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Original Article

Bioactive constituents of *Terminalia ferdinandiana* Exell: A pharmacognistic approach towards the prevention and treatment of yersiniosis

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ABSTRACT

Introduction: *Yersinia enterocolitica* is a facultatively anaerobic gram negative bacterium which contaminates meat products causing the acute gastrointestinal disease yersiniosis. *Terminalia ferdinandiana* (Kakadu plum, gubinge) is an Australian fruit with an extremely high antioxidant capacity. It was used therapeutically by the first Australians and has documented antiseptic properties against an extensive panel of bacteria. Despite this, it has not been tested for the ability to inhibit the growth of *Y. enterocolitica*. **Methods:** *T. ferdinandiana* fruit and leaf extracts were extracted by maceration and the extracts were investigated by disc diffusion assay for growth inhibitory activity against a clinical strain of *Y. enterocolitica*. The MIC values of the extracts 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 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* leaf and fruit displayed good growth inhibitory activity in the disc diffusion assay against *Y. enterocolitica*. The methanolic *T. ferdinandiana* leaf and fruit extracts, as well as the fruit ethyl acetate extract, were particularly potent growth inhibitors, with MIC values of 372, 123 and 285 µg/mL respectively. The aqueous and ethyl acetate leaf extracts also displayed good growth inhibitory activity against *Y. enterocolitica*, albeit with higher MIC values (588 and 1100 µg/mL respectively). All other extracts were either low efficacy, or completely devoid of growth inhibitory activity. All *T. ferdinandiana* leaf and fruit extracts were either nontoxic (LC_{50} values <1000 µg/mL) or of low toxicity in the *Artemia franciscana* bioassay. Non-biased GC-MS phytochemical analysis of the methanolic extracts putatively identified and highlighted several compounds that may contribute to the ability of these extracts to inhibit the growth of *Y. enterocolitica*. **Conclusion:** The lack of toxicity and the potent growth inhibitory bioactivity of the *T. ferdinandiana* fruit and leaf methanolic extracts against *Y. enterocolitica* indicates their potential as medicinal agents in the treatment and prevention of yersiniosis.

Key words: Kakadu plum, gubinge, *Yersinia enterocolitica*, Enterobacteriaceae, antioxidant, antibacterial, terpenoids, GC-MS, metabolomic profile.

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INTRODUCTION

The genus *Yersinia* (*Y.*) comprises a large group of gram-negative, facultatively anaerobic bacteria belonging to the family *Enterobacteriaceae*. Widespread in the environment and amongst animal populations, the genus is clinically important as some members are pathogenic and are the etiological agents of several diseases in humans.[1] Pathogenic members of the genus are responsible for a number of fatal and non-fatal infections, ranging from the relatively mild gastrointestinal disease yersiniosis (*Y. enterocolitica*) to the potentially fatal bubonic plague (*Y. pestis*).[2,3] Furthermore, these organisms are zoonotic pathogens and may persist in non-human reservoirs until they are able to infect humans. This poses a unique set of problems otherwise not encountered in the prevention of other diseases, as traditional methods (isolation of infected individuals, targeted immunization) are less effective in the treatment of zoonotic infections.[4]

Yersiniosis is an acute gastrointestinal infection acquired through the ingestion of contaminated food or water. Characterised by abdominal pain, diarrhoea and fever, yersiniosis is a significant disease which accounts for over 100,000 cases of gastroenteritis in the US annually.[5] The disease originates from several sources although it is a predominately food-borne illness and transmission of this type accounts for approximately 90% of all reported cases.[6] *Y. enterocolitica* has the ability to grow at 4°C and thus refrigeration is an inadequate means of protection from infection.[7] Although rarely fatal and often self-limiting, the symptoms associated with yersiniosis can result in sufferers becoming temporarily incapacitated and thus has indirect negative economic consequences. The probing of natural reservoirs for antibacterial agents effective in the growth inhibition of *Y. enterocolitica* offers an alternative approach in the prevention and treatment of these infections.

Terminalia ferdinandiana Exell (Kakadu plum, gubinge) is an endemic Australian plant which has an extremely high antioxidant content.[8,9] The fruit of this plant also has the highest ascorbic acid levels of any plant in the world, with levels reported as high as 6% of the recorded wet weight.[8,9] This is approximately 900 times higher (g/g) than the ascorbic acid content in blueberries (which are themselves considered a good source of antioxidants). The medicinal benefits of the fruit were well known by the first Australians who considered the plum both a medicine and a nutritious food source. It has been proposed that many of the health benefits of the fruit may stem from the growth inhibitory activity of its many antimicrobial compounds.[10] These include flavonoids, benzoic acid derivatives, as well as gallic acid and ellagic acid derivatives, all of which have been previously associated with microbial inhibition.[11] Indeed, the antiseptic potential of the fruit is well documented in the prevention of several disease-causing microorganisms.[12-15] Recently, *T. ferdinandiana* leaf extracts were also shown to have antibacterial activity.[16] Potent growth inhibitory activity was reported against the bacterial triggers of several auto-immune inflammatory diseases including rheumatoid arthritis

(*Proteus mirabilis*), ankylosing spondylitis (*Klebsiella pneumoniae*) and multiple sclerosis (*Acinetobacter baylyi* and *Pseudomonas aeruginosa*).[16] The study indicated that the inhibition of the bacterial growth by the leaf extracts may be due to their high tannin content. Despite these studies and the extremely high antioxidant capacity of *T. ferdinandiana*, it is yet to be evaluated for the ability to inhibit the growth of the food poisoning bacterium *Y. enterocolitica*.

MATERIALS AND METHODS

Plant source and extraction

T. ferdinandiana fruit pulp and leaves were provided and verified courtesy of David Boehme of Wild Harvest, Northern Territory (Australia). The pulp was frozen prior to transport and stored at-10°C until processing. A voucher specimen of the pulp (KP2014GD) is maintained at School of Natural Sciences, Griffith University. The pulp and leaves were extensively dehydrated in a Sunbeam food dehydrator and the desiccated material was stored at-30°C. A voucher specimen (KP2015LA) is stored at the School of Natural Sciences, Griffith University. The plant materials were comprehensively dried and ground into a coarse powder prior to use. A mass of 1 g of ground powder was extensively extracted individually by maceration in 50 mL of deionised water, methanol, hexane, ethyl acetate orchloroform for 24 h at 4°C with gentle shaking. All solvents were supplied by Ajax, Australia and were 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 subsequent pellets were dissolved in 10 mL deionised water (containing 0.5 % DMSO). The extract was passed through a 0.22 µm filter (Sarstedt) and stored at 4°C until used.

