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Original Article

Combinational Inhibitory Effects of *Terminalia arjuna* (Roxb.) Wight and Arn. Extracts and Conventional Antibiotics against Bacterial Triggers of Selected Inflammatory Diseases

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ABSTRACT

Introduction: *Terminalia arjuna* (Roxb.) Wight and Arn. is a southern Asian plant commonly used in Ayurveda to treat multiple conditions, including for the treatment of inflammation, and as a general antimicrobial agent. Despite this, few studies have tested this species for the ability to block the growth of bacterial triggers of some autoimmune inflammatory diseases. Furthermore, the ability of *T. arjuna* to potentiate the activity of conventional antibiotics is yet to be investigated. **Materials and Methods:** The minimum inhibitory concentration of the extracts and extract-antibiotic combinations was determined by disc diffusion and liquid dilution MIC methods. Fractional inhibitory concentration values were calculated to evaluate the combinational effect of combinations of extracts and conventional antibiotics. When synergistic interactions were detected, isobologram analysis was used to determine the ideal ratios for synergy. Toxicity was evaluated by *Artemia nauplii* mortality and HDF cell viability assays. **Results:** Methanolc, aqueous and ethyl acetate *T. arjuna* extracts were good inhibitors of *P. mirabilis* and *K. pneumoniae* growth when tested alone. The methanolic and aqueous extracts also synergised the inhibitory activity of ciprofloxacin against *P. mirabilis* and *K. pneumoniae* when used in combination. Additionally, numerous additive combinational effects were noted. *T. arjuna* chloroform extract was also a moderate inhibitor of a multi-drug resistant *P. aeruginosa* strain when tested alone, but did not potentiate the activity of any of the conventional antibiotics tested. All extracts and extract-antibiotic combinations were nontoxic in the *Artemia* nauplii mortality and HDF proliferation assays, indicating their suitability for therapeutic use. **Conclusion:** *T. arjuna* extracts have potential as inhibitors of bacterial triggers of selected autoimmune inflammatory diseases. Furthermore, *T. arjuna* extracts potentiate the activity of ciprofloxacin against *P. mirabilis* and *K. pneumoniae* and therefore may be beneficial in drug design against these bacteria.

Key words: Synergy, Conventional antimicrobials, Interaction, Medicinal plants, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Drug combinations.

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INTRODUCTION

When Alexander Fleming discovered penicillin in 1929, it was regarded as one of the most significant discoveries in the field of medical science. Since then, numerous other antibiotics have been discovered from natural sources. Second and subsequent generation antibiotics were subsequently developed by chemical modifications of the molecular scaffolds of existing antibiotics. These antibiotics have rendered many bacterial infections that were previously considered to be life-threatening to become of substantially less consequence, saving countless lives. However, indiscriminate and overuse of antibiotics has resulted in the widespread development of antibiotic-resistant bacterial strains.[1] As a result, antibiotics that were previously considered "gold standard" therapies are often no longer effective and there is an urgent need to develop novel antibiotic chemotherapies.

Traditional medicinal plants have great potential for antimicrobial drug development, and there has recently been a substantial increase in interest in screening traditional medicines against human pathogens.[2-6] Much of the research into traditional medicinal plant use has focused on Asian,[7-9] African,[10-12] Middle Eastern[13-15] and South American[16] plants. However, despite the potential of plants to provide us with useful pharmaceutical agents, the field is still relatively poorly studied. Only an estimated 5-10 % of the approximately 300,000-500,000 plant species worldwide have been screened for one or more bioactivities.[17]

The development of new antibiotic therapies is particularly urgent. The recent establishment of bacterial pathogens that are either extremely (XDR) or totally resistant (TDR) to common clinically used antibiotics[1] has resulted in the need to develop new and effective antibiotic chemotherapies. There are now limited therapeutic options for many diseases caused by bacterial pathogens and the situation is expected to worsen in the future as bacteria exchange resistance genes. Indeed, the development of alternative antibacterial treatment modalities has become crucial and is considered by the World Health Organisation (WHO) to be one of the most serious challenges facing medical science.[18] For reasons reviewed elsewhere,[1] it is unlikely that the previous methods of antibiotic discovery/development will be as successful in the future and new treatment modalities are urgently required. Traditional medicines and herbal remedies have great potential for antimicrobial drug development and there has recently been a substantial increase in interest in this field.[19-33]

Notably, whilst crude plant preparations often have strong antibacterial activities, purified plant compounds often only have weak growth inhibitory effects. It is likely that antibiotic plant preparations may contain relatively weak antibacterial compound(s) in combination with potentiating compounds that substantially increase the potency of the traditional medicine. Plant derived potentiator compounds may also increase the activity of conventional antibiotics. Alternatively, they may overcome bacterial resistance mechanisms, allowing antibiotics to become effective again, even in bacteria otherwise resistant to their effects.[1] Thus, the use of combinations of traditional medicinal plant extracts and conventional antibiotics may be effective in overcoming drug resistance and increasing the activity of conventional antibiotics.[1,23,25]

One of the most useful genera of therapeutic plants is *Terminalia*, which comprises approximately 250 species of flowering trees. Many species of *Terminalia* have extensive uses in multiple traditional medicinal systems in the regions in which they grow.[34-36] Many of their traditional uses relate to pathogenic diseases and the antibacterial activity of many species have already been reported. Extracts prepared from the fruit of the Australian species *Terminalia ferdinandiana* Exell. (*Kakadu plum*) have potent growth inhibitory activity against an extensive panel of pathogens including bacteria associated diarrhoea and dysentery[37] as well as the bacterial triggers of rheumatoid arthritis (*Proteus mirabilis*)[38,39] and multiple sclerosis (*Acinitobacter baylyi* and *Pseudomonas aeruginosa*).[40,41] Leaf extracts from the same species have also been shown to inhibit growth of the same bacteria, as well as a microbial trigger of ankylosing spondylitis (*Klebsiella pneumoniae*),[32,39] Notably, *T. ferdinandiana* extracts are also inhibit antibiotic resistant bacterial strains and are particularly promising against extended spectrum β-lactamase (ESBL) bacteria.[42] Similarly, African *Terminalia* spp. have been shown to be potent bacterial growth inhibitors. *Terminalia stenostachya* and *Terminalia spinosa* have strong antibacterial activity against a broad spectrum of medicinally important bacteria including several *Mycobacterium* spp., *Streptococcus faecalis*, *Staphylococcus aureus*, *Vibrio cholera*, *Bacillus anthracis*, *K. pneumoniae*, *Salmonella typhi*, *P. aeruginosa* and *Escherichia coli*.[36] Recent studies have demonstrated the growth inhibitory activity of *Terminalia sericea* and *Terminalia pruinoides* against pathogenic[28-30] and food spoilage bacteria.[31]

