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Research Article

Inhibitory activity of high antioxidant Australian native fruits against the bacterial triggers of selected autoimmune diseases

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ABSTRACT

Introduction: High antioxidant capacities have been linked to the treatment of rheumatic diseases and in the inhibition of microbial growth. Recent reports have identified several native Australian fruits with high antioxidant capacities. Despite this, several of these species are yet to be tested for the ability to inhibit the growth of the bacterial triggers of autoimmune inflammatory diseases. **Methods:** Solvent extracts prepared from selectedAustralian native fruits were analysed for antioxidant capacity by the DPPH free radical scavenging assay.Growth inhibitory activities against bacterial species associated with initiating rheumatoid arthritis and ankylosing spondylitiswere determined bydisc diffusion assay and quantified by MIC determination. Toxicity was determined by *Artemia franciscana* bioassay. **Results:** Methanolic extracts of all plant species displayed high antioxidant contents (equivalent to approximately 7-16 mg of vitamin C per gram of fruit extracted). Most aqueous extracts also contained relatively high antioxidant capacities. In contrast, the ethyl acetate, chloroform and hexane extracts for most species (except lemon aspen and bush tomato) had lower antioxidant contents (below 1.5 mg of vitamin C equivalents per gram of plant material extracted). Interestingly, the bacterial growth inhibitory activity of the extracts did not correlate with their antioxidant capacities. The fruit extracts with the highest antioxidant capacities (lemon aspen and desert lime methanolic extracts) had only low antibacterial activity, with MIC values generally >10,000 μg/ml against all bacterial species. In contrast, the Illawarra plum and desert lime ethyl acetate extracts, which had mid-range antioxidant capacities (1-6.5 mg ascorbic acid equivalents/g extracted), had potent bacterial growth inhibitory activity (200-400 μg/ml). The native tamarind ethyl acetate extract displayed low-moderate toxicity in the *Artemia franciscana* bioassay (LC50valuesbelow 1000 μg/mL).All other extracts were nontoxic. **Conclusion:** The lack of toxicity and inhibitory activity against microbial triggers of rheumatoid arthritis and ankylosing spondylitis by the fruit extracts indicates their potential in the treatment and prevention of these diseases.

Key words: Muntries, Illawarra plum, lemon aspen, native tamarind, desert lime, bush tomato, rheumatoid arthritis, ankylosing spondylitis.

INTRODUCTION

Autoimmune inflammatory disorders (e.g. rheumatoid arthritis, ankylosing spondylitis) are a group of debilitating conditions which afflict genetically susceptible individuals. 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.[1] There is a need to develop safer, more effective treatments for these conditions which will not only alleviate the symptoms, but may

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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 some 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*,[2] a normal part of the human gastrointestinal flora. Similarly, *Klebsiella pneumoniae* has been shown to initiate ankylosing spondylitis[3] and *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis.[4] *Borrelia burgdorferi* is linked with Lyme disease.[5] 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.[6] 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.

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.

Several recent studies have demonstrated the potent antimicrobial activity of several fruits with high antioxidant capacities.[16-19] In particular, *Tasmannia lanceolata,*[18] *Terminalia ferdinandiana*[19] and several Syzygium species have demonstrated potent antimicrobial activity against a wide panel of bacteria.[20-23] Despite this, many high antioxidant Australian fruits are yet to be rigorously tested for the ability to inhibit bacterial growth. The current study reports on the growth inhibitory activity of extracts of these fruits against bacterial species associated with the onset of selected autoimmune diseases.

MATERIALS AND METHODS

Plant source and extraction

All plant material was obtained from Taste Australia Bush Food as verified fruits. Voucher samples have been stored in the School of Natural Sciences, Griffith University.The fruits were thoroughly dried in a Sunbeam food dehydrator and the dried plant materials were subsequently stored at -30 ºC. Prior to use, the plant materials were thawed and freshly ground to a coarse powder. Individual 1 g quantities of the ground plant material were weighed into separate tubes and 50 ml of methanol, water, ethyl acetate, chloroform or hexane were added. All solvents were obtained from Ajax and were AR grade. The ground plant materials were individually extracted in each solvent for 24 hours at 4 ºC with gentle shaking. The extracts were subsequently filtered through filter paper (What man No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant dry extract was weighed and redissolved in 10 ml deionised water.

