RESEARCH NOTE

Anti-pathogenic potential of a classical *ayurvedic* ~~formulation-~~ *Triphala* [version 1; peer review: 1 approved with reservations]

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Abstract

A classical *ayurvedic* polyherbal formulation namely *Triphala* was assessed for its anti-pathogenic potential against five different pathogenic bacteria. Virulence of four of them towards the model host *Caenorhabditis elegans* was attenuated (by 18-45%) owing to pre-treatment with *Triphala* (≤20 µg/ml). ~~*Triphala*~~ could also exert significant therapeutic effect on worms already infected with *Chromobacterium violaceum*, *Serratia marcescens* or *Staphylococcus aureus*. Prophylactic use of ~~*Triphala*~~ allowed worms to score 14-41% better survival in face of subsequent pathogen challenge. Repeated exposure to this formulation induced resistance in *S. marcescens*, but not in *P. aeruginosa*. It also exerted a post-extract effect (PEE) on three of the test pathogens. ~~*Triphala*~~ was able to modulate production of quorum sensing (QS)-regulated pigments in three of the multidrug-resistant gram-negative test bacteria. Haemolytic activity of *S. aureus* was heavily inhibited under the influence of this formulation. *P. aeruginosa's* lysozyme-susceptibility was found to increase by ~25-43% upon ~~*Triphala*~~ -pretreatment. These results validate therapeutic potential of one of the most widely used polyherbal *ayurvedic* formulations called *Triphala*.

Keywords

Antimicrobial resistance (AMR), Quorum Sensing (QS), Triphala, Polyherbal, Post Extract Effect (PEE), Anti-virulence, Lysozyme

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	1
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Any reports and responses or comments on the article can be found at the end of the article.

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Background

Antibiotic-resistant bacterial infections are among the most serious public-health threats. Since the emergence and spread of antimicrobial resistance (AMR) is shrinking the utility spectrum of conventional bactericidal antibiotics, there is an urgent need for discovery and development of novel anti-virulence formulations. Traditional Medicine (TM) systems like *Ayurveda* offer several sophisticated formulations for a variety of disease conditions. One such classical ayurvedic formulation with a long history of safe use is *Triphala*. *Triphala* is a polyherbal formulation containing three myrobalans fruits i.e. *Phyllanthus emblica, Terminalia chebula,* and *Terminalia bellerica* (Patwardhan *et al.*, 2015). Triphala is prescribed as a general health promoter, for management of metabolic disorders, dental and skin problems, and for wound management. It has been reported to be active against bacterial pathogens of urinary tract (Bag *et al.*, 2013), and as an anticaries agent for control of gum infections (Bhattacharjee *et al.*, 2015; Prakash & Shelke, 2014). Though many popular formulations like *Triphala* have been used historically in TM and as a household remedy, their validation through modern scientific methods is necessary for their wider acceptance in modern medicine (Kothari, 2018). This study aimed to investigate the anti-pathogenic efficacy of ~~*Triphala*~~ against five different pathogenic bacteria.

Methods

Test formulation

Triphala formulation (TF) (Emami Ltd; batch no. EM0029; Proportion of 3 constituent plant species: 1:1:1) was purchased from a local market. For assay purpose, 150 mg of this formulation was suspended in 5 ml of DMSO (Merck, Mumbai), followed by vortexing for 15 min. Then it was centrifuged at 8,000 rpm for 30 min at ambient temperature, and resulting supernatant was collected in a sterile glass vial (15 ml; Borosil) and stored under refrigeration till further use. Remaining pellet was subjected to drying in an oven at 70-80°C until the solvent was completely evaporated, followed by weighing of the dried plant material. Subtracting the latter from the initial weight of 150 mg, the concentration of test formulation in supernatant was calculated to be 22.94 mg/ml. This way the whole formulation was found to contain 70% DMSO soluble fraction, which was used for our experiments.

Bacterial strains

Chromobacterium violaceum ~~(~~MTCC 2656~~)~~, *Serratia marcescens* ~~(~~MTCC 97~~)~~, *Staphylococcus aureus* ~~(~~MTCC 737~~)~~, and *Streptococcus pyogenes* ~~(~~MTCC 1924~~)~~ were procured from Microbial Type Culture Collection (MTCC), Chandigarh. *Pseudomonas aeruginosa* was available in our institutional culture collection. All the three gram-negative bacteria used in this study were multidrug resistant, and their antibiogram has previously been reported by us (Joshi *et al.*, 2019; Patel *et al.*, 2019a). Additionally, *C. violaceum* and *S. marcescens* strains mentioned here have been reported by us as beta-lactamase producers (Sarvaiya & Kothari, 2017).

In vivo assays

In vivo efficacy of ~~*Triphala*~~ against bacterial infections was tested in the nematode host *Caenorhabditis elegans* (N2-Bristol ~~strain;~~ maintained at the Institute of Science, Nirma University). Maintenance and synchronization of the worm population was done as previously described in Joshi (2019). Worms were maintained on NGM [Nematode Growing Medium; 2.5 g/L peptone (HiMedia, RM001-500G), 3 g/L NaCl (HiMedia, MB023-500G), 1 M $CaCl_2$ (HiMedia, GRM534-500G), 1 M $MgSO_4$ (Merck, 1.93645.0521), 5 mg/mL cholesterol (HiMedia, TC101-5G), 1 M phosphate buffer of pH 6, 17 g/L agar-agar (HiMedia, GRM666-500G)] agar plate with *E. coli* OP50 (LabTIE B.V. OP50 V.2; batch # 002, JR Rosmalen, the Netherlands) as food. For synchronization of the worm population, adult worms from a 4–5 days old NGM plate were first washed with distilled water, and then treated with 1 mL of bleaching solution [1N NaOH (HiMedia MB095-100G) + 4% NaOCl (Merck 61842010001730) + water in 1:1:3 proportion], followed by centrifugation ~~(~~22°C~~; 1,500 rpm)~~ for 1 min. Eggs in the resultant pellet were washed with sterile distilled water, and then transferred onto a new NGM plate seeded with *E. coli* OP50. L3-L4 stage worms appearing on this plate after 2–3 days of incubation at 22°C were used for further experiments.

Three different types of *in vivo* assays were done as under, employing the methodology described in reference cited in parenthesis following the assay name:

Anti-infective assay (Patel *et al.*, 2018a): ~~*Triphala*~~ exposed-pathogenic bacteria were allowed to infect *C. elegans* (L3-L4 stage), and their capacity to kill the worm population was compared with their ~~*Triphala*~~ unexposed counterparts, over a period of 5 days.

Prophylactic assay (Patel *et al.*, 2018b): ~~*Triphala*~~ fed worms were challenged with pathogenic bacteria (previously not exposed to the test formulation), and their ability to survive in face of pathogen challenge was compared with their ~~*Triphala*~~ unfed counterparts. *C. elegans* worms maintained on NGM were kept unfed for 24 h prior to being used for experiments. These worms were then fed with TF by mixing required concentration of this formulation (100 µL) with M9 medium (800 µL) and placed in a 24-well plate (non-treated polystyrene plates, sterile; HiMediaTPG24-1X50NO) containing 10 worms per well. Duration of exposure of worms to TF was kept to 96 h, followed by addition of pathogenic bacteria (100 µL of bacterial suspension with OD_{764} = 1.50 measured with Agilent Cary 60 UV-Vis spectrophotometer). Appropriate controls i.e. worms previously not exposed to TF, but exposed to pathogenic bacteria; worms exposed neither to TF nor bacteria; and worms exposed to TF, but not to bacterial pathogens, were also included in the experiment. Incubation was carried out at 22°C. Number of dead vs. live worms were counted every day for 5 days by putting the plate (with lid) under a light microscope (4X; Catalyst Biotech CatScope CS-U207T). Straight worms were considered to be dead. Plates were gently tapped to confirm lack of movement in the apparently-dead worms. On the last day of the experiment, when plates could be opened, their death was also confirmed by touching them with a straight wire, wherein no movement was taken as confirmation of death.

~~*Triphala*~~ as a post-infection therapy (Patel *et al.*, 2019b): Worms already infected with pathogenic bacteria (not previously exposed to the test formulation) were treated with ~~*Triphala*~~

to see whether the test formulation can exert any therapeutic effect on already infected worms. Assay methods remained the same as described in previous section, except that TF was added into assay wells after allowing bacteria either for 6 h or 24 h to establish infection.

Catechin (Sigma Aldrich; C1251-5G) and standard antibiotics (HiMedia; Ampicillin CMS645-1G; Gentamicin TC026-1G; Chloramphenicol CMS218-5G; Vancomycin CMS217-500MG) were used as positive controls. Catechin was employed at 100 µg/ml, whereas different sub-MIC concentrations (0.1–5 µg/ml) of the antibiotics were used against different organisms as per their susceptibility.

Videos of some of the *in vivo* assays were captured on the fifth day of the experiment, using an inverted microscope (Nikon Eclipse Ti) under 4X objective lens, wherein 100 µl of the liquid content from 24-well assay plate was transferred onto a large cover slip for observation and video capturing [see extended data (Patel *et al.*, 2019c)].

