Pharmacognosy Communications, 2026; 16(4):171-185.
https://www.phcogcommn.org

Original Article

The Interactive Antimicrobial Activity of *Terminalia ferdinandiana* Exell. Extracts and Conventional Antibiotics against Bacterial Triggers of Some Autoimmune Diseases

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ABSTRACT

Autoimmune inflammatory diseases are debilitating conditions for which limited safe and effective therapeutic options exist. A re-examination of traditional medicines may highlight leads for therapies against those diseases. *Terminalia ferdinandiana* has strong antibacterial activity against several bacterial pathogens, and has noteworthy COX-2 and iNOS inhibitory activity. However, the effects of *Terminalia ferdinandiana* extracts on bacterial triggers of autoimmune inflammatory diseases is relatively unexplored, and the extracts have not been tested in combinations with conventional antibiotics. *Terminalia ferdinandiana* leaf, fruit and seed extracts were screened for growth inhibitory activity against bacterial triggers of selected autoimmune inflammatory diseases and the activity was quantified using disc diffusion and liquid dilution MIC assays. Toxicity was evaluated using the *Artemia* nauplii cytotoxicity assay and therapeutic indexes were calculated. The methanolic, aqueous and ethyl acetate *T. ferdinandiana* leaf and fruit extracts displayed noteworthy growth inhibitory activity against some bacterial triggers of rheumatoid arthritis (RA), ankylosing spondylitis (AS) and multiple sclerosis (MS), with MIC values as low as 90 µg/mL. Thus, the *T. ferdinandiana* leaf and fruit extracts have potential to treat RA, AS and MS when used alone. However, the methanolic *T. ferdinandiana* extracts also potentiated the antibacterial activity of conventional antibiotics substantially when tested in combination. In total, nine combinations were synergistic, with seven of these combinations synergistic against *Proteus mirabilis*. Thus, those combinations may be particularly useful in preventing and treating RA. Notably, none of the extracts or extract-antibiotic combinations were toxic in the *Artemia* nauplii assay, indicating that they may be safe for therapeutic use, although this needs to be confirmed in mammalian cell lines and in *in vivo* toxicity assays. Further *in vivo* studies to determine the anti-inflammatory and antibacterial mechanisms are warranted.

Keywords: Ankylosing spondylitis, Antibacterial activity, Combinational therapy, Combretaceae, Kakadu plum, Multiple sclerosis, Rheumatoid arthritis.

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Received: 15-05-2026;
Revised: 09-07-2026;
Accepted: 28-09-2026.

INTRODUCTION

Exposure to specific pathogens and/or environmental antigens may stimulate the production of self-reactive antibodies in some individuals, thereby inducing autoimmune inflammatory diseases including rheumatoid arthritis (RA), ankylosing spondylitis (AS) and multiple sclerosis (MS).[1-3] Whilst environmental and dietary antigen exposure causes some autoimmune diseases (e.g. the gluten stimulus in Celiac's disease),[1] several other autoimmune inflammatory diseases can be triggered in genetically susceptible people by bacterial pathogens. Inhibition of the bacterial triggers of autoimmune diseases may provide earlier therapeutic targets for those diseases. Such a strategy may inhibit the etiology of the disease in genetically susceptible people before symptoms occur, as well as blocking the tissue damage associated with autoimmune disorders. Additionally, blocking the disease etiology would also prevent the downstream immunological and inflammatory-related symptoms of these diseases. As such, prophylactic therapies may be particularly useful in preventing autoimmune inflammatory diseases. Notably, serotyping and genotyping studies have already identified the bacterial triggers of some autoimmune inflammatory diseases.[1] *Proteus mirabilis* is a bacterial trigger of rheumatoid arthritis (RA) in genetically susceptible people.[4] Similarly, *Klebsiella pneumoniae* may trigger ankylosing spondylitis (AS) in some people,[5] whilst *Acinetobacter baylyi* and *Pseudomonas aeruginosa* infections can initiate multiple sclerosis (MS) in individuals with specific genetic markers.[6]

DOI: 10.5530/pc.2026.4.22

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A re-examination of the bioactivities of traditional medicines is promising for the discovery of new antibiotic therapies and may highlight promising leads for the treatment of bacterial-induced autoimmune inflammatory diseases. The genus *Terminalia* (family Combretaceae) is especially promising for new drug discovery as many species of this genus have well documented antibacterial and anti-inflammatory properties.[7] The Indian[8,9] and African species[10] have been most extensively studied. In contrast, the therapeutic properties of Australian *Terminalia* spp. remain relatively neglected, although recent studies from our group have reported antibacterial activity for the species *Terminalia ferdinandiana* Exell. fruit and leaf extracts against a variety of pathogenic[11,12] and food spoilage bacteria.[13] Interestingly, *T. ferdinandiana* extracts also have strong antibacterial activity against antibiotic-resistant bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and some extended-spectrum β-lactamase encoding Enteriobacteraceae species.[14] Notably, *T. ferdinandiana* extracts also are good inhibitors of some bacterial triggers of RA,[12,15] AS,[16] and.[17] However, most of these studies have focused on the therapeutic properties of the fruit and few studies have screened leaf extracts for therapeutic properties, whilst the properties of the seeds have been completely neglected. Furthermore, the antibacterial and anti-inflammatory activities of *T. ferdinandiania* leaf and seed extracts is yet to be tested against the bacterial triggers of autoimmune inflammatory diseases.

Notably, despite the widespread use of traditional and herbal medicines (including those containing *Terminalia* spp.) over a prolonged period, surprisingly few isolated phytochemicals or medicinal plant preparations are used clinically as antibiotics in allopathic medicine. Traditional medicines contain numerous compounds that may interact and their combination may synergize/potentiate the antibacterial activity of the medicine. Indeed, isolated plant compounds are often substantially less potent than the crude extracts that they are purified from and combinations of 'antibacterial' and potentiator components are required.[18,19] The potentiator compounds may overcome bacterial resistance mechanisms, thereby increasing the potency of the antibacterial components. Interestingly, these compounds may also increase the potency of conventional antibiotics and may even 'reactivate' them in bacteria otherwise resistant to their effects.[18,20,21] Additionally, conventional monotherapy drug development pathways often require years of expensive clinical trials, whereas repurposing existing antibiotic therapies may be substantially more rapid and therefore economically favourable.[19] Whilst several studies have examined the combinational effects of conventional antibiotics and *Terminalia* spp. extracts from other regions of the world,[8-10,22] the combinational effects of *T. ferdinandiana* extracts and conventional antibiotics are yet to be examined. This study was undertaken to verify the antimicrobial effects of *T. ferdinandiana* fruit extracts against some bacterial triggers of selected autoimmune inflammatory diseases, and to examine the effects of leaf and seed extracts on the same bacteria. Additionally, their interactive effects in combination with conventional antibiotics against the bacterial triggers of RA, AS and MS are reported for the first time.

MATERIALS AND METHODS

Sourcing and Preparation of plant samples

Fresh *Terminalia ferdinandiana* Exell. Fruit and leaves were provided by David Boehme of Northern Territory Wild Harvest, Australia. The fruit pulp was manually removed from the seeds using a sharp knife and processed separately. The seeds were scoured to remove any remaining fruit flesh. All plant parts were dried separately using a Sunbeam food dehydrator until uniform masses were obtained that did not decrease with further dehydration time. Voucher specimens of the leaves, fruit and seeds are deposited in the School of Natural Sciences, Griffith University, Australia (voucher numbers TFer-L-2018-L1; TFer-Fr-2018-F1; TFer-S-2018-S1 respectively). Individual quantities (1 g) of the dried plant parts were weighed into separate tubes and 50 mL of methanol, deionised water, ethyl acetate, chloroform or hexane was added. All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The leaves were extracted in each solvent for 24 h at 4ºC with gentle shaking and were then filtered through Whatman No. 54 filter paper under vacuum. The solvent extracts were subsequently air dried at room temperature, whilst the aqueous extract was lyophilised by freeze drying at -50ºC. The resultant dried extracts were weighed to determine extraction yield and dissolved in 10 mL of deionised water (containing 1% DMSO) and stored as aliquots at -30ºC until use.

Qualitative phytochemical studies

Qualitative phytochemical analyses of the *T. ferdinandioana* extracts to detect the presence of alkaloids, anthraquinones, cardiac glycosides, flavonoids, phenolic compounds, phytosteroids, saponins, tannins and triterpenoids, and to evaluate their relative abundances were performed using standard assays.[9,10]

Antibacterial analysis

Conventional antibiotics

Standard discs of ampicillin (10 µg) and chloramphenicol (10 µg) were obtained from Oxoid Ltd., Australia and used as positive controls in the disc diffusion susceptibility assays. Penicillin-G (potency of 1440-1680 µg/mg), chloramphenicol (≥98 % purity by HPLC), erythromycin (potency ≥850 µg/mg), and tetracycline (≥95% purity by HPLC) powders were purchased from Sigma-Aldrich, Australia and used for the microplate liquid dilution assay. Antibiotic powder stock solutions were prepared by solubilizing the individual antibiotics in sterile deionised water at concentrations of 0.01 mg/mL. The stock solutions were stored at 4ºC until use.

