Terminalia chebula Retz. fruit extracts inhibit bacterial triggers of some autoimmune diseases and potentiate the activity of tetracycline

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Abstract

Terminalia chebula Retz. is a northern Indian plant species known for its anti-inflammatory and antimicrobial properties. *T. chebula* fruit powder was extracted with solvents of varying polarity and screened for bacterial growth inhibition by disc diffusion assay. The minimum inhibitory concentration (MIC) was quantified by both liquid dilution and disc diffusion techniques. To screen for combinatorial effects, the *T. chebula* fruit extracts were combined with a range of conventional antibiotics and tested against each bacteria using a liquid dilution assay. Where synergy was detected, the optimal ratios were determined using isobologram analysis. Toxicity was examined using *Artemia* nauplii and HDF bioassays. *T. chebula* fruit methanolic, aqueous and ethyl acetate extracts displayed strong antimicrobial activity against the bacterial triggers of all autoimmune inflammatory diseases except *K. pneumoniae*, for which only moderate inhibition was observed. Indeed, MIC values as low as 195 μg/mL were measured for the aqueous extract against a resistant strain of *P. aeruginosa*. Of further note, both the aqueous and ethyl acetate extracts interacted synergistically in combination with tetracycline against *K. pneumoniae* (Σ FIC 0.38 and 0.25 respectively). All extracts were nontoxic in the *Artemia* and HDF toxicity assays, further indicating their potential for medicinal use.

Keywords: Synergy, multi-drug resistant bacteria, combinational therapies, rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis.

Abbreviations

ALA	brine-shrimp lethality assay
DMSO	dimethyl sulfoxide;
HDF	human dermal fibroblasts
INT	ρ-iodonitrotetrazolium chloride
LD_{50}	dose of sample necessary to have a lethal effect on 50% of test organisms or cells
MIC	minimum inhibitory concentration
ΣFIC	the sum of the fractional inhibitory concentration.

Introduction

In recent years, there has been an increase in bacterial resistance to many conventional antibiotics and several strains of important bacterial pathogens are now either extremely (XDR) or totally drug resistant (TDR) [1]. There are now limited therapeutic options for the diseases caused by these pathogens. This problem is expected to worsen in the future as bacteria exchange resistance genes and more strains become multi-drug resistant (MDR). 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 [2]. For a number of reasons reviewed elsewhere [1], it is unlikely that the previous methods of antibiotic discovery/development will be as successful in the future and therefore 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 [3-5]. The traditional Indian medicinal system Ayurveda uses a variety of natural plant and fruit products to treat numerous ailments [5-7]. Culinary fruits, herbs and spices are generally considered safe by most regulatory agencies (US Food and Drug Act, EU Standards, Food Safety and Standards Authority of India) and their consumption aids in maintaining good health. Indeed, Indian cuisine is derived from Ayurveda, accounting for the abundance of spices used in Indian foods [8].

Triphala is widely used in Ayuverda, including use as a immunostimulant, as well as for diabetes and gastrointestinal problems [6]. It also has anti-inflammatory and antibacterial properties and thus has potential against autoimmune inflammatory diseases. Triphala is derived from equal portions of the fruit from three plant species: *Terminalia bellerica* (Gaertn.) Roxb., *Terminalia chebula* Retz., and *Embilica officinalis* Gaertn. Each of the Triphala components are also used separately as anti-inflammatory, antioxidant and antimicrobial agents [7, 9]. Each individual plant component has therapeutic effects on respiratory and gastrointestinal diseases when used alone, and it has also been reported that compounds found in Triphala are effective in treating some cancers [6, 7]. Of these three plant species, *T. chebula* is perhaps the most extensively studied and it is particularly useful therapeutically. The fruits of *T. chebula* are rich in hydrolysable tannins (32%-34%), polyphenolics, flavanols, glycosides, triterpenoids and fatty acids [6; 10, 11]. These phytochemicals contribute to the high antioxidant capacity and free radical scavenging activity, anti-cancer activity, antibacterial, antifungal, antiviral and anti-inflammatory activities, as well as the wound healing and immunomodulatory activities associated with *T. chebula* fruit. *T. chebula* is also effective in the treatment and management of diabetes, hypercholesterolemia, hypertension and gastrointestinal motility [6, 10].

