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Original Article

Andrographis paniculata (Burm.f.) Nees Leaf Extracts Lack Antibacterial Activity against some Bacterial Triggers of Inflammatory Diseases and are Non-toxic *in vitro*

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ABSTRACT

Introduction: The recent development of extensively antibiotic resistant bacteria has necessitated the search for novel antibacterial compounds. An examination of aromatic plants and traditional medicines is an attractive option for drug discovery. *Andrographis paniculata* (Burm.f.) Nees is used in southern Asian traditional medicine for a variety of purposes and has been reported to have antibacterial activity against a limited panel of bacteria. **Methods:** The ability of *A. paniculata* leaf extracts to inhibit the growth of a panel of bacterial pathogens which can trigger some autoimmune diseases in genetically susceptible people was investigated by disc diffusion assays. Toxicity was examined using the *Artemia franciscana* nauplii bioassay. **Results:** *Andrographis paniculata* leaf solvent extracts of varying polarity were completely ineffective at inhibiting the growth of some bacterial triggers of autoimmune inflammatory diseases. The extracts were nontoxic in the *Artemia* nauplii bioassay following 24hr exposure. **Conclusion:** *Andrographis paniculata* leaf extracts were completely ineffective bacterial growth inhibitors against the tested pathogens. However, these extracts may have other therapeutic properties and testing against other bacterial pathogens, protozoa, viruses and tumour cells is required.

Keywords: Acanthaceae, Andrographis, Green chiretta, Antibacterial activity, Antibiotic resistant bacteria, Medicinal plants, Toxicity.

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INTRODUCTION

Traditional plant derived medicines have been used in most parts of the world for a variety of therapeutic purposes, including fighting microbial disease. Indeed, the ability of plant extracts to block the growth of pathogenic bacteria has become a focus of substantial recent study.[1-5] Much of the research into traditional medicinal plant use has focused on Asian,[6-8] African,[9-11] Middle Eastern[12-14] and South American[15] plants. However, despite the potential of plants to provide us with useful pharmaceutical agents, the field is still relatively poorly studied. Only an estimated 5-10 % of the approximately 300,000-500,000 plant species worldwide have been screened for one or more therapeutic bioactivities.[9]

The development of new antibiotic therapies is particularly urgent. The recent establishment of bacterial pathogens that are either extremely (XDR) or totally resistant (TDR) to common clinically used antibiotics[16] has resulted in the need to develop new and effective antibiotic chemotherapies. There are now limited treatments for many diseases caused by bacterial pathogens and the situation is expected to worsen in the future as bacteria exchange resistance genes. Indeed, the development of alternative antibacterial treatment modalities has become crucial and is considered by the World Health Organisation (WHO) to be one of the most serious challenges facing medical science.[17] For reasons reviewed elsewhere,[16] it is unlikely that the previous methods of antibiotic discovery/development will be as successful in the future and new treatment modalities are urgently required. Traditional medicines and herbal remedies have great potential for antimicrobial drug development and there has recently been a substantial increase in interest in this field.[18-31]

Andrographis paniculata (Burm.f.) Nees (synonyms *Justica latebrosa* Russell ex Wall., *Justica paniculata* Burm. f., *Justica stricta* Lam. ex. Steud.; commonly known as creat or green chiretta) is an erect herbaceous plant (Family Acanthaceae; Figure 1a) that is native to India and Sri Lanka, although it is now also widely cultivated throughout southern and southeastern Asia. The leaves (Figure 1b) and roots (Figure 1c) are used in Ayuverda and Siddha traditional medicinal systems to treat a variety of medical conditions including cancer, jaundice and respiratory tract infections.[32] Some studies have screened *A. paniculata* extracts and isolated compounds for therapeutic properties and antibacterial,[33] anti-inflammatory,[34] antimalarial,[35] antithrombotic,[36] and anti-anticancer[37] activities have been confirmed. However, it is noteworthy that *A. paniculata* extracts have only been tested against limited panels of pathogens and they are yet to be tested against many other bacterial species.

