

Deciphering the molecular mechanisms underlying anti-pathogenic potential of a polyherbal formulation Enteropan[®] against multidrug-resistant *Pseudomonas aeruginosa*

Sweety Parmar¹, Gemini Gajera¹, Nidhi Thakkar¹, Hanmanthrao S. Palep², Vijay Kothari¹

¹Institute of Science, Nirma University, Ahmedabad - India

²Dr. Palep's Medical Research Foundation, Mumbai - India

Sweety Parmar, Gemini Gajera and Nidhi Thakkar contributed equally.

ABSTRACT

Objective: Anti-pathogenic potential of a polyherbal formulation Enteropan[®] was investigated against a multidrug-resistant strain of the bacterium *Pseudomonas aeruginosa*.

Methods: Growth, pigment production, antibiotic susceptibility, etc., were assessed through appropriate *in vitro* assays. Virulence of the test pathogen was assessed employing the nematode worm *Caenorhabditis elegans* as a model host. Molecular mechanisms underlining the anti-pathogenic activity of the test formulation were elucidated through whole transcriptome analysis of the extract-exposed bacterial culture.

Results: Enteropan-pre-exposed *P. aeruginosa* displayed reduced (~70%↓) virulence towards the model host *C. elegans*. Enteropan affected various traits like biofilm formation, protein synthesis and secretion, quorum-modulated pigment production, antibiotic susceptibility, nitrogen metabolism, etc., in this pathogen. *P. aeruginosa* could not develop complete resistance to the virulence-attenuating activity of Enteropan even after repeated exposure to this polyherbal formulation. Whole transcriptome analysis showed 17% of *P. aeruginosa* genome to get differentially expressed under influence of Enteropan. Major mechanisms through which Enteropan exerted its anti-virulence activity were found to be generation of nitrosative stress, oxidative stress, envelop stress, quorum modulation, disturbance of protein homeostasis and metal homeostasis. Network analysis of the differentially expressed genes resulted in identification of 10 proteins with high network centrality as potential targets from among the downregulated genes. Differential expression of genes coding for five (*rpoA*, *tig*, *rpsB*, *rpsL*, and *rpsJ*) of these targets was validated through real-time polymerase chain reaction too, and they can further be pursued as potential targets by various drug discovery programmes.

Keywords: Antimicrobial resistance (AMR), Anti-virulence, *Caenorhabditis elegans*, Iron/Sulphur homeostasis, Metal homeostasis, Network analysis, Nitrosative stress, Polyherbal, Transcriptome

Introduction

Antibiotic-resistant strains of the gram-negative bacterial pathogen *Pseudomonas aeruginosa* are responsible for considerable morbidity and mortality globally (1). As per CDC's Antibiotic Resistance Threat Report (2019),

multidrug-resistant (MDR) *P. aeruginosa* was responsible for an estimated 32,600 hospitalizations, and 2,700 deaths in 2017 (2). Healthcare costs in the United States alone attributed to these infections were estimated to be 767 million USD. Later released update to this report indicated an increase in number of infections caused by hospital-onset MDR *P. aeruginosa* in 2022 as compared to that in 2019 ([Online](#)). According to a study conducted by Wattal et al (3), India holds a leading position globally with respect to consumption of antibiotics for human use. This heavy antibiotic usage contributes significantly towards antibiotic resistance, resulting in a substantial increase in mortality among newborns who contract sepsis caused by MDR pathogens. *P. aeruginosa* displays versatility with respect to types of infections it causes, as it has been involved in

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Corresponding author:

Vijay Kothari

email: vijay.kothari@nirmauni.ac.in



pneumonia, urinary tract infections, bloodstream infections, and surgical site infections. Hospitalized patients on ventilators, those with catheters, surgical wounds, or burns are particularly at higher risk of contracting *P. aeruginosa* infection (4). While treating MDR *P. aeruginosa* infections, the choice of available effective antibiotics remains quite narrow (5), and hence there is an urgent need for discovery and development of novel antibacterial agents/formulations against this notorious pathogen.

Traditional medicine (TM) formulations can be a potential source of novel leads against bacterial pathogens including *P. aeruginosa*. Since TM often relies on polyherbal formulations (6,7) for treatment, and these polyherbal formulations display 'multiplicity of targets' (8) against susceptible pathogens, investigating the effect of such formulations in the pathogens at whole metabolome or transcriptome level can result in discovery of new molecular targets in pathogens. While dearth of novel cellular and molecular targets has been one of the major hurdles in new antibiotic discovery (9,10), elucidating the anti-pathogenic potential of polyherbal formulations at molecular level can be quite relevant. Often these polyherbal formulations exhibit anti-virulence effect (11) rather than directly inhibiting growth of the target pathogens. They may do so by affecting expression of non-essential genes (i.e. other than housekeeping genes) in the pathogens.

Present study investigated one polyherbal formulation (Enteropan) for its anti-pathogenic potential against a MDR *P. aeruginosa*. This formulation or its component plant extracts are traditionally being prescribed for treatment of irritable bowel syndrome, diarrhoea, dysentery, and other gastrointestinal problems ([Online](#)).

Materials and methods

Test formulation

Test formulation Enteropan[®] was procured from Dr. Palep's Medical Research Foundation Pvt. Ltd, Mumbai. All the nine ingredient plants and their parts whose hydroalcoholic extracts have been mixed to prepare this formulation are listed in Table 1. We obtained the polyherbal mix from the manufacturer in dried powder form without any bulking agent, and mixed 4 g of it in 8 mL of dimethylsulfoxide (DMSO; Merck). The DMSO-soluble fraction of Enteropan was found to be $71.17\% \pm 3.07$. After separating the insoluble fraction through centrifugation, the remaining DMSO-dissolved fraction was stored under refrigeration.

Test organisms

P. aeruginosa strain was sourced from our internal lab culture collection. Its antibiogram (Tab. S1) generated through disc diffusion assay revealed it to be resistant to three different classes of antibiotics, that is, co-trimoxazole (combination of trimethoprim and sulfamethoxazole), streptomycin (aminoglycoside), and augmentin (combination of amoxicillin and clavulanic acid). Antibiotic susceptibility of this strain to kanamycin was classified as 'intermediate'. *Pseudomonas* broth (20 g/L peptic digest of animal tissue, 10 g/L potassium sulphate, 1.4 g/L magnesium chloride, 3% v/v glycerol, pH

TABLE 1 - Ingredients of Enteropan formulation

Scientific name	Common English name (Indian name)	Part used	Proportion in the polyherbal mix (mg/capsule)
<i>Aegle marmelos</i>	Wood apple (Bael)	Leaves	100
<i>Myristica fragrans</i>	Nutmeg (Jaiphal)	Fruit	15
<i>Zingiber officinale</i>	Ginger (Sunthi)	Rhizome	60
<i>Aconitum heterophyllum</i>	Indian Atees (Ativisha)	Bulb	30
<i>Coriandrum sativum</i>	Coriander (Dhanyak)	Seeds	40
<i>Cyperus rotundus</i>	Nutgrass (Nagarmotha)	Rhizome	25
<i>Vetiveria zizanioides</i>	Khas Khas grass (Usheer)	Roots	50
<i>Punica granatum</i>	Pomegranate (Dadim)	Rind	50
<i>Holarrhena antidysenterica</i>	Star gooseberry (Kutaj)	Bark	27

7.0 ± 0.2) or agar (HiMedia) was used to cultivate this bacterium. Inoculum density of this bacterium to be used in all experiments was adjusted at $OD_{625} = 0.08-0.10$ to achieve equivalence to McFarland turbidity standard 0.5.

Escherichia coli OP50 procured from LabTIE B.V. the Netherlands was used as food for *Caenorhabditis elegans*, while maintaining the worm on NGM (nematode growth medium; 3 g/L NaCl, 1 M $CaCl_2$, 1 M $MgSO_4$, 2.5 g/L peptone, 5 mg/mL cholesterol, 1 M phosphate buffer of pH 6, 17 g/L agar-agar).

The nematode worm *C. elegans* (N2 Bristol, procured from IIT Gandhinagar, Gujarat, India) was used as a model host for *P. aeruginosa*. The worm was maintained on NGM agar plates. Worm synchronization was done as described in literature (12) and in our previous studies (13,14) too. Prior to all *in vivo* assays, worms were kept without food for 2 days to make them gnotobiotic.

In vivo assays

Four different types of *in vivo* assays performed are described below. Each of them involved live-dead counting over a period of 5 days under a microscope (4x) with halogen light source. On the last day of the experiment, when plates could be opened, death was confirmed by touching them with a straight wire, wherein no movement was considered as confirmation of death. In each well, there were 10 worms in M9 buffer (3 g/L KH_2PO_4 ; 6 g/L Na_2HPO_4 ; 5 g/L NaCl), which were challenged with *P. aeruginosa* by adding 100 μ L ($OD_{764} = 1.50 \pm 0.05$) of bacterial culture grown in *Pseudomonas* broth for 21 ± 1 hours at $35 \pm 0.5^\circ C$.

Anti-pathogenic assay

Ten worms of L3-L4 stage contained in 900 μ L M9 buffer were challenged with *P. aeruginosa* (100 μ L of the culture broth) in absence or presence of Enteropan (50-1,000 μ g/mL), wherein neither the bacterium nor the worms were pre-exposed to Enteropan. Incubation was done at 22°C for 5 days with live-dead microscopic count once a day.

Anti-infective assay

P. aeruginosa was grown at 35°C for 20-22 hours in *Pseudomonas* broth with or without Enteropan (5-1,000 μ g/mL). Post-incubation, 100 μ L of the culture broth was mixed with 900 μ L of M9 buffer containing 10 worms (L3-L4 stage) in a 24-well plate (surface non-treated; HiMedia). This plate was incubated at 22°C for 5 days (15,16).