Qualitative phytochemical studies

Phytochemical analysis of the extracts for the presence of tannins, triterpenoids, saponins, flavonoids, phytosteroids, phenolic compounds, anthraquinones, cardiac glycosides and alkaloids were conducted by previously described assays.[17-19]

Antioxidant capacity

The antioxidant capacity of each sample was assessed using a modified DPPH free radical scavenging method.[20-22] Ascorbic acid (0-25 µg per well) was used as a reference and the absorbances were measured and recorded at 515 nm. All tests were performed in parallel with 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

Clinical *Yersinia enterocolitica* strain

The clinical isolate strain of *Yersinia enterocolitica* used in this study was supplied by Ms. Jane Gifkins of the School of Natural Sciences Griffith University, Australia. All growth studies were performed using nutrient agar (Oxoid Ltd., Australia) under aerobic conditions. Incubation was at 30°C and the stock culture was subcultured and maintained in nutrient broth at 4°C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay.[23-25] Briefly, 100 µL of *Y. enterocolitica* was grown in 10 mL of fresh nutrient broth 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 infused with 10 µL of *T. ferdinandiana* extracts, 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 chloramphenicol (10 µg) were obtained from Oxoid, Australia and were used as positive controls to compare antibacterial activity. Filter discs infused 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.[26] Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were infused with 10 µL of the extract dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted. Linear regression was used to determine MIC values.

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 *Artemia franciscana* nauplii lethality assay.[27-29] Briefly, 400 µL of seawater containing ~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. Volumes of 400 µL of the reference toxin or the diluted plant extracts were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). 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. Mortality was determined if no movement of the appendages was detected within 10 sec. 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 analysis

Separations were performed with a Shimadzu GC-2010 Plus (USA) linked to a Shimadzu MS TQ8040 (USA) mass spectrometer. The system was equipped with a Shimadzu auto-sampler AOC-5000 Plus (USA). Chromatographic separation was accomplished using a (5% phenyl, 95% dimethyl polysiloxane, 30 m×0.25×0.25 um) capillary column, obtained from (Restek USA). Helium (99.999%) was employed as a carrier gas at a flow rate of 0.75 mL/min at a split ratio of 21:1. The injector temperature was set at 280°C.

The initial column temperature was held at 40°C for 5 min, with an initial ramp at 5°C/min to acquire a temperature of 140°C which was subsequently maintained for a further minute. A second ramp was initiated at 20°C per minute, to attain a column temperature of 260°C, and held for a minute. The third ramp of 50°C/min was undertaken to reach a final temperature of 300°C, which was maintained for a duration of 5 min. The mass spectrometer was operated in the electron ionisation mode at 70eV. The analytes were then recorded in total ion count (TIC) mode. The TIC was acquired after a min and for a duration of 38.8 min utilising a mass range of 26-500 m/z.

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant capacities of the *T. ferdinandiana* 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	Combined Anthraquinones
KPLW	471	47.1	340	+++	+++	+++	++	+++	++	–	–	–	++	+++	+	+
KPLM	331	33.1	150	+++	+++	+++	+++	++	+	–	+	+	++	+++	+	+
KPLC	59	5.9	5	+	–	–	–	–	–	–	–	–	–	–	–	–
KPLH	58	5.8	0.4	+	–	–	–	–	–	–	–	–	++	+	–	–
KPLE	59	5.9	22	+++	+++	+++	–	–	–	–	–	–	++	++	–	–
KPFW	483	48.3	264	+++	+++	+++	–	+	–	–	–	–	+++	++	–	–
KPFM	359	35.9	660	+++	+++	+++	–	++	+	–	+	+	+++	++	–	–
KPFC	62	6.2	7	+	–	–	–	–	–	–	–	–	–	–	–	–
KPFH	18	1.8	1	–	–	–	–	–	–	–	–	–	–	–	–	–
KPFE	30	3	39	++	++	+	–	+	++	–	–	–	++	–	–	–

+ indicates a minor response; ++ indicates a moderate response; +++ indicates a major response.– indicates no response in the assay. **KPLW**=aqueous *T. ferdinandiana* leaf extract; **KPLM**=methanolic *T. ferdinandiana* leaf extract; **KPLC**=chloroform *T. ferdinandiana* leaf extract; **KPLH**=hexane *T. ferdinandiana* leaf extract; **KPLE**=ethyl acetate *T. ferdinandiana* leaf extract; **KPFW**=aqueous *T. ferdinandiana* fruit extract; **KPFM**=methanolic *T. ferdinandiana* fruit extract; **KPFC**=chloroform *T. ferdinandiana* fruit extract; **KPFH**=hexane *T. ferdinandiana* fruit extract; **KPFE**=ethyl acetate *T. ferdinandiana* fruit extract. Antioxidant capacity was determined by DPPH reduction and is expressed as mg ascorbic acid equivalents per gof original plant material extracted.

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

Extract	MIC	LC_{50}
KPLW	588	1,330
KPLM	372	1,133
KPLC	5900	-
KPLH	5800	-
KPLE	1100	767
KPFW	-	2,080
KPFM	123	2,115
KPFC	6200	-
KPFH	-	-
KPFE	285	-
PD	ND	186
SW	ND	-

Numbers indicate the mean MIC and LC_{50} values of triplicate determinations. - indicates no inhibition. **KPLW**=aqueous *T. ferdinandiana* leaf extract; **KPLM**=methanolic *T. ferdinandiana* leaf extract; **KPLC**=chloroform *T. ferdinandiana* leaf extract; **KPLH**=hexane *T. ferdinandiana* leaf extract; **KPLE**=ethyl acetate *T. ferdinandiana* leaf extract; **KPFW**=aqueous *T. ferdinandiana* fruit extract; **KPFM**=methanolic *T. ferdinandiana* fruit extract; **KPFC**=chloroform *T. ferdinandiana* fruit extract; **KPFH**=hexane *T. ferdinandiana* fruit extract; **KPFE**=ethyl acetate *T. ferdinandiana* fruit extract; **PD**=potassium dichromate control; **SW**=artificial seawater negative control; ND=the indicated test was not performed.