Terminalia arjuna (Roxb.) Wight & Arn. is a traditional medicinal plant used in several southern Asian medicinal systems, including ayuverda, siddha and unani.[34,35] It grows throughout tropical and subtropical regions of Asia, although it is particularly prevalent in India, Sri Lanka and Bangladesh. *Terminalia arjuna* fruit and bark have high antioxidant contents and are commonly used as anticancer, antiulcer, antiviral and antifungal therapeutics although they are perhaps best known for their cardio-protective and antibacterial activities.[34,35] Indeed, several studies have reported their growth inhibitory activity against several pathogens including *Streptococcus pyogenes*,[43] *Clostridium perfringens*[44] and *Bacillus anthracis*[33] Despite this, *T. arjuna* extracts are yet to be evaluated for growth inhibitory activity against many human pathogens. This study was undertaken to screen *T. arjuna* extracts, conventional antibiotics, and their combination on bacterial triggers of some autoimmune disease. The toxicity of the extracts was also determined to further evaluate their suitability for therapeutic use.

MATERIALS AND METHODS

Sourcing and preparation of plant material

Terminalia arjuna (Roxb.) Wight and Arn. bark was a gift from Dr Paran Rayan, Griffith University and was sourced from verified trees in southern India. A voucher specimen (PRGVA5L014A) is stored at Griffith University, Brisbane Australia. The bark was thoroughly desiccated in a Sunbeam food dehydrator and the dried materials stored at -30°C until use. Prior to usage, the materials were thawed and ground into a coarse powder. Individual 1g masses of the dried plant material was extracted extensively in 50 mL methanol, deionised water, ethyl acetate, chloroform or hexane for 24h at 4°C with gentle shaking. All solvents were purchased from Ajax Fine Chemicals, Australia and were analytical (AR) grade. The extract was filtered through filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation. The resultant pellet was resuspended in 10mL deionised water (containing 1% DMSO), passed through 0.22µm filter (Sarstedt) and stored at 4°C.

Qualitative phytochemical studies

Phytochemical analysis of the *T. arjuna* extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted using standard assays.[26,27]

Antioxidant capacity

The antioxidant capacity of each extract was assessed using the DPPH free radical scavenging method,[45] with modifications. Briefly, DPPH solution was prepared fresh each day as a 400µM solution by dissolving DPPH (Sigma) in AR grade methanol (Ajax, Australia). A 2mL aliquot of each extract was evaporated and the residue resuspended in 2mL of methanol. Each extract was added to a 96 well plate in 5, 10, 25, 50, 75µL volumes in triplicate. Methanol was added to each well to give a volume of 225µL. A volume of 75µL of the fresh DPPH solution was added to each well to give a total reaction volume of 300µL. Ascorbic acid was prepared fresh and examined across the range 0-25µg per well as a reference and the absorbances were recorded at 515nm. All tests and controls were performed in triplicate. The antioxidant capacity based on DPPH free radical scavenging ability was determined for each extract and expressed as µg ascorbic acid equivalents per gram of original plant material extracted.

Antibacterial screening

Conventional antibiotics

Penicillin-G (potency of 1440-1680 µg/mg), chloramphenicol (≥98% purity by HPLC), ciprofloxacin (≥98% purity by HPLC), erythromycin (potency ≥850µg/mg), gentamicin (potency of 600µg/mg), and tetracycline (≥95% purity by HPLC) were purchased from Sigma-Aldrich, Australia and used for the microplate liquid dilution assay. All antibiotics were prepared in sterile deionised water at stock concentrations of 0.01mg/mL and stored at 4°C until use. For the disc diffusion studies, ampicillin (2µg) and chloramphenicol discs (10µg) standard discs were obtained from Oxoid Ltd., Australia and used as positive controls.

Test micro-organisms

All bacterial strains were selected based on their ability to trigger autoimmune inflammatory diseases in genetically susceptible individuals[39-41] Reference strains of *Proteus mirabilis* (ATCC21721), *Klebsiella pneumoniae* (ATCC31488) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Type Culture Collection, USA. 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 24h and were subcultured and maintained in nutrient broth at 4°C until use.

Evaluation of antimicrobial activity

The susceptibility of the bacteria to the *T. arjuna* extracts and the conventional antibiotics was initially assessed using a modified disc diffusion assay.[4-6] Ampicillin (2µg) and chloramphenicol discs (10µg) were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

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

Microplate liquid dilution MIC assay

A standard liquid dilution MIC assay[10] was used to evaluate the bacterial growth inhibitory activity of the extracts and conventional antibiotics. Briefly, log phase bacterial cultures were diluted to produce a McFarlands inoculation culture. A 100µL volume of sterilized nutrient broth was dispensed into all wells of a 96 well micro-titre plate. A volume of 100µL of the plant extracts or conventional antibiotics was subsequently dispensed into separate wells of the top row of the plate. A negative control (nutrient broth), sterile control (broth without bacteria) and a sample-free culture control (to ensure the media was capable of supporting microbial growth) were also included on all plates. Each test sample or control was serially diluted down each column on the plate by doubling dilution. The assay culture inoculum (100µL, containing approximately $1x10^6$ colony forming units (CFU)/mL) was then added to all wells except the sterile control wells and incubated overnight at 37°C. p-Iodonitrotetrazolium violet (INT, Sigma-Aldrich, Australia) was dissolved in sterile deionised water to a concentration of 200µg/mL. A 40µL volume of the INT solution was added into all wells and the plate was incubated for a further 6h at 37°C. The MIC was visually determined as the lowest dose at which colour development was inhibited.

Disc diffusion MIC assay

The minimum inhibitory concentration (MIC) of each extract was also quantified by disc diffusion assay.[4-6] Graphs of the zone of inhibition (ZOI) versus ln concentration were plotted and MIC values were calculated by linear regression.