Bacterial cultures

The bacterial pathogens screened in this study were selected as they are all triggers autoimmune inflammatory diseases in genetically susceptible individuals.[1,2] Reference strains of *Proteus mirabilis* (ATCC21721), *Klebsiella pneumoniae* (ATCC31488), *Acinetobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Type Culture Collection, USA. All bacteria were cultured and maintained in nutrient broth (Oxoid Ltd., Australia) at 37ºC for 24 hr and stored at 4ºC until use. Streaked nutrient agar (Oxoid Ltd., Australia) plates were prepared to the manufactures specifications and were tested in parallel to ensure the purity of all bacterial cultures.

Evaluation of bacterial susceptibility to growth inhibition

Bacterial susceptibility to the *T. ferdinandiana* extracts and the conventional antibiotics was assessed using standard disc diffusion assays.[23] Preloaded ampicillin (10 µg) and chloramphenicol standard discs (10 µg) were obtained from Oxoid Ltd., Australia and included in the assays as positive controls. Filter paper discs infused with 10 µL of distilled water were included as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentrations were determined for all extracts using liquid dilution MIC assays and solid phase agar disc diffusion MIC assays.

Microplate liquid dilution MIC assay

The antibacterial activity of the individual *T. ferdinandiana* extracts and conventional antibiotics was quantified using standard liquid dilution MIC assays.[22] Briefly, 100 µL of sterilized deionised water was dispensed into each well of 96 well micro-titre plate. The *T. ferdinandiana* extracts or conventional antibiotics (100 µL) were then individually dispensed into the first row of the plate. Nutrient broth (negative control) and a sterile control (media without bacteria) were included on all plates to verify that the assay was functioning correctly. Each test well was then serially diluted down each column by doubling serial dilution. Bacterial culture (100 µL containing approximately $1x10^6$ colony forming units (CFU)/mL) was then added to all wells of the plate (excluding the sterile control wells) and incubated at 37ºC for 24 hr. The colourimetric indicator p-iodonitrotetrazolium violet (INT) (Sigma-Aldrich, Australia) was prepared in sterile deionised water as a 0.2 mg/mL INT stock solution. Following the incubation, 40 µL of the INT stock solution was dispensed into all wells and the plates were incubated for 6 hr at 30ºC to allow full colour development. The lowest dose at which colour development was completely inhibited was classified as the MIC of the test.

Disc diffusion MIC assay

The minimum inhibitory concentration (MIC) of each extract was also quantified by standard disc diffusion assays across a range of concentrations.[23] Graphs of the zone of inhibition (ZOI) versus Ln concentration were plotted and MIC values were calculated by linear regression.

Fractional inhibitory concentration (FIC) assessment

Interactive effects between combinations of the *T. ferdinandiana* extracts and conventional antimicrobials were analysed using the sum of the fractional inhibitory concentration ($^{\Sigma}$FIC). The FIC values for each component of the combination were calculated using the following equations, where (a) represents the plant sample and (b) the conventional antimicrobial sample:[22]

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

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

The $^{\Sigma}$FIC was then calculated using the equation: $^{\Sigma}FIC = FIC^{(i)} + FIC^{(ii)}$. The interactions were classified as being synergistic for $^{\Sigma}$FIC values of ≤ 0.5, additive (> 0.5 - 1.0), indifferent (> 1.0 - ≤ 4.0) or antagonistic (> 4.0).[22]

Varied ratio combination studies (isobolograms)

Synergistic combination interactions were evaluated by isobologram analysis to determine the best ratios for potentiation. Nine ratios were prepared for each synergistic combination and the MIC values determined. The components were combined at fixed concentrations of 0.01 mg/mL (antibiotic) and 32 mg/mL (plant extract), at ratios ranging from 10:90 to 90:10 (antimicrobial: plant). Data points for each ratio were plotted on an isobologram using the GraphPad Prism® software (Version 5). Ratio data points below the 0.5:0.5 line were defined as synergistic, whilst those above the 0.5:0.5 line, but below the 1.0:1.0 line were defined as additive interactions.[22] Data points above the 1.0:1.0 line (depicting indifferent and antagonistic interactions) were not depicted in the isobolograms presented herein.

Toxicity screening

Toxicity evaluations of the *T. ferdinandiana* extracts, conventional antibiotics and the reference toxin were assessed using previously optimised *Artemia franciscana* nauplii lethality assays (ALA).[24] 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. The number of dead nauplii were determined following 24 hr exposure and expressed as a % of the untreated control. The LC_{50} for each treatment was calculated using Probit analysis.

Statistical analysis

All data is expressed as the mean ± SEM of three independent experiments, each with internal triplicates (n=9). One-way ANOVA was used to calculate statistical significance between the negative control and treated groups, with a p<0.01 considered to be statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g masses of the individual *T. ferdinandiana* plant materials with solvents of varying polarity yielded dried plant extracts ranging from 20 mg (*T. ferdinandiana* fruit ethyl acetate extract) to 487 mg (*T. ferdinandiana* fruit aqueous extract) (Table 1). Qualitative phytochemical screening showed that the higher polarity solvents (methanol and water) extracted the greatest amount and widest diversity of phytochemical classes for each of the plant materials.

Bacterial growth inhibition

Inhibition of the growth of the bacterial triggers of rheumatoid arthritis (P. mirabilis)

The growth of *P. mirabilis* was susceptible to the methanolic and aqueous extracts prepared from the *T. ferdinandiana* leaves, fruit and seeds (Figure 1). The leaf extracts were stronger inhibitors of *P. mirabilis* growth (ZOIs of 17.3 and 18 mm for the methanolic and aqueous extracts respectively) than the equivalent extracts prepared from the fruit (ZOIs of 15.8 and 16.6 mm) or seeds (ZOIs of 12.5 and 10.6 mm). The inhibition of *P. mirabilis* by the methanolic and aqueous leaf and fruit extracts was particularly noteworthy as the *P. mirabilis* growth inhibition induced by those extracts were greater than that of the ampicillin and chloramphenicol controls, based on ZOI diameter (11.2 and 16.3 mm respectively). Therefore, those extracts may be particularly useful for preventing RA and for treating the condition once it has been initiated. The ethyl acetate extracts prepared from all *T. ferdinandiana* parts also inhibited *P. mirabilis* growth, albeit with substantially smaller ZOIs (≤7.6 mm). In contrast, all chloroform and hexane extracts were completely ineffective inhibitors of *P. mirabilis* growth, indicating that the *P. mirabilis* inhibitory compounds may be mid to high polarity.

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

The methanolic and aqueous *T. ferdinandiana* leaf, fruit and seed extracts were also strong inhibitors of *K. pneumoniae* growth (Figure 2). In contrast to the inhibition trends noted for *Proteus* spp., the fruit extracts were better inhibitors of *K. pneumoniae* growth (ZOIs of 9.8 and 8.9 mm for the methanolic and aqueous extracts respectively) compared to the leaf (ZOIs of 9.0 and 8.8 mm respectively) and seed extracts (ZOIs of 8.7 and 9.3 mm respectively). The ethyl acetate extracts of all plant parts also had noteworthy *K. pneumoniae* growth inhibitory activity, albeit with substantially smaller ZOIs (~7.4 mm for the leaf and seed extracts). However, the ethyl acetate extracts were substantially less concentrated than the corresponding methanolic and aqueous extracts. Therefore, the methanolic, aqueous and ethyl acetate extracts may all be useful as preventative therapies against AS (as well as other *K. pneumoniae* infections), and to

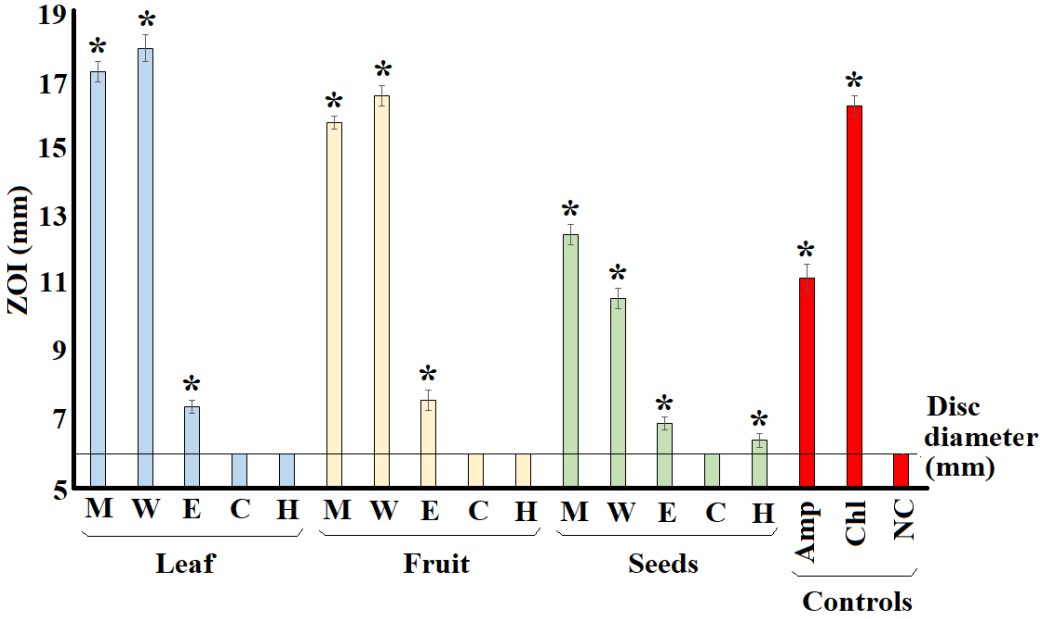


Figure 1: Antibacterial activity of the *T. ferdinandiana* extracts against *P. mirabilis* (ATCC21721) measured in mm as zones of inhibition (ZOI). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg); Chl = chloramphenicol (10 µg); NC = negative control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicates (n=9) ± SEM * indicates results that are significantly different to the negative control (p<0.01).

