Tabebuia impetiginosa (Mart. Ex DC. Mattos) Bark Extracts Inhibit the Growth Gastrointestinal Bacterial Pathogens and Potentiate the Activity of some Conventional Antibiotics

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ABSTRACT

Introduction: *Tabebuia impetiginosa* (Mart. Ex DC. Mattos) bark has been used to treat inflammation and cancer, as well as a variety of pathogenic diseases, in traditional South American healing systems. As practitioners of complementary medicine frequently use herbal medicines concurrently with conventional antibiotics, the effects of both therapies in combination needs to be evaluated. **Methods:** The growth inhibitory activity of *T. impetiginosa* bark extracts was assessed against a panel of gastrointestinal bacterial pathogens by standard disc diffusion and liquid dilution minimum inhibitory concentration (MIC) methods. Combinational effects between the extracts and conventional antimicrobials were classified using the sum of the fractional inhibitory concentration. The toxicity of the individual samples and combinations was evaluated by *Artemia* lethality and MTS HDF cell viability assays. **Results:** *T. impetiginosa* bark extracts strongly inhibited the growth of *B. cereus* but were ineffective against *Escherichia coli, Shigella sonnei, Staphylococcus aureus*. The mid polarity ethyl acetate extract was a particularly good inhibitor of *B. cereus* growth (DD and LD MIC values of 45 and 245 μg/mL respectively). However, the effects of combinations of the extracts and conventional antibiotics was of considerably more interest. Although no synergistic interactions were noted, the potency of some combinations were substantially potentiated compared to activity of the individual components. Additive potentiation was was noted for combinations containing the *T. impetiginosa* water extract and erythromycin, chloramphenicol or penicillin-G against *E. coli* and

S. aureus. Combinations containing ciprofloxacin also produced additive effects against all of the bacteria tested. Therefore, these combinations have enhanced benefits over either component alone. Of further note, antagonistic interactions were also detected in several combinations containing ciprofloxacin (particularly against *S. aureus*). These combinations should therefore be avoided against that bacterium. **Conclusion:** *T. impetiginosa* bark extracts inhibit the growth of *B. cereus* when tested alone and potentiated the activity of some conventional antibiotics against a panel of gastrointestinal pathogens when used in combination. Thus, *T. impetiginosa* bark extracts have potential in the treatment of diarrhoea and other gastrointestinal diseases.
Key words: Pau d'arco, Gastrointestinal disease, Diarrhoea, *Bacillus cereus, Escherichia coli, Shigella sonnei, Staphylococcus aureus*, Lapachol.

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INTRODUCTION

The discovery of penicillin by Alexander Flemming nearly a hundred years ago caused a major paradigm shift in the way that medical research sought to develop new antibiotic chemotherapies. Since that time, research has focussed on screening microbially derived compounds for antibiotic activity. This method of discovery has provided the majority of our first generation drugs. However, bacteria have developed resistance to all of these antibiotics, generally within a couple of years of their incorporation into clinical use.[1] Many medicinally important bacterial pathogens have become either extremely (XDR) or totally drug resistant (TDR) to commonly used antibiotics[1] and there are now limited therapeutic options to eradicate these pathogens. This problem is expected to worsen as bacteria further exchange resistance genes, rendering more bacterial strains multi-drug resistant (MDR). The development of alternative antibacterial treatment modalities has become crucial and one of the most serious challenges currently facing medical science. For a number of reasons reviewed elsewhere,[1] 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.

This problem is particularly evident for infectious gastrointestinal disease as the gastrointestinal milieu provides an ideal medium for the exchange of genetic material. As a result, nearly nine million children under the age of five die every year from diarrhoeal related illnesses.[2] Indeed, diarrhoea is the leading killer of children globally, accounting for 9% of all deaths among children under the age of five.[3] This equates

to more than 1400 young children dying each day, or about 530,000 children a year. Many gastrointestinal bacterial pathogens have developed resistance to conventional antibiotics, rendering them useless against some diarrhoea causing pathogens. There is an urgent need to develop new treatment options to combat these diseases, either through the development of novel drugs, or through new therapies enabling previously effective antibiotics to function with increased efficacy again.

