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Original Article

Terminalia bellericia (Gaertn.) Roxb. Fruit Extracts Inhibit the Growth of Bacterial Triggers of Selected Autoimmune Inflammatory Diseases and Potentiate the Activity of Conventional Antibiotics

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ABSTRACT

Introduction: An increase in antibiotic resistance and a corresponding decrease in antimicrobial drug discovery have focused researchers on alternative therapies, including plant-based medicines. However, synergistic combinations of plant extracts with conventional antibiotics may be a far more effective approach in overcoming resistance and potentiating the activity of antibiotics that are otherwise ineffective against resistant bacterial strains. **Materials and Methods:** The antibacterial activity of *Terminalia bellericia* fruit extracts was investigated by disc diffusion assays and quantified by liquid dilution and solid phase MIC assays. The extracts were also combined with a range of conventional antibiotics and tested against various microbial triggers of autoimmune diseases. The ΣFIC values obtained from these assays were used to determine the class of combinational effects. Toxicity was evaluated by *Artemia nauplii* mortality and HDF cytotoxicity assays. **Results:** Methanolic, aqueous and ethyl acetate *T. bellericia* fruit extracts had noteworthy growth inhibitory activity against the microbial triggers of several autoimmune inflammatory diseases including *P. mirabilis, P. vulgaris, K. pneumoniae, A. baylyi, P. aeruginosa* and *S. pyogenes*. Of further interest, some combinations of the *T. bellericia* fruit extracts and conventional antibiotics potentiated bacterial growth inhibition compared to the individual components alone. Seven synergistic and seven additive interactions were noted, particularly in combinations containing chloramphenicol as the antibiotic component. Notably, no antagonistic interactions were evident, indicating that all combinations could be used without decreasing the antibacterial activity of the individual components. All extracts were nontoxic in the ALA and HDF assays. **Conclusion:** *Terminalia bellericia* fruit extracts have potential as inhibitors of bacterial triggers of selected autoimmune inflammatory diseases. Furthermore, extract components may also potentiate the activity of three antibiotics that are relatively ineffective alone. Isolation and identification of these compounds may be beneficial in drug design against several bacteria, including the microbial triggers of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever.

Keywords: Combretaceae, Synergy, Conventional antimicrobials, Medicinal plants, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Drug combinations.

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INTRODUCTION

The over and misuse of antibiotics has resulted in many bacterial pathogens developing resistance towards multiple antibiotics.[1] The discovery of new antimicrobial agents has concurrently decreased dramatically in recent years, making many bacterial infections difficult to manage using current therapeutic strategies.[2] The development of alternative antibacterial treatments is considered by the World Health Organisation (WHO) to be one of the biggest challenge currently facing medical science.[3] For a multitude of reasons,[2] it is unlikely that the current methods of antibiotic discovery/development will be as successful in the future. This is particularly true for the treatment of autoimmune inflammatory diseases. These are a group of debilitating diseases including rheumatoid arthritis (RA), ankylosing spondylitis (AS), multiple sclerosis (MS), and rheumatic fever (RV).[4-6] All of these diseases result from an abnormal immune response to self-tissue as a consequence of antigen challenge, often by bacterial pathogens. There is currently no cure for any of these diseases and current treatment strategies aim to alleviate the symptoms

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with analgesics and anti-inflammatory therapies. However, as RA, AS, MS and RV are induced in genetically susceptible people by bacterial pathogens, a more effective preventative treatment may be to target the growth of the specific bacterial triggers, thereby blocking the disease etiology.[4-6] Whilst antibiotics are already available for the treatment of all of these bacteria, the increased prevalence of resistant strains in recent years has decreased their efficacy.[1] Additionally, the prophylactic use of pure antibiotics over prolonged periods may induce further antibiotic resistance, thereby rendering the bacteria refractory to their actions. A better approach may be to use combinations of antibacterial components.[2]

Traditional medicines have great potential for antimicrobial drug development. Despite this, relatively few plant derived antibiotic compounds are in common use clinically. This may be because synergistic interactions are often required to potentiate the antibacterial activity and purified compounds often have much lower activity than the crude extract.[7] A combinational approach that allows synergistic interaction between plant extracts (or pure plant compounds) and conventional antibiotics may be more effective in combatting bacterial pathogens, especially against antibiotic resistant strains.[8,9] Combinational therapies are already preferred to treat multiple life-threatening infectious diseases such as malaria, tuberculosis and HIV/AIDS due to their ability to target multiple facets of a disease and to curb resistance.[2] Combinations of plant extracts/isolated compounds with conventional antibiotics may also prove to have economic advantages.[7] Developing a new drug requires years of extensive and costly testing. However, combinational therapy can potentially restore an existing drug to a state of significantly reduced resistance, thereby bypassing the lengthy and expensive process of discovering new antimicrobial agents.[7] Furthermore, synergistic combinations may have increased efficiency, reduced side effects, increased stability and bioavailability, and require lower doses in comparison to synthetic alternatives to achieve therapeutic outcomes.[8]

Terminalia bellericia (Gaertn.) Roxb. (family Combretaceae; commonly known as baheda, bahera, beleric or bastard myrobalan) is a medicinal plant that is native to tropical and subtropical plains and lower hills regions of Southeast Asia. *Terminalia bellericia* fruit have been used for hundreds of years in traditional Ayuverdic medicine,[10] particularly as a component of the Indian rasayana herbal treatment triphala,[11] for a variety of purposes including as an analgesic, anthelmintic, antipyretic, diuretic, expectorant, and laxative, as well the treatment of coughs and colds, anaemia and malaria.[10] It also has reputed anticancer and antidiabetic properties.[10] Many of these illnesses are caused by bacterial pathogens and several studies have reported that *T. bellericia* fruit extracts inhibit the growth of multiple bacterial species. One study reported that triphala and its individual components (including *T. bellericia* fruit) strongly inhibited the growth of *S. flexneri* and *S. sonnei* in liquid dilution assays.[12,13] However, the same study reported that these extracts were ineffective in disc diffusion assays. Despite their traditional uses, the growth inhibitory properties of *T. bellericia* extracts are yet to be verified against many bacterial pathogens. Furthermore, we were unable to find any studies testing the antibacterial activity of *T. bellericia* fruit extracts in combination with conventional antibiotics, although a recent study demonstrated that the individual plant components of triphala potentiate each other's antibacterial properties.[11] This study was undertaken to investigate the antimicrobial effects of *T. bellericia* fruit extracts and their ability to potentiate the growth inhibitory properties of conventional antibiotics against the bacterial triggers of some autoimmune inflammatory diseases.