The phytochemistry of *A. paniculata* extracts has been relatively well studied and they have been reported to be relatively rich in labdane diterpenoids including andrographolide (Figure 1d), neoandrographolide (Figure 1e) and isoandrographolide (Figure 1f), and their derivatives. *Andrographis paniculata* preparations are also a relatively abundant source of xanthones including 1,2-dihydro-6,8-dimethoxyxanthone (Figure 1g) and 1,8-dihydroxy-3,7-dimethoxyxanthone (Figure 1h). Numerous other flavonoids, quinic acid derivatives and noriridoids have also been identified in *A. paniculata* extracts and these have been reviewed in detail elsewhere.[32] Several of those compounds have antibacterial activity, although most are relatively weak.[32] This study was undertaken to screen *A. paniculata* leaf extracts for the ability to inhibit the growth of a panel of bacterial triggers of autoimmune inflammatory diseases.[38,39]

MATERIALS AND METHODS

Collection of Plant Material and Extraction

Andrographis paniculata (Burm.f.) Nees leaves were purchased from Noodles Emporium, Australia (an online herbalist) and were originally sourced from India. A voucher sample (GU-APAL18a) is stored in

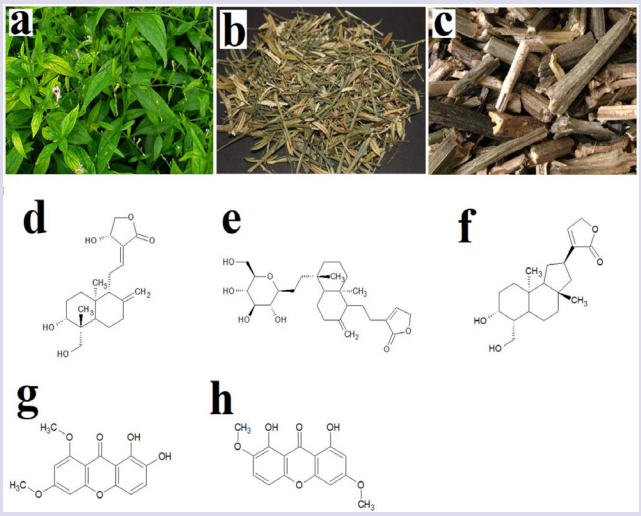

Figure 1: *Andrographis paniculata* (a) whole plant, (b) dried leaves, (c) dried roots, (d) andrographolide, (e), neoandrographolide, (f) isoandrographolide, (g) 1,2-dihydro-6,8-dimethoxyxanthone, and (h) and 1,8-dihydroxy-3,7-dimethoxyxanthone.

the School of Environment and Science, Griffith University, Australia. Individual 1g masses of the dried plant material were extracted extensively in 50mL methanol, ethyl acetate, chloroform, hexane or deionised water for 24 hr at 4°C with gentle shaking. All solvents were AR grade and were obtained from Ajax Fine Chemicals, Australia. The extracts were filtered through filter paper (Whatman No. 54) under vacuum, followed by drying in a vacuum oven at 50°C. The resultant pellet was weighed to determine the extraction yield, then resuspended in 10mL deionised water (containing 1% DMSO). The resultant extract was subsequently passed through 0.22µm filter (Sarstedt) and stored at 4°C.

Qualitative Phytochemical Studies

Phytochemical analyses of the *A. paniculata* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids were conducted by standard assays.[40-42]

Antibacterial Screening

Test microorganisms

All media was purchased from Oxoid Ltd., Australia. The reference strains of *Acinetobacter baylyi* (ATCC33304), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Tissue Culture Collection (ATCC), USA. All bacterial stock cultures were subcultured and maintained in nutrient broth at 4°C.