Many plants used in traditional medicinal systems are effective in the treatment of diarrhoea and their usage is often well documented, aiding in species selection. These traditional medicines are often used separately, or may be prescribed in combination. The multiple negative effects of diarrhoea (loose stools, cramps, loss of electrolytes and possible fever) have been targeted by combining different medicinal plants for either a synergistic effect or to treat the different symptoms. Combinational therapies including one or more plant extract, often results in synergy and a far better therapeutic outcomes than any of the components alone.[4-7]

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.[8-10] *Tabebuia impetiginosa* (Mart. Ex DC. Mattos) (synonyms *Tabebuia palmeri* Rose, *Handroanthus avellanedae* Lorentz ex Griseb.; common names Pau d'arco (Portuguese), Lapacho (Spanish) and Trumpet tree (English)) has been used by indigenous South Americans as a remedy for inflammation, cancer, syphilis, malaria,

fevers, trypanosomiasis, fungal and bacterial infections, as well as to treat stomach disorders.[11] The bark is particularly well known as an effective immunostimulant and inflammatory modulator and recent studies have demonstrated that *T. impetiginosa* bark has immunomodulatory effects. A recent study reported that *T. impetiginosa* bark were potent inhibitors of pro-inflammatory cytokine secretion, reducing the levels of both PMA-induced and LPS-induced cytokines and chemokines by greater than 95%.[12] That study reported significant modulation of IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, MCP-1 and TNF-α levels. Another study tested similar extracts against the bacterial triggers of earlier phases of the progression of some autoimmune diseases.[13] That study highlighted the potent antibacterial activity of these extracts against these autoimmune disease associated pathogens. Despite this, the growth inhibitory activity of *T. impetiginosa* extracts against other bacterial pathogens is lacking. Our study was undertaken to examine the effects of *T. impetiginosa* bark extracts on some gastrointestinal bacterial pathogens associated with diarrhoea. The aim of this project is to investigate the efficacy of the traditional South American medicinal plant (alone and in combination with conventional antibiotics) against pathogens which cause diarrhoea.

MATERIALS AND METHODS

Plant material and extraction

T. impetiginosa (Mart. Ex. DC. Mattos) bark was supplied by Noodles Emporium, Australia (an online herbal supplier) and were originally sourced from verified trees in Peru. The sample was stored at -30°C until processing. Individual 1g masses of bark were weighed and transferred into five 50 mL Falcon tubes tubes. Each tube was filled with 50mL of individual solvents of varying polarity (water, methanol, ethyl acetate, chloroform and hexane). All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The bark was extracted for 24hr with gentle oscillation. The resultant extracts were filtered into new tubes using Whatman No. 54 filter paper. The extracts were subsequently dried at 40°C in an vacuum incubator until the solvents had completely evaporated. The dried products were weighed and dissolved in 10mL deionised water (containing 1% DMSO) and syringe filtered using Millipore 0.22μm membrane filters). The extracts were stored at 4°C until further use.

Qualitative phytochemical studies

Qualitative phytochemical analysis of the *T. impetiginosa* extracts to detect the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids and evaluate their relative abundances was conducted using standard assays.[14,15]

Antibacterial screening
Conventional antibiotics

Chloramphenicol (≥98% purity), erythromycin (≥850μg/mg), gentamicin (600μg/mg), penicillin-G (1440-1680μg/mg) and tetracycline (≥95% purity) were purchased from Sigma-Aldrich, Australia. All antibiotics were prepared as 0.01mg/mL stocks in sterile deionised water. For the disc diffusion studies, pre-loaded ampicillin (2μg) and chloramphenicol (10μg) susceptibility discs (Oxoid Ltd., Australia) were used as positive controls.

Test microorganisms

All media was obtained from Oxoid Ltd., Australia and prepared as per manufacturer's instructions. A reference strain of was *E. coli* (ATCC K12) purchased from American Tissue Culture Collection, USA. The clinical strains of *Bacillus cereus, Shigella sonnei* and *S. aureus,* were kindly provided by the School of Environment and Science teaching laboratory,

Griffith University. All bacterial strains were individually cultured to log phase in nutrient broth (Oxoid Ltd., Australia). Streak nutrient agar (Oxoid Ltd., Australia) plates were cultured in parallel to ensure the purity of all bacterial cultures and to allow for sub-culturing. All cultures were incubated at 37°C for 24hr and were maintained in nutrient broth at 4°C.

Antimicrobial activity screening

The *T. impetiginosa* bark extracts were initially screened undiluted using a standard disc diffusion assay as previously described.[16,17] Briefly, individual bacterial suspensions (100μL) was spread evenly onto nutrient agar plates and 6mm filter paper discs infused with individual extracts were placed onto the surface of the plates. The plates were incubated at 37°C for 24 hr and the diameters of the zones of inhibition (ZOI) were subsequently measured and are expressed as the mean values (± SEM) of three independent experiments, each with internal triplicate measurements (*n*=9). Standard discs of ampicillin (10μg; Oxoid Ltd., Australia) and discs infused with 10μL of distilled water (containing 1% DMSO) were included on each agar plate as a positive and negative controls respectively.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using liquid dilution MIC assays and solid phase agar disc diffusion assays.