T. chebula leaf and fruit extracts have potent antibacterial activity against a number of gram-positive and gram-negative human pathogenic bacteria [10]. Despite this, no studies have yet screened the extracts for the ability to inhibit the growth of the bacterial triggers of autoimmune inflammatory diseases. Previous studies identified the bacterial triggers of some autoimmune inflammatory diseases in genetically susceptible humans, allowing drug therapies to target the initiating events of these diseases, thereby providing prophylactic chemotherapeutic options.

Acinetobacter baylyi and *Pseudomonas aeruginosa,* were identified as bacterial triggers for multiple sclerosis, *Klebsiella pneumoniae* has been linked to ankylosing spondylitis and *Proteus mirabilis* is a bacterial trigger of rheumatoid arthritis [12, 13]. *T. chebula* extracts are also yet to be tested for potentiating activity in combinational studies with conventional antibiotics. This study aimed to investigate the growth inhibitory activity of of *T. chebula* fruit extracts against *P. mirabilis, K. pneumonia, A. baylyi* and *P. aeruginosa* alone and in combination with conventional antibiotics to evaluate their interactive effects.

Materials and Methods

Plant Material and Extraction

The *Terminalia chebula* Retz. fruit used in this study were obtained from Southern India and were a gift from Dr Paran Rayan, Griffith University. Voucher samples (TCF2015b1c) have been stored at the School of Natural Sciences, Griffith University, Brisbane Australia. The fruit were thoroughly desiccated in a Sunbeam food dehydrator and the dried materials were stored at -30 ºC until use. Prior to use, the dried fruit were thawed and ground into a coarse powder. Individual 1 g quantities of the material were weighed into separate tubes and 50 mL of methanol, deionised water, ethyl acetate chloroform or hexane were added. All solvents were obtained from Ajax, Australia and were AR grade. The ground plant materials were individually extracted in each solvent for 24 hours at 4 ºC with gentle shaking. The extracts were then filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant extracts were weighed and redissolved in 10 mL deionised water (containing 1 % DMSO).

Qualitative Phytochemical Studies

Phytochemical analysis of the *T. chebula* fruit extracts for the presence of saponins, phenolic compounds, flavonoids, phytosterols, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays [14-16].

Antibacterial Screening

Conventional Antibiotics

Penicillin-G (potency of 1440-1680 µg/mg), chloramphenicol (≥98 % purity by HPLC, erythromycin (potency ≥850 µg/mg), gentamicin (potency of 600 µg/mg), and tetracycline (≥95% purity by HPLC) were purchased from Sigma-

Aldrich, Australia and were used as controls for the microplate liquid dilution assay. All antibiotics were prepared in sterile deionised water at stock concentrations of 0.01 mg/mL and stored at 4°C until use. For the disc diffusion studies, penicillin (20 µg), nystatin (100 Units), ciprofloxacin (2.5 µg) and chloramphenicol (10 µg) standard discs were obtained from Oxoid Ltd., Australia and used as positive controls.

Bacterial Cultures

All bacterial strains were selected based on their ability to trigger autoimmune inflammatory diseases in genetically susceptible individuals [12]. 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 in nutrient broth (Oxoid Ltd., Australia). Streak nutrient agar (Oxoid Ltd., Australia) plates were tested in parallel to ensure the purity of all bacterial cultures and for sub-culturing. All bacterial cultures were incubated at 37 °C for 24 h and were subcultured and maintained in nutrient broth at 4 °C until use.

Evaluation of Antibacterial Activity

Antibacterial activity screening of the *T. chebula* fruit extracts was assessed using a modified disc diffusion assay [17]. Penicillin (20 µg), nystatin (100 Units), ciprofloxacin (2.5 µg) and chloramphenicol discs (10 µg) were obtained from Oxoid Ltd., Australia and used as positive controls. Filter discs infused with 10 µL of distilled water were used as a negative control.

Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration for each extract was determined using two methods. A liquid dilution MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay [18]. Furthermore, as microplate liquid dilution MIC assays are perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method allows for comparisons with other studies. A solid phase agar disc diffusion assay was also used in this study for comparison.

Microplate Liquid Dilution MIC Assay

The MICs of the extracts were evaluated by standard methods [18, 19]. All plates were incubated at 37 °C for 24 h. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, Australia and dissolved in sterile deionised water to prepare a 0.2 mg/mL INT solution. A 40 µL volume of this solution was added into all wells and the plates

were incubated for a further 6 hours at 30 °C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

Disc Diffusion MIC Assay

The minimum inhibitory concentrations (MIC) of the extracts was also evaluated by disc diffusion assay as previously described [20]. Graphs of the zone of inhibition versus ln concentration were plotted and MIC values were determined using linear regression.

