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Kakadu plum fruit extracts inhibit growth of the bacterial triggers of rheumatoid arthritis: Identification of stilbene and tannin components

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ABSTRACT

Rheumatoid arthritis is an autoimmune disease which can be triggered in genetically susceptible individuals by Proteus spp. infections. *Terminalia ferdinandiana* (Kakadu plum) fruit extracts were investigated by disc diffusion assay against reference and clinical strains of *Proteus mirabilis* and *Proteus vulgaris* and their MIC values were determined. Polar extracts displayed potent antibacterial activity against the bacterial triggers of rheumatoid arthritis, with MIC values as low as 32 μg/ml (methanolic extract against the *P. mirabilis* reference strain). The aqueous extract was also a potent inhibitor of Proteus growth (MIC values <300 μg/ml against all bacterial species). Whilst substantially less potent, the ethyl acetate and chloroform extracts also displayed moderate to good inhibition (as determined by MIC) against both *P. mirabilis* strains. All *T. ferdinandiana* fruit extracts were nontoxic in the *Artemia franciscana* bioassay. The most potent extract (methanolic extract) was analysed by HPLC-QTOF mass spectroscopy (with screening against 3 compound databases). Five stilbenes and 7 tannins were identified in the methanolic extract. The low toxicity of the *T. ferdinandiana* fruit extracts and their potent inhibitory bioactivity against some bacterial triggers of rheumatoid arthritis indicates their potential as medicinal agents in the treatment and prevention of this disease.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease which may afflict genetically susceptible individuals. There are currently no cures for RA and current treatment strategies aim to alleviate the symptoms (particularly pain and swelling) via the use of analgesics and anti-inflammatory agents, and/or to modify the disease process through the use of disease modifying anti-rheumatic drugs (DMARDs). None of

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Chemical compounds: Chebulic acid (PubChem CID: 72284); Combretastatin A1 (PubChem CID: 6078282); Corilagin (PubChem CID: 73568); Diethylstilbestrol (PubChem CID: 448537); Ellagic acid (PubChem CID: 5281855); Ethyl gallate (PubChem CID: 13250); Gallic acid (PubChem CID: 370); Gallocatechin (PubChem CID: 9882981); Piceid (PubChem CID: 5281718); Resveratrol (PubChem CID: 445154).

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these treatments is ideal as prolonged usage of these drugs can result in unwanted side effects and toxicity (Alataha, Kapral, & Smolen, 2003). There is a need to develop safer, more effective drugs for the treatment of RA which will not only alleviate the symptoms, but which may also cure or prevent the disease.

Eradication of the cause of an inflammatory disease is an attractive target for drug design as this would not only block/decrease the late phase inflammatory symptoms, but would also block the immune response and subsequent tissue damage associated with auto-immune inflammatory disorders. Whilst the causes of RA are not comprehensively understood, it is generally accepted that it is an autoimmune disorder which is triggered by specific microbial infections in genetically susceptible individuals (individuals with the MHC class 2 allele HLA-DR4) (Nepom et al., 1989). *Proteus mirabilis* infections have been proposed as a trigger of rheumatoid arthritis as elevated serum levels of *P. mirabilis* specific cross-reactive antibodies have frequently been reported in individuals suffering from RA (Blankenberg-Sprenkels et al., 1998; Chou, Uksila, & Toivanen, 1998; Rashid et al., 1999; Senior et al., 1995; Subair et al., 1995; Wanchu et al., 1997). *P. mirabilis* infections have also been frequently reported in urine samples from patients with RA (Senior et al., 1999). Furthermore, *P. mirabilis* antibodies from RA patients have cytopathic effects on joint tissue possessing *P. mirabilis* cross-reactive antibodies (Rashid & Ebringer, 2011) and sera from rabbits immunised with HLA-DR4 positive lymphocytes bind specifically to Proteus (Ebringer et al., 1985). Amino acid sequence homologies have been identified between the 'EQ/KRRAA' motif present in RA HLA-susceptibility antigens and the 'ESRRAL' amino acid sequence present in *P. mirabilis* haemolysins (Ebringer et al., 1992). Further sequence homology between the 'LRREI' sequence of type XI collagen (present in joint cartilage) and the 'IRRET' motif present in *P. mirabilis* urease enzyme has also been reported (Wilson et al., 1995).

Many antibiotics are already known to inhibit Proteus spp. growth and/or have bactericidal effects towards Proteus spp. However, the development of super resistant bacterial strains has resulted in currently used antibiotic agents failing to end many bacterial infections. 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. Recent studies have examined the anti-*P. mirabilis* activity of conventional antimicrobials such as carbapenems (Lee et al., 2011) and of complementary and alternative therapies including nano-metallic preparations (Cock et al., 2012) and traditional South African medicinal plants (Cock & van Vuuren, 2014). A re-examination of functional foods for the treatment of inflammation and rheumatic conditions is an attractive prospect as the antiseptic qualities of medicinal plants have 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.

