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Original Article

The Interactive Antimicrobial Activities of Selected South African *Terminalia* spp. Extracts in Combination with Conventional Antibiotics against Gastrointestinal Pathogens

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ABSTRACT

Background: Recent declines in new antibiotic discovery and the increase in antibiotic resistance have resulted in failing against bacterial pathogens. To develop novel antibiotic therapies, medical researchers have begun to focus on traditional therapies. Combinational therapies consisting of medicinal plants and conventional antibiotics may reactivate current drugs that are otherwise ineffective against antibiotic-resistant bacteria. *Terminalia sericea* Burch. Ex DC, *Terminalia prunioides* Laws. and *Terminalia gazensis* Bak. f. are native South African plants with antimicrobial properties. However, combinations of *Terminalia sericea*, *Terminalia prunioides* and *Terminalia gazensis* with conventional antibiotics are yet to be evaluated for growth inhibitory activity against gastrointestinal pathogens. **Methods:** *Terminalia* spp. leaves were extracted with solvents of varying polarity. Antimicrobial screening was performed using disc diffusion and broth microdilution assays. Toxicity was measured using *Artemia franciscana* nauplii lethality assays. **Results:** All extracts (except the *T. sericea* extracts) showed low to moderate inhibitory activity against *B. cereus* and *E. faecalis*, *A. faecalis*, *A. hydrophilia*, *S. sonnei* and *S. newport* in both the disc diffusion and liquid dilution assay. Twenty-three extract/antibiotic combinations produced synergy, 26 were additive, 24 were non-interactive and seven were antagonistic. Most of the antagonist interactions occurred in combinations containing gentamicin. All extracts were either non-toxic or of low toxicity in the *Artemia* bioassay. **Conclusion:** *Terminalia* spp. extracts may mimic the actions of a resistance modifying agents, enhancing the activity of several antibiotics that are relatively ineffective alone. Further studies are required to identify the bioactive and potentiating components and their mechanisms of action.
Key words: Combretaceae, *Terminalia gazensis*, *Terminalia prunioides*, Antibiotic potentiation, Synergy, Antibiotic-resistant pathogens, Diarrhoea.

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INTRODUCTION

Overuse and misuse of antibiotics has created selective pressure on microorganisms to generate multi-antibiotic resistant strains.[1] According to a report published in the Centre for Disease Control and Prevention (CDC) (Antibiotic Resistance Threats in the United States, 2019),[2] more than 2.8 million antibiotic-resistant infections occur in the United States annually, causing 35,000 deaths due to the inability of conventional chemotherapies to combat the infections.

The emergence of multi-drug resistant pathogens, alongside a significant decline in the discovery of new antimicrobial agents in recent years, has compelled the medical community to seek alternative options to treat infectious diseases, including the re-examination of traditional medicines and medicinal plants.[3] The treatment of bacterial infections by traditional medicine has a long history. Indeed, medicinal plants were used to treat infections since before written history. Western medicine has now acknowledged the advantages of traditional medicine plants and there has been a substantial increase in interest in plants as both alternative and complementary medicines. Indeed, approximately 80% of the developing world treats pathogenic diseases using medicines derived from medicinal plants.[3,4] A survey conducted by the United Nations Conference on Trade and Development indicated that more than 33% of the total drugs produced by developed nations are plant-derived, with many others being semi-synthetic analogues of plant compounds. The World Health Organization (WHO) has recorded the names of more than 20,000 species of medicinal plants that offer a variety of potential uses.[5] Medicinal plants are often more inexpensive, safer to use, have fewer side effects and may be more readily available compared to synthetic drugs (particularly in isolated and/or developing regions).

The genus *Terminalia* (family Combretaceae) comprises approximately 200 shrubs and trees that are widely distributed in tropical regions across the world. *Terminalia* spp. have been used traditionally to treat multiple diseases including diarrhoea, cancer, inflammation, skin diseases and various other bacterial infections.[6] It has previously been suggested that the significantly high antioxidant content of the Combretaceae may be responsible for many of the therapeutic effects of these plants.[7] Extracts of *T. sericea* Burch. ex DC., have promising antidiabetic and antibacterial activities.[8,9] *Terminalia sericea* contains metabolites that are useful for the treatment of a variety of human infections, including community-acquired pathogens that are prevalent in developing countries.[10] *Terminalia prunioides* Laws. and *T. sericea* have considerable antifungal activity against the fungal species *Cryptococcus neoformans*, *Candida albicans*, *Microsporum canis*, *Aspergillus*

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fumigates and *Sporothrix schenkii*.[10] Since fungal infections cause significant morbidity and mortality, especially in immunocompromised individuals, these findings indicate their potential in the development of lifesaving treatments.[11] In contrast, many other *Terminalia* spp. are yet to be rigorously tested for antimicrobial activity. Few studies have screened the activities of *T. sericea*, *T. prunioides* and *Terminalia gazensis Bak*. f. against gastrointestinal pathogens, and their interactions with conventional antibiotics against gastrointestinal bacteria remains largely unreported. This study evaluated the activity of these species against a panel of gastrointestinal bacteria and evaluated their ability to re-activate conventional antibiotics against those pathogens.

MATERIALS AND METHODS

Materials

All solvents used in this project were of analytical grade and were obtained from the Ajax Fine-Chemicals Ltd. (Australia). All other reagents were acquired from Sigma Aldrich (Australia) unless otherwise stated.

Plant collection and extraction

Terminalia prunioides, *T. gazensis* and *T. sericea* leaves were obtained from the Lowveld Botanical Gardens, Nelspruit, South Africa in 2019 and were verified by the garden's botanist. The leaves were air dried in the shade at room temperature until a constant mass was achieved. The dried leaves were ground into a coarse powder and 1 g masses were weighed into individual Falcon tubes. A 50 mL volume of methanol or water was added to the powders and mixed thoroughly. The plant material was extracted at room temperature for 24 hours and the extracts were subsequently vacuum filtered through Whatman No. 54 filter paper into pre-weighed tubes. The extracts were dried in a vacuum incubator at 60ºC and weighed to determine the extraction yield. The dried extracts were resuspended in 10 mL deionized water (containing 1% DMSO). After a 24-hour period, the extracts were sonicated and syringe-filtered into 10 mL tubes using 0.22 µm membrane filters (Millipore Australia Pty. Ltd.). The extracts were subsequently stored at 4°C until use.