Qualitative phytochemical studies

Phytochemical analysis of the fruitextracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[24-26]

Antioxidant capacity determination

The antioxidant capacity of each sample was assessed using the DPPH free radical scavenging method[27] 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

Table 1: The bacterial triggers of the selected autoimmune inflammatory diseases as well as the bacterial antigen and host susceptibility antigen sequences.

Disease	Bacterial Trigger	Bacterial Antigen	Bacterial Sequence	Host Antigen	Host Sequence	Ref.
Rheumatoid arthritis	Proteus mirabilis and possibly also other Proteus spp.	haemolysin	ESRRAL	MHC class 2 allele HLA-DR4	EQ/KRRAA	2, 3, , 7,
		urease	IRRET	type XI collagen	LRREI	7, 9
Ankylosing spondylitis	Klebsiella pneumoniae	nitrogenase reductase enzyme	QTDRED	MHC class 1 allele HLA-B27	QTDRED	2, 10
		pullulanase	DRDE	MHC class 1 allele HLA-B27	DRED	11
		pullulanase	GxP	types I, III and IV collagen	GxP	12
Multiple sclerosis	Pseudomonas aeriginosa	□-CMLD	TRHAYG	Myelin-neuronal antigen MBP	SRFSYG	13
	Acinetobacter spp.	4-CMLD	SRFAYG	Myelin-neuronal antigen MBP	SRFSYG	13
		3-OACT-A	LTRAGK	Myelin-neuronal antigen MOG	LYRDGK	13
		Acinetobacter regulatory protein	*KKVEEI	Neurofilament-M protein	*KKVEEI	13-15

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.

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 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 *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721) and *Proteus vulgaris* (ATCC21719) 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.[28-31] 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. Inoculated plates were incubated at 30 °C for 24 hours, 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) 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.[32,33] 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 *Artemiafranciscana* nauplii bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.[34-36] Briefly, 400 µl of seawater containing approximately 44 (mean 43.8, n = 120, SD 12.1) *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 24 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.

Statistical analysis

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

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of the various dried Australian plant fruits with the solvents yielded dried plant extracts ranging from 3 mg (Illawarra plum fruit ethyl acetate extract) to 524 mg (muntries methanolic extract) (Table 2). Methanolic extracts generally gave relatively high yields of dried extracted material whilst the aqueous extracts had moderate to high yields for most species. Extraction with chloroform also generally resulted in moderate yields. Ethyl acetate and hexane extracted lower masses for most species. The dried extracts were resuspended in 10 ml of deionised water resulting in the extract concentrations shown in (Table 2).

Qualitative phytochemical studies showed that methanol and water extracted the widest range of phytochemicals for both the fruits (Table 2). All methanolic and aqueous extracts generally showed moderate to high levels of phenolics (both water soluble and insoluble phenolics), flavonoids and saponins, as well as moderate levels of triterpenoids. Muntries and Illawarra plum also showed moderate levels of tannins. Low to moderate levels of alkaloids were also noted for the methanolic and aqueous extracts desert lime and bush tomato. The ethyl acetate extracts generally had similar phytochemical profiles as the methanolic and aqueous extracts, albeit at lower levels. Few phytochemical classes were noted in the chloroform or hexane extracts. As these tests generally screen for polar phenolic compounds, this is perhaps not surprising.

Antioxidant content

Antioxidant capacity (expressed as ascorbic acid equivalence) for the fruit (Table 2) ranged frombelow the level of detection to a high of 15.9 mg ascorbic acid equivalence per gram of dried plant material extracted (lemon aspen fruit methanolic extract). The methanol extracts of all fruits and herbs had higher antioxidant capacities than the corresponding water, ethyl acetate, chloroform or hexane extracts.

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 *Proteus mirabilis* were inhibited by several of the Australian fruitextracts, albeit with relatively small zones of inhibition (Figure 1). The muntries, desert lime and bush tomato methanol extracts, the Illawarra plum, desert lime and bush tomato ethyl acetate extracts and the lemon aspen chloroform extract each inhibited both *P. mirabilis* strains. However, inhibition was weak (as judged by the zones of inhibition), with inhibition zones generally <8 mm in diameter(compared to >11 mm for the ampicillin control).