T. chebula Fruit Extract-Conventional Antibiotic Synergy Studies

Fractional Inhibitory Concentration (FIC) Assessment

Interactions between the *T. chebula* fruit extracts and the conventional antibiotics were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC) for each combination [4]. The FIC values for each component (a and b) were calculated using the following equations where a represents the plant extract sample and b represents the conventional antibiotic:

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

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

The ΣFIC was then calculated using the formula ΣFIC = FIC(a) + FIC(b). The interactions were classified as synergistic (ΣFIC ≤0.5), additive (ΣFIC >0.5-1.0), indifferent (ΣFIC >1.0-4.0) or antagonistic (ΣFIC >4.0) [19].

Varied Ratio Combination Studies (Isobolograms)

For each combination producing synergistic interactions, nine different ratios spanning the range 10:90 (extract:antibiotic) to 90:10 (extract:antibiotic) were tested. All combinations were tested in duplicate in three independent experiments, providing six replicates for each combination ratio. The data is presented as the mean of six replicates. Data points for each ratio examined were plotted on a isobologram and this was used to determine optimal combination ratios to obtain synergy. Data points on or below the 0.5:0.5 line indicated synergy; those

above the 0.5:0.5 line, up to and including the 1.0:1.0 line indicated an additive interaction; data points above the 1.0:1.0 line indicated indifferent interaction.

Toxicity screening

Two assays were used to assess the toxicity of the individual samples. The *Artemia* nauplii lethality assay (ALA) was utilised for rapid preliminary toxicity screening, whereas the MTS cellular proliferation assay was used as a cellular evaluation of toxicity.

Artemia franciscana Kellogg Nauplii Toxicity Screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4 mg/mL) and serially diluted in artificial seawater for use as a reference toxin. Toxicity of the *T. chebula* fruit extracts, the reference toxin and the conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay [21, 22]. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Cellular Viability Assay

The *T. chebula* fruit extracts and conventional antibiotics were screened individually towards normal human primary dermal fibroblasts (HDF) as previously described [23]. HDF cells were obtained from American Type Culture Collection (ATCC PCS-201-012). The cells were cultured and maintained in Dulbecco's modified eagle medium (DMEM; ThermoFisher Scientific, Australia), supplemented with 10 % foetal calf serum (Life Technologies), 50 µg/mL streptomycin (Sigma-Aldrich, Australia) and 50 IU/mL penicillin (Sigm-Aldricha, Australia). Suspensions of cells which had obtained 80% confluency were resuspended in fresh media (lacking streptomycin and penicillin supplementation) and 70 µL aliquots (containing approximately 5000 cells) were added to individual wells of a 96 well plate. A volume of 30 µL of the test extracts or cell media (for the negative control) was subsequently added to individual wells and the plates were incubated at 37ºC, 5% CO_2 for 24 hours in a humidified atmosphere. All extracts were screened at 200 µg/mL. The cells were then washed in PBS (pH 7.2) to remove interference due to sample colour. A volume of 20 µL of Cell Titre 96 Aqueous One solution (Promega) was subsequently added to each well and the plates were incubated for a further 3 hours. Absorbances were recorded at a test wavelength of 540 nm and a blank wavelength of 690 nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed in at least triplicate and triplicate 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 at least three independent experiments. One way ANOVA was used to calculate differences between the control and treated groups, with a *P* value < 0.01 considered to be significant.

Results

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g quantities of dried plant material with various solvents yielded dried plant extracts ranging from 62 mg (ethyl acetate extract) to 534 mg (methanolic extract; Table 1). Methanol and water gave relatively high yields of dried extracted material (534 and 438 mg respectively), whilst ethyl acetate, chloroform and hexane extracted substantially lower masses (62, 93 and 104 mg, respectively). The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 1. Qualitative phytochemical studies showed that methanol and water extracted the widest range and largest amount of phytochemicals. Both extracts showed moderate to high levels of phenolics, saponins, flavonoids and tannins. Similar classes of phytochemicals were detected in the ethyl acetate and chloroform extracts, although at substantially lower levels. Alkaloids were not detected in any of the extracts.