Terminalia ferdinandiana is an endemic Australian plant which has been reported to have an extremely high antioxidant content (Netzel et al., 2007). 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 (Woods, 1995). This is approximately 900 times higher (g/g) than the ascorbic acid content in blueberries (which were used as a standard). *T. ferdinandiana* has previously been shown to have strong antibacterial activity against an extensive panel of bacteria (Cock & Mohanty, 2011). 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 readily crossed 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 RA (Courtney et al., 2015). That study indicated that the inhibition of the bacterial triggers of RA 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 rigorously evaluated for the ability to inhibit Proteus spp. 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 RA aetiology and to determine if the fruit extracts have similar phytochemical compositions to the leaf extracts.

2. Materials and methods

2.1. T. ferdinandiana *fruit pulp samples*

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

2.2. *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 h 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% dimethyl sulphoxide). The extract was passed through 0.22 μm filter (Sarstedt) and stored at 4 °C.

2.3. *Qualitative phytochemical studies*

Phytochemical analysis of the *Tasmannia stipitata* extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides,

anthraquinones, tannins and alkaloids was conducted by previously described assays (Boyer & Cock, 2013; Sautron & Cock, 2014).

2.4. Antioxidant capacity

The antioxidant capacity of each sample was assessed using the DPPH free radical scavenging method with modifications (Arkhipov et al., 2014). 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 absorbances were recorded at 515. All tests were performed in triplicate and triplicate controls were included on each plate. The antioxidant capacity based on DPPH radical scavenging ability was determined for each extract and expressed as μg ascorbic acid equivalents per gram of original plant material extracted.

2.5. Antibacterial screening

2.5.1. Test microorganisms

All media were supplied by Oxoid Ltd. Reference strains of *P. mirabilis* (ATCC21721) and *Proteus vulgaris* (ATCC21719) were purchased from American Tissue Culture Collection, Manassas, VA, USA. Clinical strains of *P. mirabilis* 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.

2.5.2. Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay (Hart et al., 2014; Kalt & Cock, 2014). Briefly, 100 μl of the test bacteria were grown in 10 ml of fresh nutrient broth media until they reached a count of approximately 10^8 cells/ml. An amount of 100 μl of bacterial suspension was spread onto nutrient agar plates. The extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were impregnated with 10 μl of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4 °C for 2 h before incubation with the test microbial agents. Plates were incubated at 30 °C for 24 h, then the diameters of the inhibition zones were measured in millimetres. 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.

2.5.3. Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the extracts were determined as previously described (Vesoul & Cock, 2012; Winnett et al., 2014). 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.

2.6. Toxicity screening

2.6.1. Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Gillman, SA, 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.

2.6.2. A. franciscana nauplii toxicity screening

Toxicity was tested using a modified *A. franciscana* nauplii lethality assay (Ruebhart, Wickramasinghe, & Cock, 2009; Sirdaarta & Cock, 2008). Briefly, 400 μl of seawater containing approximately 46 (mean 46.3, n = 124, SD 12.8) *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 s. 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.

2.7. Non-targeted HPLC-MS QTOF analysis

Chromatographic separations were performed as previously described (Arkhipov et al., 2014; Courtney et al., 2015). Briefly, 2 μl of sample were injected onto an Agilent 1290 HPLC system fitted with a Zorbax Eclipse plus C18 column (2.1 × 100 mm, 1.8 μm particle size). The mobile phases consisted of (A) ultrapure water and (B) 95:5 (v/v) 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 to 30 min, followed by 3 min isocratically at 100%. Mass spectrometric analysis was performed on an Agilent 6530 quadrupole time-of-flight spectrometer fitted with a Jetstream electrospray ionisation source in both positive and negative mode.

Table 1 – 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)	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	+	–	–	–	–
	Flavonoids	+++	+++	++	–	–
	Tannins	++	++	–	–	–
Anthraquinones	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. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; AA = ascorbic acid. Antioxidant capacity determined by DPPH reduction (expressed as mg AA equivalence per g plant material extracted).

Data were analysed using the Masshunter 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 (7509 compounds). Empirical formula for unidentified compounds was determined using the Find Formula function in the software package.

2.8. *Statistical analysis*

Data are expressed as the mean ± SE of at least three independent experiments. One way ANOVA followed by Dunnett test analysis was used to calculate statistical significance between control and treated groups with a P value <0.01 considered to be statistically significant.

3. Results

3.1. *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 1). 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 1.

Qualitative phytochemical studies (Table 1) 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 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 levels 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.

3.2. *Antioxidant content*

Antioxidant capacity (expressed as ascorbic acid equivalents) for the *T. ferdinandiana* fruit extracts are shown in Table 1. 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 a high antioxidant capacity with 264 mg ascorbic acid equivalents per gram of dried plant material extracted.

