The relative abundance expressed in this table is a measure of the area under the peak expressed as a % of the total area under all chromatographic peaks.

Table 4: Qualitative GC-MS analysis of the methanolic *T. ferdinandiana* leaf extract, elucidation of empirical formulas and putative identification of each compound

Putative Identification	Molecular Mass	Empirical Formula	Retention Time (mins)	Relative Abundance (% Area)
Oxime-, methoxy-phenyl-_	151	$C_8H_9NO_2$	11.266	22.7
1-Octen-3-ol	128	$C_8H_{16}O$	13.727	2.4
Ethanol, 2-(1,1-dimethylethoxy)-	118	$C_6H_{14}O_2$	14.418	27.7
1-Hexanol, 2-ethyl-	130	$C_8H_{18}O$	15.373	1.1
Cineole	154	$C_{10}H_{18}O$	15.499	1.8
Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl carbonate	242	$C_{13}H_{22}O_4$	16.879	1
			17.04	1.3
Nonanal	142	$C_9H_{18}O$	17.873	2.1
			18.231	0.6
Oxirane, 2-methyl-2-phenyl-	134	$C_9H_{10}O$	19.56	11.4
Ethyl benzoate	150	$C_9H_{10}O_2$	20.06	0.8
2-Isopropylidene-3-methylhexa-3,5-dienal	150	$C_{10}H_{14}O$	21.031	0.5
Decanal	156	$C_{10}H_{20}O$	21.11	0.3
Benzaldehyde, 3,5-dimethyl-	134	$C_9H_{10}O$	21.527	15.6
			24.786	0.9
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4	216	$C_{12}H_{24}O_3$	26.499	2.2
2,4-Di-tert-butylphenol	206	$C_{14}H_{22}O$	31.641	1
Ethyl para-ethoxybenzoate	194	$C_{11}H_{14}O_3$	32.054	5.9
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	286	$C_{16}H_{30}O_4$	33.822	0.7

The relative abundance expressed in this table is a measure of the area under the peak expressed as a % of the total area under all chromatographic peaks.

multidrug resistant bacteria, with MIC values as low as 2 µg/mL against some species.[38] Whilst we were unable to find reports of anti-bacterial activity for the 2 furan derivatives present in in the *T. ferdinandiana* extracts, it is possible that they may contribute to the growth inhibitory activities reported in our study.

It is likely that other phytochemical classes also contribute to the growth inhibitory properties of these extracts. Our qualitative phytochemical screening studies indicate that polyphenolics, flavonoids, saponins, and terpenes were present in the *T. ferdinandiana* extracts. As our study used headspace GC-MS techniques to putatively identify the phytochemical composition of the extracts, many of the mid to higher polarity compounds may have not been identified. Recent studies have reported the LC-MS profiles of similar *T. ferdinandiana* fruit[13,15,16] and leaf extracts.[14] Several features were common to all of these studies. All reported on the diversity of tannins in both the fruit and leaf extracts. Gallic (Figure 5c) and ellagic acids (Figure 5d) and their methylated derivatives, chebulic acid (Figure 5e), galloylpyrogallol (Figure 5f), corilagen (Figure 5g), punicalin (Figure 5h), castalagin (Figure 5i) and chebulagic acid (Figure 5j) were detected in *T. ferdinandiana* extracts in each of those studies. These tannins have potent, broad spectrum growth inhibitory activity against a variety of bacterial species.[32] Gallotannins have particularly well reported inhibitory properties.[39] They function via multiple mechanisms including interacting with both cell surface proteins[40,41] and through interactions with intracellular enzymes.[42] Ellagitannins also interact with cellular proteins and induce disruptions in bacterial cell walls.[39,41]

Several recent studies also highlighted the stilbene components of a methanolic *T. ferdinandiana* fruit extracts.[14,15] Resveratrol (Figure 5k) and the glycosylated resveratrol derivative piceid (Figure 5l), diethylstilbestrol monosulfate (Figure 5m) and combretastatin A1 (Figure 5n) were putatively identified in those studies. Identification of combretastatin A1 was particularly interesting as combretastatins have attracted much recent interest due to their potent ability to block cancer cell progression and induce apoptosis by binding intracellular tubulin, thereby disrupting microtubule formation.[43] Whilst we were unable to find accounts of bacterial growth inhibition of combretastatin A1 in the literature, the growth inhibition of *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli* and *Neisseria gonorrhoeae* by several synthetic combretastatins (and synthetic resveratrol analogues) have been reported.[44] Therefore, it is likely that the *T. ferdinandiana* extract stilbene components may also contribute to the *B. anthracis* growth inhibition noted in our study.