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

T. ferdinandiana fruit methanolic extract				
Molecular Mass	**Molecular Formula**	**Putative Identification**	**Retention Time**	**Area%**
110	$C_5H_6N_2O$	1-(3H-Imidazol-4-yl)-ethanone	10.216	30.22
118	$C_6H_{14}O_2$	3-Methoxy-3-methylbutanol	10.63	0.04
132	$C_5H_8O_4$	Propanedioic acid, dimethyl ester	10.859	0.79
128	$C_8H_{16}O$	4-methyl-2-heptanone	11.191	1.26
106	C_7H_6O	Benzaldehyde	11.755	3.23
126	$C_6H_6O_3$	Methyl 2-furoate	12.325	10.07
128	$C_8H_{16}O$	Octanal	13.24	0.26
278	$C_{14}H_{30}O_3S$	Sulfurous acid, 2-ethylhexyl hexyl ester	13.705	0.15
142	$C_{10}H22$	3,3,5-Trimethylheptane	13.92	0.59
130	$C_8H_{18}O$	2-Ethylhexanol	14.105	4.81
146	$C_6H_{10}O4$	Butanedioic acid, dimethyl ester	14.205	3.4
120	C_8H_8O	Benzeneacetaldehyde	14.425	0.44
298	$C_{19}H_{38}O_2$	Oxirane, [(hexadecyloxy)methyl]-	14.85	0.06
168	$C_{12}H_{24}$	4-methyl-1-undecene	15.08	0.58
160	$C_7H_{12}O_4$	Butanedioic acid, methyl-, dimethyl ester	15.265	0.54
170	$C_{10}H_{18}O_2$	cis-Linalool oxide	15.475	3.41
120	C_8H_8O	Bicyclo[4.2.0]octa-1,3,5-trien-7-ol	15.62	0.25
126	$C_6H_6O_3$	Furylhydroxymethyl ketone	15.685	1.1
170	$C_{10}H_{18}O_2$	trans-Linalool oxide	15.96	2.1
136	$C_7H_8N_2O$	Benzhydrazide	16.08	0.53
142	$C_9H_{18}O$	Nonanal	16.45	0.35
162	$C_6H_{10}O_5$	Dimethyl dl-malate	17.09	0.43
160	$C_7H_{12}O_4$	Pentanedioic acid, dimethyl ester	17.445	0.2
110	$C_6H_6O_2$	Ethanone, 1-(2-furanyl)-	17.485	0.22
152	$C_{10}H_{16}O_2$	Camphor	17.645	0.04
111	$C_4H_5N_3O$	2-(Cyanoimino)oxazolidine	17.89	4.19
154	$C_{10}H_{18}O$	endo-Borneol	18.32	1.3
144	$C_8H_{16}O_2$	Octanoic acid	18.56	0.39
150	$C_9H_{10}O_2$	Benzeneacetic acid, methyl ester	18.61	0.27
158	$C_9H_{18}O_2$	(E)-1-(1-methoxyethoxy)-3-hexene	18.925	0.05
184	$C_{13}H_{28}$	5-(2-methylpropyl)-nonane	19.39	0.18
134	$C_9H_{10}O$	2-methyl-2-phenyl-oxirane	19.7	3.4
186	$C_{10}H_{18}O_3$	2,2-Dimethylpropanoic anhydride	19.86	0.12
126	$C_6H_6O_3$	5-Hydroxymethylfurfural	19.945	0.42
184	$C_{13}H_{28}$	4-methyl-dodecane	20.035	0.06
268	$C_{19}H_{40}$	5-methyl-octadecane	20.675	0.05
140	$C_9H_{16}O$	2,3,4,5-Tetramethylcyclopent-2-en-1-ol	20.78	0.16
190	$C_{14}H_{22}O$	Benzene, 1,3-bis(1,1-dimethylethyl)-	20.93	2.56
158	$C_9H_{18}O_2$	Nonanoic acid	21.31	1.02
168	$C_{12}H_{24}$	4-methyl-1-undecene	21.705	0.23
188	$C_{10}H_{20}O_3$	Butyl 2-butoxyacetate	21.84	0.31
114	C_8H_{18}	3,3-dimethyl-hexane	22.96	0.04
180	$C_{13}H_{24}$	1,1,6,6-Tetramethylspiro[4.4]nonane	23.35	0.36
286	$C_{16}H_{30}O_4$	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	23.615	0.12

Table 3: Continued

172	$C_{10}H_{20}O_2$	n-Decanoic acid	23.87	0.03
216	$C_{12}H_{24}O_3$	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	24.17	0.17
226	$C_{14}H_{26}O_2$	2,4,7,9-Tetramethyl-5-decyn-4,7-diol	25.39	0.12
150	$C_{11}H_{18}$	Bicyclo[2.2.1]heptane, 2-(1-buten-3-yl)-	25.49	0.06
201	$C_{10}H_{18}O_4$	Suberic acid, dimethyl ester	26.29	0.03
186	$C_{12}H_{26}O$	1-Dodecanol	27.415	0.12
206	$C_{14}H_{22}O$	3,5-bis(1,1-dimethylethyl)-Phenol	28.86	8.2
166	$C_9H_{10}O_3$	Ethyl 3-hydroxybenzoate	29.375	0.06
216	$C_{11}H_{20}O_4$	Nonanedioic acid, dimethyl ester	30.22	0.02
286	$C_{16}H_{30}O_4$	2,2,4-Trimethyl-1,3-pentanediol diisobuty	31.77	0.18
368	$C_{24}H_{48}O_2$	Heneicosanoic acid, isopropyl ester	35.92	0.02
278	$C_{16}H_{22}O_4$	Phthalic acid, diisobutylo ester	36.475	0.03
278	$C_{16}H_{22}O_4$	2-(1-oxopropyl)-benzoic acid	37.62	0.03

The relative abundance 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 methanolic *T. ferdinandiana* leaf extract, elucidation of empirical formulas and putative identification of each compound

T. ferdinandiana leaf methanolic extract				
Molecular Mass	**Molecular Formula**	**Putative Identification**	**Retention Time**	**Area%**
151	$C_8H_9NO_2$	Methyoxy-phenyl-oxime	11.266	19.7
128	$C_8H_{16}O$	1-Octen-3-ol	13.727	6.35
118	$C_6H_{14}O_2$	Ethanol, 2-(1,1-dimethylethoxy)-	14.505	1.26
130	$C_8H_{18}O$	1-Hexanol, 2-ethyl-	15.373	0.59
154	$C_{10}H_{18}O$	Cineole	15.499	0.97
242	$C_{13}H_{22}O_4$	Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2 (linalool oxide)	16.879	0.56
142	$C_9H_{18}O$	Nonanal	17.873	1.11
150	$C_{10}H_{14}O$	2-Isopropylidene-3-methylhexa-3,5-dienal	21.031	0.25
156	$C_{10}H_{20}O$	Decanal	21.11	0.14
134	$C_9H_{10}O$	Benzaldehyde, 3,5-dimethyl-	21.527	8.21
216	$C_{12}H_{24}O_3$	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4	26.499	2.5
206	C14 H22 O	2,4-Di-tert-butylphenol	31.641	0.54
194	$C_{11}H_{14}O_3$	Ethyl para-ethoxybenzoate	32.054	3.16
286	$C_{16}H_{30}O_4$	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	33.822	0.4

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

Statistical analysis

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

RESULTS

Liquid extraction yields and qualitative phytochemical screening

T. ferdinandiana solvent extractions yielded dried plant extracts ranging from 58 mg to 471 mg (leaf extracts) and from 18 mg to 483 mg (fruit extracts) (Table 1). Methanolic and aqueous extracts provided considerably greater yields of extracted material relative to the ethyl acetate, chloroform and hexane counterparts, which gave low to moderate yields. The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the concentrations presented in Table 1.

