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Original Article

Antibacterial Activity of *Harpagophytum procumbens* (Burch.) DC. ex Meisn. Root Extracts against Gastrointestinal Pathogens and Bacterial Triggers of Autoimmune Diseases

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ABSTRACT

Background: Recent increases in the numbers of antibiotic resistant bacteria and corresponding decreases in antimicrobial discovery have focussed drug discovery efforts towards plant based medicines. *Harpagophytum procumbens* has been used in southern African traditional medicine for a variety of conditions including inflammation, and to treat bacterial infections. Despite this, investigations of the antibacterial activity of *H. procumbens* root extracts have been relatively neglected. **Materials and Methods:** The antimicrobial activity of *H. procumbens* root extracts was assessed using disc diffusion and liquid dilution minimum inhibitory concentration (MIC) assays against gastrointestinal pathogens and bacterial triggers of some autoimmune diseases. The toxicity of the individual samples was assessed using the *Artemia* nauplii lethality assay (ALA) and an MTS HDF cell viability assay. **Results:** *Harpagophytum procumbens* root extracts displayed notable antibacterial activity against several bacterial triggers of autoimmune diseases, and against several gastrointestinal bacterial pathogens. The methanolic and aqueous extracts were particularly good inhibitors of *Proteus* spp. (MIC 125-313 µg/mL), *K. pneumonia* (MIC 313 µg/mL), *P. aeruginosa* (MIC 625 µg/mL) and *S. pyogenes* (MIC 313 µg/mL). Substantially higher MIC values were recorded against *A. baylyi* and the gastrointestinal bacteria. None of the extracts were toxic in the ALA or MTS assays, indicating their suitability for therapeutic use. **Conclusion:** The *H. procumbens* root extracts were effective inhibitors of the growth of several bacterial triggers of autoimmune inflammatory diseases and had noteworthy activity against some gastrointestinal bacteria. Isolation and identification of the bioactive compounds may be beneficial in the development of new antibiotic drugs.

Key words: Devil's claw, Pedaliaceae, Medicinal plants, Conventional antimicrobials, Synergy, Interaction, Toxicity.

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INTRODUCTION

The development of multiple bacterial strains that are resistant to several classes of clinical antibiotics has rendered many of those antibiotics of decreased efficacy, or even rendered them completely ineffective.[1] New antibiotics are urgently needed and the development of alternative chemotherapies is considered by the World Health Organisation (WHO) to be one of the biggest challenge facing medical science.[2] For a number of reasons reviewed elsewhere,[1] it is unlikely that many previously successful pathways for antibiotic discovery/development will be substantially less successful in the future and alternatives sources of new drugs are increasingly being explored. In recent years, the development of novel natural compounds from traditional medicines has attracted substantial interest for the development of new antibiotics. However, despite the rapidly increasing number of studies published each year exploring the antibacterial properties of traditional plant-based medicines, there are still relatively few plant-derived antibiotic compounds in clinical usage and the vast majority of plants that were used to treat bacterial infections are yet to be tested.

Harpagophytum procumbens (Burch.) DC. ex Meisn. (family Pedaliaceae; commonly known as Devil's claw, grapple plant, wood spider) is a tuberous perennial plant with creeping stems and dark pink flowers (Figure 1a) that is native to arid southern regions of the African continent. They are particularly prevalent in Angola, Botswana, Namibia and the Kalahari Desert region of South Africa. The common name Devil's claw derives from the appearance of its woody fruit, which are covered with small hook-like projections (Figure 1b). *Harpagophytum procumbens* is noted for its large water storing tuborous roots (Figure 1c), which are important in several southern African traditional healing systems to treat a variety of ailments. However, the roots are particularly well known for the treatment of pain and inflammation.[3,4] Indeed, multiple studies have examined the anti-inflammatory effects of *H. procumbens* roots and have linked the anti-inflammatory activity to the iridoid glycoside, harpagoside (Figure 1d).[4,5] *Harpagophytum procumbens* root extracts supress inflammation via multiple mechanisms, including the suppression of lipoxygenase and cyclooxygenase enzymes, thereby reducing leukotriene and prostaglandin synthesis, and by down-regulating the production of pro-inflammatory cytokines (as reviewed by Grant L *et al.*)[4]

The roots have also been used for thousands of years to treat blood diseases, fever, dyspepsia, urinary tract infections, wounds, burns, and multiple skin diseases.[4,5] Notably, bacterial pathogens cause many of these diseases. Despite this, surprisingly few studies have examined *H. procumbens* root extracts for the ability to inhibit the growth of bacteria. One recent study screened *H. procumbens* leaf extracts and isolated compounds against *Staphylococcus aureus* and reported antibacterial activity.[6] Notably, the authors of that study tested a single, high extract concentration and did not quantify the MIC value. Furthermore, the authors used a agar well diffusion assay in that study, making it difficult to compare the activity with different studies, which more frequently utilise different methods. Additionally, that study tested leaf extracts for antibacterial activity, whilst the roots are traditionally used medicinally. In contrast, an earlier study screened *H. procumbens* root extracts against a broader panel of bacteria, including several gastrointestinal and skin pathogens and quantified the MIC and MBC values.[7] That study reported noteworthy activity against