Antibacterial studies

Antibacterial susceptibilities and MIC values of each extract were determined *via* two methods. The disc diffusion assay served as a rapid screening tool to measure the antibacterial efficacy. The liquid dilution MIC assay was also used as it is generally considered to be more sensitive and it is perhaps the most commonly used bacterial growth inhibitory assay, allowing quantitative investigations of the antimicrobial activity of extracts, antibiotics and their combinations and comparison with other studies.

Bacterial cultures

Antibacterial screening was performed using nutrient agar and nutrient broth purchased from Oxoid Ltd. (Australia). The nutrient agar and broth were prepared according to the manufacturer specifications and sterilized by autoclaving. Initially, bacterial stock cultures were streaked individually onto nutrient agar plates and incubated at 37°C for 24 hours. After incubation, a single pure colony of the bacteria was transferred into fresh nutrient broth and incubated until log phase of the bacteria life cycle was achieved. Subsequently, a 100 µL aliquot of the bacterial suspension was transferred into fresh nutrient medium and incubated for 24 hours at 37°C. The stock culture was maintained in nutrient broth at 4°C until use.

Disc diffusion assays

The antimicrobial activity of the plant extracts on agar was determined using a modified Kirby Bauer's disc diffusion assay.[12] Nutrient agar plates were inoculated with 100 µL of individual log phase bacterial suspensions and allowed to stand for two hours at room temperature to allow the bacteria to absorb into the agar surface. Filter discs infused with the extracts or the conventional antibiotics were placed onto the agar surface. Following bacterial growth at 37°C for 24 hours, the diameters of the zones of inhibition were measured to the closest whole mm. Standard discs of ampicillin (2 µg) and chloramphenicol (10 µg) (Sigma-Aldrich, Australia) served as positive controls, while discs infused with 10 µL of sterile deionized water served as negative controls. Each of the antimicrobial assays were performed in triplicate over two independent experiments (n = 6) and the results are reported as mean values (± SEM). Zones of inhibition (ZOIs) were measured. The extracts were tested across a range of concentrations until MIC values could be obtained. Graphs of zone of inhibition versus Ln concentration were plotted and the MIC values quantified by linear regression.

Liquid dilution assays

The MIC values of the extracts, both alone and in combination with the conventional antibiotics, were determined using standard liquid dilution assays.[13,14] Briefly, individual bacterial cultures were added dropwise to fresh nutrient broth flasks to produce a 0.5 MacFarland's standard culture. This culture was further diluted in nutrient broth at a ratio of 1:100, giving an approximate inoculum count of ~1 x 10^6 colony forming units/mL. A 100 µL volume of sterile nutrient broth was added to all wells of a 96-well plate, then 100 µL of test solution (either antibiotic, extract, or extract-antibiotic combined at a ratio of 1:1) was added and mixed. Serial two-fold dilutions were performed, yielding a final volume of 100 µL per well. A volume of 100 µL of individual bacterial inoculum was then added to each well, and the plates were incubated at 37 °C for 24 h. *p*-Iodonitrotetrazolium violet (INT; Sigma-Aldrich, Australia) was dissolved in sterile deionised water to prepare a 0.2 mg/mL stock solution. A volume of 40 µL of the INT stock solution was added into all wells of a sterile 96 well plate and incubated for a further 6 h at 37 °C. MICs were visually determined as the lowest dose at which colour development was inhibited. Extract MIC values >5000 µg/mL were considered inactive; MIC values between 2000 and 5000 µg/mL were considered as low activity; 1000-2000 ug/ml were considered as moderate activity; 400-1000 µg/mL were considered as noteworthy activity; 100-400 µg/mL were considered as good activity; and <100 µg/mL were considered to be high activity.

Determination of combinational effects

The combinational effects between the plant extracts and the conventional antibiotics were determined by calculating the sum of fractional inhibitory concentration (ΣFIC) for each combination.[15] FIC values for the individual components were calculated using the following equations, where a represents the plant extract sample and b represents the conventional antibiotic:

FIC (a) = (MIC of plant extract a combination with antibiotic b) / (MIC of plant extract an independently)

FIC (b) = (MIC of antibiotic b in combination with plant extract a) / (MIC of antibiotic b independently)

The ΣFIC was subsequently calculated using the formula ΣFIC = FIC (a) + FIC (b).

Interactions were classified as either antagonistic (≥ 4.0), indifferent (>1.0≤ 4.0), additive (>0.5≤ 1.0) or synergistic (≤ 0.5).

Toxicity studies

The *Artemia franciscana* nauplii toxicity assay was used to determine the concentration that induced mortality in 50% of the nauplii (LC_{50}). A volume of 400 µL artificial seawater (Red Sea Pharm Ltd., Israel) containing approximately 50 *A. franciscana* nauplii was added to all wells of a 48 well plate. Then, 400 µL of test solution (either plant extract,

reference toxin or sea water control) was added to respective wells and the plates were incubated at 25 ± 1 °C for 24h. On each plate, a positive control of 400 µL reference toxin (potassium dichromate prepared in deionized water at a concentration of 1 mg/mL) and 400 µL of artificial seawater (Red Sea Pharm Ltd., Israel) as the negative control were included on each plate. Each of the tests were performed three times in triplicate (n = 9). Following 24 hours' exposure, the number of dead nauplii were counted. Nauplii was considered dead if no movement of appendages was observed for 10 seconds. After 24 hours of exposure, all nauplii were sacrificed by the addition of 50 µL glacial acetic acid and a final count was used to determine the percentage mortality per well. The LC_{50} was then determined by linear regression.

Statistical analysis

All data is expressed as the mean ± SEM of three independent experiments, each with triplicate internal repeats (n = 9), except where otherwise stated. One-way analysis of variance (ANOVA) was used to calculate statistical significance between the negative control and treated groups, with $p < 0.05$ considered to be statistically significant.