3.3. *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

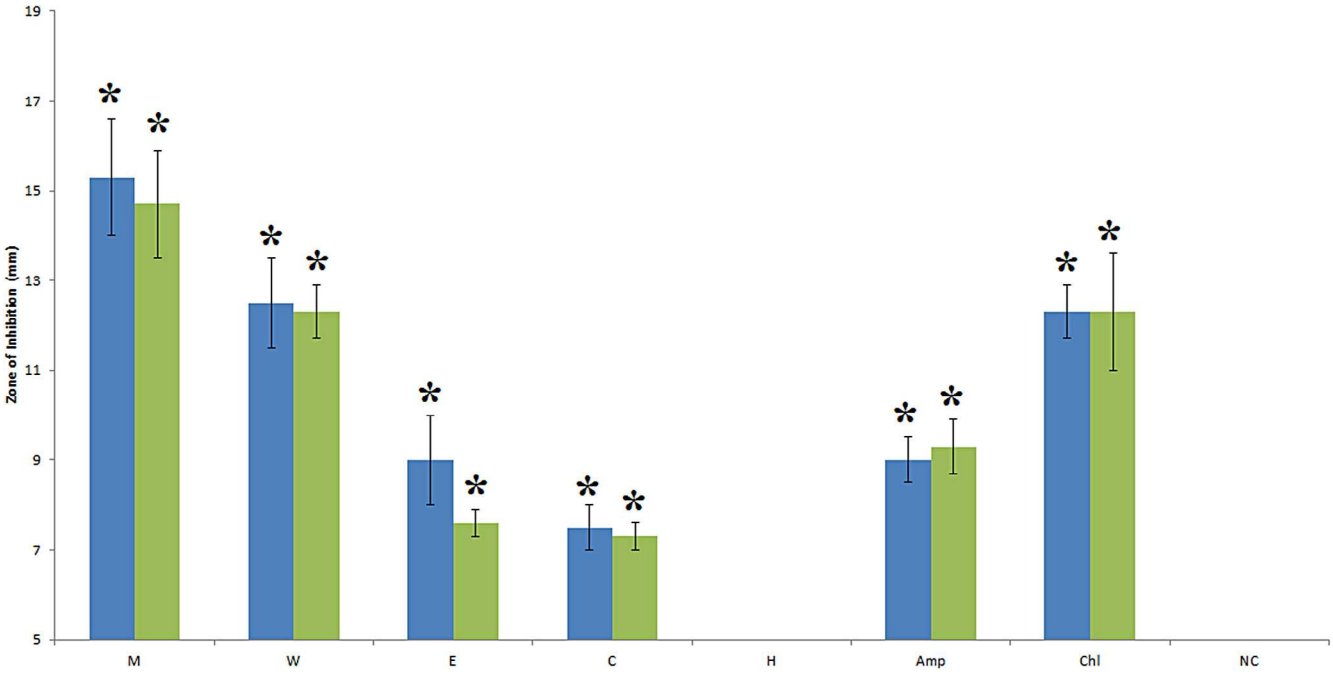

Fig. 1 – Antibacterial activity of *T. ferdinandiana* fruit extracts against *P. mirabilis* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:21721) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 μg) control; Chl = chloramphenicol (10 μg) control; NC = negative control (deionised water). Results are expressed as mean zones of inhibition ± SE. *Indicates results that are significantly different to the negative control ($P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as microbial triggers of RA. Both reference and clinical strains of *P. mirabilis* were strongly inhibited by the methanol and water *T. ferdinandiana* fruit extracts (Fig. 1). Indeed, these extracts inhibited *P. mirabilis* growth of both strains more effectively than the ampicillin and chloramphenicol controls, with zones of inhibition generally >12 mm against both strains (compared to approximately 9 mm for ampicillin and 12 mm for chloramphenicol). The ethyl acetate and chloroform extracts also significantly inhibited *P. mirabilis* growth, albeit with smaller zones of inhibition.

The *P. mirabilis* reference strain was more susceptible to the methanol and water extracts than was the clinical strain (as determined by the zones of inhibition) (Fig. 1). The methanol extract was the most potent bacterial growth inhibitor, with zones of inhibition of 15.3 and 14.7 mm for the reference and clinical strains respectively. The aqueous extract was also a potent inhibitor of the reference strain of *P. mirabilis* growth with zones of inhibition of 12.5 and 12.3 mm for the reference and clinical strains respectively.

P. vulgaris growth was also susceptible to the methanol, water, ethyl acetate and chloroform extracts (Fig. 2). In contrast with the inhibition of *P. mirabilis* growth where the methanol extract was the most potent growth inhibitor, the ethyl acetate extract was the most potent inhibitor of *P. vulgaris* growth. Zones of inhibition of 10.3, 8.7, 11 and 6.6 mm were noted for *P. vulgaris* against the methanol, water, ethyl acetate and chloroform extracts 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 2). 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). The methanolic extract was particularly potent, with MIC values <75 μg/ml against all Proteus strains (<0.8 μg impregnated in the disc), indicating the potential of these extracts in inhibiting the microbial triggers of RA and limiting its impact. Water was similarly potent, with MIC values <300 μg/ml against all Proteus strains (<3 μg impregnated in the disc).