Extract-conventional antibiotic interaction studies

Fractional inhibitory concentration (FIC) assessment

Interactions between the combinations of plant samples and conventional antimicrobials were further classified using the sum of the fractional inhibitory concentration (ΣFIC). The FIC was calculated using the following equation, where (a) represents the plant sample and (b) the conventional antimicrobial sample:[10]

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

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

The ΣFIC was then calculated using the equation: $\Sigma FIC = FIC^{(i)} + FIC^{(ii)}$. The interactions were classified as being synergistic for ΣFIC values of ≤ 0.5, additive (> 0.5 – 1.0), indifferent (> 1.0 – ≤ 4.0) or antagonistic (> 4.0).[10]

Varied ratio combination studies (isobolograms)

For any detected synergistic interactions, nine different ratios of the combination were prepared and the MIC values determined. The samples were combined at fixed concentrations of 0.01 or 0.1mg/mL for the antibiotic component and 32mg/mL for the plant extract, at various volume ratios (antimicrobial: plant), resulting in varied concentrations for each ratio (Table 1). Data points for each ratio were plotted on an isobologram using the GraphPad Prism® software (Version 5). The construction of isobolograms allowed for the identification of the agent (plant or antimicrobial sample) most responsible for the synergistic effects within the combination. Data points falling below the 0.5:0.5 line indicated synergy, while those above the 0.5:0.5 line, but below the 1.0:1.0 line indicated an additive interaction. Data points above the 1.0:1.0 line, but below the 4.0:4.0 line indicated a non-interactive or indifferent interaction and data points falling above the 4.0:4.0 line indicated antagonism.[10]

Toxicity studies

Artemia franciscana nauplii toxicity screening

Toxicity was tested using an adapted *Artemia franciscana* nauplii lethality assay.[46,47] Briefly, *A. franciscana* nauplii were incubated in the presence of the extracts, reference toxin (1mg/mL potassium dichromate) or artificial seawater (negative control) at 25±1°C under artificial light. All treatments were performed three times, each with three replicates (n=9). The number of dead were counted in each well at 24h then sacrificed and the total number of nauplii in each well were counted and used to calculate the % mortality per well. LC_{50} values were calculated for each treatment using probit analysis.

MTS Cellular viability assay

All *T. arjuna* extracts and conventional antibiotics were screened individually and in combination towards normal human primary dermal fibroblasts (HDF) by standard assays.[45] Briefly, the HDF cells were obtained from American Type Culture Collection (ATCC PCS-201-012) and were cultured and maintained in Dulbecco's modified eagle medium (DMEM; ThermoFisher Scientific, Australia), supplemented with 10% foetal calf serum (Life Technologies), 50µg/mL streptomycin (Sigma-Aldrich, Australia) and 50IU/mL penicillin (Sigm-Aldricha, Australia). The cells were maintained as monolayers in 75mL flasks at 37°C, 5% CO_2 in a humidified atmosphere until approximately 80% confluent. Once confluency was achieved, 1mL of trypsin (Sigma, Australia) was added to the culture flasks and incubated at 37°C, 5% CO_2 for 15min to dislodge the HDF cells. The cell suspensions were then transferred to a 10mL centrifuge tube and sedimented by centrifugation. The supernatant was discarded and the cells were resuspended in 9mL of fresh media (lacking streptomycin and penicillin supplementation). Aliquots of the resuspended cells (70µL, containing approximately 5000 cells) were added to individual wells of a 96 well plate. A volume of 30µL of the test extracts or cell media (for the negative control) was added to individual wells and the plates were incubated at 37°C, 5% CO_2 for 24 hrs in a humidified atmosphere. All extracts were screened at 200µg/mL. The cells were then washed in PBS (pH 7.2) to remove interference due to sample colour. A volume of 20µL of Cell Titre 96 Aqueous One solution (Promega) was subsequently added to each well and the plates were incubated for a further 3 hrs. Absorbances were recorded at a test wavelength of 540nm and a blank wavelength of 690nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed in triplicate and triplicate

Table 1: The concentration ratios used for antibiotic and plant extract combination studies.

Volume ratio of extract: antibiotic (µL)	Concentration of plant extract in combination (mg/mL)	Concentration of antibiotic[a] in combination (µg/mL)
90:10	90.00	3.20
80:20	80.00	6.40
70:30	70.00	9.60
60:40	60.00	12.80
50:50	50.00	16.00
40:60	40.00	19.20
30:70	30.00	22.40
20:80	20.00	25.60
10:90	10.00	28.80

a = penicillin G / chloramphenicol / ciprofloxacin / erythromycin / gentamicin / tetracycline.

controls were included on each plate. The % cellular viability of each test was calculated using the following formula:

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

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

Statistical analysis

Data are expressed as the mean ± SEM of three independent experiments with internal triplicates (n=9). One way ANOVA was used to calculate statistical significance between control and treated groups, with a P value < 0.01 considered to be statistically significant.

RESULTS

Liquid extraction yields, qualitative phytochemical screening and antioxidant capacity

Extraction of 1g of dried plant material with various solvents yielded dried plant extracts ranging from approximately 42mg (ethyl acetate extract) to 240mg (methanolic extract; Table 2). The dried extracts were resuspended in 10mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 2. Phytochemical studies (Table 2) showed that methanol and water extracted the widest range and largest amount of phytochemicals. Both of these extracts contained high levels of total phenolics (water soluble and insoluble phenolics), flavonoids and tannins, as well as low to moderate levels of saponins. The ethyl acetate and chloroform extracts contained similar classes of phytochemicals, albeit at substantially lower relative abundances. Notably, none of the classes of phyto-compounds was detected in the hexane extract. As hexane extracts contain highly lipophilic compounds and these classes of compounds were not evaluated in these assays, this is not be surprising. Further qualitative assays are required to detect the presence of those classes of compounds. Antioxidant capacity (expressed as ascorbic acid equivalence) for the *T. arjuna* extracts are also presented in Table 2. The antioxidant capacity ranged from levels below the detection sensitivity of the assay (ethyl acetate, chloroform and hexane extracts) to a high of approximately 14mg ascorbic acid equivalence per gram of dried plant material extracted (methanolic *T. arjuna* extract).

Bacterial growth inhibition activity screening

Aliquots (10µl) of each extract were tested in the disc diffusion assay against a panel of bacterial pathogens (Figures 1-3). The *P. mirabilis, K. pneumonia* and *P. aeruginosa* bacterial strains screened were selected based on their ability to trigger rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis respectively in genetically susceptible individuals.[39-41] All of the *T. arjuna* extracts displayed noteworthy inhibitory activity against *P. mirabilis,* based on the measured zones of inhibition (ZOIs) (Figure 1). Indeed, the methanolic extract produced substantially larger ZOIs against *P. mirabilis* than the ampicillin and nystatin controls, indicating the potent antimicrobial activity of these extracts against this bacterium.