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

Plant Part	Extract	Mass of Dried Extracted Material (mg)	Concentration of extract (mg/mL)	Ph			CG	SP	TT	PS	Alk		Fl		Ta	Aq	
				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
Leaf	Methanol	293	29.3	+++	+++	++	-	++	+	-	+	-	++	++	+++	-	-
	Water	237	23.7	+++	+++	++	-	+++	+	-	-	-	++	++	+++	-	-
	Ethyl Acetate	38	3.8	+	+	-	-	-	-	-	-	-	++	+	++	-	-
	Chloroform	47	4.7	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	Hexane	24	2.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fruit	Methanol	367	36.7	+++	+++	+	-	++	+	-	-	-	+++	+++	+++	-	-
	Water	487	48.7	+++	+++	++	-	+++	-	-	-	-	+++	+++	+++	-	-
	Ethyl Acetate	20	2	+	+	-	-	+	-	-	-	-	+	+	++	-	-
	Chloroform	44	4.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hexane	23	2.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Seed	Methanol	273	27.3	++	+++	+	-	++	+	-	-	-	++	++	++	-	-
	Water	319	32	+++	+++	+	-	++	+	-	-	-	++	++	++	-	-
	Ethyl Acetate	26	2.6	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	Chloroform	32	3.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hexane	25	2.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Ph = polyphenolics; CG = cardiac glycosides; SP = saponins; TT = triterpenoids; PS = phytosterols; Alk = alkaloids; Fl = flavonoids; Ta = tannins; Aq = anthraquinones; +++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

treat the condition once it is triggered. Notably, the strain of *K. pneumoniae* screened in our study was completely resistant to ampicillin, although it was susceptible to chloramphenicol control (ZOI=9.3 mm), indicating that these extracts may be particularly useful against β-lactam resistant infections. In contrast, all lower polarity chloroform and hexane extracts were generally completely ineffective *K. pneumoniae* inhibitors.

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

The growth inhibitory activity of the *T. ferdinandiana* leaves, fruit and seed extracts was also tested against some bacterial triggers of MS (*A. baylyi,* Figure 3; *P. aeruginosa*, Figure 4). The mid to higher polarity methanolic, aqueous and ethyl acetate extracts were effective inhibitors of *A. baylyi* growth (Figure 3). Similar potency was noted for the leaf and fruit extracts (based on ZOIs), although the seed extracts were generally substantially less effective. As *A. baylyi* can induce multiple sclerosis in genetically susceptible people, these extracts may be useful in preventing and treating this disease, although studies are required to test if these effects are also apparent *in vivo*. Both of the positive controls also inhibited *A. baylyi* growth, although the chloroform extract was substantially more effective than ampicillin (ZOIs of 15.6 and 8.2 mm respectively). In contrast, the chloroform and hexane extracts produced from all plant parts were completely ineffective inhibitors of *A. baylyi* growth, indicating that the antibacterial components may be relatively polar.

Whilst, the *T. ferdinanadiana* leaf, fruit and seed extracts also inhibited the growth of *P. aeruginosa* (Figure 4), substantially different trends were noted compared to the inhibitory activity against the other bacteria tested. Greater potency (based on the size of the ZOIs) was evident for the mid to lower polarity extracts, compared to the high polarity extracts. Indeed, ZOIs of

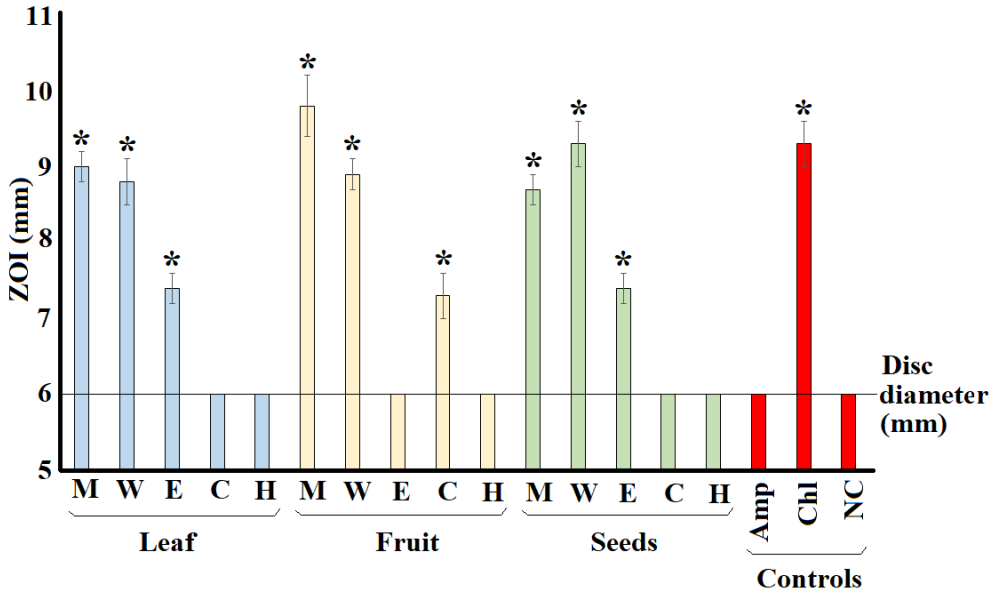


Figure 2: Antibacterial activity of the *T. ferdinandiana* extracts against *K. pneumoniae* (ATCC31488) measured in mm as zones of inhibition (ZOI). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg); Chl = chloramphenicol (10 µg); NC = negative control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicates (n=9) ± SEM * indicates results that are significantly different to the negative control (p<0.01).

9.2 and 8.6 mm were measured for the chloroform leaf and fruit extracts against *P. aeruginosa*. Similarly, a ZOI of 9.3 mm was measured for the fruit ethyl acetate extract against this bacterium. In contrast, ZOIs of 8.8 and 8.3 mm were measured for the methanolic extracts, indicating that different extract components may be responsible for *P. aeruginosa* growth inhibition than for the other bacteria studied herein, although this remains to be verified. The *P. aeruginosa* growth inhibitory activities of the *T. ferdinandiana* extracts indicate that they may be useful for preventing and treating multiple sclerosis (and other diseases caused by *P. aeruginosa* infections).

Quantification of minimum inhibitory concentration (MIC)

The bacterial growth inhibitory activity of the *T. ferdinandiana* extracts and the conventional antibiotics was quantified by liquid dilution MIC assays (Table 2). For this assay, MIC values >1 µg/mL for pure antibiotic compounds are defined as indicating resistance to the antibiotic.[22,23] Notably, all of the bacterial strains tested herein were resistant to penicillin-G, chloramphenicol, erythromycin and tetracycline, with MIC values ranging from 1.25 to 3.3 µg/mL. These results are consistent with previous studies that have also reported that these bacterial strains are resistant to these antibiotics.[9,10,23] Therefore, extracts with noteworthy growth inhibitory activity against these bacteria may be particularly useful to prevent and treat RA, AS and MS.

The *T. ferdinandiana* leaf and fruit extracts displayed noteworthy growth inhibitory activity (MICs <1000 µg/mL) against all of the bacterial strains tested in our study (Table 2). The leaf extracts were generally stronger bacterial growth inhibitors than the corresponding fruit extracts, with substantially lower MIC values. The aqueous leaf extract was a particularly good bacterial growth inhibitor, with liquid dilution (LD) MIC values of 350, 940, 90 and 90 µg/mL against *P. mirabilis, K. pneumonia, A. baylyi* and *P. aeruginosa* respectively. The methanolic *T. ferdinandiana* leaf extract had similar, albeit slightly less potent antibacterial activity LD MICs of 470, 470, 120 and 90 µg/mL respectively, whilst the LD MIC values of the leaf ethyl acetate extracts were slightly higher than those values. The methanolic, aqueous and ethyl acetate fruit extracts also displayed noteworthy growth inhibitory activity against the bacterial strains screened, although many of the LD MIC values calculated for the fruit extracts were up to twice those of the corresponding leaf extracts, indicating substantially lower activity. In contrast, the *T. ferdinandiana* seed extracts were less potent again, with LD MIC values generally substantially greater than 1000 µg/mL against all of the tested bacteria. The exception to this trend was the inhibition *of P. mirabilis* and *K. pneumoniae* growth by the seed ethyl acetate extract (LD MICs of 650 µg/mL against both bacteria), which indicates that this extract may be useful in the prevention and treatment of RA and AS.

