Tannin components and inhibitory activity of Kakadu plum leaf extracts against microbial triggers of autoimmune inflammatory diseases

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ABSTRACT

Introduction: Autoimmune inflammatory diseases can be triggered by specific bacteria in susceptible individuals. *Terminalia ferdinandiana* (Kakadu plum) has documented therapeutic properties as a general antiseptic agent. However, the high ascorbic acid levels in Kakadu plum fruit may interfere with this activity. **Methods:** *T. ferdinandiana* leaf solvent extracts were investigated by disc diffusion assay against a panel of bacteria known to trigger autoimmune inflammatory diseases.Their MIC values were determined to quantify and compare their efficacies.Toxicity was determined using the *Artemia franciscana* nauplii bioassay. Non-targeted HPLC separation of crude extracts coupled to high resolution time-of-flight (TOF) mass spectroscopy with screening against 3 compound databases was used 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 rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis. The ethyl acetate extract had the most potent inhibitory activity, with MIC values less than 120 μg/ml against *P. mirabilis* and *A. baylyi* (both reference and clinical strains). The ethyl acetate extract had similar potency against *K. pneumonia* (both reference and clinical strains), but had higher MIC values (2733 μg/ml) against *P. aeruginosa*. The methanolic extract was also a potent inhibitor of bacterial growth, with MIC values generally < 1000 μg/ml. In comparison, the water, chloroform and hexane leafextracts were all substantially less potent antibacterial agents, with MICs values generally well over 1000 μg/ml. All *T. ferdinandiana* leaf extracts were either nontoxic or of low toxicity in the *Artemia fransiscana* bioassay.Non-biased phytochemical analysis of the ethyl acetate extract revealed the presence of high levels of tannins (exifone (4-galloylpyrogallol), ellagic acid dehydrate, trimethylellagic acid, chebulic acid, corilagin, punicalin, castalagin and chebulagic acid). **Conclusion:** The low toxicity of the *T. ferdinandiana* leaf extracts and their potent inhibitory bioactivity against the bacterial triggers of autoimmune inflammatory disorders indicates their potential as medicinal agents in the treatment and prevention of these diseases.

Key words: *Terminalia ferdinandiana,* rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis, *Proteus mirabilis, Klebsiella pneumoniae , Acinetobacter baylyi, Pseudomonas aeruginosa.*

INTRODUCTION

Autoimmune inflammatory disorders are a group of debilitating conditions including rheumatoid arthritis, ankylosing spondylitis, lupus and multiple sclerosis, which afflict genetically susceptible individuals. There is no common susceptibility profile for these disorders. Rheumatoid arthritis for example is most prevalent in middle aged to older women, whereas the onset of ankylosing spondylitis occurs most frequently in younger males.[1] There are no cures for any of these conditions. Instead, current treatment strategies aim to alleviate the symptoms (particularly pain, swelling and inflammation) with analgesics and anti-inflammatory agents and/or to modify the disease process through the use of disease modifying drugs. None of these treatments is ideal as prolonged usage of these drugs is often accompanied by unwanted side effects and toxicity.[2] There is a need

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to develop safer, more effective treatments for these conditions which will not only alleviate the symptoms, but may also cure or prevent the disease. A greater understanding of the onset and progression of these disorders should greatly assist in more relevant drug discovery and development.

The causes of the autoimmune inflammatory disorders are currently not well understood. However, it is generally accepted that they are immune disorders triggered in susceptible individuals by specific microbial infections. Recent serotyping studies have identified several of the bacterial triggers of these conditions and the bacterial antigens responsible for the induction of an immune response (Table 1). The major microbial trigger of rheumatoid arthritis has been identified as *Proteus mirabilis*,[3] a normal part of the human gastrointestinal flora. Similarly, *Klebsiella pneumoniae* has been shown to initiate ankylosing spondylitis[4] and *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis.[5] *Borrelia burgdorferi* is linked with Lyme disease.[6] Whilst microbial triggers have also been postulated for lupus, the specific causative agents are yet to be identified. Similarly, members of the Enterobacteriaceae family are associated with Graves' disease and Kawasaki syndrome and *Mycoplasma pneumoniae* is associated with several demyelinating diseases.[7] The development of antibiotic agents targeted at the specific bacterial triggers of autoimmune inflammatory disorders would enable afflicted individuals to target these microbes and thus prevent the onset of the disease and reduce the severity of the symptoms once the disease has progressed.

Herbal medicines have been used for thousands of years in a wide variety of cultures to treat inflammatory disease. A re-examination of traditional medicines for the treatment of inflammation and rheumatic conditions is an attractive prospect as the antiseptic qualities of medicinal plants have also been long recognised and recorded. Furthermore, there has recently been a revival of interest in herbal medications due to a perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals. Antimicrobial plant extracts with high antioxidant contents are particularly attractive as they may treat the symptoms of inflammation as well as blocking the microbial trigger and thus have pleuripotent effects.

Terminalia ferdinandiana is an endemic Australian plant which has been reported to have an extremely high antioxidant content.[17, 18] 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.[19,20] 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).[21]

Terminalia ferdinandiana has strong antibacterial activity against an extensive panel of bacteria. In a recent study, solvent extracts of various polarities were tested against both Gram positive and Gram negative bacteria.[22] The polar extracts proved to be more effective antibacterial agents, indicating that the antibacterial components were polar compounds. 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 readily crossed the Gram negative cell wall.