Microplate liquid dilution MIC assay

A standard liquid dilution MIC assay[18,19] was used to evaluate the bacterial growth inhibitory activity of the extracts and conventional antibiotics, both independently and as combinations. Briefly, log phase bacterial cultures were diluted to produce a McFarlands inoculation culture.[18,19] A 100μL volume of sterilized nutrient broth was dispensed into all wells of a 96 well micro-titre plate. Then, 100μL of the plant extracts or conventional antibiotics were dispensed into separate wells of the top row of the plate. A negative control (nutrient broth), sterile control (broth without bacteria) and a sample-free culture control (to ensure the media was capable of supporting microbial growth) were also included on all plates. Each test sample or control was serially diluted down each column on the plate by doubling dilution. The assay culture inoculum (100μL, containing approximately 1×10^6 colony forming units (CFU)/mL) was then added to all wells except the sterile control wells and incubated overnight at 37°C. p-Iodonitrotetrazolium violet (INT, Sigma-Aldrich, Australia) was dissolved in sterile deionised water to a concentration of 0.2 mg/mL. A volume of 40μL of the INT solution was added into all wells and the plate was incubated for a further 6 hr at 37°C. The MIC was visually determined as the lowest dose at which colour development was inhibited.

Disc diffusion MIC assay

The minimum inhibitory concentration (MIC) of each extract was also quantified by disc diffusion assay.[16,17] Graphs of the zone of inhibition (ZOI) versus ln concentration were plotted and MIC values were calculated by linear regression.

Extract-conventional antibiotic interaction studies: Fractional inhibitory concentration (FIC) assessment

Interactions between the plant samples and conventional antimicrobials were detected and classified via determination of the sum of the fractional inhibitory concentration (ΣFIC). The FIC was calculated using the following equation, where (a) represents the plant sample and (b) the conventional antibiotic sample:[20,21]

$$FIC^{(i)} = \frac{\text{MIC (a) in combination with (b)}}{\text{MIC (a) independently}}$$

$$FIC^{(ii)} = \frac{MIC\ (b)\ in\ combination\ with\ (a)}{MIC\ (b)\ independently}$$

The ΣFIC was then calculated using the equation: $\Sigma FIC = FIC^{(i)} + FIC^{(ii)}$. The interactions were classified as being synergistic for ΣFIC values of $\leq$ 0.5, additive (>0.5–1.0), indifferent (>1.0–$\leq$4.0) or antagonistic (>4.0).[7]

Toxicity evaluation

Two assays were used to evaluate and quantify the toxicity of the extracts. The *Artemia* lethality assay (ALA) was utilised for rapid preliminary toxicity assessment. An MTS-based cellular proliferation assay was used as a cellular evaluation of the extract's toxicity.

Artemia franciscana nauplii toxicity screening

Toxicity was evaluated using an *A. franciscana* nauplii (brine shrimp) lethality assay, modified for testing plant extracts.[22,23] Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in distilled water as a 1.6mg/mL solution and used as a reference toxin in the *Artemia franciscana* nauplii bioassay. *A. franciscana* nauplii were incubated in the presence of the test extracts, reference toxin or artificial seawater (negative control) at 25±1°C under artificial light (1000 Lux). All treatments were performed three times in triplicate (*n*=9). The number of dead were counted in each well at 24hr and the remaining live nauplii were then 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.

Cellular viability assay

The *T. impetiginosa* bark extracts were screened against normal human primary dermal fibroblasts (HDF; American Type Culture Collection (ATCC PCS-201-012)) using conventional assays.[24] Briefly, aliquots of the HDF cells (70μL, containing approximately 5000 cells) were added to individual wells of a 96 well plate. The test extracts or controls (30μL) were added to individual wells and the plates were incubated at 37°C, 5% CO_2 for 24 hr in a humidified atmosphere. All extracts were initially tested at 200μg/mL. Following a wash in PBS (pH 7.2) to remove interference due to sample colour, 20μL of Cell Titre 96 Aqueous One solution (Promega) was added to all wells and re-incubated at 37°C for a further 3 hr. Absorbances were recorded at a test wavelength of 540nm and a blank wavelength of 690nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed three times, each with internal triplicates (*n*=9). The % cellular viability of each test was calculated using the following formula:

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

Less than 50% cellular viability indicated toxicity, whereas tests with >50% untreated control viability were defined as non-toxic.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) 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 *P* values <0.01 considered statistically significant.