Prophylactic assay

Gnotobiotic worms were incubated in M9 buffer supplemented with Enteropan (5-1,000 μ g/mL) for 96 hours. Following incubation, these worms were washed with M9 buffer twice, and then challenged with *P. aeruginosa* not pre-exposed to Enteropan (100 μ L of the culture broth) in 24-well plates (HiMedia). Worm survival was monitored over a 5-day period under microscope (17).

Post-infection assay

Ten worms contained in M9 buffer were first challenged with pathogen, and after allowing *P. aeruginosa* (100 μ L of the culture broth) for 3 or 6 hours to establish infection, Enteropan (50-1,000 μ g/mL) was added into the well as a possible post-infection therapy. Survival of worms was observed through a live-dead count under microscope over 5 days (18).

Appropriate controls were included in all the above experiments as relevant:

Sterility Control: Sterile M9 buffer containing neither bacteria nor worms

Survival Control: M9 buffer containing 10 worms (no bacteria added)

Toxicity Control: 10 worms in M9 buffer supplemented with Enteropan

Infection Control: 10 worms in M9 buffer + 100 μ L of the *P. aeruginosa* culture broth ($OD_{764} = 1.50 \pm 0.05$). These wells did not contain any plant extract.

Vehicle Control: 0.5% v/v DMSO was used in place of Enteropan.

Positive Control: Standard antibiotics employed as positive controls are detailed in the figure legends.

In vitro assays

Growth and pigment quantification

The broth dilution assay was used to evaluate *P. aeruginosa*'s growth and quorum sensing (QS)-regulated pigment synthesis in the presence or absence of the test formulation.

Different concentrations (ranging from 5 to 1,000 μ g/mL) of Enteropan formulation were used to challenge the organism. The growth media employed was *Pseudomonas* broth, into which bacterial inoculum set to 0.5 McFarland turbidity standard was added at 10% v/v, followed by incubation at 35°C for 20-22 hours, with intermittent shaking. The experiment also contained an appropriate vehicle control with DMSO (0.5% v/v) and an abiotic control with extract and growth medium but no inoculum.

Bacterial growth was measured photometrically at the end of the incubation by measuring the culture density at 764 nm (Agilent Cary 60 UV-visible spectrophotometer) (19). Following this, pigment was extracted and quantified in accordance with the procedure outlined for each pigment.

One mL of culture broth was mixed in a 2:1 ratio with chloroform (Merck, Mumbai), followed by centrifugation (15,300 g) for 10 minutes. This resulted in the formation of two immiscible layers. OD of the upper aqueous layer containing the yellow-green fluorescent pigment pyoverdine was measured at 405 nm. Pyoverdine Unit was calculated as OD_{405}/OD_{764} . The lower chloroform layer containing the blue pigment pyocyanin was mixed with 0.1 N HCl (20% v/v; Merck). This caused a change of colour from blue to pink. This was followed by centrifugation (15,300 g) for 10 minutes, and OD of upper layer acidic liquid containing pyocyanin was quantified at 520 nm. Pyocyanin Unit was calculated as OD_{520}/OD_{764} .

Biofilm assays

Biofilm formation is an important virulence trait, and hence the effect of Enteropan on biofilm forming ability of *P. aeruginosa*, as well as on pre-formed biofilm was investigated. A flow diagram depicting all four different biofilm assays is included in supplementary file (Fig. S2). Biofilm quantification was achieved through crystal violet assay (20). Biofilm viability was assessed through 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (21).

For the crystal violet assay, the biofilm-containing tubes (after discarding the inside liquid) were washed with phosphate-buffered saline (PBS) in order to remove all non-adherent (planktonic) bacteria, and air-dried for 15 minutes. Then, each of the washed tubes was stained with 1.5 mL of 0.4% aqueous crystal violet (Central Drug House, Delhi) solution for 30 minutes. Afterwards, each tube was washed twice with 2 mL of sterile distilled water and immediately de-stained with 1.5 mL of 95% ethanol. After 45 minutes of de-staining, 1 mL of de-staining solution was transferred into separate tubes, and read at 580 nm (Agilent Cary 60 UV-Vis).

For the MTT assay, the biofilm-containing tubes (after discarding the inside liquid) were washed with PBS in order to remove all non-adherent (planktonic) bacteria, and air-dried for 15 minutes. Then 1.8 mL of minimal media (sucrose 15 g/L, K_2HPO_4 5 g/L, NH_4Cl 2 g/L, NaCl 1 g/L, $MgSO_4$ 0.1 g/L, yeast extract 0.1 g/L, pH 7.4 \pm 0.2) was added into each tube, followed by addition of 200 μ L of 0.3% MTT [HiMedia]. Then after 2 hours of incubation at 35°C, all liquid content was discarded, and the remaining purple formazan derivatives were dissolved in 2 mL of DMSO and measured at 540 nm.



Nitrite estimation

Quantification of nitrite in bacterial culture was achieved through a colorimetric assay using modified Griess reagent (22,23). Supernatant (250 μ L) obtained from centrifugation (13,500 g; 25°C; 10 minutes) of *P. aeruginosa* culture grown in the presence or absence of Enteropan was mixed with 250 μ L of Griess reagent (1 \times ; Sigma-Aldrich), followed by 15 minutes of incubation in dark at room temperature. Absorbance of the resulting pink colour was measured at 540 nm. This absorbance was plotted on the standard curve prepared using NaNO₂ (0.43–65 μ M) to calculate nitrite concentration. Sodium nitroprusside (Sigma-Aldrich) was used as a positive control, as it is known to generate nitrosative stress in bacteria (24). Deionized water was used as negative control.

Antibiotic susceptibility test

Antibiogram of *P. aeruginosa*'s overnight grown culture in *Pseudomonas* broth in the presence or absence of Enteropan was generated through disc diffusion assay in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (25). Cells grown in *Pseudomonas* broth were separated through centrifugation (13,600 g) and washed with phosphate buffer (pH 7.0 \pm 0.2) followed by centrifugation. The resulting cell pellet was used to prepare inoculum for subsequent disc diffusion assay by suspending the cells in normal saline and adjusting the OD₆₂₅ between 0.08 and 0.10. This inoculum (100 μ L) was spread onto cation-adjusted Mueller-Hinton agar (HiMedia) plates (Borosil; 150 mm) followed by placing the antibiotic discs (Icosa G-I MINUS; HiMedia, Mumbai) on the agar surface. Incubation at 35°C was made for 18 \pm 1 hours, followed by observation and measurement of zone of inhibition.

Protein estimation

Extracellular protein present in bacterial culture (grown in the presence or absence of Enteropan) supernatant, and intracellular protein in the cell lysate was quantified through Folin-Lowry method (26,27). After measuring cell density, 1 mL of *P. aeruginosa* culture was centrifuged (13,600 g), and the resulting supernatant was used for extracellular protein estimation. The remaining cell pellet was subjected to lysis (28) for release of intracellular proteins. Briefly, the cell pellet was washed with phosphate buffer (pH 7.4) and centrifuged (13,600 g). Resulting pellet was resuspended in 1 mL of chilled lysis buffer (0.876 g NaCl, 1 mL of Triton X-100, 0.5 g sodium deoxycholate, 0.1 g sodium dodecyl sulphate, and 0.60 g Tris HCl, in 99 mL of distilled water) and centrifuged (500 rpm) for 30 minutes at 4°C for agitation purpose. This was followed by further centrifugation (16,000 g at 4°C) for 20 min. Resulting cell lysate (supernatant) was used for protein estimation. Kanamycin (HiMedia; at IC₅₀: 200 μ g/mL), an aminoglycoside antibiotic known to inhibit bacterial protein synthesis (29,30), was used as a positive control.

Whole transcriptome analysis

To gain insight into the molecular mechanisms by which Enteropan attenuates bacterial virulence and modulates

various traits like pigment production, antibiotic susceptibility, nitrogen metabolism, protein synthesis/excretion, etc., we compared the gene expression profile of Enteropan-pre-treated *P. aeruginosa* with that of control culture at the whole transcriptome level.

RNA extraction

Trizol (Invitrogen Bioservices; 343909) was used to extract RNA from bacterial cells (31). RNA was dissolved in nuclease-free water after precipitation with isopropanol and washing with 75% ethanol. Using the RNA HS assay kit (Thermo Fisher; Q32851) and adhering to the manufacturer's instructions, the extracted RNA was quantified using a Qubit 4.0 fluorometer (Thermo Fisher; Q33238). RNA concentration and purity were evaluated using Nanodrop 1000. Finally, RNA was checked on the TapeStation using HS RNA ScreenTape (Agilent) to yield RIN (RNA Integrity Number) values (Tab. S2).

Library preparation

Final libraries were measured using a Qubit 4.0 fluorometer (Thermo Fisher; Q33238), a DNA HS assay kit (Thermo Fisher; Q322851), and a TapeStation 4150 (Agilent) using high-sensitivity D1000 ScreenTapes (Agilent; 5067-5582). The acquired sizes of all libraries are reported in Table S3.

Genome annotation and functional analysis

FastQC v.0.11.9 (default parameters) was used to undertake a quality assessment of the sample's raw fastq readings (32). The reads' quality was then reevaluated using Fastq v.0.20.1 (33) after pre-processing the raw fastq reads with Fastq v.0.20.1.