RESULTS

Liquid extraction yields

Extraction of the *T. prunioides*, *T. gazensis* and *T. sericea*. plant materials (1 g) with solvents of varying polarity yielded the dried plant extract concentrations presented in Table 1.

Antibacterial studies

Inhibitory activity of the plant extracts against Gram-positive bacteria: *Terminalia gazensis* and *T. prunioides* methanol and water extracts inhibited the growth of *B. cereus* and *E. faecalis,* except for the *T. prunioides* water extract, which lacked inhibitory activity against *B. cereus* (Figure 1). The inhibitory activity of the *T. prunioides* methanol extract was slightly greater against *E. faecalis* compared to the *B. cereus*, based on the zone of inhibition (ZOI) (8 ± 0.6 mm for *E. faecalis*, compared to 7.3 ± 0.6 mm against *B. cereus*). Similarly, the antimicrobial activity of the *T. gazensis* methanol extract was slightly greater against *E. faecalis* (ZOI = 9.3 ± 0.6 mm) compared to *B. cereus* (ZOI = 8 ± 0.6 mm). Notably, both the methanol and water extracts of *T. sericea* lacked inhibitory activity against *B. cereus* and *E. faecalis*. Additionally, the *T. prunioides* water extract lacked inhibitory activity against *B. cereus*, but inhibited *E. faecalis* growth (ZOI = 8 ± 1.1 mm). The *T. gazensis* water extract activity was greater against *E. faecalis*, (ZOI =10 ± 1.3 mm), which was also substantially greater than the ZOI recorded against *B. cereus* (8 ± 0.6 mm). In comparison, the ZOI of the positive control chloramphenicol against *B. cereus* was 21.3 ± 1.7 mm and 12 ± 0.6 mm against *E. faecalis*. However, the other conventional antibiotic tested in this study (ampicillin) was ineffective against *B. cereus* and *E. faecalis*, confirming that these bacterial strains were antibiotic-resistant.

Inhibitory activity of plant extracts against Gram-negative bacteria: All of the *T. prunioides* and *T. gazensis* extracts inhibited the growth of *A. faecalis* and *A. hydrophilia* (Figure 2). The activity of the *T. prunioides* methanol extract was slightly greater against *A. hydrophilia* compared to *A. faecalis* (based on the ZOI). The ZOI of the *T. prunioides* methanol extract against *A. faecalis* was 8 ± 0.6 mm, compared to 9.3 ± 1.1 mm against *A. hydrophilia*. The activity of the *T. gazensis* methanol extract was greater against *A. faecalis*, (ZOI = 9.3 ± 0.6 mm), compared to *A. hydrophilia* (ZOI = 8 ± 0.6 mm). Both the methanol and water *T. sericea* extracts lacked inhibitory activity against *A. faecalis* and *A. hydrophilia*. The *T. prunioides* water extract was equally effective against both *A. faecalis* and *A. hydrophilia* (8 ± 0.6 mm). The *T. gazensis* water extract activity was a better inhibitor of *A. hydrophilia* growth (ZOI = 9.3 ± 1.1 mm), compared to *A. faecalis* (ZOI = 8 mm ± 0.6 mm). The activity of the chloramphenicol positive control was similar against *A. faecalis* (ZOI = 21.3 ± 0.6 mm) and *A. hydrophilia* (ZOI = 22.6 ± 1.1 mm). In contrast, the ampicillin control was inactive towards *A. faecalis* or *A. hydrophilia*.

Similarly, both of the *T. prunioides* and *T. gazensis* methanol and water extracts inhibited the growth of *S. newport* and *S. sonnei* (Figure 3). The inhibitory activity of the *T. prunioides* methanol extract against *S. newport* (ZOI = 8.6 ± 0.6 mm) and *S. sonnei* (ZOI = 9.3 ± 0.6 mm) was noteworthy. The activity of *T. gazensis* methanol extract, against both *S. newport* and *S. sonnei* was the same (ZOI = 10 ± 1.34 mm). Both the methanol and water *T. sericea* extracts lacked inhibitory activity against *S. newport* and *S. sonnei,* whilst the *T. prunioides* water extract had similar inhibitory activities against both pathogens (ZOI = 7.3 ± 0.6 mm).

The *T. gazensis* water extract had slightly greater inhibitory activity against *S. sonnei* (ZOI = 9.3 ± 1.3 mm), compared to *S. newport* (ZOI = 8.6 ± 0.6 mm). The activity of the chloramphenicol positive control was substantially greater against *S. newport* (ZOI = 24 ± 1.5 mm). In contrast, a ZOI value of 10 ± 0.6 mm was recorded for chloramphenicol against *S. sonnei*. The ampicillin control completely lacked inhibitory activity against these pathogens indicating that they are β-lactam antibiotic-resistant strains.

Table 1: Liquid extraction yields of *T. prunioides, T. gazensis* and *T. sericea*.

Plant	Extract type	Mean mass of dried extract (mg)	Mean concentration of resuspended extract (mg/ml)
T. prunioides	Methanol	106 ± 3.1	10.6 ± 1
	Water	179 ± 6.1	17.9 ± 1.2
T. gazensis	Methanol	138 ± 6.7	13.8 ± 1.4
	Water	171 ± 5.8	17.1 ± 1.6
T. sericea	Methanol	99 ± 4.4	9.9 ± 1.2
	Water	264 ± 4.9	26.4 ± 1.8

Mass of dried extract (mg) and concentration of resuspended extract (mg/mL) are expressed as means of four experiments (±SEM) (n=4).

MIC determinations

The antimicrobial efficacies of the plant extracts were further evaluated by determining the MIC values for each extract against the individual pathogens. The MIC of each extract was determined using two methods. A liquid dilution (LD) MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay[15]. In addition, the liquid dilution assay is commonly used for analysing bacterial growth inhibition efficacy, allowing comparisons with other studies. The MIC values were also determined by disc diffusion (DD) assay across a range of concentrations, using linear regression. The majority of the *T. gazensis* methanol and water extracts inhibited the growth of all the bacterial species, albeit to varying extents. The *T. prunioides* extracts inhibited the most bacterial species, although the MIC was substantially higher (indicating lower activity) than measured for the *T. gazensis* extracts. In contrast, the *T. sericea* extracts was unable to inhibit the growth of any of the bacterial species tested in this study.