RESULTS

Extraction yields and qualitative phytochemical screening

Extraction of 1g masses of dried *T. impetiginosa* bark with various solvents yielded dried plant extracts ranging from 51.3mg (water extract) to 79.3mg (hexane extract) (Table 1). Methanol and hexane gave the highest yields of dried extract material, whilst all other solvents extracted

lower masses. The dried extracts were resuspended in 10mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 1. Qualitative phytochemical studies (Table 1) showed that methanol extracted the widest range of phytochemicals, with high levels of phenolic compounds, cardiac glycosides, triterpenoids, flavonoids and alkaloids detected. The water extract had high levels of phenolics, cardiac glycosides, saponins, triterpenoids and alkaloids. Ethyl acetate extracted high levels of water insoluble phenols, saponins, triterpenoids and alkaloids, as well as moderate levels of flavonoids and low levels of phenolic compounds. Similarly, the chloroform extract contained high levels of saponins, triterpenoids, alkaloids, as well as low levels of phenolic compounds and flavonoids. Hexane extracted the narrowest range of phytochemicals, with water insoluble phenols and alkaloids present in the highest relative abundance. Low levels of phenolic compounds, triterpenoids and flavonoids were also detected in the hexane extract.

Antibacterial activity

To examine the growth inhibitory activity of the *T. impetiginosa* bark extracts, a series of disc diffusion assays were conducted on nutrient agar plates inoculated with diarrhoea causing bacterial strains (*B. cereus, E. coli, S. sonnei* and *S. aureus*). *B. cereus* growth was inhibited by all of the *T. impetiginosa* bark extracts except the aqueous extract (Figure 1). The methanolic extract had the strongest antibacterial effects, with zones of inhibition of 8.6 ± 0.4mm. The zones of inhibition of the other extracts ranged from approximately 7.2-7.6mm. These zones of inhibition are indicative of low to moderate growth inhibition. However, this bacterial strain was also somewhat resistant to ampicillin. Therefore, these extracts may still have potential for the prevention and treatment of *B. cereus* infections. In contrast, chloramphenicol was a relatively good inhibitor of *B. cereus* growth. No growth inhibitory activity was observed for any of the extracts against any of the other bacterial species.

The minimum inhibitory concentration for each extract was determined by further analysing the extracts that showed antimicrobial activity against *B. cereus* across a range of concentrations in the disc diffusion and liquid dilution MIC assays (Table 2). MIC values for the antibiotic controls are only provided for the liquid dilution assays as the standard antibiotic control discs used for the disc diffusion assay were only tested at a single dose. The *T. impetiginosa* bark extracts only inhibited the growth of *B. cereus*. No inhibition was noted against any of the other bacteria in either assay system so no MIC values are recorded here. Similarly, the aqueous extract was completely ineffective against *B. cereus* so an MIC value is also not recorded. In contrast, the methanolic, ethyl acetate, chloroform and hexane extracts displayed MIC values substantially <1000μg/mL against *B. cereus*, indicating good growth inhibitory properties. Indeed, the ethyl acetate extract had MIC values of 54 and 245μg/mL in the disc diffusion and liquid dilution MIC assays respectively. The hexane extract was the next strongest inhibitor of *B. cereus* growth, followed by the methanol and chloroform extracts. These results indicate that the active component(s) within these extracts may be mid to low polarity.

Combinational effects on bacterial gastrointestinal pathogens

The LD assay was also used to study combinational effects of the *T. impetiginosa* bark extracts in combination with six conventional antibiotics (tetracycline, gentamycin, erythromycin, chloramphenicol, penicillin-G and ciprofloxacin). The ΣFIC values calculated in this study (Table 3) were used to distinguish between synergistic, additive, non-interactive and antagonistic combinational effects. None of the combinations resulted in synergy. The majority of the combinations had little benefit when

Table 1: Extraction yields and qualitative phytochemical screenings of *T. impetiginosa* bark extracts.

Extract	H₂O	MeOH	ETAC	CHCl₃	HEX
Mass of extract (mg)	51.3	76.1	63.8	65.5	79.3
Concentration of the extract (mg/mL)	5.13	7.61	6.38	6.55	7.93
Phenolic compounds	+++	+++	+	+	+
Water soluble phenols	+	++	-	-	-
Water insoluble phenols	-	+++	+++	+++	+++
Cardiac glycosides	+++	+++	-	-	-
Polysteroids	-	-	-	-	-
Saponins	+++	-	+++	+++	-
Triterpenoids	+++	+++	+++	+++	+
Flavonoids	-	+++	++	+	+
Free anthraquinones	-	-	-	-	-
Combined anthraquinones	-	-	-	-	-
Mayer reagent test (alkaloids)	+++	+++	+++	+++	+++
Wagner's reagent test (alkaloids)	+++	+++	+++	+++	+++