Several important terpenoids have also been reported in *T. ferdinandiana* extracts in recent studies.[15] Monoterpenoids were particularly prevalent, with isomyocorene, cineol, cuminol, camphor, iso-menthol reported in *T. ferdinandiana* fruit ethyl acetate extract examined in that study. Notably, cineole was also putatively identified in the anti- *B. anthracis* leaf methanolic extract examined in our study. Many of these terpenoids have potent broad spectrum antibacterial activity[45] and therefore may contribute to the *B. anthracis* growth inhibition reported in our study. Interestingly, several of these monoterpenes have also been reported to suppress NF-κB signalling (the major regulator of inflammatory diseases).[46-49] Thus, the terpene components may have a pleuripotent mechanism in blocking anthrax, by inhibiting the growth of the causative bacterium, as well as relieving the downstream inflammatory symptoms evident with the most common (cutaneous) form of the disease.

With the exception of the *T. ferdinandiana* ethyl acetate leaf extract, the findings reported here demonstrate that the *T. ferdinandiana* extracts

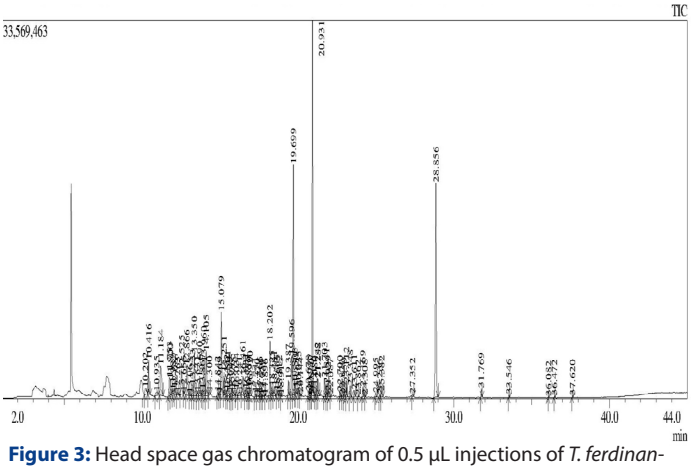

Figure 3: Head space gas chromatogram of 0.5 µL injections of *T. ferdinandiana* ethyl acetate fruit extract. The extract were dried and resuspended in methanol for analysis.

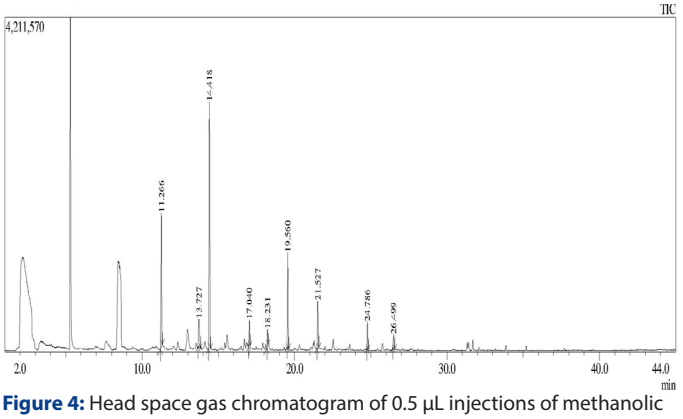

Figure 4: Head space gas chromatogram of 0.5 µL injections of methanolic T. ferdinandiana leaf extract. The extract were dried and resuspended in methanol for analysis.