In vitro assays

After confirming the *in vivo* anti-pathogenic efficacy of the ~~*Triphala* formulation~~, we performed following *in vitro* assays to gain insight into interaction of this formulation with the pathogenic bacteria, as per the methodology described in respective references mentioned in the parenthesis:

a. Broth dilution assay (Joshi *et al.*, 2016) to investigate effect of ~~*Triphala*~~ on bacterial growth and quorum sensing (QS)-regulated pigment production: *C. violaceum*, and *S. marcescens* were inoculated in nutrient broth (HiMedia MV002-500G) supplemented with TF. Media used for *S. aureus* and *P. aeruginosa* were Staphylococcus broth (HiMedia M578-500G) and Pseudomonas broth [10 g/l potassium sulfate (SRL 44277), 1.4 g/l magnesium chloride (Merck 1.9366.30521), 16 g/l peptone (HiMedia RM001-500G)] respectively. *S. pyogenes* was grown in BHI (brain heart infusion; HiMedia MV210-500G) broth. Following incubation, cell density was measured at 750 nm (Biorad 680). Pigment from these culture broths were extracted as previously described by us in Joshi *et al.* (2016). Cell pellets of *C. violaceum*, *S. marcescens*, and *S. aureus* were dissolved in DMSO [(Merck 1.07046.2521), acidified methanol [4 ml HCl (HiMedia AS003-500ML) into 96 ml of methanol], and methanol (Merck 1.94516.2521) respectively. This allowed their pigments to be extracted in the solvent applied. In case of *P. aeruginosa*, pigment extraction was achieved by mixing chloroform (Merck 1.67024.0521) with culture broth (2:1 ratio). Quantification of each pigment was done at the wavelength nearest to its $\lambda_{max,}$ available in the microplate reader (Biorad 680) used by us.

b. Effect of ~~*Triphala*~~ on biofilm formation and its possible potential to eradicate pre-formed biofilm was assessed through crystal violet assay (Patel *et al.*, 2013); and its effect on biofilm viability was assessed through MTT assay (Trafny *et al.*, 2013). For the crystal violet assay, the biofilm-containing tubes (after discarding the inside liquid) were washed with PBS in order to remove all nonadherent bacteria and air-dried for 15 min. Then, each of the washed tubes was stained with 1.5 mL of aqueous crystal violet solution (0.4%; SRL 54862-9) for 30 min. Afterwards, each tube was washed twice with 2 ml of sterile distilled water and immediately destained with 1500 µL of ethanol (95%). After 45 min of destaining, 1 mL of destaining solution was transferred into separate tubes and read at 570 nm (Biorad 680). For the MTT assay, the biofilm-containing tubes (after discarding the inside liquid) were washed with PBS in order to remove all nonadherent bacteria and air-dried for 15 min. Then, 900 µL of minimal media was added into each tube, followed by addition of 100 µL of 0.3% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HiMedia MB186-100MG). After 2 h incubation at 37°C, resulting purple formazan derivatives were dissolved in DMSO and measured at 570 nm (Biorad 680).

c. Effect of ~~*Triphala*~~ on haemolytic potential of the test pathogens (Neun *et al.*, 2015): Small volume of human blood required for this assay was obtained from the authors, who each gave their written informed consent. The use of this blood was approved by the Institutional Ethics Committee of the Institute of Science, Nirma University (approval no: EC/NU/18/IS/4). Blood collection was executed by one of the authors (AA) on three different times in heparinized vials. OD_{750} of the overnight grown (in presence or absence of TF) culture was standardized to 1.00. Cell-free supernatant was prepared by centrifugation at 15,300 g for 10 min. 10 µl of human blood was incubated with this cell-free supernatant for 2 h at 37°C, followed by centrifugation at 800 g for 15 min. OD of the supernatant was read at 490 nm, to quantify the amount of hemoglobin released. 1% Triton X-100 (CDH, New Delhi; CDH030632) was used as a positive control. Phosphate buffer saline was used as a negative control.

d. Effect of ~~*Triphala*~~ on lysozyme-susceptibility of test pathogens: Bacterial cells were first inoculated in a TF-containing media for 24 h, and then the cell pellet was separated by centrifugation ~~[~~15,000 rpm ~~(21130 g)~~ for 15 min~~]~~ to be resuspended into phosphate buffer saline (PBS; pH 7.4), so as to attain OD_{750}=1 (Biorad 680). 200 µl of this bacterial suspension was mixed with lysozyme (750 µg/ml; Sigma Aldrich L6876-1G) prepared in PBS, and then incubated for 24 h at appropriate temp for each organism. At the end of incubation OD_{750} was measured.

Effect of TF on probiotic strains

Bifidobacterium bifidum ~~(~~NCDC 255~~)~~, *Enterococcus faecium* ~~(~~NCIM 5366~~)~~, and *Lactobacillus plantarum* ~~(~~MTCC

2621) were grown in Lactobacillus MRS broth (HiMedia GM369-500G) containing TF, in screw capped tubes at 37°C for 22-24 h. For *B. bifidum*, this medium was supplemented with 0.05% cysteine (HiMedia PCT0305-25G). Effect of TF (10–100 µg/ml) on these bacteria was interpreted by comparing their cell density [OD_{655} measured with microplate reader (Biorad 680)] in TF-supplemented media to that in TF-free media.

Statistical analysis

All the experiments were performed in triplicate, and measurements are reported as mean ± standard deviation (SD) of 3 independent experiments. Statistical significance of the data was evaluated by applying *t*-test using Microsoft Excel® 2013. *p* values ≤0.05 were considered to be statistically significant. Trial version of GraphPad Prism 7 was used to make Kaplan-Meier survival curve for worms.

Results

In vivo experiments

Anti-infective assay. When all the five pathogens were pre-treated with *Triphala* (0.5-100 µg/ml) before being allowed to attack *C.elegans*, *Triphala* formulation (TF) was able to attenuate virulence of all test pathogens except *S. pyogenes* at ≤20 µg/ml [Figure 1; underlying data (Patel *et al.*, 2019c)]. Worms challenged with TF-treated pathogens demonstrated 18–45.50% better survival than those challenged with TF-unexposed pathogens. Effect of catechin and standard antibiotics (both used as positive controls) on bacterial virulence is shown in Figure 2 [underlying data (Patel *et al.*, 2019c)].

After confirming the anti-infective activity of TF, we investigated (as described in Patel *et al.*, 2019a) whether this formulation exerts any post-extract effect (PEE - https://doi.org/10.32388/359873) on the test pathogens i.e. whether the virulence-attenuating effect suffered by the parent bacterial culture is retained even in their daughter population never receiving any direct exposure to TF. When the TF-treated bacteria were subsequently subcultured on TF-free media, their daughter populations were unable to exert virulence at par with that of control (DMSO-treated parent culture). In case of *P. aeruginosa* and *S. aureus*, this PEE lasted up to the second subculturing, whereas in the case of *S. marcescens* PEE lasted until first subculturing [Figure 3; underlying data (Patel *et al.*, 2019c)].

TF as a post-infection therapy. To test the therapeutic efficacy of TF in already-infected worm population, we first allowed different pathogenic bacteria (not previously exposed to TF) to infect *C. elegans* either for 6 h or 24 h, and then exposed the infected worms to two different concentrations of TF. TF failed to exert any therapeutic effect on worms infected with *P. aeruginosa*. However, it could exert significant (p≤0.05) therapeutic effect on worms infected with *C. violaceum* or *S. marcescens*. Against *S. aureus*, TF was effective only if the worms were given TF-treatment early (i.e. 6 hour-post infection) [Figure 4; underlying data (Patel *et al.*, 2019c)]. TF could also not rescue the worms if they already had a mixed infection (*S. aureus* and *P. aeruginosa*).

Prophylactic potential of TF. To investigate whether previous feeding with TF can make the worm population tolerate subsequent challenge with pathogenic bacteria (not treated with TF) better; worms were first maintained in a TF-containing M9 buffer for 96 h, and then challenged with different bacterial pathogens. Such TF-fed worms scored 14.50–41.50% better survival in face of pathogen challenge [Figure 5; underlying data (Patel *et al.*, 2019c)]. However, TF did not confer any prophylactic benefit on the worm population against mix-culture challenge of *P. aeruginosa* and *S. aureus*. Since prophylactic activity of any formulation can be said to stem mainly from its effect on the host, we also compared whether TF imparts any extension of longevity on the worm. Worms fed with TF (10-20 µg/ml) registered marginally better survival up to 11 days (Figure 6).

Repeated exposure of test pathogens to TF. Since one of the major challenges with even the most potent antimicrobial agents/formulations is development of resistance against them by the pathogen populations, we tested whether repeated exposure of the test pathogens to TF can induce any resistance in them. For this, we subcultured two of the gram-negative test pathogens (*P. aeruginosa* and *S. marcescens*) in TF (50 µg/ml)-containing broth for 10 subsequent times, and then the 'TF-habituated' cultures thus obtained were tested for their virulence towards the nematode host. Repeated TF-exposure was found to induce resistance in *S. marcescens* [Figure 7A; underlying data (Patel *et al.*, 2019c)]. Though TF-habituated *P. aeruginosa* could kill more worms than its counterpart receiving single TF-exposure, it still could not kill as many worms as TF-unexposed *P. aeruginosa* [Figure 7B; underlying data (Patel *et al.*, 2019c)]. These results indicate that it may be difficult for the pathogenic bacteria to develop complete resistance against polyherbal formulations like *Triphala*, but not impossible. Though our previous results on other polyherbal formulations (Joshi *et al.*, 2019; Patel *et al.*, 2019a) and multicomponent crude plant extracts (Joshi, 2019) have indicated that the probability of pathogens developing resistance against multi-component anti-virulence preparations is low, such a probability can certainly not be ruled out (Kalia *et al.*, 2014; Singh, 2014)

In vitro experiments

Since TF showed significant *in vivo* anti-pathogenic potential in the *C. elegans* model, we performed various *in vitro* experiments to gain insight into its interaction with the target pathogens. TF was able to modulate production of quorum sensing (QS)-regulated pigments in all the three gram-negative bacteria [Figure 8; underlying data (Patel *et al.*, 2019c)]. It did so with *S. marcescens* without affecting its growth, which is characteristic of an ideal anti-virulence agent i.e. attenuation of virulence without exerting any selection pressure on the susceptible pathogen. However, TF did exert a growth-inhibitory effect on *P. aeruginosa*, wherein its IC_{50} was observed to be near 50 µg/ml. The quorum modulatory effect of TF on pigment production in *P. aeruginosa* was observed not to be amenable to be described by a linear dose-response curve. It seems to fall within the realm of hormesis (Calabrese, 2004). For example, production of both pigments was not affected