Several studies have reported that high levels of vitamin C may inhibit eukaryotic cell death due to inhibition of oxidative stress.[23,24] Whilst similar studies are lacking for prokaryotic cells, it is possible that vitamin C may also have a protective effect, antagonising the antibacterial action of the extracts and protecting against bacterial cell death. Thus, whilst potent antibacterial activity has previously been reported for Kakadu plum fruit,[22] the growth inhibition may be more pronounced in extracts with lower vitamin C levels. The current study was undertaken to test the ability of Kakadu plum leaf extracts to inhibit the growth of bacterial associated with autoimmune inflammatory diseases.

MATERIALS AND METHODS

Plant source and extraction

T. ferdinandiana leaves were obtained from David Bohme of Northern Territory Wild Harvest, Australia. The leaves were thoroughly dried in a Sunbeam food dehydrator and the dried plant materials were subsequently stored at -30°C. Prior to use, the plant materials were thawed and freshly ground to a coarse powder. Individual 1 g quantities of the ground leaves were weighed into separate tubes and 50ml of methanol, water, ethyl acetate, chloroform or hexane were added. All solvents were obtained from Ajax and were AR grade. The ground leaveswere individually extracted in each solvent for 24 hours at 4 °C with gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301.

Table 1: The bacterial triggers of the autoimmune inflammatory diseases examined in this study as well as the bacterial antigen and host susceptibility antigen sequences.

Disease	Bacterial Trigger	Bacterial Antigen	Bacterial Sequence	Host Antigen	Host Sequence	References
Rheumatoid arthritis	*Proteus mirabilis* and possibly also other Proteus spp.	haemolysin	ESRRAL	MHC class 2 allele HLA-DR4	EQ/KRRAA	3, 4, 8,9
		urease	IRRET	type XI collagen	LRREI	8,10
Ankylosing spondylitis	Klebsiella pneumoniae	nitrogenase reductase enzyme	QTDRED	MHC class 1 allele HLA-B27	QTDRED	3,11
		pullulanase	DRDE	MHC class 1 allele HLA-B27	DRED	12
		pullulanase	GxP	types I, III and IV collagen	GxP	13
Multiple sclerosis	Pseudomonas aeriginosa	γ-CMLD	TRHAYG	Myelin-neuronal antigen MBP	SRFSYG	14
	Acinetobacter spp.	4-CMLD	SRFAYG	Myelin-neuronal antigen MBP	SRFSYG	14
		3-OACT-A	LTRAGK	Myelin-neuronal antigen MOG	LYRDGK	14
		Acinetobacter regulatory protein	*KKVEEI	Neurofilament-M protein	*KKVEEI	14-16

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

The resultant dry extract was weighed and redissolved in 10 ml deionised water.

Qualitative phytochemical studies

Phytochemical analysis of the *T. ferdinandiana* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[25-27]

Antioxidant capacity determination

The antioxidant capacity of each sample was assessed using the DPPH free radical scavenging method[28] 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), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC21719) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Tissue Culture Collection, USA.All other clinical microbial strains 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.[29-32] 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 the bacterial species *Klebsiella pneumoniae* , *Proteus mirabilis*, *Proteus vulgaris* and*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) 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.[33,34] 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/mlsolution 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.[35-37] Briefly, 400 µl of seawater containing approximately 47 (mean 47.6, n = 128, SD 14.9) *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 24h 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.

Statistical analysis

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

Non-targeted HPLC-MS/MS QTOF analysis

Chromatographic separations were performed as previously described.[28] Briefly, 2 µL of each sample was injected onto an Agilent 1290 HPLC system fitted with a Zorbax 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 isocratic ally at 5% B, a gradient of (B) from 5% to 100% was applied from 5 min to 30 min, followed by 3 min isocratic ally 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 (650 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.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried *T. ferdinandiana* leaves with various solvents yielded dried plant extracts ranging from 59 mg (ethyl acetate extract) to 471 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 extractswere 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 extractedthe widest range of phytochemicals.Both showed high levels of phenolics (both water soluble and insoluble phenolics)and tannins, as well as well as high to moderate to high levels of cardiac glycosides, saponins and flavonoids. Triterpenes and anthraquinones were also present in low levels in both extracts and alkaloids were detetected in the methanol extract. The ethyl acetate extract also had high levels of phenolics and moderate levels of flavonoids and tannins. Only low levels of phenolics, flavonoids and tannins were detected in the chloroform and hexane extracts.

Antioxidant content

Antioxidant capacity (expressed as ascorbic acid equivalence) for the *T. ferdinandiana* leaf extracts are shown in (Table 2). The antioxidant capacity ranged from a low of 0.4 mg ascorbic acid equivalence per gram of dried plant material extracted (hexane extract) to a high of 340 mg ascorbic acid equivalence per gram of dried plant material extracted (aqueous extract).Whilst significantly lower than the aqueous extract, the methanolic extract also had a high antioxidant capacity with 150 mg ascorbic acid equivalence per gram of dried plant material extracted (aqueous extract).