MATERIALS AND METHODS

Plant source and extraction

Terminalia bellericia (Gaertn.) Roxb. fruit material was obtained from Noodles Herbal Emporium, Australia and a voucher specimen (GU2018aTBF_1) was deposited in the School of Environment and Science, Griffith University, Australia. Individual 1 g masses of the ground plant material were weighed into separate 50 mL Falcon tubes and 50 mL of methanol, deionised water, ethyl acetate, chloroform or hexane were individually added. 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 filtered through Whatman No. 54 filter paper under vacuum and 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 to determine the extraction yield and then dissolved in 10 mL deionised water (containing 1% DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the *T. bellericia* extracts for the presence of alkaloids, cardiac glycosides, flavonoids, phenolic compounds, phytosterols, saponins, tannins and triterpenoids were achieved as previously described.[14,15]

ANTIBACTERIAL SCREENING

Conventional Antibiotics

Penicillin-G (1440-1680 µg/mg), chloramphenicol (≥98% purity), erythromycin (≥850 µg/mg), and tetracycline (≥95% purity) 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 (10 µg) standard discs were obtained from Oxoid Ltd., Australia and used as positive controls.

Bacterial cultures

All bacterial strains were selected based on their ability to trigger autoimmune inflammatory diseases in genetically susceptible individuals.[16-18] Reference strains of Proteus mirabilis (ATCC21721), *Proteus vulgaris* (ATCC21719), *Klebsiella pneumoniae* (ATCC31488), *Acinetobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Type Culture Collection, USA. A clinical isolate strain of Streptococcus pyogenes was provided by the School of Environment and Science teaching laboratory at 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 antibacterial activity

Antibacterial activity screening of the *T. bellericia* fruit extracts was assessed using a modified disc diffusion assay.[19,20] 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 (containing 1% DMSO) 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 facilitates comparisons with other studies. A solid phase agar disc diffusion assay was also used in this study for comparison as it more accurately represents the growth patterns of the bacteria on solid surfaces.

Microplate liquid dilution MIC assay

The MICs of the extracts were evaluated by standard methods.[22-24] All plates were incubated at 37°C for 24 hr. 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 37°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.[25,26] Graphs of the zone of inhibition versus Ln concentration were plotted and MIC values were achieved using linear regression.

Sum of Fractional Inhibitory Concentration (ΣFIC) assessment

Interactions between the *T. bellericia* fruit extracts and the conventional antibiotics were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC) for each combination.[21] The FIC values for each component (a and b) were

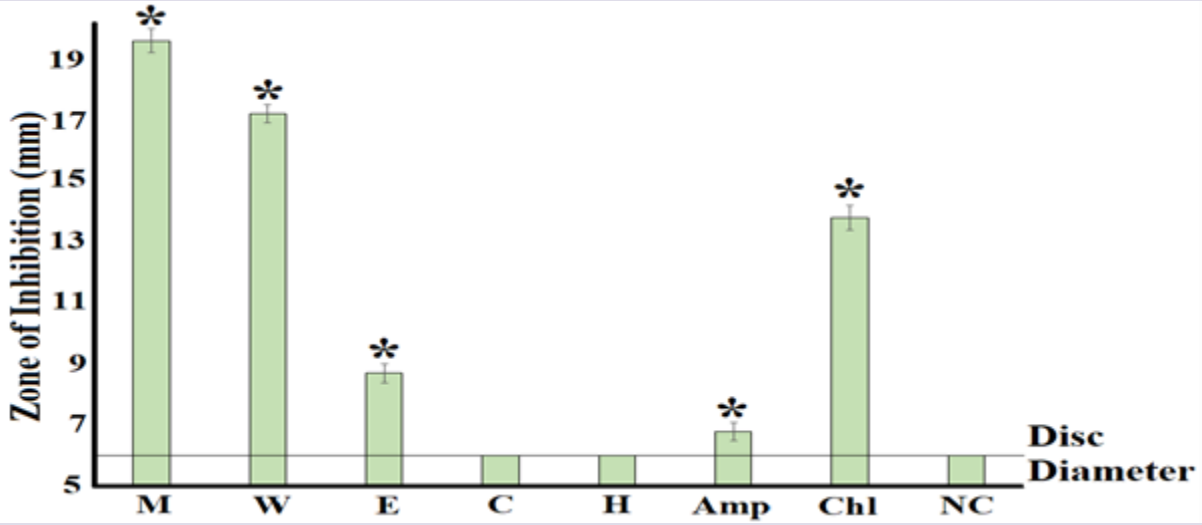

Figure 1: Antibacterial activity of *T. bellericia* fruit extracts against *P. mirabilis* (ATCC21721) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract. The positive controls were Amp (ampicillin 10 µg) and Chl (chloramphenicol 10 µg). Negative Control (NC)=water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates (n=6)±SEM. * indicates results that are significantly different to the negative control (p<0.01).

calculated using the following equations where a represents the plant extract sample and b represents the conventional antibiotic:

$$FIC(a) = \left(\frac{MIC[a \text{ in combination with b}]}{MIC[a \text{ independently}]}\right)$$

$$FIC(b) = \left(\frac{MIC[b \text{ in combination with a}]}{MIC[b \text{ independently}]}\right)$$

The ΣFIC was then calculated using the formula ΣFIC=FIC(a)+FIC(b). The interactions were classified as synergistic (ΣFIC≤0.5), additive (ΣFIC>0.5-1.0), indifferent (ΣFIC >1.0-4.0) or antagonistic (ΣFIC >4.0).[21]

Toxicity screening

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.