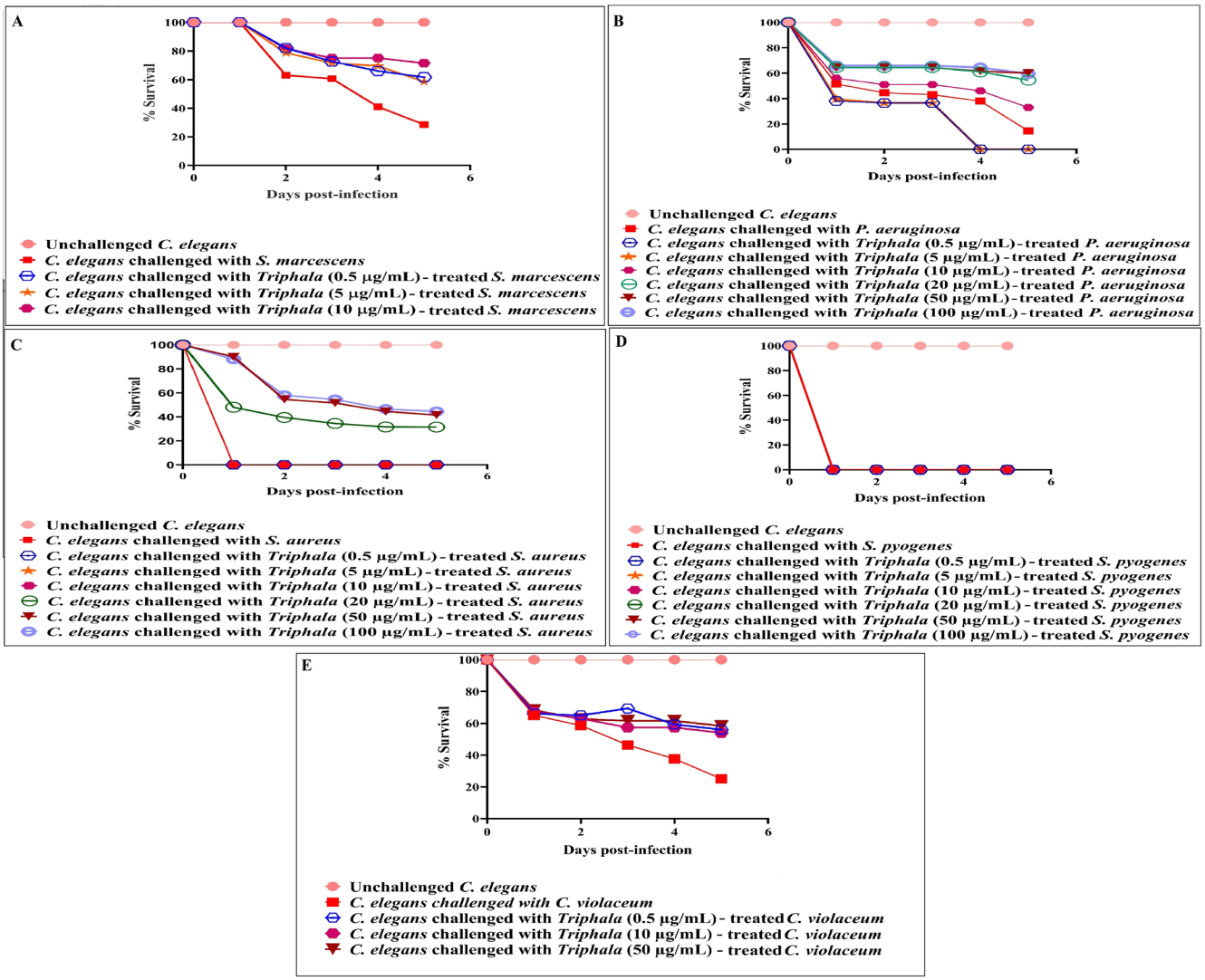

Figure 1. Anti-infective activity of *Triphala* formulation against test pathogens. TF-treatment attenuated virulence of four of the test pathogens towards *C. elegans*. DMSO present in the 'vehicle control' at 0.5%v/v did not affect virulence of any of the bacterium towards *C. elegans*; DMSO (0.5%v/v) and TF at tested concentrations showed no toxicity towards the worm. (**A**) TF at 0.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml, and 100 µg/ml allowed 32%±5.10, 29%***±9.60, 41.9%***±2.40, 38.4%***±7.10, 39.9%***±9.40, and 41.4%***±7.10 better survival of the worm population respectively, when challenged with *S. marcescens*. Also see videos a-b submitted as part of extended data. (**B**) TF at 10 µg/ml, 20 µg/ml, 50 µg/ml, 100 µg/ml allowed 18%***±5.01, 40%***±7.01, 45.5%***±0, and 45%***±5.00 better survival of the worm population respectively, when challenged with *P. aeruginosa*. (**C**) TF at 20 µg/ml, 50 µg/ml and 100 µg/ml allowed 31.5%±***2.35, 41.5%***±2.35and 44.5%***±14.14 better survival of the worm population respectively, when challenged with *S. aureus*. (**D**) TF-treatment did not attenuate virulence of *S. pyogenes* towards the worms. (**E**) TF at 0.5µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml, 100 µg/ml allowed 31%***±5.77, 29.5%***±8.81, 29%***±1.92, 29%***±1.92, 32.3%***±1.92, and 32.3%**±5.09 better survival of the worm population respectively, when challenged with *C. violaceum*. *p<0.05, **p<0.01, ***p<0.001; TF: *Triphala* Formulation.

~~maximally at the highest test concentration~~. Pyocyanin production was inhibited ~~more~~ at 0.5 µg/ml ~~than at 20~~ µg/ml. ~~Effect of TF on pyoverdine production followed a linear threshold model within concentration range of 0.5-50 µg/ml, but it took an inverted-U shape over 20-100 µg/ml. Though the exact mechanism responsible for a non-linear dose-response relationship is not known, it may be the varying magnitude of bacterial adaptive response at different concentrations of the test agent, which generates such non-linear response curves (Lushchak, 2014).~~

We also tested the effect of TF on two important virulence traits of the bacterial pathogens i.e. haemolytic activity, and biofilm. Though TF could not curb haemolytic activity of any of the gram-negative bacteria, this activity of *S. aureus* was heavily inhibited under the influence of TF [Figure 9; underlying data

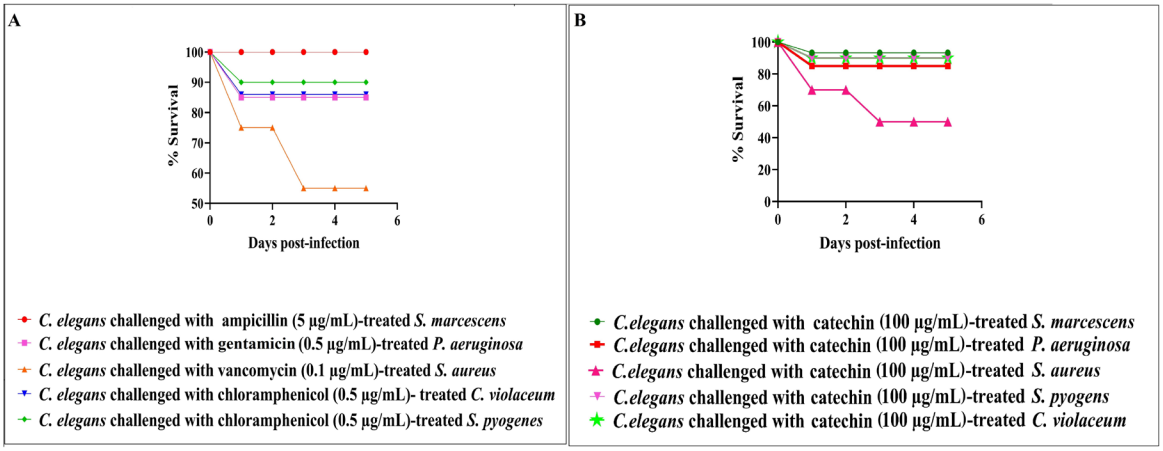

Figure 2. Anti-infective activity of positive controls (catechin and antibiotics) against test pathogens. Catechin was able to reduce virulence of different test bacteria towards *C. elegans* by 50–100% ($p \leq 0.05$). Various standard antibiotics at sub-MIC level could reduce bacterial virulence by 55–100% ($p \leq 0.05$).