H₂O = water extract; **MeOH** = methanol extract; **ETAC** = ethyl acetate extract; **CHCl₃** = chloroform extract; **HEX** = hexane extract; +++ indicates a strong response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay
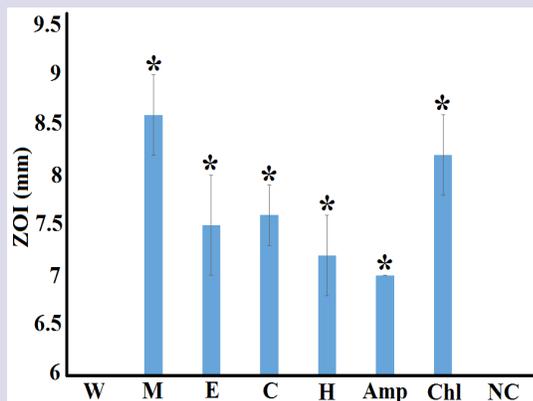
Figure 1: Antibacterial activity of the *T. impetiginosa* bark extracts against a clinical *B. cereus* strain measured as zones of inhibition (mm). W = aqueous extract; M = methanolic extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. Positive controls: Amp = ampicillin (10µg); Chl = chloramphenicol (10µg); Negative control (NC) = water. 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).
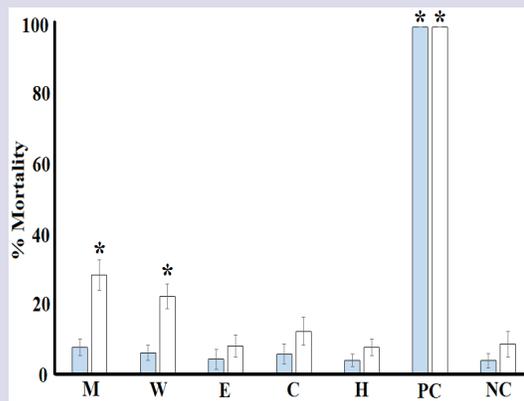
Figure 2: The lethality of the *T. impetiginosa* bark extracts, potassium dichromate control (1000µg/mL) and seawater (negative control). Shaded bars represent the mortality induced by the *T. impetiginosa* extracts following 24hr exposure; open bars represent the mortality induced by the *T. impetiginosa* extracts following 48hr exposure. All bioassays were performed three times in triplicate (*n*=9) and are expressed as mean ± SD. * indicates results that are significantly different to the untreated (seawater) control at the equivalent exposure time (*P*< 0.01).

tested against *E. coli*. When the extracts were combined with gentamicin, all combinations were classified as indifferent (ΣFICs ranging from 1.62-2.09; Table 3). Similarly, the ethyl acetate and chloroform extracts produced indifferent interactions when combined with ciprofloxacin (ΣFICs of 3.03 and 1.62 respectively). However, additive reactions were observed when combining the polar aqueous and methanolic extracts with erythromycin, chloramphenicol or penicillin-G, with ΣFIC values of 0.6 recorded. When the aqueous or methanolic extracts were combined with ciprofloxacin, an ΣFIC of 1 was observed, also indicating additive effects. Therefore, these combinations may have some benefits

in treating diarrhoea caused by these pathogens, compared to the effects of the extracts or the antibiotic when used alone.

When the *T. impetiginosa* bark extracts were tested in combination with the conventional antibiotics against *B. cereus,* most had no activity. Of the combinations that inhibited *B. cereus* growth, most produced indifferent effects. Indeed, all of the combinations containing gentamicin were non-interactive. Interestingly, when the ethyl acetate or chloroform extracts were combined with ciprofloxacin, additive effects were noted (ΣFICs of 0.51 and 1 respectively). These combinations have enhanced efficacy compared to either component when used alone. Therefore, the use of

Table 2: Disc diffusion (DD) and liquid dilution (LD) MIC values for *T. impetiginosa* bark extracts against *E. coli* (ATCC: K12) and the clinical strains of *B. cereus*, *S. sonneii* and *S. aureus* growth (μg/mL).

EXTRACT	E. coli (ATCC: K12)		B. cereus (CI)		S. sonneii (CI)		S. aureus (CI)	
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC
TIH$_2$O	-	-	-	-	-	-	-	-
TIMeOH	-	-	643	480	-	-	-	-
TIETAC	-	-	53.6	245	-	-	-	-
TICHCl$_3$	-	-	797.2	840	-	-	-	-
TIHEX	-	-	425.5	755	-	-	-	-
Positive controls								
Tetracycline	ND	-	ND	-	ND	2.5	ND	-
Gentamicin	ND	0.4	ND	0.3	ND	0.3	ND	0.3
Erythromycin	ND	0.01	ND	-	ND	-	ND	-
Chloramphenicol	ND	-	ND	-	ND	-	ND	-
Penicillin G	ND	-	ND	-	ND	-	ND	-
Ciprofloxacin	ND	0.2	ND	0.2	ND	0.08	ND	0.2
Negative control								
1% DMSO (DD)/ nutrient broth (LD)	ND	-	ND	-	ND	-	ND	-

