Use of specific combinations of the triphala plant component extracts to potentiate the inhibition of gastrointestinal bacterial growth

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ABSTRACT

Ethnopharmacological relevance: Triphala is used in Ayurveda to treat a wide variety of diseases, including numerous bacterial infections. Interestingly, the plant components of triphala (*Terminalia bellirica*, *Terminalia chebula* and *Emblica officinalis*) are also good inhibitors of bacterial growth when used individually, yet plant preparations are generally used in combination in traditional medicine. Surprisingly, no previous studies have addressed the reason why the combination is preferred over the individual components to treat bacterial infections.

Aim of the study: To test and compare the antibacterial efficacy of triphala and its component parts to quantify their relative efficacies. The individual plant components will also be tested as combinations, thereby determining whether combining the individual components potentiates the antibacterial activity of the components used alone.

Materials and methods: Triphala and the three individual plant components were extracted using solvents of varying polarity (methanol, water, ethyl acetate) and the antibacterial activity of the aqueous resuspensions was quantified by disc diffusion and broth microdilution MIC assays. Combinations of extracts produced from the individual components were also tested against each bacterial species and the ΣFICs was calculated to determine the class of interaction. Where synergy was detected, isobologram analysis was used to determine the optimal component ratios. The *Artemia* nauplii bioassay was used to test for toxicity and GC-MS headspace profiling analysis was used to highlight terpenoid components that may contribute to the antibacterial activity of triphala.

Results: The aqueous and methanolic triphala, *T. bellirica*, *T. chebula* and *E. officinalis* extracts displayed good inhibitory activity against all bacterial strains, with MICs often in the 250-750 µg/mL range. The methanolic extracts were generally more potent than the aqueous extracts and *T. chebula* was the most potent of the individual plant components. Combining the extracts of the different plant species resulted in potentiation of the growth inhibitory activity of most combinations compared to that of the individual components. Indeed, with the exception of *S. flexneri*, all bacterial species were potentiated by at least one combination of methanolic plant extracts, with a substantial proportion of these displaying synergistic interactions. All extracts were found to be either non-toxic, or of low to moderate toxicity in *Artemia* nauplii assays.

Conclusion: Whilst the individual plant components of triphala all inhibit the growth of multiple pathogenic bacteria, the activity is potentiated for multiple combinations. Therefore,

the traditional usage of the combination of the three plant materials in triphala not only extends the activity profile of the mixture over that of the individual components, but it also substantially potentiates the inhibitory activity towards multiple bacteria, partially explaining the preference of triphala compared to the individual components.

Keywords: Ayurveda, Triphala, antibacterial, synergy, GC-MS analysis

1. Introduction

Triphala (TA) is a popular polyherbal Ayurvedic medicine which has been used for more than 1000 years (Mukherjee et al., 2017). It comprises three separate dried fruit powders mixed in equal proportion: *Terminalia bellirica* (Gaertn.) Roxb. (TB), *Terminalia chebula* Retz. (TC) and *Emblica officinalis* L. (EO), all of which are native to the Indian subcontinent. The formula possesses significant ethnopharmacological importance due to its broad array of therapeutic applications that include treatments for obesity, diabetes, cardiovascular conditions, cancer, cataracts, inflammation and ageing, as well as many diseases triggered by microorganisms (Baliga et al., 2012).

Triphala is a treatment option for various ailments associated with the gastrointestinal system. Interestingly, it has been found to possess both anti-diarrhoeal (Biradar et al., 2008) and laxative (Mukherjee et al., 2006) properties. The mechanisms via which these conditions are ameliorated remains unclear, although it is possible in cases of diarrhoea caused by enteric infections that antimicrobial properties inherent in the triphala formulation are responsible. Diarrheagenic *Escherichia coli* (Cabrera-Sosa & Ochoa, 2020) is a common trigger of diarrhoea especially in children, while *Staphylococcus aureus* has been identified as a causative agent involved in antibiotic-associated nosocomial diarrhoea (Polage et al., 2012). *Bacillus cereus* releases diarrheagenic toxins in food poisoning cases (Bhunia, 2018). Shigellosis is triggered by *Shigella* spp. including *Shigella flexneri* and *Shigella sonnei* (Kotloff et al., 2018). A recent outbreak of fatal diarrhoea was attributed to *Aeromonas hydrophila* (Liu et al., 2019), whilst other food-borne strains such as *Salmonella* Typhimurium (Wotzka et al., 2017) inhabit the lower gut and cause acute diarrhoea.

There have been reports of antimicrobial activity of triphala towards these strains, although the activity determinations vary widely among the individual studies. As an example, a 2007 study showed that the aqueous extract of *E. officinalis* was inactive against *S. aureus* on agar disc diffusion assays but possessed a low minimum inhibitory concentration (MIC) of 100 μg/mL in broth assays (Srikumar et al., 2007). In contrast, a later study reported a

substantially lower MIC value (25 μg/mL) for these extracts against the same strain (Mehrotra et al., 2010). Other studies have shown antibacterial activity of triphala (and its separate plant components) against *E. coli*, *S. aureus*, and *S.* Typhimurium in disc diffusion experiments, although these studies employed very large concentrations or quantities of the extracts (3-10 mg per disc) in the assays (Amanullah et al., 2010; Tambekar and Dahikar, 2011; Sheta et al., 2016). Furthermore, these studies tested a limited number of extract doses and MIC values were not determined, making comparisons between studies impossible. In some cases, gram quantities of extract were used in assays (Mahajan and Jain, 2015) or the amount of extracts used was not stated (Parveen et al., 2018). Maji et al. (2010) documented the activity of *E. officinalis* aqueous extracts against *B. cereus*, although the other plant components and the triphala formulation were not tested. Activities against *S.* Typhimurium have been reported although the results are dubious as the extracts were resuspended in 100% DMSO for testing (Tambekar et al., 2007). Dimethyl sulfoxide itself is toxic to bacteria and it is likely that this accounts for the growth inhibitory activity reported in that study. Another study showed low MIC values for aqueous extracts of triphala and each of its three components in a broth microdilution assay against *S. flexneri* and *S. sonnei*, although zones of inhibition were not observed for *T. bellirica*, *E. officinalis* and the Triphala formulation using agar disc diffusion assays (Srikumar et al., 2007; Amanullah et al., 2010). These inconsistencies between the activities of triphala and its components, and/or the techniques used to determine the activities, warrant further investigation. Additionally, we were unable to find reports of the effects of triphala against *A. hydrophila* in the literature.