All extracts (with the exception of both *T. sericea* extracts) showed low to moderate inhibitory activity against the Gram-positive bacteria *B. cereus* and *E. faecalis* in both the disc diffusion and liquid dilution assay. The activity was greatest for the *T. gazensis* methanol extract in both assays against *B. cereus* (DD MIC = 6900 µg/mL; 13800 µg/mL) (Table 2). Similarly, the extracts also showed low to moderate inhibitory activity against the Gram-negative bacteria *A. faecalis*, *A. hydrophilia*, *S. sonnei* and *S. newport* in both the disc diffusion and liquid dilution assays. The activity of the *T. gazensis* methanol extract was the strongest in the liquid dilution assay (LD MIC = 1725 µg/mL) against *A. faecalis* and *S. newport* (Table 3). Neither of the *T. sericea* extracts showed any activity against the Gram-negative bacteria in either the disc diffusion or liquid dilution assays.

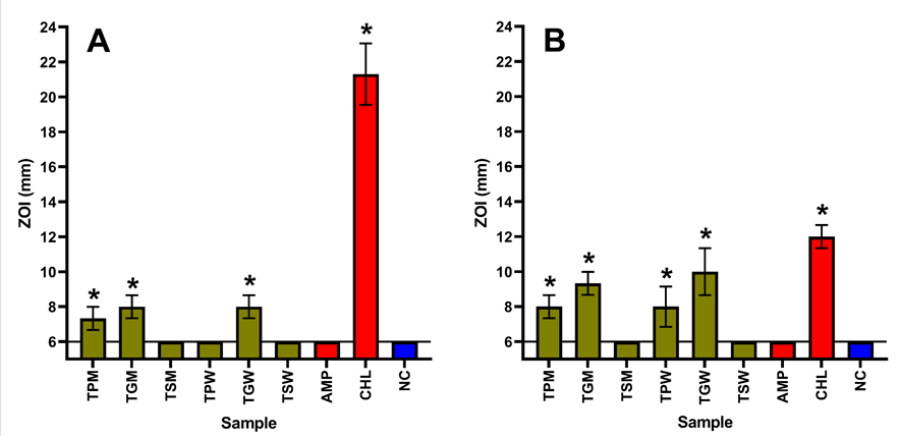

Figure 1: Antibacterial activities of plant extracts (green bars), positive control antibiotics (red bars) and negative control (blue bars) against *B. cereus* (A) and *E. faecalis* (B). TPM = *T. prunioides* methanol extract; TGM = *T. gazensis* methanol extract; TSM = *T. sericea* methanol extract; TPW = *T. prunioides* water extract; TGW = *T. gazensis* water extract; TSW = *T. sericea* water extract; AMP = ampicillin (2 µg); CHL = chloramphenicol (10 µg). NC = negative control (sterile water). Results are expressed as mean zones of inhibition (ZOI) of at least six replicates ± SEM. The horizontal line at y = 6 mm indicates the size of the discs used in the assay. Asterisks above bars indicate statistical difference to the negative control ($p < 0.05$).

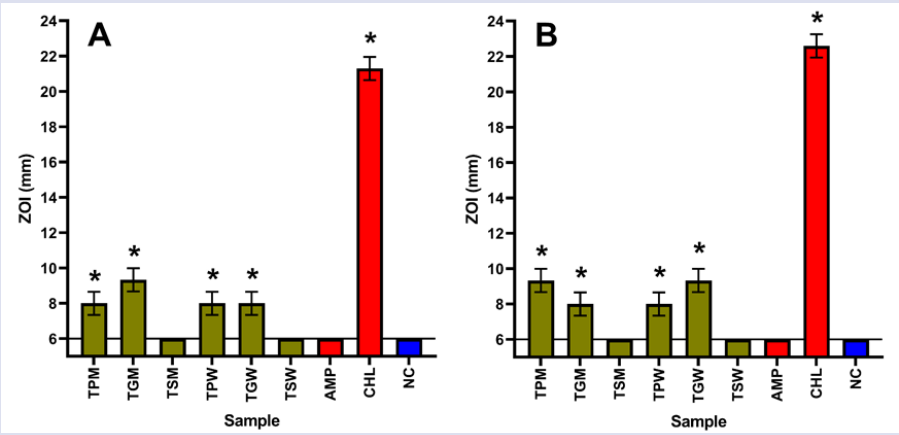

Figure 2: Antibacterial activities of plant extracts (green bars), positive control antibiotics (red bars) and negative control (blue bars) against *A. faecalis* (A) and *A. hydrophilia* (B). TPM = *T. prunioides* methanol extract; TGM = *T. gazensis* methanol extract; TSM = *T. sericea* methanol extract; TPW = *T. prunioides* water extract; TGW = *T. gazensis* water extract; TSW = *T. sericea* water extract; AMP = ampicillin (2 µg); CHL = chloramphenicol (10 µg). NC = negative control (sterile water). Results are expressed as mean zones of inhibition (ZOI) of at least six replicates ± SEM. The horizontal line at y = 6 mm indicates the size of the discs used in the assay. Asterisks above bars indicate statistical difference to the negative control ($p < 0.05$).

