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RESEARCH ARTICLE

Identifying the Molecular Targets of an Anti-pathogenic Hydroalcoholic Extract of *Punica granatum* Peel Against Multidrug-resistant *Serratia marcescens*

Chinmayi Joshi[1], Pooja Patel[1], Pawan Godatwar[2], Sanjeev Sharma[2] and Vijay Kothari[1,*]

[1]*Institute of Science, Nirma University, Ahmedabad- 382481, India;* [2]*National Institute of Ayurveda, Jaipur, India*

Abstract: ***Background*:** Antibiotic-resistant members of the family *Enterobacteriaceae* are among the serious threats to human health globally. This study reports the anti-pathogenic activity of *Punica granatum* peel extract (PGPE) against a multi-drug resistant, beta-lactamase producing member of this family i.e. *Serratia marcescens*.

***Objective*:** This study aimed at assessing the anti-pathogenic activity of PGPE against the gram-negative bacterial pathogen *S. marcescens* and identifying the molecular targets of this extract in the test bacterium.

***Methods*:** Effect of PGPE on *S. marcescens* growth and quorum sensing (QS)-regulated pigment production was assessed through broth dilution assay. *In vivo* anti-infective and prophylactic activity of PGPE was assessed employing the nematode worm *Caenorhabditis elegans* as a model host. Differential gene expression in PGPE-exposed *S. marcescens* was studied through a whole transcriptome approach.

***Results*:** PGPE was able to modulate QS-regulated pigment production in *S. marcescens* without exerting any heavy growth-inhibitory effect at concentrations as low as ≥2.5 µg/mL. It could attenuate the virulence of the test bacterium towards the worm host by 22-42% ($p \leq 0.01$) at even lower concentrations (≥0.5 µg/mL). PGPE also exerted a post-extract effect on *S. marcescens*. This extract was found to offer prophylactic benefit too, to the host worm, as PGPE-pre-fed worms scored better (34-51%; $p \leq 0.001$) survival in face of subsequent bacterial attack. Differential gene expression analysis revealed that PGPE affected the expression of a total of 66 genes in *S. marcescens* by ≥1.5 fold.

***Conclusion*:** The anti-virulence effect of PGPE against *S. marcescens* is multifaceted, affecting stress-response machinery, efflux activity, iron homeostasis, and cellular energetics of this bacterium notably. Among the major molecular targets identified in this study are LPS export transporter permease (LptF), t-RNA pseudouridine synthase (TruB), *etc*.

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1. INTRODUCTION

Antibiotic-resistant bacteria have now been well-recognized as a major factor impacting our health as well as the social and economic aspects of life [1]. Antimicrobial resistance (AMR) is a serious economic and security threat, which needs immediate action. If left unchecked, the estimated 'cost of inaction' maybe 100 trillion USD. Though a variety of gram-positive and gram-negative bacterial pathogens harbor AMR trait, gram-negative bacteria are particularly believed to be more troublesome. The antibiotic pipeline lacks new leads and new mechanisms against antibiotic-resistant gram-negative bacteria (bugworksresearch.com). It is difficult to find new 'hits' against gram-negative bacteria, partly due to the presence of an additional barrier in the form of the 'outer membrane' in their cell envelope [2].

Many gram-negative bacteria of the family *Enterobacteriaceae* are frequently isolated from clinical samples. Carbapenem and cephalosporin (third-generation) resistant,

*Address to correspondence to this author at the Institute of Science, Nirma University, Ahmedabad- 382481, India;
E-mail: vijay.kothari@nirmauni.ac.in

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ESBL (extended-spectrum beta-lactamase) producing members of this family have been listed by WHO as 'critical' priority pathogens [https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf]. CDC (Centers for Disease Control and Prevention) has listed this group as 'serious threats'[https://www.cdc.gov/drugresistance/biggest_threats.html].The bacterium *Serratia marcescens* is a member of the *Enterobacteriaceae*. It is a notable nosocomial pathogen involved in pneumonia, intravenous catheter-associated infections, urinary tract infections, and endocarditis [3, 4]. *S. marcescens* is considered an important emerging pathogen [5] with the capacity of rapid spread within hospitals, and a systemic mortality rate of ~41% [6]. Despite the undisputed clinical relevance of this bacterial pathogen, not much is known regarding its mechanism of pathogenesis and the host immune response. Thus, there is a knowledge-gap, which needs to be addressed.

To blunt the impact of antibiotic-resistant gram-negative infections on patients and mitigate the rising costs of treating them, novel approaches, mechanisms, and targets are urgently needed. One of the under-exploited strategies is tapping the potential of traditional medicine (TM) for combating AMR. TM practices prevalent in various geographic locations involve the use of different herbal/ herbo-mineral extracts/ formulations to manage different diseases. One such widely mentioned item in these ancient systems of complementary and alternative medicine, including the Indian system *Ayurved*, is *Punica granatum* L. extracts. This plant belonging to the family Lythraceae (Punicaceae), bears common English and Hindi names as pomegranate and *Anar*, respectively. In Egyptian culture, common ailments such as inflammation, diarrhea, intestinal worms, cough, and infertility are treated using pomegranate peel extract [7]. *P. granatum* has been indicated in traditional Iranian as well as Indian medicine for its antimicrobial activity, for treatment of throat infections, diarrhea, wound healing, *etc* [8]. In *Ayurvedic* texts, this plant has been described as 'dadim' (its *Sanskrit* name) and is suggested to cure parasitic infections [9]. For the management of many health problems (vaginal trichomoniasis, pharyngitis, periodontitis, paracoccodioidomycosis, malaria, leshminasis, *Klebseiella* Pneumonia, *Helicobacter pylori* infection, gonorrhea, gingivitis, genital herpes, dysentery, diarrhea, conjunctivitis, cholera, *etc*.) that involve microbial infections, *P. granatum* has been prescribed in TM (https://cb.imsc.res.in/imppat/basicsearch/therapeutics). Ayurvedic pharmacopeia of India mentions formulations containing rind of *P. granatum* to be useful in conditions such as fever, dysentery, bacteremia, and infections of the oral cavity [10]. Despite their wide fame in medicinal texts of various cultures, the efficacy of pomegranate extracts needs to be validated through appropriate experiments for their wider acceptance in the modern world.