The *P. aeruginosa* genome (GCA_000006765.1_ASM676v1) was indexed using bowtie2-build (34) v2.4.2 (default parameters). The processed reads were mapped to the *P. aeruginosa* genome using bowtie2 v2.4.2. Gene counts were determined using feature count v.0.46.1 (35) to quantify the aligned reads from the individual samples. Differential expression was estimated using the exact test (parameters: dispersion 0.1) with these gene counts as inputs in edgeR (36). The up- and downregulated sequences were extracted from the *P. aeruginosa* coding file and annotated using Blast2GO (37) to obtain the Gene Ontology (GO) keywords. These GO terms were used to create GO bar graphs with the wego tool (38).

All the raw sequence data has been submitted to the Sequence Read Archive. The relevant accession number is SRX15248092 ([Online](#)).

Network analysis

Network analysis was carried out for Enteropan-exposed *P. aeruginosa*'s differentially expressed genes (DEG) fulfilling the dual criteria of log fold change \geq 2 and false discovery rate (FDR) \leq 0.001. List of DEG was fed into the database STRING (v.11.5) (39) for generating the Protein-Protein Interaction (PPI) network for the genes were arranged in decreasing order of 'node degree' (a measure of connectivity with other genes or proteins), and those above an empirically selected



threshold value (19 and 53 for up- and downregulated genes, respectively) were subjected to ranking by cytoHubba (v.3.9.1) plugin (40) of Cytoscape (41). Since cytoHubba uses 12 different ranking methods, we considered the DEG being top-ranked by ≥ 6 different methods (i.e. 50% of the total ranking methods) for further analysis. These top-ranked shortlisted proteins were further subjected to network cluster analysis through STRING, and those that were part of multiple clusters were considered as ‘hubs’ which can be taken up for further confirmation of their targetability. Here ‘hub’ refers to a gene or protein interacting with many other genes/proteins. Hubs thus identified were further subjected to co-occurrence analysis to see whether an anti-virulence agent targeting them is likely to satisfy the criterion of selective toxicity (i.e. targeting the pathogen without harming host). This sequence analysis allowed us to end with a limited number of proteins which satisfied various statistical and biological significance criteria simultaneously, that is, (1) log fold change ≥ 2 ; (2) FDR ≤ 0.001 ; (3) relative higher node degree; (4) top-ranking by at least six cytoHubba methods; (5) member of more than one local network cluster; (6) high probability of the target being absent from the host.

Real-time polymerase chain reaction analysis

Polymerase chain reaction (PCR) was used to confirm the differential expression of the possible hubs discovered by network analysis of the DEG reported from whole transcriptome analysis (WTA). Primer3Plus (42) was used to design primers for the target genes (Tab. 2). These primer sequences were verified for their ability to specifically bind only to the target gene sequence throughout the whole genome file of *P. aeruginosa*. RNA extraction and purity check was executed as described in the previous section. The SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen Biosciences) was used to generate complementary DNA. Using gene-specific primers purchased from Sigma-Aldrich, the PCR experiment was carried out employing the temperature profile shown in Table S4. The gene PA3725 (*recJ*) was kept as an endogenous control. The reaction mix

used was FastStart Essential DNA Green Master mix (Roche; 06402712001). Real-time PCR (RT-PCR) assay was performed on QuantStudio 5 RT-PCR machine (Thermo Fisher Scientific, USA). Sample generation for PCR validation was done independent of that for transcriptome assay.

Statistical analysis

All results reported are means of three or more independent experiments, each performed in triplicate. Statistical significance was assessed through t-test performed using Microsoft Excel®, and data with $p \leq 0.05$ was considered to be significant.

Results and discussion

In vivo assays

P. aeruginosa displayed reduced virulence towards *C. elegans* in the presence of Enteropan

When *C. elegans* was challenged with *P. aeruginosa* in the presence of Enteropan, the bacterium could kill lesser worms than in the absence of Enteropan (Fig. 1A; Supplementary videos: A-E). The most effective concentration of Enteropan with respect to offering protection to the worm population from bacterial attack was found to be 250 $\mu\text{g}/\text{mL}$. Since higher concentrations offered either at par or lesser protection to the worms, the dose-response relationship here can be said to be nonlinear. Irrespective of the magnitude of protection offered to worm population in the presence of Enteropan®, progeny worms were observed (third day onwards) in all experimental and positive control wells, but not in the wells pertaining to vehicle control. It might have occurred that the virulence-attenuated *P. aeruginosa* were used by the worms as food, and that allowed them to reproduce.

Enteropan pre-treatment reduced bacterial virulence towards *C. elegans*

Enteropan (5-1,000 $\mu\text{g}/\text{mL}$)-pre-treated *P. aeruginosa* was found to exert lesser virulence against *C. elegans* than its not-exposed counterpart. Enteropan concentrations ≤ 250 $\mu\text{g}/\text{mL}$ could not compromise *P. aeruginosa*'s ability to kill *C. elegans*; however, concentrations ≥ 500 $\mu\text{g}/\text{mL}$ did compromise bacterial virulence significantly (Fig. 1B; Supplementary videos: F-I). Enteropan pre-treatment of the pathogen at these effective concentrations not only attenuated bacterial virulence but also supported worm fertility as evidenced by the appearance of numerous progenies in the experimental wells by the fifth day. The most effective concentration of Enteropan with respect to virulence-attenuating effect was found to be 600 $\mu\text{g}/\text{mL}$, as the effect of higher concentrations till 1 mg/mL was statistically not different than that of 600 $\mu\text{g}/\text{mL}$.

After confirming the anti-virulence activity of Enteropan against *P. aeruginosa*, we asked whether this pathogen can develop resistance upon repeatedly getting exposed to the test formulation. To answer this, we subcultured *P. aeruginosa* in *Pseudomonas* broth supplemented with Enteropan (600 $\mu\text{g}/\text{mL}$) multiple times. Enteropan-pre-exposed *P. aeruginosa* thus obtained after fifth and tenth such subculturing

TABLE 2 - Primer sequences for the target genes

Gene ID	Primers	Amplicon size (bp)
PA4238 (<i>rpoA</i>)	FP: 5'-CGCTGAACATGAAGCTGAAG-3' RP: 5'-CAGGACCAGTTTGTCCAGGT-3'	194
PA1800 (<i>tig</i>)	FP: 5'-ACCGAAGTCAACAAGCGTCT-3' RP: 5'-GGATTCAGCTTCTGCTCGAC-3'	208
PA3656 (<i>rpsB</i>)	FP: 5'-GCGCAACAAGATCCATATCA-3' RP: 5'-GATCGACTGACGGATGGTCT-3'	232
PA4268 (<i>rpsL</i>)	FP: 5'-TACATCGGTGGTGAAGGTCA-3' RP: 5'-TACTTCGAACGACCCTGCTT-3'	155
PA4264 (<i>rpsJ</i>)	FP: 5'-GATTCGGTTGAAGGCTTTT-3' RP: 5'-TACTGATCACGCGCATCTTT-3'	174
Control gene PA3725 (<i>recJ</i>)	FP: 5'-CCAGTTGAGCATCCAGGAGT-3' RP: 5'-TTTCAGCACCAGCTTCAGGT-3'	157