Evaluation of Antimicrobial Activity

Antimicrobial activity of the *A. paniculata* leaf extracts was determined using a modified disc diffusion assay.[43-45] Briefly, 100µL of each microbial suspension in log phase was spread onto individual nutrient agar plates and the extracts were tested for antimicrobial activity using 6mm sterilised filter paper discs. The discs were each infused with 10µL of the individual plant extract, allowed to dry and placed onto the inoculated plates. The plates were allowed to stand at 4°C for 2hr before incubation at 37°C for 24hr. The diameters of the zones of inhibition (ZOIs) were measured to the closest whole millimetre. Each assay was performed three times in triplicate (n=9). Mean values (± SEM) are reported in this study. Standard discs of ampicillin (10µg) and chloramphenicol (10µg) were obtained from Oxoid, Australia and were used as positive controls to compare antibacterial activity. Filter discs infused with 10µL of distilled water were used as a negative control.

Artemia franciscana Nauplii Toxicity Screening

Toxicity was tested using an adapted *Artemia franciscana* nauplii lethality assay.[46-48] Briefly, *A. franciscana* nauplii were incubated in the presence of the extracts, reference toxin (1mg/mL potassium dichromate) or artificial seawater (negative control) at 25±1°C under artificial light. All treatments were performed three times in triplicate (n=9). The number of dead were counted in each well at 24hr and 48hr. At the completion of the 48hr exposure period, the remaining live nauplii were sacrificed and the total number of nauplii in each well were counted and used to calculate the % mortality per well. LC_{50} values were calculated for each treatment using probit analysis.

Statistical Analysis

Data are expressed as the mean ± SEM of three independent experiments, each with internal triplicates (n=9). 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 1g of dried and powdered *A. paniculata* leaf with solvents of varying polarity yielded dried plant extracts ranging from 6mg (*A. paniculata* leaf ethyl acetate extract) to 126mg (*A. paniculata* leaf aqueous extract) (Table 1). The extracts were resuspended in 10mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 1. Qualitative phytochemical screening (Table 1) showed that the higher polarity solvents (methanol and water) extracted the greatest amount and widest diversity of phytochemical classes.

Antimicrobial Activity

To determine the growth inhibitory activity of the *A. paniculata* leaf extracts, aliquots (10µL) of each extract were screened in the disc diffusion assay. The *A. paniculata* leaf extracts were ineffective at inhibiting the growth of *P. mirabilis* (Figure 2), *K. pneumoniae* (Figure 3), *A. baylyi* (Figure 4) and *P. aeruginosa* (Figure 5). In contrast, both positive control antibiotics (ampicillin and chloramphenicol) were generally effective growth inhibitors against most bacterial strains tested, with ZOI's of up to 15.6 mm (ampicillin against *A. baylyi*) and 14.5mm (chloramphenicol against *E. coli*). Most of the bacteria were substantially more susceptible to chloramphenicol than the ampicillin control. Indeed, both *K. pneumoniae* and *P. aeruginosa* were completely resistant to that antibiotic, whilst *P. mirabilis* was only relatively weakly inhibited by ampicillin. As all bacteria species tested were resistant to the *A. paniculata* leaf extracts, we were unable to determine the MIC values for any extract.

Quantification of Toxicity

The toxicity of the *A. paniculata* leaf extracts was initially tested at 1000µg/mL in the *A. franciscana* nauplii bioassay (Figure 6). The mortality in the presence of the ethyl acetate, chloroform and hexane extracts was substantially <50% at 24hr when 1000µg/mL concentrations were tested. Thus, those extracts were deemed to be non-toxic. Extracts with 24hr LC_{50} values >1000µg/mL have previously been defined as non-toxic.[45-47] In contrast, the methanol and aqueous extracts both induced .50% mortality at 2000µg/mL so those extracts were also

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *A. paniculata* leaf extracts.