Antioxidant content

Antioxidant capacity for the plant extracts (Table 1) ranged from 0.4 mg (hexane leaf extract) to a high of 660 mg (methanolic fruit extract) ascorbic acid equivalence per gram of dried plant material extracted. The methanolic and aqueous extracts typically had higher antioxidant capacities than the corresponding ethyl acetate, hexane and chloroform extracts.

Antimicrobial activity

To determine the ability of the *T. ferdinandiana* fruit and leaf extracts to inhibit *Y. enterocolitica* growth, 10 µL of each extract was screened using a disc diffusion assay. Bacterial growth was strongly inhibited by 8 of the 10 extracts screened (80%) (Figure 1). The aqueous and methanolic leaf extracts were the most potent inhibitors of growth (as judged by zone of inhibition), with inhibition zones of 18 ± 0.6 and 17.3 ± 1.5 mm respectively. This compares positively with the chloramphenicol control (10 µg;

inhibition zone=9 ± 0.6 mm). The methanolic fruit extract also displayed good inhibition of *Y. enterocolitica* growth, with a zone of inhibition ≥ 15 mm. Typically, the leaf extracts were more potent inhibitors of *Y. enterocolitica* growth than the corresponding fruit extracts (as judged by inhibition zone).

The antimicrobial efficacy was further quantified through the determination of MIC values against the *T. ferdinandiana* extracts (Table 2). Most of the extracts were effective at inhibiting *Y. enterocolitica* growth with MIC values <1000 µg/mL determined for several of the extracts (<10 µg impregnated in the disc). The methanolic extracts of both the leaf and fruit were particularly potent, with MIC values of 372 µg/mL (approximately 3.7 µg infused into the disc) and 123 µg/mL(approximately 1.2 µg infused into the disc) respectively. These results compare well with the growth inhibitory activity of the chloramphenicol controls which were tested at 10 µgper disc. The aqueous and ethyl acetate leaf extracts, as well as the fruit ethyl acetate extract, were also potent *Y. enterocolitica* growth inhibitors (MIC values of 588, 1100 and 285 µg/mL respectively). Whilst less potent, the leaf chloroform (MIC=5900 µg/mL) and hexane extracts (MIC=5800 µg/mL), as well as the fruit chloroform extract (MIC=6200 µg/mL), also inhibited *Y. enterocolitica* growth. Their MIC values indicate moderate to low inhibitory activity.

Quantification of toxicity

All extracts were initially screened in the assay at 2000 µg/mL (Figure 2). As a reference toxin, potassium dichromate was also tested in the bioassay. The reference toxin was prompt in its onset of mortality, inducing nauplii death within the first 3 h of exposure and 100 % mortality was evident within 4-5 h (unpublished results). All methanolic, aqueous extracts and ethyl acetate leaf extracts showed > 90 % mortality rates at 24 h. All other extracts exhibited< 10% mortality rates at 24 h with the exception of the chloroform leaf extract.

To further assess the effects of toxin concentration on the initiation of mortality, the extracts were serially diluted in artificial seawater to test across a series of concentrations in the *Artemia* nauplii bioassay. The LC_{50} values of the *T. ferdinandiana* extracts towards *A. franciscana* following 24 h exposure are displayed in Table 2. No LC_{50} values are reported for the chloroform or hexane extracts, nor for the ethyl acetate fruit extract as < 50 % mortality was seen in all tested concentrations. Extracts with an LC_{50} greater than 1000 µg/mL towards *Artemia* nauplii have previously been defined as nontoxic in this assay.[30] As only the ethyl acetate fruit extract had an LC_{50} value of < 1000 µg/ml, all other extracts were considered nontoxic. Whilst the LC_{50} value for the ethyl acetate leaf extract is < 1000 µg/mL, a value of 767 µg/mL is representative of low to moderate toxicity.

GC-MS head space analysis

As the *T. ferdinandiana* leaf and fruit methanolic extracts were the most potent inhibitor of *Y. enterocolitica* (as judged by MIC), they were considered the most promising extracts for further phytochemical analysis. Optimised GC-MS parameters were developed and used to examine these extracts. Numerous overlapping peaks were evident in the methanolic fruit extract chromatogram (Figure 3). A total of 57 peaks were detected and putatively identified in that extract, with prominent peaks eluting at 10.2 min (1-(3H-imidazol-4-yl)-ethanone), 12.3 min (methyl-2-furoate), 14.1 min (2-ethylhexanol), 15.5 min (cis-linalool oxide), 16.0 min (trans-linalool oxide), 17.9 min (2-(cyanoimino) oxazolidine, 19.7 min (2-methyl-2-phenyl-oxirane), 20.9 min (1,3-bis(1,1-dimethylethyl)-benzene) and 28.9 min (3,5-bis(1,1-dimethylethyl)-phenol). Numerous overlapping peaks were also evident throughout the chromatogram, with a broad range of retention times, particularly between 10 and 25 min, attesting to the wide range of compounds of widely varying polarities in the methanolic fruit extract.

The *T. ferdinandiana* methanolic leaf extract GC-MS chromatogram (Figure 4) was substantially less complex than the corresponding fruit extract chromatogram. Indeed, only 15 peaks were detected and putatively identified in this chromatogram. Major peaks were evident at 11.3 min (methoxy-phenyl-oxime), 13.8 min (1-octen-3-ol), 14.4 min (2-(1,1-dimethylethoxy)-ethanol, 21.5 min (3,5-dimethyl-benzaldehyde) and 26.5 min (2-methyl-3-hydroxy-2,2,4-propanoic acid). Further prominent peaks were present at 17.0, 18.2, 19.6 and 24.8 min. However, the compound database used in our studies was unable to putatively identify these compounds. Table 4 lists only the compounds which were putatively identified by comparison with the compound database.

DISCUSSION

Many *Terminalia* spp. have a history of therapeutic usage to treat microbial infections and numerous recent investigations have reported on their antibacterial properties.[31] The Australian species *T. ferdinandiana* has proven to be particularly effective, with growth inhibitory activity reported against a broad panel of bacterial pathogens,[32] including several bacteria associated with diarrhoea and dysentery.[32,33] *T. ferdinandiana* extracts also inhibit the growth of bacterial triggers of rheumatoid arthritis[14,16] and multiple sclerosis.[13] Furthermore, *T. ferdinandiana* has also recently been reported to inhibit the proliferation of the gastrointestinal protozoan parasite *Giardia duodenalis,*[11] indicating its therapeutic potential against both prokaryotic and eukaryotic pathogens. However, despite the wealth of information into the therapeutic potential of *T. ferdinandiana*, it is yet to be comprehensively studied for antibacterial activity against many bacterial species associated with food poisoning. Here we report, growth inhibitory activity for *T. ferdinandiana* extracts against the bacterial cause of the gastrointestinal disease yersiniosis, an acute infection acquired through the ingestion of food (particularly pork)[34] contaminated with *Y. enterocolitica* bacteria.