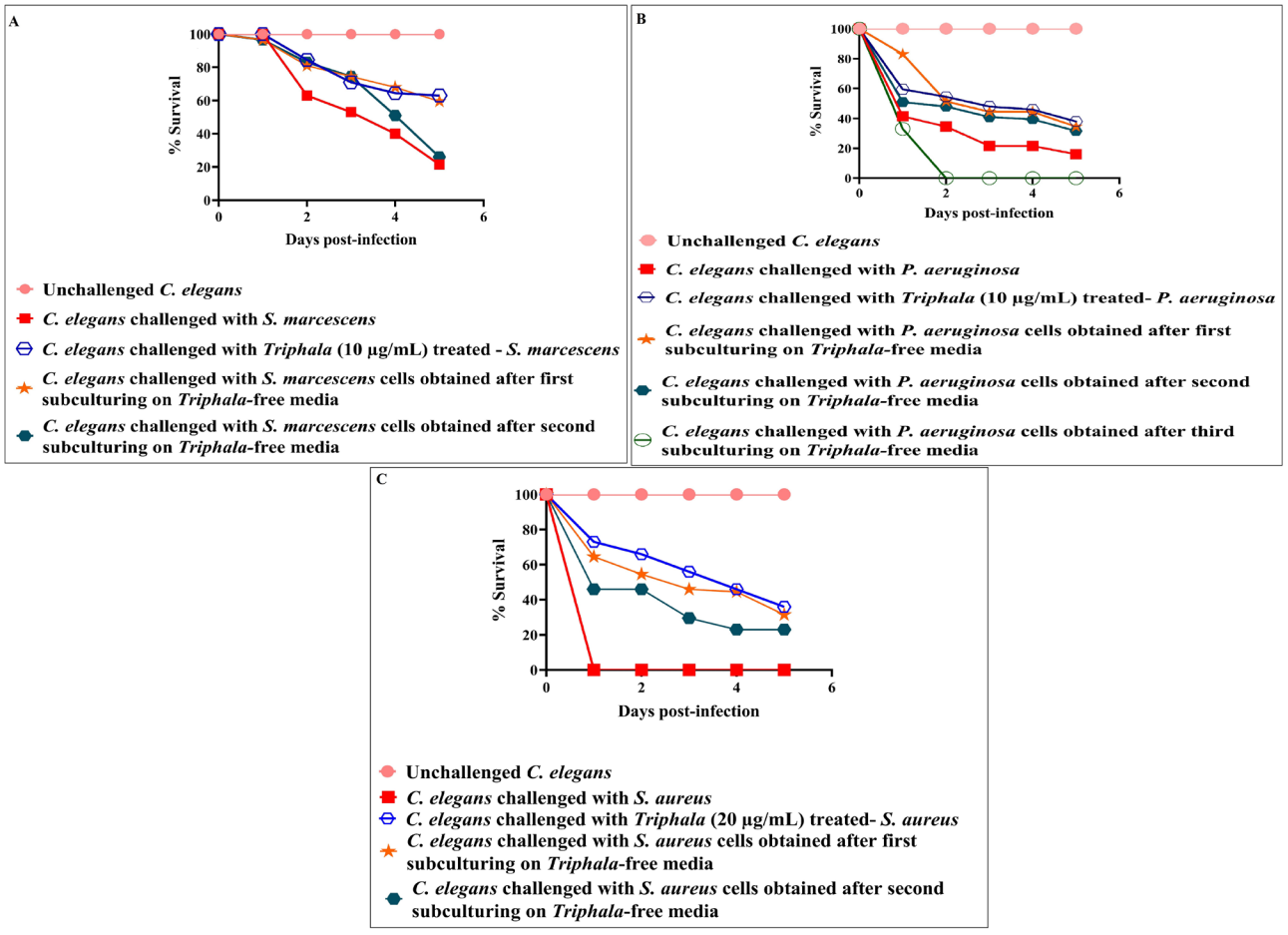

Figure 3. Post extract effect of *Triphala* on test pathogens. TF-treatment reduces the virulence of all the test pathogens towards *C. elegans* even after subculturing of cells in TF-free media. DMSO present in the 'vehicle control' at 0.5% v/v did not affect virulence of the bacterium towards *C. elegans*; DMSO (0.5%v/v) and TF at tested concentrations showed no toxicity towards the worm. (**A**) *S. marcescens* obtained after first subculturing on TF-free media were able to kill 38%***±4.71 lesser worms than control; (**B**) *P. aeruginosa* and obtained after first and second subculturing on TF-free media were able to kill 18.5%**±2.35 and 15.5%*±2.35 lesser worms respectively, than control; (**C**) *S. aureus* obtained after first and second subculturing on TF-free media were able to kill 31.5%***±2.35 and 23%***±0 lesser worms respectively, than control. *p<0.05, **p<0.01, ***p<0.001; TF: *Triphala* Formulation.

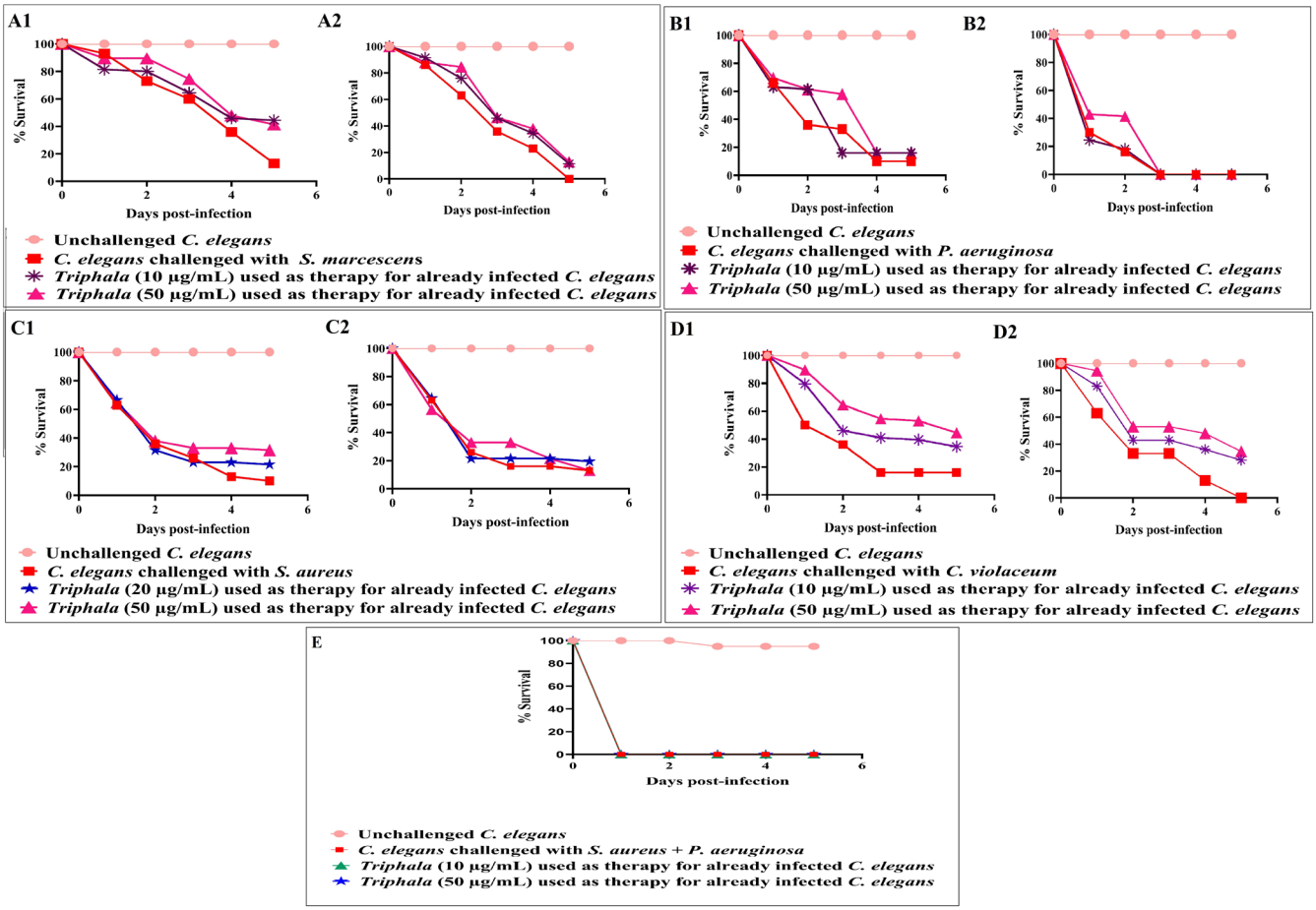

Figure 4. Assessing efficacy of *Triphala* as a post-infection therapy in pre-infected worms. DMSO (0.5%v/v) did not affect survival of pre-infected worms. DMSO (0.5%v/v) and TF at tested concentrations showed no toxicity towards the worm. **A1**–**D1**: TF employed 6 h post-infection **A2**–**D2**: TF employed 24 h post-infection (**A1**) TF at 10 µg/ml, 50 µg/ml used as therapy for already *S. marcescens* infected *C. elegans* after 6 h incubation conferred 31.5%***±2.35 and 28.5%***±2.35 survival benefit, respectively; (**A2**) TF at 10 µg/ml, 50 µg/ml used as therapy for already *S. marcescens* infected *C. elegans* after 24 h incubation conferred 11.5%***±2.35 and 13%*** survival benefit, respectively; (**B1**–**B2**):TF could not rescue *P. aeruginosa*-infected worms when tested as post-infection remedy. (**C1**) TF at 20 µg/ml, 50 µg/ml used as therapy for already *S. aureus* infected *C. elegans* after 6 h incubation conferred 11.5%***±2.35 and 21.5%***±2.35 survival benefit, respectively; (**C2**) TF could not rescue *S. aureus* infected worms when tested as post-infection remedy. (**D1**) TF at 10 µg/ml, 50 µg/ml used as therapy for already *C. violaceum* infected *C. elegans* after 6 h incubation conferred 18.5%***±2.35 and 28.5%***±2.35 survival benefit, respectively; (**D2**) TF at 10 µg/ml, 50 µg/ml used as therapy for already *C. violaceum* infected *C. elegans* after 24 h incubation conferred 22%***±2.35 and 28.5%***±2.35 survival benefit, respectively. (**E**) TF could not rescue the worms in face of mix-culture infection by *S. aureus* and *P. aeruginosa*, when tested as post-infection remedy. Survival benefit refers to the difference between number of worms surviving in experimental and control wells. *p<0.05, **p<0.01, ***p<0.001; TF: *Triphala* Formulation.