			Leaf				
			M	W	E	C	H
	Mass of extracted material (mg)		67	126	6	33	11
	Concentration of resuspended extract (mg/mL)		6.7	12.6	0.6	3.3	1.1
Qualitative Phytochemical Tests	Phenols	Total phenols	+++	+++	+	+	-
		Water soluble phenols	+++	+++	+	+	-
		Insoluble phenols	+	+	-	-	-
	Saponins	Froth persistence	+	++	-	-	
		Emulsion test	+	+	-	-	
	Cardiac glycosides	Keller-Kiliani Test	-	-	-	-	-
	Triterpenoids	Salkowski Test	-	-	-	-	-
	Phytosterols	Acetic Anhydride Test	-	-	-	-	-
	Alkaloids	Meyer's Test	-	-	-	-	
		Wagner's Test	-	-	-	-	
		Draggendoff's Test	-	-	-	-	-
	Flavonoids	Kumar Test	+++	+++	++	+	-
	Tannins	Ferric Chloride Test	++	++	-	-	-
		Lead Acetate Test	++	+	-	-	-
	Anthraquinones	Free	-	-	-	-	-
		Combined	-	-	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

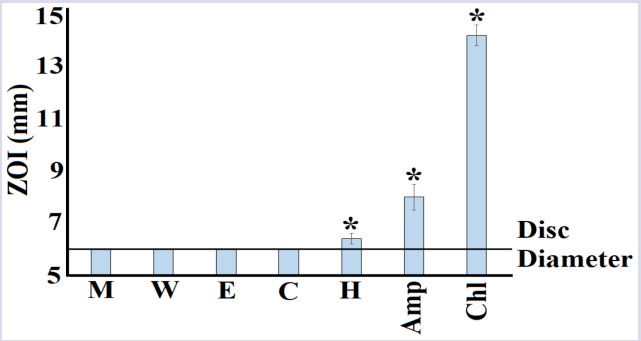

Figure 2: Growth inhibitory activity of *A. paniculata* leaf extracts and reference antibiotics against *P. mirabilis* measured as ZOIs (mm) ± SEM. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10µg); Chl = chloramphenicol (10µg); NC = negative control. All assays were completed three times, each with internal triplicates (*n*=9) and the results are expressed as mean zones of inhibition (mm) ± SEM. * = results significantly different to the negative control ($p<0.05$).

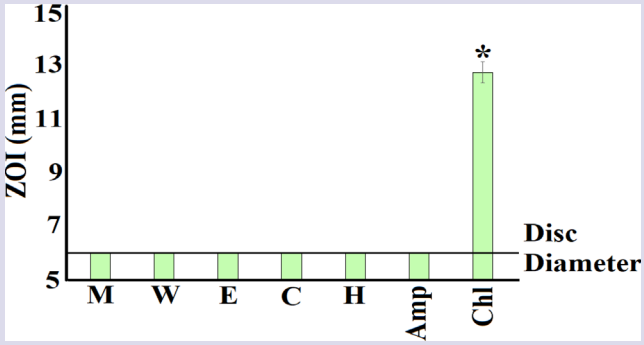

Figure 3: Growth inhibitory activity of *A. paniculata* leaf extracts and reference antibiotics against *K. pneumoniae* measured as ZOIs (mm) ± SEM. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10µg); Chl = chloramphenicol (10µg); NC = negative control. All assays were completed three times, each with internal triplicates (*n*=9) and the results are expressed as mean zones of inhibition (mm) ± SEM. * = results significantly different to the negative control ($p<0.05$).

tested at 1000µg/mL. As both induced substantially <50% mortality at that concentration (results not shown), they were also deemed to be nontoxic. The potassium dichromate positive control induced substantial mortality within 4hr (results not shown), with 100% mortality induction seen by 24hr, confirming that the assay was functioning properly. Whilst toxicity was assessed in this study with the test organism *A. franciscana*, toxicity towards *A. franciscana* has previously been shown to correlate well with toxicity towards human cells for many toxins.[45-47] However, further studies are required to confirm whether this is also true for the *A. paniculata* leaf extracts examined in these studies.