Potent *Y. enterocolitica* growth inhibitory activity was evident in the *T. ferdinandiana* fruit and leaf extracts. The methanolic extracts were the most potent growth inhibitors, with MIC values of 123 and 372 µg/mL for the fruit and leaf extracts respectively. Qualitative GC-MS analysis of these extracts identified a number of interesting compounds which may contribute to this activity. The presence of the furan compounds methyl 2-furoate (Figure 5c), linalool oxide (Figure 5e), furylhydroxymethyl ketone (Figure 5g),1-(2-furanyl)-ethanone (Figure 5i) 5-hydroxymethylfurfural (Figure 5o) in the methanolic *T. ferdinandiana* extracts is particularly noteworthy 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.[35] Thus, the furan compounds in this extract may have cytostatic *Y. enterocolitica* growth inhibitory mechanisms although this is yet to be investigated. Further studies reported synthetic furan derivatives (modified by the addition of a rhodanine moiety) to be potent inhibitors of the growth of a panel of multidrug resistant bacteria, with MIC values as low as 2 µg/mL against some species.[36] Whilst we were unable to find reports of anti-bacterial activity for the furan derivatives present in the *T. ferdinandiana* extracts, it is possible that they may contribute to the growth inhibitory activities reported in our study.

Also noteworthy, an imidazole compound,1-(3H-imidazol-4-yl)-ethanone (Figure 5a), was putatively identified in the methanolic *T. ferdinandiana* fruit extract. Several imidazo-compounds (e.g. metronidazole, azanidazole tinidazole) have notable antibiotic activity.[37] Interestingly, similar imidazole compounds also have inhibitory activity against eukaryotic parasites.[40] Indeed, anti-giardial activity has been reported for *T. ferdinandiana* fruit extract.[11] A variety of benzene and benzoic acid derivatives including benzaldehyde (Figure 5b), benzene acetaldehyde (Figure 5d), bicyclo[4.2.0]octa-1,3,5-trien-7-ol (Figure 5f), benzhydrazide

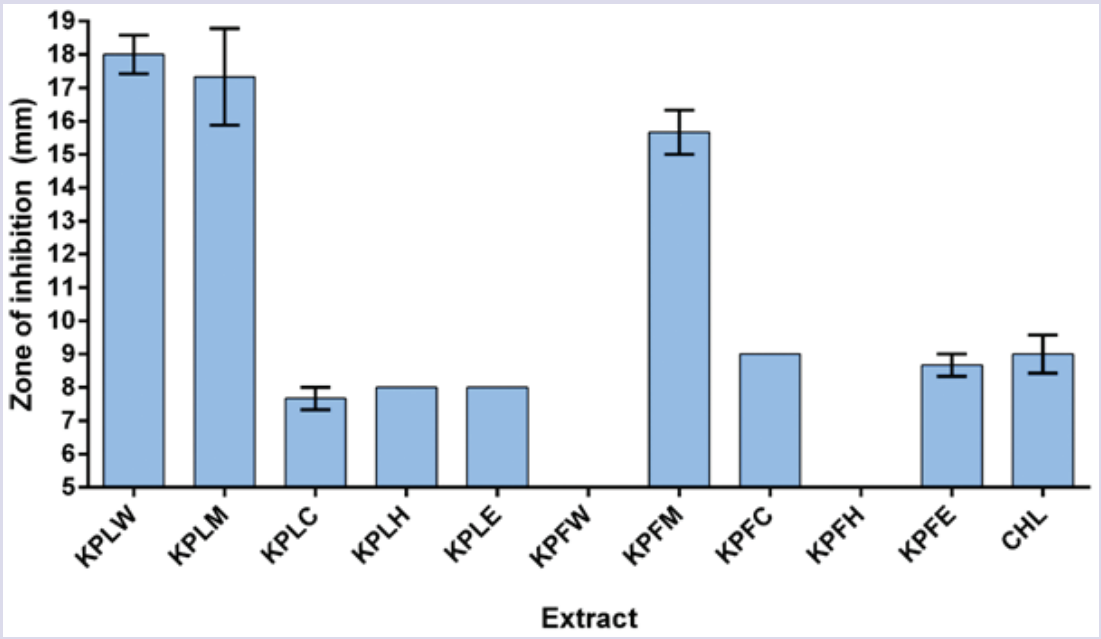

Figure 1: Growth inhibitory activity of *T. ferdinandiana*plant extracts against the *Y. enterocolitica* environmental isolate measured as zones of inhibition (mm). **KPLW**=aqueous *T. ferdinandiana* leaf extract; **KPLM**=methanolic *T. ferdinandiana* leaf extract; **KPLC**=chloroform *T. ferdinandiana* leaf extract; **KPLH**=hexane *T. ferdinandiana* leaf extract; **KPLE**=ethyl acetate *T. ferdinandiana* leaf extract; **KPFW**=aqueous *T. ferdinandiana* fruit extract; **KPFM**=methanolic *T. ferdinandiana* fruit extract; **KPFC**=chloroform *T. ferdinandiana* fruit extract; **KPFH**=hexane *T. ferdinandiana* fruit extract; **KPFE**=ethyl acetate *T. ferdinandiana* fruit extract; **CHL**=chloramphenicol (10 µg). Results are expressed as mean zones of inhibition ± SEM.