Fractional inhibitory concentration (FIC) assessment

Four *T. ferdinandiana* leaf extract and conventional antibiotic combinations produced synergistic interactions when tested together (Table 3). Interestingly, all of the synergistic interactions were noted in combinations that contained the methanolic leaf extract as the extract component of the combination. Furthermore, three of the four (75%) synergistic combinations containing the methanolic leaf extract were against *P. mirabilis* as the test bacterium. Indeed, only the methanolic leaf and erythromycin combination was not synergistic. However, even

that combination yielded a low ΣFIC value (0.558) in the additive interaction range. Therefore, that combination would also be substantially more effective than using either the methanolic leaf extract or erythromycin alone and therefore would be beneficial in preventing and treating *P. mirabilis* infections. Whilst several resistance mechanisms may account for bacterial resistance to these antibiotics, efflux pumps are relatively common and often a single efflux pump may confer resistance to multiple antibiotics. Therefore, extract efflux pump inhibitor (EPI) compounds may 'reactivate' multiple antibiotic compounds towards the same bacterium. Such compounds would be particularly useful for antibiotic combination therapies. Further studies are required to determine whether the synergistic interactions reported herein are due to EFIs, and if so, to identify those compounds. Additionally, a further synergistic interaction was noted for the methanolic leaf and tetracycline combination against *P. aeruginosa*. As tetracycline-resistance is most frequently due to the development of tetracycline-specific efflux pumps,[18,25] this result provides further confirmation that the potentiation of tetracycline's antibacterial activity is likely to be due to EPI(s) in the extract. Furthermore, as *P. aeruginosa* is resistant to a wide range of conventional antibiotics, this combination may be particularly useful.

The combinations containing the seed extracts generally mirrored those containing the leaf extracts, with all three synergistic combinations containing the methanolic seed extract, and either penicillin, chloramphenicol or tetracycline as the antibiotic component. Thus, the potentiating components may be common between the leaf and seed extracts, although this remains to be verified. Two further synergistic interactions were evident for combinations containing the *T. ferdinandiana* fruit extracts. One of the synergistic fruit combinations contained the methanolic fruit extract and tetracycline against *P. mirabilis*. In contrast, the other synergistic fruit extract combination did not follow the previous trends. Instead, that combination contained aqueous fruit extract and tetracycline against *A. baylyi*. These results are particularly noteworthy as these combinations have substantially higher antibacterial activity than for either component separately (>4 times higher activity). These combinations may be particularly useful in preventing and treating RA, as well as other *P. mirabilis* infections.

Additionally, fifty combinations produced additive effects and would therefore be beneficial in treating *P. mirabilis, K. pneumoniae, A. baylyi* and *P. aeruginosa* infections. Notably, none of the combinations were antagonistic towards the inhibition of growth of any of the bacteria tested, indicating that it is safe to use all of these combinations without decreasing the antibacterial activity of either component. This is an important finding as traditional and herbal therapies are often used concurrently, without the patient informing their medical practitioner about their traditional medicine usage. Instead, the remainder of the combinations were non-interactive. Whilst there is no benefit of using these therapies in combination, their concurrent use would not decrease the potency of either medicine.

Varied ratio combination studies (isobolograms)

Four synergistic combinations were detected for combinations containing *T. ferdinandiana* methanolic leaf extracts. These combinations were further examined using isobologram analysis across a range of extract-antibiotic ratios to identify which ratios

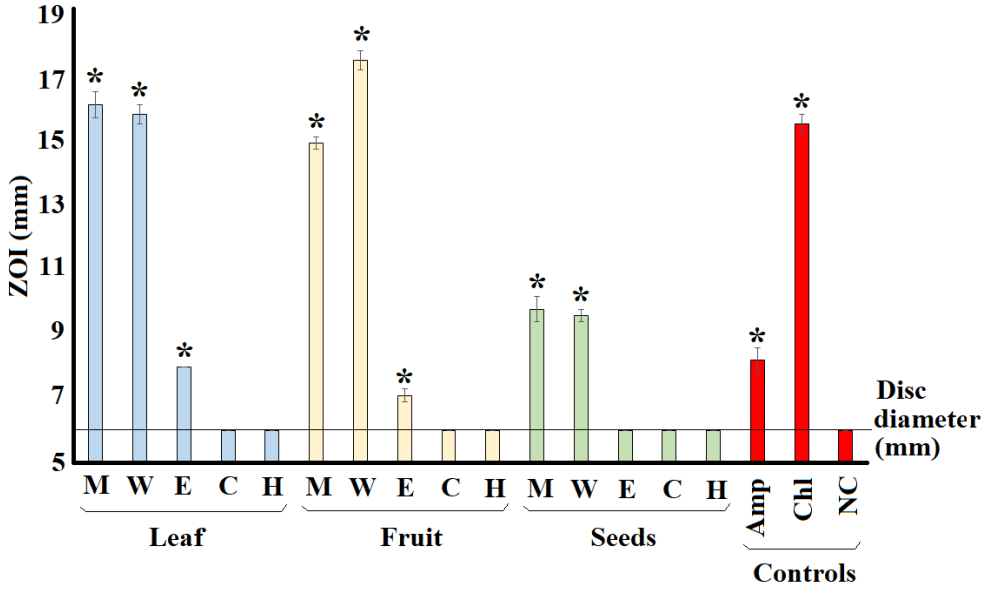


Figure 3: Antibacterial activity of the *T. ferdinandiana* extracts against *A. baylyi* (ATCC33304) measured in mm as zones of inhibition (ZOI). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg); Chl = chloramphenicol (10 µg); NC = negative control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicates (n=9) ± SEM * indicates results that are significantly different to the negative control (p<0.01).

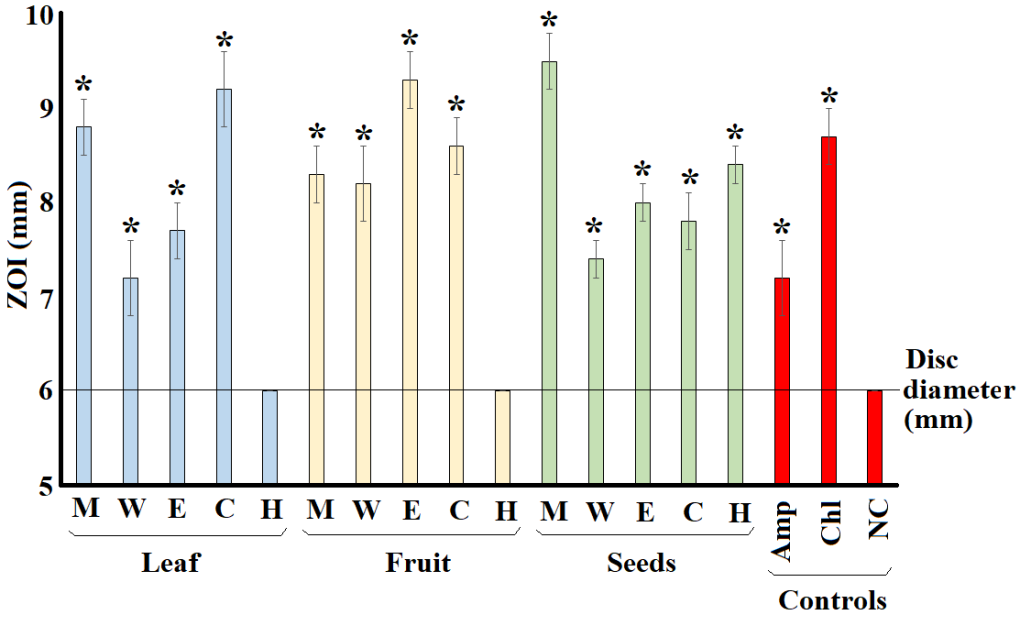


Figure 4: Antibacterial activity of the *T. ferdinandiana* extracts against *P. aeruginosa* (ATCC39324) measured in mm as zones of inhibition (ZOI). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg); Chl = chloramphenicol (10 µg); NC = negative control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicates (n=9) ± SEM * indicates results that are significantly different to the negative control (p<0.01).

induce synergy (Figure 5). Interestingly, similar trends were noted for all of the combinations containing the methanolic *T. ferdinandiana* methanolic leaf extract, with synergy generally evident for ratios of ≥40% extract, up to 60-70 % extract (Figure 5). All other ratios resulted in additive effects and thus would also be beneficial for inhibiting *P. mirabilis* and *P. aeruginosa* growth and thereby preventing and treating RA and MS respectively. These results indicate that extract component(s) may be competing with the antibiotic component for interaction with the bacterial resistance mechanism (e.g. an efflux pump or antibiotic inactivating enzyme). As the % of extract in the ratio increases, extract component(s) may outcompete the antibiotic component for interaction with the resistance mechanism, effectively blocking its ability to inhibit the antibiotics effects.

Additionally, two combinations containing *T. ferdinandiana* fruit extracts also produced synergistic interactions. In particular, the combination of the methanolic *T. ferdinandiana* fruit extract with tetracycline was synergistic for *P. mirabilis* growth inhibition (Figure 6a). Isobologram analysis of this combination determined that combinations containing ≥30% extract up to 60 % were synergistic and were therefore deemed to be the preferred ratios for the prevention and treatment of RA, and other illnesses caused by *P. mirabilis* infections. In contrast, combinations containing the aqueous *T. ferdinandiana* fruit extract and tetracycline produced synergistic effects at a wider range of extract-antibiotic ratios. Indeed, all ratios except those containing 10% and 90% extract were synergistic (Figure 6b). The ability of the extract components to overcome the bacterial resistance mechanisms even when low extract % are present may indicate that compounds in the aqueous *T. ferdinandiana* fruit extract may function as irreversible inhibitors of the bacterial resistance mechanism(s). Similar isobologram trends have previously been reported for Augmentin® (a antibiotic therapy containing a combination of amoxicillin and clavulanic acid).[14] Notably, the synergistic activity ratios reported for the Augmentin® components is the basis of the 15% clavulanic acid and 85% amoxicillin composition of that the clinical antibiotic drug.