3.4. *Quantification of toxicity*

T. ferdinandiana fruit extracts were initially screened at 2000 μg/ml in the assay (Fig. 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 h of exposure and 100% mortality was evident following 4–5 h (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.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in artificial seawater to test across a range of concentrations in the Artemia nauplii bioassay at 24 h. Table 3 shows the LC50 values

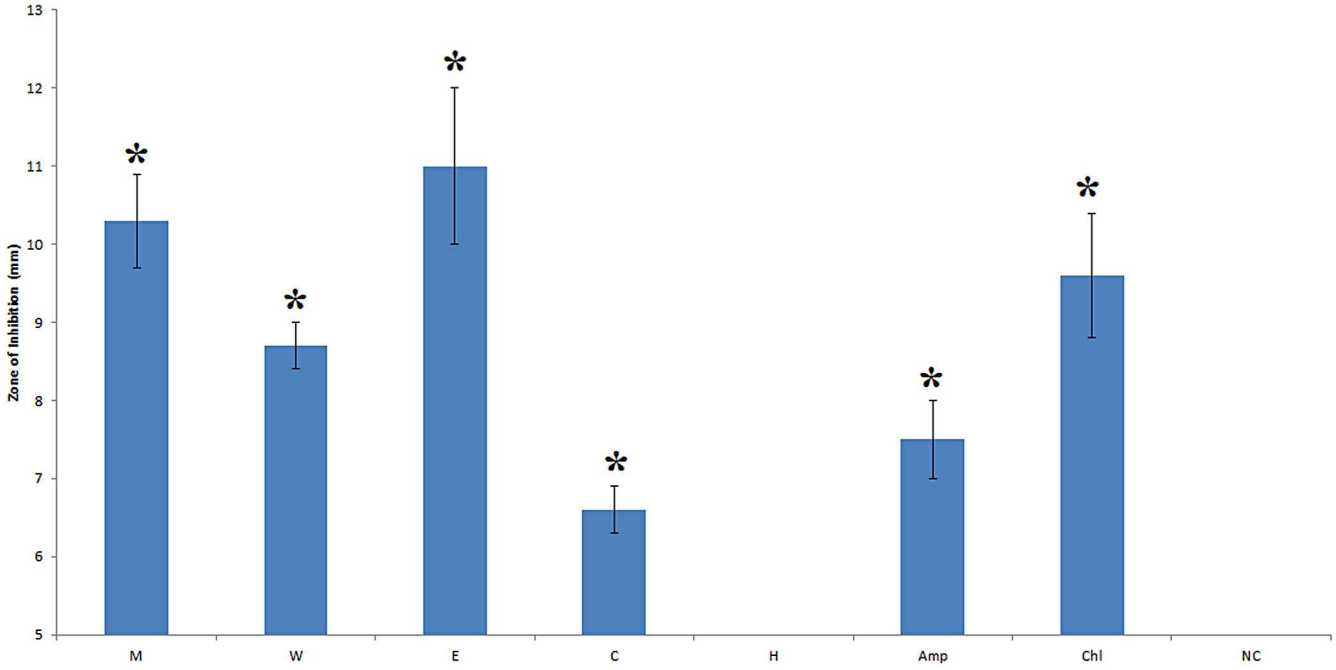

Fig. 2 – Antibacterial activity of *T. ferdinandiana* fruit extracts against the *P. vulgaris* reference strain (ATCC:21719) measured as zones of inhibition (mm). M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 μg) control; Chl = chloramphenicol (10 μg) control. NC = negative control (deionised water). Results are expressed as mean zones of inhibition ± SE. *Indicates results that are significantly different to the negative control (P < 0.01).

of the *T. ferdinandiana* 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 (Cock & Ruebhart, 2009). As none of the extracts had a LC50 <1000 μg/ml, all were considered nontoxic.

3.5. *HPLC-MS QTOF analysis*

As the methanol extract had the greatest antibacterial efficacy against all Proteus strains (as determined by MIC), it was deemed the most promising extract for further phytochemical analysis. This extract was examined for the presence of tannins and stilbenes as both of these classes of compound have been reported to be potent antibacterial agents (Paulo et al., 2010; Wu-Yuan, Chen, & Wu, 1988). Furthermore, stilbenes have also been reported to be potent anti-inflammatory agents. Therefore, if present, they may provide further therapeutic effects against other phases of the disease progression.

Optimised HPLC-MS QTOF parameters used previously for the analysis of *T. ferdinandiana* leaf extracts (Courtney et al., 2015) were also used to determine the methanolic fruit extract compound profile in this study. The resultant total compound chromatograms for the positive ion and negative ion chromatograms are presented in Fig. 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* methanolic extract positive ion base peak chromatogram (Fig. 4a) revealed multiple overlapping peaks in the early to middle stages of the chromatogram corresponding to the elution of polar compounds. Most of the extract compounds had eluted by 10 minutes of the chromatogram, corresponding to approximately 20% acetonitrile. However,

Table 2 – Minimum inhibitory concentration of Kakadu plum fruit extracts and LC50 values in the Artemia nauplii bioassay.