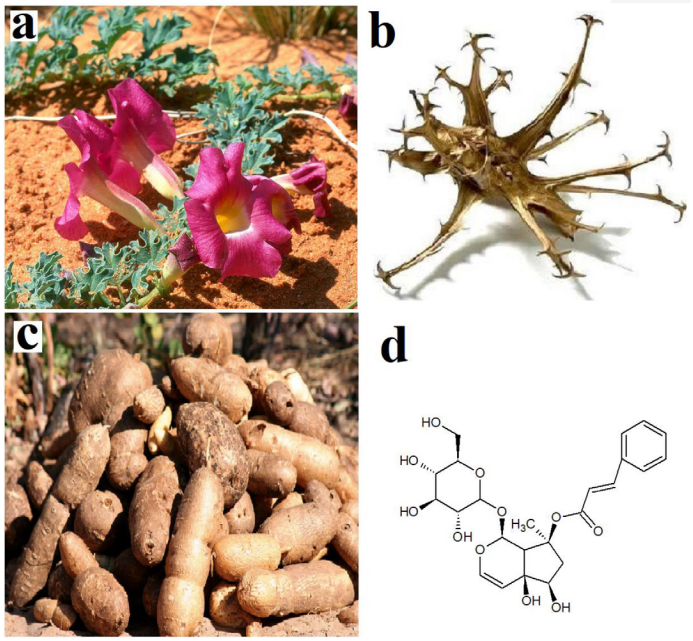

Figure 1: *Harpagophytum procumbens* (a) whole plant with flower, (b) dried fruit, (c) roots, and (d) harpagoside.

most of the bacteria screened, partially validating the use *H. procumbens* root extracts for the treatment of bacterial gastrointestinal and skin infections. Our study aimed to extend these earlier studies by evaluating the growth inhibitory properties of *H. procumbens* root extracts against bacterial triggers of some autoimmune inflammatory diseases, as well as some bacterial gastrointestinal pathogens. Additionally, the toxicity of the extracts was tested to evaluate the safety of the root extracts for medicinal usage.

MATERIALS AND METHODS

Sourcing and preparation of plant samples

Dried and finely ground *Harpagophytum procumbens* (Burch.) DC. ex Meisn. root material was purchased from Noodles Emporium, Australia. Voucher specimens are deposited in the School of Natural Sciences, Griffith University, Australia (voucher number GUHGRa1-2016-1/1). Individual quantities (1 g) of the ground plant material were weighed into separate tubes and 50 mL of deionised methanol, water, ethyl acetate, chloroform or hexane were added and mixed thoroughly. All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The ground plant materials were extracted in each solvent for 24 hr at 4°C with gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum. The solvent extracts were air dried at room temperature in the shade. The aqueous extracts were lyophilised by freeze drying at -50°C. The resultant dried extracts were weighed and dissolved in 10 mL deionised water (containing 1 % DMSO).

Qualitative phytochemical analysis

Phytochemical analysis of the *H. procumbens* root extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved as previously described.[8,9]

Antioxidant capacity

The antioxidant capacity of each extract was assessed using standard DPPH free radical scavenging assays.[10,11] Briefly, a 400 µM DPPH (Sigma, Australia) solution was freshly prepared in AR methanol (Ajax, Australia) each day prior to use. A 2 mL volume of each extract and the ascorbic acid standards were dried by evaporation and resuspended in 2 mL of AR grade methanol. Each extract was tested across a range of concentrations on a 96 well plate in triplicate. Extra methanol was subsequently added to each well to yield a total volume of 225 µL. Fresh DPPH solution (75 µL) was then added to each well. The absorbances of all tests and the ascorbic acid controls were recorded at 515 nm in triplicate. An ascorbic acid standard curve was established and use to calculate the antioxidant capacity for each extract as µg ascorbic acid equivalents per gram of original plant material extracted.

Antibacterial analysis

Conventional antibiotics

Penicillin-G (potency of 1440-1680 µg/mg), chloramphenicol (≥98 % purity by HPLC, erythromycin (potency ≥850 µg/mg), ciprofloxacin (potency of 600 µg/mg), and tetracycline (≥95% purity by HPLC) were purchased from Sigma-Aldrich, Australia and used for the microplate liquid dilution assay. All antibiotics were prepared in sterile deionised water at stock concentrations of 0.01 mg/mL and stored at 4°C until use. For the disc diffusion studies, ampicillin (10 µg) and chloramphenicol discs (10 µg) standard discs were obtained from Oxoid Ltd., Australia and used as positive controls.

Bacterial cultures

All bacterial strains were selected either on the basis of their ability to trigger autoimmune inflammatory diseases in genetically susceptible individuals,[12-14] or as they are common gastrointestinal bacteria.[15-17] Reference strains of *Proteus mirabilis* (ATCC21721), *Klebsiella pneumoniae* (ATCC31488), *Acinetobacter baylyi* (ATCC33304), *Pseudomonas aeruginosa* (ATCC39324) and *E. coli* (ATCC0157) were purchased from American Type Culture Collection, USA. Clinical isolate strain of *Streptococcus pyogenes, Alcaligenes faecalis*, *Bacillus cereus, Enterobacter aerogenes*, *Salmonella newport* and *Shigella sonneii* were obtained from the School of Natural Sciences teaching laboratory, Griffith University, Australia. All bacteria were cultured in nutrient broth (Oxoid Ltd., Australia). Streak nutrient agar (Oxoid Ltd., Australia) plates were tested in parallel to ensure the purity of all bacterial cultures and for sub-culturing. All bacterial cultures were incubated at 37°C for 24 hr and were subcultured and maintained in nutrient broth at 4°C until use.