The interactive effects determined for the *T. ferdinandiana* methanolic seed extracts were similar to those previously described for the methanolic leaf extract containing combinations, although with a wider range of extract % capable of inducing synergy. For the methanolic seed extract and penicillin combination, all ratios between 10 to 90% extract were synergistic (Figure 7a), indicating that extract components may be irreversibly inhibiting the bacteria's penicillin-resistance mechanism. In contrast, combinations of the methanolic seed extract with either chloramphenicol (Figure 7b) or tetracycline (Figure 7c) were only synergistic for combinations containing between 30-40% to 60-70% extract. Such isobologram profiles are more consistent with reversible competitive inhibition of the bacterial antibiotic-resistance mechanisms, although the resistance mechanism is yet to be determined. As bacteria would be less likely to develop resistance when combinations are used in ratios that minimize the use of conventional antibiotic, combinations containing 60-70% *T. ferdinandiana* methanolic seed extract would be preferred for long-term prophylactic therapy to prevent RA. However, the % extract that maximizes the efficacy of the treatment (i.e. 30-40% extract) would be preferred for the treatment of acute *P. mirabilis* infections (e.g. urinary tract infections).

Toxicity studies

All of the *T. ferdinandiana* extracts (1000 µg/mL) and the conventional antibiotics (10 µg/mL) were tested individually in the *Artemia* lethality assay (ALA) (Table 4). The extracts were also tested across a range of concentrations and LC_{50} values were determined by linear regression. In this assay, extracts and pure compounds are considered toxic if they induced percentage mortalities greater than 50 % at the initial screening concentrations following 24 hr of exposure to the *Artemia nauplii*.[24] Notably, all of the conventional antibiotics and *T. ferdinandiana* extracts induced substantially <50% mortality following 24 hr exposure. Therefore, all extracts and antibiotics were deemed nontoxic. In contrast, the positive control potassium dichromate induced 100% mortality in the ALA, indicating that the assay was functioning correctly. Combinations of the extracts and antibiotics were also screened for toxicity to determine whether the combination decreases the safety of the therapy. In this study, the LC_{50} values of the combinations refer to the toxicity of the extract component of the combination. Interestingly, combining the extracts with conventional antibiotics did not substantially affect the mortality induction and all combinations were deemed to be nontoxic.

As a further measure of the safety of the extracts, individual antibiotics and combinations were further analysed by calculating the therapeutic index (TI). For this study, TI values ≥3.0 were considered to be noteworthy and are highlighted in Table 4. The methanolic, aqueous and ethyl acetate leaf extracts displayed notable TI values against *P. mirabilis, A. baylyi* and *P. aeruginosa,* indicating that they may be safe to use at therapeutic doses, although further studies are required to confirm the safety *in vivo*. The aqueous extract appeared particularly safe, with TI values of 19.5 calculated for both *A. bayli* and *P. aeruginosa* growth inhibition. The methanolic and aqueous *T. ferdinandiana* fruit extracts also had noteworthy TI values against *P. mirabilis, A. baylyi* and *P. aeruginosa*, albeit with substantially lower TI values (3.2-4.2). In contrast, all TI values calculated for the *T. ferdinandiana* fruit extracts were substantially <3.0. Therefore, caution is required for these extracts and substantially more work is required to evaluate their safety before they can be used therapeutically. Interestingly, addition of conventional antibiotics to the extracts did not substantially affect the TI values for any combination tested.

Table 2: MIC values of the *T. ferdinandiana* extracts and conventional antibiotics (µg/mL) against some bacterial triggers of selected autoimmune anti-inflammatory diseases.

Extract or Antibiotic	DD MIC	*P. mirabilis* (ATC21721)		*K. pneumoniae* (ATCC31488)		*A. baylyi* (ATCC33304)		*P. aeruginosa* (ATCC39324)	
		LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	
Leaf	M	543	470	556	470	134	120	134	90
	W	440	350	1667	940	100	90	100	90
	E	633	450	987	718	643	450	643	340
	C	-	-	-	-	-	-	1580	1200
	H	-	-	-	-	-	-	-	-
Fruit	M	730	580	1273	870	519	440	1180	870
	W	1264	760	1667	1264	653	500	785	500
	E	337	250	-	-	958	500	1262	500
	C	-	-	1880	1100	-	-	1894	1100
	H	-	-	-	-	-	-	-	-
Seed	M	1514	1260	1640	1260	2855	1700	3030	2550
	W	1771	1500	2259	1500	>5000	>5000	>5000	>5000
	E	1437	650	1146	650	-	-	1670	650
	C	-	-	-	-	-	-	1755	800
	H	1537	630	-	-	-	-	1426	630
Conventional Antibiotics	Pen	ND	1.25	ND	2.5	ND	2.5	ND	1.25
	Chlor	ND	3.3	ND	2.5	ND	2.5	ND	1.25
	Ery	ND	2.5	ND	1.25	ND	1.25	ND	1.25
	Tet	ND	1.25	ND	1.25	ND	1.25	ND	1.25

DD MIC = MIC in the disc diffusion assay; LD MIC = MIC in the liquid dilution assay; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Pen = penicillin-G; Chlor = chloramphenicol; Ery = erythromycin; Tet = tetracycline; - indicates no inhibition at any dose tested; ND = no MIC was determined as the antibiotic was tested at a single dose only in the DD assay.

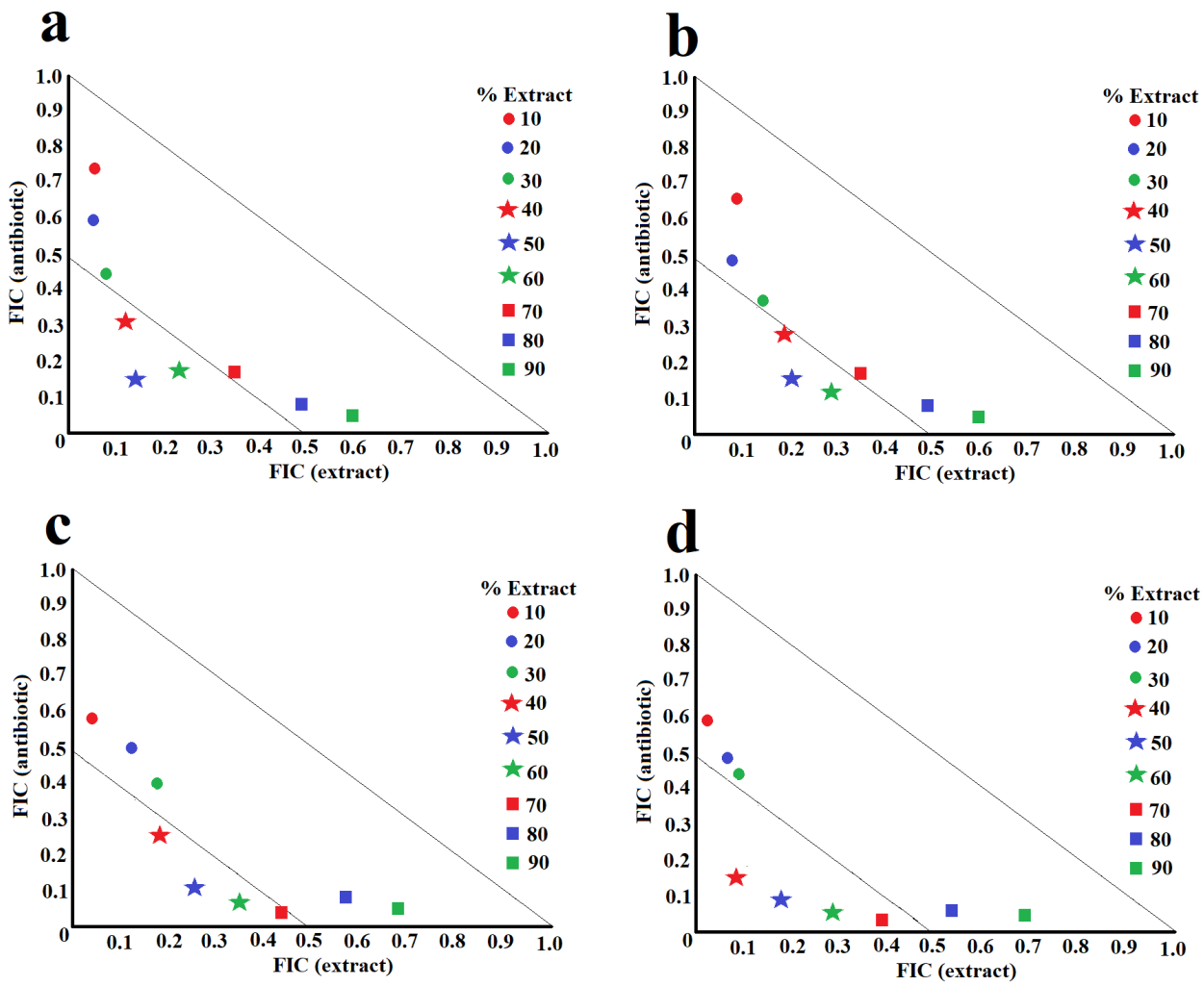


Figure 5: Isobolograms for combinations of (a) *T. ferdinandiana* methanolic leaf extract + penicillin against *P. mirabilis*, (b) *T. ferdinandiana* methanolic leaf extract + chloramphenicol against *P. mirabilis*, (c) *T. ferdinandiana* methanolic leaf extract + tetracycline against *P. mirabilis*, and (d) *T. ferdinandiana* methanolic leaf extract + tetracycline against *P. aeruginosa*. The combinations were tested at various ratios between 10% and 90% extract. Results represent the mean FIC values of four replicates. Ratios lying on or below the 0.5:0.5 line are considered to be synergistic. Values between the 0.5:0.5 and 1.0:1.0 lines are considered to be additive.