		Methanol	Water	Ethyl Acetate	Chloroform	Hexane
MIC (μg/ml)	*P. mirabilis* (reference strain)	32.4	87.8	513.6	783.9	–
	P. mirabilis (clinical strain)	46.3	188.7	843.3	2289	–
	P. vulgaris (reference strain)	73.8	294.5	1798	–	–
	LC50 (μg/ml)	2115	2080	–	–	–

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

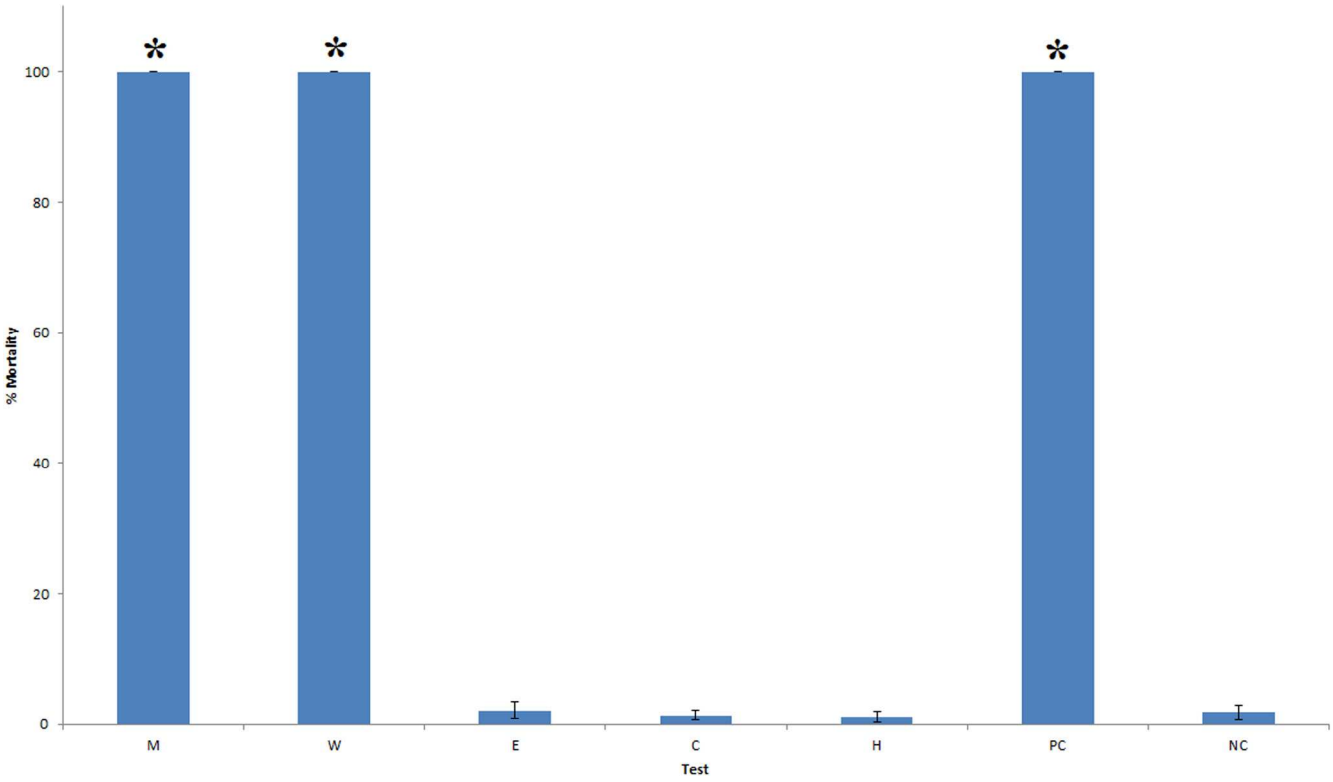

Fig. 3 – The lethality of *T. ferdinandiana* fruit extracts (2000 μg/ml) and potassium dichromate control (1000 μg/ml) towards *Artemia franciscana* nauplii after 24 hour exposure. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; PC = potassium dichromate control; NC = negative (seawater) control. Results are expressed as mean ± SE. *Indicates results that are significantly different to the negative control ($P < 0.01$).

several small peaks and 1 prominent peak in the positive ionisation mode chromatogram between 11 and 16 min indicate the broad spread of polarities of the compounds in this extract. This later eluting peak was not apparent in the *T. ferdinandiana* fruit methanol extract negative ion chromatogram (Fig. 4b).

In total, 556 unique mass signals were noted for the *T. ferdinandiana* fruit methanol extract. Putative empirical formulas were achieved for all of these compounds. Of the 556 unique molecular mass signals detected, 7 tannin compounds were putatively identified by comparison to the Metlin metabolomics, forensic toxicology (Agilent) and phytochemicals (developed in this laboratory) databases (Table 3). Three different ethyl gallate isomers (Figs. 5a–c) were detected. However, as mass spectral analysis cannot distinguish between different isomeric forms, we were unable to assign individual

Table 3 – Tannin and stilbene compounds detected in the Kakadu plum methanolic extract.

Compound Class	Putative Identification	Molecular Formula	Molecular Mass	Retention Time (min)	Detection Mode	
					Positive mode	Negative Mode
Tannins	Ethyl gallate isomer 1	$C_9H_{10}O_5$	198.0529	2.33	X	X
	Ethyl gallate isomer 2	$C_9H_{10}O_5$	198.0529	4.871	X	X
	Ethyl gallate isomer 3	$C_9H_{10}O_5$	198.0529	6.065	X	X
	Gallocatechin	$C_{15}H_{14}O_7$	306.0768	6.142	X	X
	Trimethyl ellagic acid	$C_{17}H_{12}O_8$	344.0536	9.798	X	X
	Chebulic acid	$C_{14}H_{12}O_{11}$	356.0389	1.256	X	X
	Corilagin	$C_{27}H_{22}O_{18}$	634.0815	4.943		X
Stilbenes	Resveratrol	$C_{14}H_{12}O_3$	228.2433	6.235	X	
	Combretastatin	$C_{18}H_{22}O_6$	334.1403	8.001	X	
	Combretastatin A-1	$C_{18}H_{20}O_6$	332.1245	6.94	X	
	Diethylstilbestrol monosulphate	$C_{18}H_{20}O_5S$	348.1036	1.805	X	
	Resveratrol glucoside	$C_{20}H_{22}O_8$	390.1333	6.924	X	

X indicates the ionisation mode that each compound was detected in.