Despite the traditional use of triphala as a 1:1:1 formulation of the three plant components, little is known about the effectiveness of these plants as antibacterial agents when *T. bellirica*, *T. chebula* and *E. officinalis* are used in different combinations and proportions. It may be possible that different mixtures of the individual components address different aspects of the disease progression or deliver an expanded antibacterial spectrum. Alternatively, in some cases, the combinations may synergise antibacterial activities against specific microorganisms (van Vuuren & Viljoen, 2011). This has been demonstrated in the past where combinations of extracts or essential oils prepared from different plants synergistically inhibit bacterial or fungal growth (Sibanze et al., 2010; de Rapper et al., 2013; Mabona et al., 2013; Orchard et al., 2018a; Orchard et al 2018b; Orchard et al, 2019). While triphala and its individual components possess antibacterial activities against a number of pathogens, as previously stated, the benefits

of combining the individual components rather than using them in isolation are hitherto unknown.

In the present study, we aim to verify the activities of these plants against a panel of seven bacterial pathogens known to trigger and/or exacerbate gastrointestinal diseases, and to determine whether combinations of *T. bellirica*, *T. chebula* or *E. officinalis* alter the spectrum and efficacy of the therapy. *E. faecalis* was also included in the study as a non-pathogenic species present within the human alimentary tract (Murray, 1990) in order to compare the effects of the extracts on this strain with those on pathogenic bacteria. Furthermore, we also sought to detect additive or synergistic interactions and determine the nature of those interactions. Thereby, we aimed to elucidate options to optimise therapeutic formulations to treat enteric infections that trigger diarrhoea. This would be particularly useful where activities can be significantly enhanced using specific combinations of the components. Therefore, this study aimed to provide an ethnomedicinal basis for adjusting formulations targeted against specific illnesses that are triggered by pathogenic gastrointestinal strains.

2. Materials and Methods

2.1. Source of plant samples

Triphala*, T. bellirica, T. chebula* and *E. officianalis* plant materials were sourced commercially from Noodles Emporium, Australia and supplied as dried and ground powdered forms. All plants were sourced from India and were verified by the supplier. Voucher samples have also been stored at the School of Environment and Science, Griffith University (Nathan, QLD). Voucher numbers are GU-TA19, GU-TB19, GU-TC19 and GU-EO19 for triphala*, T. bellirica, T. chebula* and *E. officianalis*, respectively.

2.2. Preparation of extracts

A 1 g mass of triphala, *T. bellirica*, *T. chebula* and *E. officinalis* powders were weighed into individual tubes and either deionised water, methanol or ethyl acetate added to a volume of 50 mL. All solvents were supplied by Ajax Fine Chemicals, Australia and were AR grade. This ultimately produced 12 different extracts (four botanical samples extracted with three different solvents). The samples were mixed by continuous rolling at 30 rpm for 24 h at room temperature. Samples were then filtered through Whatman No. 54 filter paper under vacuum into fresh, pre-weighed 50 mL tubes. Aqueous samples were frozen at -80 °C for 30 min and dried by lyophilisation for 48 h. Organic solvents (methanol and ethyl acetate) were evaporated in a drying oven at 50 °C for up to 48 h. Dried extracts were weighed and resuspended in 10

mL 1% DMSO and sonicated three times (20 s pulses at 1 kHz with 30 s rest between pulses). Samples were sterilised by passage through 0.22 µm filters (Sarstedt) and stored at room temperature until use.

2.3. Qualitative phytochemical analysis

Phytochemical analyses of the triphala, *T. bellirica*, *T. chebula* and *E. officinalis* extracts were conducted in order to test for the presence of saponins, phenolic compounds, flavonoids, phytosterols, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids using previously described assays (Vesoul and Cock, 2011).

2.4. Antibiotics and bacterial strains

Penicillin-G (potency of 1440-1680 µg/mg), chloramphenicol (≥98% purity by HPLC), erythromycin (potency ≥850 µg/mg), ciprofloxacin (≥98% purity by HPLC) and tetracycline (≥95% purity by HPLC) were purchased from Sigma-Aldrich (Australia) and were used as controls for the disc diffusion and microplate broth microdilution assays. Reference bacterial strains of *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 19433), *Shigella sonnei* (ATCC 25931), *Aeromonas hydrophila* (ATCC 7966), *Salmonella* Typhimurium (*Salmonella enterica* serovar Typhimurium; ATCC 14028), *Shigella flexneri* (ATCC 12022), *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 14579) were purchased from the American Type Culture Collection (ATCC; USA). All strains were sub-cultured and maintained in nutrient broth and on nutrient agar (Oxoid Ltd., Australia), and the antibacterial test conditions conformed to CLSI standardised methods (Clinical and Laboratory Standards Institute, 2019).

2.5. Disc diffusion assays

A modified Kirby-Bauer disc diffusion method was used to determine the antibacterial activity of all plant extracts on agar (Cock, 2008). Individual bacterial colonies for each strain were inoculated into 40 mL of fresh nutrient broth and incubated at 37 °C with orbital oscillation at 150 rpm until the cell count reached approximately 10^8 cells/mL. The cultures were then used to prepare 0.5 McFarland standards for each strain and 100 µL of the cultures spread on nutrient agar. Ten microlitres of resuspended extracts (tested undiluted at the concentrations provided in Table 1), the resuspension solvent (1% DMSO), or the reference antibiotics were infused into filter discs (Whatman #1 paper, 6 mm in diameter), allowed to dry and then attached to the agar surface. All samples were tested using triplicate assays. The

discs were allowed to dry on the plate for 30 min and the plates incubated at 37 °C for 18-24 h. Diameters of the zone of inhibition (ZOI) were measured to the nearest whole millimetre and values included the 6 mm diameter of the filter discs. Mean values (± SEM) are reported in this study.