DISCUSSION

The studies reported herein evaluated and quantified the bacterial growth inhibitory properties of *T. ferdinandiana* leaf, fruit and seed extracts against bacterial triggers of RA, AS and MS.[1,2] There are no widely available safe, highly effective and affordable chemotherapies to prevent and treat these diseases and the development of new therapies is urgently required. Whilst multiple treatments are clinically available, the majority of these use anti-inflammatory drugs (particularly non-steroidal anti-inflammatory drugs (NSAIDs)) and analgesics to target the later phase disease symptoms (particularly inflammation and pain). These treatments alleviate the patient's pain and discomfort, although the prolonged use of many of these drugs induces toxicity and is associated with many unwanted side-effects.[26] For example, use of cyclooxygenase-2 (COX-2) inhibitors over extended periods can increase the incidence of myocardial infarction.[27] Additionally, most of the current clinical treatments only target the late-phase inflammatory events and fail to alter the disease progression, and therefore generally do not significantly inhibit autoimmune tissue damage. In contrast, targeting autoimmune inflammatory diseases via inhibition of the disease etiology with prophylactic/preventative treatments may limit autoimmune tissue degradation by reducing the need for an immunological response, thereby inhibiting the subsequent inflammatory symptoms of these diseases.

Most previous studies aimed at developing new therapies have focused on inhibiting the inflammatory symptoms, or on modulation of the immunological signals and inflammatory cascades. Biological therapies such as Brodalumab, Ixekizumab and Secukinumab that inhibit the pro-inflammatory cytokine interleukin 17 (IL-17) have proven to be particularly effective therapies for some autoimmune diseases.[28] However, the same study also reported increased incidence of bacterial infections, which may increase the duration of the autoimmune disease

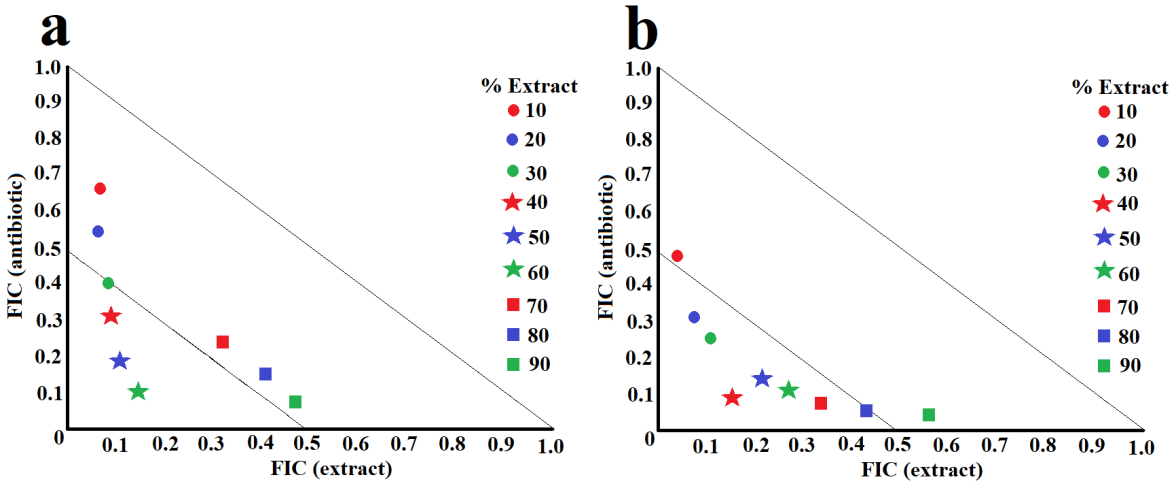


Figure 6: Isobolograms for combinations of (a) *T. ferdinandiana* methanolic fruit extract + tetracycline against *P. mirabilis*, (b) *T. ferdinandiana* aqueous fruit extract + tetracycline against *A. baylyi*. The combinations were tested at various ratios between 10% and 90% extract. Results represent the mean FIC values of four replicates. Ratios lying on or below the 0.5:0.5 line are considered to be synergistic. Values between the 0.5:0.5 and 1.0:1.0 lines are considered to be additive.

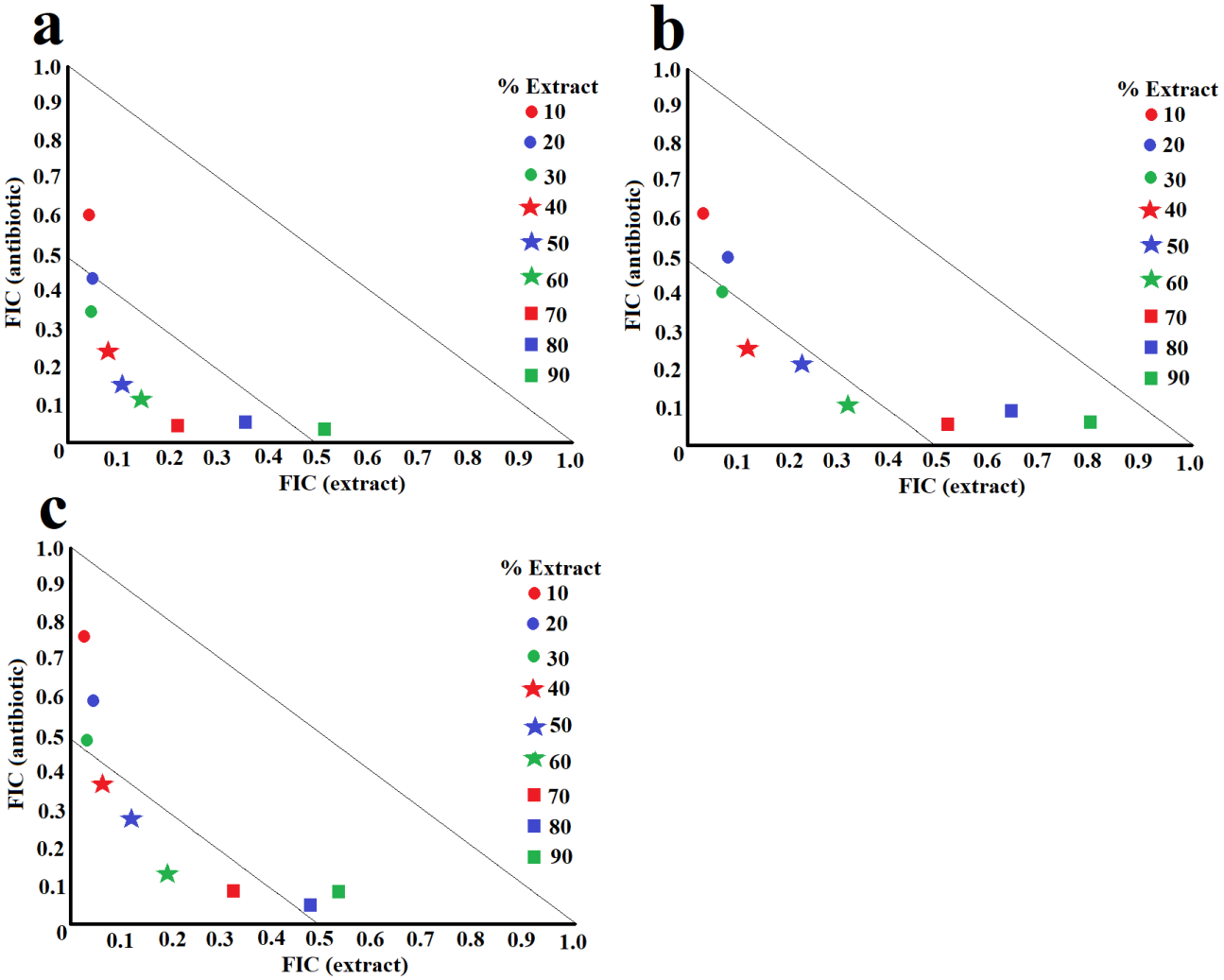


Figure 7: Isobolograms for combinations of (a) *T. ferdinandiana* methanolic seed extract + penicillin against *P. mirabilis*, (b) *T. ferdinandiana* methanolic seed extract + chloramphenicol against *P. mirabilis*, (c) *T. ferdinandiana* methanolic seed extract + tetracycline against P. mirabilis. The combinations were tested at various ratios between 10% and 90% extract. Results represent the mean FIC values of four replicates. Ratios lying on or below the 0.5:0.5 line are considered to be synergistic. Values between the 0.5:0.5 and 1.0:1.0 lines are considered to be additive.

Table 3: ΣFIC values for the *T. ferdinandiana* leaf, fruit and seed extracts in conbination with conventional antibiotics against some bacterial triggers of autoimmune diseases.