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 bacteriapreviously identified as microbial triggers of autoimmune inflammatory diseases. Both reference and clinical strains of *Proteus mirabilis* were strongly inhibited by the methanol, water and ethyl acetate *T. ferdinandiana* leaf extracts (Figure 1). Indeed, the methanol extract inhibited *P. mirabilis*growth of both strains more effectively than the ampicillin control, with zones of inhibition of >17.5 mm against both strains(compared to approximately 14 mm for the ampicillin control). The methanol, water and ethyl acetate extracts also inhibited *P. vulgaris* growth (Figure 2), with zones of inhibition of up to 15 mm (for the ethyl acetate extract), compared to 7mm for the ampicillin control.

The methanol and ethyl acetate extracts (but not the water extract) were similarly potent inhibitors of *K. pnuemoniae* growth (Figure 3). In general, the clinical strain was significantly more susceptible to the extracts, with the inhibition zone of the clinical *K. pneumonia* being nearly 3mm greater than for the ATCC reference strain. Whilst zones of inhibition were also noted for the water, chloroform and hexane extracts, these were only 6mm in diameter, indicating that the anti-Klebsiella compounds in these extractswere weak, in low concentrations and/or nonpolar.

In contrast(with the exception of the water extract), the *A. baylyi* reference strain was significantly more susceptible to the extracts than was the clinical strain (as determined by the zones of inhibition) (Figure 4). The methanol extract was the most potent bacterial growth inhibitor, with zones of inhibition of 19 and 16.3 mm for the reference and clinical strains respectively.The water and ethyl acetate extracts were also potent inhibitors of *A. baylyi* growth with >10 mm zones of inhibition.

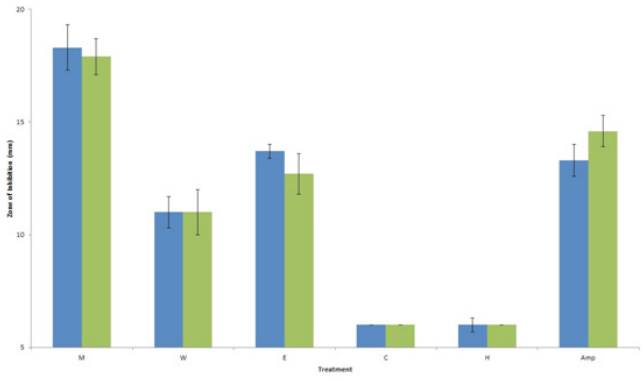

Figure 1: *Antibacterial activity of T. ferdinandia Leaf extracts against P. mirabilis measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC: 21721) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.*

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

		M	W	E	C	H
	Mass of extract (mg)	331	471	59	59	58
	Concentration of extract (mg/mL)	33.1	47.1	5.9	5.9	5.8
Phenolics	Total phenolics	+++	+++	+++	+	+
	Water soluble phenolics	+++	+++	+++	-	-
	Water insoluble phenolics	+++	+++	+++	-	-
Alkaloids	Cardiac glycosides	+++	++	-	-	-
	Saponins	++	+++	-	-	-
	Triterpenes	+	++	-	-	-
	Polysteroids	-	-	-	-	-
	Meyer test	+	-	-	-	-
	Wagner test	+	-	-	-	-
Anthraquinones	Flavonoids	++	++	++	-	++
	Tannins	+++	+++	++	-	+
	Free	+	+	-	-	-
	Combined	+	+	-	-	-
	Antioxidant content by DPPH reduction (expressed as mg AA equivalence per g plant material extracted)	150	340	22	5	0.4

M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; +++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. AA = ascorbic acid.

P. aeruginosa growth was also susceptible to the methanol, water and ethyl acetate extracts (Figure 5). The clinical strain was significantly more susceptible to the extracts than was the reference strain. Zones of inhibition of 14.3 and 8.3 mm were noted for the clinical and reference *P. aeruginosa* strains respectively against the methanol extract. The zones of inhibition for the aqueous and ethyl acetate extracts were approximately 7.5 and 10.5 mm for the reference and clinical strains respectively.

The Antimicrobial efficacy was further quantified by determining the MIC values for each extract against the microbial species 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

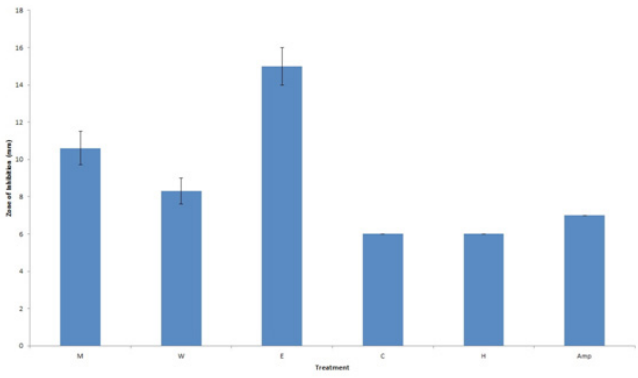

Figure 2: *Antibacterial activity of T. ferdinandia leafextracts against P. vulgaris measured as zones of inhibition (mm). M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.*