Antibacterial activity

To examine the growth inhibitory activity of the *T. chebula* fruit extracts, a series of disc diffusion assays were conducted on agar plates inoculated with autoimmune inflammatory disease initiating bacterial strains (*P. mirabilis, K. pneumoniae, A. baylyi* and *P. aeruginosa*). *P. mirabilis* growth was particularly susceptible to the mid to high polarity methanolic, aqueous and ethyl acetate *T. chebula* fruit extracts (Fig. 1), with zones of inhibition ranging from approximately 17 to 20 mm. Notably, both the methanolic and aqueous extracts produced substantially larger zones of inhibition than the penicillin, nystatin and chloramphenicol controls. Indeed, this *P. mirabilis* strain was

completely resistant to nystatin. In contrast, ciprofloxacin was a potent inhibitor of *P. mirabilis* growth (as judged by the size of the zone of inhibition). No growth inhibitory activity was observed for the lower polarity chloroform and hexane extracts.

A similar trend was noted for the bacterial trigger of ankylosing spondylitis (*K. pneumoniae*). The methanolic, aqueous and ethyl acetate *T. chebula* fruit extracts were deemed to be good inhibitors of *K. pneumoniae* growth based on their zones of inhibition (Fig. 2), with similar potency to that determined for the inhibition of *P. mirabilis.* This is noteworthy as the *K. pneumoniae* strain tested in these studies was completely resistant to penicillin and nystatin and has previously been reported to be resistant to multiple other antibiotics [4]. In contrast, this bacterium was highly susceptible to chloramphenicol and ciprofloxacin, with zones of inhibition between 22 and 32 mm. No growth inhibition was noted for the lower polarity chloroform and hexane extracts. The mid to high polarity *T. chebula* methanolic, aqueous and ethyl acetate fruit extracts were similarly strong inhibitors of *A. baylyi* (Fig. 3a) and *P. aeruginosa* (Fig. 3b). Thus, the methanolic, aqueous and ethyl acetate extracts have potential as prophylactic therapies to inhibit some bacterial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis.

Quantification of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration for each extract was determined by further analysing the extracts that showed antimicrobial activity against susceptible bacterial strains in the disc diffusion assays. The *T. chebula* extracts were tested across a range of concentrations against *P. mirabilis, K. pneumonia, A. baylyi* and *P. aeruginosa* in microplate liquid dilution and disc diffusion assays to determine the MIC of each extract. The results of the disc diffusion and liquid dilution MIC assays were consistent with the antimicrobial screening studies where the higher polarity extracts displayed antimicrobial activity, whereas the lower polarity extracts showed little or no inhibition (Table 2). Antibiotic control MIC's are only provided for the liquid dilution assays as the standard antibiotic discs were only tested at a single dose. Interestingly, the *P. aeruginosa* strain used in the study was resistant to all of the conventional antibiotic controls except ciprofloxacin. In addition, both penicillin-G and erythromycin were ineffective against *P. mirabilis, K. pneumoniae* and *A. baylyi.* Whilst chloramphenicol and tetracycline did inhibit bacterial growth, their relatively high MIC values indicate that each of the bacterial strains tested were at least partially resistant to all of the conventional antibiotics screened in this study.

The water extract displayed the lowest MIC values for all bacterial strains with the exception of *K. pneumonia*, in which the ethyl acetate extract produced the lowest MIC (LD MIC 1060 µg/mL). The *T. chebula* fruit water extract was a potent inhibitor of *P. aeruginosa*, displaying the lowest recorded MIC of all extracts (LD

MIC 195 µg/mL). Interestingly, *P. aeruginosa* was susceptible to low concentrations of all extracts (water - LD MIC 195 µg/mL; methanol - LD MIC 245 µg/mL; ethyl acetate - MIC LD 265 µg/mL; chloroform - LD MIC 750 µg/mL) with the exception of hexane extract, for which no inhibitory activity was observed. This is particularly noteworthy as this *P. aeruginosa* strain was resistant to all conventional antibiotics tested in this assay except ciproflaxacin, indicating that the bioactive components within these extracts may function via different inhibitory mechanisms to these antibiotics. Alternatively, the extracts may contain compounds which potentiate the antibacterial components, allowing them to function in bacteria that would be otherwise resistant to their actions. The water extract was an effective inhibitor of *P. mirabilis* growth (LD MIC 390 µg/mL), with good inhibition against *A. baylyi* (LD MIC 781 µg/mL) and moderate potency against *K. pneumoniae* (LD MIC 1570 µg/mL). The methanol and ethyl acetate extracts shared similar potency across all bacterial species tested, with moderate to high levels of antimicrobial activity. However, *K. pneumoniae* displayed lower levels of susceptibility in comparison with the other bacterial species (Table 2). Surprisingly, the chloroform extract had moderate antimicrobial activity against *P. aeruginosa* (LD MIC 750µg/mL), although it was completely devoid of growth inhibitory activity against all other bacterial strains.