Artemia franciscana Kellogg nauplii toxicity screening

Potassium Dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4 mg/mL) and serially diluted in artificial seawater as a reference toxin. Toxicity of the *T. bellericia* extracts, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.[27,28] The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Cellular viability assay

All extracts and conventional antibiotics were screened for toxicity towards normal human primary dermal fibroblasts (HDF; ATCC PCS-201-012) by standard methods.[29,30] The HDF cells were cultured and screened in Dulbecco's modified eagle medium (DMEM; ThermoFisher Scientific, Australia), supplemented with 10% foetal calf serum (Life Technologies), 50 µg/mL streptomycin (Sigma-Aldrich, Australia) and 50 IU/mL penicillin (Sigm-Aldricha, Australia). All extracts were screened at 200 µg/mL with incubation at 37°C and 5% CO_2 in a humidified atmosphere following standard protocols.[25] Following the incubation, 20 µL of Cell Titre 96 Aqueous One solution (Promega) was added to each well and the plates were incubated for a further 3 hr. Absorbances were recorded at a test wavelength of 540 nm and a blank wavelength of 690 nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed three time, each with internal triplicates (n=9). Triplicate controls

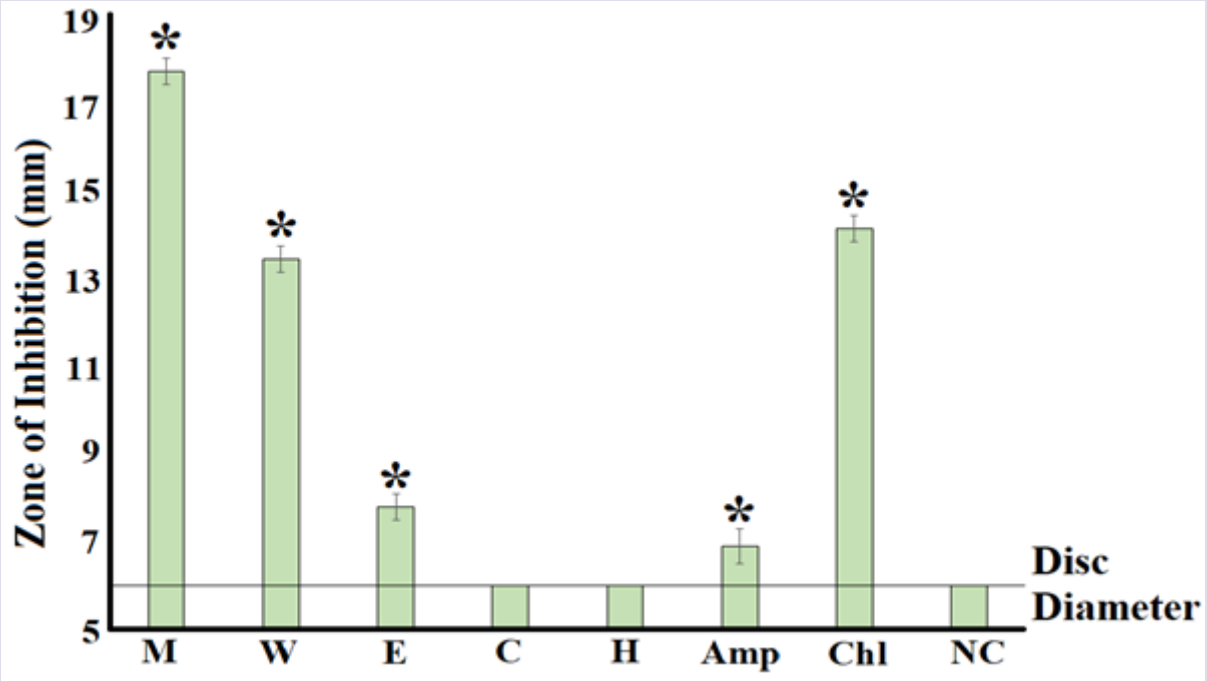

Figure 2: Antibacterial activity of *T. bellericia* fruit extracts against *P. vulgaris* (ATCC21719) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract. The positive controls were Amp (ampicillin 10 µg) and Chl (chloramphenicol 10 µg). Negative Control (NC)=water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates (n=6)±SEM. * indicates results that are significantly different to the negative control (p<0.01).

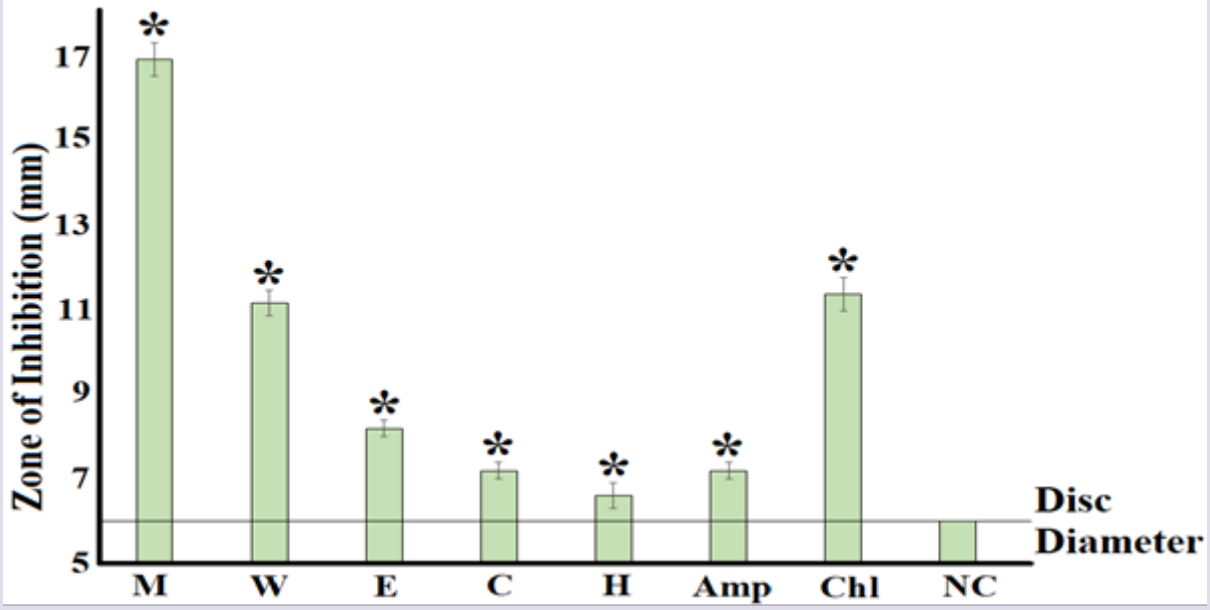

Figure 3: Antibacterial activity of *T. bellericia* fruit extracts against extracts against *K. pneumoniae* (ATCC31488) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract. The positive controls were Amp (ampicillin 10 µg) and Chl (chloramphenicol 10 µg). Negative Control (NC)=water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates (n=6)±SEM. * indicates results that are significantly different to the negative control (p<0.01).