(Patel *et al.*, 2019c)]. While *P. aeruginosa* biofilm was not affected by TF, TF was able to reduce biofilm formation by *S. marcescens*, and *S. aureus*. When TF was applied on pre-formed bacterial biofilms, it seemed to enhance synthesis of the biofilm matrix material (quantified thorough crystal violet assay), and also the metabolic activity (measured in terms of organism's ability to reduce MTT) of the bacterial biofilm [Figure 10; underlying data (Patel *et al.*, 2019c)]. It may be speculated that TF-treatment induces stress in the bacterial population residing in biofilm form, and this causes the bacteria to mount stress-response. Slow metabolism is a general characteristic of bacterial biofilms (Singh *et al.*, 2017), but TF seems to have forced the biofilms of two of our test bacteria to enhance the rate of their metabolic activity, as well as synthesis/secretion of biofilm matrix components (e.g. polysaccharides, proteins, and extracellular DNA). Enhanced production of exopolysaccharide and e-DNA is believed to occur in stressed bacterial populations (Chang *et al.*, 2007; Zatorska *et al.*, 2018). Sub-inhibitory concentrations of beta-lactam antibiotics have been reported to induce extracellular

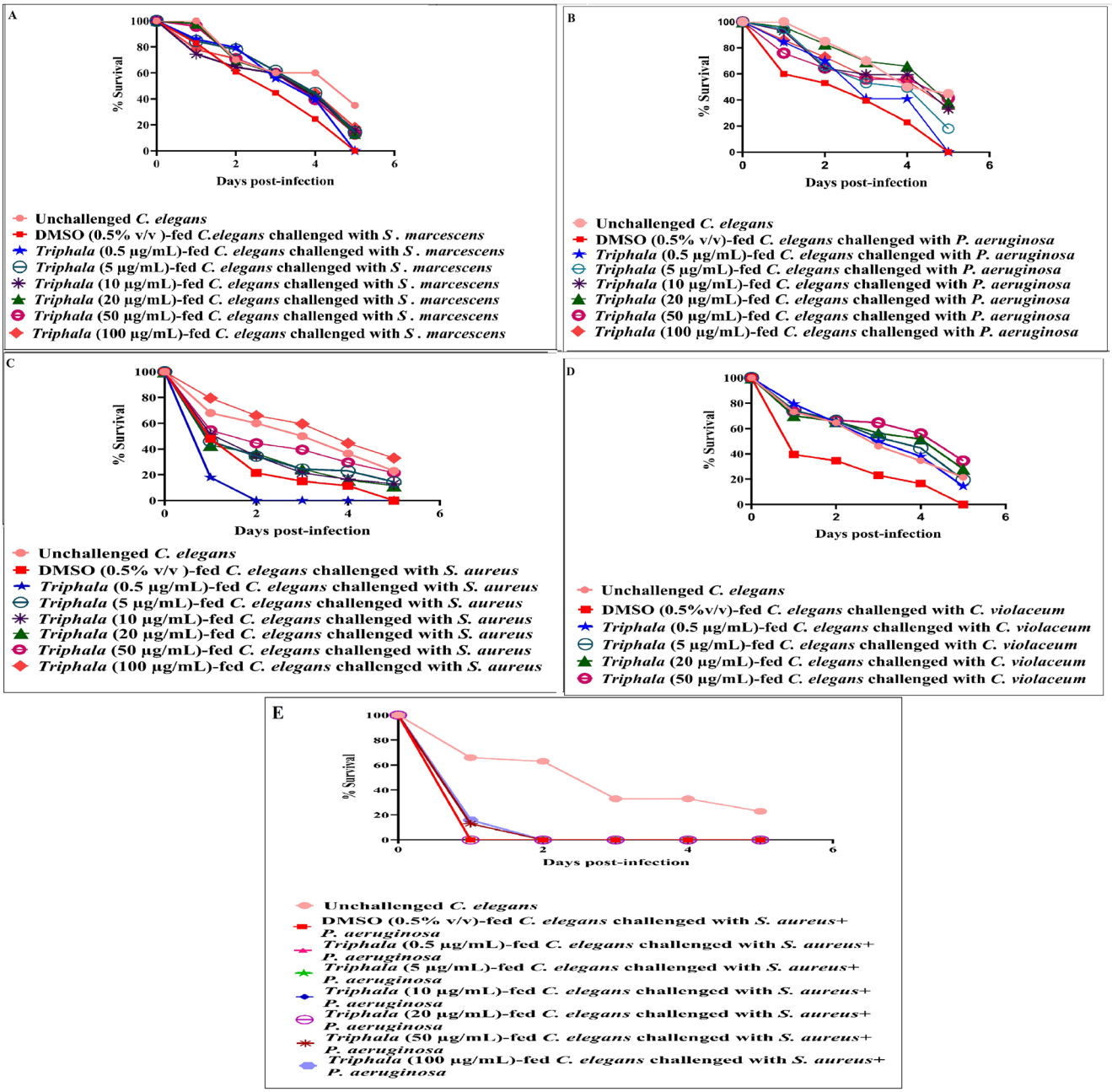

Figure 5. TF-pre-exposed *C. elegans* exhibit better resistance to subsequent bacterial challenge. Pre-treatment of worms with DMSO (0.5%v/v) did not alter their susceptibility to subsequent challenge with pathogenic bacteria. DMSO (0.5%v/v) and TF at tested concentrations showed no toxicity towards the worm. Among positive controls, catechin (100 µg/ml) pre-treatment conferred 23%±0 protection on worm population against subsequent *S. marcescens, P. aeruginosa, S. aureus,* and *C. violaceum* challenge; (**A**) Ampicillin (5 µg/ml) pre-treatment conferred 26%±0 protection on worm populations against subsequent *S. marcescens* challenge; TF (100 µg/ml) pre-treatment conferred 18%***±2.35 protection on fifth day worm population against subsequent *S. marcescens* challenge; (**B**) Gentamicin (0.5 µg/ml) pre-treatment conferred 26% protection on worm populations against subsequent *P. aeruginosa* challenge; TF pretreatment at 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml, 100 µg/ml, conferred 18%***±0, 33%***±2.35, 38%***±2.35, 41.5%**±0 and 34.5%***±2.35 protection on worm population against subsequent *P. aeruginosa* challenge (**C**) Vancomycin (0.1 µg/ml) pre-treatment conferred 26% protection on worm population, TF pretreatment at 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml, 100 µg/ml, conferred 14.5%***±2.35, 13%***±4.71, 11.5%***±2.35, 21.5%***±2.35, and 33%***±0 protection on worm population against *S. aureus* (**D**) Chloramphenicol (0.5 µg/ml) pre-treatment conferred 26% protection on worm population against *C. violaceum*, TF pre-treatment at 0.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml, 100 µg/ml, conferred 14.5%***±0, 19.5%***±4.71, 14.5%***±2.35, 28%***±7.07, 34.5%***± 2.35 and 35.5%***±0 protection on worm population against *C. violaceum*. *p<0.05, **p<0.01, ***p<0.001; TF: *Triphala* Formulation.

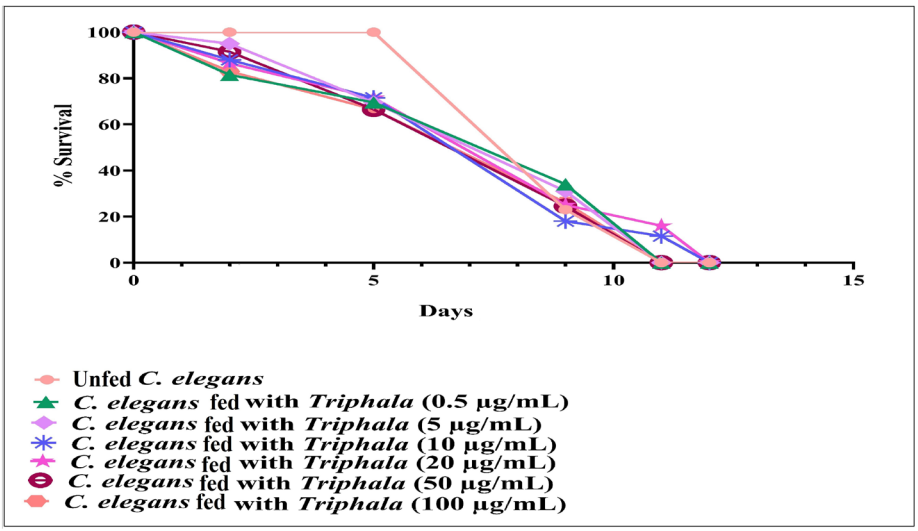

Figure 6. *Triphala* imparts marginal longevity extension on *C. elegans*. Worms fed with TF (10 µg/ml and 20 µg/ml) scored 11.5%***±1.20, and 16%***±0.96 better survival on 11th day. All worms (not fed with TF) in control were dead by the 11th day. DMSO (0.5%v/v) and TF at tested concentration had no effect on worm longevity. ***p≤0.001; TF: *Triphala* Formulation.