Determination of combinational effects: Fractional inhibitory concentration (FIC) assessment

Fractional inhibitory concentration (FIC) determination was performed using a 1:1 ratio of each *T. chebula* extract to conventional antibiotic and sums of FIC (ΣFIC) were calculated for any combinations containing extracts that inhibited bacterial growth on their own (Table 3). As previously shown, the *P. aeruginosa* strain used in this study was resistant to all antibiotics in the liquid dilution assay, so no ΣFIC values could be calculated for any combinations against this bacteria. Furthermore, the absence of antimicrobial activity from penicillin-G and erythromycin against all bacterial strains tested prevented the calculation of ΣFIC values for these combinations. Approximately 33% of the *T. chebula* extract:conventional antibiotic combinations were non-interactive with tetracycline or chloramphenicol. In contrast, only two synergistic interactions were noted in combinations containing the *T. chebula* water extract (ΣFIC = 0.38) and ethyl acetate extract (ΣFIC = 0.25) against *K. pneumoniae* when tested in combination with tetracycline. This is an interesting result as this bacteria displayed partial resistance against tetracycline alone (MIC 0.63 µg/mL). Further studies are warranted to examine the synergistic mechanism and to identify the synergising component(s) in the extracts. Although ΣFIC values could not be determined for the remaining interactive combinations (Table 3), it is noteworthy that no interactions were antagonistic, indicating that

the *T. chebula* extracts can be used in combination with the conventional antibiotics tested in this study without reducing the effects of the antibiotic component of the combinations.

Varied ratio combination studies (isobolograms)

As two synergistic interactions were noted against *K. pneumoniae*, ratios of these combinations were tested in order to identify the optimal ratios at which synergy occurs (Figure 4). Of the nine ratios of the aqueous *T. chebula* fruit hexane extract in combination with tetracycline, six were synergistic (10-60% extract: Fig. 4a). Therefore, all of these ratios would be effective for inhibiting the growth of *K. pneumoniae*. The other three ratios yielded additive interactions. The ideal synergistic ratio for the treatment and prevention of ankylosing spondylitis was therefore identified to be 60% *M. oleifera* leaf hexane extract and 40% tetracycline, as this ratio would minimise the amount of tetracycline in the combination and thus reduce the chances of developing further resistance with long term prophylactic usage. In contrast, the preferred ratio for acute infections (the highest antibiotic % to maximise the efficacy of the treatment) is the 10% *T. chebula* fruit hexane extract and 90% tetracycline ratio. Interestingly, 8 of the 9 ratios of the ethyl acetate *T. chebula* fruit extract and tetracycline combination also produced synergistic interactions against *K. pneumoniae* (Fig. 4b). Only the combination containing the lowest percentage of tetracycline was not synergistic and even combinations containing the lowest levels of extract induced synergistic effects. This is particularly interesting as it is similar to the effects produced by clavulanic acid in combination with β-lactam antibiotics and may indicate that the potentiation of tetracycline's antibiotic effects is via irreversible inhibition of the bacterial resistance mechanisms [1].

Quantification of toxicity

To evaluate and quantify the effect of the extracts on the induction of mortality, each was diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay. Table 4 shows the concentration required to induce 50% mortality (LC_{50} value)s of the extracts towards *A. franciscana*. No LC_{50} values are reported for the chloroform, hexane and ethyl acetate extracts as <50 % mortality was seen for all concentrations tested. The methanolic and aqueous extracts were also determined to be nontoxic, with LC_{50} values substantially greater than 1000 µg/mL following 24 h exposure. Extracts with an LC_{50} of greater than 1000 µg/mL towards *Artemia* nauplii have been defined as being nontoxic [21]. Similarly, the HDF cell viability was >50% for all extract treatments, confirming that all extracts were nontoxic.