Evaluation of bacterial susceptibility to growth inhibition

The susceptibility of the bacteria to the *H. procumbens* root extracts and the conventional antibiotics was initially assessed using a modified disc diffusion assay.[18-20] Ampicillin (10 µg) and chloramphenicol discs (10 µg) were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using two methods. A liquid dilution MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay.[21] Furthermore, as microplate liquid dilution MIC assays are perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method allows for comparisons with other studies. A solid phase agar disc diffusion assay was also used

in this study for comparison, and as a model for bacterial infections on surfaces.

Microplate liquid dilution MIC assay

A standard liquid dilution MIC assay[21-23] was used to evaluate the antimicrobial activity of the plant samples and the conventional antimicrobials. Briefly, 100 µL of sterilized distilled water was dispensed into each well of 96 well micro-titre plate. The plant samples and conventional antibiotics (100 µL) were then added into separate wells of the first row of the plate. The conventional antibiotics were introduced at a starting concentration of 0.01 mg/mL. A negative control (nutrient broth), a sterile control (without bacteria) and a sample-free culture control (to ensure the media was capable of supporting microbial growth) were included on all plates. After addition of the test samples to the plate, each was serially diluted by doubling dilution. Each bacterial culture inoculum (100 µL) was then added individually to all wells of the plate except the sterile control wells. Each inoculum contained approximately $1x10^6$ colony forming units (CFU)/mL. All plates were subsequently incubated at 37°C. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, Australia and dissolved in sterile deionised water to prepare a 0.2 mg/mL INT solution. A 40 µL volume of this solution was added into all wells and the plates were incubated for a further 6 hr at 30°C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

Disc diffusion MIC assay

The minimum inhibitory concentrations (MIC) of the extracts was also evaluated by disc diffusion assay as previously described.[18-20] Graphs of the zone of inhibition versus Ln concentration were plotted and MIC values were achieved using linear regression.

Toxicity studies

Two assays were used to assess the toxicity of the individual samples. The *Artemia* nauplii lethality assay (ALA) was utilised for rapid preliminary toxicity screening, whereas the MTS cellular proliferation assay was used to determine a cellular evaluation of toxicity.

Brine-shrimp lethality assay (ALA)

Toxicity of the *H. procumbens* root extracts, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* Kellogg nauplii lethality assay as previously described.[24,25] Samples producing than 50% mortality were considered to be toxic. These samples were subsequently serially diluted and tested across the concentration range 1- 0.032 mg/mL to obtain a log-sigmoid dose response curve, generated with GraphPad Prism® software (Version 5), from which the LD_{50} values were determined.

MTS cellular viability assay

All *H. procumbens* root extracts and conventional antibiotics were screened individually and in combination towards normal human primary dermal fibroblasts obtained from American Type Culture Collection (ATCC PCS-201-012) using standard assays.[10,26] Quinine (Sigma, Australia) was included on each plate as a positive control. All tests were performed in at least triplicate and triplicate controls were included on each plate. The % cellular viability of each test was calculated using the following formula:

$$\% \text{ cellular viability} = \frac{\text{Abs test sample} - (\text{mean Abs control} - \text{mean Abs blank})}{(\text{mean Abs control} - \text{mean Abs blank})}$$

Cellular viability ≤50% of the untreated control indicated toxicity, whereas extracts or controls with >50% untreated control viability were deemed to be nontoxic.

Statistical analysis

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

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extractions of the *H. procumbens* root material (1 g) with solvents of varying polarity yielded dried plant extracts ranging from 13 mg (*H. procumbens* root ethyl acetate extract) to 401 mg (*H. procumbens* root methanolic extract) (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. Similarly, the methanolic extract also contained the highest antioxidant capacity (1.01 mg/mL ascorbic acid equivalents), compared to all other extracts, which had substantially <0.4 mg/mL ascorbic acid equivalents.

Inhibition of a bacterial triggers selected autoimmune diseases

Notably, all of the bacterial triggers of autoimmune diseases that were screened in this study were only susceptible to the higher polarity methanolic and aqueous extracts, with no inhibitory activity recorded for the ethyl acetate, chloroform or hexane extracts against any of the bacteria tested. This may correlate to the lower concentrations of those extracts (1.3-2.6 mg/mL) compared to the concentrations of the methanolic (40.1 mg/mL) and aqueous extracts (36.8 mg/mL). *Proteus mirabilis* growth was particularly susceptible to the methanolic *H. procumbens* root extract, with a zones of inhibition (ZOI) of approximately 14 mm. Substantially smaller (~7.5 mm) ZOIs were recorded for the aqueous extract (Figure 2a). Similar inhibitory activity was note against both the reference and clinical isolate strains. Similar, albeit smaller ZOIs were measured for *P. vulgaris*, with ZOIs of 10.7 and 7.2 mm for the methanolic and aqueous extracts respectively (Figure 2b). As *Proteus* spp. have been linked with the etiology of rheumatoid arthritis in genetically susceptible people,[12] these extracts may be useful in the prevention and treatment of that disease.

The methanolic and aqueous extracts were also good inhibitors of *K. pneumoniae* growth (Figure 2c). As noted for the *Proteus* spp., the *H. procumbens* extracts both inhibited the growth of *K. pneumoniae* with similar potency (as judged by the size of the ZOI). The methanolic extract was a particularly good growth inhibitor, with 9.8 and 9.2 mm ZOIs measured against the reference and clinical isolate bacterial strains respectively. ZOIs of approximately 8 mm were also measured for the aqueous extract against both *K. pneumoniae* stains. As *K. pneumoniae* can trigger ankylosing spondylitis in genetically susceptible people,[12] the *H. procumbens* root extracts may also be useful for preventing and treating that disease.