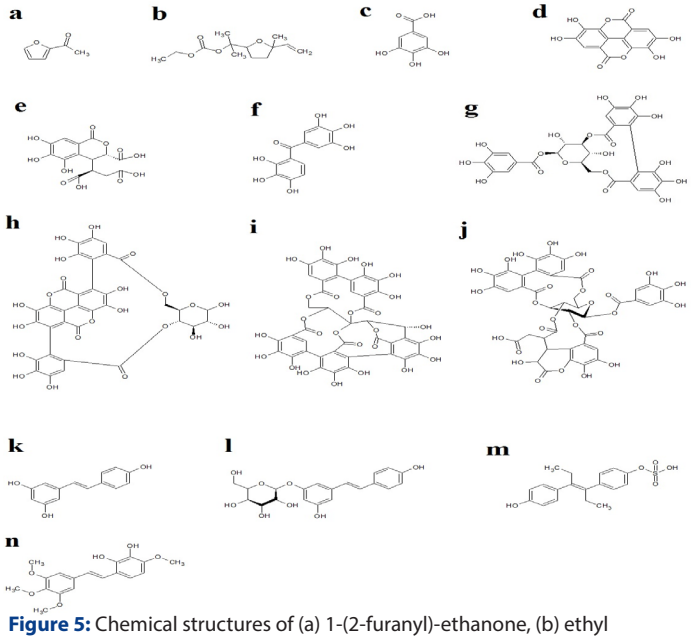

Figure 5: Chemical structures of (a) 1-(2-furanyl)-ethanone, (b) ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl) carbonate, (c) gallic acid, (d) ellagic acid; (e) chebulic acid, (f) galloyl pyrogallol, (g) corilagen, (h) punicalin, (i) castalagin, (j) chebulagic acid, (k) resveratrol, (l) piceid, (m) diethylstilbestrol monosulfate, (n) combretastatin A1.

were nontoxic towards *Artemia franciscana* nauplii, with LC_{50} values substantially > 1000 µg/mL. Extracts with LC_{50} values > 1000 µg/mL towards *Artemia* nauplii are defined as being nontoxic.[31] Even the ethyl acetate leaf extract which induced significant mortality was deemed low to moderate toxicity due to its moderate LC_{50} value. Whilst our preliminary toxicity studies indicate that these extracts may be safe for use as *B. anthracis* growth inhibitors, studies using human cell lines are required to further evaluate the safety of these extracts. Furthermore, whilst the results of our study are promising, it must be noted that the growth inhibitory studies screened against vegetative cells. As *Bacillus* spp. are spore formers, further studies are required to determine whether extracts with *B. anthracis* growth inhibitory activity also affect bacterial growth from the spores.

CONCLUSION

The results of this study demonstrate the potential of *T. ferdinandiana* in the inhibition of *B. anthracis* growth. The fruit ethyl acetate and methanolic leaf extracts were particularly potent growth inhibitors. Further investigations aimed at the purification of the bioactive components are needed to assess the mechanisms of action of these agents.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide, **LC_{50}:** The concentration required to achieve 50 % mortality, **MIC:** minimum inhibitory concentration, **PYE:** peptone yeast extract.

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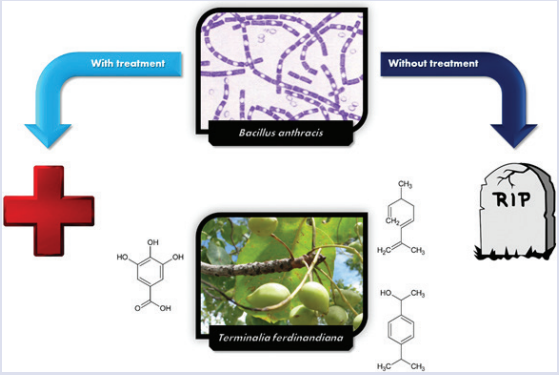
GRAPHICAL ABSTRACT



HIGHLIGHTS OF PAPER

- *T. ferdinandiana* fruit and leaf extractswere inhibitors of *Bacillus anthracis* growth.
- The fruit ethyl acetate and methanolic leaf extracts were particularly potent growth inhibitors with MIC's of 451 and 377μg/mL respectively.
- All inhibitory extracts were non-toxic in the *Artemia* nauplii assay.
- GC-MS headspace profiling of the inhibitory extracts revealed distinct phytochemical profiles for the fruit and leaf extracts.
- Phytochemical profiling highlighted several nonpolar compounds as potentially contributing to the *B. anthracis* growth inhibitory activity.

AUTHOR PROFILE

Dr Wright: Received his PhD in 2014, for his work investigating the manganese reduction and oxidation characteristics of environmental bacteria. He is currently a postdoctoral researcher at Griffith University, Australia, where he is working on several projects both in the areas of geomicrobiology and pharmocognosy. His present research interests are the use of biogenic manganese oxides in the bioremediation of metal-contaminated sites as well as the use of Australian native plants in the treatment and prevention of various pathogenic bacteria.

Dr Anthony Greene: Is a senior lecturer and researcher at Griffith University, Brisbane Australia. He obtained his PhD 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.

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 *Scaevolaspinescens*, *Pittosporum phylliraeoides, Terminalia ferdinandiana* (Kakadu plum), Australian Acacias, Syzygiums, Petalostigmas and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in nearly 200 publications in a variety of peer reviewed journals.

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