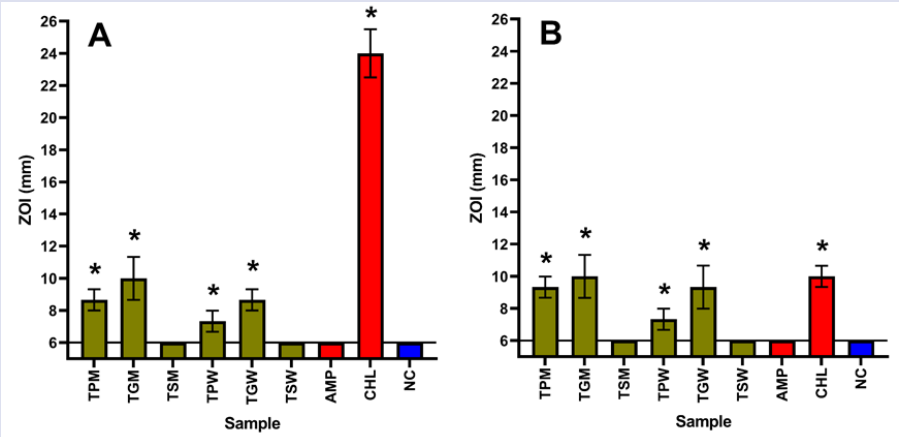

Figure 3: Antibacterial activities of plant extracts (green bars), positive control antibiotics (red bars) and negative control (blue bars) against *S. newport* (A) and *S. sonnei* (B). TPM = *T. prunioides* methanol extract; TGM = *T. gazensis* methanol extract; TSM = *T. sericea* methanol extract; TPW = *T. prunioides* water extract; TGW = *T. gazensis* water extract; TSW = *T. sericea* water extract; AMP = ampicillin (2 µg); CHL = chloramphenicol (10 µg). NC = negative control (sterile water). Results are expressed as mean zones of inhibition (ZOI) of at least six replicates ± SEM. The horizontal line at y = 6 mm indicates the size of the discs used in the assay. Asterisks above bars indicate statistical difference to the negative control ($p < 0.05$).

Table 2: Disc diffusion and liquid dilution MIC values (µg/mL) against selected gram-positive bacteria.

	B. cereus		*E. faecalis*	
	DD MIC	LD MIC	DD MIC	LD MIC
TPM	10600	2650	13800	3450
TGM	6900	1725	13800	3450
TSM	-	-	-	-
TPW	17900	4475	17900	4475
TGW	17100	4275	17100	4275
TSW	-	-	-	-
Positive controls				
Penicillin	ND	-	ND	-
Chloramphenicol	ND	-	ND	-
Gentamicin	ND	0.035	ND	0.035
Ciprofloxacin	ND	5	ND	5
Tetracycline	ND	5	ND	5
Erythromycin	ND	5	ND	5
Negative control	ND	-	ND	5

Disc diffusion and liquid dilution MIC values for *T. prunioides* methanol = TPM; *T. gazensis* methanol = TGM; *T. sericea* methanol = TSM; *T. prunioides* water = TPW; *T. gazensis* water = TGW; *T. sericea* water extracts = TSW against *B. cereus* and *E. faecalis.* DD = Disc diffusion; LD = Liquid dilution; - indicates no inhibition at any dose observed; ND = MIC could not be determined due to the nature of the disc diffusion assay. Mean DD MIC and LD MIC values of triplicate determinations are shown and are expressed in units of µg/mL.

Table 3: Disc diffusion and liquid dilution MIC values (µg/mL) of selected gram-negative bacteria.

Extracts	*A. faecalis*		*A. hydrophilia*		*S. newport*		*S. sonnei*	
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC
TPM	10600	2650	10600	2650	5300	2650	10600	2650
TGM	13800	1725	13800	1725	6900	1725	13800	3450
TSM	-	-	-	-	-	-	-	-
TPW	17900	2237.5	17900	2237.5	8950	2237.5	17900	4475
TGW	17100	2137.5	17100	2137.5	8550	4275	17100	4275
TSW	-	-	-	-	-	-	-	-
Positive controls								
Penicillin	ND	-	ND	-	ND	-	ND	-
Chloramphenicol	ND	-	ND	-	ND	-	ND	-
Gentamicin	ND	0.625	ND	0.078	ND	0.625	ND	0.625
Ciprofloxacin	ND	2.5	ND	0.625	ND	2.5	ND	1.25
Tetracycline	ND	-	ND	0.625	ND	2.5	ND	2.5
Erythromycin	ND	-	ND	0.625	ND	-	ND	-
Negative control	ND	-	ND	-	ND	-	ND	-

Disc diffusion and liquid dilution MIC values for *T. prunioides* methanol = TPM; *T. gazensis* methanol = TGM; *T. sericea* methanol = TSM; *T. prunioides* water = TPW; *T. gazensis* water = TGW; *T. sericea* water extracts = TSW against *A. faecalis*, *A. hydrophilia, S. newport* and *S. sonnei.* DD = Disc diffusion; LD = Liquid dilution; - indicates no inhibition at any dose observed; ND= MIC could not be determined due to the nature of the disc diffusion assay. Mean DD MIC and LD MIC values of triplicate determinations are shown and are expressed in units of µg/mL.

Table 4: Σ FIC values calculated for the plant extracts and antibiotic combinations.

Bacteria	Extract	Gentamicin	Ciprofloxacin	Tetracycline	Erythromycin
B. cereus	TPM	4.06	1.00	0.25	0.50
	TGM	4.12	0.75	0.37	1.50
	TSM	-	-	-	-
	TPW	4.06	1.00	0.25	0.50
	TGW	2.03	0.50	0.25	0.50
	TSW	-	-	-	-
E. faecalis	TPM	4.30	1.30	0.40	0.60
	TGM	4.19	0.90	0.60	1.90
	TSM	-	-	-	-
	TPW	4.25	0.98	0.40	0.75
	TGW	2.50	0.70	0.35	0.65
	TSW	-	-		

A. faecalis	TPM	1.25	0.25	-	-
	TGM	1.50	0.37	-	-
	TSM	-	-	-	-
	TPW	1.50	0.75	-	-
	TGW	1.50	0.18	-	-
	TSW	-	-	-	-
A. hydrophilia	TPM	2.06	1.25	1.25	0.62
	TGM	1.06	1.50	0.75	0.37
	TSM	-	-	-	-
	TPW	8.50	3.00	1.50	0.75
	TGW	1.06	1.50	0.75	0.75
	TSW	-	-	-	-
S. newport	TPM	0.62	0.25	0.50	-
	TGM	0.75	1.50	1.50	-
	TSM	-	-	-	-
	TPW	0.75	1.50	1.50	-
	TGW	0.73	1.50	1.50	-
	TSW				
S. sonnei	TPM	0.18	0.75	0.50	-
	TGM	0.08	0.75	0.75	-
	TSM	-	-	-	
	TPW	0.62	0.75	0.25	-
	TGW	0.62	0.18	0.18	-
	TSW	-	-	-	-

ΣFIC values of *T. prunioides* methanol extract= TPM; *T. gazensis* methanol extract =TGM; *T. sericea* methanol extract = TSM; *T. prunioides* water extract = TPW; *T. gazensis* water extract = TGW; *T. sericea* water extract = TSW in combination with conventional antibiotics against selected gastrointestinal pathogens. Synergy = ≤ 0.5; additive = > 0.5- 1.0; indifferent = > 1.0 - ≤ 4; antagonistic = > 4.0. FIC values were performed in duplicate. - indicates no inhibition at any dose tested.