Similarly, the higher polarity *H. procumbens* root extracts also inhibited the growth of *A. baylyi* and *P. aeruginosa* (Figures 2d and 2e respectively), which can both trigger multiple sclerosis in genetically susceptible people.[12,13] Whilst the methanolic extract was the stronger growth inhibitor of *A. baylyi* (8.2 and 8 mm against the reference and clinical strains respectively), the aqueous extract was the stronger inhibitor of *P. aeruginosa* growth (as judged by ZOI diameter), with ZOIs of approximately 8.2 mm against both strains of that bacterium.

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the H. procumbens root extracts.

Extract	Mass of Dried Extracted Material (mg)	Conventration of ectract (mg/mL)	Phenols			CG	Sap	Tri	Phyt	Alk		Flav		Tan	Anth		Antiox Cap
			Total Phenolics	Water Soluble	Water Insoluble	Keller-Kiliani Test	Froth Persistence	Salkowski Test	Acetic Anhydride	Meyers Test	Wagners Test	Shinoda Test	Kumar test	Ferric Chloride	Free	Combined	Ascorbic Acid Equivalents (mg/mL)
M	401	40.1	+++	++	++	-	-	-	-	-	-	+++	+++	++	-	-	1.02
W	368	36.8	+++	++	+	-	-	-	-	+	-	+++	+++	+++	-	-	0.36
EA	13	1.3	+	+	-	-	-	-	-	-	-	+	+	+	-	-	0.16
C	26	2.6	+	-	-	-	-	-	-	-	-	+	+	-	-	-	0.17
H	15	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.09

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay; M = methanolic extract; W = aqueous extract; EA = ethyl acetate extract; C = chloroform extract; H = hexane extract; Phenols = polyphenolics; CG = cardiac glycosides; Sap = saponins; Tri = triterpenoids; Phyt = phytosterols; Alk = alkaloids; Flav = flavonoids; Tan = tannins; Anth = anthraquinones.

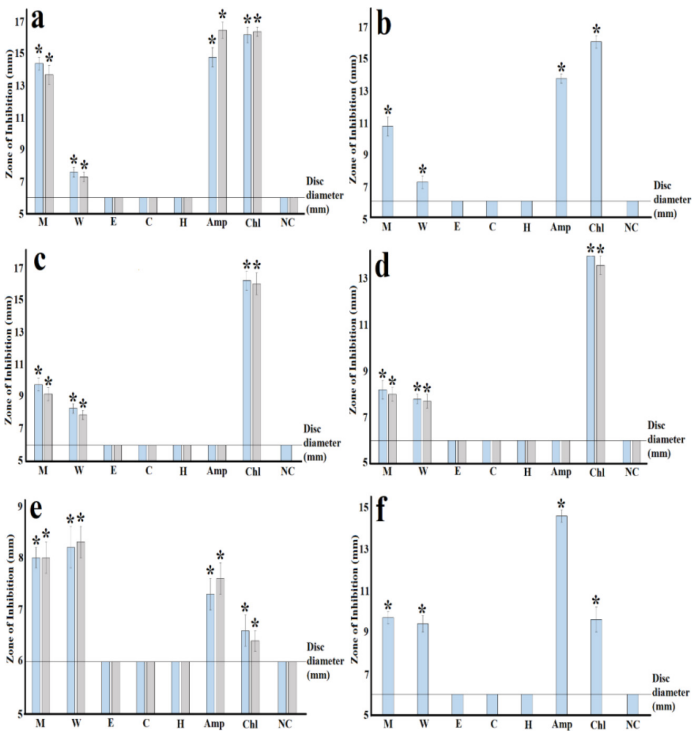

Figure 2: Antibacterial activity of *H. procumbens* root extracts against (a) *P. mirabilis* (ATCC21721) and clinical isolate strain; (b) *P. vulgaris* (ATCC21719); (c) *K. pneumoniae* (ATCC31488) and clinical strain; (d) *A. baylyi* (ATCC33304) and clinical strain; (e) *P. aeruginosa* (ATCC: 39324) and clinical strain; (f) *S. pyogenes* clinical strain, measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg); Chl = chloramphenicol (10 µg); NC = negative control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates (two repeats) ± SEM. * indicates results that are significantly different to the negative control (P<0.01).

The inhibition of this bacterium is particularly noteworthy as both strains are particularly antibiotic resistant. Indeed, ZOIs of ~6.5 mm were measured for both strains of *P. aeruginosa* against chloramphenicol, indicating noteworthy resistance against chloramphenicol. Slightly larger ZOIs were measured against ampicillin (~7.5 mm), although these are also indicative of antibiotic resistance. Notably, the resistance of both of these strains has also previously been reported in other studies.[27-30] The growth of *S. pyogenes* was also strongly inhibited by the *H. procumbens* methanolic and aqueous extracts (ZOIs of 9.7 and 9.4 mm respectively; Figure 2f). As *S. pyogenes* can induce rheumatic fever in genetically susceptible people, these extracts may be useful in preventing and treating this disease in genetically susceptible people, as well as treating other diseases caused by infections of this bacterium.

