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Research Article

GC-MS and LC-MS analysis of Kakadu plum fruit extracts displaying inhibitory activity against microbial triggers of multiple sclerosis

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ABSTRACT

Introduction: Multiple sclerosis is an autoimmune disease which can be triggered in genetic susceptible individuals by Acinetobacter spp. and *Pseudomonas aeruginosa* infections. *Terminalia ferdinandiana* (Kakadu plum) fruit has documented therapeutic properties as a general antiseptic agent. Extracts prepared from the leaves have also been shown to block several microbial triggers of autoimmune inflammatory diseases. This study examines the ability of Kakadu plum fruit extracts to inhibit some microbial triggers of multiple sclerosis. **Methods:** *T. ferdinandiana* fruit solvent extracts were investigated by disc diffusion assay against reference and clinical strains of *A.baylyi* and *P. aeruginosa*. Their MIC values were determined to quantify and compare their efficacies. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. Active extracts were analysed by non-targeted HPLC-QTOF mass spectroscopy (with screening against 3 compound databases) and by GC-MS (with screening against 1 compound databases) for the identification and characterisation of individual components in crude plant extracts. **Results:** Methanolic, aqueous and ethyl acetate *T. ferdinandiana* leaf extracts displayed potent antibacterial activity in the disc diffusion assay against the bacterial triggers of multiple sclerosis (*A.baylyi* and *P. aeruginosa*). The methanol and ethyl acetate extracts had the most potent growth inhibitory activity, with MIC values less than 1000 μg/ml against *A. baylyi* and *P. aeruginosa* (both reference and clinical strains). In comparison, the water extract was substantially less potent. Neither the chloroform nor hexane extracts inhibited the growth of any of the bacterial strains tested. All *T. ferdinandiana* fruit extracts were nontoxic in the *Artemia fransiscana* bioassay. Non-biased phytochemical analysis of the ethyl acetate extract revealed only low levels of the tannins gallic acid and chebulic acid and no other tannins. **Conclusion:** The low toxicity of the *T. ferdinandiana* fruit extracts and their potent inhibitory bioactivity against the bacterial triggers of multiple sclerosis indicates their potential as medicinal agents in the treatment and prevention of this disease. Phytochemical studies indicate that this activity is likely to be due to phytochemicals other than tannins.

Key words: Antioxidant, Autoimmune inflammatory disease, Acinetobacter, Complementary and alternative therapies, Demyelinating disease, Kakadu plum, *Pseudomonas aeruginosa, Terminalia ferdinandiana*.

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune inflammatory disease which results in demyelination of central nervous system cells. MS afflicts approximately 0.03 % of the world's population, and is particularly prevalent in populations of Northern European descent where the incidence has been estimated to be as high as 0.2 % of the population.[1] Furthermore, MS has been proposed to have a latitudinal correlation, with higher prevalence's in far northern regions of the Northern Hemisphere and far south of the Southern Hemisphere than in tropical regions.[2] It was proposed in that study that the latitudinal

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Table 1: The bacterial triggers of multiple sclerosis as the bacterial antigen and host susceptibility antigen sequences.

Bacterial Trigger	Bacterial Antigen	Bacterial Sequence	Host Antigen	Host Sequence	References
Pseudomonas aeriginosa	ϒ-CMLD	TRHAYG	Myelin-neuronal antigen MBP	SRFSYG	12-14
Acinetobacter spp.	ϒ-CMLD	SRFAYG	Myelin-neuronal antigen MBP	SRFSYG	12-14
	3-OACT-A	LTRAGK	Myelin-neuronal antigen MOG	LYRDGK	12-14
	Acinteobacter regulatory protein	*KKVEEI	Neurofilament-M protein	*KKVEEI	12-14

MOG = myelin oligodendrocyte glycoprotein; MBP = myelin basic protein; 4-CMLD = 4-carboxy-muconolactone decarboxylase; 3-OACT-A = 3-oxoadipate CoA-transferase; ϒ-CMLD = ϒ-carboxy-muconolactone decarboxylase. * indicates the sequence likely to be responsible for cross reactivity although this is yet to be confirmed.

correlation may be linked to the increased incidence of respiratory infections at these latitudes. The disease onset is most predominant in young adults in their 20s and 30s and rarely begins in childhood or in people over 50.[3] As with several other autoimmune disorders including rheumatoid arthritis (RA), MS is significantly more common in women than in men.[3] Indeed, a recent study by the World Health Organization (WHO) estimates that MS is approximately twice as prevalent in women than in men.[4]

Pathologically, MS is characterized by the formation of central nervous system (CNS) lesions (also called plaques), inflammation and widespread areas of demyelinated neurons.[1] Clinically, MS is characterized by a wide variety of neurological signs and symptoms with autonomic, motor and sensory affects being the most common.[5] The specific symptoms in each individual appear to be determined by the location of the lesions in the CNS and may include loss of sensitivity, a 'tingling' sensation (paresthesias, numbness), muscle weakness, spasms, uncoordinated movement, pronounced reflexes, vocal and speech problems, difficulty with swallowing, visual disturbances, chronic fatigue, sexual dysfunction, pain and heat intolerance.[1] Difficulty with cognitive reasoning and emotional issues (e.g. depression, unstable moods) are also common amongst MS sufferers.[5] The progression of MS is highly variable. The majority of patients initially present with isolated (relapsing) attacks, followed by complete or partial recovery from their symptoms.[6] In the relapse-remitting form of the disease (RRMS), individual attacks may be characterized by new symptoms or a recurrence of previously expressed symptoms. Alternatively, 10-15 % of MS patients accumulate neurological disabilities continuously from the disease etiology, without periods of remission.

There is currently no known cure for MS. Current treatment strategies using anti-inflammatory drugs aim to alleviate the symptoms. Administration of high doses of corticosteroids, whilst effective for short term relief, does not have a significant impact on long term recovery.[7] Furthermore, these pharmacological treatments are not ideal as prolonged usage of these drugs is often accompanied by significant side effects and toxicity.[8] Alternatively, MS may be controlled through complementary and alternative therapies including diet, yoga, acupuncture, hyperbaric oxygen therapy, reflexology[9] and herbal medicines (including cannabis),[9,10] with varied success. There is a need to find and develop safe, effective drugs for the treatment of MS which will not only alleviate the symptoms, but which may also prevent progression of the disease. Greater understanding of the disease's etiology and progression should allow more relevant drug discovery and development.