Klebsiellia pneumoniae was substantially less susceptible to the *T. arjuna* extracts than *P. mirabilis* was. Indeed, only the methanolic, aqueous and ethyl acetate extracts inhibited *K. pneumoniae* growth. The chloroform and hexane extracts were completely ineffective. Despite the narrower susceptibility range of *K. pneumonia* towards the extracts, the inhibition by the methanolic extract was particular noteworthy (ZOIs >8mm ZOIs). Indeed, this bacterium was completely resistant to the ampicillin and nystatin antibiotic controls, although it was highly susceptible to chloramphenicol (as determined by the size of the ZOI).

The *Pseudomonas aeruginosa* strain evaluated in this study was also relatively resistant to both the *T. arjuna* extracts and to the conventional antibiotics. This bacterium was completely resistant to the aqueous, ethyl acetate and hexane extracts, as well as the nystatin control. It was also relatively resistant to the ampicillin control (ZOI~7mm). *P. aeruginosa* showed low susceptibility to the methanolic extract (ZOI~6.5mm) and substantially higher susceptibility to the chloroform extract (ZOI~9.5mm). This inhibition is particularly noteworthy as this strain is resistant to the antibiotic controls. Indeed, the chloroform *T. arjuna* extract was a better inhibitor of this bacterium than the chloroamphenicol control (as judged by ZOI). Of further note, the higher growth inhibitory activity that was noted in the chloroform extract compared to the other extracts may indicate that the *P. aeruginosa* inhibitory compounds are of low polarity.

Table 2: The mass of dried extracted *T. arjuna* material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant contents.

		M	W	E	C	H
	Mass of extract (mg)	240	144	42	92	136
	Concentration of extract (mg/mL)	24	14.4	4.2	9.2	13.6
Phenolics	**Total phenolics**	+++	+++	+	+	-
	Water soluble phenolics	+++	+++	+	+	-
	Water insoluble phenolics	+++	+++	-	-	-
	Cardiac glycosides	-	-	-	-	-
	Saponins	++	+	-	-	-
	Triterpenes	-	-	-	-	-
	Phytosterols	-	-	-	-	-
Alkaloids	**Meyer test**	-	-	-	-	-
	Wagner test	-	-	-	-	-
	Flavonoids	+++	+++	+	-	-
	Tannins	+++	+++	+	+	-
Anthraquinones	**Free**	-	-	-	-	-
	Combined	-	-	-	-	-
	Antioxidant capacity	13.6	11.5	BDT	BDT	BDT

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. AA = ascorbic acid; BDT = below detection threshold. Antioxidant capacity determined by DPPH reduction (expressed as mg AA equivalence per g plant material extracted).

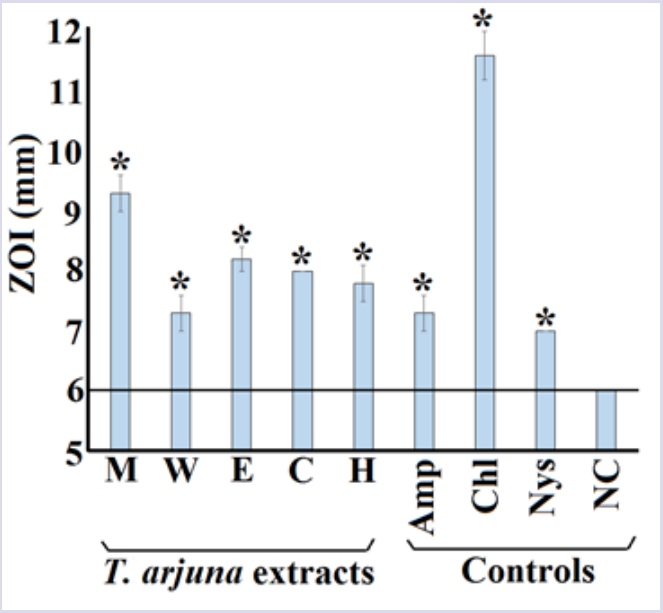

Figure 1: Antibacterial activity of *T. arjuna* extracts and ampicillin (2µg), chloramphenicol (10µg) and nystatin (10µg) controls measured as zones of inhibition (mm) against *P. mirabilis*. Results are expressed as mean ± SEM of at three experimental repeats, each with internal triplicates (n=9). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin; Chl = chloramphenicol; Nys = nystatin; NC = negative control. * = results that are significantly different to the negative control (p<0.01). The 6mm line indicates the diameter of the disc.

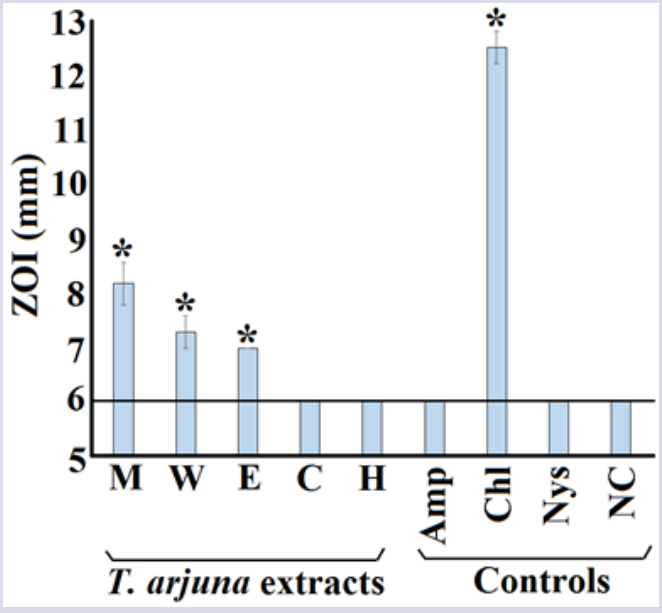

Figure 2: Antibacterial activity of *T. arjuna* extracts and ampicillin (2µg), chloramphenicol (10µg) and nystatin (10µg) controls measured as zones of inhibition (mm) against *K. pneumoniae*. Results are expressed as mean ± SEM of at three experimental repeats, each with internal triplicates (*n*=9). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin; Chl = chloramphenicol; Nys = nystatin; NC = negative control. * = results that are significantly different to the negative control (p<0.01). The 6mm line indicates the diameter of the disc.