2.6. Determination of minimum inhibitory concentration (MIC) values

The MICs for each extract and antibiotic was determined using a 96-well micro-titre plate broth microdilution assay (Hübsch et al, 2014; Ilanko and Cock, 2019). Briefly, samples were serially diluted followed by addition of 100 µL of a 1:100 dilution of a 0.5 McFarland cell suspension, and the plates incubated at 37 °C for 20-24 h. Aliquots (40 µL) of *p*-iodonitrotetrazolium violet (INTZ; Sigma Aldrich, Australia) dye solution (0.4 mg/mL) were added into each micro-titre plate well and incubated for 6 h at room temperature. MIC values were determined by visual inspection as the lowest concentration of extract or antibiotic that completely inhibited bacterial growth as defined as the lack of red-pink colour development. Extract MIC values >5000 µg/mL were considered inactive; MIC values between 2000-5000 µg/mL were considered as low activity; 1000-2000 µg/mL were considered as moderate activity; 400-1000 µg/mL were considered as noteworthy activity; 100-400 µg/mL were considered as good activity; and <100 µg/mL were considered to be high activity.

2.7. Fractional inhibitory concentration (FIC) and ∑FIC determinations

Extracts showing appreciable antibacterial activity (<2000 µg/mL) were tested in combination with each other, to test whether interactions occurred between the different plant component extracts. Initially, 50:50 ratios of two different extracts were tested. Interactions between extracts were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC) for each combination. The FIC values for each component (a and b) were calculated using the following equations where a and b represent the two plant extracts being tested:

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

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

∑FIC was then calculated using the formula ∑FIC = FIC(a) + FIC(b). The interactions were classified as synergistic (∑FIC ≤0.5), additive (∑FIC >0.5–≤1.0), indifferent (∑FIC >1.0-≤4.0) or antagonistic (∑FIC >4.0) (Doern, 2014).

In instances where the 50:50 ratios of two of the combined components produced synergistic interactions, the combinations were examined further by testing nine different ratios ranging from 10:90 (a:b) to 90:10 (a:b). All combinations were tested in duplicate in two independent experiments (n=4). Data points for each ratio examined were plotted on isobologram graphs, and these were used to determine the optimal combination ratios that elicited synergy. Data points on or below the 0.5:0.5 line indicated synergy; those above the 0.5:0.5 line, and 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 interactions.

2.8. Artemia nauplii lethality screening

The toxicity of the extracts and controls was quantified using standard *Artemia franciscana* Kellogg nauplii lethality assays (ALA) (Ruebhart et al., 2009). Briefly, 400 µL volumes of the diluted plant extracts or the controls were added 400 µL of artificial seawater containing 40–60 newly hatched *A. franciscana* nauplii (within 1 day of hatching) in individual wells of a 48 well plate. Negative controls (32 g/L artificial seawater; Sigma Australia) and positive controls (1 mg/mL potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) were included on all plates. After a 24 h incubation at 25±1 ºC, the percentage of dead nauplii was determined. The LC_{50} values were calculated as the concentration of extract or control required to kill 50 % of the *A. franciscana* nauplii, and were determined using Probit analysis. The results are expressed as the mean of three independent experiments, each with internal triplicates (n=9). To determine the suitability of the extracts as potential therapeutic agents, their therapeutic index was calculated using the formula:

Therapeutic index (TI) = (LC_{50})/(MIC)

2.9. GC-MS profiling analysis

Separation, identification and quantification of the relative abundance of individual extract components putative was performed using a Shimadzu GC-2010 plus (USA) chromatography system linked to a Shimadzu MS TQ8040 (USA) mass selective detector system using previously optimised parameters (Shalom & Cock, 2018). The mass spectrometer was operated in the electron ionisation mode at 70 eV and the individual mass signals were recorded in total ion count (TIC) mode. The TIC was acquired for 45 mins utilising a mass range of 45-450 *m/z.* Individual compounds were putatively identified by comparison with the ChemSpider database.

2.10. Statistical analysis

ZOI data were expressed as the mean ± SEM of at least three independent experiments. One-way ANOVA analysis was used to calculate statistical significance between control and treatment groups. Although statistical analysis could not be performed with the 96-well microtitre broth microdilution assays, the reliability of MIC values was ensured by repeating the broth microdilution assays twice on separate days, with two replicates per assay, to confirm that the results were reproducible for all extracts, antibiotics and combinations tested.

3. Results

3.1. Liquid extraction yields and phytochemical screening

The extraction of 1 g quantities of dried triphala and its botanical ingredients produced varying yields, ranging from 2-5% (ethyl acetate) to 40-70% (water or methanol). All extracts were resuspended in 1% DMSO to produce the final extract concentrations shown in Table 1. All extracts were found to be rich in flavonoids, tannins and phenolic compounds. However, the methanolic extracts possessed a wider profile of phytochemicals, which included triterpenoids and cardiac glycosides in low to moderate abundances, respectively (Table 1). Alkaloids were present in the aqueous *E. officinalis* and methanolic *T. chebula* extracts only. All extracts were devoid of phytosterols and anthraquinones. Saponins were present in high levels in methanolic *T. chebula* extracts and in low levels in aqueous *T. chebula* samples but were absent in all other extracts. Alkaloids were detected in low levels in aqueous *E. officinalis* and methanolic *T. chebula* extracts.

3.2. Antimicrobial activity

Volumes of 10 µl were used directly in the agar disc diffusion assay to produce the activities shown in Fig. 1. The aqueous and methanolic triphala, *T. bellirica*, *T. chebula* and *E. officinalis* extracts exhibited inhibitory activity against most of the bacterial species tested, with some ethyl acetate extracts also inhibiting the growth of some bacteria. It is likely that the much lower yields obtained from the ethyl acetate extractions contributed to their smaller ZOIs on agar plates for samples showing any activity. Furthermore, the levels of inhibition observed on disc diffusion assays were generally concordant with inhibition in broth microdilution assays for both extracts and positive control antibiotics (Fig. 1 and Table 2), in that higher ZOI values (on disc diffusion assays) for any given extract against a bacterial strain produced lower MIC values. No activity was observed against the non-pathogenic species, *E. faecalis* on agar plates with low activities detected in broth microdilution assays. However, inhibition of the growth of this strain was observed for some of the positive control antibiotics, indicating that the assays

were functioning correctly. As such, the plant extracts were not tested further against *E. faecalis*.