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 at least three independent experiments. One way ANOVA was used to calculate statistical significance between the negative control and treated groups with a p<0.01 considered to be statistically significant.

RESULTS

Liquid extraction yields ranged from 13 mg (ethyl acetate *T. bellericia* fruit extract) to 267 mg (aqueous *T. bellericia* fruit extract) (Table 1). Qualitative phytochemical screening (Table 1) showed that the higher polarity solvents (methanol and water) extracted the greatest mass and widest diversity of phytochemical classes.

Bacterial growth inhibition screening

Inhibition of bacterial triggers of rheumatoid arthritis (P. mirabilis and P. vulgaris)

Proteus mirabilis growth was inhibited by the mid to high polarity *T. bellericia* fruit methanol, aqueous and ethyl acetate extracts (Figure 1). The methanolic extract was the strongest inhibitor of *P. mirabilis* growth (as judged by ZOI), with a ZOI of 19.6 mm. A volume of 10 µL of this extract was infused into the disc, which equates to approximately 230 µg of extract infused into the disc. The ZOI for this extract is substantially larger than that of the ampicillin and chloramphenicol controls (6.8 and 13.8 mm respectively). Notably, the ampicillin and chloramphenicol control antibiotics were pure and were tested at relatively high dose (10 µg/disc). In contrast, the extracts were crude mixtures and the antimicrobial compounds would be expected to account for a small % of the total extract mass. Therefore, the methanolic extract was considered to be a particularly effective inhibitor of *P. mirabilis* growth and may be effective in the prevention and treatment of rheumatoid arthritis. The aqueous and ethyl acetate extracts were also good inhibitors of *P. mirabilis* growth, albeit with substantially smaller ZOIs than the methanolic extract (17.2 and 8.7 mm respectively). In contrast, the chloroform and hexane extracts were completely ineffective against *P. mirabilis* growth. Similar inhibitory trends were noted for *P. vulgaris* growth (Figure 2), although smaller ZOIs were measured. The methanolic extract was again the strongest inhibitor of *P. vulgaris* growth, (ZOI=17.8 mm). The aqueous and ethyl acetate extracts, whilst also a good inhibitor of *P. vulgaris* growth, induced slightly smaller ZOIs (13.5 and 7.8 m respectively). All other extracts were completely ineffective at inhibiting *P. vulgaris* growth.

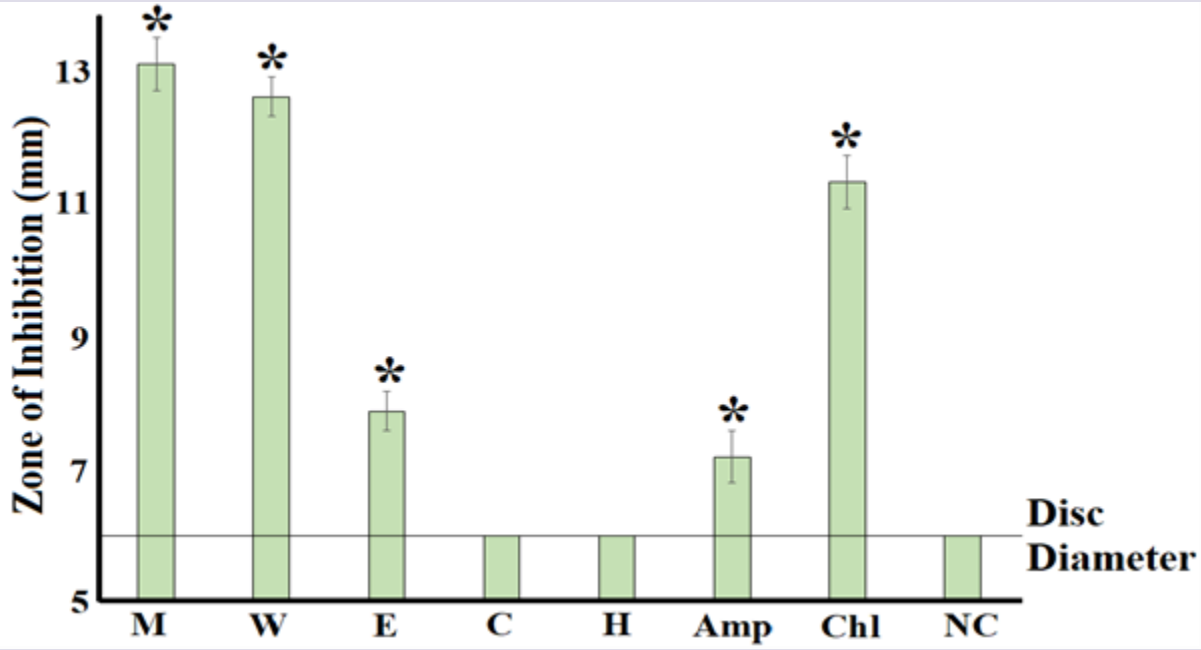

Figure 4: Antibacterial activity of *T. bellericia* fruit extracts against *A. baylyi* (ATCC33304) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract. The positive controls were Amp (ampicillin 10 µg) and Chl (chloramphenicol 10 µg). Negative Control (NC)=water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates (n=6)±SEM. * indicates results that are significantly different to the negative control (p<0.01).

Inhibition of a bacterial trigger of ankylosing spondylitis (*K. pneumoniae*)

All *T. bellericia* fruit extracts also inhibited the growth of *K. pneumoniae*, with ZOIs ranging from 6.6 to 17 mm (Figure 3). The inhibition by the methanolic extract was particularly impressive, with a ZOI of 17 mm. The aqueous extract was also a good inhibitor of *K. pneumoniae* growth, albeit with a lower ZOI (11.2 mm). These ZOIs were noteworthy compared to those recorded for the ampicillin and chloramphenicol controls (7.2 and 11.4 mm respectively). In contrast, the ethyl acetate, chloroform and hexane *T. bellericia* fruit extracts were less effective against *K. pneumoniae* (ZOIs=8.2, 7.2 and 6.6 mm respectively). As *K. pneumoniae* can induce ankylosing spondylitis in genetically susceptible individuals,[4,5] the *T. bellericia* fruit extracts (particularly the methanolic extract) may be beneficial in the prevention and treatment of that disease.