Table 5: LC_{50} of the plant extracts in the Artemia nauplii bioassay after 24 hours exposure.

Extracts	LC_{50} value (µg/mL)	Toxicity evaluation
T. prunioides methanol	>2000	Non-toxic
T. gazensis methanol	>2000	Non-toxic
T. sericea methanol	>1000	Non-toxic
T. prunioides water	>2000	Non-toxic
T. gazensis water	>2000	Non-toxic
T. sericea water	>2000	Non-toxic

100% mortality was induced by potassium dichromate 4mg/ml (positive control) and 0% by the negative control (seawater).

Determination of combination effects: FICs

A wide range of interactions were observed for *T. sericea*, *T. prunioides* and *T. gazensis* extracts in combination with conventional antibiotics against the tested pathogens (Table 4). Twenty-three combinations produced synergistic effects, twenty-six produced additive effects, twenty-four produced non-interactive effects and seven were antagonistic. The extracts were not tested for combinational effects with penicillin and chloramphenicol as those antibiotics were completely inactive alone at the highest concentration tested in the liquid dilution assay. Therefore, it was not possible to determine FIC values (and hence ΣFIC) for those combinations. Notably, all of the *T. prunioides* and *T. gazensis* extracts yielded synergistic interactions in combination with tetracycline against *B. cereus*.

Interestingly, all of the antagonistic interactions detected were produced in combinations containing gentamicin. This is perhaps not unsurprising, as gentamicin is a relatively new drug and most bacteria have not developed resistance against it yet. Therefore, it is important to be cautious when using *T. prunioides* and *T. gazensis* plant extracts in combination with gentamicin to not decrease its activity.

Quantification of toxicity

The extracts were tested against *Artemia franciscana* nauplii at concentrations ranging from 62.5 to 2000 µg/mL to evaluate toxicity (Table 5) and the mean % mortality of the triplicate results was used to calculate the LC_{50} values. Extracts that produced <50% mortality at a particular concentration were deemed to be non-toxic at that concentration. Extracts that produced >50% mortality were further diluted and tested across a range of concentrations to determine the concentration at which <50% mortality occurred. Almost all extracts were non-toxic at 2000 µg/mL, except the *T. sericea* methanol extract, which produced >50% mortality at that concentration. Therefore, the *T. sericea* methanol extract was further diluted to determine the LC_{50}. At 1000 µg/mL, the *T. sericea* methanol extract was non-toxic and produced a mortality of <50%. Therefore, this extract was also deemed to be non-toxic.

DISCUSSION

Thousands of medicinal plants are used globally for treating a wide variety of diseases. However, only a small proportion of these plants have been investigated to determine their pharmacological and phytochemical characteristics.[17] Furthermore, relatively little work has examined the antimicrobial properties of *T. prunioides*, *T. gazensis* and *T. sericea* in combination with conventional antibiotics against gastrointestinal pathogens, highlighting the need to further evaluate the antimicrobial activities of these plants.

The first aim of the study was to determine the antimicrobial effects of methanol and water extracts of *T. prunioides*, *T. gazensis* and *T.*

sericea extracts against a panel of gastrointestinal pathogens. The initial antibacterial screenings (Figures 1-3) demonstrated that the methanol and water extracts of *T. prunioides* and *T. gazensis* were effective inhibitors of bacterial growth. However, substantial differences were evident between the antimicrobial activity of *T. prunioides*, *T. gazensis* and *T. sericea* extracts. In contrast, the *T. sericea* extracts lacked significant inhibitory activity. This was unexpected given the close taxonomic relationship shared between these plant species and previous reports of antibacterial activity of similar extracts against other bacterial pathogens. It is likely that compounds in the *T. sericea*, *T. prunioides* and *T. gazensis* extracts target mechanisms in the bacteria screened in the previous studies that are absent in the pathogens tested in our study.

Another aim of this study was to determine the MIC values of the plant extracts in both the liquid dilution and disc diffusion assays, and to benchmark their potency against other plant extracts, as well as against pure antibiotics. A liquid dilution MIC assay was used in this study, as it is considered to be more sensitive than the disc diffusion assay. However, in some cases the disc diffusion method may provide a better evaluation of inhibitory activity than the liquid dilution assay. This may be due to the separation the inhibitory molecules from the active compounds as they diffuse through the agar gel at different rates.[18] Thus, the disc diffusion assay may allow for visualization of inhibitory activity that may not otherwise be visible in the liquid dilution assays. This demonstrates the importance of utilizing multiple methodologies to assess the antimicrobial activity. Notably, there is a clear difference between the MIC values obtained from the two different methods. The disc diffusion assay relies on the diffusion of the phytochemicals through agar gel and is influenced by the multiple factors that may affect the determination of apparent MIC values. For example, the diffusion of complex large phytochemicals (including tannins, which are components of all *Terminalia* spp.) within the gel may be hindered and thus may provide a false evaluation of the efficacy of some plant extracts.