The growth of *P. vulgaris* was substantially more susceptible to inhibition by the fruit extracts than were the *P. mirabilis* strains (as judged by the zones of inhibition). Interestingly, the nonpolar hexane fruit extracts appeared to be much more potent bacterial growth inhibitors than the more polar extracts such as the methanolic and aqueous extracts (Figure 2). Indeed, zones of inhibition significantly greater than 10 mm were seen for the lemon aspen, desert lime and bush tomato hexane extracts. This is particularly noteworthy when compared to the inhibition of the ampicillin control antibiotic (< 8mm). The

Table 2: The mass of dried extracted plant material, the concentration after resuspension, qualitative phytochemical screenings and antioxidant contents of fruit extracts.

Plant Species	Extract	Mass of Dried Extract (mg)	Resuspended Extract Concentration (mg/ml)	Total Phenolics	Water Soluble	Water Insoluble	Cardiac Glycosides	Saponins	Triterpenes	Polysteroids	Alkaloids (Meyer test)	Alkaloids (Wagners test)	Flavanoids	Tannins	Free Anthraquinones	Combined Anthraquinones	Antioxidant capacity (mg AA equivalency)
Muntries	M	524	52.4	+++	+++	-	-	+++	++	-	-	-	+++	++	-	-	6.9
	W	350	35	+++	+++	+++	-	+++	++	-	-	-	+++	++	-	-	2.9
	E	19	1.9	+	-	+++	-	-	-	-	-	-	+	++	-	-	1.2
	C	120	12	+	-	-	-	-	-	-	-	-	+	-	-	-	0.4
	H	20	2	-	-	-	-	++	-	-	-	-	-	-	-	-	0.2
Illawarra Plum	M	314	31.4	+++	+++	+++	-	+++	++	-	-	-	++	++	+	++	6.8
	W	195	19.5	+++	++	+++	-	+++	++	-	-	-	++	++	++	++	2.7
	E	3	0.3	+	-	+	-	-	-	-	-	-	++	+	-	-	1.2
	C	140	14	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3
	H	50	5	-	-	-	-	++	-	-	-	-	-	-	-	-	0.2
Native Tamarind	M	107	10.7	+++	-	+++	-	++	-	-	-	-	+	-	-	-	9.2
	W	52	5.2	++	-	+++	-	-	-	-	-	-	+	-	++	-	8.4
	E	27	27	+	-	+	++	-	++	-	-	-	+	-	++	++	0.7
	C	120	12	-	-	-	+	++	-	-	-	-	-	-	-	-	0.2
	H	70	7	-	-	-	+	-	-	-	-	-	-	-	-	-	BDT
Lemon Aspen	M	360	36	+++	-	-	+	-	++	-	-	-	+++	-	-	-	15.9
	W	162	16.2	+++	-	-	+	+++	++	-	-	-	+++	-	-	-	7.2
	E	66	6.6	+++	-	+	+++	-	++	-	-	-	-	-	-	-	6.4
	C	180	18	+++	-	+	+	-	-	-	-	-	-	-	-	-	6
	H	70	7	+	-	-	++	-	-	-	-	-	-	-	-	-	3.2
Desert Lime	M	247	24.7	+++	++	++	+	-	++	-	-	++	+++	-	-	-	11.7
	W	182	18.2	+	-	-	+	++	++	-	-	-	+++	-	-	-	6.3
	E	4	0.4	+	-	-	-	-	++	-	-	+	++	-	-	-	1.1
	C	240	24	+	-	-	-	-	++	-	-	+	+	-	-	-	0.7
	H	140	14	-	-	-	-	++	-	-	-	+	-	-	-	-	0.3
Bush Tomato	M	313	31.3	+++	-	+++	-	-	++	-	-	++	++	-	-	-	9.1
	W	79	7.9	+++	++	+++	-	+++	++	-	-	++	+++	++	-	-	5.6
	E	81	8.1	-	-	-	+	-	-	-	-	-	-	-	-	-	3.5
	C	280	28	+	-	-	+	-	++	-	-	+	+	-	-	-	3.7
	H	80	8	-	-	-	-	-	-	-	-	+	-	-	-	-	2.2

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. BDT = below detection threshold.