Extract or Antibiotic		*P. mirabilis*				*K. pneumoniae*				*A. baylyi*				*P. aeruginosa*			
		Chl	Eryth	Tet	Pen	Chl	Eryth	Tet	Pen	Chl	Eryth	Tet	Pen	Chl	Eryth	Tet	
Leaf	M	0.467	0.452	0.558	0.44	1.375	0.95	1.118	0.73	0.67	1.47	1.18	0.92	0.95	0.62	0.88	0.387
		(SYN)	(SYN)	(ADD)	(SYN)	(IND)	(ADD)	(IND)	(ADD)	(ADD)	(IND)	(IND)	(ADD)	(ADD)	(ADD)	(ADD)	(SYN)
	W	0.67	0.88	0.92	0.66	1.762	2.15	2.771	1.28	1.15	1.93	1.76	0.77	1.376	1.228	1.43	0.88
		(ADD)	(ADD)	(ADD)	(ADD)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(ADD)	(IND)	(IND)	(IND)	(ADD)
	E	1.25	1.46	1.933	1.075	1.484	1.96	2.54	1.447	1.8	2.36	2.45	2.29	1.072	1.335	1.821	1.15
		(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)
	C	CND	CND	CND	CND	CND	CND	CND	CND	CND	CND	CND	CND	1.552	1.776	2.07	1.883
														(IND)	(IND)	(IND)	(IND)
Fruit	M	0.88	1.08	0.56	0.438	1.116	1.054	1.229	0.58	0.56	0.64	0.56	0.68	0.71	0.96	0.55	0.66
		(ADD)	(IND)	(ADD)	(SYN)	(IND)	(IND)	(IND)	(ADD)	(ADD)	(ADD)	(ADD)	(ADD)	(ADD)	(ADD)	(ADD)	(ADD)
	W	0.56	0.84	0.56	0.51	1.995	2.526	3.33	1.924	0.56	0.72	0.56	0.23	1.06	1.184	1.193	0.56
		(ADD)	(ADD)	(ADD)	(ADD)	(IND)	(IND)	(IND)	(IND)	(ADD)	(ADD)	(ADD)	(SYN)	(IND)	(IND)	(IND)	(ADD)
	E	1	1.15	1.46	0.83	CND	CND	CND	CND	0.83	1.14	0.83	1.67	1.495	1.883	2.2	2.168
		(ADD)	(IND)	(IND)	(ADD)					(ADD)	(IND)	(ADD)	(IND)	(IND)	(IND)	(IND)	(IND)
	C	CND	CND	CND	CND	2.387	2.831	3.105	2.256	CND	CND	CND	CND	CND	CND	CND	CND
						(IND)	(IND)	(IND)	(IND)								
Seed	M	0.074	0.47	0.67	0.38	0.82	1.146	1.302	0.72	2.06	2.46	3.15	1.06	0.88	0.92	1.258	0.55
		(SYN)	(SYN)	(ADD)	(SYN)	(ADD)	(IND)	(IND)	(ADD)	(IND)	(IND)	(IND)	(IND)	(ADD)	(ADD)	(IND)	(ADD)
	W	0.67	0.85	0.67	0.56	2.26	2.883	3.572	2.22	2.06	2.72	3.3	1.06	0.82	1.18	1.392	0.83
		(ADD)	(ADD)	(ADD)	(ADD)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(ADD)	(IND)	(IND)	(ADD)
	E	0.94	1.07	1.11	1.074	2.416	3.085	3.77	2.449	CND	CND	CND	CND	1.884	2.365	3.395	2.63
		(ADD)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)					(IND)	(IND)	(IND)	(IND)
	C	CND	CND	CND	CND	CND	CND	CND	CND	CND	CND	CND	CND	2.43	2.885	3.6	3.122
														(IND)	(IND)	(IND)	(IND)
	H	1.585	1.665	1.852	1.448	1.766	1.893	2.108	1.85	CND	CND	CND	CND	2.23	2.762	3.287	2.69
		(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)					(IND)	(IND)	(IND)	(IND)

M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; CND = could not determine as one component of the combination was inactive at all concentrations tested; SYN = synergistic interaction; ADD = additive interaction; IND = indifferent interaction; Where an extract was inactive against all bacteria, combinations containing that extract are not shown in this table.

whilst blocking the symptoms. Additionally, the usage of monoclonal antibiodies targeting key steps in inflammation are also effective against some autoimmune inflammatory diseases. Infliximab (an antibody targeting NF-κB) substantially inhibits pro-inflammatory cytokine production in AS sufferers, thereby decreasing symptoms by >50 %.[29] Glatiramer acetate inhibits the immune response in sufferers of autoimmune diseases by down-regulating pro-inflammatory Th1 T-cells, thereby decreasing the disease symptoms.[30] Similarly, Teriflunomide inhibits pyrimidine synthesis, thereby reducing the levels of activated T-cells and blocking autoimmune damage to self-tissue.[31] However, whilst these biological therapies are often more effective and cause less side-effects than other clinical therapies for autoimmune diseases, they are not frequently used as they are far more expensive than other existing therapies and are generally only prescribed when other therapies fail. Plant-derived anti-infective and/or immunomodulatory compounds may provide cheaper therapies that are safe and effective.

Several recent studies have examined the use of traditional medicines to prevent the etiological events of some autoimmune diseases by inhibiting the antigenic triggers of those diseases.[8,22,23,32] Our study extended the earlier studies by screening the effects of *T. ferdinandiana* leaf, fruit and seed extracts as inhibitors some bacterial triggers of selected autoimmune diseases. *Proteus mirabilis* was selected for screening as it induces self-reactive antibody production in people susceptible to rheumatoid arthritis.[1] Similarly, *K. pneumoniae* was included in this study as infections of this bacterium stimulate the production of self-reactive antibodies in people with ankylosing spondylitis. *Acinitobacter baylyi* and *P. aeruginosa* can induce autoimmune tissue damage in people genetically susceptible to multiple sclerosis,[1,2] and therefore those bacteria were also screened in our study. Methanolic, aqueous and ethyl acetate *T. ferdinandiana* leaf and fruit extracts were effective growth inhibitors against all of the bacterial triggers of RA, AS and MS tested in this study. The *T. ferdinandiana* ethyl acetate seed extracts also had noteworthy

Table 4: Toxicity of the *T. ferdinandiana* extracts, the conventional antibiotics and combinations expressed as the % mortality and LC_{50}'s (µg/mL) and therapeutic indexes.

Tests		% Mortality in ALA	LC_{50} (µg/mL) *P. mirabilis*	TI *K. pneumoniae*	TI *A. bayli*	TI *P. aeruginosa*	TI	Tests		% Mortality *P. mirabilis*	LC_{50} (µg/mL) *K. pneumoniae*	TI *A. bayli*	TI *P. aeruginosa*	TI	TI
Conventional Antibiotics	Pen	4.2±1.4	ND	ND	ND	ND	ND	Fruit extract + antibiotic mixtures	F M + Pen	42.6 ± 1.8	1780	3.1	2	4	2
	Chlor	5.5±1.6	ND	ND	ND	ND	ND		F M + Chlor	44 ± 2.8	1689	2.9	1.9	3.8	1.9
	Ery	3.3±0.7	ND	ND	ND	ND	ND		F M + Ery	38.4 ± 3.3	1954	3.4	2.2	4.4	2.2
	Tet	4.6±1.5	ND	ND	ND	ND	ND		F M + Tet	42.9 ± 3	1755	3	2	4	2
Leaf	M	34.6 ± 2.7	1228	2.6	2.6	10.2	13.6		F W + Pen	33.7 ± 3.3	1643	2.2	1.3	3.3	3.3
	W	39.5 ± 3.3	1754	5	1.9	19.5	19.5		F W + Chlor	35.8 ± 4.1	1505	2	1.2	3	3
	E	22.8 ± 3.4	1486	3.3	2.1	3.3	4.4		F W + Ery	31.7 ± 2.7	1719	2.3	1.4	3.4	3.4
	C	9.7 ± 3.7	-	-	-	-	-		F W + Tet	36.5 ± 2.6	1557	2	1.2	3.1	3.1
	H	7.8 ± 2.4	-	-	-	-	-		F E + Pen	6.2 ± 2	-	-	-	-	-
Fruit	M	41.2 ± 1.6	1863	3.2	2.1	4.2	2.1		F E + Chlor	6.7 ± 2.9	-	-	-	-	-
	W	35 ± 2.8	1588	2.1	1.3	3.2	3.2		F E + Ery	5.1 ± 2	-	-	-	-	-
	E	6.7 ± 2	-	-	-	-	-		FE + Tet	7.2 ± 2.5	-	-	-	-	-
	C	5.4 ± 1.5	-	-	-	-	-		FC + Pen	5.2 ± 2.6	-	-	-	-	-
	H	3.9 ± 2.4	-	-	-	-	-		FC + Chlor	6.1 ± 1.9	-	-	-	-	-
Seed	M	38.4 ± 2.6	1447	1.2	1.2	0.9	0.6		FC + Ery	4.7 ± 2.6	-	-	-	-	-
	W	42.9 ± 3.3	1506	1	1	<0.3	<0.3		FC + Tet	6.4 ± 2.9	-	-	-	-	-
	E	4.4 ± 1.7	-	-	-	-	-	Seed extract + antibiotic mixtures	SM + Pen	36.7 ± 2.9	1628	1.3	1.3	1	0.6
	C	5.2 ± 2.6	-	-	-	-	-		SM + Chlor	38.6 ± 3.5	1440	1.1	1.1	0.8	0.6
	H	3.7 ± 2.2	-	-	-	-	-		SM + Ery	34.8 ± 3	1754	1.4	1.4	1	0.7
Combinations									SM + Tet	38.4 ± 2.2	1483	1.2	1.2	0.9	0.6
Leaf extract + antibiotic mixtures	L M + Pen	35.8 ± 2.4	1408	3	3	11.7	15.6		SW + Pen	40.9 ± 3.1	1604	1.1	1.1	<0.3	<0.3
	L M + Chlor	35.6 ± 2.5	1663	3.5	1.8	13.8	18.5		SW + Chlor	41.8 ± 2.4	1552	1	1	<0.3	<0.3
	L M + Ery	31.6 ± 3.6	1766	3.8	1.9	14.7	19.6		SW + Ery	38.7 ± 2.7	1726	1.2	1.2	<0.3	<0.3
	L M + Tet	36.6 ± 3.3	1620	3.4	3.4	13.5	18		SW + Tet	40.3 ± 3.5	1637	2.5	2.5	<0.3	<0.3
	L W + Pen	38.5 ± 2.7	1685	4.8	1.8	18.7	18.7		SE + Pen	4.2 ± 2.7	-	-	-	-	-
	L W + Chlor	35.2 ± 3	1558	4.5	1.7	17.3	17.3		SE + Chlor	4.6 ± 2	-	-	-	-	-
	L W + Ery	37.7 ± 3	1863	5.3	2	20.7	20.7		SE + Ery	3.7 ± 1.8	-	-	-	-	-
	L W + Tet	39.1 ± 1.8	1588	4.5	1.7	17.6	17.6		SE + Tet	4.4 ± 2.1	-	-	-	-	-
	L E + Pen	20.4 ± 2.2	1425	3.2	2	3.2	4.2		SC + Pen	6.1 ± 2.6	-	-	-	-	-
	L E + Chlor	21.6 ± 3.4	1572	3.5	2.2	3.5	4.6		SC + Chlor	6.8 ± 2.7	-	-	-	-	-
	L E + Ery	24.8 ± 1.5	1506	3.3	2.1	3.3	4.4		SC + Ery	5 ± 1.8	-	-	-	-	-
	L E + Tet	27.1 ± 2.7	1550	3.4	2.2	3.4	4.6		SC + Tet	6.9 ± 3.2	-	-	-	-	-
	L C + Pen	8.6 ± 2.5	-	-	-	-	-		SH + Pen	3.5 ± 1.8	-	-	-	-	-
	L C + Chlor	9.8 ± 2.6	-	-	-	-	-		SH + Chlor	5.2 ± 2.6	-	-	-	-	-
	L C + Ery	10.7 ± 2.8	-	-	-	-	-		SH + Ery	3.7 ± 2.3	-	-	-	-	-
	L C + Tet	11.3 ± 3.4	-	-	-	-	-		SH + Tet	5.4 ± 2.7	-	-	-	-	-