The methanolic extracts exerted consistently greater efficacies against the pathogenic strains on both agar and in liquid assays. The water and ethyl acetate extracts usually showed either lower levels of activity or fewer of the components were active, although there are instances of high activities for extracts prepared with these solvents (Fig. 1 and Table 2). Interestingly, some of the ethyl acetate extracts that failed to inhibit the growth of some of the strains on agar also produced low MIC values (e.g. *S. flexneri* and *B. cereus*, Fig. 1F and 1G and Table 2).

Among the different plant species, the effects of *T. chebula* extracts on bacterial growth on agar was generally stronger against the pathogenic strains than the other plant extracts, with the exception of *E. coli,* where *T. bellirica* was more active than *T. chebula*. It should be noted that in most cases, the methanolic extracts possessed similar activities between the plants, particularly in agar disc diffusion experiments (Fig. 1). Similar trends were noted in broth microdilution assays (Table 2). *T. bellirica* and *T. chebula* proved to generally have the best activity for species such as *B. cereus*, *A. hydrophila*, *S. flexneri*, and *S. aureus*. Low MIC values were also obtained for some of the ethyl acetate extracts against these bacterial strains may have been revealed due to the greater sensitivity of this method.

3.3. Extract combinations

Since the methanolic extracts were generally more active, all were tested in combination in two-component systems. The remaining extracts were tested only if both extracts used in combination possessed an MIC value less than 2000 µg/ml. These selections were made in order to focus the study on higher potency extracts.

Although *E. coli* and *S. sonnei* were less susceptible to growth inhibition by the methanolic extracts, combinations of some of the extracts in 50:50 ratios boosted activities (Table 3). Combinations of *T. bellirica* and *T. chebula* led to synergistic inhibition of growth of *E. coli* and *A. hydrophila*, while additive effects were found against *S. sonnei* and *B. cereus*. The same effects were found when combinations of *T. bellirica* and *E. officinalis* were tested, whilst an additive effect on *S. aureus* was found using this mixture. When *T. chebula* and *E. officinalis* were combined, synergistic inhibition of *A. hydrophila* was produced, while additive effects were observed against *E. coli*, *S. sonnei*, *S.* Typhimurium and *B. cereus*.

There were only a limited number of cases where either water or ethyl acetate extracts were sufficiently active for testing combinations. There was a single case for the aqueous extracts, where *T. bellirica* and *T. chebula* combinations produced synergistic activity on *S. flexneri* (FIC_{TB} = 0.250, FIC_{TC} = 0.250, ∑FIC = 0.500). There were three cases for the ethyl acetate extracts, again involving *T. bellirica* and *T. chebula*, although no interactions were observed against the relevant strains (FIC_{TB} = 0.501, FIC_{TC} = 2.179, ∑FIC = 2.680 for *S. flexneri*; FIC_{TB} = 0.500, FIC_{TC} = 0.500, ∑FIC = 1.000 for *S. aureus*; and FIC_{TB} = 0.500, FIC_{TC} = 1.198, ∑FIC = 1.698 for *B. cereus*).

The synergistic combinations were particularly interesting because they not only show enhanced efficacy but a vastly greater efficacy than either component alone. Therefore, isobologram analysis was conducted in order to determine which ratio(s) produced synergistic (or additive) effects. These graphs are shown in Fig. 2. Mixtures of *T. bellirica* and *T. chebula*, or *T. bellirica* and *E. officianalis*, methanolic extracts showed additive inhibition of the growth of *E. coli* up until 50:50 ratios. However, ratios containing a larger proportion of the second extract produced synergy (Fig. 2A and 2B). The same was observed for *T. bellirica* and *T. chebula* mixtures against *A. hydrophila* (Fig. 2C). Almost all combination ratios of *T. bellirica* and *E. officianalis* elicited synergistic inhibition of *A. hydrophila* growth (Fig. 2D). In contrast to Fig. 2A and Fig. 2B, mixtures of *T. chebula* and *E. officinalis* methanolic extracts against *A. hydrophila* (Fig. 2E), or *T. bellirica* and *T. chebula* aqueous extracts against *S. flexneri* (Fig. 2F) require a lesser proportion of the second extract, and thus more of the first extract, in order to achieve synergistic inhibition of bacterial growth.

3.4. Artemia lethality assay (ALA)

All extracts were tested in the *Artemia* nauplii bioassay across a range of concentrations and were deemed to be toxic if they possessed LC_{50} values <1000 µg/mL following 24 h exposure (Ruebhart et al., 2009). LC_{50} values could not be calculated for the methanolic *T. bellirica* and *T. chebula* extracts or the ethyl acetate triphala and *T. bellirica* extracts, as nauplii death did not occur at any concentration screened. However, an LC_{50} value of 750 µg/mL was found for all aqueous extracts, the methanolic triphala and *E. officianalis* extracts, and the ethyl acetate *T. chebula* and *E. officianalis* extracts, indicating a mild level of toxicity of these preparations under these assay conditions.

3.4.1. Therapeutic index (TI) of the extracts

To evaluate the suitability of the tested extracts as therapeutic agents, the therapeutic index (TI) was calculated for all extracts against each bacterial pathogen. We were unable to calculate TI values for the methanolic *T. bellirica* and *T. chebula* extracts or the ethyl acetate triphala and *T. bellirica* extracts, as they did not display toxicity at any concentration tested and are indicated as by the term "High" in Table 4. Due to their lack of toxicity, these were assumed to have high TI values. The TIs of these extracts are therefore noteworthy and indicate that these extracts are suitable as oral as well as topical formulations to treat infections of these bacteria. The calculated TI values for all other extracts are shown in Table 4.