Quantification of minimum inhibitory activity (MIC)

The relative level of antimicrobial activity was further evaluated by determining the MIC values (Table 3) against the bacterial pathogens. The ethyl acetate *T. arjuna* extract was a particularly good inhibitor of *P. mirabilis* and *K. pneumoniae* growth (LD MIC values of 175 and 452µg/mL respectively), although this extract was completely ineffective against *P. aeruginosa*. The methanolic bark extract was also a good inhibitor of *P. mirabilis* and *K. pneumoniae* growth (LD MIC values of 250 and 913µg/mL respectively), but was a poor inhibitor of *P. aeruginosa* growth (LD MIC >5000 µg/mL). As *P. mirabilis* and *K. pneumonia* can induce rheumatoid arthritis and ankylosing spondylitis respectively in genetically susceptible individuals,[39] the methanolic and ethyl acetate extracts have potential in treating these diseases, as well as other diseases caused by these pathogens.

In contrast, only the chloroform *T. arjuna* extract gave appreciable activity towards *P. aeruginosa*, with an MIC of 1280µg/mL. Whilst this MIC indicates moderate anti-*P. aeruginosa* activity, it is noteworthy that this bacterium was also resistant to the control antibiotics. Indeed, only gentamycin gave MIC vales indicative of good activity. Whilst MIC values were also determined for chloramphenicol and ciprofloxacin, these were consistently >1µg/mL, which (for a pure antibiotic) is indicative of resistance. Thus, despite only having moderate activity against *P. aeruginosa*, the *T. arjuna* chloroform extract has potential in the prevention and treatment of multiple sclerosis,[40,41] as well as other diseases caused by this bacterium. Notably, *Acinitobacter baylyi* can also induce multiple sclerosis in genetically susceptible people.[41] Thus, this study alone cannot completely evaluate the potential of the *T. arjuna* chloroform extract for the prevention of multiple sclerosis. Further studies screening against *A. baylyi* are also required.

Fractional Inhibitory concentration (FIC) assessment

Synergistic interactions were noted for two (5%) of the *T. arjuna* bark extracts and conventional antibiotic combinations when tested against *P. mirabilis* (Table 4). A further eight (20%) combinations produced additive effects against that bacterium. As these combinations produce effects greater than either the individual extract or conventional antibiotic components alone, these combinations would be beneficial in the prevention and treatment of rheumatoid arthritis. All of the other combinations were non-interactive, or were inactive. Whilst the non-interactive combinations provide no added benefit over that of the individual components alone, the components do not antagonise each other's effects and are therefore safe to use concurrently without lessening the efficacy of either component. Notably, no antagonistic combinations were noted against *P. mirabilis* (or against any of the other bacteria screened).

Similarly, two *T. arjuna* bark extract and conventional antibiotic combinations also induced synergy against *K. pneumoniae*, whilst another six combinations showed additive effects. Therefore, as *K. pneumoniae* can induce ankylosing spondylitis in genetically susceptible people,[39] these combinations would be beneficial in the prevention and treatment of that disease (as well as other illnesses caused by that bacterium). In contrast, no potentiating combinational effects (synergy or additive effects) were noted for any combination against *P. aeruginosa*. For the other combinations that showed growth inhibitory activity against that bacterium, only non-interactive combinations were noted. Whilst these combinations provide no added benefit over that of the individual therapies, the components do not antagonise each other's effects and are therefore safe to use in combination without decreasing the activity of either component.

Table 3: Minimum inhibitory concentrations (µg/mL) of *T. arjuna* extracts against susceptible microbial species.

Solvent	Minimum Inhibitory Concentration (µg/mL)					
	P. mirabilis		*K. pneumoniae*		*P. aeruginosa*	
	DD	LD	DD	LD	DD	LD
Methanol	1144	250	1413	913	>5000	>5000
Water	2173	1800	1394	2940	-	-
Ethyl Acetate	893	175	1452	452	-	-
Chloroform	1922	1300	-	-	2104	1280
Hexane	1734	1400	-	-	-	-
Positive Controls						
Penicillin	ND	-	ND	-	ND	-
Chloramphenicol	ND	1.25	ND	1.25	ND	2.5
Ciprofloxacin	ND	0.63	ND	1.25	ND	1.25
Erythromycin	ND	2.5	ND	-	ND	-
Gentamycin	ND	1.25	ND	0.63	ND	0.31
Tetracycline	ND	-	ND	2.5	ND	-

Numbers indicate the mean MIC values of triplicate determinations expressed in µg/mL. DD = disc diffusion; LD = liquid dilution; ND = MIC values were not determined as only a single dose was screened; - indicates no inhibition at any concentration tested; Bold and italics text indicates noteworthy MIC values.

Varied ratio combination studies (isobolograms)

Four synergistic combinations were detected (two each against *P. mirabilis* and *K. pneumoniae*). Notably, all of these combinations included ciprofloxacin as the conventional antibiotic component. These combinations were further examined using isobologram analysis across a range of extract:ciprofloxacin ratios to identify the ideal ratios to obtain synergy. Interestingly, both the methanolic and ethyl acetate *T. arjuna* bark extracts synergised the activity of ciprofloxacin against *P. mirabilis*, even at low ratios (Figure 4). Indeed, all combinations containing ≥10%-≤90% extract:antibiotic ratios resulted in synergistic interactions. Ratios outside this range produced additive effects and would thus also be beneficial for inhibiting *P. mirabilis* growth.

Similarly, the methanolic (Figure 5a) and ethyl acetate (Figure 5b) *T. arjuna* bark extracts also produced synergistic interactions in combination with ciprofloxacin against *K. pneumoniae* at a wide range of ratios tested (generally between 40 and 70% extract). Thus, all these combination ratios would be particularly beneficial to enhance *K. pneumoniae* growth inhibition. However, bacteria would be less likely to develop resistance when combinations are used in ratios which minimise the amount of conventional antibiotic used. Thus, for long term prophylactic treatment (as would be required to prevent and treat ankylosing spondylitis), the ideal extract: ciprofloxacin ratio may be 70:30. However, when used for the treatment of acute infections (e.g. pulmonary infections), the ratio which maximises the efficacy of the treatment (i.e. the 40:60 ratio) may be the preferred option.

Table 4: ΣFIC values of *T. arjuna* extracts combined with conventional antibiotics against *P. mirabilis, K. pneumonia* and *P. aeruginosa*.