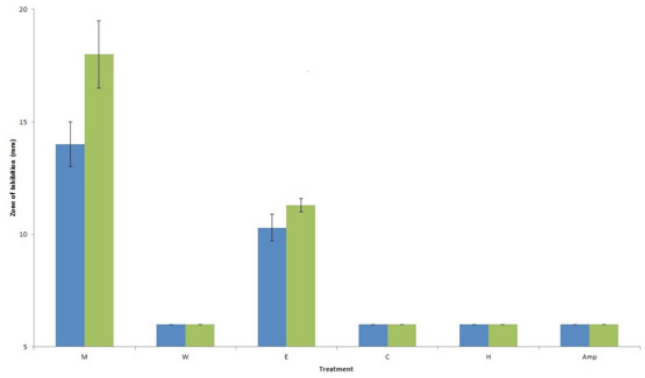

Figure 3: *Antibacterial activity of T. ferdinandia leafextracts against K. pneumoniae measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:31488) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.*

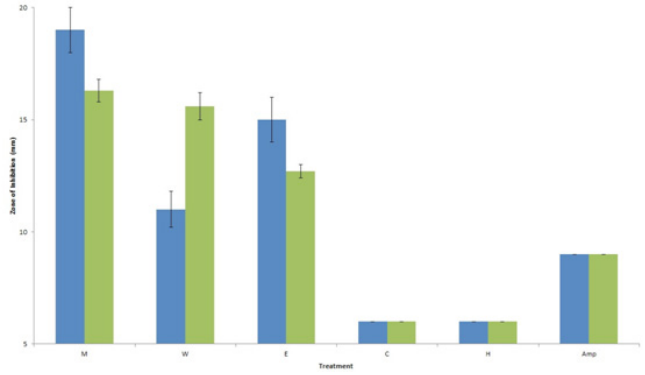

Figure 4: *Antibacterial activity of T. ferdinandia leafextracts against A. baylyi measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:33304) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.*

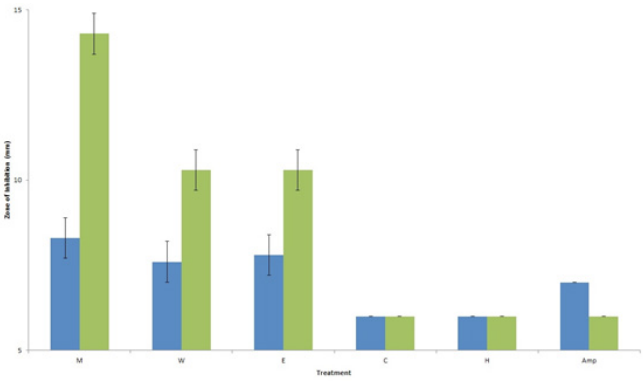

Figure 5: *Antibacterial activity of T. ferdinandia leafextracts against P. aeruginosa measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:39324) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.*

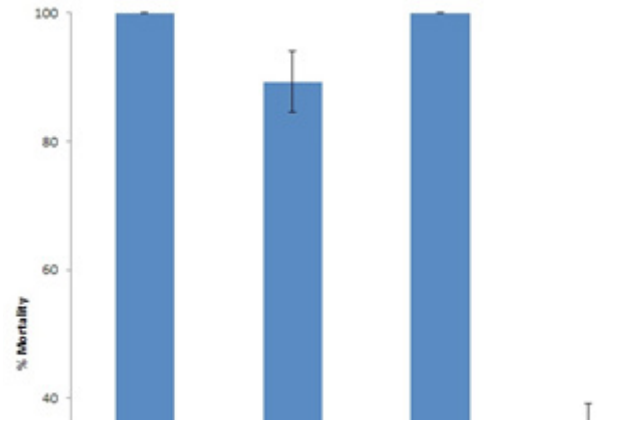

Figure 6: *The lethality of T. ferdinanianaleaf extracts (2000 µg/ml) and the potassium dichromate control (1000 µg/mL) towards Artemia nauplii following 24 hours exposure. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.*

controlling multiple autoimmune inflammatory disorders. The MIC values determined for the ethyl acetate extract were particularly noteworthy, with most MIC values 100-200µg/ml (1-2 µg impregnated in the disc) against most of the bacterial triggers of autoimmune diseases tested.

Quantification of toxicity

T. ferdinandia leaf extracts were initially screened at 2000 µg/ml in the assay (Figure 6). 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, water and ethyl acetate extracts also induced significant mortality following 24 h exposure, indicating that they were toxic at the concentration tested. The chloroform and hexane extracts did not induce mortality significantly different to the seawater control and were therefore deemed to be nontoxic.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in