Inhibition of the growth of some gastrointestinal bacterial pathogens

The *H. procumbens* methanolic and aqueous root extracts also inhibited the growth of most of the gastrointestinal bacteria (Figure 3). As noted for the autoimmune disease bacterial triggers, the methanolic extract was a better growth inhibitor of *A. faecalis* (Figure 3a), *B. cereus* (Figure 3b) *S. newport* (Figure 3e) and *S. sonnei* (Figure 3f). Interestingly, the aqueous *H. procumbens* root extract was a stronger inhibitor of the growth of *E. aerogenes* (Figure 3c) and *E. coli* (Figure 3d), indicating that different (higher polarity) compounds may be responsible for the antibacterial activity against those bacterial species. Notably, all of the gastrointestinal bacteria screened herein were completely resistant to ampicillin, indicating the potential of the *H. procumbens* methanolic and aqueous root extracts to treat gastrointestinal diseases caused by these bacteria. Previous studies have also reported that these bacteria are resistant to a variety of β-lactam antibiotics.[31-33] Notably, *faecalis*, *B. cereus*, *E. coli*, *S. newport* and *S. sonneii* were also resistant to chloramphenicol, with ZOIs generally <7.5 mm. Only *E. aerogenes* was highly susceptible to chloramphenicol, with a ZOI of 14.5 mm. As all of the gastrointestinal bacterial species screened in our study were relatively susceptible to the

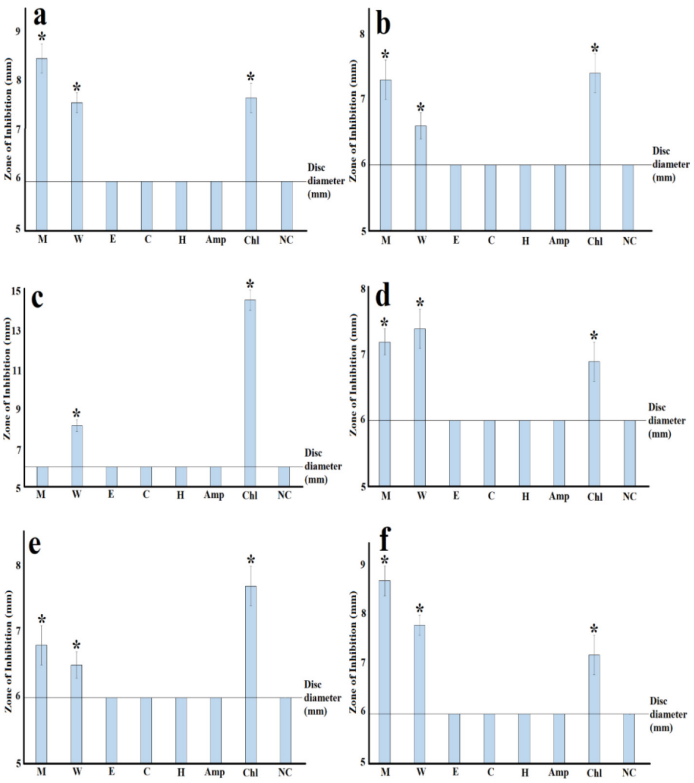

Figure 3: Antibacterial activity of *H. procumbens* root extracts against (a) *A. faecalis* clinical isolate strain; (b) *B. cereus* clinical isolate strain; (c) *E. aerogenes* clinical strain; (d) *E. coli* (ATCC:0157); (e) *S. newport* clinical strain; and (f) *S. sonneii* clinical strain, measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg); Chl = chloramphenicol (10 µg); NC = negative control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates (two repeats) ± SEM. * indicates results that are significantly different to the negative control (P<0.01).

polar *H. procumbens* root extracts despite those bacteria being antibiotic-resistant species, the extracts may be particularly useful in the treatment of antibiotic-resistant bacterial infections.

Quantification of minimum inhibitory concentration (MIC)

The relative level of antimicrobial activity was further evaluated by determining the MIC values using two methods: the liquid dilution MIC assay and the disc diffusion MIC assay (Table 2 and Table 3 for the autoimmune bacterial triggers and gastrointestinal bacteria respectively). Consistent with the antibacterial screening assays, only the higher polarity methanolic and aqueous *H. procumbens* root extracts inhibited all of the bacteria tested. In comparison, the ethyl acetate, chloroform and hexane extracts were completely ineffective growth inhibitors of these bacteria. The MIC values of the conventional antibiotic controls were only determined for the liquid dilution assay as commercial discs containing a fixed mass of antibiotic were used in the disc diffusion assay. Thus, the zones of only single doses was recorded for that assay and we were unable to determine MIC values. Ciprofloxacin was the most potent antibiotic against all bacteria (as judged by its MIC). Notably, both *P. mirabilis* and *K. pneumoniae* strains and the clinical *S. pyogenes* isolate, as well as all of the gastrointestinal pathogens strains tested, were strongly inhibited by ciprofloxacin. In contrast, with the exception of the erythromycin inhibition of *A. faecalis*, all of the bacterial strains tested were strongly resistant to all other antibiotics, with MIC values substantially >1 µg/mL. Indeed, the gastrointestinal pathogens were all completely resistant to penicillin and chloramphenicol, with no inhibition noted at any concentration tested.