TI= *T. impetiginosa;* H$_2$O= water; MeOH= methanol; ETAC= ethyl acetate; CHCl$_3$= chloroform; HEX= hexane; DD=Disc Diffusion; LD= Liquid Dilution; - = No inhibition; **numbers** indicate the mean DD MIC and LD MIC values of triplicate determinations, expressed in μg/mL; ND= MIC could not be determined as only standard discs containing a fixed dose were used.

these combinations is beneficial in the treatment of diarrhoea caused by *B. cereus*. Interestingly, when the water extract was combined with ciprofloxacin, antagonism was observed (ΣFIC of 5, Table 3). Therefore, this combination should be avoided when treating diarrhoea.

Four additive interactions were observed when the methanol, ethyl acetate, chloroform or hexane extracts were tested with ciprofloxacin (ΣFICs of 1) against *S. sonnei*, whereas most of the combinations with gentamicin were indifferent. No other combination showed combinational effects against *S. sonnei*. Similarly, when the *T. impetiginosa* bark extracts were combined with the antibiotics and tested against *S. aureus,* most of the reactions were classified as indifferent. However, when the water extract was combined with erythromycin, chloramphenicol or penicillin-G, additive effects were observed, with ΣFICs of 1. Therefore, the use of these combinations would be beneficial for the treatment of *S. aureus* induced gastrointestinal disease. Notably, when combining the aqueous, methanolic or the ethyl acetate extract with ciprofloxacin, antagonistic effects were observed (ΣFICs of 4.5, 8.1 and 8.1 respectively). Therefore, these combinations should be avoided when treating diarrhoea.

Quantification of toxicity

All extracts were initially screened undiluted in the assay in parallel with the reference toxin potassium dichromate (1000μg/mL) (Figure 2). The toxic effects of potassium dichromate were evident within the first 4 hr of exposure, with 100% mortality achieved following 6hr exposure (results not shown). In contrast, all extracts induced substantially <50% mortality at all concentrations screened and were therefore deemed to be nontoxic and LC$_{50}$ values could not be determined. The *T. impetiginosa* bark extracts were also screened against human dermal fibroblasts (HDF) in the cell viability assay. In this assay, extracts producing <50%

cell viability at 200μg/mL are deemed to be toxic.[25,26] None of the extracts induced changes in % cell viability that were significantly different from the untreated control values. Therefore, LC$_{50}$ values could not be determined for these extracts. All extracts were deemed to be non-toxic. In contrast, toxicity of the positive control (quinine) was confirmed, with a LC$_{50}$ value of 46μg/mL calculated, indicating that the assay was functioning properly.

DISCUSSION

Diarrhoea remains a leading killer of children, accounting for 8% of all death among children under the age of 5 die.[27] Indeed, more than half a million children in this age group die each year from diarrhoeal disease,[28] which equates to over 1400 deaths per day. Furthermore, as bacterial pathogens develop further resistance to conventional antibiotics, medicines are expected to have reduced efficacy against diarrhoeal diseases and increases in mortality rates are expected.[28,29] There is an urgent need to develop new therapeutic options to combat these illnesses, either through novel drug molecules or by utilizing new therapy regimens that enable (or re-purpose) previously effective antibiotics that have lost their potency due to the emergence of highly resistant infections to become useful again.

Many plants used in traditional medicinal systems are effective in the treatment of diarrhoea. *T. impetiginosa* bark has been used for thousands of years by indigenous South Americans to treat inflammation, cancer, syphilis, malaria, fevers, trypanosomiasis, fungal and bacterial infections, as well as stomach disorders.[11] Studies on the antibacterial properties of *T. impetiginosa* bark are relatively lacking and much more work is required in this area. Recent studies have reported that *T. impetiginosa* bark extracts are potent inhibitors of pro-infammatory cytokine

Table 3: Combinational effects of the *T. impetigosa* extracts and conventional antibiotics on the bacterial gastrointestinal pathogens.