3.5. Non-targeted GC-MS headspace analysis

Volatile terpenoids are major contributors to antibacterial activity of many plant extracts (Guimaräes et al., 2019). Therefore, we chose to examine the volatile terpenoid contents of the extracts prepared from triphala and its component plants. GC-MS headspace analysis was selected to identify components in these extracts. Although we were most interested in identifying terpenoid components, our analysis was unbiased towards that phytochemical class and instead sought to identify all volatile compounds in the extracts. (Table 5) Optimised GC-MS parameters were previously developed in our group (Shalom & Cock, 2018) and those parameters were employed in this study. Several peaks identified as monoterpenoids were evident in multiple of the extracts prepared from triphala and its component species. A peak at 13.92 min was particularly prevalent, being detected in all of the extracts analysed. Comparison of the mass spectra of this peak with the ChemSpider database identified this compound as methoxycitronellal. Similarly, a peak was present in all chromatograms at 16.75 min. This compound was putatively identified as carvone. A further peak was detected at approximately 13.6 min in the triphala and *T. bellirica* methanolic and aqueous extracts, the *T. chebula* methanolic extract and the *E. officianalis* extracts. Analysis of the mass spectra of this peak identified this compound as camphor. A peak at 14.23 min in multiple extracts was identified as endo borneol. A further peak at 11.99 min was noted in the triphala, *T. bellirica* and *T. chebula* methanolic extracts, as well as the *T. bellirica* aqueous extract chromatograms. This compound was putatively identified as fenchone. The peaks present at 15.73 and 16.33 min in multiple extracts were identified as hyscylene and p-cumic aldehyde respectively. A peak was also detected at 14.56 min in all methanolic and aqueous extracts except the aqueous triphala extract. In contrast, this peak was lacking in all ethyl acetate extracts. Comparisons of the mass spectra of this peak with the ChemSpider data base putatively identified this compound as terpinen-4-ol.

Several other monoterpenoids were also identified, although most of these were present in fewer of the analysed extracts. The peak at 14.95 min in the *T. chebula* methanolic extract was identified as terpineol by comparing its mass spectra with the ChemSpider database. A peak at 12.23 min was only present in the *T. bellirica* methanolic and aqueous chromatograms as well as in the *T. chebula* aqueous extract. This compound was putatively identified as linalool. Pinocarveol was only identified as the compound at 13.41 min in the aqueous triphala extract chromatogram. The sesquiterpenoid patchoulane was also identified in all methanolic extracts analysed by comparison of its mass spectra with the ChemSpider database.

4. Discussion

The extracts were tested at the concentration in which they were prepared as an approximation of how they have been used traditionally. This provided an initial, semi-quantitative measure of antibacterial activity on a solid surface (agar), followed by a more sensitive determination of MIC in the broth microdilution assays. Although triphala is often used, and is a proportionate mixture of *T. bellirica*, *T. chebula* and *E. officianalis*, the separate components themselves have been shown to possess antimicrobial activity (Srikumar et al., 2007; Amanullah et al., 2010; Maji et al., 2010; Mehrotra et al., 2010; Hutchings and Cock, 2018; Mandeville and Cock, 2018). We set out to determine why triphala is commonly used as a formula and to investigate the activities and spectrum of activities against a panel of gastrointestinal bacteria that trigger a disease (diarrhoea) that may be traditionally treated by ingestion of concoctions of these plants.

The methanolic extracts are generally the most efficacious, with the best activities typically arising from the *T. chebula* component. Perhaps more interestingly are the effects on bacteria when specific combinations of active extracts are used. Potentiation of the activity of one extract with another extract occurred in several cases, providing a basis for the medicinal benefits of the triphala formulation. These interactions are complex, since they appear to be influenced not only by the solvent that is used to prepare the extract, but also the individual plant components that are combined and the bacterial species that are affected. Isobologram plots also revealed insights into the nature of the interactions. In some cases, one of the two components tested acts as the potentiator, while in others it eliminates synergy, possibly acting in an irreversible or non-competitive manner. In yet another case, a very small proportion of one component is all that is necessary to produce synergistic inhibition of growth. Together, these findings suggest that individually tailored plant formulations may be designed to treat specific illnesses, based on the combinations of extract types used and the bacterial species

involved in the disease. Additionally, it validates the use of the triphala components in mixtures, as they have been used traditionally for centuries, by providing a basis for implementing specific combinations of the plant extracts to boost activities against microbial triggers of disease.

Future investigations will be geared towards determining an expanded spectrum of activity against other bacterial pathogens, and to provide additional targeted therapies by altering the ratios of the components used. This may be further enhanced by studying the effects of a three-component system that involves *T. bellirica*, *T. chebula* and *E. officianalis* in specific ratios on bacterial growth, and to determine the mechanisms behind their bacteriostatic or bactericidal properties, with particular emphasis on the potentiation of these activities when extracts are used in mixtures.

Qualitative GC-MS analysis of the triphala, *T. bellirica*, *T. chebula* and *E. officianalis* methanolic, aqueous and ethyl acetate extracts identified a number of interesting compounds which may contribute to the antibacterial and antibiotic growth inhibitory activities reported in our study. Notably, multiple monoterpenoids were putatively identified in in the extracts. Methoxycitronellal, endo-borneol, terpinen-4-ol and cumic aldehyde were particularly prevalent, being detected in at least one extracts prepared from triphala and from each of the component plants. Similarly, eucalyptol and fenchone were present in triphala, *T. bellirica* and *T. chebula*, but not in *E. officianalis*. Many of these terpenoids have potent broad-spectrum antibacterial activity (Rajput et al., 2017) and therefore may contribute to the bacterial growth inhibitory activity reported here. The sesquiterpenoid compound patchoulane was also detected in triphala, as well as in all component plants and may also contribute to the growth inhibitory activity, or to the potentiating activity of the triphala formulation. It is likely that other phytochemical classes also contribute to the growth inhibitory properties these extracts. The qualitative phytochemical analysis studies reported that polyphenolics, flavonoids and tannins were present in relative abundance in triphala and all of the component plants. As our study used GC-MS techniques to putatively identify the phytochemical composition of the extracts, it is likely that many of the mid to higher polarity compounds may have not been identified in this study. High levels and a diversity of tannins are a common feature of all *Terminalia* spp., included those examined in this study (Cock, 2015). For example, gallic acid, ellagic acid and their methylated derivatives, chebulic acid, galloyl pyrogallol, corilagen, punicalin, castalagin and chebulagic acid have previously been reported in similar *T. chebula* extracts to those tested in our study (Cock et al., 2019). These tannins have potent, broad spectrum growth inhibitory