Discussion

This study examined whether *T. chebula* fruit extracts could inhibit the growth of selected bacterial triggers of autoimmune inflammatory diseases, both alone and in combination with conventional antibiotics. The high to mid polar methanol, water and ethyl acetate *T. chebula* fruit extracts were good bacterial growth inhibitors, whereas the less polar chloroform and hexane extracts were generally devoid of inhibitory activity. Thus, the methanolic, aqueous and ethyl acetate extracts have potential as prophylactic therapies to inhibit some bacterial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis, thereby inhibiting the onset of these diseases. In particular, the water extract was a strong inhibitor of *P. aeruginosa* and *P. mirabilis* with MIC values as low as 195 µg/mL. However, when tested against *K. pneumoniae* the water extract was less effective, with an MIC value of 1570 µg/mL. Surprisingly, the MIC values determined for extracts tested against *P. aeruginosa* were the lowest recorded against all bacterial strains tested. This is very interesting as this *P. aeruginosa* strain was resistant to all conventional antibiotics tested in the liquid dilution assay. Earlier studies have also confirmed the multidrug resistance of this strain [4, 12]. Definitive identification of the phytochemicals in each extract was not undertaken within this study, although previous research suggests the higher polarity solvent extracts of *T. chebula* contain bioactive components responsible for the antimicrobial activity [6]. *T. chebula* fruit is a good source of chebulic and ellagic acids and these tannins have been shown to inhibit the growth of a variety of bacteria [6, 11, 12]. This inhibitory activity has been attributed to range of intra and extracellular mechanisms, including enzyme precipitation and inhibition, complexing with bacterial cell walls, and they have also been shown to suppress DNA synthesis [6, 24]. However, whilst it is likely that tannins may contribute to the antibacterial activity reported in our study, further studies are required to evaluate the mechanism and identify the bioactive compounds responsible for the observed growth inhibition.

Although the inhibition of the growth of some bacterial triggers of autoimmune inflammatory diseases was noteworthy, the results of the combinational studies were perhaps of greater significance. Two combinations displayed greater potential against *K. pneumoniae* than either of the extracts or conventional antibiotics alone, highlighting possible future therapies for treating and preventing ankylosing spondylitis. When used in combination with tetracycline, both the ethyl acetate and water extracts induced synergistic interactions against *K. pneumoniae*. Interestingly, interactions between the lower polarity extracts (chloroform and hexane) in combination with tetracycline, chloramphenicol or erythromycin were also observed. However, due to antibiotic resistances in these bacterial strains, only the FIC's of tetracycline and chloramphenicol could be calculated. The remaining extracts

were non-interactive when tested in combination with the conventional antibiotics. This is itself a noteworthy finding as it indicates that it is safe to co-administer these extracts with tetracycline or chloramphenicol without compromising their inhibitory activity.

Synergistic interactions allow for greater efficacy in drug administration by increasing a drug's effectiveness, or reducing the potential for the development of further microbial resistance by allowing lower doses of the conventional antibiotic to be prescribed [19]. Furthermore, combinations of the fruit extracts and conventional antibiotics could potentially repurpose antibiotics which would otherwise be ineffective against resistant bacterial strains. Examples of combinational medicines repurposing conventional antibiotics already exist. In particular, the drug Augmentin takes advantage of the synergising activity of clavulanic acid in combination with β-lactam antibiotics [1]. Clavulanic acid alone has negligible inherent antibacterial activity. However, it binds irreversibly to bacterial β-lactamase enzymes, thereby inactivating them and overcoming bacterial resistance to the β-lactams [1, 25, 26]. Research has identified several strains of *K. pneumoniae* that possess extended-spectrum β-lactam resistance [27]. Our data from the antimicrobial screening was consistent with these studies as penicillin was ineffective as a growth inhibitor against *K. pneumoniae.* However, our combinational studies indicate that the synergy was unlikely to be due to β-lactamase inhibition as no antimicrobial activity was observed in extract:penicillin combinations. Therefore, other resistance mechanisms are more likely.