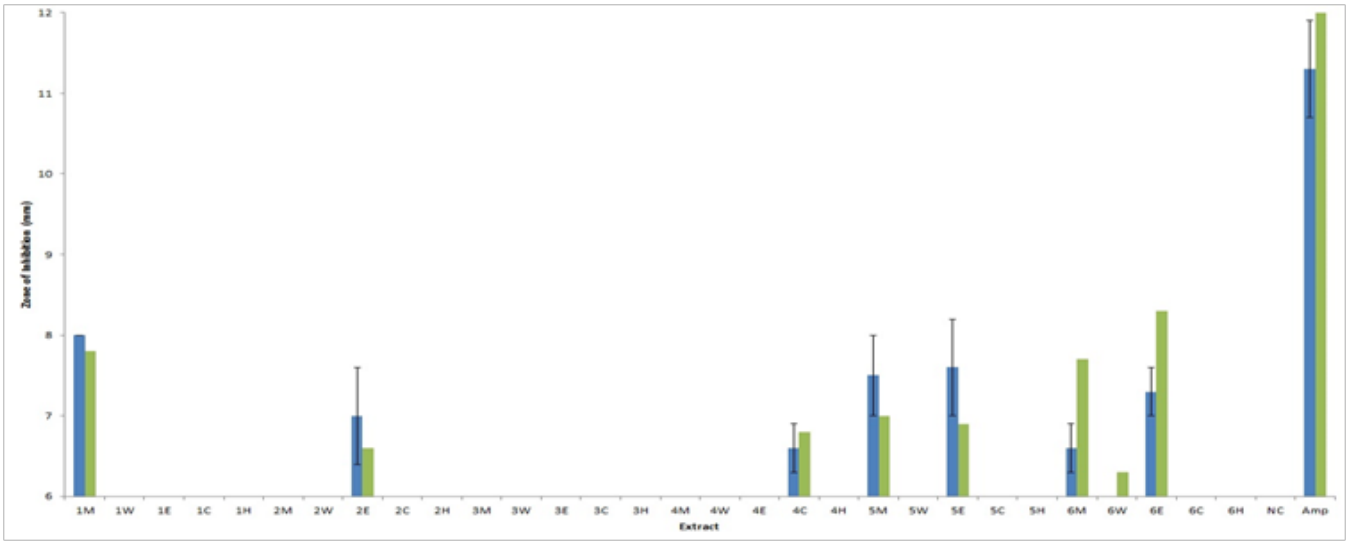

Figure 1: Antibacterial activity of the Australian fruit extracts against P. mirabilis measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC: 21721) and the green bars represent the zones of inhibition against the clinical strain. 1 = muntries; 2 = Illawarra plum; 3 = native tamarind; 4 = lemon aspen; 5 = desert lime; 6 = bush tomato; M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = 0.5% DMSO; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.