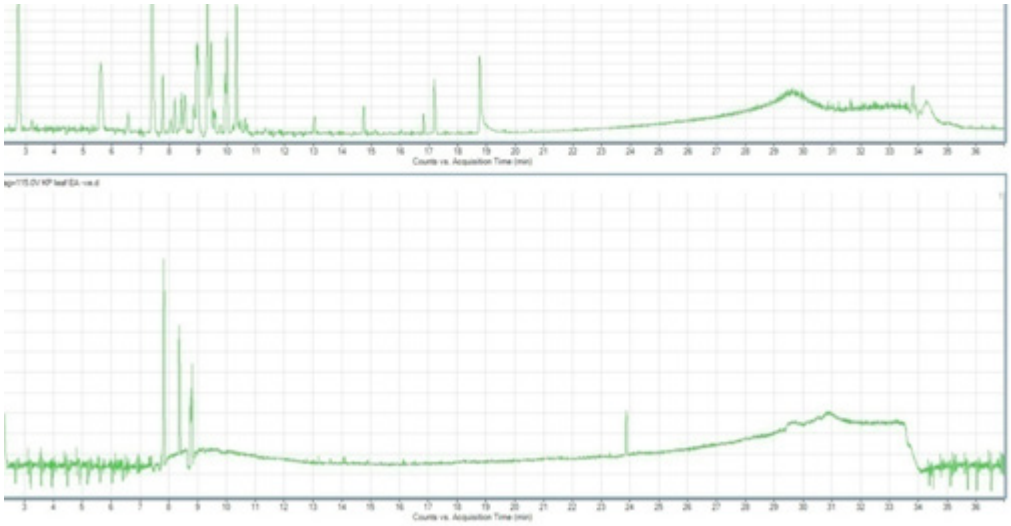

Figure 7:*(a) Positive and (b)negative ionRP-HPLC base peak chromatogram (BPC) of 2 µl injections ofT. ferdinandianaleafethyl acetate extract.*

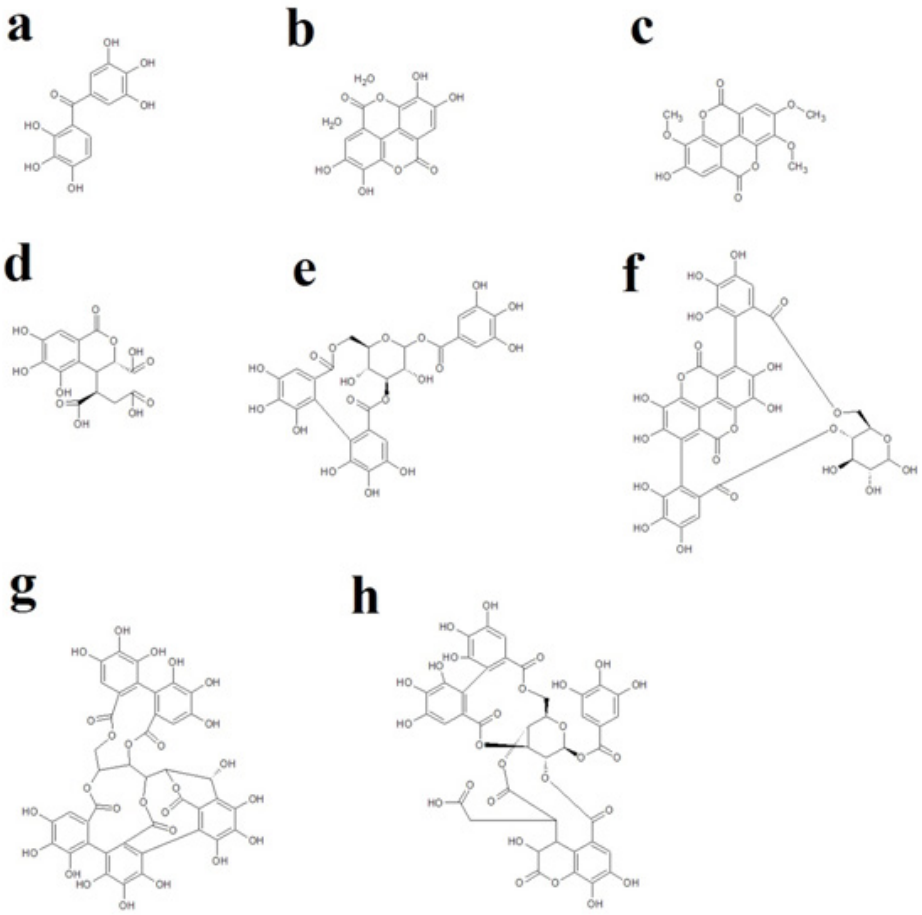

Figure 8:*Chemical structures of T. ferdinandiana leaf compounds detected in the ethyl acetate extract: (a) exifone (4-galloylpyrogallol); (b) ellagic acid dehydrate; (c) trimethylellagic acid; (d) chebulic acid; (e) corilagin; (f) punicalin; (g) castalagin; (h) chebulagic acid.*

artificial seawater 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* leaf extracts towards *A. franciscana*. No LC50 values are reported for The 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.[38] As only the ethyl acetate extract had a LC50 <1000 µg/ml, all other extracts were considered nontoxic. Whilst the LC50 value for ethyl acetate is below 1000 µg/ml, the value of 767 µg/ml indicates low to moderate toxicity.