The causes of MS are currently not fully understood. It is generally accepted to be an autoimmune disorder which may be triggered by specific microbial infections in genetically susceptible individuals with specific antigenic sequences (Table 1).[11] There has been some conjecture as to the nature of the infection(s) and several infective agents have been proposed. However, the roles of most of these organisms in the pathogenic mechanisms that lead to MS have been ruled out as causative agents because of contradictory evidence.

Recent studies have documented the presence of elevated serum levels of antibodies specific to Acinetobacter spp. and *Pseudomonas aeruginosa* in individuals suffering from MS.[11] Furthermore, amino acid sequence homologies have been identified between the‘ TRHAYG’ and ‘SRFAYG’ sequence motifs present in the *P. aeruginosa* ϒ-carboxy-muconolactone decarboxylase (ϒ-CMLD) enzyme and them yelin-neuronal basic protein (MBP) ‘SRFSYG’sequence present in susceptible individuals (Table 1).[12-14] Further peptide similarities were also identified between the ‘LTRAGK’ motif in *P. aeruginosa* 3-oxoadipate CoA-transferase (3-OACT-A) and a‘LYRDGK’ myelin oligodendrocyte glycoprotein (MOG) amino acid sequence in susceptible individuals. A further sequence homology has also been detected between the KKVEE-Isequences of both Acinetobacter spp. regulatory proteinand host neurofilament-M protein.

Many antibiotics are already known to inhibit Acinetobacter spp. and *P. aeruginosa* growth (e.g. amino glycosides, fluoroquinolones, tetracyclines, chloramphenicol). However, the development of super-resistant bacterial strains has reduced their value for treating many diseases. The search is ongoing for new antimicrobials, either by (a) the design and synthesis of new agents, or (b) re-searching the repertoire of natural resources for as yet unrecognised or poorly characterised antimicrobial agents.The antiseptic qualities of medicinal plants have long been 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 end biotic phytochemicals compared to synthetic xenobiotic pharmaceuticals.

Terminalia ferdinandiana is an endemic Australian plant which has been reported to have an extremely high antioxidant content.[15,16] Furthermore, it was reported that 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.[17,18] This is approximately 900 times higher (g/g) than the ascorbic acid content in blueberries (which were used as a standard). As a further comparison, oranges and grapefruit (which are considered good sources of ascorbic acid) only contain approximately 0.007% wet weight (0.5% dry weight).[19]

T. ferdinandiana has previously been shown to have strong antibacterial activity against an extensive panel of bacteria.[20] Solvent extracts of various polarities were tested against both Gram positive and Gram negative bacteria. The polar extracts proved to be more effective antibacterial agents, indicating that the antibacterial components were polar. Indeed, the polar extracts inhibited the growth of nearly every bacteria tested. Both Gram positive and Gram negative bacteria were susceptible, indicating that the inhibitory compounds may readily cross the Gram negative cell wall.

Recently, *T. ferdinandiana* leaf extracts were shown to have potent inhibitory activity against the bacterial triggers of several auto immune inflammatory diseases including multiple sclerosis.[21] That study indicated that the inhibition of the bacterial triggers of multiple sclerosis by the leaf extracts may be due to their high tannin content. Despite this, and the extremely high antioxidant capacity of *T. ferdinandiana* fruit, the fruit extracts have not been evaluated for the ability to inhibit Acinetobacter spp. and *P. aeruginosa* growth, nor has the phytochemistry of these extracts been extensively examined. The current study was undertaken to test the ability of *T. ferdinandiana* fruit extracts to inhibit the growth of bacteria associated with multiple sclerosis etiology and to determine if the fruit extracts have similar phytochemical compositions to the leaf extracts.

MATERIALS AND METHODS

T. ferdinandiana fruit pulp samples

T. ferdinandiana fruit pulp was a gift from David Boehme of Wild Harvest, Northern Territory, Australia. The pulp was frozen for transport and stored at -10°C until processed.

Preparation of extracts

T. ferdinandiana fruit pulp was thawed at room temperature and dried in a Sunbeam food dehydrator. The dried pulp material was subsequently ground to a coarse powder. A mass of 1 g of ground dried pulp was extracted extensively in 50 ml of methanol, deionised water, ethyl acetate, chloroform or hexane for 24 hours at 4°C with gentle shaking. All solvents were supplied by Ajax and were AR grade. The extracts were filtered through filter paper (Whatman No. 54). The solvent extracts were air dried at room temperature. The aqueous extract was lyophilised by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellets were dissolved in 10 ml deionised water (containing 0.5% DMSO). The extract was passed through 0.22 μm filter (Sarstedt) and stored at 4°C.

Qualitative phytochemical studies

Phytochemical analysis of the *T. stipitata* 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[25] with modifications. Briefly, DPPH solution was prepared fresh each day 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 evaporated 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 reference and the absorbance's were recorded at 515. 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

Test microorganisms

All media was supplied by Oxoid Ltd. Reference strains of *Acinetobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Tissue Culture Collection, USA. Clinical strains of these microbial species were obtained from the School of Natural Sciences teaching laboratory, Griffith University. All stock cultures were 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.[26-29] 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 hours before incubation with the test microbial agents. Plates inoculated with *Pseudomonas aeuroginosa*, were incubated at 30°C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Acinetobacter baylyi*, were incubated at 37°C for 24 hours, then the diameters of the inhibition zones were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (2 µg) and chloramphenicol (10 µg) were obtained from Oxoid 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 were determined as previously described.[30,31] 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 1.6 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.[32-34] 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 72 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.

Non-targeted HPLC-MS QTOF analysis

Chromatographic separations were performed as previously described.[21,25] Briefly, 2 µL of sample was injected onto an Agilent 1290 HPLC system fitted with a Zorbax

Table 2: The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant capacities of *T. ferdinandiana* leaf extracts.