activity against a variety of bacterial species and function via multiple mechanisms, including interactions with cell surface proteins and intracellular enzymes (Hogg and Embery, 1982; Wu-Yuan et al., 1988; Buzzini et al., 2008). Therefore, it is likely that the tannin components may also contribute to the bacterial growth inhibitory activity noted herein. Future studies will further examine the antibacterial and antibiotic potentiating activity of these compounds.

5. Conclusions

Our study shows that combining the plant components of triphala does indeed have substantial potentiating effects, greatly increasing the antibacterial activity of the mixture against some bacteria. As triphala and the individual components each inhibited the growth of all bacterial pathogens tested, we were unable to determine whether the combination also extends the antibacterial spectrum. However, our study tested against a limited panel of bacteria (8 species) and future studies testing against further bacterial species may also provide evidence of further benefits of using triphala compared to the use of the individual components.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

Mr Gagan Tiwana, Dr Matthew Cheesman and Dr Ian Cock performed the antimicrobial, phytochemical and toxicity studies. Mr Alan White performed the GC-MS analysis. Dr Matthew Cheesman and Dr Ian Cock conceived and supervised the study and all authors were involved in the preparation of the manuscript.

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Abbreviations

DMSO	Dimethyl sulfoxide
EO	*Emblica officinalis*
FIC	Fractional inhibitory concentration
GC-MS	Gas chromatography-mass spectrometry
kHz	Kilohertz
LC_{50}	50% lethal concentration
MIC	Minimum inhibitory concentration

SEM Standard error of the mean

TA Triphala

TB *Terminalia bellirica*

TC *Terminalia chebula*

TI Therapeutic index

TIC Total ion count

ZOI Zone of inhibition

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Fig. 1. Antibacterial activities of extracts and reference antibiotics against *E. coli* (A), *S. sonnei* (B), *A. hydrophila* (C), *S.* Typhimurium (D), *S. flexneri* (E), *S. aureus* (F), *B. cereus* (G) and *E. faecalis* (H) by disc diffusion assays measured as ZOI in mm. The negative control discs (N) contained 1% DMSO. Values are expressed as mean ± SEM of triplicate assays. Results are shown as significantly different to the negative control if $p < 0.05$ (*) or $p < 0.01$ (**), and highly statistically significant if $p < 0.001$ (***). TA = triphala, TB = *T. bellirica*; TC = *T. chebula*; EO = *E. officinalis*; Pen = penicillin, Ery = erythromycin, Tet = tetracycline, Chl = chloramphenicol and Cip = ciprofloxacin.

Fig. 2. Isobolograms for combinations of plant component extracts at various ratios that showed synergistic antibacterial activity as shown in Table 4. TA = triphala, TB = *T. bellirica*; TC = *T. chebula*; EO = *E. officinalis.* The graphs are: TB + TC (A) and TB + EO (B) methanol extract combinations against *E. coli*; TB + TC (C), TB + EO (D) and TC + EO (E) methanol extracts against *A. hydrophila*, and TB + TC (F) aqueous extract combinations against *S. flexneri*. Results were generated from mean MIC values of four replicates for each extract. Ratio = % extract: % antibiotic. Ratios lying on or underneath the 0.5:0.5 line are considered to be synergistic (Σ FIC $\leq$ 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).

Table 1

Mass of dried extracted material, concentration after resuspension in 1 % DMSO, and qualitative phytochemical screenings of the resuspended extracts. TA = triphala, TB = *Terminalia bellirica*; TC = *Terminalia chebula*; EO = *Emblica officinalis*. +++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

Extract	Mass of Dried Extract (mg)	Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble phenols	Water Insoluble phenols	Cardiac Glycosides	Saponins	Triterpenoids	Phytosterols	Alkaloids	Flavonoids	Tannins	Anthraquinones
Aqueous													
TA	686	68.6	+++	+++	+++	-	-	-	-	-	+++	+++	-
TB	474	47.4	+++	+++	+++	-	-	-	-	-	+++	+++	-
TC	567	56.7	+++	+++	+++	-	+	-	-	-	+++	+++	-
EO	361	36.1	+++	+++	+++	-	-	-	-	+	+++	+++	-
Methanolic													
TA	404	40.4	+++	+++	+++	+	-	++	-	-	+++	+++	-
TB	368	36.8	+++	+++	+++	+	-	++	-	-	+++	+++	-
TC	707	70.7	+++	+++	+++	+	+++	++	-	+	+++	+++	-
EO	544	54.4	+++	+++	+++	+	-	++	-	-	+++	+++	-
Ethyl acetate													
TA	19	1.9	+++	+++	+++	-	-	-	-	-	++	+++	-
TB	52	5.2	+++	+++	+++	-	-	-	-	-	++	+++	-
TC	44	4.4	+++	+++	+++	-	-	-	-	-	+++	+++	-
EO	34	3.4	+++	+++	+++	-	-	-	-	-	+	+++	-

Table 2

MIC values (µg/mL) for the aqueous, methanolic and ethyl acetate triphala, *T. bellirica*, *T. chebula* and *E. officinalis* extracts and the reference (positive control) antibiotics against the eight bacterial strains tested in this study.