HPLC-MS/MS analysis

As the ethyl acetate extract had the greatest antibacterial efficacy (as determined by MIC) yet contained the least extracted material (Table 1), it was deemed the most promising extract for further phytochemical analysis. Optimised HPLC-MS/MS parameters were developed and used to determinethe ethyl acetate extract compound profile. The resultant base peak chromatograms for the positive ion and negative ion chromatograms are presented in (Figure 7a and 7b) 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 7a) revealed numerous peaks, particularly in the early and middle stages of the chromatogram corresponding to the elution of polar compounds.Nearly all of the methanol extract compounds had eluted by 11 minutes (corresponding to approximately 30 % acetonitrile).Indeed, several peaks eluted in the first 1 minute with 5 % acetonitrile. However, several prominent peaks between 13 and 20 min, and a minor peak eluting later in the chromatogram (at nearly 34 min)indicates the broad spread of polarities of the compounds in this extract.

Qualitative mass spectral analysis of Kakadu plum leaf extracts

In total,63 unique masssignals were noted for the Kakadu plum leaf ethyl acetateextract (Table 4). Putative empirical formulas were achieved for all of these compounds. Of the 63 unique molecular mass signals detected,42 compounds (66.7 %) were putatively identified by comparison to the Metlin metabolomics, forensic toxicology (Agilent) and phytochemicals (developed in this laboratory) databases.

DISCUSSION

Previous studies within our laboratory reported broad spectrum antibacterial activity for *T. ferdianadiana* fruit extracts.[22] Whilst that study reported potent growth inhibitory properties for the fruit extracts, it did not determine the phytochemical(s) responsible for this activity. Whilst much of the phytochemistry of *T. ferdianadiana* has yet to be determined, the high antioxidant capacity and the extremely high levels of ascorbic acid in the fruit have been frequently reported.[17,18] Indeed, *T. ferdinandiana* fruit

Table 3: Minimum inhibitory concentration (µg/ml) of T. ferdinandiana leaf extracts and LC50 values (µg/mL) in the Artemia nauplii bioassay.

		Methanol	Water	Ethyl Acetate	Chloroform	Hexane
MIC (ug/mL)	*P. mirabilis* (reference strain)	356	925	119	1571	1571
	P. mirabilis (clinical strain)	494	1207	119	1571	1571
	P. vulgaris (reference strain)	734	1752	188	1571	1571
	K. pneumoniae (reference strain)	902	4667	192	1571	1571
	K. pneumoniae (clinical strain)	1352	4980	164	1571	1571
	A. baylyi (reference strain)	780	1188	125	1571	1571
	A. baylyi (clinical strain)	578	1779	108	1571	1571
	P. aeruginosa (reference strain)	739	1239	2733	1571	1571
	P. aeruginosa (clinical strain)	1020	1711	2733	1571	1571
	LC50 (ug/mL)	1133	1330	767	-	-

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

has been reported to have ascorbic acid levels as high as 6% of the recorded wet weight.[19,20]

The high antioxidant capacity of *T. ferdinandiana* fruit has been postulated as being responsible (at least in part) to potential medicinal properties.[39,40] In particular, ascorbic acid has been linked to antibacterial, antifungal and antiviral activities, as well as anticancer properties.[39, 40] However, other studies have indicated that ascorbic acid may protect cells from oxidative stress and thus protect against cell death.[23,24] Whilst those studies examined the effects of ascorbic acid on eukaryotic cells, it is possible that ascorbic acid may have a similar protective effect against bacterial cell death. *T. ferdinandiana* leaves were examined in the current study in an attempt to minimise the effects of the high ascorbic acid contents present in the fruit.

The leaf extracts examined in this study displayed potent growth inhibitory activity against the bacterial species tested. Indeed, in several cases, we report similar or better antibacterial activities for the *T. ferdinandiana* leaf extracts compared to those previously reported for the equivalent fruit extracts.[22] MIC values <200 μg/ml are reported here for the leaf ethyl acetate extract against a bacterial trigger of ankylosing spondylitis (*K. pnuemoniae*). In the previous study, no inhibition of *K. pnuemoniae* growth by the fruit ethyl acetate extract was noted, indicating the greater potency of the leaf extract. The leaf ethyl acetate extract was also a much more potent inhibitor of *P. mirabilis* than the fruit ethyl acetate extract reported in the previous study (MIC values of 120 μg/ml and 500 μg/ml respectively).

As the ethyl acetate extract had the most potent antibacterial activity yet the least amount of extracted material, it was deemed the best extract for phytochemical analysis. A total of 63 compounds were detected in the ethyl acetate extract and 44 of these compounds were putatively identified. Of these compounds, 8 were identified as tannins. As well as having a wide diversity of tannin components, the tannins were present as major components. The corilagen, castalagin and the chebulagic acid chromatographic peaks accounted for approximately 11 %,1 % and 4 % of the total peak areas respectively for the negative ionisation mode chromatogram (Table 4). Puncialin accounted for approximately 6 % of the total chromatographic peak area in positive ionisation mode, with ellagic acid dehydrate, trimethylellagic acid and chebulic acid combined represent ingapproximately a further 5 % of the total peak area.