Inhibition of some bacterial triggers of multiple sclerosis (*A. baylyi* and *P. aeruginosa*)

The methanolic, aqueous and ethyl acetate *T. bellericia* fruit extracts also inhibited *A. baylyi* growth, with ZOIs of 13.2, 12.7 and 7.9 mm respectively (Figure 4). This *A. baylyi* strain was resistant to ampicillin (ZOI=7.2 mm), but was highly susceptible to chloramphenicol (ZOI=11.4 mm). In contrast, the lower polarity chloroform and hexane extracts were completely devoid of *A. baylyi* growth inhibitory activity. Surprisingly, all of the *T. bellericia* fruit extracts also strongly inhibited the growth of the *P. aeruginosa* strain tested in this study (Figure 5). This was noteworthy as previous studies have reported that this is a particularly antibiotic-resistant strain.[31-34] Furthermore, our study confirmed that this *P. aeruginosa* strain is ampicillin resistant, although it was relatively sensitive to chloramphenicol (ZOI=8.6 mm). Therefore, due to their noteworthy growth inhibitory activity against *A. baylyi* and *P. aeruginosa*, the *T. bellericia* fruit extracts may be useful in preventing and treating multiple sclerosis in genetically susceptible people.[4-6]

Inhibition of a bacterial trigger of rheumatic fever (*S. pyogenes*)

The methanolic, aqueous and ethyl acetate *T. bellericia* fruit extracts also inhibited *S. pyogenes* growth, with ZOIs of 16.7, 7.8 and 7.2 mm respectively (Figure 6). This *S. pyogenes* strain was resistant to ampicillin (ZOI=6.5 mm), but was susceptible to chloramphenicol (ZOI=8.6 mm). The methanolic extract was a particularly good inhibitor of *S. pyogenes* growth, with a ZOI of 16.7 mm. The aqueous and ethyl acetate extracts were moderate inhibitors of *S. pyogenes* growth, albeit with a lower ZOI (7.8 and 7.2 mm respectively). However, the inhibition by these extracts was still noteworthy compared to the ampicillin and chloramphenicol controls (6.5 and 8.6 mm respectively). In contrast, chloroform and hexane *T. bellericia* fruit extracts were completely ineffective against *S. pyogenes*. As *S. pyogenes* can induce rheumatic fever in genetically susceptible individuals,[4-6]

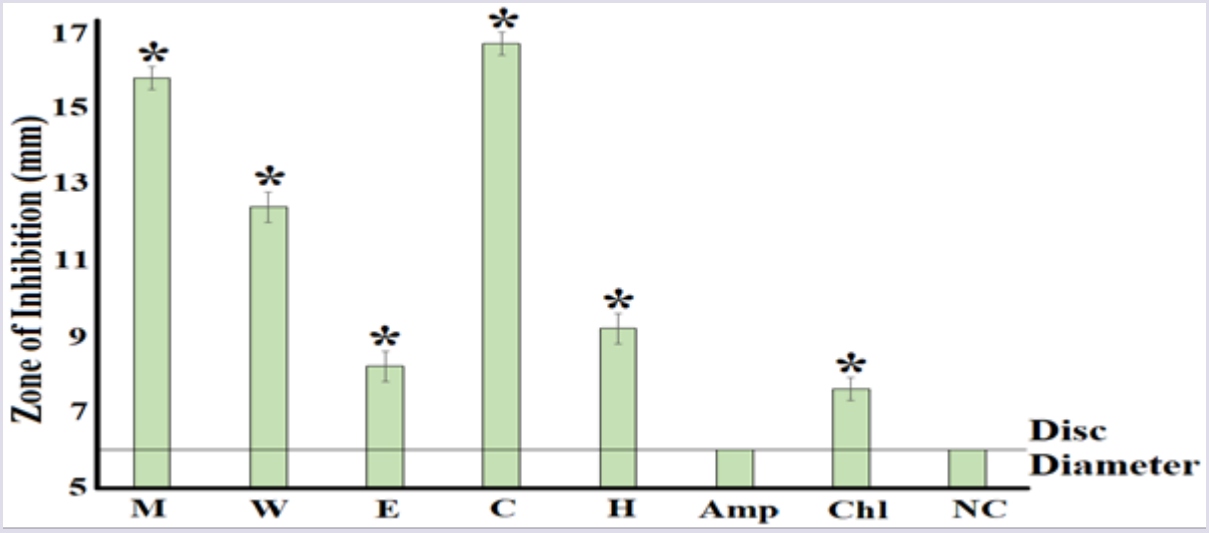

Figure 5: Antibacterial activity of *T. bellericia* fruit extracts against *P. aeruginosa* (ATCC39324) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract. The positive controls were Amp (ampicillin 10 µg) and Chl (chloramphenicol 10 µg). Negative Control (NC)=water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates (n=6)±SEM. * indicates results that are significantly different to the negative control (p<0.01).