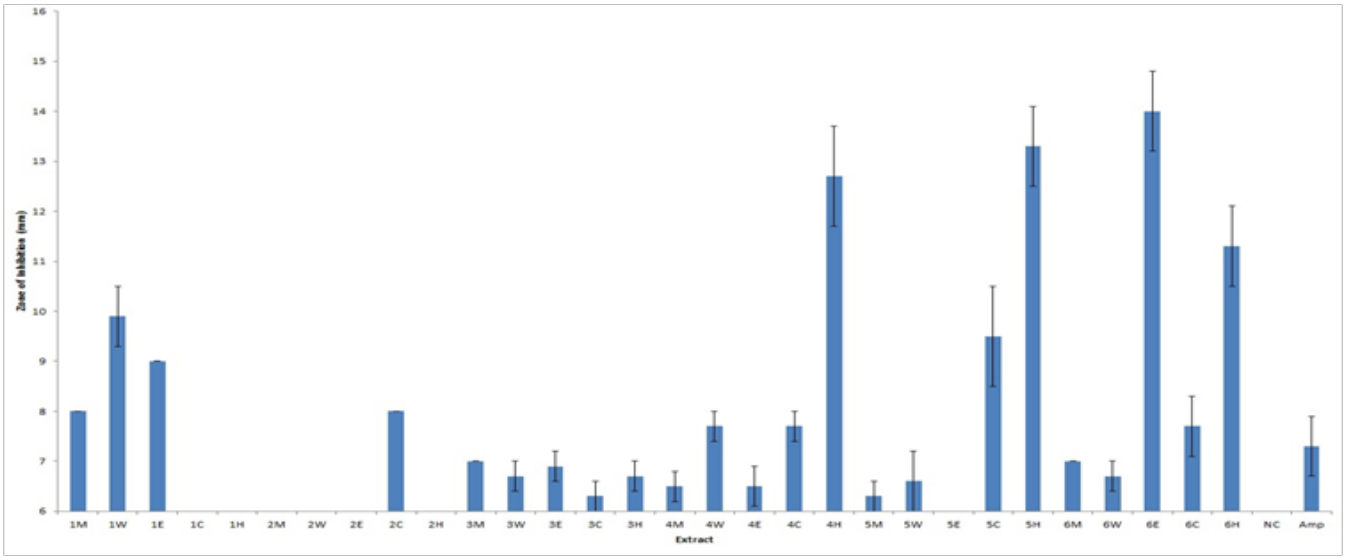

Figure 2: Antibacterial activity of the Australian fruit extracts against P. vulgaris (ATCC21719)measured as zones of inhibition (mm). 1 = muntries; 2 = Illawarra plum; 3 = native tamarind; 4 = lemon aspen; 5 = desert lime; 6 = bush tomato; M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = 0.5% DMSO; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.

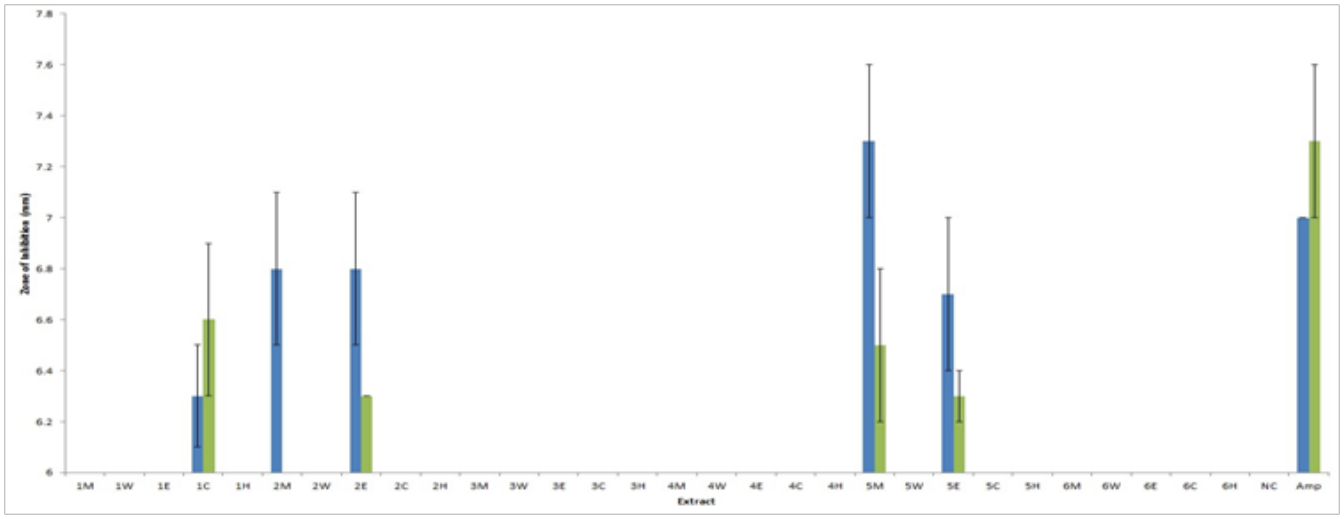

Figure 3: Antibacterial activity of the Australian fruit extracts against K. pneumoniae measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC31488) and the green bars represent the zones of inhibition against the clinical strain. 1 = muntries; 2 = Illawarra plum; 3 = native tamarind; 4 = lemon aspen; 5 = desert lime; 6 = bush tomato; M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = 0.5% DMSO; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.

low growth inhibition by the ampicillin control indicates that this strain is resistant to ampicillin. Thus it is likely that these hexane extracts are inhibiting by a mechanism different to that of ampicillin, although this needs testing. If this is determined to be the case, these extracts may be particularly useful against resistant and persistent infections.

Both reference and clinical strains of *Klebsiella pneumoniae* were inhibited by several of the Australian fruitextracts, albeit with relatively small zones of inhibition (Figure 3). In general the reference *K. pneumoniae* strain was more susceptible to the fruit extracts than was the clinical strain. The muntries chloroform extract, Illawarra plum ethyl acetate extracts and the desert lime methanolicand ethyl acetate extracts each inhibited both *K. pneumonia* strains. In addition, the Illawarra plum methanolic extract inhibited the reference but not the clinical *K. pneumonia* strain. Inhibition was weak (as judged by the zones of inhibition), with inhibition zones generally <7.5 mm in diameter. However, it is noteworthy that both *K. Pneumonia* strains were also not greatly inhibited by the antibiotic control (2 µg ampicillin), with only small zones of inhibition also noted.