The methanolic and aqueous *H. procumbens* root extracts were good inhibitors of the growth of the bacterial triggers of autoimmune diseases, with MIC values as low as 125 µg/mL. The exception was *A. baylyi*, for which low inhibition was noted, with MIC values >2500 µg/mL. Generally, the methanolic extract was a stronger growth inhibitor than the corresponding aqueous extract against most bacterial strains. However, a different trend was evident for *P. aeruginosa*, with lower MIC values determined for the aqueous extract compared to the methanolic extract. Particularly low MIC values were recorded for the methanolic

Table 2: Disc diffusion (DD) and liquid dilution (LD) MIC values (µg/mL) for *H. procumbens* root extracts against microbial triggers of some autoimmune inflammatory diseases.

Bacterial Species	Methanol Extract		Aqueous Extract		Ethyl Acetate Extract Pen		Chloroform Extract Chlor		Hexane Extract Tetra		Controls					
											NC					
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	LD MIC	LD MIC	LD MIC	LD MIC	LD MIC	LD MIC
***P. mirabilis* (ATCC: 33304)**	500	125	>5000	250	-	-	-	-	-	-	2.5	1.25	3.3	2.5	0.63	-
***P. mirabilis* clinical isolate**	625	250	>5000	250	-	-	-	-	-	-	2.5	1.25	3.3	2.5	0.63	-
***P. vulgaris* (ATCC21719)**	625	313	>5000	625	-	-	-	-	-	-	2.5	1.25	3.3	1.25	1.25	-
***K. pneumoniae* (ATCC: 31488)**	1250	313	625	156	-	-	-	-	-	-	3.3	1.9	1.9	1.9	0.3	-
***K. pneumoniae* clinical isolate**	1250	313	625	313	-	-	-	-	-	-	2.5	1.9	1.6	1.9	0.3	-
***A. baylyi* (ATCC: 21721)**	>5000	2500	>5000	>5000	-	-	-	-	-	-	3.3	2.5	2.5	1.25	1.25	-
***A. baylyi* clinical isolate**	>5000	2500	>5000	>5000	-	-	-	-	-	-	3.3	3.3	2.5	1.25	1.25	-
***P. aeruginosa* (ATCC: 39324)**	1316	625	625	313	-	-	-	-	-	-	3.3	1.25	3.3	1.25	1.25	-
***P. aeruginosa* clinical isolate**	1316	625	1316	625	-	-	-	-	-	-	3.3	1.25	3.3	2.5	1.25	-
***S. pyogenes* clinical isolate**	1316	313	1316	313	-	-	-	-	-	-	3.3	2.5	3.3	2.5	0.63	-

M = methanol extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane; DD = disc diffusion; LD = liquid dilution; Pen = penicillin-G; Chlor = chloramphenicol; Eryth = erythromycin; Tetra = tetracycline; Cip = ciprofloxacin. - indicates no inhibition at any dose tested.

Table 3: Disc diffusion (DD) and liquid dilution (LD) MIC values (µg/mL) for *H. procumbens* root extracts against bacterial gastrointestinal pathogens.

Bacterial Species	Methanol Extract		Aqueous Extract		Ethyl Acetate Extract Pen		Chloroform Extract Chlor		Hexane Extract Tetra		Controls					
							Eryth		Cip		NC					
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	LD MIC	LD MIC	LD MIC	LD MIC	LD MIC	LD MIC
***A. faecalis* clinical isolate**	>5000	625	>5000	1250	-	-	-	-	-	-	-	-	0.63	1.25	0.31	-
***B. cereus* clinical isolate**	>5000	1250	>5000	2500	-	-	-	-	-	-	-	-	-	-	0.63	-
***E. aerogenes* clinical isolate**	-	-	>5000	-	-	-	-	-	-	-	-	-	-	-	0.63	-
***E. coli* (ATCC: 0157)**	>5000	2500	>5000	1250	-	-	-	-	-	-	-	-	2.5	2.5	0.7	-
***S. newport* clinical isolate**	>5000	2500	>5000	2500	-	-	-	-	-	-	-	-	-	2.5	0.31	-
***S. sonneii* clinical isolate**	>5000	625	>5000	1250	-	-	-	-	-	-	-	-	2.5	2.5	0.16	-

M = methanol extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane; DD = disc diffusion; LD = liquid dilution; Pen = penicillin-G; Chlor = chloramphenicol; Eryth = erythromycin; Tetra = tetracycline; Gent = gentamycin. - indicates no inhibition at any dose tested.

extract against the *Proteus* spp., *K. pneumoniae* and *S. pyogenes*, indicating that this extract may be particularly useful for preventing rheumatoid arthritis, ankylosing spondylitis and rheumatic fever, as well as other diseases caused by these bacteria. Higher MIC values were recorded against the bacterial triggers of multiple sclerosis (*A. baylyi* and *P. aeruginosa*). However, these bacterial strains were particularly antibiotic-resistant. Therefore, the methanolic and aqueous *H. procumbens* root extracts may still be useful for preventing and treating multiple sclerosis. Future studies are required to test these extracts against an extended panel of *A. baylyi* and *P. aeruginosa* strains for a greater understanding of their therapeutic potential.

In contrast, the methanolic and aqueous extracts were less potent inhibitors of the growth of the gastrointestinal bacteria. Indeed, with the exception of the inhibition of *A. faecalis* and *S. sonneii* growth by the methanolic extract (MIC = 625 µg/mL for each), the MICs against all other bacteria were substantially >1000 µg/mL and therefore are classified as low to moderate growth inhibitory activity. However, all of the gastrointestinal bacteria were resistant (MIC <1 µg/mL) to all conventional antibiotics tested in this study, except ciprofloxacin. Therefore, the methanolic and aqueous *H. procumbens* root extracts may be useful for the treatment of gastrointestinal infections. Screening of these extracts against multiple strains of these bacteria is warranted to further evaluate the therapeutic potential of these extracts.