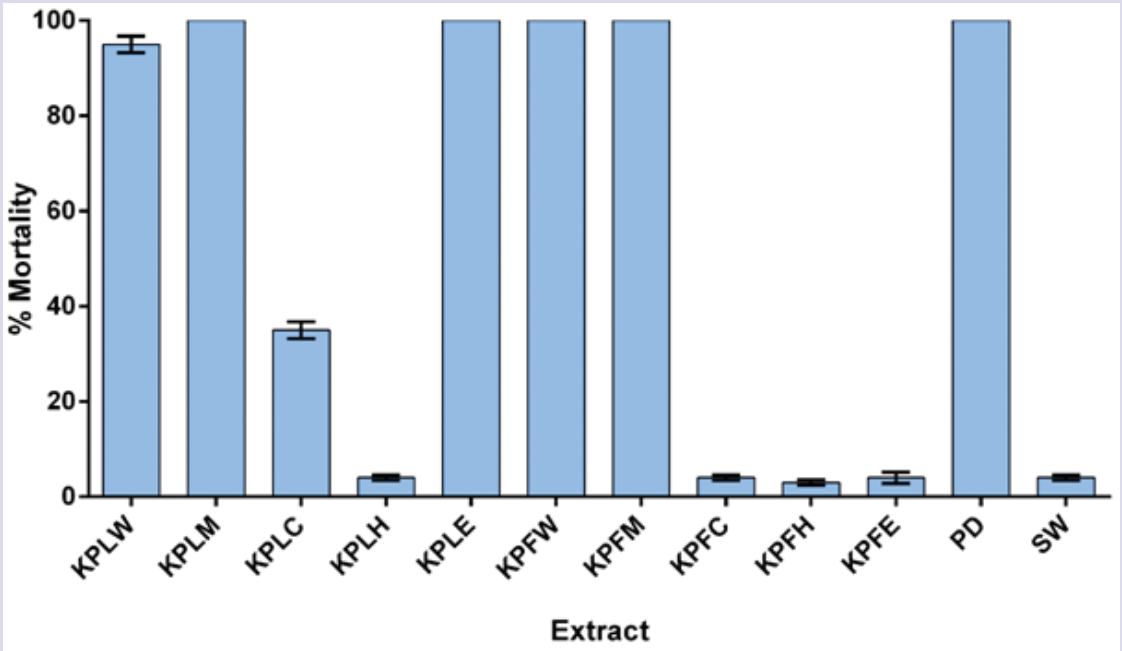

Figure 2: The lethality of the *T. ferdinandiana* extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *Artemia franciscana* nauplii after 24 h exposure. **KPLW**=aqueous *T. ferdinandiana* leaf extract; **KPLM**=methanolic *T. ferdinandiana* leaf extract; **KPLC**=chloroform *T. ferdinandiana* leaf extract; **KPLH**=hexane *T. ferdinandiana* leaf extract; **KPLE**=ethyl acetate *T. ferdinandiana* leaf extract; **KPFW**=aqueous *T. ferdinandiana* fruit extract; **KPFM**=methanolic *T. ferdinandiana* fruit extract; **KPFC**=chloroform *T. ferdinandiana* fruit extract; **KPFH**=hexane *T. ferdinandiana* fruit extract; **KPFE**=ethyl acetate *T. ferdinandiana* fruit extract; **PD**=potassium dichromate control; **SW**=seawater control. Results are expressed as mean % mortality ± SEM.

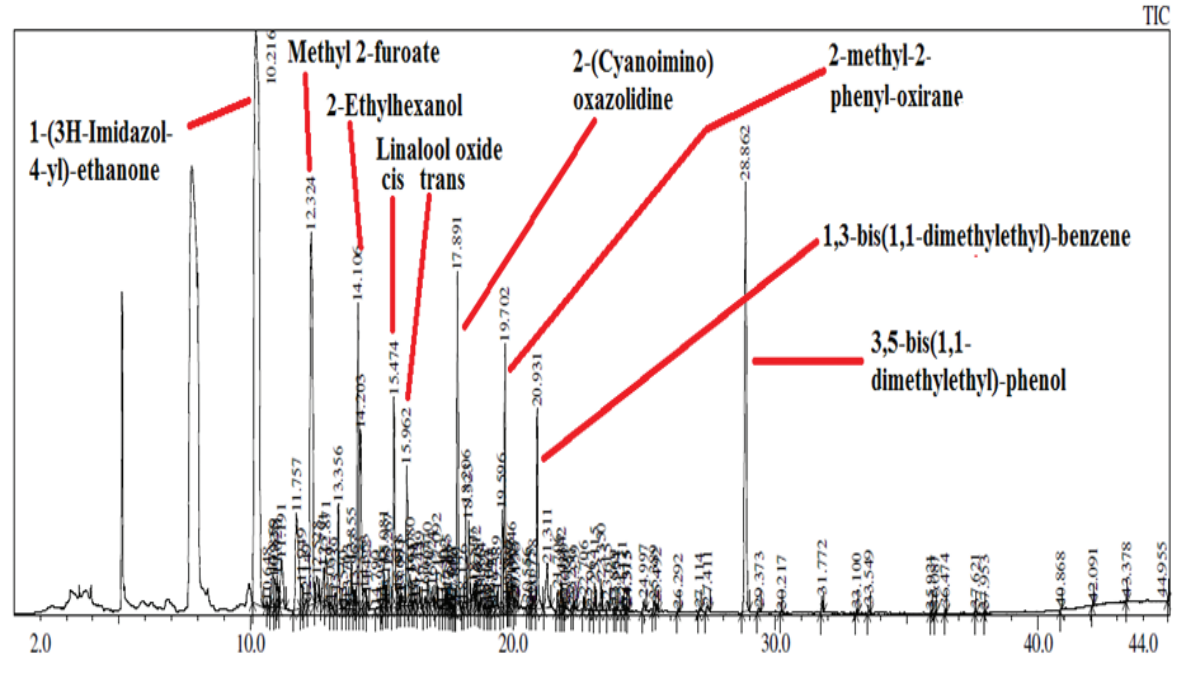

Figure 3: Gas chromatogram of a 0.5 µL injection of *T. ferdinandiana* methanolic fruit extract. The extract was dried and resuspended in methanol for analysis.

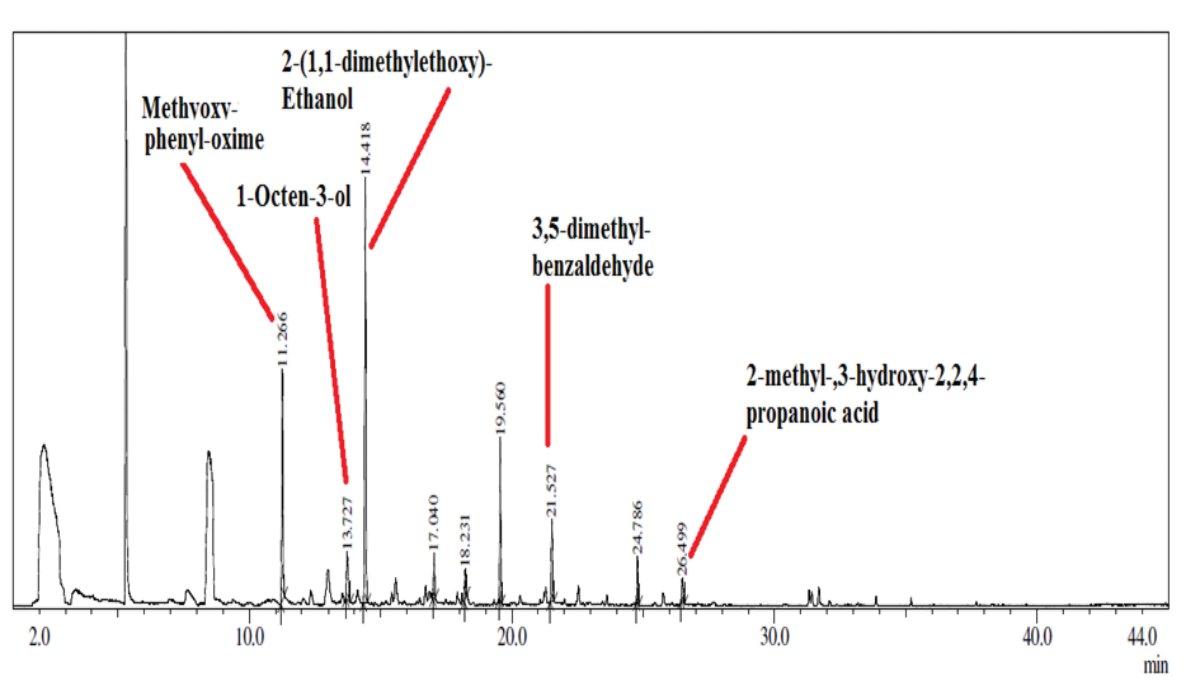

Figure 4: Gas chromatogram of a 0.5 µL injection of *T. ferdinandiana* methanolic leaf extract. The extract was dried and resuspended in methanol for analysis.