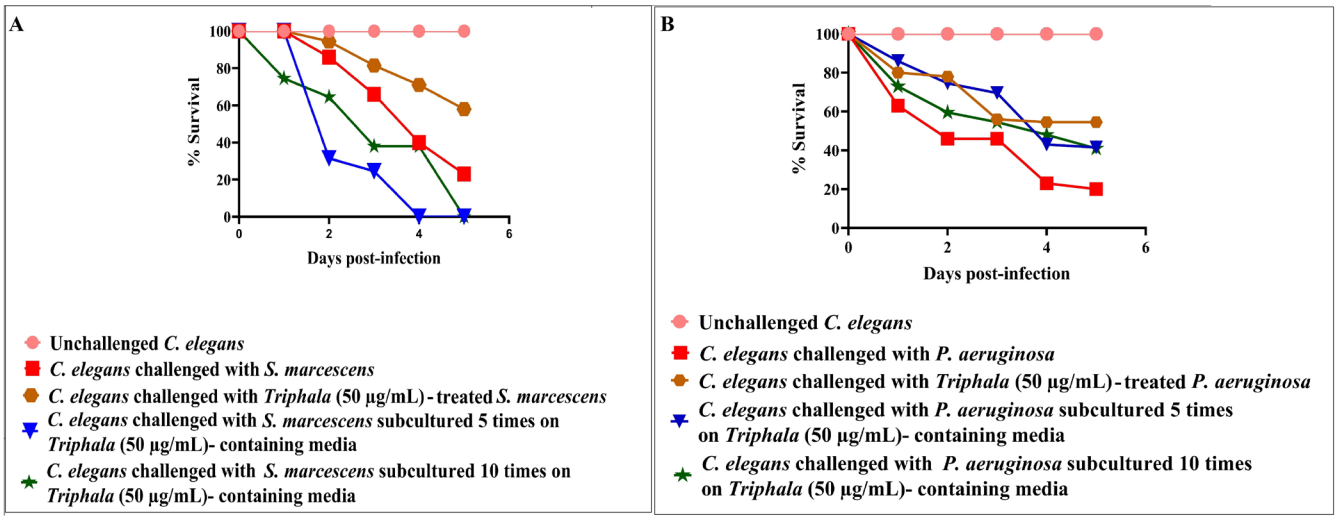

Figure 7. Effect of repeated exposure of *Triphala* on test pathogens. DMSO present in the 'vehicle control' at 0.5%v/v did not affect virulence of the bacterium towards *C. elegans*; DMSO (0.5%v/v) and TF at tested concentrations showed no toxicity towards the worm. (**A**) *C. elegans* challenged with TF (50 µg/ml) treated *S. marcescens* conferred 35%***±2.35 survival benefit. *C. elegans* could not conferred survival benefit when challenged with *S. marcescens* which subcultured 5 times or 10 times on TF containing media could not conferred survival benefit. (**B**) *P. aeruginosa* obtained after 5 and 10 subculturings in TF (50 µg/ml)-containing media were able to kill 21.5%***±2.35 and 21%***±7.07 lesser worms respectively, as compared to control (DMSO-treated) bacterial population. *p<0.05, **p<0.01, ***p<0.001; TF: *Triphala* Formulation.

DNA release and biofilm formation in some *S. aureus* strains (Kaplan *et al.*, 2012).

During the host-pathogen interaction, host defense mechanisms play a determinant role in deciding the outcome of this interaction. Since lysozyme is an important component of the innate defense machinery of human immune system against invading microbes (Herbert *et al.*, 2007), we also studied whether TF can have any effect on susceptibility of the test pathogens to lysozyme. TF-treated cells of *S. marcescens* and *S. aureus*

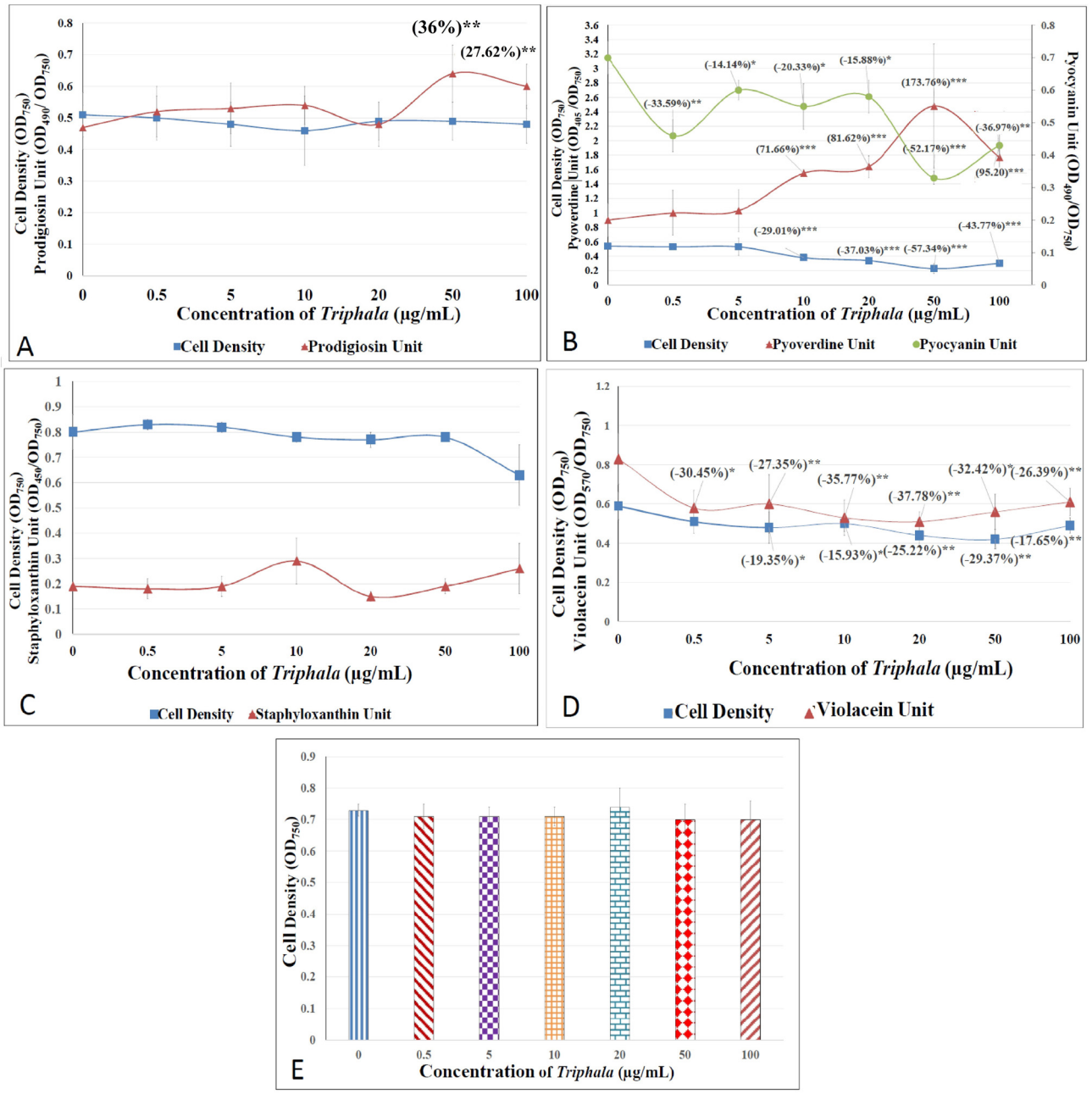

Figure 8. Effect of *Triphala* on growth and QS-regulated pigment production in test pathogens. Bacterial cell density was quantified as OD_{750}. DMSO (0.5%v/v) in the vehicle control did not affect growth and pigment production in any of the test pathogens. (**A**) *S. marcescens*: OD of prodigiosin was measured at 490 nm, and Prodigiosin Unit was calculated as the ratio OD_{490}/OD_{750} (an indication of prodigiosin production per unit of growth). Catechin (100 µg/ml) inhibited prodigiosin production by 13.05%*±0.10 without affecting bacterial growth; Ampicillin (5 µg/ml) inhibited growth and prodigiosin production by 8.48%**±0.02 and 40.60%*±0.23, respectively. (**B**) *P. aeruginosa*: OD of pyoverdine and pyocyanin was measured at 405 nm and at 490 nm. Pyoverdine Unit and Pyocyanin Unit was calculated as the ratio OD_{405}/OD_{750} and OD_{490}/OD_{750} (an indication of pyoverdine and pyocyanin production per unit of growth). Catechin (100 µg/ml) inhibited, pyoverdine and pyocyanin production by 3.85%*± 0.38 and 12.74%*± 2.60 without affecting bacterial growth; Gentamicin (0.5 µg/ml) inhibited, pyoverdine, pyocyanin production by 10.53%*±2.07 and 57.93%***±6.47 without affecting bacterial growth; (**C**) *S. aureus*: OD of staphyloxanthin was measured at 450 nm, and Staphyloxanthin Unit was calculated as the ratio OD_{450}/OD_{750} (an indication of staphyloxanthin production per unit of growth). Catechin (100 µg/ml) and vancomycin (0.1 µg/ml) did not affect growth as well as staphyloxanthin pigment production. (**D**) *C. violaceum*: OD of violacein was measured at 570 nm, and Violacein Unit was calculated as the ratio OD_{570}/OD_{750} (an indication of violacein production per unit of growth). Catechin (100 µg/ml) not affect growth as well as violacein pigment productions; Chloramphenicol (0.5 µg/ml) inhibited growth by 40.31**%±0.44 without affecting violacein pigment production. (**E**) *S. pyogenes*: TF and catechin (100 µg/ml) did not affect the growth when measured as OD_{655}; Chloramphenicol (0.5 µg/ml) inhibited growth by 7.56%**±3.46. *$p<0.05$, **$p<0.01$, ***$p<0.001$; TF: *Triphala* Formulation; QS: Quorum Sensing.