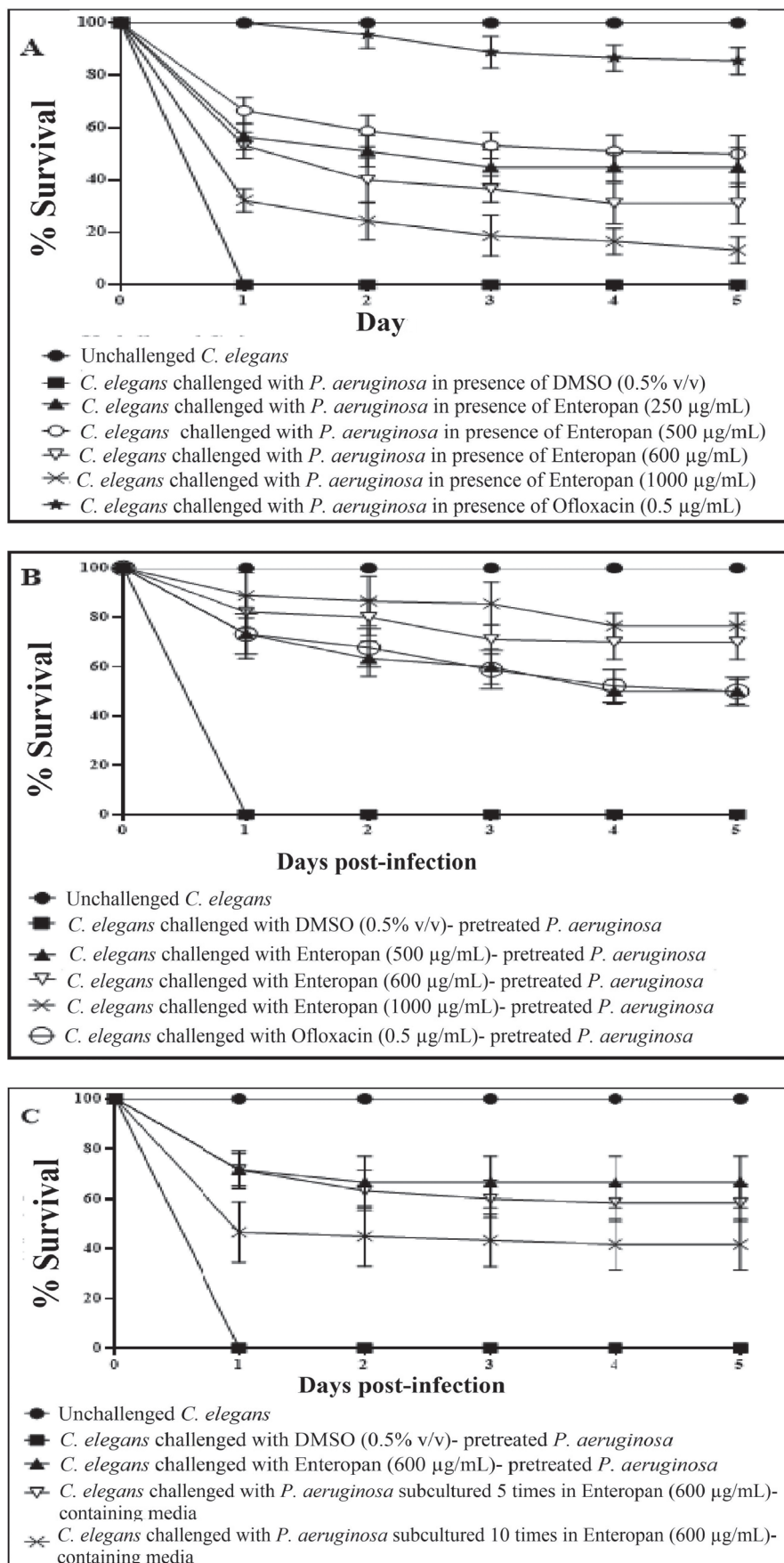


FIGURE 1 - Enteropan attenuates *Pseudomonas aeruginosa*'s virulence towards the model host *Caenorhabditis elegans*. Dimethylsulfoxide (DMSO) present in the 'vehicle control' at 0.5% v/v did not affect bacterial virulence. Neither DMSO nor Enteropan showed any toxicity towards the worm population at tested concentrations. To avoid overcrowding in the figures (A, B), we have not shown lines corresponding to concentrations which had no effect on bacterial virulence, and also that for 750 µg/mL, as its virulence-attenuating effect was statistically a part of that of 600 µg/mL. Supplementary videos pertaining to these experiments are available at: osf.io/fnywk. All the % values reported in this figure legend are statistically significant at $p < 0.001$.

A) *P. aeruginosa*'s virulence towards the host worm gets attenuated in the presence of Enteropan. Enteropan conferred a survival benefit on host *C. elegans* at concentrations of 250, 500, 600, 750, and 1,000 µg/mL with survival rates of $45\% \pm 5.47$, $50\% \pm 7.07$, $31.11\% \pm 7.8$, $35\% \pm 8.36$, and $13.33\% \pm 5$, respectively. Ofloxacin (0.5 µg/mL) was employed as a positive control and conferred $85.55\% \pm 5.27$ survival benefit on host worm. Progenies (TNTC – too numerous to count) were observed on the third day in experimental wells and positive control. See supplementary videos A-E. **B)** Enteropan pre-treatment reduced bacterial virulence towards *C. elegans*. Pre-treatment of bacteria with Enteropan at concentrations of 500, 600, 750, and 1000 µg/mL reduced its virulence towards host worm by $50\% \pm 5$, $70\% \pm 7.07$, $78.88\% \pm 6$, and $76.66\% \pm 5$, respectively, as per the fifth day end-point. Ofloxacin (0.5 µg/mL) pre-treatment reduced bacterial virulence towards the host worm by $50\% \pm 5.77$. Progenies were observed on the third day in experimental wells corresponding to ≥ 500 µg/mL Enteropan as well as positive control. See supplementary videos F-I. **C)** *P. aeruginosa* did not develop complete resistance even after repeated exposure to Enteropan. *P. aeruginosa* obtained after fifth and tenth subculturing in Enteropan (600 µg/mL)-containing media displayed $58.33\% \pm 4.04$ and $43.33\% \pm 10.3$ lesser virulence, respectively, than extract-non-exposed pathogen.

were allowed to attack *C. elegans* in M9 buffer (containing no Enteropan). No resistance seemed to have evolved in *P. aeruginosa* till fifth subculturing; however, 10 subculturings in Enteropan-supplemented media seemed to allow the pathogen to overcome this formulation's anti-virulence effect marginally (23.3%; Fig. 1C). This inability of the pathogen to develop complete resistance against Enteropan might be attributable to the polyherbal nature of the formulation. As the polyherbal formulations can have multiple bioactive compounds in them, they may exert a multiplicity of targets against the susceptible pathogen. To develop resistance in this scenario, the pathogen would be required to develop multiple simultaneous mutations, and that is a quite less probable event biologically as well statistically.

Enteropan offered prophylactic protection to C. elegans

To investigate whether Enteropan pre-feeding can offer any prophylactic benefit to worm population in the face of subsequent pathogen challenge, we allowed *P. aeruginosa* to attack worms pre-fed with Enteropan (5-1,000 $\mu\text{g}/\text{mL}$). Enteropan at ≥ 50 $\mu\text{g}/\text{mL}$ did confer prophylactic benefit on worm population. While concentrations till 500 $\mu\text{g}/\text{mL}$ supported worm survival (13%-26%) till 48 hours post-pathogen challenge (Fig. 2A), higher concentrations supported worm survival (20%-23%) till the fifth day (Fig. 2B; Supplementary videos: J-M). Considering the first-day end-point (i.e. by the time the pathogen killed 100% worms in control wells), certain Enteropan concentrations (500-750 $\mu\text{g}/\text{mL}$) performed at par to the positive control ofloxacin.

Enteropan is effective as a post-infection therapy

To investigate whether Enteropan is effective as a post-infection therapy, we added Enteropan (50-1,000 $\mu\text{g}/\text{mL}$) after 3 or 6 hours of mixing bacteria with the worms. While Enteropan addition post 6 hours of bacterial attack on worms could not rescue the host (Fig. S1), its addition post 3 hours of bacterial attack could rescue 10%-27% of the worms (Fig. 2C; Supplementary videos: N-R). As a post-infection therapy, Enteropan's performance did not improve with increase in concentration.

To have a mechanistic insight into the Enteropan-*P. aeruginosa* interaction, we checked the effect of Enteropan on various virulence traits of this pathogen *in vitro*, and also compared the gene expression profile of the Enteropan-treated *P. aeruginosa* with that of extract-non-exposed control at the whole transcriptome level.

In vitro experiments

Enteropan forced overproduction of QS-regulated pigments without affecting the bacterial growth heavily

Enteropan till 100 $\mu\text{g}/\text{mL}$ had no effect on *P. aeruginosa* growth. Though from 250 $\mu\text{g}/\text{mL}$ onwards it had some growth inhibitory effect, the magnitude of this inhibitory effect did not increase much with increase in concentration (Fig. 3A). Except 5 $\mu\text{g}/\text{mL}$, Enteropan at all tested concentrations enhanced production of QS-regulated pigments (pyoverdine and pyocyanin).

Hence concentrations of 25-100 $\mu\text{g}/\text{mL}$ can be said to have pure quorum-modulatory effect on *P. aeruginosa*. Though in general Enteropan's effect on pigment production seemed to be dose-dependent, bacterium's response at 600 $\mu\text{g}/\text{mL}$ seemed to deviate from this pattern in case of both the pigments.

Enteropan had a moderately negative effect on pre-formed biofilm

Though Enteropan's presence could not compromise *P. aeruginosa*'s ability to form biofilm, when Enteropan was added onto the pre-formed biofilm, it could eradicate the biofilm partly and also reduced the metabolic activity within the biofilm (Fig. 3B).

Enteropan disturbed nitrogen metabolism in P. aeruginosa

Since multiple genes associated with detoxification of reactive nitrogen species were upregulated (transcriptome data described later), we hypothesized that Enteropan-treated *P. aeruginosa*'s ability to overcome nitrosative stress is compromised. To check this hypothesis, we quantified nitrite concentration in extract-treated *P. aeruginosa* culture supernatant, wherein it was found to have 47.10% higher nitrite concentration as compared to control (Fig. 3C). This higher accumulation of nitrite can be taken as an indication of compromised denitrification efficiency, since nitrite is an important intermediate in denitrification pathway ahead of the toxic nitric oxide (43). Nitrosative stress can impact the overall bacterial fitness negatively in multiple ways (44). Reactive nitrogen species can damage biomolecules like DNA, lipids, and proteins. Resistance to nitrosative stresses is of crucial importance towards the survival of bacteria in the environment as well as inside the host. In gram-negative bacteria, several mechanisms protecting against oxidative and nitrosative stresses are present in the envelope. Excessive nitrosative stress can disturb the envelope homeostasis, and this in fact is reflected in the transcriptome of Enteropan-exposed *P. aeruginosa*, wherein 32 cell envelope (cell wall and lipopolysaccharide, LPS)-associated genes are expressed differently.