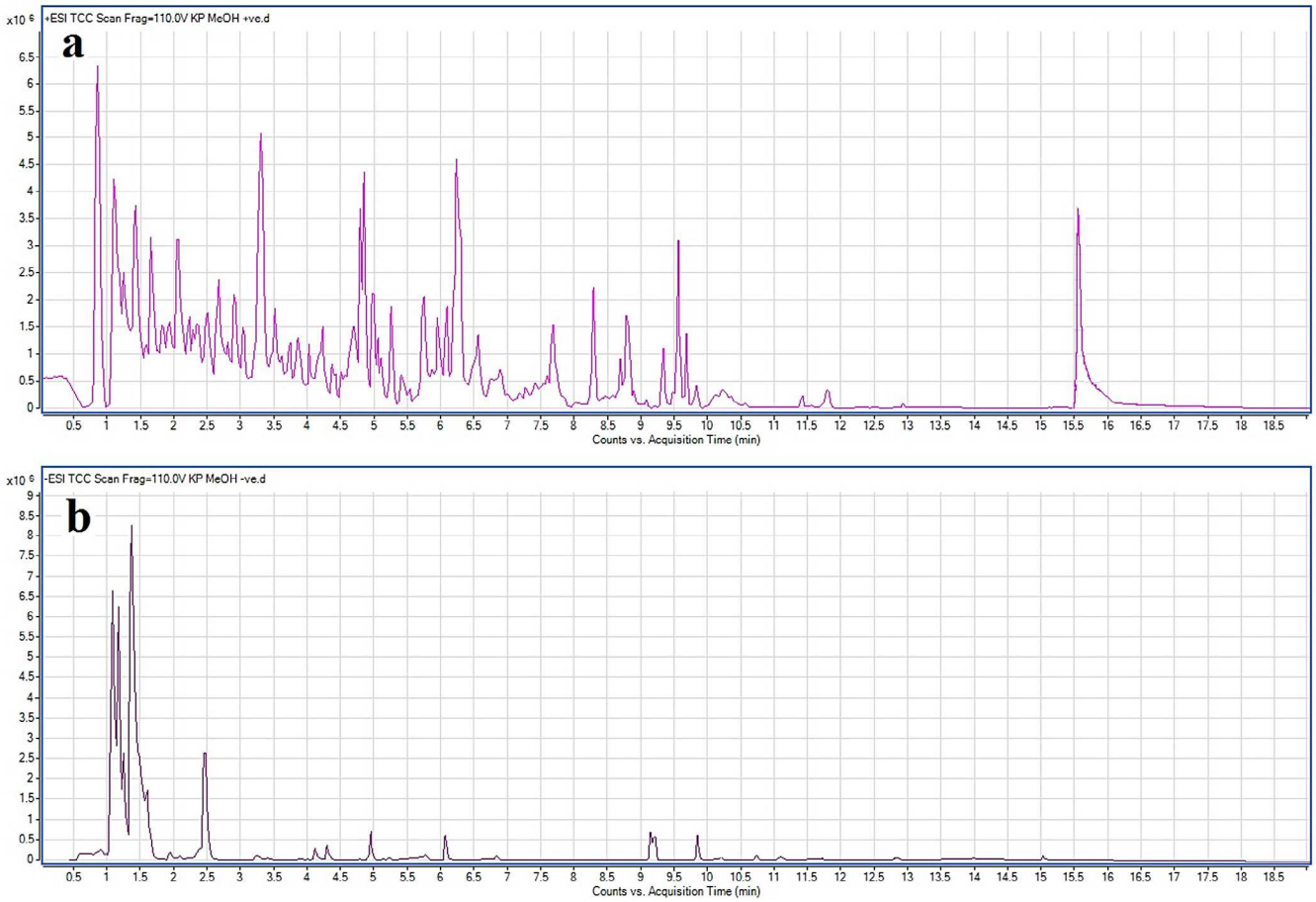

Fig. 4 – (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 μl injections of *T. ferdinandiana* fruit methanolic extract.

structures to each of the compounds. The tannins gallocatechin (Fig. 5d), trimethyl-ellagic acid (Fig. 5e), chebulic acid (Fig. 5f) and corilagin (Fig. 5g) were also putatively identified in the methanolic *T. ferdinandiana* fruit extract. The stilbenes resveratrol (Fig. 5h), combretastatin (Fig. 5i) and combretastatin A1 (Fig. 5j) were also detected and putatively identified. Furthermore, the sulphated stilbene diethylstilbestrol monosulphate (Fig. 5k) and glycosylated resveratrol (piceid) (Fig. 5l) were also putatively identified.