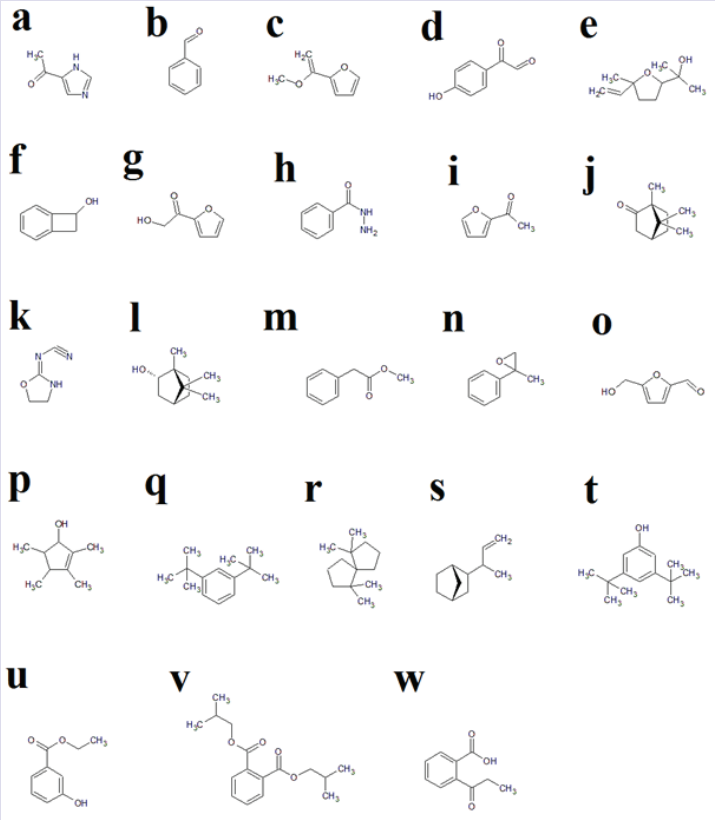

Figure 5: Chemical structures of *T. ferdinandiana* fruit methanolic extract compounds: (a) 1-(3H-imidazol-4-yl)-ethanone; (b) benzaldehyde; (c) methyl 2-furoate; (d) benzeneacetaldehyde; (e) linalool oxide; (f) bicyclo[4.2.0]octa-1,3,5-trien-7-ol; (g) furylhydroxymethyl ketone; (h) benzhydrazide; (i) 1-(2-furanyl)-ethanone; (j) camphor; (k) 2-(cyanoimino) oxazolidine; (l) borneal; (m) benzeneacetic acid, methyl ester; (n) 2-methyl-2-phenyl-oxirane; (o) 5-hydroxymethylfurfural; (p) 2,3,4,5-tetramethylcyclopent-2-en-1-ol; (q) 1,3-bis(1,1-dimethylethyl)-benzene; (r) 1,1,6,6-tetramethylspiro[4.4]nonane; (s) 2-(1-buten-3-yl)-bicyclo[2.2.1] heptane; (t) 3,5-bis(1,1-dimethylethyl)-phenol; (u) ethyl 3-hydroxybenzoate; (v) phthalic acid, diisobutylo ester; (w) 2-(1-oxopropyl)-benzoic acid.

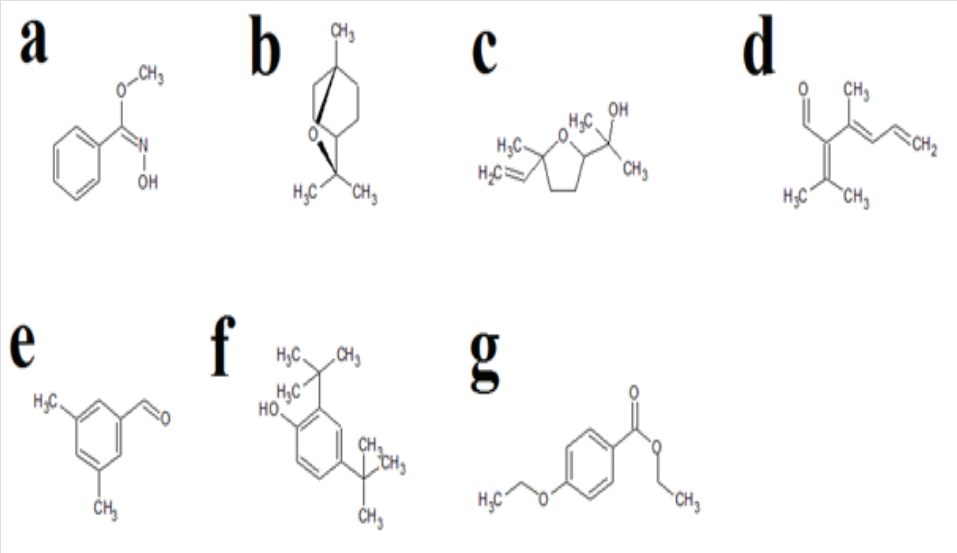

Figure 6: Chemical structures of *T. ferdinandiana* leaf methanolic extract compounds: (a) methyoxy-phenyl-oxime; (b) cineole; (c) linalool oxide; (d) 2-isopropyl-idene-3-methylhexa-3,5-dienal; (e) 3,5-dimethyl-benzaldehyde; (f) 2,4-di-tert-butylphenol; (g) ethyl p-ethoxybenzoate.