Toxicity studies

Two assays (ALA and the MTS cell viability assay) were used to assess the toxicity of the individual extracts and conventional antibiotics. The ALA was undertaken for the preliminary toxicity screening whilst the MTS cell viability assay provided a cellular evaluation of toxicity.

Artemia lethality assay (ALA)

All extracts were screened at 1 mg/mL in the ALA assay as an initial toxicity screen (Table 4). The extracts were only considered toxic if they induced percentage mortalities greater than 50% (LD_{50}) following 24 hr of exposure to the *Artemia* nauplii.[25] The conventional antibiotics demonstrated no toxicity in the ALA (Table 4). Similarly, none of the *H. procumbens* root extracts produced mortality significantly different to that of the negative control. Therefore, all extracts were deemed to be nontoxic. In contrast, the positive control potassium dichromate induced 100% mortality in the ALA, confirming that the assay was functioning correctly.

Table 4: Mortality (%) and cellular viability (%) results for samples tested individually in the ALA and MTS cell viability assay, respectively (*n*=6).

	Sample	Mortality ± SD (%) [a]		Cell viability ± SD (%) [b]
		After 24 hr:	After 48 hr:	After 24 hr:
Antimicrobials	Penicillin G	5.8 ± 2.2	8.6 ± 3.7	97.1 ± 2.6
	Chloranphenicol	2.6 ± 2.1	9.3 ± 3.6	91.2 ± 4.4
	Erythromycin	3.7 ± 1.6	6.4 ± 3.3	94.5 ± 4.8
	Tetracycline	4.2 ± 2.6	7.3 ± 3.2	92.7 ± 3.9
	Gentamicin	5.3 ± 2.8	9.2 ± 3.8	92.9 ± 3.7
Extracts	M	8.4 ± 4	24.8 ± 3.3	80.5 ± 4.6
	W	6.6 ± 3.5	18.4 ± 3.9	82.7 ± 4.7
	E	2.8 ± 1.4	8 ± 2.7	96.5 ± 4.2
	C	5.3 ± 2.7	10.4 ± 4.5	89.4 ± 4.5
	H	2.6 ± 1.5	5.8 ± 3.1	101.3 ± 4.7
Controls	Deionised water	2.7 ± 1.7	3.6 ± 2.5	96.8 ± 5.7
	Quinine	2.3 ± 1.1 [a]	4.6 ± 2.7[a]	31.4 ± 4.8 [b]
	Potassium dichromate	100.00 ± 0.00 [a]		NT

[a] = Tested at a concentration of 1 mg/mL; [b] = Tested at a concentration of 200 µg/mL; M =methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; SD = standard deviation; NT = control not tested in the assay.

MTS cell viability assay

The *H. procumbens* root extracts and the conventional antibiotics were also each individually screened at 200 µg/mL against HDF in the cell viability assay. In this assay, extracts which produce <50% cell at 200 µg/mL are deemed to be toxic.[10] None of the extracts or conventional antibiotics displayed <50% HDF viability and thus all were deemed to be non-toxic (Table 4). In contrast, exposure to the positive control (quinine) reduced HDF cell viability by approximately 70%,

DISCUSSION

This study investigated the ability of *H. procumbens* root extracts to inhibit the growth of some bacterial triggers of autoimmune inflammatory diseases and selected gastrointestinal bacterial pathogens. The methanolic and aqueous *H. procumbens* root extracts were identified as effective bacterial growth inhibitors against multiple bacterial pathogens, with MIC values as low as 125 µg/mL determined against some bacterial strains. Interestingly, only the methanolic and aqueous *H. procumbens* root extracts inhibited the growth of the bacteria, indicating that the antibacterial compounds were relatively high polarity. The methanolic extract was a good growth inhibitor of the bacterial triggers of autoimmune diseases, particularly against *P. mirabliis* (MIC = 125 µg/mL), *K. pneumonia* (MIC = 313 µg/mL) and *S. pyogenes* (MIC = 313 µg/mL) growth. The inhibitory activity of the methanolic extract was slightly lower but still noteworthy against *P. aeruginosa* (MIC = 625 µg/mL). In contrast, the MIC values of 2500 µg/mL calculated for *A. baylyi* is indicative of only low inhibitory activity.

In contrast to the autoimmune disease bacterial triggers, the *H. procumbens* root extracts were substantially less potent inhibitors of the gastrointestinal bacterial pathogens. Indeed, whilst methanolic extract produced MIC values of 625 µg/mL against *A. faecalis* and *S. sonneii*, these were the only MIC values <1000 µg/mL measured against the gastrointestinal bacterial panel of bacteria. The MIC values against all other gastrointestinal bacteria indicated only weak inhibitory activity. Notably, these inhibitory profiles were consistent with those reported in a previous study, which screened *H. procumbens* root extracts against a panel of skin and gastrointestinal bacteria.[7] The earlier study quantified antibacterial activity using an agar dilution/colony forming units (CFU) assay and defined the MIC as the extract concentration that gave <10 colonies, whereas our study used a liquid dilution assay and defined the MIC as the concentration at which no visible bacterial growth was evident. Whilst a comparison of gastrointestinal bacteria inhibition MICs between these studies is therefore difficult, it is noteworthy that similar trends were still apparent.