4. Discussion

Previous studies within our laboratory have reported potent antibacterial activity for *T. ferdinandiana* fruit extracts (Cock & Mohanty, 2011). Recently, we also reported growth inhibitory activity of *T. ferdinandiana* leaf extracts against some microbial triggers of selected autoimmune inflammatory diseases (Courtney et al., 2015). 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, corilagin, 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 lipoteichoic acid and proline-rich cell surface proteins (Hogg & Embery, 1982; Machado et al., 2003), and by inhibiting glucosyltransferase enzymes (Buzzini et al., 2008). In contrast to our previous leaf study, the only gallotannins detected in the *T. ferdinandiana* fruit extracts by LC–MS analysis were the 3 isomeric forms of ethyl gallate and gallocatechin. Whilst these tannins have been reported to be potent antibacterial agents (Buzzini et al., 2008; Hogg & Embery, 1982; Machado et al., 2003), they were detected at much lower relative levels than in the leaf extracts (as determined by peak area).

The ellagitannins ellagic acid and corilagin and the ellagitannin component chebulic acid were also putatively identified in this extract. Ellagitannins are highly potent inhibitors of bacterial growth, with MIC values as low as 62.5 μg/ml (Machado et al., 2003; Wu-Yuan et al., 1988). Ellagitannins have been reported to function via several antibiotic mechanisms including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls (Buzzini et al., 2008; Hogg & Embery, 1982). It is likely that both the gallotannin and ellagitannin components contribute to the anti-Proteus activity of the methanolic *T. ferdinandiana* fruit extract.

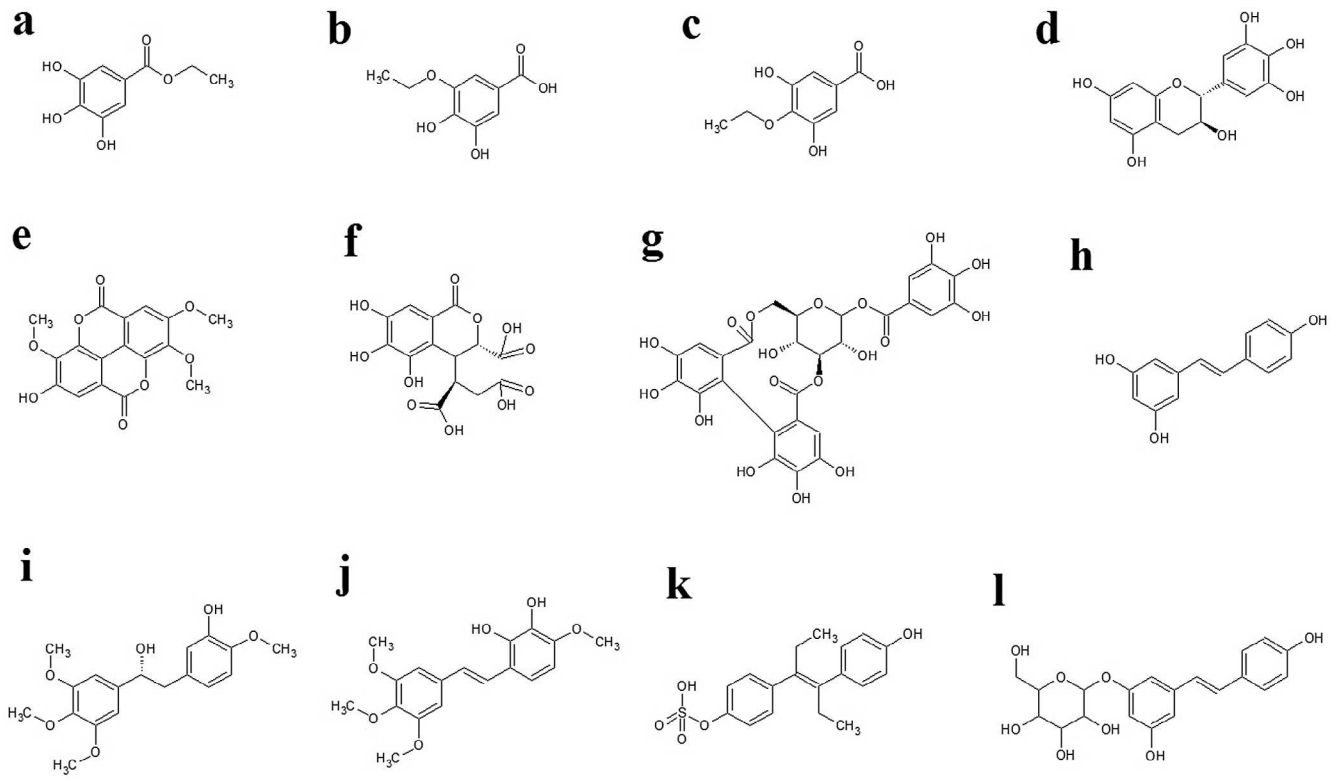

Fig. 5 – Chemical structures of *T. ferdinandiana* fruit compounds detected in the methanolic extract: (a–c) ethyl gallate isomers; (d) gallocatechin; (e) trimethyl ellagic acid; (f) chebulic acid; (g) corilagin; (h) resveratrol; (i) combretastatin; (j) combretastatin A1; (k) Diethylstilbestrol monosulphate; (l) resveratrol glucoside (piceid).