It is likely that the high tannin contents in the *T. ferdinandiana* ethyl acetate leaf extract contributes to the inhibitory activity against the microbial triggers of autoimmune inflammatory diseases. Gallotannins including exifone (4-galloylpyrogallol) (Figure 8a) have been reported to inhibit the growth of a broad spectrum of bacterial species[41] through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins,[42,43] and by inhibiting glucosyl transferase enzymes.[44] Elligitannins are also potent inhibitors of bacterial growth. Ellagic acid dehydrate (Figure 8b), trimethylellagic acid (Figure 8c), chebulic acid (Figure 8d), corilagin (Figure 8e), punicalin (Figure 8f) and castalagin (Figure 8g) have been reported to be highly potent antibiotics, with MIC values as low as 62.5 μg/ml.[41, 45, 46] Ellagitannins have also been reported to function via several antibiotic mechanisms including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls.[41,45] Interestingly, the benzopyran tannin chebulagic acid (Figure 8h) has been reported to inhibit a multi-resistant strain of *Acinetobacter baumannii*,[47] a species taxonomically related to *A. baylyi*, examined in our study.

It is likely that other phytochemical classes may also contribute to the anti-inflammatory properties of these extracts. Alkaloids, anthraquionones, flavonoids, polyphenolics, phytosterols, saponins, stilbenes and terpenes have also been linked with anti-bacterial activity in different plant species and thus may be responsible (at least in part) for the bacterial growth inhibitory activities reported here. Several terpenoids previously reported in *T. ferdinandiana* fruit extracts have been reported to suppress NF-kB signaling (the major regulator of inflammatory diseases).[48] The monoterpenes limonene[49,50] and α-pinene[51] have been reported to inhibit NF-κBsignaling pathways. α-Pinene affects inflammation by inhibiting p65 translocation into the nucleus in LPS-induced NF-κB signalling.[51] Furthermore, many other sesquiterpenes and sesquiterpene lactones also have well established anti-inflammatory activities.[48] Whilst much work is still needed to characterize the mechanisms of action of these compounds, it appears that NF-kxxxxxxB inhibitory activities may be responsible.

Whilst none of these terpenoid compounds were detected in our study, it is possible that they may be present and may contribute to the antibacterial activity of the *T. ferdinandiana* leaf extracts. Our study examined the phytochemical composition of the extracts using HPLC-MS/MS, whereas the previous studies used GC-MS analysis. Generally, HPLC-MS/MS is a good choice for metabolomic profiling studies as it detects a larger amount of compounds of varying polarities than does GC-MS. However, HPLC-MS/MS analysis is limited to studies of the mid-highly polar compounds

Table 4: Qualitative HPLC-MS/MS analysis of the T. ferdinandiana leaf extracts, elucidation of empirical formulas and identification (where possible) of the compound.

Putative identification	Empirical formula	Molecular mass	Retention time	% peak area	
				Negative ionisation mode	Positive ionisation mode
protocatechuic acid	$C_7 H_6 O_4$	154.0272	0.522	2.34	
Ethyl 4-hydroxybenzoate	$C_9 H_{10} O_3$	166.063	0.632	0.69	
(1S,5R)-4-Oxo-6,8-dioxabicyclo [3.2.1]oct-2-ene-2-carboxylic acid	$C_7 H_6 O_5$	170.0219	0.417	7.22	
Gabapentin	$C_9 H_{17} NO_2$	171.126	8.996		1.3
shikimic acid	$C_7 H_{10} O_5$	174.0539	0.395	11.54	
2-tert-Butyl-4-methoxyphenol	$C_{11} H_{16} O_2$	180.1153	14.724		1.18
Mannitol	$C_6 H_{14} O_6$	182.0793	0.505		0.56
Valdipromide	$C_{11} H_{23} NO$	185.1785	0.91		1.41
Benzenemethanol, 2-(2-aminopropoxy) -3-methyl-	$C_{11} H_{16} O_3$	196.1106	9.988	1.31	
11-amino-undecanoic acid	$C_{11} H_{23} N O_2$	201.1734	0.691		6.67
jasmonic acid	$C_{12} H_{18} O_3$	210.1257	11.536		0.15
vanilpyruvic acid	$C_{10} H_{10} O_5$	210.0529	0.632	0.58	
	$C_9 H_6 O_7$	226.0117	0.871	0.59	
Heptylheptanoate	$C_{14} H_{28} O_2$	228.2092	20.989	0.51	
	$C_{11} H_8 O_7$	252.0272	1.808	0.93	
Diprophylline	$C_{10} H_{14} N_4 O_4$	254.1012	0.512		1.5
palmitic acid	$C_{16} H_{32} O_2$	256.2412	23.868	3.38	
2-cyclohexylpiperidine oxalate	$C_{13} H_{23} N O_4$	257.1631	3.205		0.7
	$C_{15} H_{21} N_5$	271.1794	7.743		1.66
Exifone	$C_{13} H_{10} O_7$	278.0433	9.32	1	
	$C_{12} H_{10} O_8$	282.0382	0.406	0.39	
N-(2,3-Dimethylphenyl)-1,3,5-triazaspiro[5.5]undeca-1,4-diene-2,4-diamine	$C_{16} H_{23} N_5$	285.1943	4.277		0.56
	$C_{14} H_4 N_4 O_4$	292.0228	7.434		1.51
	$C_{14} H_4 N_4 O_4$	292.0229	2.254	5.15	
gingerol	$C_{17} H_{26} O_4$	294.1836	13.017	1.25	
	$C_{17} H_{25} N_5$	299.211	8.158		1.09
TEGASEROD	$C_{16} H_{23} N_5 O$	301.19	2.726		9.12
	$C_{13} H_8 O_9$	308.017	0.751	1.04	
	$C_{14} H_4 N_4 O_5$	308.0173	0.978	1.02	
9,13-dihydroxy-11-octadecenoic acid	$C_{18} H_{34} O_4$	314.2462	20.261	0.26	
	$C_{17} H_{25}$ N5 O	315.2059	7.359		15.57
Naphtho[2'',3'':4',5']imidazo[2',1':2,3] [1,3]thiazolo[4,5-b]quinoxaline	$C_{19} H_{10} N_4 S$	326.0647	0.555	1.42	