(Figure 5h), benzeneacetic acid, methyl ester (Figure 5m), 2-methyl-2-phenyl-oxirane (Figure 5n), 1,3-bis(1,1-dimethylethyl)-benzene (Figure 5q); 3,5-bis(1,1-dimethylethyl)-phenol (Figure 5t), ethyl 3-hydroxybenzoate (Figure 5u), phthalic acid, diisobutylo ester (Figure 5v) and 2-(1-oxopropyl)-benzoic acid (Figure 5w) were also detected in the methanolic *T. ferdinandiana* fruit extract. Similarly, methyoxy-phenyl-oxime (Figure 6a), 3,5-dimethyl-benzaldehyde (Figure 6e),2,4-di-tert-butylphenol (Figure 6f) and ethyl p-ethoxybenzoate (Figure 6g) were putatively identified in the leaf methanolic extract. Several studies have previously reported potent antibacterial activity for phenolic benzaldehyde and benzoic acid derivatives.[38] Thus, these compounds may also contribute to the *Y. enterocolitica* growth inhibitory activity reported in our study.

Several monoterpenoids were also putatively identified in the methanolic *T. ferdinandiana* extracts, with cis- and trans-linalool oxide (Figure 5e), camphor (Figure 5j) and borneol (Figure 5l) identified in the fruit extract. Linalool oxide was also present in the leaf methanolic extract, together with cineole (Figure 6b). Several other important terpenoids have also been reported in *T. ferdinandiana* extracts in recent studies using different analytical techniques.[13] Monoterpenoids were particularly prevalent in those studies, with isomyocorene, cineole, cuminol, camphor andisomenthol reported in *T. ferdinandiana* fruit extracts. Many of these terpenoids have potent broad spectrum antibacterial activity[39,42] and therefore may contribute to the *Y. enterocolitica* growth inhibitory activity.

It is also likely that other phytochemical classes 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 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[11,14] and leaf extracts.[16] Several features were common to all of these studies. All reported a diversity of tannins in both the fruit and leaf extracts. Gallic acid, ellagic acid and their methylated derivatives, chebulic acid, galloyl pyrogallol, corilagen, punicalin, castalagin and chebulagic acid were previously reported in similar *T. ferdinandiana* extracts to those tested in our study. These tannins have potent, broad spectrum growth inhibitory activity against a variety of bacterial species.[40] Gallotannins have particularly well reported inhibitory properties.[41] They function via multiple mechanisms including interacting with both cell surface proteins[43,44] and through interactions with intracellular enzymes.[45] Ellagitatannins also interact with cellular proteins and induce disruptions in bacterial cell walls.[41,43]

Recent studies also highlighted the stilbene components of *T. ferdinandiana* fruit extracts.[13,14] Resveratrol and the glycosylated resveratrol derivative piceid, diethylstilbestrol monosulfate and combretastatin A1 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.[42] 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.[43] Therefore, it is likely that the *T. ferdinandiana* extract stilbene components may also contribute to the *Y. enterocolitica* growth inhibition noted in our study.

The findings reported here also indicate that the *T. ferdinandiana* fruit and leaf extracts were generally nontoxic, with LC_{50} values substantially >1000 µg/mL in the *Artemia* nauplii bioassay. Indeed, only the *T. ferdinandiana* leaf ethyl acetate extract had an LC_{50} value<1000 µg/mL. Whilst this extract inhibited *Y. enterocolitica* growth, the MIC value of 1100 µg/mL is indicative of moderate growth and thus has lower therapeutic potential in the treatment of yersiniosis compared to the methanolic extracts. Furthermore, an LC_{50} value of 767 µg/mL indicate low to moderate toxicity. However, this toxicity may impact on the usefulness of the leaf ethyl acetate extract as a medicinal antiseptic agent. Similar extracts prepared from fruits of these species have previously been reported to have high ascorbic acid levels.[9] Whilst these ascorbic acid levels may have beneficial therapeutic effects, they may also be responsible (at least in part) for the toxicity reported here. Whilst *A. franciscana* have generally been reported to be a robust and hardy organism for toxicity screening, they are susceptible to pH changes.[30] The levels of ascorbic acid previously reported in the *T. ferdinandiana* leaf ethyl acetate-extract[13,14] would be expected to have a significant impact on the pH of the seawater in the tests and this change may be responsible for the mortality induction reported in our study. Indeed, studies in our laboratory have shown that testing pure ascorbic acid in the concentrations previously reported to be in these extracts results in mortality similar to that reported here (unpublished results).

Toxicity was assessed in this study with the test organism *A. franciscana*. The lack of toxicity of these extracts indicates that they are likely to be safe for medicinal usage. This is hardly surprising as *T. ferdinandiana* is a food which is reputed to be highly nutritious. However, whilst the extracts examined in this report have potential as *Y. enterocolitica* growth inhibitory agents, caution is needed before these compounds can be applied to medicinal purposes. Toxicity towards *A. franciscana* has previously been shown to correlate well with toxicity towards human cells for many toxins.[30] However, further studies are required to determine whether this is also true for the *T. ferdinandiana* extracts examined in these studies. The results of this study indicate that the *T. ferdinandiana* extracts examined in this study warrant further study due to their *Y. enterocolitica* growth inhibitory activity. Purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents.

CONCLUSION

The results of this study demonstrate the potential of *T. ferdinandiana* fruit and leaf extracts to inhibit *Y. enterocolitica* growth. The methanolic *T. ferdinandiana* leaf and fruit extracts, as well as the fruit ethyl acetate extract, were particularly potent growth inhibitors, with MIC values generally <350 µg/mL. However, before being acceptable for therapeutic uses, further cell line toxicity studies are required to verify the safety of these extracts. Furthermore, studies aimed at the purification and identification of the bioactive components are required to examine the mechanisms of action of these extracts.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS USED

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

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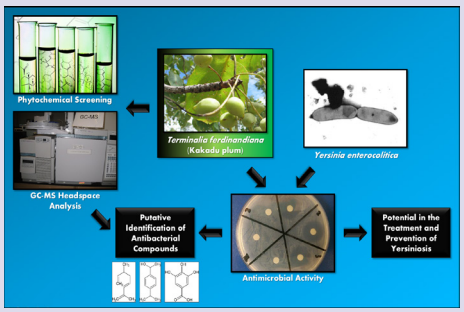
PICTORIAL ABSTRACT



SUMMARY

- *T. ferdinandiana* fruit and leaf extracts were potent inhibitors of *Yersinia enterocolitica* growth.
- Methanolic leaf and fruit extracts were particularly potent, with MIC's of 372 and 123 µg/mL respectively.
- The ethyl acetate fruit extract was also a potent growth inhibitor (MIC 285 µg/mL).
- All inhibitory extracts were either non-toxic or of low toxicity in the *Artemia* nauplii assay.
- GC-MS analysis identified several compounds that may contribute to the inhibitory activity.

ABOUT AUTHORS

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 pharmacognosy. His present research interests are the use of bacteriogenic 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.

Megan Arnold: Is currently undertaking her PhD in Tropical Parasitology at Griffith University's Eskitis Institute for Drug Discovery with a focus on the identification and development of novel chemoprophylactic agents for malaria. Her other research interests include investigating Australian high antioxidant plants for their antibacterial capabilities.

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

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