As much lower tannin amounts and diversity were detected in the *T. ferdinandiana* fruit methanolic extract compared to the leaf extract (Courtney et al., 2015), it is likely that other phytochemical classes may contribute to the antibacterial and anti-inflammatory properties of this extract. Stilbenes are phytoalexins produced by plants for protection against microbes and thus may contribute to the bacterial growth inhibitory activities reported here. Resveratrol, which was identified in the methanolic *T. ferdinandiana* fruit extract in our study, has been particularly well studied and has been reported to have inhibitory activity against multiple bacterial species (Paulo et al., 2010). However, pure resveratrol was unable to inhibit the growth of *P. mirabilis* in our studies, even at high concentrations (unreported results). Furthermore, we were unable to find any reports of *in vitro* anti-Proteus growth inhibition in the published literature. Several studies have reported on the ability of resveratrol to inhibit *P. mirabilis* swarming and virulence factor expression *in vivo*, so it is likely that resveratrol does affect *P. mirabilis* colonisation and infection of the urinary tract (Wang et al., 2006), albeit possibly by mechanisms other than bactericidal or growth inhibition mechanisms.

Furthermore, resveratrol is a potent specific inhibitor of NF-κB activation via its induction by TFN-α and IL-1β (Elmali et al., 2007). Thus, resveratrol treatment directly blocks cytokine production and inflammation via its inhibition of NF-κB activation. Any extract capable of inhibiting Proteus spp. growth and also containing resveratrol would be likely to have anti-RA activity via several mechanisms (growth inhibition, colonisation blocking, cytokine production inhibition) and therefore would be particularly effective in treating RA.

Qualitative HPLC-MS/MS analysis of the *T. ferdinandiana* fruit methanolic extract also detected the resveratrol glycoside piceid (2-[3-hydroxy-5[(E)-2-(4-hydroxyphenyl) ethenyl] phenoxy]-6-(hydroxymethyl) oxane-3,4,5-triol). Glycosylated stilbenes may be hydrolysed *in vivo* to remove glucose. Thus the presence of piceid in the extract is likely to result in the release of the resveratrol moiety *in vivo*. Piceid (and other glycosylated stilbenes) has also been shown to block inflammation by decreasing IL-17 production in stimulated human mononuclear cells (Lanzilli et al., 2012).

Other stilbenes were also detected in the *T. ferdinandiana* fruit methanolic extract. Combretastatin and combretastatin A-1 were detected in the methanolic fruit extract. Combretastatins are well known for their potent ability to block cancer cell progression and induce apoptosis by binding intracellular tubulin, thereby disrupting microtubule formation (Dark et al., 1997). Accounts of direct anti-inflammatory activity of combretastatins are lacking. However, it is believed that they act by a similar mechanism to that of colchicine (N-[(7S)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptlen-7-yl] acetamide) by binding the colchicine binding site on the tubulin peptide and inhibiting polymerisation (Bhardwaj et al., 2010). It is likely that they may have a similar anti-inflammatory activity and mechanism to colchicine.

We were also unable to find accounts of the antibacterial activities of natural combretastatins in the literature. However,

recent studies have examined the growth inhibitory activity of several synthetic combretastatin and resveratrol structural analogues (Pettit et al., 2009). These studies reported potent growth inhibition towards a panel of bacteria including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli* and *Neisseria gonorrhoeae*. Especially interesting was the low MIC values of some analogues against *N. gonorrhoeae*, although several other species were also highly susceptible to the modified stilbenes. The same study also reported potent inhibitory effects of the panel of modified stilbenes against the fungal species *Candida albicans* and *Cryptococcus neoformans*.

A number of other inflammatory stilbenes have also previously been reported in other plant species. For example, 2,3,4,5-tetrahydroxystilbene-2-O-β-D-glucoside (TSG) inhibits inflammation by suppressing the induction of pro-inflammatory mediators by reducing NF-κB binding to DNA (Huang et al., 2013). The same study detected TSG in numerous herbs used to treat inflammation in Chinese traditional medicine. Furthermore, nine stilbene and stilbene derivatives isolated from the roots of Cicer spp. (chickpeas) were shown to inhibit bacterial and fungal growth (Aslam et al., 2009). Potent anti-inflammatory effects have been reported for pterostilbene, a methylated resveratrol analogue (Choo et al., 2014). Indeed, pterostilbene is a considerably more potent inhibitor of arthritic synovial fibroblast proliferation than resveratrol. The same study also reported the suppression of NF-κB and several interleukins by pterostilbene. Whilst these compounds were not detected in the compound databases used in our studies, it is possible that they may still be present in small quantities.

Whilst our studies provide insight into the bioactivity and 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 *A. franciscana*. Indeed, the LC50 values for all extracts were well in excess of 1000 μg/ml and are therefore nontoxic.

5. Conclusions

The results of this study demonstrate the potential of *T. ferdinandiana* fruit extracts to block the growth of bacterial species associated with the onset of RA. Thus, *T. ferdinandiana* fruit extracts have potential in the prevention and treatment of RA 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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