		M	W	E	C	H
	Mass of extract (mg)	359	483	30	62	18
	Concentration of extract (mg/mL)	35.9	48.3	3	6.2	1.8
Phenolics	Total phenolics	+++	+++	++	+	-
	Water soluble phenolics	+++	+++	++	-	-
	Water insoluble phenolics	+++	+++	+	-	-
	Cardiac glycosides	-	-	-	-	-
	Saponins	++	+	+	-	-
	Triterpenes	+	-	++	-	-
	Polysteroids	-	-	-	-	-
Alkaloids	Meyer test	+	-	-	-	-
	Wagner test	+	-	-	-	-
Anthraquinones	Flavonoids	+++	+++	++	-	-
	Tannins	++	++	-	-	-
	Free	-	-	-	-	-
	Combined	-	-	-	-	-
	Antioxidant capacity	660	264	39	7	1

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. Antioxidant capacity determined by DPPH reduction (expressed as mg ascorbic acid equivalence per g plant material extracted)

Eclipse plus C18 column (2.1 x 100 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.

Non-targeted GC-MS head space analysis

Separation and quantification were performed with a Shimadzu GC-2010 plus (USA) linked to a Shimadzu MS TQ8040 (USA) mass selective detector system. 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 mins 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.

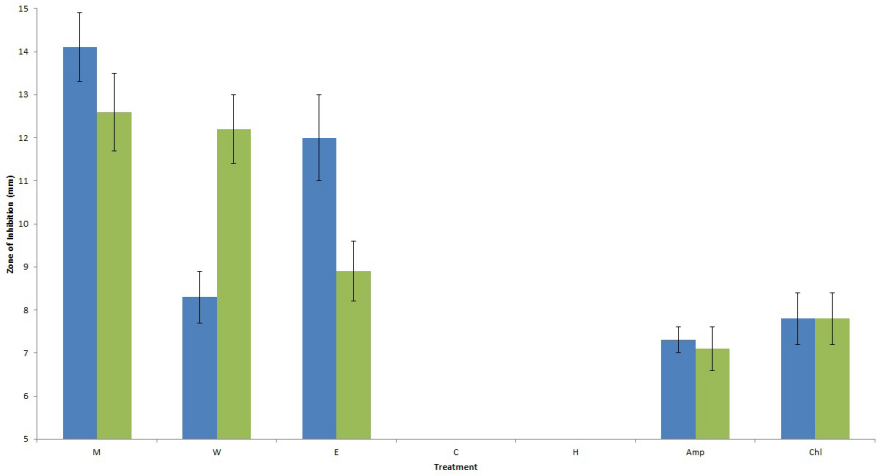

Figure 1: Antibacterial activity of *T. ferdinandia* fruit extracts against *A. baylyi* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC: 33304) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W=water extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; Amp=ampicillin (2 μg) control; Chl=chloramphenicol (10 μg) control. Results are expressed as mean zones of inhibition ± SEM.

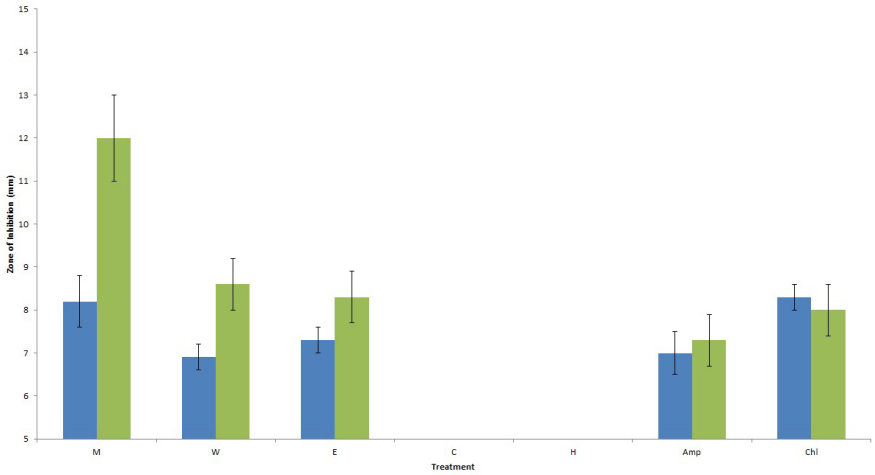

Figure 2: Antibacterial activity of *T. ferdinandia* fruit extracts against *P. aeruginosa* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:33304) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 μg) control; Chl = chloramphenicol (10 μg) control. Results are expressed as mean zones of inhibition ± SEM.

Statistical analysis

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

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried *T. ferdinandiana* fruit with various solvents yielded dried plant extracts ranging from 30 mg (ethyl acetate extract) to 483 mg (water extract) (Table 2). Deionised water and methanol gave relatively high yields of dried extracted material, whilst all other solvents extracted lower masses. The dried extracts were resuspended in 10 ml of deionised water resulting in the extract concentrations shown in Table 2.

Qualitative phytochemical studies (Table 2) showed that methanol and water extracted the widest range of phytochemicals. Both showed high levels of phenolics (both water soluble and insoluble phenolics) and flavonoids, as well as high to moderate to high levels of tannins. Saponins were also present in low to moderate levels. Triterpenes and alkaloids were also present in low levels in the methanol extract. The ethyl acetate extract also had moderate lev-

Table 3: Minimum inhibitory concentration (μg/mL) of Kakadu plum fruit extracts and LC50 values (μg/mL) in the *Artemia* nauplii bioassay.

		Methanol	Water	Ethyl Acetate	Chloroform	Hexane
MIC (ug/mL)	***A. baylyi*** **(reference strain)**	186	648	351	-	-
	A. baylyi **(clinical strain)**	263	306	782	-	-
	P. aeruginosa **(reference strain)**	882	1258	985	-	-
	P. aeruginosa **(clinical strain)**	692	924	887		-
	LC50 (ug/mL)	2115	2080	-	-	-

Numbers indicate the mean MIC and LC50 values of triplicate determinations. - indicates no inhibition.