The antimicrobial efficacy was further quantified by determining the MIC values for each extract against the microbial species which were determined to be susceptible. Many of the fruit extracts were effective at inhibiting microbial growth (Table 3), with MIC values against many of the susceptible bacteria<1000 µg/ml (<10 µg impregnated in the disc), indicating the potential of these extracts in controlling multiple autoimmune inflammatory disorders.The MIC values determined for the Illawarra plum and desert lime ethyl acetate extracts were particularly noteworthy, with MIC values generally 200-400 µg/ml (2-4 µg impregnated in the disc) against most of the bacterial triggers of autoimmune diseases tested.

Quantification of toxicity

All extracts were initially screened undiluted in the assay (Figure 4). For comparison, the reference toxin potassium dichromate (1000 µg/ml) was also tested in the bioassay. The potassium dichromate reference toxin was rapid in its onset of mortality, inducing nauplii death within the first 3 hours of exposure and 100 % mortality was evident following 4-5 hours (results not shown). Similarly, most of the fruit extracts displayed > 50 % mortality rates at 24 h and 48 h.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in artificial seawaterto testacross a range of concentrations in the Artemia nauplii bioassay. (Table 4) shows the LC 50 values of the fruit extracts towards *A. franciscana*. No LC 50 values are reported for the muntries aqueous and hexane extracts, the Illawarra plum and native tamarind hexane extracts or the desert lime methanolic and ethyl acetate extracts as less than 50 % mortality was seen for all concentrations tested. All fruit extracts except

Table 3: Minimum inhibitory concentration (µg/ml) of the Australian fruit extracts (µg/mL).

Plant Species	Extract	P. mirabilis (reference strain)	P. mirabilis (clinical strain)	P. vulgaris (reference strain)	K. pneumoniae (reference strain)	K. pneumoniae (clinical strain)
Muntries	M	>10000	2315	1494	-	-
	W	-	-	743	-	-
	E	-	-	710	-	-
	C	-	-	-	8231	7282
	H	-	-	-	-	-
Illawarra Plum	M	-	-	-	>10000	-
	W	-	-	-	-	-
	E	256	210	-	187	293
	C	-	-	9235	-	-
	H	-	-	-	-	-
Native Tamarind	M	-	-	6703	-	-
	W	-	-	4166	-	-
	E	-	-	1830	-	-
	C	-	-	>10000	-	-
	H	-	-	5891	-	-
Lemon Aspen	M	-	-	>10000	-	-
	W	-	-	>10000	-	-
	E	-	-	2758	-	-
	C			796	-	-
	H	-	-	5096	-	-
Desert Lime	M	2316	-	>10000	>10000	>10000
	W	-	-	>10000	-	-
	E	183	267	-	265	387
	C	-	-	387	-	-
	H	-	-	587	-	-
Bush Tomato	M	>10000	8733	8128	-	-
	W	-	7347	2287	-	-
	E	2151	1582	1127	-	-
	C	-	-	>10000	-	-
	H	-	-	287	-	-

Numbers indicate the mean MIC values of triplicate determinations. - indicates no inhibition. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract.

native tamarind ethyl acetate extract were determined to be nontoxic with LC 50 values much greater than 1000 µg/ml following 24 h exposure. Extracts with an LC 50 of greater than 1000 µg/ml towards *Artemia* nauplii have been defined as being nontoxic.[37]

DISCUSSION

Plant remedies are becoming increasingly sought after in the treatment of a myriad of diseases and disorders due both to their perception of greater safety than synthetic drugs, and the failure of current drug regimens to effectively treat many diseases. This is especially true for the autoimmune inflammatory diseases. The current treatments utilising disease modifying anti-rheumatic drugs (DMARDs) to alleviate the symptoms of these diseases and/or alter the disease progression are not entirely effective and have been associated with numerous adverse effects.[1] Furthermore, many of the current treatments are aimed at treating the symptoms without addressing the underlying causes and pathogenic mechanisms. A better understanding of the mechanisms for initiation and progression of the autoimmune inflammatory diseases is important for developing of new drugs to target specific processes and thus more effectively treat autoimmune inflammatory diseases.