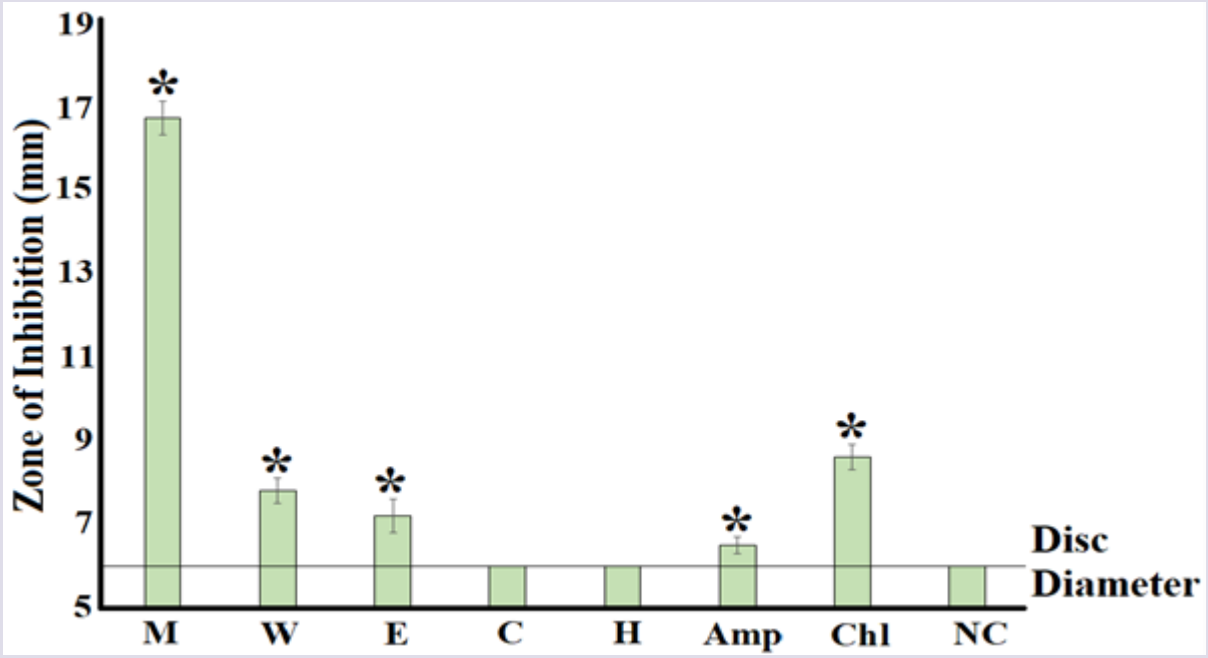

Figure 6: Antibacterial activity of *T. bellericia* fruit extracts against *S. pyogenes* (clinical isolate) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract. The positive controls were Amp (ampicillin 10 µg) and Chl (chloramphenicol 10 µg). Negative Control (NC)=water (1% DMSO). Results are expressed as mean zones of inhibition of two independent repeats, each with internal triplicates (n=6)±SEM. * indicates results that are significantly different to the negative control (p<0.01).

Table 1: Disc Diffusion (DD) and Liquid Dilution (LD) MIC values (µg/mL) for the *T. bellericia* extracts against microbial triggers of some autoimmune inflammatory diseases.

Extract	Mass of Dried Extracted Material (mg)	Concentration of extract (mg/mL)	Phenols			Cardiac Glycosides	Saponins	Triterpenes	Phytosterols	Alkaloids		Flavonoids'		Tannins	Anthraquinones	
			Total Phenolics	Water Soluble	Water Insoluble	Keller-Kiliani Test	Froth Persistence	Salkowski Test	Acetic Anhydride Test	Meyers Test	Wagners Test	Shinoda Test	Kumar test	Ferric Chloride Test	Free	Combined
Methanol	232	23.2	+++	+++	++	-	+	-	-	-	-	+++	+++	+++	-	-
Water	267	26.7	+++	+++	+	-	+	-	-	-	-	+++	++	+++	-	-
Ethyl Acetate	13	1.3	+	+	-	-	-	-	-	-	-	+	+	+	-	-
Chloroform	62	6.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	62	6.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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

Table 2: Disc Diffusion (DD) and Liquid Dilution (LD) MIC values (µg/mL) for the *T. bellericia* extracts against microbial triggers of some autoimmune inflammatory diseases.

Extract	*P. mirabilis*		*P. vulgaris*		*K. pneumoniae*		*A. baylyi*		*P. aeruginosa*		*S. pyogenes*	
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC
M	257	104	832	208	2145	416	1456	362	1859	724	1023	181
W	1213	417	1179	834	3543	517	1796	1668	2098	1668	2846	1668
E	683	388	705	388	1896	450	1693	827	1187	234	1205	256
C	-	-	-	-	2258	873	-	-	1545	256	-	-
H	-	-	-	-	2375	1028	-	-	2356	520	-	-
Controls												
Penicillin-G	ND	2.5	ND	2.5	ND	-	ND	-	ND	-	ND	1.25
Erythromycin	ND	-	ND	-	ND	-	ND	2.5	ND	-	ND	-
Tetracycline	ND	-	ND	-	ND	1.25	ND	1.25	ND	2.5	ND	2.5
Chloramphenicol	ND	2.5	ND	1.25	ND	1.25	ND	2.5	ND	-	ND	-

M=methanol extract; W=water extract; E=ethyl acetate extract; C=chloroform extract; H=hexane; DD=disc diffusion; LD=liquid dilution; - indicates no inhibition at any dose tested.

the methanolic *T. bellericia* fruit extract may be beneficial in the prevention and treatment of that disease.

Quantification of minimum inhibitory concentration (MIC)

The relative antimicrobial strength of the extracts was further evaluated by determining the MIC values using two methods: the liquid dilution MIC assay and the disc diffusion MIC assay (Table 2). Consistent with the antibacterial screening assays, the mid to higher polarity methanol, aqueous and ethyl acetate *T. bellericia* fruit extracts were the most effective at inhibiting the growth of the bacterial triggers of the selected autoimmune diseases. The MIC values of the conventional antibiotic controls were only determined for the liquid dilution assay. Commercially manufactured discs with set amounts of antibiotics loaded were used for the disc diffusion assay and thus the zones of only single doses were recorded. Chloramphenicol and tetracycline were the most versatile antibiotics as they each inhibited the growth of all except two of the bacterial species tested. Notably, the *P. aeruginosa* strain used in these studies was completely resistant

Table 3: ΣFIC values for the *T. bellericia* fruit extracts and conventional antibiotic combinations against susceptible bacteria.