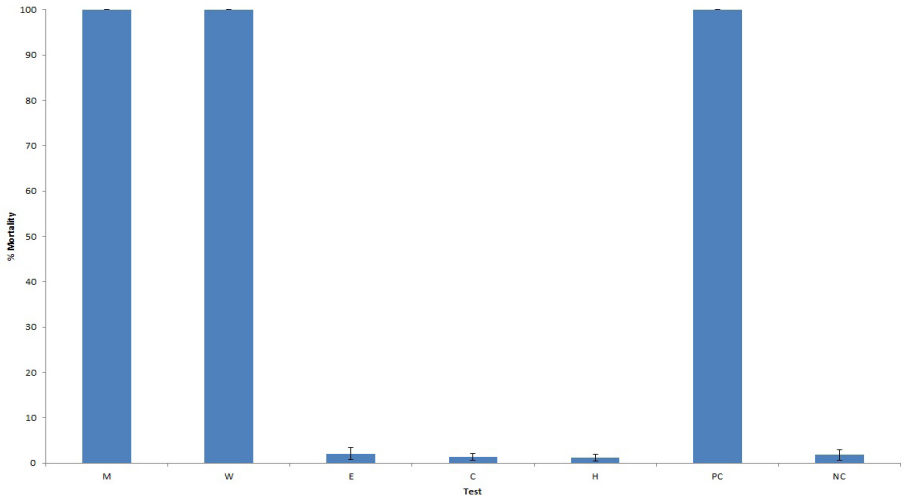

Figure 3: The lethality of *T. ferdinandiana* fruit extracts (2000 μg/mL) and potassium dichromate control (1000 μg/mL) towards *Artemia franciscana* nauplii after 24 hours exposure. M=methanolic extract; W=water extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; PC=potassium dichromate control; NC=negative (seawater) control. Results are expressed as mean ± SEM of at least triplicate determinations.

els of phenolics, flavonoids and triterpenes as well as low levels of saponins. Low levels of phenolics were detected in the chloroform extract whilst no phytochemical class was present in detectable levels in the hexane extract.

Antioxidant content

Antioxidant capacity (expressed as ascorbic acid equivalence) for the *T. ferdinandiana* fruit extracts are shown in Table 2. The antioxidant capacity ranged from a low of 1 mg ascorbic acid equivalence per gram of dried plant material extracted (hexane extract) to a high of 660 mg ascorbic acid equivalence per gram of dried plant material extracted (methanol extract). Whilst significantly lower than the methanol extract, the aqueous extract also had high antioxidant capacity with 264 mg ascorbic acid equivalence per gram of dried plant material extracted.

Antimicrobial activity

To determine the antimicrobial activity of the crude plant extracts, aliquots (10 μL) of each extract were tested in the disc diffusion assay against a panel of bacteria previously identified as microbial triggers of autoimmune inflammatory diseases. Both reference and clinical strains of *Acinetobacter baylyi* were strongly inhibited by the methanol, water and ethyl acetate *T. ferdinandiana* fruit extracts (Figure 1). Indeed, all 3 of these extracts inhibited *A.baylyi* growth of both strains more effectively than the ampicillin and chloramphenicol controls, with zones of inhibition generally>10 mm for against both strains (compared to < 8 mm for the ampicillin and chloramphenicol controls). The *A. baylyi* reference strain was significantly more susceptible to the methanol and ethyl acetate extracts than was the clinical strain (as determined by the zones of inhibition) (Figure 1). The methanol extract was the most potent bac-

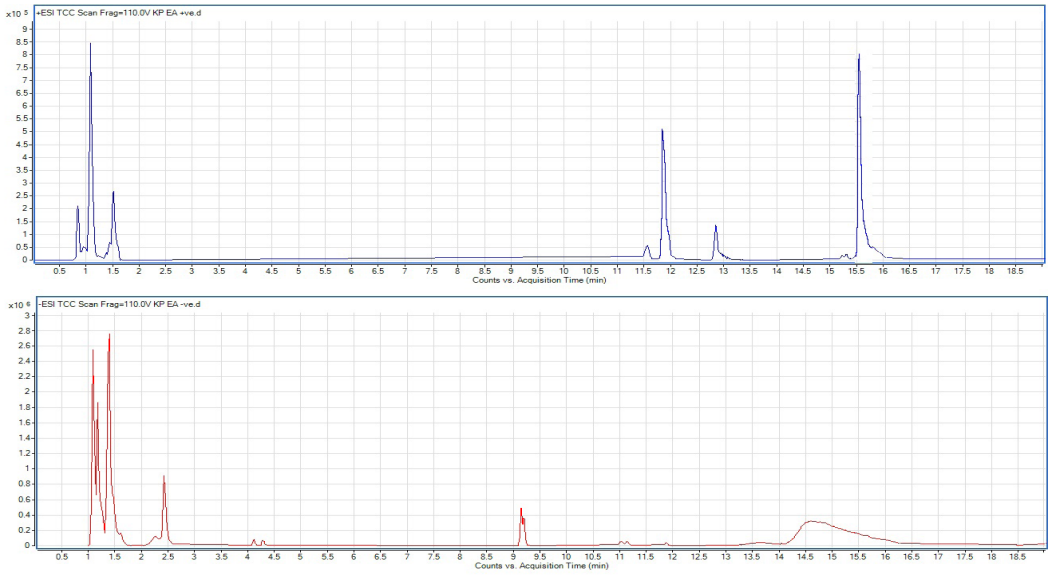

Figure 4: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µl injections of *T. ferdinandiana* fruit ethyl acetate extract

Table 4: Qualitative HPLC-MS QTOF analysis of the *T. ferdinandiana* ethyl acetate fruit extract, elucidation of empirical formulas and putative identification (where possible) of the compounds.

Putative Identification	Empirical formula	Molecular mass	Retention time	% Relative abundance	
				Negative ionisation mode	Positive ionisation mode
Purine	C5 H4 N4	120.0436	1.502		18.59
Gallic acid (3,4,5-trihdroxybutanoic acid)	C4 H8 O5	136.0374	1.382	6.69	
1-Hydroxy-4,6-dimethylpyridin-2(1H)-one (Metipirox)	C7 H9 N O2	139.061	1.077		21.79
Ribonolactone	C5 H8 O5	148.0372	1.159	6.2	
Erythorbic acid (Isoascorbic acid)	C5 H8 O6	164.032	1.473	2.17	4.1
Apionic acid	C5 H10 O6	166.0478	1.371	6.33	
(1S,5R)-4-Oxo-6,8-dioxabicyclo[3.2.1]oct-2-ene-2-carboxylic acid	C7 H6 O5	170.0214	2.419	5.28	
ascorbic acid	C6 H8 O6	176.0301	1.372	1.01	
gluconolactone	C6 H10 O6	178.0475	1.158	2.43	
methyl eugenol	C11 H14 O2	178.0988	11.136	0.39	
glucose	C6 H12 O6	180.0634	1.073	6.72	
2,4-DINITROPHENOL	C6 H4 N2 O5	184.0137	1.17	2.9	
quinic acid	C7 H12 O6	192.0629	1.379	1.51	
glucuronic acid	C6 H10 O7	194.0428	1.357	4.37	
(3S,4S,5R)-2-(Dihydroxymethyl) tetrahydro-2H-pyran-2,3,4,5-tetrol	C6 H12 O7	196.0578	1.353	1.22	
	C4 H6 N6 O4	202.0455	1.066		8.58
Glucoheptonic acid-1,4-lactone	C7 H12 O7	208.0579	1.237	1.25	
xanthotoxin	C12 H8 O4	216.0403	1.076	5.34	
Eujavanoic acid	C14 H22 O3	238.1562	11.034	0.48	