Antibiotic	Solvent	ΣFIC		
		P. mirabilis	*K. pneumoniae*	*P. aeruginosa*
Chloramphenicol	Methanol	*0.74*	*0.91*	2.7
	Water	1.43	1.85	NT
	Ethyl Acetate	*0.52*	*0.76*	NT
	Chloroform	1.29	NT	3.2
	Hexane	2.24	NT	NT
Ciprofloxacin	Methanol	**0.07**	**0.32**	1.32
	Water	*0.58*	**0.32**	NT
	Ethyl Acetate	**0.02**	*0.66*	NT
	Chloroform	*0.8*	NT	1.56
	Hexane	*0.79*	NT	NT
Erythromycin	Methanol	*0.88*	NT	NT
	Water	1.94	NT	NT
	Ethyl Acetate	1.16	NT	NT
	Chloroform	2.4	NT	NT
	Hexane	2.76	NT	NT
Gentamycin	Methanol	*0.83*	1.77	3.61
	Water	1.48	2.46	NT
	Ethyl Acetate	*0.78*	1.2	NT
	Chloroform	1.95	NT	1.79
	Hexane	2.44	NT	NT
Tetracycline	Methanol	NT	*0.85*	NT
	Water	NT	*0.85*	NT
	Ethyl Acetate	NT	*0.63*	NT
	Chloroform	NT	NT	NT
	Hexane	NT	NT	NT

Bold = synergistic interaction; italics = additive interaction; normal text = indifferent interaction; NT = not tested as one or both combination components was inactive against that bacterium.

Quantification of toxicity

Artemia lethality assay (ALA)

All plant extracts and antibiotics were individually screened at 2mg/mL in the *Artemia* nauplii lethality assay. The extracts were only considered toxic if they induced percentage mortalities greater than 50% (LD_{50}) following 24 hrs of exposure to the *Artemia* nauplii.[51,52] The ethyl acetate, chloroform and hexane extracts all induced substantially <50% mortality in this assay (Figure 6) and were thus deemed to be nontoxic. In contrast, both the methanolic and aqueous extracts induced 100% mortality at

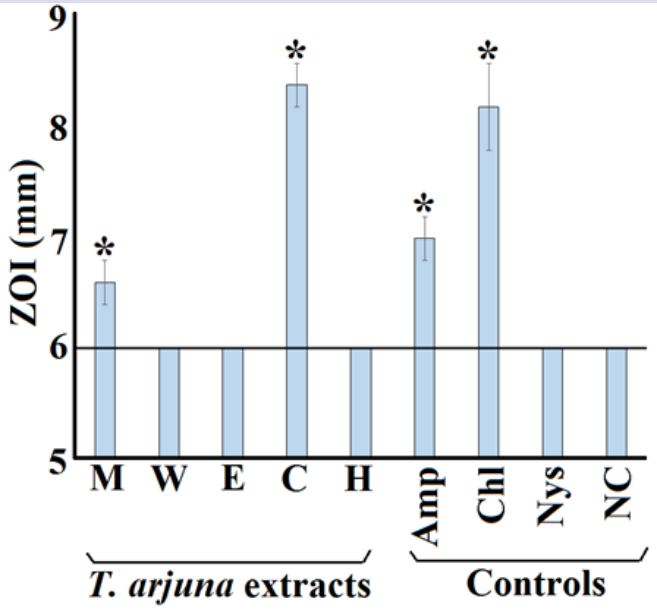

Figure 3: Antibacterial activity of *T. arjuna* extracts and ampicillin (2µg), chloramphenicol (10µg) and nystatin (10µg) controls measured as zones of inhibition (mm) against *P. aeruginosa*. Results are expressed as mean ± SEM of at three experimental repeats, each with internal triplicates (n=9). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin; Chl = chloramphenicol; Nys = nystatin; NC = negative control. * = results that are significantly different to the negative control (p<0.01). The 6mm line indicates the diameter of the disc.

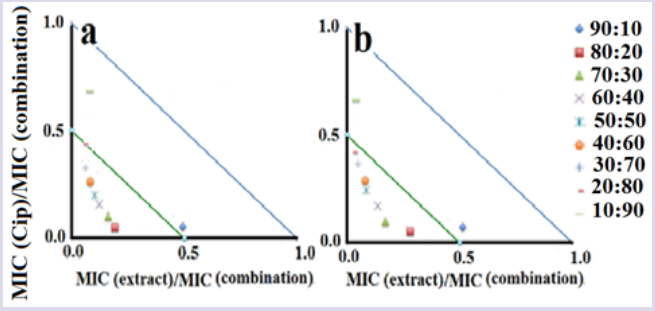

Figure 4: Isobolograms for combinations of ciprofloxacin with *T. arjuna* (a) methanolic extract and (b) ethyl acetate extract, tested at various ratios against *P. mirabilis*. Results represent mean MIC values of four replicates. Ratio = % extract : % antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic (Σ FIC ≤ 0.5). Any points between the 0.5:0.5 and 1.0:1.0 lines are deemed additive (Σ FIC > 0.5-1.0).

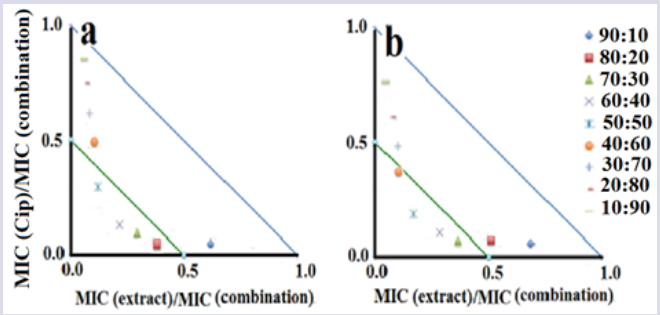

Figure 5: Isobolograms for combinations of ciprofloxacin with *T. arjuna* (a) methanolic extract and (b) aqueous extract, tested at various ratios against *K. pneumoniae*. Results represent mean MIC values of four replicates. Ratio = % extract : % antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic (Σ FIC ≤ 0.5). Any points between the 0.5:0.5 and 1.0:1.0 lines are deemed additive (Σ FIC > 0.5-1.0).

2mg/mL. Therefore, these extracts were further evaluated across a range of concentrations to determine their LC_{50} values (Table 5). Interestingly, LC_{50} values >1000µg/mL were determined for both the methanolic and aqueous *T. arjuna* extracts. Therefore, these extracts were deemed to be non-toxic.

When tested individually in the *Artemia* nauplii assay, none of the antibiotics demonstrated significant toxicity (Table 5). Similarly, when tested in combination with the *T. arjuna* extracts in the *Artemia* nauplii bioassay, none of the extract-antibiotic combinations produced LC_{50} values <1000µg/mL. Therefore, all combinations were also deemed to be non-toxic. In contrast, the positive control potassium dicrhromate induced 100% mortality following 24h exposure.