Whilst a detailed investigation of the phytochemistry of the *H. procumbens* root extracts was beyond the scope of this study, the qualitative phytochemical studies highlighted several phytochemical classes that may contribute to the bacterial growth inhibitory activity. The methanolic and aqueous extracts had relatively high abundances in polyphenolics, flavonoids and tannins. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.[34] This has been attributed to a variety of mechanisms, including their ability to complex with extracellular and soluble proteins, as well as bacterial cell wall proteins.[35] Similarly, multiple tannins have broad spectrum antibacterial activity via a variety of intra- and extracellular mechanisms, including the precipitation of microbial proteins.[36] Phenolics are toxic to microorganisms via enzyme inhibition mechanisms, possibly through non-specific interaction with proteins or by reaction with sulfhydryl groups.[37] It is also likely that other phytochemical classes may also contribute to the growth inhibitory properties of these extracts. Therefore, phytochemical evaluation studies and bioactivity driven isolation of active components are required to evaluate the mechanism of the *H. procumbens* root extracts growth inhibition.

There is a common misconception amongst consumers that all natural products are safe. However, like synthetic drugs, many traditional medicines and natural products may induce side effects and are not devoid of toxicity, even when used correctly.[38-40] It is surprising given the widespread use of traditional medicines that relatively few have been thoroughly evaluated for safety and substantially further work is required. Notably, none of the *H. procumbens* root extracts, displayed toxicity in either the ALA or HDF MTS assays, confirming their potential for therapeutic use. The non-toxicity of the conventional antibiotics is hardly surprising as these drugs have a long history of therapeutic use. Additionally, our results correlate well with previous reports from other groups.[41,42] Indeed, a recent study[43] reported an LC_{50} of 20 mg/mL in a similar ALA assay as that used in our study. As LC_{50} values >1 mg/mL are generally regarded as nontoxic in that assay,[24,25] the aqueous root extracts examined in that study were classified as nontoxic. Similarly, several *in vitro* cytotoxicity studies have also reported ethanol and aqueous *H. procumbens* root extracts to be nontoxic towards RAW267.4 macrophages, C2C12 mouse myoblasts and HCT116 human colorectal adenocarcinoma cells.[44,45] Whilst the cell lines used in those studies differ from the HDF cells used in our study, they confirm the lack of cytotoxicity of the *H. procumbens* root extracts. Whilst the lack of toxicity detected in our study and in the previous studies indicate their potential for therapeutic usage, further *in vitro* studies using a wider panel of human cell lines are required to verify their safety. Furthermore, *in vivo* testing is also required to confirm that the extracts remain nontoxic in complex biological systems.

CONCLUSION

Whilst the findings reported herein indicate the potential of methanolic and aqueous *H. procumbens* root extracts for the prevention and treatment of selected autoimmune inflammatory diseases and to treat gastrointestinal infections, testing against a wider panel of bacterial strains is warranted. Additionally, further *in vivo* investigations are required to support these *in vitro* findings and to determine whether the extracts are also useful in complex multicellular systems. Furthermore, studies to determine the possible mechanism of action resulting in the observed growth inhibitory activities are warranted, and bioactivity driven compound isolation and/or metabolomics studies are also required to determine the active compound(s) within the *H. procumbens* root extracts.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ABBREVIATIONS

ALA: brine-shrimp lethality assay; **DMSO:** Dimethyl sulfoxide; **INT:** ρ-iodonitrotetrazolium chloride; **LD_{50}:** dose of sample necessary to have a lethal effect on 50% of test organisms or cells; **MIC:** minimum inhibitory concentration; ΣFIC: The sum of the fractional inhibitory concentration.

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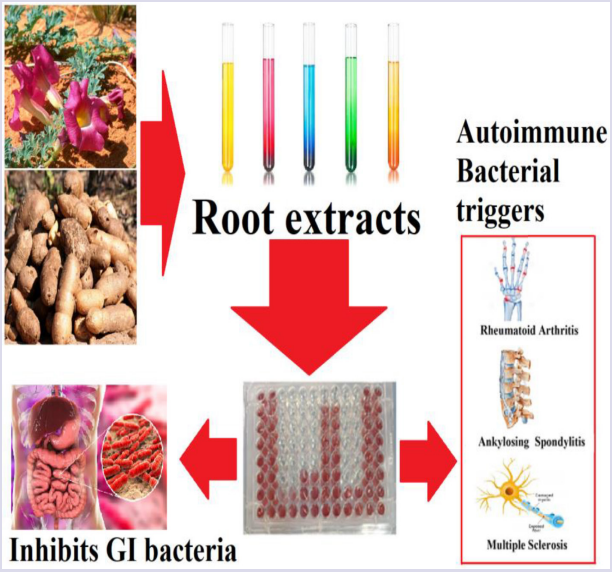
PICTORIAL ABSTRACT



SUMMARY

- *Harpagophytum procumbens* root extracts were screened for the ability to block the growth of a panel of bacterial triggers of selected autoimmune diseases.
- The extracts were also screened for the ability to block the growth of a panel of gastrointestinal bacterial pathogens.
- The antibacterial activity was quantified by determining the MIC values of each extract.
- Toxicity of *H. procumbens* root extracts was determined using the *Artemia* nauplii and HDF cell viability toxicity bioassays.

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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 nearly 250 publications in a variety of peer reviewed journals.

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