Mono-N-depropylprobenecid	C10 H13 N O4 S	243.0593	1.077	0.22	
Glucosamine 6-sulfate	C6 H13 N O8 S	259.0376	1.069		3.69
5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-Pentanoic acid (Gemfibrozil M1)	C15 H22 O4	266.1517	9.159	3.44	
CARPROFEN	C15 H12 Cl N O2	273.0529	2.225	0.74	
p-Hydroxytiaprofenic acid	C14 H12 O4 S	276.0455	1.358	0.52	
	C7 H6 O10 S	281.9702	14.76	32.02	
	C17 H22 N4	282.1835	11.562		2.55
	C16 H33 N3 O	283.2593	13.697	3.42	
SAPPANONE A 7-METHYL ETHER	C17 H14 O5	298.0879	1.366	0.14	
	C19 H29 N5	327.2414	11.561		2.25
	C11 H14 O10	306.0556	2.378	0.99	
ferulic acid dehydrodimer	C19 H18 O6	342.1159	1.075	0.33	
Gibberellin A34	C19 H24 O6	348.1543	9.157	0.27	
chebulic acid	C14 H12 O11	356.0378	2.26	0.85	
LeuAlaArg	C15 H30 N6 O4	358.2336	12.849		5.74
REBAMIPIDE	C19 H15 Cl N2 O4	370.0742	1.616	0.62	
	C20 H39 N5 O2	381.3095	12.853		5.01
	C11 H34 N10 O S4	450.1807	11.874	0.22	
cyanidin-3-glucoside chloride	C21 H21 Cl O11	484.0849	4.283	1.44	
	C25 H33 N11	487.2919	11.875		2.71
	C32 H55 N5 O5	589.4203	15.317		1.28
	C31 H51 N15	633.4454	15.23		1.27
	C23 H24 N14 O15	736.1535	2.228	0.51	

terial growth inhibitor, with zones of inhibition of 14.1 and 12.6 mm for the reference and clinical strains respectively. The ethyl acetate extract was also a potent inhibitor of the reference strain of *A. baylyi* growth with zones of inhibition of 12 and 8.9 mm for the reference and clinical strains respectively. In contrast, the clinical strain was significantly more susceptible to the aqueous extract than was the reference strain (12.2 and 8.2 mm respectively). Neither the chloroform nor the hexane extract displayed any inhibitory activity against either *A. baylyi* strain.

P. aeruginosa growth was also susceptible to the methanol, water and ethyl acetate extracts, albeit to a lesser extent (Figure 2). The clinical strain was significantly more susceptible to these extracts than was the reference strain. Zones of inhibition of 12.0, 8.6 and 8.3 mm were noted for the clinical *P. aeruginosa* strain against the methanol, water and ethyl acetate extracts respectively. In contrast, the zones of inhibition for these extracts against the reference strain were approximately 8.2, 6.9 and 7.3 mm respectively.

The antimicrobial efficacy was further quantified by determining the MIC values for each extract against the microbial species/strains which were determined to be susceptible (Table 3). Most of the extracts were effective at inhibiting microbial growth, with MIC values against the susceptible bacteria generally <1000 μg/mL (<10 μg impregnated in the disc), indicating the potential of these extracts in inhibiting the microbial triggers ofmultiple sclerosis and limiting its impact.

Quantification of toxicity

T. ferdinandia fruit extracts were initially screened at 2000 μg/mL in the assay (Figure 3). 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 mortality within the first 3 hours of exposure and 100 % mortality was evident following 4-5 hours (unpublished results).The methanol and water extracts also induced significant mortality following 24 h exposure, indicating that they were toxic at the concentration tested. The ethyl acetate, chloroform and hexane extracts did not induce mortality significantly different to the seawater control and were therefore deemed to be nontoxic.

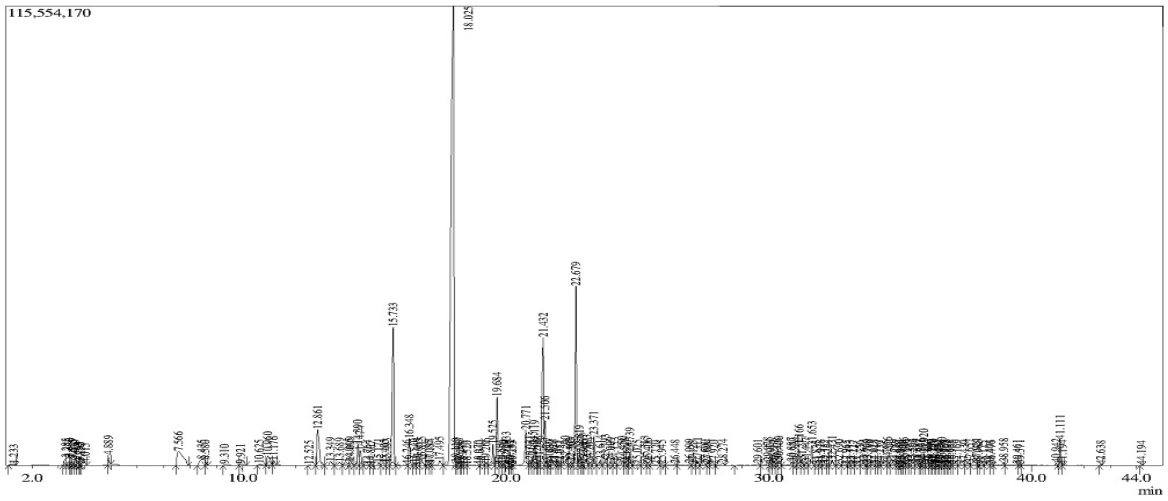

Figure 5: Head space gas chromatogram of 0.5 µL injections of *T. Ferdinandiana* ethyl acetate fruit extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in artificial sea water to test across a range of concentrations in the *Artemia* nauplii bioassay at 24 hours. Table 3 shows the LC50 values of the *T. ferdianadiana* fruit extracts towards *A. franciscana*. No LC50 values are reported for the ethyl acetate, chloroform and hexane extracts as less than 50 % mortality was seen for all concentrations tested. Extracts with an LC50 greater than 1000 µg/mL towards *Artemia* nauplii have been defined as being nontoxic in this assay.[35] As none of the extracts had a LC50 <1000 µg/mL, all were considered nontoxic.