According to the disc diffusion assay results, nearly all of the methanol and water extracts showed moderate to weak inhibitory activity against the gastrointestinal bacteria tested in this study. These results suggest that the phytochemicals responsible for the antimicrobial activity of the extracts were less polar, or larger in size and hence were unable to efficiently diffuse through the solid agar. Furthermore, agar gels also do not accommodate volatile compounds and they rapidly evaporate from the surface of the agar gel,[19] thereby reducing their concentration and efficacy. Disc diffusion assays are also substantially influenced by the solubility of the extract compounds. Generally, polar compounds diffuse through the gel much more rapidly than less soluble compounds, which remain concentrated around the disc. Therefore, disc diffusion assays may provide falsely high MIC values when nonpolar compounds contribute to the inhibition of growth.[20]

The antimicrobial activity of the plant extracts in combination with the conventional antibiotics was also determined. Any activity of the *T. prunioides*, *T. gazensis* and *T. sericea* extracts, either alone or synergistically in combination with conventional antibiotics, may contribute to the development of new antibiotic chemotherapies against gastrointestinal pathogens. Synergistic combinations that contain the lowest possible amount of antibiotic compared to extract may be the most suitable choice as a future chemotherapeutic agent against these gastrointestinal pathogens as they would have good activity yet minimize the development of further resistance. In this study, twenty-three combinations produced synergistic effects, twenty-six produced additive effects, twenty-four produced non-interactive effects and seven were antagonistic.

Synergistic interactions greatly enhanced the efficacy of the treatment, allowing the use of low dose administration, which may decrease the side effects and reduce the establishment of further antimicrobial resistance.[15] Synergistic reactions have previously been reported for other plant species. For example, *Helichrysum longifolium* DC extracts behave synergistically when combined with the range of antibiotics against several bacterial species.[21] The authors of that study suggested that the crude leaf extract of *H. longifolium* contains a range of antibiotic resistance modifying compounds.[21] Gram-negative bacteria may acquire resistance to antibiotics by three main mechanisms: (1) efflux of the antibiotic, (2) modification of the target site that prevent binding of antibiotics and (3) inactivation of the drug.[22] Bacterial species use these approaches to protect themselves from external threats. Antibiotics can be rendered inactive by a variety (or a combination) of those mechanisms. For example, penicillin inhibits the biosynthesis of the bacterial cell wall, while erythromycin and chloramphenicol inhibit the synthesis of protein.

In Gram-negative bacteria, the cell wall and outer membrane function as selective barriers. Any modification or loss of the outer membrane proteins may make the bacteria resistant to the antibiotics due to reduction in membrane permeability. Hence, the bacteria essentially limit the uptake of the antibiotics into the cells, thereby blocking its effects.[23] Antibiotics such as chloramphenicol and penicillin are particularly susceptible to this form of resistance. Some plant extract compounds may diffuse into the cell membrane and therefore increase the permeability of bacteria to antibiotics.[23] Some compounds that may function in this way include polyphenolics and flavonoids, both of which are relatively common in all *Terminalia* species.[6] Perturbation of the cell membrane, in combination with β-lactams, may enhance the antimicrobial effect of this antibiotic class.[21] Additionally, some plant-derived compounds can enhance the *in vitro* activity of antibiotics that inhibit peptidoglycan by directly attacking the same site in the cell wall.[21] Extract components (like antibiotics) may be predisposed to inactivation by efflux pumps.[24] The presence of a single efflux pump may provide bacteria with resistance to an array of structurally and chemically diverse compounds and it is not unusual for bacteria to code for more than one efflux pump.[23,24] Hence, it is important to identify agents that can inhibit efflux mechanisms, or change the process of efflux, and can therefore enhance the efficacy of current antibiotic therapies. Plants produce multiple secondary metabolites that they use as defence mechanisms against bacterial pathogens. Some plants produce antimicrobials and other compounds that prevent the efflux of these antimicrobials from bacterial cells. For example, *Berberis* spp. produce the antimicrobial alkaloid compound berberine.[25] They also produce an *S. aureus* efflux pump inhibitor, which substantially decreases the MIC of berberine,[25] thereby substantially increasing its efficacy. Several other plant-derived phytoconstituents have also been identified as efflux pump inhibitors.[17]

It is also possible that in addition to multi-drug resistant efflux pumps, the resistant bacteria tested in this study may have acquired genes that code for reduced-affinity penicillin-binding protein 2a (PBP2a) that can render the β-lactam antibiotics ineffective.[26] By using high performance liquid chromatography (HPLC), F-10 (a bioactive fraction) was identified in a *Duabanga grandiflora* (Roxb. ex DC.) extract. F-10 acts synergistically in combination with ampicillin, significantly inhibiting the growth of MRSA.[26] Western blot analysis confirmed that F-10 and ampicillin suppressed the expression of the PBP2a in MRSA.[26] It was postulated that the presence of F-10 interferes with regulatory genes that are involved in the expression of PBP2a.[26] Phytochemical analysis revealed the presence of tannins and flavonoids in F10. It is likely that these phytochemicals may also interact non-specifically with penicillin binding proteins and affect the biosynthesis of bacterial cell components. The antibacterial plant extracts identified in the present study may also contain β-lactamase inhibitors, although this remains to be confirmed.[24] Clavulanic acid is an irreversible β-lactamase inhibitor

that, in the presence of β-lactam antibiotics, can inhibit the bacterial antimicrobial resistance mechanism. Clavulanic acid has a β-lactam ring, which allows it to compete with the antibiotic for binding to β-lactamase enzymes.[27] Similar studies have shown antagonistic effects of *Rumus versicarius* on β-lactamases.[27] Plant extracts that have activity in the present study may contain compounds that function similarly to clavulanic acid (i.e. as an irreversible competitive inhibitor). Therefore, high performance liquid chromatography (HPLC-MS) metabolomics finger printing studies are required to determine whether there are compounds in the extract that possess similar chemical and biological characteristics as clavulanic acid (i.e., the presence of a β-lactam ring).

Twelve combinations containing *T. prunioides* methanol and water extracts, and eleven different combinations of *T. gazensis* methanol and water extracts with conventional antibiotics were synergistic against several pathogens (Table 4). The active phytochemicals that induce synergistic effects may be present in greater amounts in the *T. prunioides* methanol and water extracts compared to other extracts, accounting for its greater strength, although this remains to be verified.