Bacteria	Extract	Penicillin-G	Chloramphenicol	Erythromycin	Tetracycline
P. mirabilis	M	1.163	0.313	-	-
		(IND)	(SYN)		
	W	2.17	0.748	-	-
		(IND)	(ADD)		
	E	1.328	0.75	-	-
		(IND)	(ADD)		
P. vulgaris	M	1.635	0.749	-	-
		(IND)	(IND)		
	W	2.288	1.065	-	-
		(IND)	(IND)		
	E	1.427	1.248	-	-
		(IND)	(ADD)		
K. pneumoniae	M	-	0.078	-	1.763
			(SYN)		(IND)
	W	-	0.313	-	1.849
			(SYN)		(IND)
	E	-	0.487	-	2.002
			(SYN)		(IND)
	C	-	2.684	-	1.928
			(IND)		(IND)
	H	-	1.933	-	1.57
			(IND)		(IND)
A. baylyi	M	-	0.141	0.68	1.27
			(SYN)	(ADD)	(IND)
	W	-	3.05	1.43	1.39
			(IND)	(IND)	(IND)
	E	-	0.42	0.86	1.822
			(SYN)	(ADD)	(IND)
P. aeruginosa	M	-	-	-	0.865
					(ADD)
	W	-	-	-	1.24
					(IND)
	E	-	-	-	1.13
					(IND)
	C	-	-	-	1.73
					(IND)
	H	-	-	-	2.244
					(IND)

Bacteria	Extract	Penicillin-G	Chloramphenicol	Erythromycin	Tetracycline
S. pyogenes	M	2.214 (IND)	-	-	0.485 (SYN)
	W	2.642 (IND)	-	-	1.362 (IND)
	E	1.89 (IND)	-	-	0.858 (ADD)

M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; H=hexane extract; SYN=synergistic interaction; ADD=additive interaction; IND=indifferent interaction; -=a ΣFIC could not be determined as at least one component of the combination was inactive.

Table 4: LC_{50} values determined for *T. bellericia* fruit extracts in the *Artemia nauplii* and HDF bioassays following 24 hr exposure.

Extract	LC_{50} value (µg/mL)	
	ALA	HDF assay
M	1136	-
W	1873	-
E	-	-
C	-	-
H	-	-
PC	56	NT

- indicates that less than 50% mortality was induced by the extract at all concentrations tested. ALA=artemia nauplii toxicity assay; HDF=human dermal fibroblast toxicity assay; M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; NT=not tested.

to all antibiotics except tetracycline. Furthermore, all bacteria except *A. baylyi* were completely resistant to erythromycin. As MIC values >1 µg/mL for pure antibiotics in this assay indicates resistance,[31-34] all of these bacteria were considered resistant to all of the conventional antibiotics tested.

The MIC values determined for the *T. bellericia* fruit extracts differed substantially between the disc diffusion and liquid dilution assays. Generally, lower MIC values were recorded using the liquid dilution MIC assay. This is because the rate and extent of diffusion of molecules through agar gels is dependent on their polarity and molecular masses. Lower polarity compounds, with low solubility in aqueous solutions, do not readily diffuse in the aqueous environment of the gel. Additionally, the diffusion of larger molecules within the gel matrix is retarded spatially. Thus, the disc diffusion assay may be relatively insensitive when used to monitor extracts with large amounts of large, low polarity molecules, whereas these factors have minimal influence in the liquid dilution assay.

All of the bacterial species tested displayed noteworthy susceptibility to the methanolic and ethyl acetate extracts (MIC<1000 µg/mL). Good inhibitory activity was also noted for the aqueous extract against *P. mirabilis, P. vulgaris* and *K. pneumoniae*. The methanolic extract was a particularly good growth inhibitor of the Proteus spp. (MIC values of 104 and 208 µg/mL against for *P. mirabilis* and *P. vulgaris* respectively), and *S. pyogenes* (MIC=181 µg/mL). Therefore, the *T. bellericia* fruit extracts (particularly the methanolic and ethyl acetate extracts) may be useful in the prevention and treatment of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever in genetically susceptible people. Notably, all of the bacterial strains tested in our study were resistant against all control antibiotics. Therefore, the extracts may be particularly useful against these bacteria and testing against other strains of these bacteria is required.

Fractional inhibitory concentration (FIC) assessment

Combinations of the *T. bellericia* fruit extracts with conventional antibiotics against the bacterial pathogens were tested to determine the classes of interactions for these combinations (Table 3). ΣFIC values could not be determined for many of the combinations as one or both of the components in the combination were ineffective against the tested bacteria when tested alone. Of the effective combinations, the majority of were non-interactive (approximately 67% of the inhibitory combinations). Whilst these combinations have no additional benefit over the individual monotherapies, the lack of antagonism indicates that taking these therapies in combination would not have detrimental effects. This is important information as allopathic and complementary therapies are often taken concurrently. Notably, seven synergistic combinations were evident. Interestingly, with the exception of methanolic extract-tetracycline combination tested against *S. pyogenes*, all synergistic combinations contained chloramphenicol as the antibiotic component. Furthermore, all synergistic combinations contained the mid to high polarity methanolic, aqueous or ethyl acetate extracts as the other component. Additionally, seven combinations also produced additive effects. As the synergistic and additive combinations have enhanced effects compared to individual components alone, they would be beneficial for the treatment and prevention of these autoimmune diseases. Notably, none of the combinations produced antagonistic effects. Therefore, all combinations are safe to use without decreasing the activity of either component.

Quantification of toxicity

No LC_{50} values were determined for the ethyl acetate, chloroform or hexane extracts as <50 % mortality was seen in all tested

concentrations (Table 4). In contrast, LC_{50} values of 1136 and 1873 µg/mL were determined for the methanolic and aqueous extracts respectively. As extracts with LC_{50} values <1000 µg/mL towards *Artemia nauplii* have previously been defined as being toxic in this assay,[27,28] all extracts were deemed to be nontoxic. Furthermore, all plant extracts demonstrated a lack of toxicity towards normal human primary dermal fibroblasts, with cellular viability for all tests substantially >50% of the untreated control. All extracts were therefore deemed to be nontoxic.

DISCUSSION

This study investigated the ability of *T. bellericia* fruit extracts to inhibit the growth of some bacterial triggers of autoimmune inflammatory diseases, both alone and in combination with conventional antibiotics. Several *T. bellericia* fruit extracts were identified as effective bacterial growth inhibitors. The methanolic and ethyl acetate extracts were particularly good inhibitors of the growth of all bacterial species tested, with MIC values generally substantially <500 µg/mL. Therefore, the methanolic and ethyl acetate *T. bellericia* fruit extracts may be useful in the prevention and treatment of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever in genetically susceptible people. Noteworthy activity was also noted for the aqueous extract against the Proteus spp. and *K. pneumoniae*. Whilst a detailed investigation of the phytochemistry of the *T. bellericia* fruit 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. Interestingly, the methanolic and aqueous *T. bellericia* fruit extracts had relatively high abundances of polyphenolics, flavonoids and tannins. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.[35-37] This has been attributed to a variety of mechanisms, isncluding their ability to complex with extracellular and soluble proteins, as well as bacterial cell walls.[37] Similarly, multiple tannins have broad-spectrum antibacterial activity via a variety of intra- and extra-cellular mechanisms, including the precipitation of microbial proteins.[38] It is 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 the active components are required to evaluate the mechanism of the *T. bellericia* fruit extracts growth inhibitory activity.