HPLC-MS QTOF analysis

As the ethyl acetate extract had the greatest antibacterial efficacy (as determined by MIC) yet contained a low mass of extracted material (Table 1), it was deemed the most promising extract for further phytochemical analysis. Optimised HPLC-MS QTOF parameters used previously for the analysis of *T. ferdinandiana* leaf extracts[21] were also used to determine the ethyl acetate fruit extract compound profile in this study. The resultant total compound chromatograms for the positive ion and negative ion chromatograms are presented in Figure 4a and 4b respectively. The negative ion chromatograms had significantly higher background absorbance levels than the positive ion chromatogram, due to ionisation of the reference ions in this mode, possibly masking the signal for some peaks of interest.

The *T. ferdinandiana* ethyl acetate extract positive ion base peak chromatogram (Figure 4a) revealed several overlapping peaks in the early stages of the chromatogram corresponding to the elution of polar compounds. Most of the extract compounds had eluted in the first couple of minutes during the isocratic stage of the chromatogram (5 % acetonitrile). However, several prominent peaks in the positive ionisation mode chromatogram between 11 and 16 min indicates the broad spread of polarities of the compounds in this extract.

In total, 42 unique mass signals were noted for the *T. ferdinandiana* fruit ethyl acetate extract (Table 4). Putative empirical formulas were achieved for all of these compounds. Of the 42 unique molecular mass signals detected, 31 compounds (73.8 %) were putatively identified by comparison to the Metlin metabolomics, forensic toxicology (Agilent) and phytochemicals (developed in this laboratory) databases.

GC-MS Head space analysis

Optimised GC-MS parameters were developed and used to further examine the ethyl acetate fruit extract compound profile. The resultant gas chromategram is presented in Figure 5. Several major peaks were noted at approximately 15.7, 18.0, 19.7, 21.4 and 22.7 min. numerous overlapping peaks were also evident in all stages of the chromate gram. In total, 140 unique mass signals were noted for the *T. ferdinandiana* fruit ethyl acetate extract (Table 5). Putative empirical formulas and identifications were achieved for all of these compounds.

DISCUSSION

Previous studies within our laboratory reported potent antibacterial activity for *T. ferdianadiana* fruit extracts (Cock and Mohanty 2011). Recently, we also reported growth inhibitory activity of *T. ferdianadiana* leaf extracts against some microbial triggers of selected autoimmune inflam-

Table 5: Qualitative GC-MS analysis of the *T. ferdinandiana* ethyl acetate extract, elucidation of empirical formulas and putative identification of each compound.

Putative Identification	Molecular Mass	Empirical Formula	Retention Time	Relative Abundance (% Area)
Monomethylolacetone	88	C4H8O2	3.875	0.3
2-Hexenal, (E)-	98	C6H10O	9.255	0.18
1,1-Dimethylcyclopentane	98	C7H14	16.725	0.39
Hexanal	100	C6H12O	7.46	2.51
cis-2-Methylcyclopentanol	100	C6H12O	14.955	0.15
1-Hexanol	102	C6H14O	9.86	0.17
Pentanoic acid	102	C5H10O2	13.8	0.27
1-Ethyl-1-methylcyclopentane	112	C8H16	12.48	0.05
2-Heptenal, (E)-	112	C7H12O	12.78	2.43
2-n-Butylacrolein	112	C7H12O	13.12	0.3
4,4-Dimethylpent-2-enal	112	C7H12O	21.96	0.04
2-Heptanone	114	C7H14O	10.56	0.11
Heptanal	114	C7H14O	10.89	0.78
3-Methylhexanol	116	C7H16O	19.975	0.43
2-Ethyl-2-methyl-1,3-propanediol	118	C6H14O2	3.675	0.32
3,3-Dimethyl-1,2-butanediol	118	C6H14O2	18.265	0.04
Phthalan	120	C8H8O	15.83	0.16
2-n-Butylfuran	124	C8H12O	18.095	0.1
1,7-Octadien-3-ol	126	C8H14O	13.49	0.29
3-Octen-2-one	126	C8H14O	15.59	6.63
2-Octenal, (E)-	126	C8H14O	16.28	1.12
3-Aminopyrazole-4-carboxylic acid	127	C4H5N3O2	19.02	0.05
(2Z)-n-Butyl-2-buten-1-amine	127	C8H17N	25.315	0.27
Octanal	128	C8H16O	14.435	0.73
2,4,4-Trimethylcyclopentanol	128	C8H16O	20.22	0.02
Acetic acid, pentyl ester	130	C7H14O2	3.375	0.54
3-Methyllevulinic acid	130	C6H10O3	3.5	0.19
1,2,4,5-Tetramethylbenzene	134	C10H14	18.4	0.04
2,4-Dimethylbenzaldehyde	134	C9H10O	21.505	1.56
Isomyocorene	136	C10H16	14.58	0.08
2,4-Nonadienal, (E,E)-	138	C9H14O	21.43	8.12
1,3-Cyclohexadiene, 5,6-dimethoxy-1,3-cyclohexadiene	140	C8H12O2	3.285	0.38
2-Nonyn-1-ol	140	C9H16O	19.21	0.13
2-Nonenal, (E)-	140	C9H16O	19.595	2.28
2,3,4,5-Tetramethylcyclopent-2-en-1-ol	140	C9H16O	24.655	0.04
2-Nonanone	142	C9H18O	17.185	0.42
N,N'-Diacetylethylenediamine	144	C6H12N2O2	4.41	0.4
2,3-Anhydro-d-galactosan	144	C6H8O4	23.795	0.37
2-Chloroethyl 1-propynyl sulfoxide	150	C5H7ClOS	3.62	0.22
p-Cymen-7-ol	150	C10H14O	19.865	0.07
Methyl N-hydroxybenzenecarboximidoate	151	C8H9NO2	11.135	0.21
2,4-Decadienal, (E,E)-	152	C10H16O	14.055	0.31
Camphor	152	C10H16O	22.47	0.12