Enteropan-modulated P. aeruginosa's susceptibility to imipenem and augmentin

When Enteropan-pre-treated *P. aeruginosa* cells were subsequently challenged with different antibiotics in a disc diffusion assay, these cells exhibited marginal increase in their susceptibility to imipenem; however, their susceptibility to augmentin disappeared following Enteropan pre-treatment (Tab. S5; Fig. 3D, E). This effect of Enteropan pre-treatment on imipenem susceptibility was also confirmed in liquid culture, wherein Enteropan-pre-treated cells were observed to exhibit up to 21.43% higher susceptibility to imipenem (Fig. 3F). Imipenem belongs to the carbapenem class of beta-lactams (45), and carbapenem resistance among *P. aeruginosa* isolates are being viewed as a serious problem (46). Since this class of antibiotics are looked as a last resort for treatment of MDR *P. aeruginosa* (47), resistance modifiers capable of making this bacterium more susceptible to them can be of help in extending the lifespan of these antibiotics



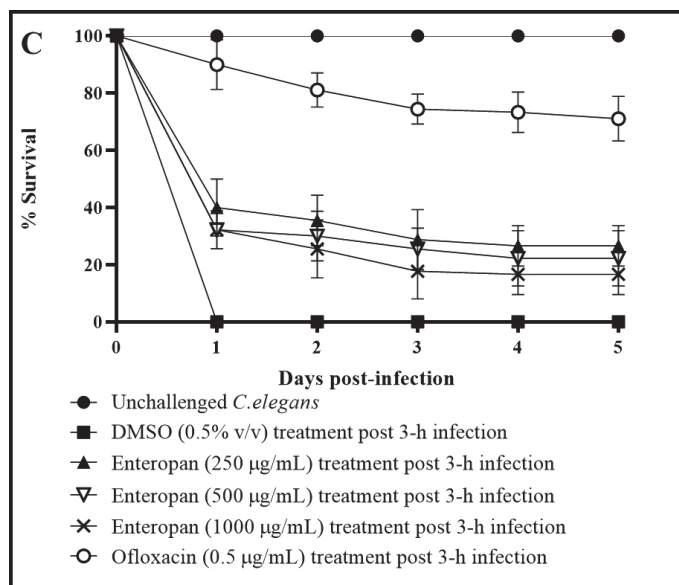
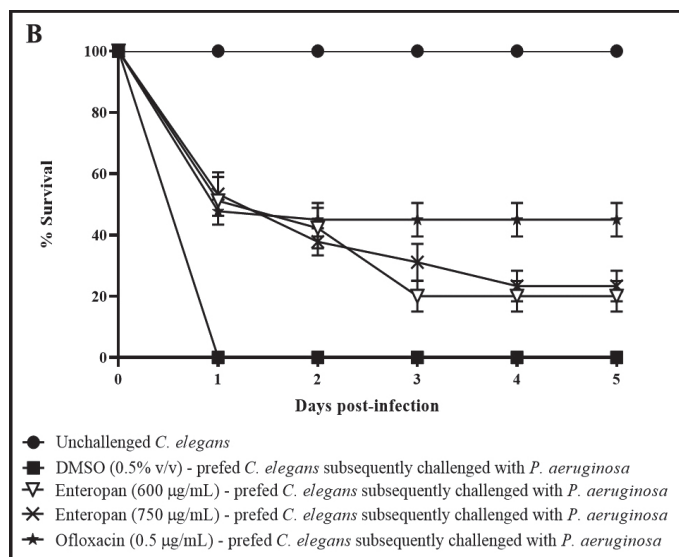
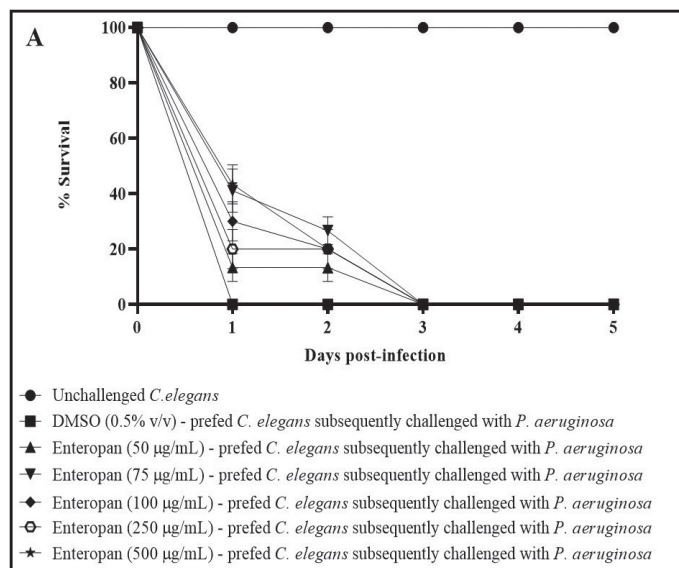


FIGURE 2 - Prophylactic and post-infection therapeutic potential of Enteropan. All the % values reported in this figure legend are statistically significant at $p < 0.001$. **A-B)** Enteropan offered prophylactic protection to the worm population against subsequent bacterial challenge. Worms pre-fed with Enteropan concentrations of 50, 75, 100, 250, and 500 µg/mL registered 13.3% ± 5, 26.6% ± 5, 20% ± 8.66, 17% ± 6.66, and 20% ± 8.66 better survival, respectively, till the second day in the face of subsequent pathogen challenge. Worms pre-fed with higher concentrations of Enteropan at 600, 750, and 1000 µg/mL registered 51.11% ± 7.8, 53.33 ± 7.07, and 43.33% ± 8.66 survival, respectively, till the end of first day of pathogen challenge. Magnitude of the prophylactic benefit as per fifth day point was 20% ± 5, 23.33 ± 5, and 20% ± 7.07, respectively. Pre-feeding the worms with dimethylsulfoxide (DMSO, 0.5% v/v) did not alter their susceptibility to subsequent bacterial challenge. Ofloxacin (0.5 µg/mL) employed as a positive control conferred 44.9% ± 5.49 prophylactic benefit on the host worms. See supplementary videos J-M. **C)** Enteropan could partially rescue worm population when used as a post-infection therapy. When pre-infected worms were exposed to Enteropan at 250, 500, 600, 750, and 1000 µg/mL, they scored 26.66% ± 7.0, 22.22% ± 9.71, 11.11% ± 3.33, 13.33% ± 6, and 16.66% ± 7.07 better survival, respectively, than control worms. Ofloxacin (0.5 µg/mL) employed as positive control, 3 hours post-infection, rescued 71.11% ± 7.81 worms. DMSO (0.5% v/v) did not confer any survival benefit when added post-infection. See supplementary videos N-R.