The *K. pneumoniae* strain examined in our study may have multiple resistance mechanisms. Many bacteria have acquired multidrug resistance mechanisms to withstand antibiotics that would otherwise be lethal. Efflux pumps have been identified as one of the major contributors to drug resistance and research has found that not only do the efflux pumps expel antibiotics from the bacterial cell, but they also promote additional resistance mechanisms through accumulation of mutations [1, 28]. In our study, *K. pneumoniae* was resistant toward tetracycline (MIC 0.63 μg/mL) when compared with the MIC's measured against the other bacterial strains (Table 2). The most common resistance mechanism against tetracycline is via specific efflux pumps [29, 30]. Thus, the water and ethyl acetate extracts may contain tetracycline efflux pump inhibitors (EPI), thereby increasing *K. pneumoniae*'s susceptibility to tetracycline, although further studies are required to confirm this. Whilst any such tetracycline EPI remains to be identified, a gallotannin 1,2,6-tri-*0*-galloyl-β-D-glucopyranose isolated from hydroalcoholic extracts of *T. chebula* fruit has previously been reported to be a potent inhibitor of a multi-drug resistant strain of *E. coli* [31]. That study isolated the tannin and determined that it was responsible for the increased antimicrobial activity. Similarly, ellagic acid can also synergize the activity of some antibiotics [32]. Although these earlier studies support the proposal that

T. chebula components may function as tetracycline EPI's, further investigations are required to confirm the mechanism and identify the potentiating compound(s).

In this study, we have reported the effects of combinations antibiotics with crude extracts. For many applications (particularly for acute conditions), combining the antibiotic with a single potentiating compound may be preferred as such combinations are much less complex, easier to standardize and have lower chances of unwanted side effects. However, when treating chronic illness, or using a combinational approach to prevent illness (as would be required in preventing autoimmune inflammatory diseases), the use of a pure potentiator compound in combination with the antibiotic may not be desirable. Continuous exposure of bacteria to a pure antibiotic (or to a combination of a single antibiotic and single potentiator) is likely to induce resistance to one or both of the compounds in the bacteria [1]. The use of crude extracts in these preparations is also effective and may still be acceptable to treat some diseases. Indeed, some *E.coli* strains are now resistant to amoxicillin-clavulanic acid combinations [33]. However, crude plant extracts often contain numerous antibacterial compounds which may affect multiple bacterial targets. Thus, using a plant extract (rather than pure plant compounds) in combination with an antibiotic is less likely to result in resistant bacteria. Indeed, we were unable to find any reports of any bacteria developing resistance to a crude plant extract. For this reason, the lowest extract:highest antibiotic ratio which produced synergy may be ideal ratio for treating acute bacterial infections, whilst the highest extract:lowest antibiotic ratio which produced synergy would be preferred for preventing and treating chronic disease.

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Figure Legends

Figure 1: Antibacterial activity of the *T. chebula* fruit extracts against *P. mirabilis* (ATCC: 21721) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. Positive controls: Pen = penicillin-G (20µg); Nys = nystatin (100 Units); Cip = ciprofloxacin (2.5µg); Chl = chloramplenicol (10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of at least six replicates (two repeats) ± SEM. * indicates results that are significantly different to the negative control ($P<0.01$).

Figure 2: Antibacterial activity of the *T. chebula* fruit extracts against *K. pneumoniae* (ATCC: 39324) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. Positive controls: Pen = penicillin-G (20µg); Nys = nystatin (100 Units); Cip = ciprofloxacin (2.5µg); Chl = chloramplenicol (10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of at least six replicates (two repeats) ± SEM. * indicates results that are significantly different to the negative control ($P<0.01$).

Figure 3: Antibacterial activity of the *T. chebula* fruit extracts against (a) *A. baylyi* (ATCC:21721) and (b) *P. aeruginosa* (ATCC:31488) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. Positive controls: Pen = penicillin-G (20µg); Nys = nystatin (100 Units); Cip = ciprofloxacin (2.5µg); Chl = chloramplenicol (10µg). Negative control (NC) = water. Results are expressed as mean zones of inhibition of at least six replicates (two repeats) ± SEM. * indicates results that are significantly different to the negative control ($P<0.01$).

Figure 4: Isobologram for combinations of tetracycline and (a) aqueous *T. chebula* fruit extracts and (b) ethyl acetate *T. chebula* fruit extracts tested at various ratios against *K. pneumoniae* (ATCC: 39324). Results represent mean MIC values of four replicates. Ratio = % extract: % antibiotic. Ratios lying on or underneath the 0.5:0.5 (green) line are considered to be synergistic (Σ FIC ≤ 0.5). Any points between the 0.5:0.5 (green) and 1.0:1.0 (blue) line or on the 1.0:1.0 (blue) line are deemed additive (Σ FIC > 0.5-1.0).