DISCUSSION

The development of new antibiotic chemotherapies is a high priority for medical science due to the recent development of large numbers of antibiotic resistant bacterial strains[16,17] A parallel decrease in the discovery of new antibiotic medicines by conventional strategies has increased interest in re-evaluating medicinal plants for new antibiotic chemotherapies.[16] This is especially true for chronic disorders such as the autoimmune inflammatory diseases. The current treatments utilising disease modifying anti-rheumatic drugs (DMARDs) to alleviate the

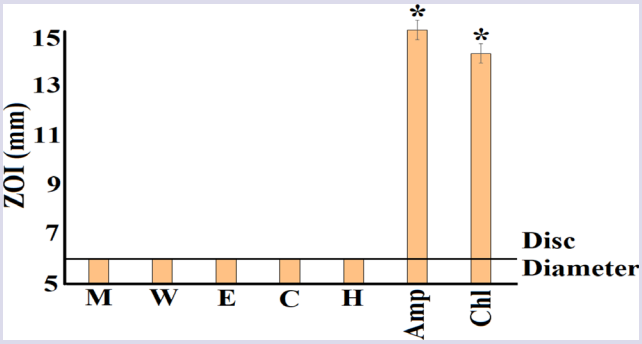

Figure 4: Growth inhibitory activity of *A. paniculata* leaf extracts and reference antibiotics against *A. baylyi* measured as ZOIs (mm) ± SEM. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10µg); Chl = chloramphenicol (10µg); NC = negative control. All assays were completed three times, each with internal triplicates (*n*=9) and the results are expressed as mean zones of inhibition (mm) ± SEM. * = results significantly different to the negative control ($p<0.05$).

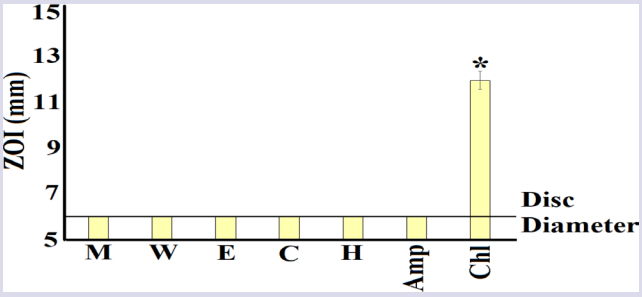

Figure 5: Growth inhibitory activity of *A. paniculata* leaf extracts and reference antibiotics against *P. aeruginosa* measured as ZOIs (mm) ± SEM. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10µg); Chl = chloramphenicol (10µg); NC = negative control. All assays were completed three times, each with internal triplicates (*n*=9) and the results are expressed as mean zones of inhibition (mm) ± SEM. * = results significantly different to the negative control ($p<0.05$).

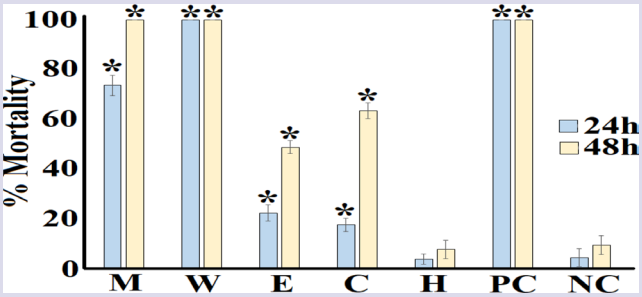

Figure 6: The lethality of the *A. paniculata* leaf extracts (2000µg/mL), potassium dichromate control (PC; 1000µg/mL) and seawater negative control (NC) following 24 and 48 hr of exposure. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; PC = potassium dichromate control; NC = seawater (negative) control. All bioassays were performed three times in triplicate (*n*=9) and are expressed as mean ± SEM. * indicates results that are significantly different to the untreated (seawater) control at the equivalent exposure time ($P<0.01$).