E. coli (ATCC K12)						
Extracts	**Tetracycline**	**Gentamycin**	**Erythromycin**	**Chloramphenicol**	**Penicillin-G**	**Ciprofloxacin**
TIH$_2$O	-	2.1	*0.6*	*0.6*	*0.6*	*1*
TIMeOH	-	1.8	*0.6*	*0.6*	-	*1*
TIETAC	-	1.6	-	-	-	3
TICHCl$_3$	-	1.8	-	-	-	1.6
TIHEX	-	1.8	-	-	-	*1*
B. cereus clinical strain						
Extracts	Tetracycline	Gentamycin	Erythromycin	Chloramphenicol	Penicillin-G	Ciprofloxacin
TIH$_2$O	-	2.5	1.1	1.1	1.1	<u>5</u>
TIMeOH	-	2.3	-	-	-	2.3
TIETAC	-	1.1	-	-	-	*0.51*
TICHCl$_3$	-	1.1	-	-	-	*1*
TIHEX	-	1.1	-	-	-	2
S. sonnei clinical strain						
Extracts	Tetracycline	Gentamycin	Erythromycin	Chloramphenicol	Penicillin-G	Ciprofloxacin
TIH$_2$O	-	2.1	-	-	-	-
TIMeOH	-	2.3	-	-	-	*1*
TIETAC	-	2.1	-	-	-	*1*
TICHCl$_3$	-	2.1	-	-	-	*1*
TIHEX	-	-	-	-	-	*1*
S. aureus						
Extracts	Tetracycline	Gentamycin	Erythromycin	Chloramphenicol	Penicillin-G	Ciprofloxacin
TIH$_2$O	-	2.5	*1*	*1*	*1*	<u>4.5</u>
TIMeOH	-	2.1	-	-	-	<u>8.1</u>
TIETAC	2.1	-	-	-	-	<u>8.1</u>
TICHCl$_3$	2.1	2.1	-	-	-	-
TIHEX	-	2.1	-	-	-	*1*

TI= *T. Impetiginosa* bark; H$_2$O= water; MeOH= methanol; ETAC= ethyl acetate; CHCl$_3$= chloroform; HEX= hexane; Numbers indicates the mean FIC values calculated in duplicate; - = indicates that ΣFIC could not be determined; **Synergy = ≤ 0.5**; *Additive = > 0.5-1.0*; Non-Interactive= > 1.0 - ≤ 4; <u>Antagonistic= > 4.0</u>.

secretion[12] and the growth of bacterial mediators of some autoimmune diseases.[13] This report aimed to extend these previous studies by screening the extracts against bacterial gastrointestinal pathogens (alone and in combination with conventional antibiotics). Whilst we did detect some antibacterial activity in this study, most bacterial species examined were refractory to inhibition by the extracts. However, activity was observed for the methanolic, ethyl acetate, chloroform and hexane *T. impetiginosa* bark extracts towards *B. cereus*. This suggests that *T. impetiginosa* bark extracts contains components that could be used to treat diarrhoeal illness caused by *B. cereus*, despite the fact that the bacteriostatic/cidal effects are relatively mild. To our knowledge, this represents the first evidence that *T. impetiginosa* bark possesses bacterial growth inhibitory properties against this specific strain.

The combinational studies reported in this manuscript screened the *T. impetiginosa* bark extracts extracts for interactions with the conventional antibiotics. These studies are not only important to identify possible antimicrobial alternatives to overcome bacterial antibiotic resistance; they may also provide valuable information for clinical use of these therapies, where herbal therapy-conventional antibiotic interactions may occur.

Many people use herbal therapies and conventional antibiotics concurrently, without any understanding of the interactions that may occur between the different medicines. Natural products may have severe interactions in combination with conventional medicines, even when either component is safe when used alone.[30] Also of concern, the efficacy of a therapy may be affected in combination with other medicines. Whilst, some combinations may have increased potency, other combinations may antagonize each other's effects, thereby decreasing the efficacy of the therapy. It is important to identify such combinations so that they can be avoided.

The *T. impetiginosa* bark extracts could potentiate the activity of some conventional antibiotics. Several additive combinatorial interactions were noted. In particular, the water and methanolic extracts potentiated the activity of erythromycin, chloramphenicol and ciprofloxacin against *B. cereus*. The aqueous extract also potentiated the activity of penicillin-G against the same bacterium. Similarly, the aqueous extract also produced additive effects when combined with erythromycin, penicillin-G and chloramphenicol against *S. aureus*. Interestingly, all extracts (except the aqueous extract) also produced additive effects in combination with

ciprofloxacin against *S. sonnei*. Similarly, the mid-polarity ethyl acetate and chloroform extracts also produced additive interactions with ciprofloxacin against *B. cereus*. As such, these combinations would be beneficial for the treatment of diarrhoea, as they have greater efficacy than that of either component alone. Of the remaining combinations, the majority were either non-interactive, or were unable to be determined as at least one of the components in the combination showed no inhibition of bacterial growth when tested alone. Therefore, whilst using these combinations would have no additional therapeutic benefit, the components would not impede the activity of the other component.