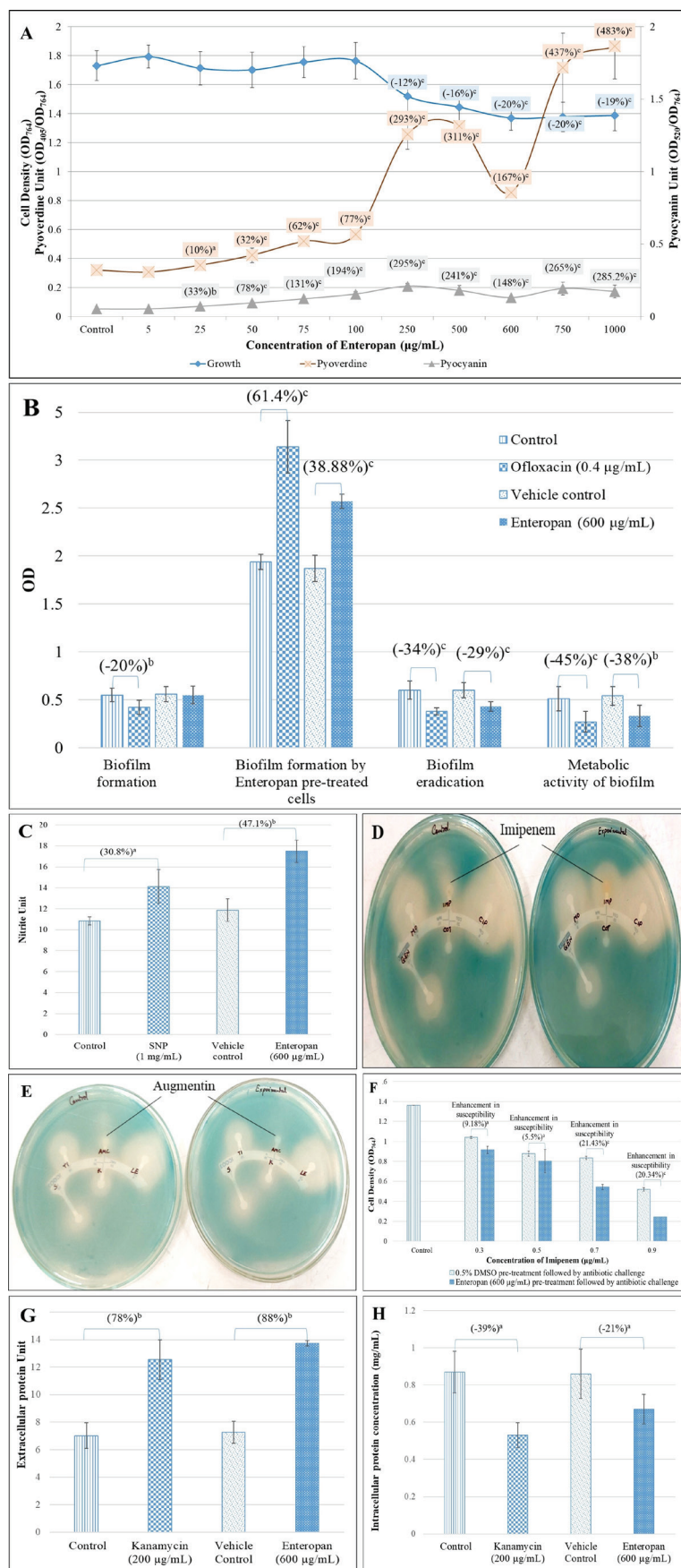


FIGURE 3 - Enteropan's effect on various phenotypic traits of *Pseudomonas aeruginosa* revealed through different *in vitro* assays. **A)** Enteropan enhances production of quorum-regulated pigments in *P. aeruginosa*, while exhibiting a mild growth inhibitory effect. Bacterial growth was measured as OD₇₆₄. Pyoverdine Unit and Pyocyanin Unit were calculated as the ratio OD₄₀₅/OD₇₆₄ and OD₅₂₀/OD₇₆₄ (an indication of pyoverdine and pyocyanin production per unit of growth), respectively; 'Control' shown in this figure is the vehicle control (0.5% v/v dimethylsulfoxide (DMSO)), which affected neither growth nor pigment production. Ofloxacin (0.5 µg/mL) inhibited growth by 65.6% ±5.32, while inhibiting pigment production completely. **B)** Enteropan's effect on *P. aeruginosa*'s biofilm formation capability and on pre-formed biofilm. While *P. aeruginosa*'s biofilm formation ability remained unaffected in the presence of Enteropan, Enteropan-pre-exposed cells subsequently allowed to form biofilm on glass surface accumulated higher biomass. Enteropan when added onto pre-formed biofilm could eradicate the biofilm partially, and also reduced the biofilm metabolic activity notably. DMSO (0.5% v/v) used as vehicle control did not affect biofilm of the *P. aeruginosa* in any of these four assays. **C)** *P. aeruginosa* culture accumulated higher extracellular nitrite in the presence of Enteropan. While nitrite concentration in vehicle control (*P. aeruginosa* supplemented with 0.5% v/v DMSO) was at par to that without DMSO, Enteropan caused nitrite concentration in *P. aeruginosa* culture supernatant to rise. Sodium nitroprusside used as positive control caused 30.8% higher nitrite build-up in *P. aeruginosa* culture. Nitrite Unit (i.e. Nitrite concentration:cell density ratio was calculated to nullify any effect of cell density on nitrite production). **D, E)** Enteropan-pretreated cells responded to certain antibiotics differently. Enteropan-pre-exposed *P. aeruginosa* experienced an increased or decreased susceptibility to imipenem and augmentin, respectively, as revealed in disc diffusion assay. **F)** Enteropan pre-treatment enhanced *P. aeruginosa*'s susceptibility to imipenem, as revealed in the broth dilution assay. **G)** Increased extracellular protein content in *P. aeruginosa* culture grown in the presence of Enteropan. Protein Unit was calculated as ratio of OD₇₅₀/OD₇₆₄ (an indication of protein production per unit of growth). **H)** Reduced intracellular protein content in *P. aeruginosa* grown in the presence of Enteropan. Protein content reported in mg/mL are cell density neutralized values, wherein OD₇₆₄ was adjusted to 1.00 prior to cell lysis. Kanamycin employed as a positive control at its sub-minimum inhibitory concentration level also generated response similar to that of Enteropan from bacterial culture with respect to extracellular and intracellular protein content. DMSO (0.5% v/v) used as 'vehicle control' affected neither extracellular nor intracellular protein content. ^ap ≤ 0.05, ^bp ≤ 0.01, ^cp ≤ 0.001.

by allowing their use at lower doses. However as seen with augmentin in this study, the effect of herbals on antibiotic susceptibility of pathogen may not always be favourable.

Enteropan alters protein synthesis and secretion in P. aeruginosa

Extracellular protein concentration (after nullifying cell density) in *P. aeruginosa* culture supernatant in the presence of Enteropan was found to be 1.89-fold higher than that in the absence of Enteropan (Fig. 3G). Cell density-neutralized intracellular protein concentration of *P. aeruginosa* cells grown in the presence of Enteropan was found to be 1.40-fold lower than cells grown in the absence of Enteropan (Fig. 3H). It seems that Enteropan exerted an inhibitory effect on protein synthesis in *P. aeruginosa* and promoted protein export. This might have caused even some of the essential proteins to leave the cell. The increased export of proteins by Enteropan-treated cells may be assumed to have originated from overexpression of efflux pump/transport machinery (as suggested by the transcriptome data too described later) and a compromised cell envelope integrity suggested by differential expression of 32 genes involved in cell wall or LPS synthesis. Kanamycin, a known inhibitor of protein synthesis in bacteria, was employed as a positive control in this assay. Kanamycin belongs to the aminoglycoside group of antibiotics, which at sub-minimum inhibitory concentration (MIC) level caused *P. aeruginosa* culture supernatants to have 1.79-fold higher extracellular protein. Such increase in extracellular protein concentration in *P. aeruginosa* exposed to sub-MIC level of kanamycin was also reported by Takahashi et al (48).

Enteropan treatment causes large-scale differential gene expression in P. aeruginosa

A whole transcriptome level comparison of the gene expression profile of Enteropan (600 µg/mL)-treated *P. aeruginosa* with that of control revealed a total of 952

genes getting expressed differentially (log fold change ≥ 2 and FDR ≤ 0.001). This amounted to differential expression of 17% of genome, wherein 616 genes were upregulated (Tab. S6) and 336 were downregulated (Tab. S9). Corresponding volcano plot (Fig. S3) is given in supplementary file. A function-wise categorization of all the DEG is presented in Figure 4. While all DEG pertaining to cell division were downregulated (and majority of DEG associated with translation too), the majority of DEG associated with efflux pump/transport were upregulated. Overexpression of efflux machinery is known to compromise bacterial fitness by causing physiological dysregulation (49). Owing to the important physiological roles of efflux pumps in various functions such as intercellular communication, bacterial pathogenicity and virulence, and biofilm formation, expression of majority of them is subject to tight control by different transcriptional regulators. Any mischief with this regulation leading to overexpression of the efflux function may result in leaking of even essential items. An empirical look at the list of DEG suggested that Enteropan attenuated virulence of *P. aeruginosa* by causing dysregulation of metal homeostasis, nitrogen metabolism, transcription, amino acid and protein synthesis, carbon metabolism, motility, efflux, etc. Results of various *in vitro* assays presented in the preceding section corroborates well with the transcriptome data.

Network analysis of DEG in Enteropan-exposed P. aeruginosa

We created PPI network for up- and downregulated genes separately. PPI network for upregulated genes generated through STRING is presented in Figure 5A, which shows 610 nodes connected through 2,272 edges with an average node degree of 7.45. Since the number of edges in this PPI network is 2.06-fold higher than expected (1,101) with a PPI enrichment p-value $< 1.0e-16$, this network can be said to possess

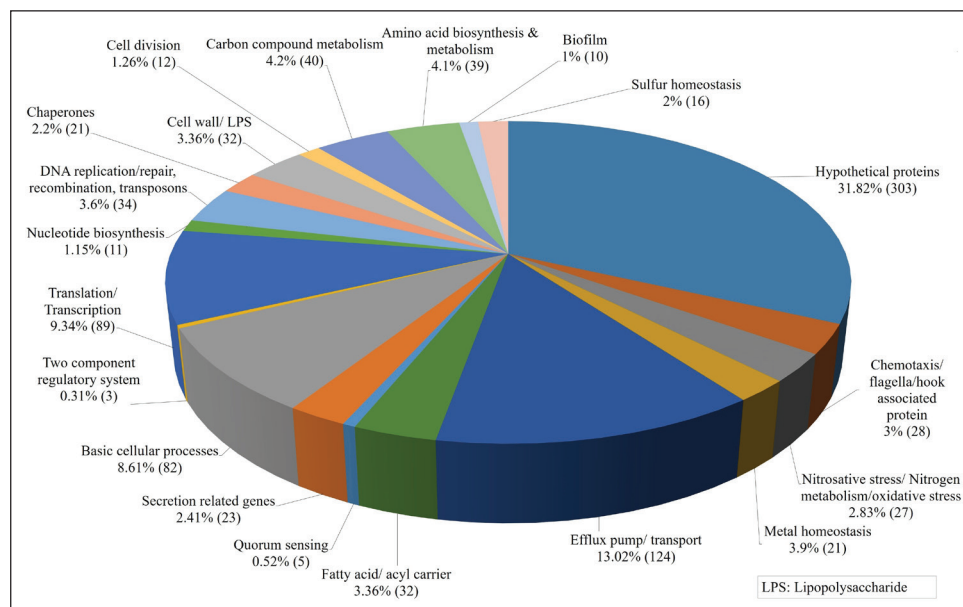


FIGURE 4 - Function-wise categorization of the differentially expressed genes (DEG) in Enteropan-treated *Pseudomonas aeruginosa*. Percent values reported are calculated considering the total number of differently expressed genes as 100%. Values in parenthesis are number of DEG belonging to that particular category.