cis-7-Decen-1-al	154	C10H18O	22.82	0.59
Cyclodecanol	156	C10H20O	17.635	37.93
(2E,6E)-4-Methyl-2,6-octadiene-4,5-diol	156	C9H16O2	20.12	0.03
Decanal	156	C10H20O	21.07	0.84
Isomenthol	156	C10H20O	39.46	0.13
5-Ethoxy-3-ethyl-1,3,4-oxadiazol-2(3H)-one	158	C6H10N2O3	15.26	0.12
2,2-Dimethyl-1-octanol	158	C10H22O	19.76	0.07
Nonanoic acid	158	C9H18O2	23.095	0.43
Eusterol	163	C10H13NO	33.115	0.06
tert-Butyl-p-benzoquinone	164	C10H12O2	20.985	0.34
4,6,8-Trimethyl-1-nonene	168	C12H24	31.98	0.08
4-Methyl-1-undecene	168	C12H24	36.465	0.05
3,7-Dimethyldecane	170	C12H26	23.615	0.22
Dodecane	170	C12H26	29.6	0.27
2-Methylundecane	170	C12H26	34.875	0.05
Methyl n-nonanoate	172	C10H20O2	21.67	0.03
1-Undecanol	172	C11H24O	27	0.18
Phenacylthiocyanate	177	C9H7NOS	16.595	0.18
(4,6,6-Trimethylbicyclo[3.1.1]hept-3-en-2-yl)acetaldehyde	178	C12H18O	18.325	0.03
1-(tert-Butylsulfonyl)-2-propanol	180	C7H16O3S	18.545	0.1
Dodecanenitrile	181	C12H23N	24.53	0.15
7-Dodecen-6-one	182	C12H22O	22.96	0.1
2,5,6-Trimethyldecane	184	C13H28	14.825	0.04
4,7-Dimethylundecane	184	C13H28	16.475	0.1
Tridecane	184	C13H28	20.88	0.2
2,8-Dimethylundecane	184	C13H28	22.05	0.03
2,4-Dimethylundecane	184	C13H28	22.875	0.46
3-Methyl-5-propylnonane	184	C13H28	24.01	0.19
4,7-Dimethylundecane	184	C13H28	24.45	0.23
2-Dodecanone	184	C12H24O	27.13	0.05
4,6-Dimethylundecane	184	C13H28	29.98	0.25
2,2,4,6-Tetramethyl-3,5-heptanedione	184	C11H20O2	33.455	0.05
Difuranylglyoxal	190	C10H6O4	8.57	0.14
1,3-Di-tert-butylbenzene	190	C14H22	22.68	6.58
4-Hydroxy-3,3-dimethyl-4-phenyl-2-butanone	192	C12H16O2	16.96	0.08
2,3,5,8-Tetramethyldecane	198	C14H30	23.37	0.99
Tridecanal	198	C13H26O	27.695	0.12
Tetradecane	198	C14H30	27.905	0.15
4,6-Dimethyldodecane	198	C14H30	32.155	0.11
Tetradecane	198	C14H30	35.375	0.06
3,3,7-Trimethyl-oct-6-enoic acid, methyl ester	198	C12H22O2	36.71	0.04
2,3-Dimethyldodecane	198	C14H30	36.78	0.04
2-(5-[1,3]Dioxolan-2-yl-pentyl)-3-methylaziridine	199	C11H21NO2	3.82	0.13
Lilial	204	C14H20O	27.605	0.11

cis-7-Decen-1-al	154	C10H18O	22.82	0.59
Cyclodecanol	156	C10H20O	17.635	37.93
(2E,6E)-4-Methyl-2,6-octadiene-4,5-diol	156	C9H16O2	20.12	0.03
Decanal	156	C10H20O	21.07	0.84
Isomenthol	156	C10H20O	39.46	0.13
5-Ethoxy-3-ethyl-1,3,4-oxadiazol-2(3H)-one	158	C6H10N2O3	15.26	0.12
2,2-Dimethyl-1-octanol	158	C10H22O	19.76	0.07
Nonanoic acid	158	C9H18O2	23.095	0.43
Eusterol	163	C10H13NO	33.115	0.06
tert-Butyl-p-benzoquinone	164	C10H12O2	20.985	0.34
4,6,8-Trimethyl-1-nonene	168	C12H24	31.98	0.08
4-Methyl-1-undecene	168	C12H24	36.465	0.05
3,7-Dimethyldecane	170	C12H26	23.615	0.22
Dodecane	170	C12H26	29.6	0.27
2-Methylundecane	170	C12H26	34.875	0.05

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.