The combinational studies combining the *T. bellericia* fruit extracts with conventional antibiotics also yielded interesting results. Several combinations displayed enhanced potential as therapeutic agents compared with the inhibitory activity of either the extract or antibiotic components alone. Indeed, seven synergistic and seven additive interactions were noted against, in our studies. Notably, nearly all of these potentiating combinations contained chloramphenicol as the antibiotic component, in combination with either the methanolic, aqueous or ethyl acetate *T. bellericia* fruit extracts. The implications of these potentiating combinations include enhanced efficacy, the requirement for lower dose administration and a reduction in side effects, as well as possibly reduced antimicrobial resistance.[2,21] Importantly, none of the combinations produced antagonistic effects. This is an important finding as it indicates that it is safe to use the *T. bellericia* fruit extracts and conventional antibiotics in combination without decreasing the efficacy of either component.

A further trend was evident in our study: the extract-antibiotic combinations that did not produce potentiating effects, generally did not greatly affect the efficacy of the antibiotic i.e. they appear to not counter-indicate with the antibiotics tested in this study. This is important as many users of herbal and traditional medicines self-diagnose/treat, often with multiple therapies concurrently. Thus, an understanding of drug herbal medicine interactions is important. This is an important finding as it indicates that all of the combinations tested are safe to use without decreasing the efficacy of either component.

Microbes have developed numerous resistance mechanisms to avoid the effects of antibiotics. A common method is through the use of multi-drug resistant (MDR) efflux pumps that are encoded chromosomally and are used to rapidly remove antibiotics that have entered the bacterial cells, thus rendering them resistant to the effects of the antibiotic.[39,40] A single pump may allow the bacteria to escape several types of antimicrobials. When these efflux pumps are inhibited, the intracellular concentration of antibiotic will increase, allowing the treatment to once again be effective. Interestingly, many plants possess multi-drug resistance (MDR) pump inhibitors in order to enhance the activity of their own natural antimicrobial compounds. Such MDR pump inhibitors become effective tools when used in combination with some previously ineffective/resistance prone antibiotic compounds and several examples have previously been reported.[40] Isoflavones isolated from *Lupinus argenteus* Pursh potentiate the activity of the natural plant antibiotic berberine as well as the synthetic fluoroquinoline antibiotic, norfloxacin as inhibitors of *S. aureus* growth.[40] That study reported that the isoflavone allows a greater concentration of berberine to occur inside the bacteria by inhibiting the efflux mechanism (MDR pump). Similarly, *Mezoneuron benthamianum* Baill. and *Securinega virosa* (Roxb. Ex Willd) Baill. extracts act as efflux pump inhibitors for fluoroquinolone, tetracycline and erythromycin in resistant strains of *S. aureus* (MRSA).[41] As a consequence, the *M. benthamianum* ethanol extract and chloroform extract of *S. virosa* reduce the MIC (minimum inhibitory concentration) of norfloxacin against *S. aureus* by a factor of 4.

In our study, all bacterial species were resistant to penicillin-G, chloramphenicol, erythromycin and tetracycline, with only low susceptibility or complete resistance to each antibiotic. All of these antibiotics are susceptible to resistance due to efflux pumps.[41,42] A single pump can provide bacteria with resistance to

a wide array of chemically and structurally diverse antibiotics and it is not uncommon for an organism to code for more than one efflux pump.[41,42] It is therefore imperative to identify agents that can block the efflux mechanism (efflux pump inhibitors-EPIs) or alter the process of efflux, and in so doing, extend the life of existing antibacterial drugs. Plants produce various secondary metabolites that are used as defense mechanisms against pathogenic invaders. Some plants produce antimicrobials which, along with other compounds, inhibit the efflux of those antimicrobials from a bacterial cell. There are currently no EPI/antimicrobial drug combinations on the market, although research into identifying potential EPIs is ongoing.[41] The synergistic and additive interactions noted in our study suggest the possibility of a common EPI in the *T. bellericia* fruit extracts that could be inhibiting a MDR efflux pump in these bacteria.

None of the *T. bellericia* fruit extracts or conventional antibiotics were toxic, indicating their potential for therapeutic use. The non-toxicity of the *T. bellericia* fruit extracts is unsurprising as this species has long been used in several traditional medicine systems to treat a wide variety of diseases.[10,11] However, in vitro studies using further human cell lines are required to verify their safety. Furthermore, in vivo testing is also required to confirm that the extracts and combinations retain efficacy and remain nontoxic in complex biological systems.

CONCLUSION

The results of this study demonstrate the potential of the *T. bellericia* fruit extracts in inhibiting the growth of some bacterial triggers of autoimmune inflammatory diseases. Extract components may also potentiate the activity of antibiotics that are relatively ineffective alone. Therefore, a combinational approach not only increases the effectiveness of these antibiotics, but may also potentially reduce the side effects and reduce the development of drug-resistant pathogens. Isolation of the bioactive and potentiating compounds may be beneficial in drug design against several bacteria including the microbial triggers of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ALA: *Artemia* lethality assay; **DMSO:** Dimethyl sulfoxide; **EPI:** Efflux pump inhibitor; **FIC:** Fractional inhibitory concentration; **HDF:** Human dermal fibroblasts; **LC_{50}:** The concentration required to achieve 50% mortality; **MIC:** Minimum inhibitory concentration; **MDR:** Multi-drug resistant; **ZOI:** Zone of inhibition.

SUMMARY

- *Terminalia bellericia* fruit extracts were screened for the ability to block the growth of a panel of bacterial triggers of autoimmune inflammatory diseases.
- 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 *T. bellericia* fruit extracts was determined using the *Artemia nauplii* and HDF cell viability toxicity bioassays.

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