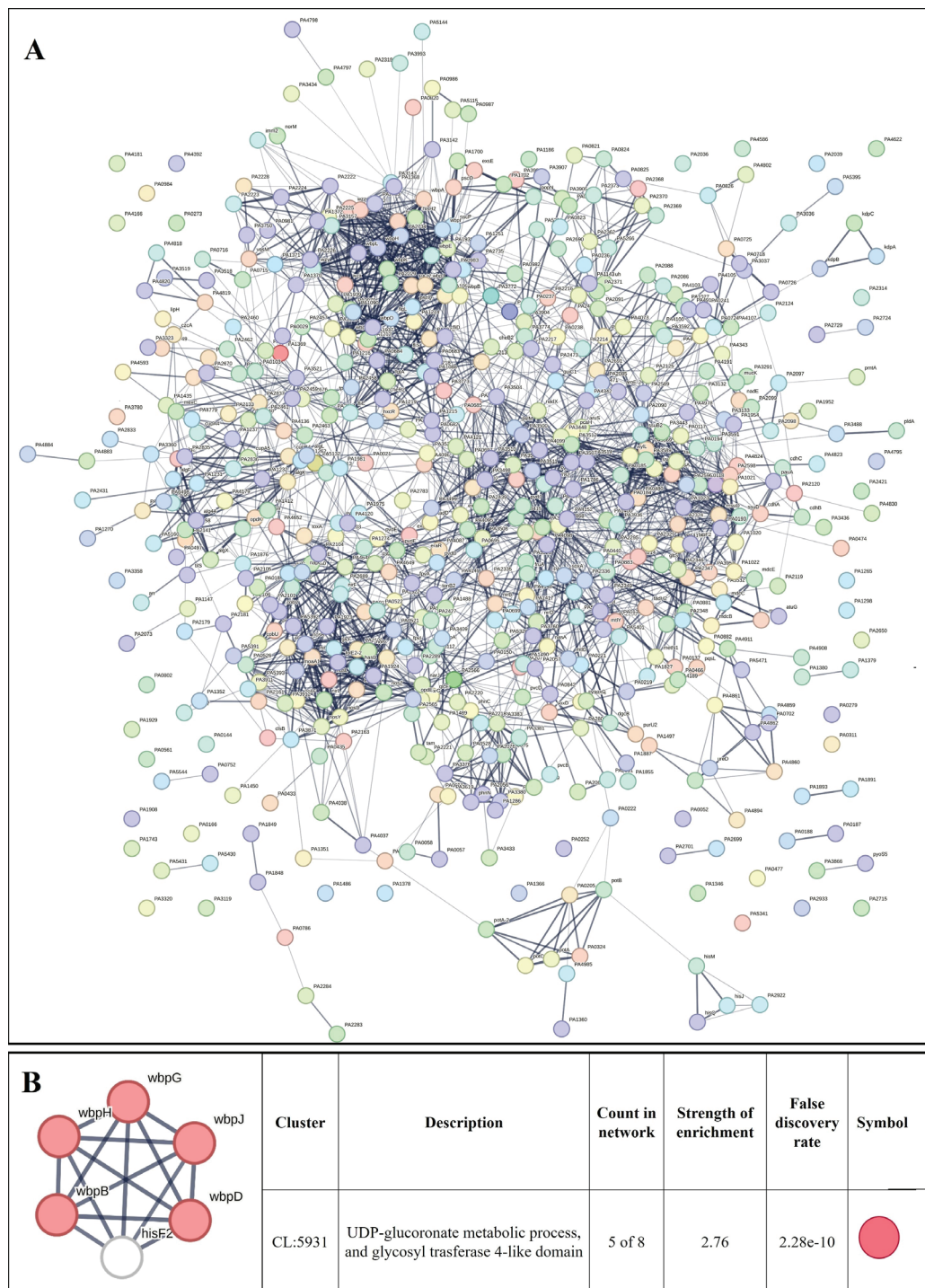


FIGURE 5 - A) Protein-Protein Interaction (PPI) network of upregulated genes in Enteropan-exposed *Pseudomonas aeruginosa*. Edges represent protein-protein associations that are meant to be specific and meaningful, that is, proteins jointly contribute to a shared function. This does not necessarily mean they are physically binding to each other. Network nodes represent all the proteins produced by a single, protein-coding gene locus. **B)** PPI network of top-ranked genes revealed through cytoHubba among upregulated differentially expressed genes (DEG) in Enteropan-exposed *P. aeruginosa*.

significantly more interactions among the member proteins than what can be expected for a random set of proteins of the identical sample size and degree distribution. Such enrichment can be taken as an indication of the member proteins being at least partially biologically connected. When we arranged all the upregulated DEGs in decreasing order of node degree, 572 nodes were found to have a non-zero score (Tab. S7), and we selected top 52 genes with a node

degree ≥ 19 for further ranking by different cytoHubba methods. Then we looked for genes which appeared among top-6 ranked candidates by ≥ 6 cytoHubba methods (Tab. S8), and six such genes were further checked for interactions among themselves by cluster analysis (Fig. 5B), whose overexpression can be hypothesized to disturb pathogen physiology. Interaction map of these six potential hubs showed them to be strongly networked as the average node degree score

was 5. This network possessed 15 edges as against expected (zero) for any such random set of proteins. The PPI network showed five of these six potential hubs to be part of a single local network cluster (Fig. 5B). Co-occurrence analysis showed all of these six hubs being absent from humans (Tab. 3) and

hence agonists of these hubs may be expected to target pathogen selectively without interfering with host system functioning.

PPI network for downregulated genes is presented in Figure 6A, which shows 327 nodes connected through 3,206

TABLE 3 - Co-occurrence analysis of genes coding for potential targets in *P. aeruginosa*

Organism	Potential hubs up-regulated in <i>P. aeruginosa</i>						Potential hubs down-regulated in <i>P. aeruginosa</i>									
	hisF2	wbpG	wbpD	wbpJ	wbpH	wbpB	rpsL	rpIE	rpsD	rpsJ	rpIT	rpoA	rpsB	rpIN	rpIL	rpIF
<i>Pseudomonas aeruginosa</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
<i>Homo sapiens</i>							■	■			■		■	■	■	■
<i>Acinetobacter baumannii</i>	■		■	■			■	■	■	■	■	■	■	■	■	■
<i>Enterobacteriaceae</i>	■		■	■		■	■	■	■	■	■	■	■	■	■	■
<i>Salmonella Serotype typhi</i>	■		■	■			■	■	■	■	■	■	■	■	■	■
<i>Shigella Spp.</i>	■		■	■			■	■	■	■	■	■	■	■	■	■
<i>Mycobacterium tuberculosis</i>	■		■	■			■	■	■	■	■	■	■	■	■	■

The color of the squares denotes, for each gene of interest, the degree of similarity of its best hit in a given STRING genome.

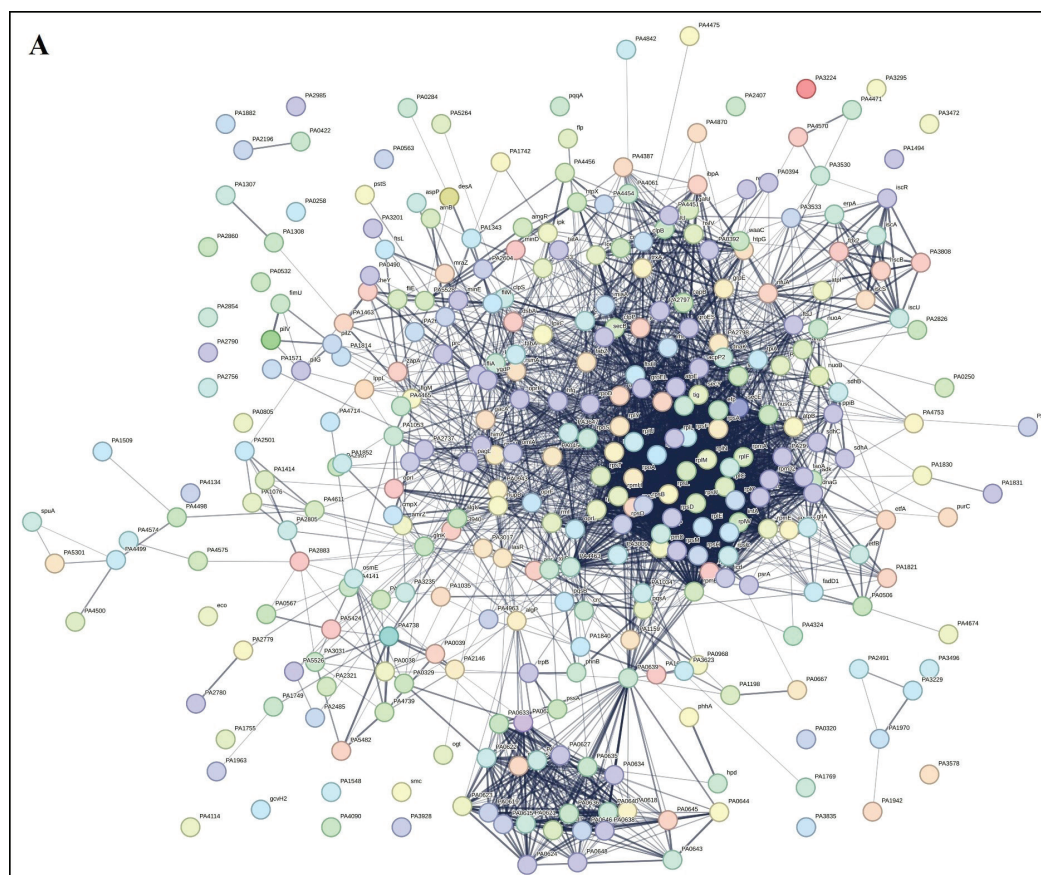


FIGURE 6 - A) Protein-Protein Interaction (PPI) network of downregulated genes in Enteropan-exposed *Pseudomonas aeruginosa*. **B)** PPI network of top-ranked genes revealed through cytoHubba among downregulated differentially expressed genes (DEG) in Enteropan-exposed *P. aeruginosa*.

Cluster	Description	Count in network	Strength of enrichment	False discovery rate	Symbol
CL:150	Small ribosomal subunit	2 of 7	2.2	0.0072	●
CL:96	rRNA-binding	4 of 15	2.17	1.65e-06	●
CL:93	Cytosolic large ribosomal subunit and small ribosomal subunit	6 of 25	2.13	3.22e-10	●
CL:149	Small ribosomal subunit and zinc-binding ribosomal protein	3 of 13	2.11	0.00018	●
CL:90	Ribosomal subunit	9 of 49	2.01	1.39e-15	●
CL:84	Ribosome	10 of 60	1.97	6.58e-17	●



edges with an average node degree of 19.6. Since the number of edges in this PPI network is 1.93-fold higher than expected (1,658) with a PPI enrichment p-value $<1.0e-16$, this network can be said to possess significantly more interactions among the member proteins than what can be expected for a random set of proteins of the identical sample size and degree distribution. Such enrichment can be taken as an indication of the member proteins being at least partially biologically connected. When we arranged all the downregulated DEGs in decreasing order of node degree, 298 nodes were found to have a non-zero score, and we selected top 50 genes with a node degree ≥ 53 (Tab. S10) for further ranking by different cytoHubba methods. Then we looked for genes which appear among top-10 ranked candidates by ≥ 6 cytoHubba methods, and 10 such genes (Tab. S11) were identified as potential hubs, whose downregulation can be hypothesized to attenuate *P. aeruginosa* virulence. Interaction map of these 10 potential hubs (Fig. 6B) showed them to be strongly networked as the average node degree score was 9. This network possessed 45 edges as against expected (15) for any such random set of proteins. The PPI network showed these 10 genes to be distributed among six different local network clusters, whose strength score ranged from 1.97 to 2.2 (Fig. 6B). Co-occurrence analysis (Tab. 3) of these 10 hub proteins indicated four (*rpsD*, *rpsJ*, *rpoA*, *rplF*) of them to be absent from humans, and hence they can be said to possess high targetability with respect to discovery of new antibiotics satisfying the criteria of selective toxicity. Since all the 10 predicted hubs are indicated by co-occurrence analysis to be present in other important bacterial pathogens too, antagonists of these proteins are likely to be useful as broad-spectrum antibiotics.