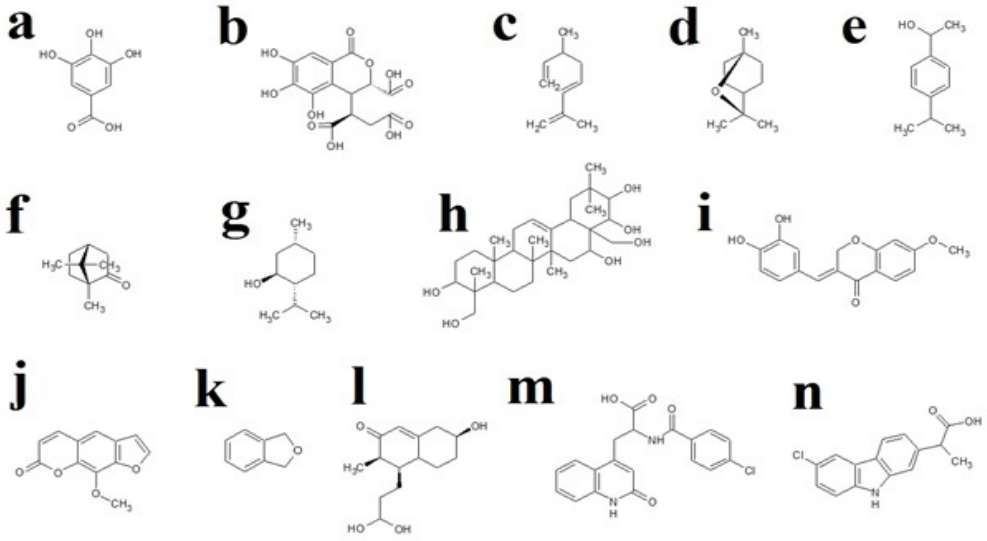

Figure 6: Chemical structures of *T. ferdinandiana* fruit compounds detected in the ethyl acetate extract: (a) gallic acid; (b) chebulic acid; (c) isomyocorene; (d) cineole (eucalyptol); (e) cuminol; (f) camphor; (g) isomenthol; (h) (3β,4α,16α,21β,22α) Olean-12-ene-3,16,21,22,23,28-hexol; (i) sappanone A-7 methyl ether; (j) xanthotoxin; (k) phthalane; (l) eujavanoic acid; (m) rebamipide (n) carpofen.

matory diseases (Courtney *et al.* in press). That study also screened the phytochemical profile of the bioactive ethyl acetate extract and determined that the extract contained relatively high levels of a number of tannin components including exifone (4-galloylpyrogallol), ellagic acid dehydrate, trimethyl ellagic acid, chebulic acid, corilagen, castalagin and chebulagic acid. Gallotannins have been reported to inhibit the growth of a broad spectrum of bacterial species (Buzzini *et al.* 2008) through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins (Wolinsky and Sote 1984; Hogg and Embery 1982), and by inhibiting glucosyltransferase enzymes (Wu-Yuan *et al.* 1988). Elligitannins are also highly potent inhibitors of bacterial growth, with MIC values as low as 62.5 µg/ml (Buzzini *et al.* 2008; Machado *et al.* 2003; Hogg and Embery 1982). Ellagitannins have also been reported to function via several antibiotic mechanisms including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls (Buzzini *et al.* 2008; Hogg and Embery 1982).

In contrast to the previous leaf study, only 2 tannin compound (gallic acid (Figure 6a) and chebulic acid (Figure

6b)) were detected in the *T. ferdianadiana* fruit extracts by LC-MS analysis. Whilst both of these tannins have been reported to be potent antibacterial agents (Cock 2013) they were detected at much lower relative levels than in the leaf extracts (Courtney *et al*. in press).

The fruit extracts examined in this study displayed potent growth inhibitory activity against *A. baylyi* and *P. aeruginosa*. MIC values <1000 μg/ml are reported for the fruit ethyl acetate extract against all bacterial triggers of MS tested (*A. baylyi* and *P. aeruginosa* reference and clinical strains). In our previous study screening *T. ferdianadiana* leaf extracts, lower MIC values were noted against *A. baylyi* than are reported here for the fruit extracts, indicating the greater potency of the leaf ethyl acetate extract against these bacteria. In contrast, the lower MIC values towards the *P. aeruginosa* determined for the *T. ferdianadiana* fruit extracts in this study compared to the leaf extracts in the previous study indicate that these 2 bacterial species may be inhibited via different mechanisms.

As much lower tannin amounts and diversity were detected in the *T. ferdianadiana* fruit ethyl acetate extract compared to the leaf extract, is likely that other phytochemical classes may contribute to the anti-inflammatory properties of these extracts. 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.

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 fingerprinting, 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 non-polar compounds. Thus, many nonpolar phytosterols, saponins, stilbenes and terpenes which may contribute to the inhibitory activity of the ethyl acetate extract may escape detection by HPLC-MS. For this reason, we also utilised GC-MS analysis to detect many of the less polar compounds. This enabled us to obtain the broadest possible metabolomic profile of the bioactive extract.

A number of monoterpenoids including isomyocorene (Figure 6c), cineole (eucalyptol) (Figure 6d), cuminol (Figure 6e), camphor (Figure 6f) and isomenthol (Figure 6g) were detected in the ethyl acetate fruit extract by GC-MS analysis. The amyrintriterpenoid (3β,4α,16α,21β, 22α) Olean-12-ene-3, 16, 21, 22, 23, 28-hexol (Figure 6 h) was also detected by GC-MS. Many of these terpenoids have been previously reported to have potent broad spectrum antibacterial activity[41] and therefore may contribute to the inhibitory activity against *A. baylyi* and *P. aeruginosa* in our study. Interestingly, several of these monoterpenes have also been reported to suppress NF-ϰB signalling (the major regulator of inflammatory diseases).[42-45] Thus, the terpene components may have a pleuripotent mechanism in blocking multiple sclerosis and relieving its symptoms by acting on both the initiator and downstream inflammatory stages of the disease.

Several flavonoids includings appanone A-7 methyl ether (Figure 6i), xanthotoxin (Figure 6j), phthalane (Figure 6k), eujavanoic acid (Figure 6l) and rebamipide (Figure 6m) were also putatively identified in our study. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.[46] Thus, it is likely that multiple compounds within the ethyl acetate extract are contributing to the growth inhibition of the microbial triggers of MS. The putative identification of rebamipidine is particularly interesting as it has also been reported to suppress bladder[47] and gastrointestinal inflammation.[48] The propionic acid derivative carpofen (Figure 6n) also has anti-inflammatory properties and has been reported to inhibit cyclooxygenase 2 (COX-2) and thus block the synthesis of pro-inflammatory prostaglandins.[49]

Whilst our studies provide insight into the phytochemical composition of this extract, it is noteworthy that 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. Our findings demonstrate that *T. Ferdinandiana* fruit extracts display low toxicity towards *Artemia franciscana*. Indeed, the LC50 values for all extracts were well in excess of 1000 μg/ml and are therefore nontoxic.

CONCLUSION

The results of this study demonstrate the potential of *T. Ferdianadiana* fruit extracts to block the growth of bacterial species associated with the onset of MS. Thus, *T. Ferdianadiana* fruit extracts have potential in the prevention and treatment of MS in genetically susceptible individuals. Further studies aimed at the purification and identification of the bioactive components are needed to examine the mechanisms of action of these agents.

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