Solvent extractant	Plant or antibiotic	MIC (µg/mL)							
		E. coli	*E. faecalis*	*S. sonnei*	*A. hydrophila*	*S. typhimurium*	*S. flexneri*	*S. aureus*	*B. cereus*
Aqueous	**TA**	8575	8575	4287	4287	4287	4287	8575	8575
	TB	5925	5925	2962	*370*	2962	1481	**741**	5925
	TC	7087	7087	3544	3544	3544	1772	3544	1772
	EO	9025	4512	2256	2256	2256	2256	4512	2256
Methanolic	**TA**	2525	2525	2525	**631**	2525	**631**	1262	1262
	TB	1150	2300	1150	**575**	1150	*287*	**575**	**575**
	TC	2209	2209	2209	1105	2209	*276*	**552**	1105
	EO	3400	3400	3400	3400	3400	1700	1700	1700
Ethyl acetate	**TA**	>10000	>10000	>10000	**475**	>10000	**475**	>10000	>10000
	TB	>10000	>10000	>10000	*325*	>10000	*325*	1300	1300
	TC	>10000	>10000	>10000	**550**	1100	**550**	1100	**550**
	EO	>10000	>10000	>10000	>10000	>10000	>10000	>10000	>10000
Reference antibiotics	**Pen**	>2.5	>2.5	>2.5	>2.5	2.5	>2.5	>2.5	>2.5
	Ery	0.625	>2.5	>2.5	>2.5	>2.5	>2.5	0.625	0.078
	Tet	0.625	0.625	0.313	0.313	0.625	0.625	0.313	0.313
	Chl	>2.5	1.25	1.25	0.625	1.25	0.625	>2.5	>2.5
	Cip	0.625	<0.02	<0.02	<0.02	<0.02	<0.02	0.156	0.156

TA = triphala, TB = *T. bellirica,* TC = *T. chebula*, EO = *E. officinalis.* MIC values for extracts with noteworthy activity (400-1000 µg/ml) are in **bold** while those with good activity (100-

400 µg/ml) are *italicised*. Pen = penicillin; Ery = erythromycin; Tet = tetracycline; Chl = chloramphenicol; Cip = ciprofloxacin. The range of concentrations used in the assays was 0.01 – 15 mg/ml for the plant extracts and 0.01 – 2.5 µg/ml for the reference antibiotics.

Table 3

FIC and ∑FIC values, where relevant, for the combinations of the methanolic *T. bellirica*, *T. chebula* and *E. officinalis* extracts against seven of the bacterial strains used in this study.

Extract solvent	Combination tested	FIC or ∑FIC values	Bacterial strain						
			E. coli	*S. sonnei*	*A. hydrophila*	*S. typhimurium*	*S. flexneri*	*S. aureus*	*B. cereus*
Methanol	**TB + TC**	FIC_{TB}	0.225	0.134	0.224	0.500	0.500	0.500	0.500
		FIC_{TC}	0.219	0.468	0.219	0.500	1.000	1.000	0.499
		∑FIC	*0.444*	**0.602**	*0.443*	1.000	1.500	1.500	**0.999**
	TB + EO	FIC_{TB}	0.225	0.500	0.318	1.000	1.000	0.500	0.500
		FIC_{EO}	0.103	0.250	0.125	0.523	0.250	0.250	0.250
		∑FIC	*0.328*	**0.750**	*0.431*	1.523	1.250	**0.750**	**0.750**
	TC + EO	FIC_{TC}	0.421	0.500	0.219	0.500	2.000	1.000	0.499
		FIC_{EO}	0.103	0.250	0.063	0.250	0.250	0.250	0.250
		∑FIC	**0.523**	**0.750**	*0.281*	**0.750**	2.250	1.250	**0.749**

Synergistic ∑FIC values (≤0.5) are shown in *italics*, and additive values (>0.5-≤1.0) in **bold**. Indifferent values are >1.0-≤4.0. Antagonistic interactions (>4.0) were not observed. TB = *T. bellirica,* TC = *T. chebula*, EO = *E. officinalis.*

Table 4

Therapeutic index values (TI) for extracts of triphala and each of the botanical components against the tested bacterial species.

Plant	Extract solvent	*E. coli*	*E. faecalis*	*S. sonnei*	*A. hydrophila*	*S. typhimurium*	*S. flexneri*	*S. aureus*	*B. cereus*
TA	**M**	0.3	0.3	0.3	1.19	0.3	1.19	0.59	0.59
	W	0.09	0.09	0.18	0.18	0.18	0.18	0.09	0.09
	E	High	High	High	High	High	High	High	High
TB	**M**	High	High	High	High	High	High	High	High
	W	0.13	0.13	0.25	2.03	0.25	0.51	1.01	0.13
	E	High	High	High	High	High	High	High	High
TC	**M**	High	High	High	High	High	High	High	High
	W	0.11	0.11	0.21	0.21	0.21	0.42	0.21	0.42
	E	CND	CND	CND	1.36	0.68	1.36	0.68	1.36
EO	**M**	0.22	0.22	0.22	0.22	0.22	0.44	0.44	0.44
	W	0.08	0.17	0.33	0.33	0.33	0.33	0.17	0.33
	E	CND	CND	CND	CND	CND	CND	CND	CND

Numerical values were determined by LC_{50}/MIC. High = TI could not be determined as no toxicity was observed at the highest concentrations; these were therefore deemed to have high TI values. CND indicates that a TI could not be determined as the extract was inactive against the indicated bacterial species. TA = triphala, TB = *T. bellirica,* TC = *T. chebula*, EO = *E. officinalis.*

Table 5

Qualitative GC-MS headspace analysis of extracts prepared from triphala and its components, elucidation of empirical formulas, relative abundances and putative identification of each compound where possible. TA = triphala, TB = *T. bellirica,* TC = *T. chebula*, EO = *E. officinalis.*