Several combinations produced an antagonistic effect against *B. cereus* and *S. aureus*. Notably, all of these antagonistic combinations contained ciprofloxacin. Antagonistic interactions often occur when the chemical agent that stops bacteria from reproducing (bacteriostatic) reaches the site of infection before the one that kills bacteria (bactericidal).[31] These specific combinations should be avoided in the treatment of diarrhoea and any other diseases caused by these bacteria. The mechanisms of action regarding additive or antagonistic effects of the combinations against bacterial growth were not examined in this study. However, they may involve the alteration of specific cellular components by the plant extract to facilitate changes in the bacterial growth properties. Further investigations are necessary in order to more definitively identify the mechanism(s) involved.

Whilst a detailed investigation of the phytochemistry of the *T. impetiginosa* bark extracts was beyond the scope of this study, the qualitative phytochemical screening allowed the major classes of secondary chemical components in the plant to be determined. Additionally, recent studies have identified several interesting compounds in *T. impetiginosa* bark extracts. Most of these studies have focused on lapachol due to its antineoplastic and antitumor effects.[32,33] However, other studies have also identified furanonaphthoquinones,[34,35] quinones,[36] naphthoquinoes,[37] benzoic acid, benzaldehyde derivatives[38] as well as flavonoids.[39] Further phytochemical purification and structural analysis studies are required to evaluate the growth inhibitory mechanism(s) and to identify the bioactive and potentiating (or antagonizing) extract components.

Our studies only examined the effects of the *T. impetiginosa* bark extracts against planktontic bacteria. Bacterial infections are generally much more difficult to treat if the bacteria form biofilms. Biofilms occurs when bacteria enter the body and attach to the surface of the infected tissue. They subsequently coordinate and synthesise adhesion sites between cells to form a three dimensional biofilm. The cells communicate via quorum sensing to regulate gene expression within the bacterium and increase the bacteria's resistance to antibiotic treatments.[40] The biofilm community begins to grow through cell division and recruitment (of other bacteria, yeasts, minerals and host proteins) making it resistant to the immune system and exogenous antibiotics.[40] This aspect of bacterial colonisation *in vivo* may greatly affect susceptibility and this requires attention. Furthermore, as bacteria in biofilms generally have MIC values at least an order of magnitude higher than in liquid cultures, it would be interesting to test whether *T. impetiginosa* bark extracts can also block biofilm formation by the bacterial strains reported in this manuscript and future studies are planned to examine this.

CONCLUSION

The results of this study demonstrate the potential of *T. impetiginosa* bark extracts as inhibitors of the growth of some bacterial species associated with gastrointestinal disaease and diarrhoea. All *T. impetiginosa* bark extracts were nontoxic, further indicating their suitability for prophylactic therapeutic usage. Further studies are required to isolate the inhibitory compounds and identify their antimicrobial mechanisms.

CONFLICT OF INTEREST

The authors report no conflicts 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
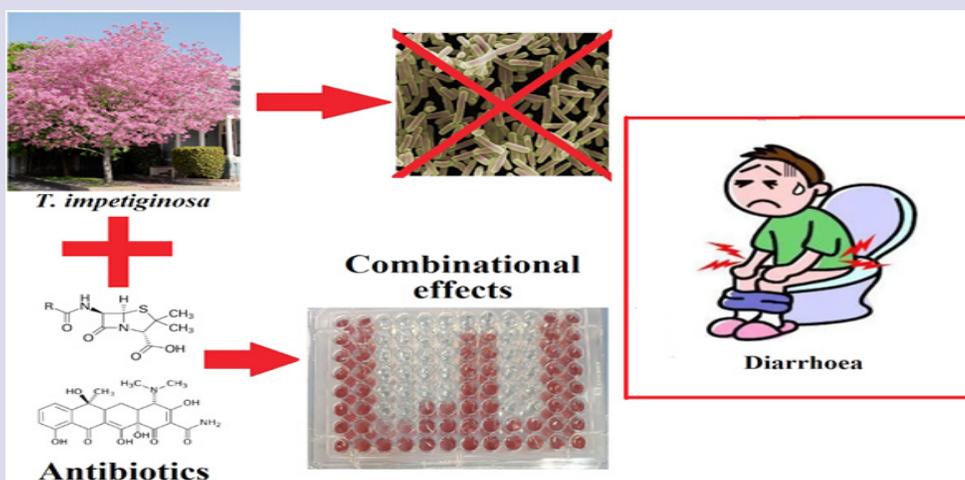
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Dr. Ian Cock leads a research team in the Environmental Futures Research Institute and the School of Environment and Science at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species 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 200 publications in a variety of peer reviewed journals.

SUMMARY

- *T. impetiginosa* bark extracts were screened for the ability to block the growth of bacterial gastrointestinal pathogens.
- The antibacterial activity was quantified by determining the MIC values of each extract.
- The extracts were also tested in combination with conventional antibiotics and the class of interaction was determined.
- Toxicity of the *T. impetiginosa* bark extracts was determined using the Artemia nauplii and HDF viability toxicity bioassays.