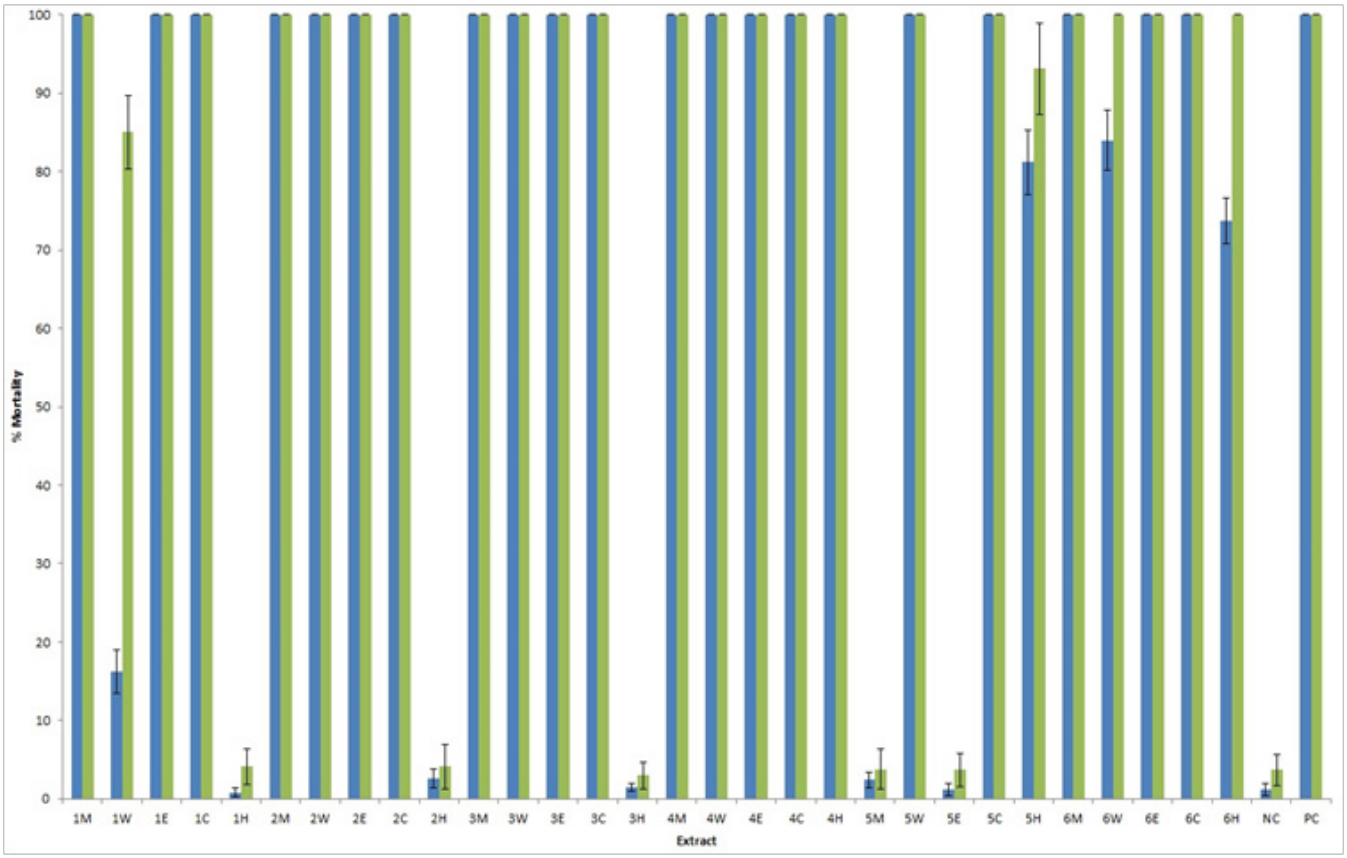

Figure 4: The lethality of the undiluted fruit extracts and the potassium dichromate control (1000 µg/mL) towards Artemia nauplii. The blue bars represent the mortality after 24h exposure and the green bars represent the mortality after 48 h exposure. 1 = muntries; 2 = Illawarra plum; 3 = native tamarind; 4 = lemon aspen; 5 = desert lime; 6 = bush tomato; M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = negative (seawater) control; PC = positive control (1000 µg/ml potassium dichromate). All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