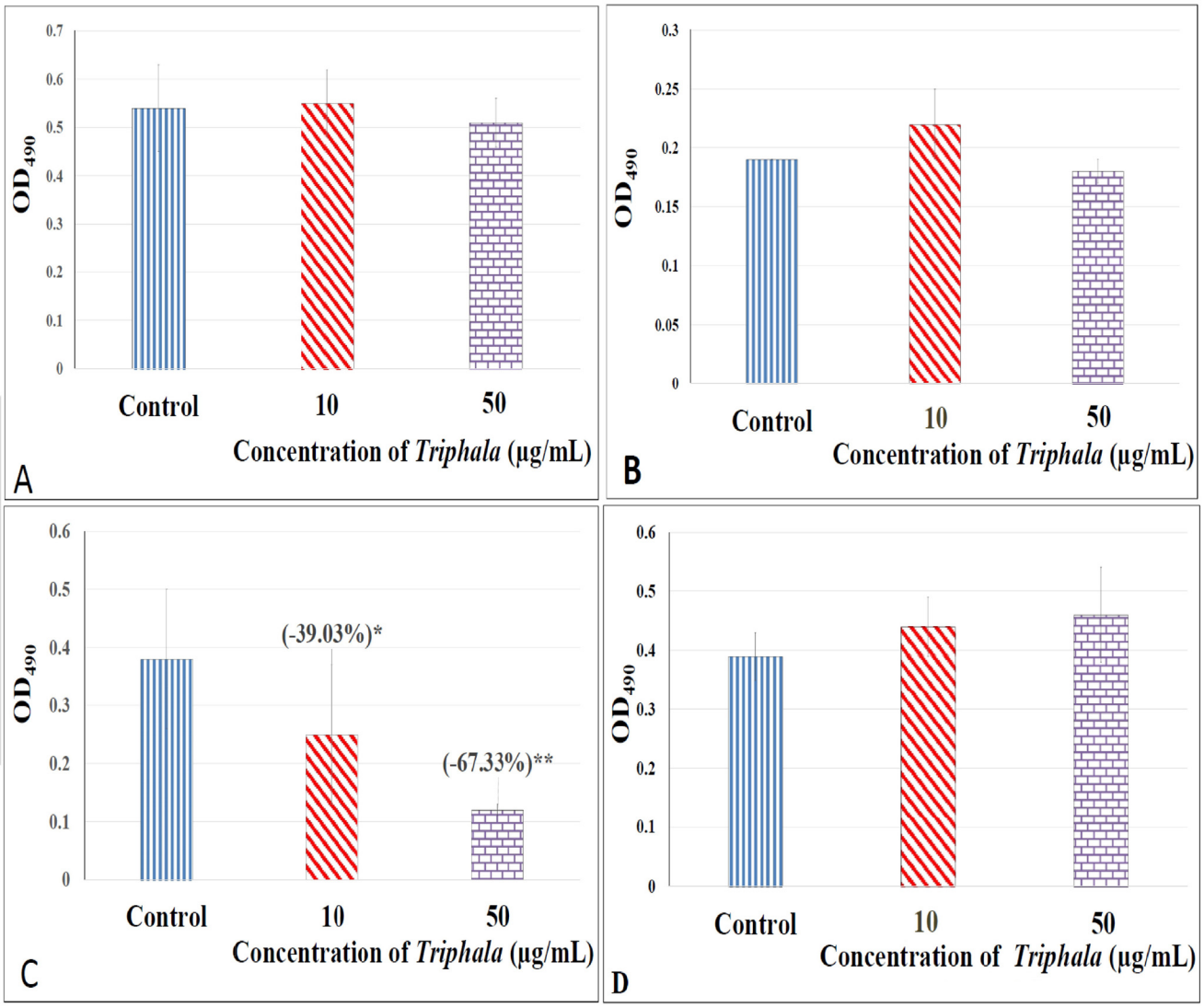

Figure 9. Effect of *Triphala* on haemolytic activity of test pathogens. TF had no effect on hemolytic activity of *S. marcescens* (**A**), *P. aeruginosa* (**B**), and *C. violaceum* (**D**). However, it curbed haemolytic potential of *S. aureus* notably. Hemoglobin released as a result of haemolysis was quantified as OD_{490}; 1% triton (OD_{490} = 1.2), and PBS (pH 7.4) were used as positive and negative control respectively; *p<0.05, ***p<0.001; TF: *Triphala* Formulation; PBS: Phosphate Buffer Saline.

were found to suffer marginal (albeit statistically significant; p≤0.05) increase in their susceptibility to lysis by lysozyme. *P. aeruginosa's* lysozyme-susceptibility was found to increase heavily (by ~25-43%) upon TF-pretreatment [Figure 11; underlying data (Patel *et al.*, 2019c)].

Most conventional antibiotics suffer from an inherent limitation of not being selectively inhibitory to pathogenic bacteria, and they simultaneously inhibit resident bacterial members of the human microbiome; which may lead to gut dysbiosis (Wipperman *et al.*, 2017). Thus an ideal anti-pathogenic formulation should exert anti-pathogenic effects without inhibiting indigenous members of human microbiome. We tested TF's effect on three such bacteria (*Enterococcus faecium*, *Bifidobacterium bifidum*, and *Lactobacillus plantarum*) which are part of human microbiome, and also used as probiotic strains. Though TF did not exert any prebiotic potential by promoting growth of the probiotic bacteria, it also had no negative effect on them [Extended data: Figure S1 (Patel *et al.*, 2019c)].

Conclusion

This study has found the classical *Triphala* formulation to possess significant anti-infective potential against ~~various~~ gram-positive and gram-negative pathogenic bacteria. It was

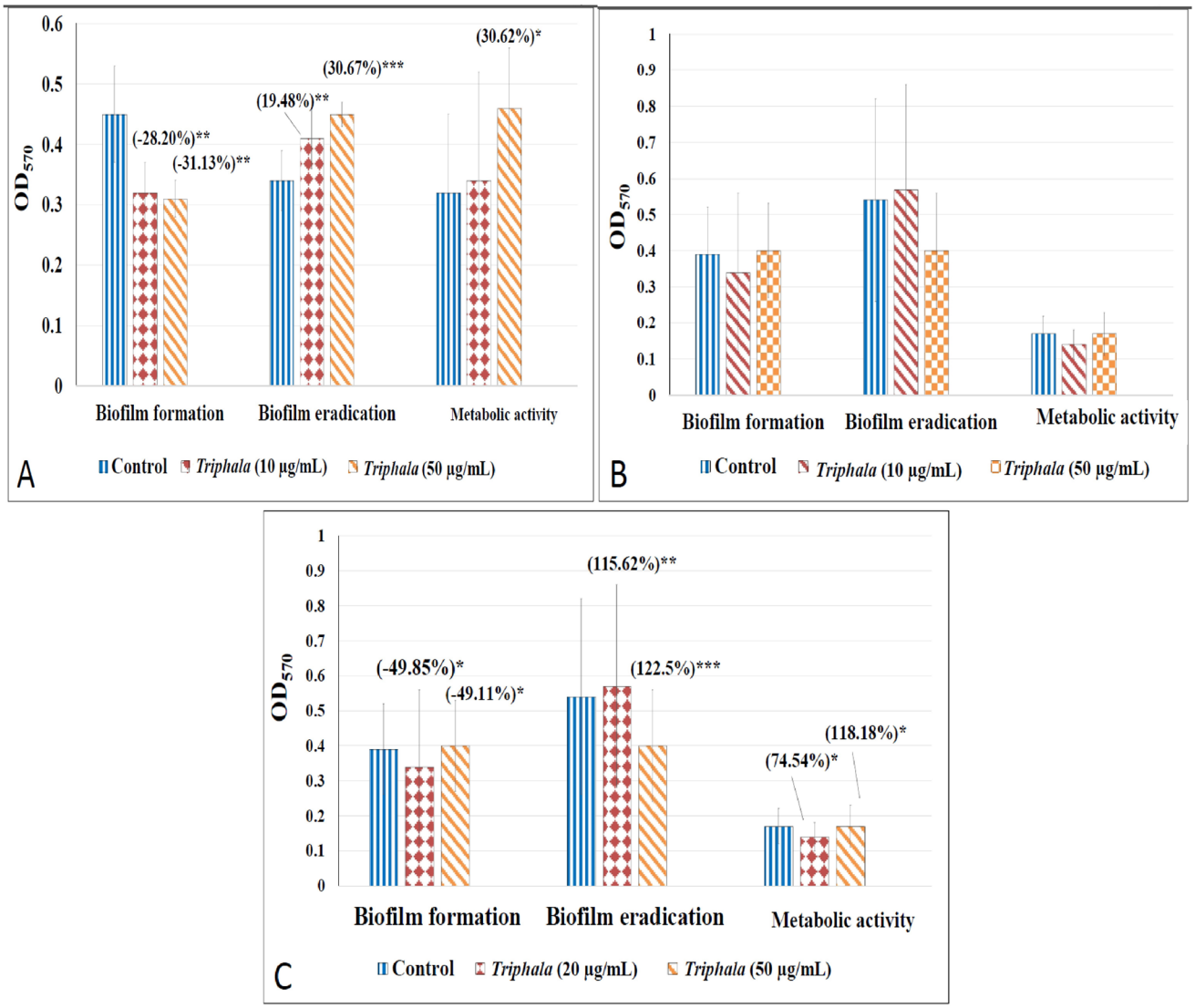

Figure 10. Effect of *Triphala* on biofilm of test pathogens. Crystal violet assay was performed to quantify biofilm formation or eradication, wherein amount of this dye retained by the biofilm was read at 570 nm after extracting it in ethanol. MTT assay was performed to quantify biofilm viability (metabolic activity), wherein change in colour of the MTT dye owing to bacterial metabolism was read at 570 nm. Catechin (100 µg/ml) for all test bacteria, ampicillin (5 µg/ml) for *S. marcescens,* gentamicin (0.5 µg/ml) for *P. aeruginosa,* and vancomycin(0.1 µg/ml) for *S. aureus* were used as positive controls. (**A**) Effect of TF on *S. marcescens* biofilm. Catechin inhibited *S. marcescens* biofilm formation by (29.80±9.38)%***, eradicated pre-formed biofilm by (49.60 ±12.00)%*, and reduced metabolic activity of biofilm by (86.20 ±0.88)% ***. Ampicillin inhibited all three by 29.25*** ±7.30, 54.23*** ±3.67, 86.11*** ±0.21 for *S. marcescens*. (**B**) Effect of TF on *P. aeruginosa* biofilm. Catechin inhibited *P. aeruginosa* biofilm formation by (22.18±1.16)%***, eradicated pre-formed biofilm by (30.54 ±3.50)%**, and enhanced metabolic activity of biofilm by (32.27 ± 4.74**)%***. Gentamicin did not affect biofilm formation, eradicated pre-formed biofilm by (23.27±4.91)%***, reduced metabolic activity of biofilm by (123.99 ±26.81)% ***. (**C**) Effect of TF on *S. aureus* biofilm. Catechin inhibited *S. aureus* biofilm formation by (26.24±7.35)%***, enhanced pre-formed biofilm by (22.54 ±10.90)%**, and enhanced metabolic activity of biofilm by (177.71 ± 16.49)% ***. Vancomycin inhibited biofilm formation by (41.53±5.49)% ***, eradicate pre-formed biofilm by (42.37±11.21)%***, enhanced metabolic activity of biofilm by (70.90 ±5.10)% ***. *p<0.05, **p<0.01, ***p<0.001; TF: *Triphala* Formulation.