MTS cell viability assay

The plant extracts and conventional antibiotics were each individually screened at 200µg/mL against HDF in the cell viability assay. In this assay, extracts which produce <50% cell at 200µg/mL are deemed to be toxic.[45] None of the extracts or conventional antibiotics displayed <50% HDF viability and thus all were deemed to be non-toxic (Table 5). Similarly, all combinations provided substantially >50% cell viability and were thus also deemed to be toxic. In contrast, exposure to the positive control (quinine) reduced HDF cell viability by approximately 70%.

DISCUSSION

Members of genus *Terminalia* have been used for a broad range of medicinal purposes by traditional healers from a wide variety of ethnic and cultural groupings. The best documented of these are the traditional Indian medicinal systems, particularly Ayurveda. Ayurvedic practitioners employ multiple *Terminalia* spp. for a wide variety of therapeutic purposes including abdominal and back pain, coughs and colds, conjunctivitis, diarrhoea and dysentery, fever, headache, heart disorders, inflammation, leprosy, pneumoniae, sexually transmitted diseases, worms, wounds, haemorrhages, ulcers, and as a general tonic.[34] Many of these diseases are caused by microbial pathogens, indicating the potential of these plants as antiseptic agents. Numerous recent investigations have reported on the antibacterial properties of these species. *T. arjuna* leaf and bark extracts have previously been reported to have antibacterial activity against a wide panel of microbes.[34,35] Our study was performed to investigate the antimicrobial activity of the *T. arjuna* extracts and conventional antibiotics against some bacterial triggers of some autoimmune inflammatory diseases. All of the bacteria tested in this study were selected based on their role as triggers of autoimmune inflammatory diseases.[39-41] *Proteus mirabilis, Klebsiella pneumonia* and *Pseudomonas aeruginosa* are known to induce rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis respectively in genetically susceptible individuals.[39-41]

The methanolic and aqueous *T. arjuna* extracts were good inhibitors of *P. mirabilis* and *K. pneumoniae* when tested alone and thus have potential in the prevention and treatment of rheumatoid arthritis and ankylosing spondylitis (as well as other diseases caused by these pathogens). The

Table 5: Mortality (%) and cellular viability (%) results for *T. arjuna* extracts and conventional antibiotics tested individually and as combinations in the *Artemia* nauplii and MTS cell viability assays respectively.

	Sample	Mortality ± SEM (%) [a]	ALA LC_{50} (µg/mL)	Cell viability ± SEM (%) [b]
Antimicrobials	Penicillin	1.8 ± 1.4	-	98.3 ± 3.4
	Chloramphenicol	2.7 ± 1.3	-	102.2 ± 3.7
	Ciprofloxacin	4.6 ± 2.4	-	93.1 ± 4.7
	Erythromycin	1.2 ± 0.6	-	97.7 ± 5.5
	Gentamicin	2.4 ± 1.5	-	95.8 ± 4.7
	Tretracycline	3.1 ± 1.8	-	94.7 ± 4.6
Extracts	M	100 ± 0	1094	83.6 ± 4.3
	W	100 ± 0	1452	84.8 ± 3.7
	E	36.7 ± 3.3	-	96.4 ± 5.5
	C	6.9 ± 2.8	-	88.3 ± 4.1
	H	8.6 ± 3.2	-	101.9 ± 4.7
Combinations	M + Penicillin G	4.3 ± 2.8	1287	93.6 ± 2.7
	M + Chloramphenicol	4.8 ± 3.1	1342	89.5 ± 4.5
	M + Ciprofloxacin	5.3 ± 2.9	-	91.6 ± 4.3
	M + Erythromycin	3.6 ± 2.4	-	92.7 ± 3.0
	M + Gentamicin	5.6 ± 2.8	-	89.0 ± 3.5
	M + Tetracycline	7.4 ± 3.3	-	84.8 ± 4.8
	W + Penicillin G	4.7 ± 2.5	1105	84.6 ± 3.7
	W + Chloramphenicol	6.9 ± 4.3	1280	80.5 ± 3.8
	W + Ciprofloxacin	5.5 ± 3.1	-	82.8 ± 5.5
	W + Erythromycin	4.2 ± 2.6	-	88.3 ± 3.1
	W + Gentamicin	5.7 ± 1.9	-	87.7 ± 4.8
	W + Tetracycline	7.8 ± 2.6	-	76.9 ± 4.6
	E + Penicillin G	4.6 ± 2.4	-	94.6 ± 3.1
	E + Chloramphenicol	5.5 ± 3.6	-	91.2 ± 4.3
	E + Ciprofloxacin	6.9 ± 4.7	-	87.7 ± 5.3
	E + Erythromycin	2.8 ± 2.1	-	102.6 ± 4.2
	E + Gentamicin	6.4 ± 4.2	-	91.6 ± 3.2
	E + Tetracycline	8.9 ± 4.6	-	87.2 ± 6.1
	C + Penicillin G	5.6 ± 3.4	-	96.4 ± 2.1
	C + Chloramphenicol	4.5 ± 3.3	-	92.1 ± 3.4
	C + Ciprofloxacin	6.2 ± 3.8	-	88.6 ± 4.7
	C + Erythromycin	3.8 ± 1.9	-	103.8 ± 4.7
	C + Gentamicin	5.7 ± 3.8	-	92.8 ± 3.6
	C + Tetracycline	7.6 ± 4.2	-	87.4 ± 5.3
	H + Penicillin G	3.8± 1.9	-	106.7 ± 3.9
	H + Chloramphenicol	5.4 ± 3.1	-	94.4 ± 6.2
	H + Ciprofloxacin	4.6 ± 3.2	-	97.3 ± 4.8
	H + Erythromycin	5.7 ± 4.3	-	93.7 ± 2.7
	H + Gentamicin	5.1 ± 3.9	-	93.5 ± 4.8
	H + Tetracycline	2.7 ± 1.5	-	91.9 ± 5.7
Controls	Deionised water	3.2 ± 1.7	-	96.8 ± 5.7
	Quinine	4.6 ± 2.1 [a]	NT	31.4 ± 4.8 [b]
	Potassium dichromate	100.00 ± 0.00 [a]	58.6	NT

[a] = Tested at a concentration of 1 mg/mL; [b] = Tested at a concentration of 200 µg/mL; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; - = LC_{50} could not be determined as mortality did not exceed 50% at any concentration tested; NT = control not tested in the assay. Results represent means ± SEM of 3 independent experiments, each preformed in triplicate (n = 9).