symptoms of these diseases and/or alter the disease progression are not entirely effective and have been associated with numerous adverse effects.[48] Furthermore, many of the current treatments are aimed at treating the symptoms without addressing the underlying causes and pathogenic mechanisms. The studies reported herein examined the ability of *A. paniculata* leaf extracts to block microbial triggers of three autoimmune inflammatory disorders (*Proteus* spp.: rheumatoid arthritis; *K. pneumonia*: ankylosing spondylitis; *A. baylyi* and *P. aeurginosa*: multiple sclerosis). Notably, the *A. paniculata* leaf extracts completely lacked inhibitory activity against all bacterial species tested. However, these extracts may have other therapeutic properties and testing against other bacterial pathogens, protozoa, viruses and tumour cells is required

A single assay technique was used to screen for antibacterial activity in this study. We chose to use the disc diffusion assay as it is a rapid method and it has previously been widely utilised in other studies. Therefore, comparisons between studies are relatively simple. However, as the disc diffusion method is reliant on the diffusion of a molecule through the aqueous environment of an agar gel, this 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. Diffusion of molecules within an agar gel is also affected by the size of the molecules. The movement of large, complex phytochemicals (e.g. tannins, triterpenoids) through agar gels by diffusion would also be retarded and may provide a false idea of the efficacy of an extract. As many tannins and triterpenoids have well described antibiotic properties, screening for growth inhibition using agar diffusion techniques may give a fallacious view of an extract's inhibitory potential. For this reason, whilst this assay is a rapid and simple way to detect antimicrobial susceptibility in polar extracts, it may not be ideal for nonpolar compounds. For examining nonpolar mixtures, other techniques such as liquid dilution assays may be preferred to screen *A. paniculata* leaf extracts for activity and future studies are planned to re-examine the *A. paniculata* leaf extracts using these methods.

CONCLUSION

All *A. paniculata* leaf extracts were completely ineffective inhibitors of the growth of the bacterial triggers of selected autoimmune inflammatory diseases. The extracts were also nontoxic towards *Artemia* nauplii.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DMSO: dimethyl sulfoxide; **LC_{50}:** the concentration required to achieve 50 % mortality; **MIC:** minimum inhibitory concentration; **ZOI:** zone of inhibition.

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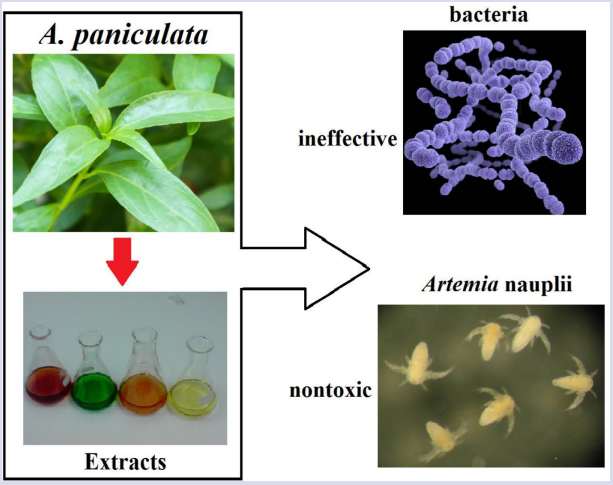
PICTORIAL ABSTRACT



SUMMARY

- *Andrographis paniculata* leaf extracts were screened for the ability to block the growth of a panel of human bacterial pathogens.
- No inhibitory activity was evident against any of the bacterial species tested.
- Toxicity of the *A. paniculata* leaf extracts was determined using the *Artemia* nauplii toxicity bioassay.
- All extracts were nontoxic.

About Authors

Dr. Ian Cock leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin, including *Aloe vera*, South Asian and South American tropical fruits, as well as Australia plants including *Scaevola spinescens, Pittosporum phylliraeoides, Terminalia ferdinandiana* (Kakadu plum), Australian *Acacias, Syzygiums, Petalostigmas* and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in more than 200 publications in a variety of peer reviewed journals.

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