9,12,13-trihydroxy-10,15-octadecadienoic acid	$C_{18}H_{32}O_5$	328.2251	11.03	0.25	
ellagic acid dihydrate	$C_{14}H_{10}O_{10}$	338.0281	1.085		0.77
trimethylellagic acid	$C_{17}H_{12}O_8$	344.0538	14.237	1.3	0.85
chebulic acid	$C_{14}H_{12}O_{11}$	356.0391	0.356	1.84	2.99
	$C_{15}H_{38}N_{10}O_2$	390.3178	29.379	3	
Val His Phe	$C_{20}H_{27}N_5O_4$	401.2059	7.411		0.67
PheLeu His	$C_{21}H_{29}N_5O_4$	415.222	8.951		5.37
Pro Trp Lys	$C_{22}H_{31}N_5O_4$	429.238	10.297		4.1
vitexin	$C_{21}H_{20}O_{10}$	432.1064	9.397	0.77	5.59
1alpha,25-dihydroxy-26,27-dimethyl-22,22,23,23-tetradehydro vitamin D3 / 1alpha,25-dihydroxy-26,27-di	$C_{29}H_{44}O_3$	440.3262	6.929		0.14
luteolin	$C_{21}H_{20}O_{11}$	448.1025	8.73	2.84	7.99
	$C_{21}H_{12}N_4O_9$	464.0606	8.394		4.73
Meclocycline	$C_{22}H_{21}ClN_2O_8$	476.0962	13.012		0.78
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	478.1112	9.854	0.27	
	$C_{20}H_{18}O_{14}$	482.0701	0.593	0.7	
	$C_{26}H_{16}O_{16}$	584.0442	2.249	0.74	
	$C_{31}H_{48}O_4$	484.3525	4.256		0.66
	$C_{31}H_{48}O_4$	484.3529	6.545		1.18
	$C_{29}H_{20}N_4O_{10}$	584.1174	10.667		0.57
	$C_{28}H_{14}N_{14}O_3$	594.1377	12.355	0.26	
NORSTICTIC ACID PENTAACETATE	$C_{28}H_{24}O_{15}$	600.1113	9.468	0.37	0.48
	$C_{26}H_{16}N_{10}O_{10}$	628.1062	14.188	0.3	
corilagin	$C_{27}H_{22}O_{18}$	634.0819	7.803	10.74	0.69
Diethyl 1,7-bis(2,4-dinitrophenyl)-1,7-dihydrodipyrazolo[3,4-b:4',3'-e] pyrazine-3,5-dicarboxylate	$C_{24}H_{16}N_{10}O_{12}$	636.0976	8.345	9.67	
	$C_{23}H_{16}N_{10}O_{13}$	640.0908	1.623	0.15	
	$C_{32}H_{56}N_{20}$	720.5001	31.075		1.2
Diadenosine triphosphate	$C_{20}H_{27}N_{10}O_{16}P_3$	756.0815	11.293		0.41
punicalin	$C_{34}H_{22}O_{22}$	782.0621	9.363		5.65
castalagin	$C_{41}H_{26}O_{26}$	934.0715	8.692	1.1	
chebulagic acid (isomer 1)	$C_{41}H_{30}O_{27}$	954.0957	7.654	1.32	
chebulagic acid (isomer 2)	$C_{41}H_{30}O_{27}$	954.0965	9.878	2.25	

and is not as useful for studies aimed at highly non-polar compounds. The terpenoids are relatively nonpolar compounds and it is possible that our analysis protocol was unable to detect them. However, this is unlikely as we have used this protocol routinely in our lab and have previously detected low polarity compounds including several of these terpenoids. It is more likely that if present, their levels were below the threshold of detection in our system and that they did not contribute significantly to the potent antibacterial activity reported here.

Our findings also indicate that *T. ferdinandiana* leaf extracts displayed low toxicity towards *Artemia franciscana*. Indeed, the methanol, water, chloroform and hexane extracts all had LC50 values well in excess of 1000 μg/ml. Only the ethyl acetate extract displayed significant toxicity with an LC50 value of 767 μg/ml. As an LC50of ≥ 1000 μg/ml is defined as nontoxic,[38] this extract is considered to be of only low to moderate toxicity.

CONCLUSION

The results of this study demonstrate the potential of *T. ferdianadiana* leaf extracts to block the growth of bacterial species associated with the onset of several autoimmune inflammatory diseases. Thus, *T. ferdianadiana* leaf extracts have potential in the prevention and treatment of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis 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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