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water (containing 1% DMSO) and qualitative phytochemical screenings of the *T. chebula* fruit extracts.

Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
M	534	53.4	+++	+++	+++	-	+++	-	-	-	-	+++	+++	+	-
W	438	43.8	+++	+++	+++	-	+++	-	-	-	-	+++	+++	+	-
E	62	6.2	+++	++	+	-	-	-	-	-	-	++	+	-	-
C	93	9.3	+	-	-	-	-	-	-	-	-	-	+++	-	-
H	104	10.4	-	-	-	-	-	-	-	-	-	-	-	+	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay; M = methanolic *T. chebula* fruit extract; W = aqueous *T. chebula* fruit extract; E = ethyl acetate *T. chebula* fruit extract; C = chloroform *T. chebula* fruit extract; H = hexane *T. chebula* fruit extract.

Table 2: Disc diffusion and liquid dilution MIC values (µg/mL) against the bacterial triggers of some selected autoimmune inflammatory diseases.

Extract	Minimun Inhibitory Concentration (µg/mL)							
	P. mirabilis		***K. pneumoniae***		***A. baylyi***		***P. aeruginosa***	
	DD	**LD**	**DD**	**LD**	**DD**	**LD**	**DD**	**LD**
Methanol	984	490	7875	1900	3938	980	984	245
Water	1563	390	3125	1570	6250	781	3125	195
Ethyl acetate	2125	530	8500	1060	4250	1060	2125	265
Chloroform	-	-	-	-	-	-	-	750
Hexane	-	-	-	-	-	-	-	-
Positive control								
Penicillin	ND	-	ND	-	ND	-	ND	-
Erythromycin	ND	-	ND	-	ND	-	ND	-
Chloramphenicol	ND	2.5	ND	2.5	ND	0.625	ND	-
Tetracycline	ND	0.125	ND	0.63	ND	0.313	ND	-

The values represent the MIC value in µg/mL. DD = disc diffusion; LD = liquid dilution; ND = not determined; - = no inhibition was observed.

Table 3: ΣFIC values for combinations of the *T. chebula* fruit extracts in combination with chloramphenicol and tetracycline against the bacterial triggers of some autoimmune inflammatory diseases.

Antibiotic	Extract	MIC values of individual components in the mixture and FIC Index (ΣFIC) of the mixture											
		P. mirabilis			*K. pneumoniae*			*A. baylyi*			*P. aeruginosa*		
		MIC (em)	MIC (am)	ΣFIC	MIC (em)	MIC (am)	ΣFIC	MIC (em)	MIC (am)	ΣFIC	MIC (em)	MIC (am)	ΣFIC
Chloramphenicol	Methanol	260	1.34	1.06	1172	1.41	1.13	620	1.58	1.26	CND	CND	CND
	Water	217	1.28	1.06	898	1.41	1.13	798	2.43	2.02	CND	CND	CND
	Ethyl acetate	354	1.56	1.25	684	1.56	1.25	1060	0.63	2	CND	CND	CND
	Chloroform	CND	CND	CND	CND	CND	CND	CND	CND	ACT	CND	CND	ACT
	Hexane	CND	CND	CND	CND	CND	ACT	CND	CND	ACT	CND	CND	CND
Tetracycline	Methanol	277	0.1	1.13	1425	0.47	1.5	740	0.22	1.51	CND	CND	CND
	Water	220	0.09	1.13	298	0.09	0.38	596	0.24	1.5	CND	CND	CND
	Ethyl acetate	398	0.11	1.5	134	0.08	0.25	795	0.24	1.5	CND	CND	CND
	Chloroform	CND	CND	ACT	CND	CND	CND	CND	CND	CND	CND	CND	CND
	Hexane	CND	CND	ACT	CND	CND	ACT	CND	CND	CND	CND	CND	CND

Numbers indicate the mean MIC or ΣFIC values of 6 determinations; MIC(em) = MIC of the extract component in the mixture; MIC(am) = MIC of the antibiotic component in the mixture; CND (grey highlighting) = could not be determined as one or both components of the conbination were inactive; ACT (red highlighting) = activity was detected but an MIC or ΣFIC could not be determined; Synergy (blue highlighting) = ΣFIC ≤0.5; Indifferent effects (green highlighting) = >1.0-≤4.0.