also found to be efficacious as a post-infection therapy as well as a prophylactic measure against bacterial infection. ~~*Triphala*~~ can be said to possess a broad-spectrum of anti-pathogenic activity, which seems to partly arise from its ability to interfere with bacterial quorum-sensing. Its prophylactic efficacy indicates that it is not only exerting inhibitory effect on the susceptible bacteria, but also beneficial effect on the host worm, and thus can be described as a combination of immunomodulatory and

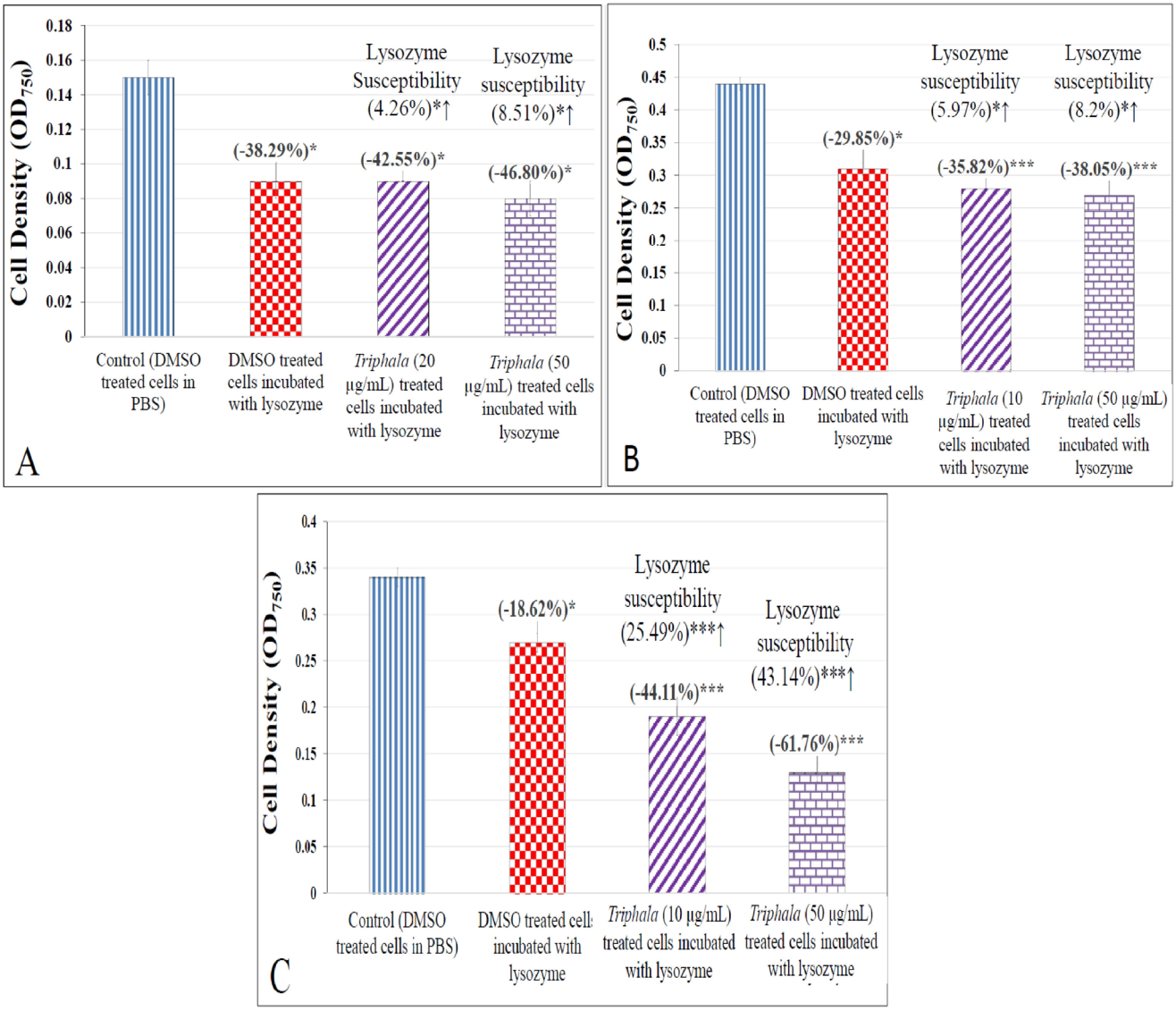

Figure 11. *Triphala* increases susceptibility of test pathogens to lysozyme. (**A**) *S. aureus*; (**B**) *S. marcescens*; (**C**) *P. aeruginosa* *p≤0.05 ***p≤0.001.

anti-pathogenic activities in one formulation. Exerting such combined efficacy without displaying any negative effect on beneficial members of human microbiome are key attributes for 21st century antimicrobials (Laxminarayan *et al.*, 2013). Further investigation for elucidating the molecular mechanisms associated with the biological effects of ~~*Triphala*~~ are warranted, with special emphasis on its role in combating AMR. Such traditional medicine polyherbal formulations need not necessarily be thought of as replacement of conventional antibiotic treatments, but more realistically as adjunctive therapies boosting our efforts to tackle AMR effectively.

Data availability

Underlying data

Figshare: Anti-pathogenic potential of a classical ayurvedic ~~formulation~~ Triphala. https://doi.org/10.6084/m9.figshare.8052143.v2 (Patel *et al.*, 2019c)

- Raw data_Figures 1-11_S1.rar

Extended data

Figshare: Anti-pathogenic potential of a classical ayurvedic ~~formulation~~ Triphala. https://doi.org/10.6084/m9.figshare.8052143.v2 (Patel *et al.*, 2019c)

This project contains the following extended data:

- Video (a).avi (Video of *C. elegans* challenged with *S. marcescens*)
- Video (b).avi (Video of *C. elegans* exposed to TF-treated *S. marcescens*)
- Figure S1.jpg (effect of TF treatment on probiotic bacterial strains)

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgement

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Reviewer Report 15 August 2019

https://doi.org/10.5256/f1000research.21707.r51849

?

Shibabrata Pattanayak
ARD (Vet. Research & Investigation), Government of West Bengal, Kolkata, West Bengal, India

Following remarks are to be considered.
A.Test formulation
Triphala formulation (TF) (Emami Ltd; batch no. EM0029; Proportion of 3 constituent plant species: 1:1:1) was purchased from a local market. For assay purpose, 150 mg of this formulation was suspended in 5 ml of DMSO (Merck, Mumbai), followed by vortexing for 15 min. Then it was centrifuged at 8,000 rpm for 30 min at ambient temperature, and resulting supernatant was collected in a sterile glass vial (15 ml; Borosil) and stored under refrigeration till further use. Remaining pellet was subjected to drying in an oven at 70-80°C until the solvent was completely evaporated, followed by weighing of the dried plant material. Subtracting the latter from the initial weight of 150 mg, the concentration of test formulation in supernatant was calculated to be 22.94 mg/ml. This way the whole formulation was found to contain 70% DMSO soluble fraction, which was used for our experiments.

Remark
DMSO is having some effect on at least some microorganisms. What concentration of DMSO was used? Please add report / reference of non – germicidal effect of DMSO at the concentration used in your experiment.
You may consult the following articles:

1. Kirkwood ZI, Millar BC, Downey DG, Moore JE. Antimicrobial effect of dimethyl sulfoxide and *N, N*-Dimethylformamide on *Mycobacterium abscessus*: Implications for antimicrobial susceptibility testing. Int J Mycobacteriol 2018;7:134-136.[1]

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3. Ashraf S. Hassan (2014) The Antibacterial Activity of Dimethyl Sulfoxide (DMSO) with and without of Some Ligand Complexes of the Transitional Metal Ions of Ethyl Coumarin against Bacteria

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4. Howard C. Ansel, William P. Norred, Ivan L. Roth (1969) Antimicrobial activity of dimethyl sulfoxide against *Escherichia coli, Pseudomonas aeruginosa*, and *Bacillus megaterium.* J Pharmaceutical sciences 58(7): 836-839.[4]

B. Some portions are appeared unclear. Please modify the sentences:

1. **Page 2, Column 2 Paragraph 4.**

   Prophylactic assay (Patel *et al*., 2018b): …..These worms were then fed with TF by mixing required concentration of this formulation (100 µL) with M9 medium (800 µL) and placed in a 24-well plate (non-treated polystyrene plates….

   Remark:

   Is it the DMSO –TRIFALA mixed supernatant?

2. **Page 4 Column 1 Paragraph 4.**
   …..at par with that of control (DMSO treated parent culture). –

   Remark:
   Point is not clear.

Overall Comment:

The research work is very good. The writing style is also good. After the mentioned modifications, the article may be accepted.

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Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Ethno- Pharmacology, Immunology, Bacteriology, Virology, Biochemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.