Nine multidrug efflux systems have been recognized in *Acinetobacter* spp., including the Tet (A) tetracycline efflux protein.[28] Baicalein, which is a weak antibacterial trihydroxy flavone isolated from the *Thymus vulgaris* L., possesses a strong synergistic activity in combination with tetracycline against MRSA strains that express Tet (K).[25,28] Bioassay-guided isolation of the plant extract components revealed several diterpenes (including carnosic acid), that act as potentiators of tetracycline against bacteria that possess Tet (K) multidrug efflux pumps.[24] Similarly, the plant alkaloid reserpine, which was isolated from the *Rauwolfia vomitoria* L., is also an effective efflux-pump inhibitor against the Bmr efflux pump that mediates tetracycline efflux in *Bacillus subtilis*.[24] In order to identify compounds that induce synergy, HPLC-MS and GC-MS metabolomic fingerprinting and/or profiling studies, (followed by NMR studies on the isolated compounds) are required. Notably, all of the *Terminalia* spp. extracts were non-toxic in the *Artemia* nauplii assay except for the *T. sericea* methanol extract. However, future studies should also use mammalian cell line toxicity assays to further validate the safety of these extracts.

CONCLUSIONS

This study identified several extracts of *T. prunioides*, *T. gazensis* and one extract of *T. sericea* that are effective in inhibiting the growth of several gastrointestinal pathogens. However, combinations of plant extract with conventional antibiotics may have greater potential as therapeutic agents against bacterial triggers of gastrointestinal pathogens than the extract alone. Although, the exact mechanisms of synergy are unclear, compounds within *Terminalia* spp. extracts may mimic the actions of a resistance modifying agents, thus enhancing the activity of several antibiotics that are relatively ineffective alone.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

WHO	World Health Organisation
MRSA	methicillin resistant *Staphylococcus aureus*
PBP	penicillin-binding protein
CDC	Center for Disease Control and Prevention
DMSO	dimethyl sulfoxide
MIC	minimum inhibitory concentration
DD	disc diffusion
LD	liquid dilution
ZOI	zone of inhibition
SEM	standard error of the mean
FIC	fractional inhibitory concentration
LC_{50}	concentration inducing 50% lethality
ANOVA	one-way analysis of variance
HPLC	high-performance liquid chromatography.

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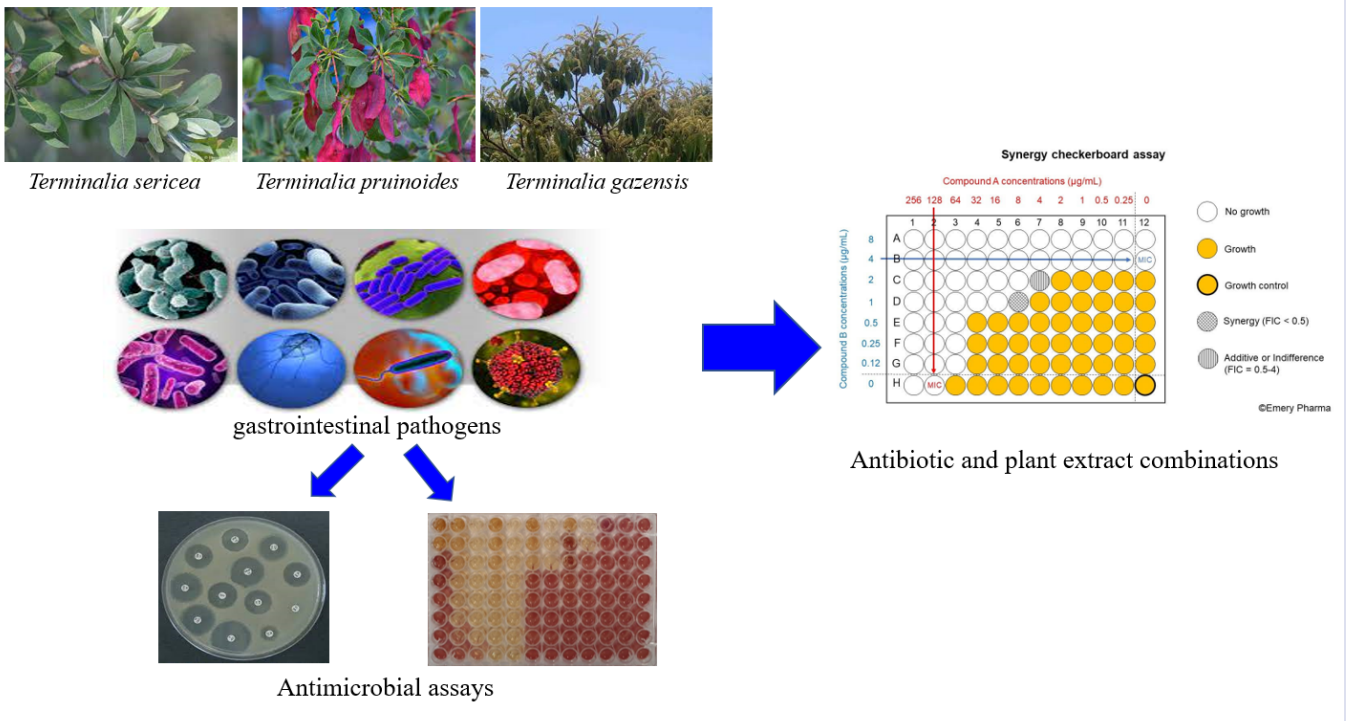
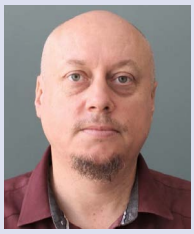
GRAPHICAL ABSTRACT



ABOUT AUTHORS

Mr Muhammad Jawad Yousaf Zai is a postgraduate student at Griffith University under the supervision of Dr Ian Cock and Dr Matthew Cheesman. His project examines the antibacterial properties of some traditional medicine plants, particularly against antibiotic-resistant pathogens. He is also interested in the use of combinational therapies to treat bacterial infections.



Dr. Matthew Cheesman is a Senior Lecturer from Griffith University and is a member of the Quality Use of Medicines (QUM) Network. His laboratory group studies the antimicrobial properties of medicinal plants and the mechanisms underlying microbial resistance.

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 over 250 publications in a variety of peer-reviewed journals.

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