Target validation through RT-PCR

From the 10 identified hubs among the downregulated genes, we selected five (*rpoA*, *tig*, *rpsB*, *rpsL*, *rpsJ*) for further validation through RT-PCR. From the 10 genes shown in Figure 6B, four (*rpoA*, *rpsB*, *rpsL*, *rpsJ*) passing the dual criteria of node degree ≥ 70 (Tab. S10) and been part of ≥ 3 clusters were selected for RT-PCR. Though *rpsD* (node degree 70) also passed these dual criteria, since already three *rps* genes were selected for PCR, we preferred *rpoA* (node degree 75) over it. Additionally, we included one gene (*tig*; 3.73-fold \downarrow ; node degree 70) for PCR validation, though it was not among the identified hubs, because *tig* is a trigger factor involved in protein export, and we did observe a heavy increase in extracellular protein content in Enteropan-exposed *P. aeruginosa*. PCR did confirm downregulation of all the selected five genes in Enteropan-exposed *P. aeruginosa* (Fig. 7), and thus they can be considered as potential antibacterial targets worthy of attention by drug discovery programmes.

Conclusion

The polyherbal formulation Enteropan was found to have virulence-attenuating effect against an important gram-negative bacterial pathogen *P. aeruginosa*, without affecting

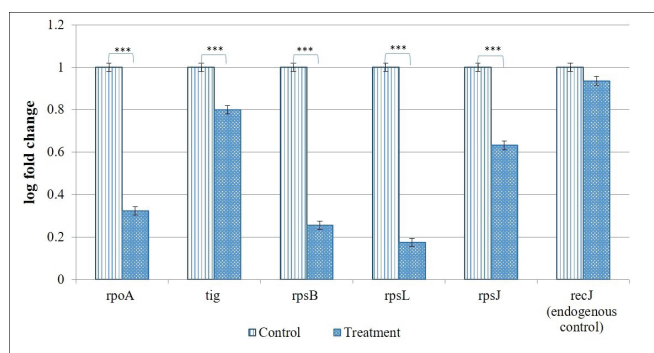


FIGURE 7 - Confirmation of differential expression of selected genes in Enteropan-treated *Pseudomonas aeruginosa* through real-time polymerase chain reaction (RT-PCR). *recJ* selected as an endogenous control was not expressed differently (false discovery rate 1) between control and experimental bacterial cultures. *** $p \leq 0.001$.

its growth heavily. As can be expected from any multicomponent polyherbal formulation, Enteropan also exerted multiplicity of targets against the test pathogen. A large fraction of the bacterial genome was expressed differently under influence of this anti-pathogenic formulation, which corroborated well with the altered phenotypic traits in extract-exposed bacterial culture. Major mechanisms revealed various *in vitro/in vivo* assays and transcriptome analysis through which Enteropan exerted its anti-virulence activity were found to be generating nitrosative stress, oxidative stress, quorum modulation, disturbance of protein homeostasis, and metal homeostasis. A wholistic summary depicting the mechanistic details associated with the anti-pathogenic potential of Enteropan against *P. aeruginosa* is presented in Figure 8, with particular attention on Enteropan's effect on QS machinery and virulence regulators of this notorious pathogen. Our results validate the anti-pathogenic potential of Enteropan, and also the concept of polyherbalism and its relevance in combating antimicrobial resistance.

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Disclosures

Conflict of interest: HSP, is involved in manufacturing and marketing of the formulation Enteropan[®]; however, that in no way has affected the design of experiments or interpretation of results. Other authors declare no competing interest.

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Data availability statement: All the relevant data has been included in the main manuscript and supplementary files.



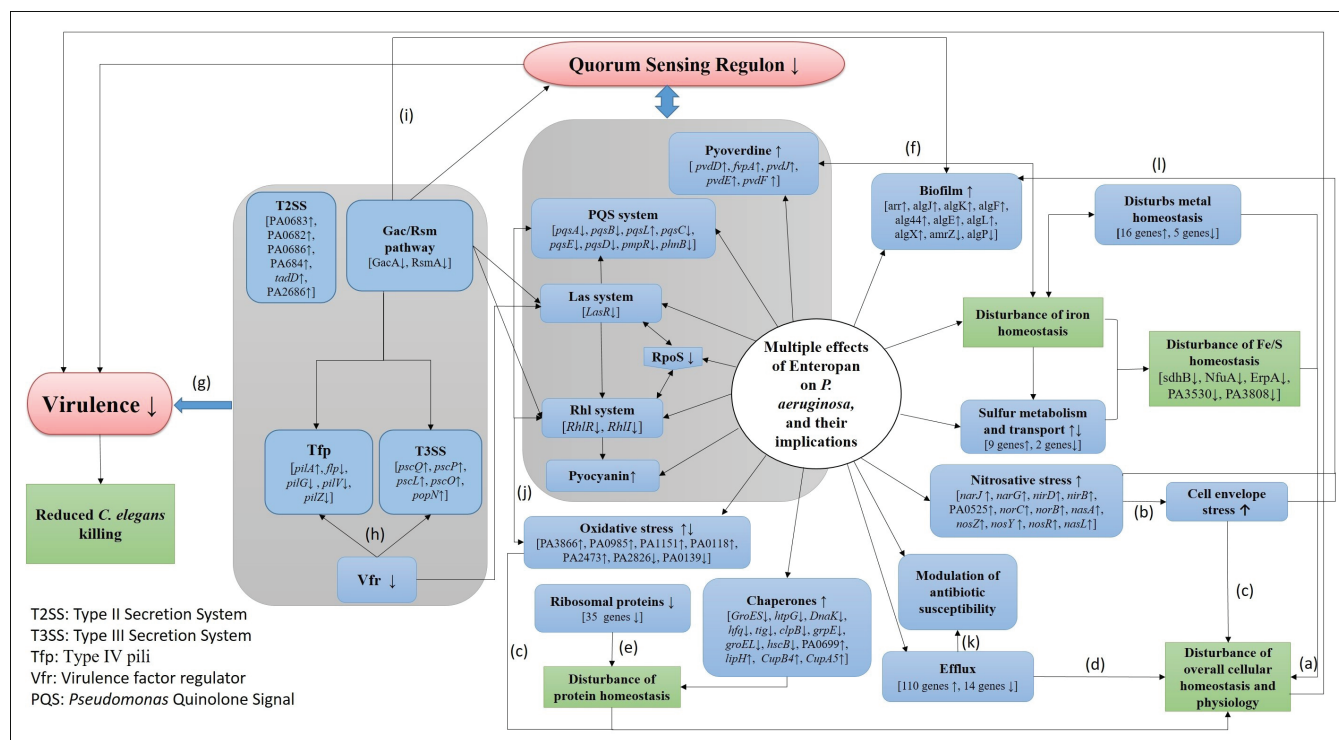


FIGURE 8 - Large-scale disturbance of transcriptional regulation in *Pseudomonas aeruginosa* caused by Enteropan compromises its overall cellular homeostasis and virulence. This figure presents a wholistic summary of multiple effects exerted by Enteropan against *P. aeruginosa*. Various cellular, physiological, and virulence-associated traits of *P. aeruginosa* expressed differently under the influence of Enteropan are depicted. The genes shown with an up or down arrow are those getting differentially expressed with a log fold change of ≥ 1.5 and false discovery rate (FDR) ≤ 0.05 . The *Gac/Rsm pathway* inversely regulates the expression of virulence factors (*T3SS*, *Tfp*, exopolysaccharides) associated with acute and chronic disease (50). *T2SS* is responsible for secreting many secretory proteins like alkaline phosphatase, lipase, exotoxin A, phospholipase, and proteases. *T3SS* is largely involved in secretion of virulence determinants associated with acute infection. *Vfr* is a global regulator of virulence gene expression, which allows coordinated production of related virulence functions (*Tfp*, *T3SS*) necessary for adherence to an intoxication of host cells. *LasR* is a transcriptional activator of multiple virulence-associated genes in *P. aeruginosa*. It represents a central checkpoint, with the highest degree of interconnection in the network. *RpoS*, the stationary phase sigma factor, influences the expression of more than one-third of all the quorum-regulated genes. It is a central regulator of many stationary phase-inducible genes and a master stress-response regulator under a variety of stress conditions (51). *Tfp*, a major surface adhesin, mechanistically regulates virulence factors in *P. aeruginosa* (52). The *Rhl* system is a quorum sensing system acquired by *P. aeruginosa* through lateral gene transfer. *PQS* is an essential mediator of the shaping of the population structure of *P. aeruginosa* and of its response to and survival in stress conditions. ^a(53); ^b(44); ^c(54); ^d(55); ^e(56); ^f(57); ^g(58); ^h(59); ⁱ(60); ^j(61, 62); ^k(63); ^l(64).

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