Retention Time (min)	MW (Da)	Empirical Formula	Putative Identification	TA			TB			TC			EO		
				Relative abundances (% of peak area compared to total area under chromatogram)											
				M	W	E	M	W	E	M	W	E	M	W	E
4.29	100	$C_{10}H_{12}O$	Hexanal	-	1.37	-	-	0.74	-	-	0.61	-	-	-	-
4.59	116	$C_6H_{12}O_2$	n-Butyl acetate	-	-	0.09	-	-	-	-	-	1.25	-	-	-
4.78	78	C_2H_6OS	DMSO	68.11	85.34	97.56	19.47	88.15	98.52	58.25	94.93	90.36	49.04	95.78	92.83
6.646	118	$C_6H_{14}O_2$	2-Butoxy-ethanol,	27.29	0.49	0.07	19.81	0.41	0.08	33.05	0.17	-	44.44	0.21	-
8.018	112	$C_7H_{12}O$	(E)-2-Heptenal	-	1.31	-	-	0.25	-	0.41	-	-	-	0.19	-
8.647	158	$C_{10}H_{22}O$	n-Propyl heptyl ether	0.35	0.52	0.05	0.18	0.33	-	0.42	0.25	0.09	-	0.23	-
9.346	130	$C_8H_{18}O$	Octanal	0.18	0.51	0.11	0.1	0.48	0.06	0.46	0.15	0.77	0.24	0.4	0.3
10.122	130	$C_8H_{18}O$	2-Ethyl-1-hexanol	1.17	0.19	0.45	0.32	0.02	0.08	1.5	0.05	3.64	1.66	-	1.26
10.221	154	$C_{10}H_{18}O$	Eucalyptol	-	0.57	-	0.38	1.23	-	0.22	-	-	-	-	-
10.963	126	$C_8H_{14}O$	2-Octenal	-	0.31	-	-	-	-	0.13	-	-	-	-	-
11.355	130	$C_8H_{18}O$	1-Octanol	0.39	0.26	-	0.23	0.55	-	0.75	-	0.16	0.24	-	-
11.667	125	$C_8H_{15}N$	Octanenitrile	-	-	-	0.06	-	-	-	-	-	-	-	-
11.986	152	$C_{10}H_{16}O$	L-Fenchone	0.18	-	-	0.44	1.71	-	0.35	-	-	-	-	-
12.226	154	$C_{10}H_{18}O$	Linalool	-	-	-	0.05	0.36	-	-	0.05	-	-	-	-
12.344	142	$C_9H_{18}O$	Nonanal	0.24	0.08	-	0.29	0.47	0.02	0.82	0.16	0.14	0.21	0.07	-

12.72		Unable to determine		0.03	0.06	-	-	0.05	-	0.08	0.08	-	0.05	0.05	-
13.41	152	$C_{10}H_{16}O$	Pinocarveol	-	0.03	-	-	-	-	-	-	-	-	-	-
13.597	152	$C_{10}H_{16}O$	Camphor	0.16	0.46	-	0.25	1.07	-	0.05	-	-	-	0.04	-
13.917	186	$C_{11}H_{22}O_2$	Methoxycitronellal	0.34	3.16	0.17	0.22	0.45	0.15	0.36	0.62	0.21	0.35	0.37	0.43
14.231	154	$C_{10}H_{18}O$	Endo-borneol	-	0.06	-	0.02	0.1	-	0.16	-	-	0.08	0.07	-
14.559	154	$C_{19}H_{18}O$	Terpinen-4-ol	0.06	-	-	0.16	0.11	-	0.26	0.46	-	0.03	0.04	-
14.952	154	$C_{19}H_{18}O$	Terpineol	-	-	-	-	-	-	0.03	-	-	-	-	-
15.158	156	$C_{11}H_{24}$	2-Methyl-decane	0.11	0.19	0.05	0.05	0.16	0.02	0.19	0.07	0.05	0.06	0.07	0.12
15.447		Unable to determine		0.1	0.09	0.18	0.05	0.07	0.07	0.26	-	0.57	0.25	0.05	0.68
15.549	138	$C_9H_{14}O$	(E,E)-2,4-Nonadienal	-	0.02	-	-	-	0.08	0.02	-	0.14	-	-	0.08
15.726	166	$C_{10}H_{14}O_2$	Hyscylene	0.04	-	0.04	-	0.06	-	0.62	0.08	0.04	-	-	0.22
16.334	148	$C_{10}H_{12}O$	p-Cumic aldehyde	-	-	-	0.11	0.19	-	0.08	0.13	-	0.04	-	-
16.745	150	$C_{10}H_{14}O$	Carvone	0.29	0.11	0.13	0.17	0.34	0.12	0.28	0.34	0.18	0.03	0.31	0.42
16.901	154	$C_{10}H_{18}O$	(E)-2-Decenal	0.04	0.11	-	0.02	0.05	-	0.2	-	-	0.32		-
17.029	158	$C_9H_{18}O_2$	Nonanoic acid	0.02	-	-	0.02	-	-	0.03	-	-	-	-	-
17.228	148	$C_{10}H_{12}O$	2-Phenylbutanal	0.11	-	0.11	0.05	0.07	0.04	0.13	0.03	0.32	0.21	0.04	1.55
18.66				-	0.03	0.03	0.04	0.04	0.03	0.03	-	0.14	0.09	0.1	0.12
19.426	286	$C_{16}H_{30}O_4$	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	-	0.46	0.16	0.02	0.39	0.06	-	0.25	0.31	0.09	0.4	0.19
19.984	216	$C_{12}H_{24}O_3$	2-Ethyl-3-hydroxyhexyl 2-methylpropanoate	0.04	0.79	0.26	0.04	0.66	0.11	0.03	0.45	0.51	0.1	0.71	0.29
22.919	206	$C_{15}H_{26}$	Patchoulane	0.08	-	-	0.04	-	-	0.07	-	-	0.39	-	-
24.671		Unable to determine		0.21	0.15	0.22	0.13	0.22	0.18	0.51	0.12	0.4	1.25	0.05	0.72
27.504		Unable to determine		0.11	0.31	0.05	-	-	0.04	0.08	-	0.06	0.1	0.09	0.12

27.599	286	C_{16} H_{18} O_3	1-Isobutyl 4-isopropyl 3-isopropyl-2,2-dimethylsuccinate	0.15	0.38	0.15	0.26	0.11	0.11	0.16	0.19	0.18	0.42	0.45	0.39

The relative abundance is a measure of the area under the peak expressed as a % of the total area under all chromatographic peaks. – indicates that a compound was not detected in that extract.