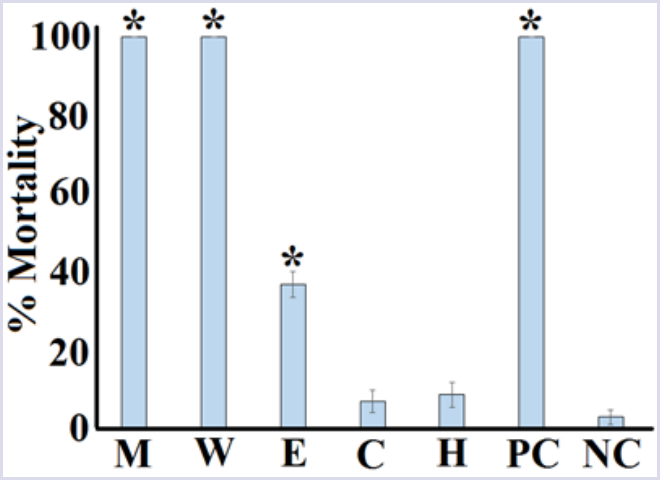

Figure 6: The lethality of the *T. arjuna* extracts (2000µg/mL) and the potassium dichromate (1000µg/mL) and seawater controls towards *Artemia franciscana* nauplii after 24 hrs exposure. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. Results are expressed as mean % mortality ± SEM. * indicates results that are significantly different to the negative control (p<0.01).

chloroform extract was also found to have noteworthy activity against a multi-resistant strain of *P. aeruginosa* and thus has potential in the prevention and treatment of multiple sclerosis. Of perhaps greater interest, the methanolic and aqueous *T. arjuna* bark extracts also potentiated the growth inhibitory activity of ciprofloxacin against *P. mirabilis* and *K. pneumoniae*. These combinations may be particularly useful in the prevention and treatment of rheumatoid arthritis and ankylosing spondylitis. Notably, the majority of the extract:conventional antibiotic combinations tested in our study demonstrated indifferent interactions. Whilst use of these combinations would have no benefit over using the conventional antibiotic (or extract) alone, they do alleviate some concerns related to concurrent use of the two forms of allopathic healthcare and traditional medicines as these interactions indicate that neither therapy reduces the efficacy of the other therapy.

Whilst a detailed examination of the phytochemistry of the *T. arjuna*

extracts was beyond the scope of our study, a commonality of this genus is their relatively high levels of a number of tannin components including exifone (4-galloylpyrogallol), ellagic acid dehydrate, trimethyl ellagic acid, chebulic acid, corilagen, castalagin and chebulagic acid.[38,39,40,45] Gallotannins have been reported to inhibit the growth of a broad spectrum of bacterial species[48] through a variety of mechanisms including binding cell surface molecules including lipoteichoic acid and proline-rich cell surface proteins,[49,50] and by inhibiting glucosyltransferase enzymes.[51] Ellagitannins are also highly potent inhibitors of bacterial growth, with MIC values as low as 62.5**µ**g/mL.[48] Ellagitannins have also been reported to function via several antibiotic mechanisms, including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls.[48]

It is likely that other phytochemical classes also contribute to the growth inhibitory properties of the *T. arjuna* extracts. Our qualitative phytochemical screening studies indicate that polyphenolics, flavonoids and saponins were present in the *T. arjuna* extracts. Many studies have also reported potent antibacterial activities for a wide variety of flavonoids.[52] Further phytochemical evaluation studies and bioactivity driven isolation of active components is required to further evaluate the mechanism of bacterial growth inhibition.

The findings reported here also demonstrate that the *T. arjuna* extracts tested in our study were nontoxic towards *Artemia franciscana* nauplii, with LC_{50} values substantially >1000µg/mL. Extracts with LC_{50} values >1000µg/mL towards *Artemia* nauplii are defined as being nontoxic.[46,47] Whilst our preliminary toxicity studies indicate that these extracts may be safe for use as growth inhibitors of bacterial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis, studies using further human cell lines are required to further evaluate the safety of these extracts. Furthermore, studies to determine the mechanism(s) of action resulting in the observed interactions are warranted, and bioactivity driven compound isolation and/or metabolomics studies are also required to determine the active compound(s) (as well as those responsible for the antibiotic potentiation) within the *T. arjuna* bark extracts.

CONCLUSION

Whilst the findings reported here indicate the potential of *T. arjuna* bark extracts (particularly in combination with ciprofloxacin) as preventative and therapeutic options against bacterial triggers of some autoimmune inflammatory diseases, further *in vivo* investigations are required to support these *in vitro* findings. Additionally, bioactivity driven purifications of the active components and an examination of the mechanisms of action of these agents is required.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS

ALA: *Artemia* lethality assay; **DMSO:** Dimethyl sulfoxide; **FIC:** Fractional inhibitory concentration; **ΣFIC:** Sum of fractional inhibitory concentrations; **HDF:** Human dermal fibroblasts; **LC_{50}:** The concentration required to achieve 50 % mortality; **MIC:** Minimum inhibitory concentration; **MTS:** 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; **ZOI:** Zone of inhibition.

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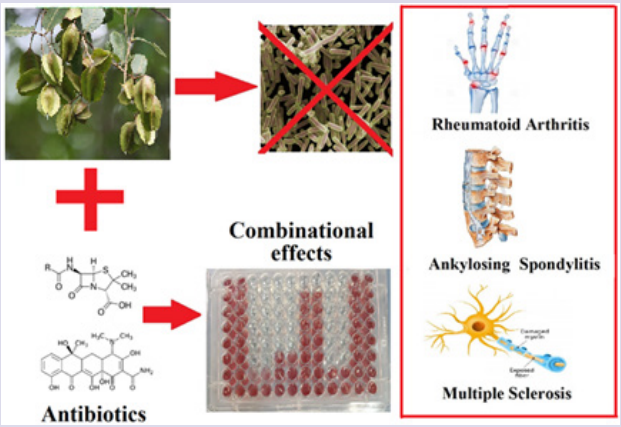
PICTORIAL ABSTRACT



SUMMARY

- *T. arjuna* extracts were screened for the ability to block the growth of a panel of human bacterial pathogens.
- Combinations of *T. arjuna* extracts were tested in combination with conventional antibiotics and the class of interaction was determined.
- Synergistic combinations were analysed by isobologram analysis to determine the optimal combination ratios.
- Toxicity of the *T. arjuna* extracts was evaluated using the *Artemia nauplii* mortality and HDF proliferation bioassays.

ABOUT AUTHORS

Dr Ian Cock leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin, including *Aloe vera*, South Asian and South American tropical fruits, as well as Australia plants including *Scaevola spinescens, Pittosporum phylliraeoides, Terminalia ferdinandiana* (Kakadu plum), Australian Acacias, Syzygiums, Petalostigmas and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in nearly 200 publications in a variety of peer reviewed journals.