The studies reported here examined the ability of Australian native fruitextracts to block microbial triggers of 2 autoimmune inflammatory disorders (Proteus spp.: rheumatoid arthritis; *K. pneumonia*: ankylosing spondylitis). Several fruit extracts were identified displaying potent inhibition against these bacteria. Interestingly, the bacterial growth inhibitory activity of the extracts did not correlate with their antioxidant capacities. The fruit extracts with the highest antioxidant capacities (lemon aspen and desert lime methanolic extracts) had only low antibacterial activity, with MIC values generally >10,000 µg/ml against all bacterial species. In contrast, the Illawarra plum and desert lime ethyl acetate extracts, which had mid-range antioxidant capacities (1-6.5 mg ascorbic acid equivalents/g extracted), had potent bacterial growth inhibitory activity.

Interestingly, whilst the measured MIC values indicate good inhibitory activity, the initial spot tests were not as promising. Only small zones of inhibition were measured for many extracts, including some of those which were determined to be potent growth inhibitors by MIC determination. For example, *K. pneumonia* growth inhibition zones of ≤7.5 mm were recorded for the Illawarra plum and desert lime ethyl acetate extracts in the initial screenings, yet MICs of approximately 200-400 µg/ml were determined when the extract was tested across a range of concentrations. This illustrates several important points:

• As the disc diffusion method is reliant on the diffusion of a molecule through the aqueous environment of an agar gel, the results of a single spot assay may be affected by the solubility of the extract compounds in the aqueous environment. Polar compounds that are highly soluble in water would be expected to diffuse easily in the gel, whereas less soluble compounds would not diffuse as readily and thus be concentrated around the disc. For this reason, whilst this is a handy assay for screening aque-

ous extracts, this technique may not be ideal if nonpolar compounds are thought to be the active components (e.g. when screening essential oils). For examining highly nonpolar mixtures, other techniques such as liquid dilution assays may be preferred.

• Diffusion of molecules within a gel is also affected by the size of those molecules. Thus the diffusion of large, complex phytochemicals (e.g. complex tannins) within agar gels would also be retarded and may provide a false idea of the efficacy of an extract. As many tannins have well described antibiotic properties, screening for growth inhibition using a single concentration may give a distorted view of its inhibitory potential.

• The extracts were tested at different concentrations in the initial screening experiments. This was to examine the efficacy of each extract in an approximation of the form in which it would be taken therapeutically. However, for extracts such as the ethyl acetate and hexane extracts (which had low extraction yields compared to many of the other extracts), this may not provide a good indication of the inhibitory activity of the extract.

Thus, extracts displaying growth inhibitory activity should be tested across a range of concentrations as was performed in our study. This allows for a comparison of the efficacy of each extract with other extracts and with control antibiotics. Ideally, different techniques (e.g. semi-quantitative liquid diffusion MIC assays) more suited to nonpolar compounds should also be employed if nonpolar compounds are suspected as contributing to the growth inhibition. Such assays are currently planned to extend our studies. These studies are expected to be particularly useful to further examine the inhibitory activity of the chloroform and hexane extracts.

Whilst these studies have demonstrated the potential of several Australian fruit to treat autoimmune disease, much more work is required. This study has only tested these extracts against some microbial triggers of 2 autoimmune diseases (rheumatoid arthritis and ankylosing spondylitis). The microbial triggers for several other autoimmune inflammatory disorders are also known. *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis[4] and *Borrelia burgdorferi* is linked with Lyme disease.[15]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. *Mycoplasma pneumoniae* is associated with several demyelinating diseases.[6] It would be interesting to extend our studies to also screen for the ability of the extracts to block these microbial triggers of autoimmune diseases.

Our findings also indicate that the native Australian fruit-extracts examined in this study displayed low toxicity towards *Artemia franciscana*. Indeed, all extracts had LC50 values well in excess of 1000 μg/ml. As an LC50of ≥ 1000 μg/ml is defined as nontoxic,[37] this extract is considered to be of only low to moderate toxicity.

CONCLUSION

The results of this study demonstrate the potential of these high antioxidant Australian fruit extractsto block the growth of bacterial species associated with the onset of several autoimmune inflammatory diseases.Thus, these extracts have potential in the prevention and treatment of rheumatoid arthritis and ankylosing spondylitis 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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CONFLICT OF INTEREST

All authors declare no conflicts of interest.

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