All antibiotics and extracts were initially tested at 2 µg/mL and 1000 µg/mL respectively. The LC_{50} values represent the toxicity of the extract component only as it comprises the majority of the combination. Pen = penicillin; Chlor = chloramphenicol; Ery = erythromycin; Tet = tetracycline; L = leaf; F = fruit; S = seed; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; - indicates that an LC_{50} or TI could not be determined as the mortality did not exceed 50% at the highest concentration tested; ND = LC_{50} value was not determined. Ti values were calculated by dividing the LC_{50} by the MIC of the relevant extract. Noteworthy TI values ≥3) are highlighted.

inhibitory activity against *P. mirabilis, K. pneumoniae* and *P. aeruginosa*, but was completely ineffective against *A. baylyi*. Notably, the *T. ferdinandiana* aqueous leaf extracts were the strongest inhibitors of bacterial growth, with LD MIC values of as low as 90 µg/mL against several bacteria. Similar, albeit slightly higher LD MICs were also measured for the methanolic leaf extract against the same bacteria, indicating that both of these extracts may be useful for preventing and treating RA, AS and MS in genetically susceptible people and further studies are warranted. Similarly, the LD MIC values of the methanolic, aqueous and ethyl acetate fruit extracts also indicate that further study is warranted into their therapeutic potential against autoimmune inflammatory diseases.

The combinational studies provided further insight into the potential of *T. ferdinandiana* extracts in combination with conventional antibiotics against antibiotic-resistant bacterial pathogens. The bacterial strains selected for screening in our study were each resistant to all of the conventional antibiotics tested, with measured MIC values >1 µg/mL. Notably, several extract-antibiotic combinations displayed substantially increased growth inhibitory activity towards the bacterial triggers of RA, AS and MS than the extracts or antibiotics alone. Nine synergistic combinations were identified in our study, with the most noteworthy of these containing methanolic *T. ferdinandiana* leaf extract and either penicillin, chloramphenicol or tetracycline against *P. mirabilis*. These combinations may be especially useful as synergistic combinations have substantial advantages over antibiotic monotherapies, particularly for prolonged usage. The enhanced potency of the combination would provide greater efficacy, yet allow for the use of lower doses of the conventional antibiotic component, thus reducing the risk of side effects. The use of less conventional antibiotics may also reduce the development of further antimicrobial resistant bacterial strains. Our study identified several combinations that may 'reactivate' the conventional antibiotic components of the mixture towards antibiotic-resistant bacteria and substantially increase their antibacterial efficacy.

Notably, whilst our study screened *T. ferdinandiana* extracts for potential to inhibit the onset of RA, AS and MS by blocking some bacterial triggers of those diseases, we did not screen the extracts for therapeutic effects against other phases of these diseases. However, several *Terminalia* spp. have substantial anti-inflammatory effects.[7] and therefore may modulate several phases of autoimmune disease progression. If this also proves to be the case for this species, *T. ferdinandiana* extracts may be particularly useful for treating these diseases and further study is warranted. Interestingly, a recent study reported that a hydroalcohol *T. ferdinandiana* fruit extract (400 µg/mL) significantly inhibited the key inflammatory enzymes cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) *in vitro* in murine RAW 264.7 macrophage cells.[33] Interestingly, the extract did not significantly affect the expression of COX-1. However, there is a lack of studies examining the effects of *T. ferdinandiana* fruit extract on other inflammatory biomarkers, and the effects of *T. ferdinandiana* leaf and seed extracts remain completely unreported. Further studies are required to screen these and other *T. ferdinandiana* extracts (both alone and in combination with conventional anti-inflammatory drugs) against other inflammatory biomarkers. It is noteworthy that all of the *T. ferdinandiana* extracts and combinations tested in this study were nontoxic in the ALA toxicity assay, indicating their safety for therapeutic usage. However, further *in vitro* toxicity studies using other human cell lines are required to verify the safety of these extracts prior to clinical usage. Additionally, studies to examine chronic toxicity are also required before the extracts, combinations, or purified components can be deemed to be safe for prolonged prophylactic usage. Future *in vivo* toxicity assays are also required to confirm the safety of these compounds and combinations in complex biological systems.

CONCLUSION

The findings of this study highlight the potential of *T. ferdinandiana* extracts (especially the leaf extracts) to inhibit the etiological events of RA, AS and MS. However, further *in vivo* investigations are required to verify these *in vitro* findings and studies to determine the therapeutic mechanisms of the extracts are warranted. Additionally, studies to isolate and identify the potentiator and antibacterial components of the extract may provide useful targets for further antibiotic chemotherapy development. Further studies are also required to determine whether *T. ferdinandiana* extracts also affect other phases of the progression of these autoimmune diseases, including the immunological and inflammatory cascades.

ACKNOWLEDGEMENT

We are grateful to David Boehme of Northern Territory Wild Harvest for providing the *T. ferdinandiana* fruit, leaves and seeds used in this study. The financial assistance of the Centre for Planetary Health and Food Security, and the School of Environment and Science, Griffith University is hereby acknowledged.

ABBREVIATIONS

ALA: Brine-shrimp lethality assay; **COX:** Cyclooxygenase; **DMSO:** Dimethyl Sulfoxide; **EPI:** Efflux pump inhibitor; **ESBL:** Extended-spectrum β-lactamase; **ΣFIC:** Sum of the fractional inhibitory concentration; **HDF:** Human Dermal Fibroblasts; **IL-17:** Interleukin-17; **iNOS:** Inducible nitric oxide synthase; **INT:** ρ-Iodonitrotetrazolium chloride; **LC_{50}:** Concentration of sample necessary to have a lethal effect on 50% of test organisms or cells; **MIC:** Minimum inhibitory concentration; **MRSA:**

Methicillin-resistant *Staphylococcus aureus*; **ZOI:** Zone of inhibition.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

Terminalia ferdinandiana leaf fruit and seed extracts were screened for growth inhibitory activity against bacterial triggers of some autoimmune diseases. The antibacterial activity of the extracts was quantified by disc diffusion and liquid dilution MIC assays. The interactive effects of combinations of extracts and conventional antibiotics were assessed by ΣFIC determination. The preferred extract: antibiotic ratios of the synergistic combinations were determined by isobologram analysis. Toxicity of the individual compounds and combinations was evaluated using the Artemia nauplii bioassay.

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Cite this article: Guo X, Liu J, Cock IE. The Interactive Antimicrobial Activity of *Terminalia ferdinandiana* Exell. Extracts and Conventional Antibiotics against Bacterial Triggers of Some Autoimmune Diseases. Pharmacognosy Communications. 2026;16(4):171-85.