This study investigated the effect of hydroalcoholic extract of *P. granatum* peel on *S. marcescens* growth and various virulence traits, besides characterizing the differential gene expression of extract-exposed *S. marcescens.*

2. MATERIALS AND METHODS

2.1. Test Organism

S. marcescens (MTCC 97) was procured from MTCC (Microbial Type Culture Collection, Chandigarh). Incubation temperature was kept 28 ºC to support pigment production [11]. Pigment (prodigiosin) production in *S. marcescens* is a trait, which is reported to be under control (largely but not fully) of its quorum sensing (QS) machinery [4]. Incubation time was kept 22-24 h. Antibiotic resistance profile of the bacterial strain used in this study was generated using the antibiotic discs-Dodeca Universal – I, Dodeca G - III – Plus, and Icosa Universal -2 (HiMedia, Mumbai). *S. marcescens* was found to be resistant to cefadroxil (30 µg), ampicillin (10 µg), cloxacillin (1 µg), penicillin (10 µg), and vancomycin (30 µg).

2.2. Plant Material

Peels of *P. granatum* were procured from the fruits purchased from the local market in the city of Ahmedabad. Plant material (ref no: GU/Bot/29/4/2015) was authenticated for its unambiguous identity by Dr. Archana Mankad, Department of Botany, Gujarat University, Ahmedabad. The plant name was also checked with http://www.theplantlist.org on April 20, 2018. Collected peels were shade dried before being used for extract preparation.

2.3. Extract Preparation

A hydroalcoholic extract of the plant material was prepared in 50% ethanol using the microwave-assisted extraction (MAE) method [12]. Dry peel powder (total 5 g; 1 g in each vessel) was soaked in the solvent in a ratio of 1:50, and subjected to microwave heating (Electrolux EM30EC90SS) at 720 W. Total heating time was kept 120 seconds, with intermittent cooling (first heating of 40 sec followed by 40-sec cooling, and then all subsequent heating periods of 10 sec with intermittent 40-sec cooling). This was followed by centrifugation (at 10,000 rpm for 15 min.) and filtration with Whatman paper # 1 (Axiva, Haryana). The solvent was evaporated from the filtered extract and then the dried extract was reconstituted in dimethyl sulfoxide (DMSO (absolute); Merck) for broth dilution assay. The reconstituted extract was stored under refrigeration for further use. The stability of this extract under refrigerated storage with respect to its anti-infective activity was also ensured: https://doi.org/10.1101/511733. Extraction efficiency was calculated as the percentage weight of the starting dried plant material, and its value was 42% w/w. Details on the chromatographic and mass spectrometric characterization of this extract can respectively be accessed at: https://shodhganga.inflibnet.ac.in/jspui/bitstream/10603/245830/10/13_chapter4.pdf, and https://shodhganga.inflibnet.ac.in/bitstream/10603/245830/14/17_annexure.pdf.

2.4. Broth Dilution Assay

Assessment of QS-regulated pigment production by test pathogen in the presence or absence of the test extract was done using broth dilution assay [13]. The organism was challenged with different concentrations (0.5-500 µg/mL) of *P. granatum* peel extract (PGPE). The nutrient broth was used as a growth medium. Inoculum standardized to 0.5 McFarland turbidity standard was added at 10%v/v to the media supplemented with a required concentration of PGPE, followed by incubation at 28 ºC. Appropriate vehicle control containing DMSO was also included in the experiment,

along with abiotic control (containing extract and growth medium, but no inoculum). Catechin (50 µg/mL; Sigma-Aldrich, USA) and Chloramphenicol (5 µg/mL; HiMedia, Mumbai) were used as positive controls since these are already known QS inhibitor [14, 15].

2.4.1. Measurement of Bacterial Growth and Pigment Production

At the end of the incubation, bacterial growth was quantified at 764 nm [16, 17]. This was followed by pigment extraction and quantification, as per the method described in the following paragraph. The purity of the extracted pigment was confirmed by running a UV-vis scan (Agilent Cary 60 UV-visible spectrophotometer). The appearance of a single major peak (at the λ_{max} reported in the literature, *i.e*. 535 nm) was taken as an indication of purity.

2.4.2. Prodigiosin Extraction

One mL of the culture broth was centrifuged at 10,600 g for 10 min. Centrifugation was carried out at 4°C, as prodigiosin is a temperature-sensitive compound. The resulting supernatant was discarded. The remaining cell pellet was resuspended in 1 mL of acidified methanol (4 mL of HCl into 96 mL of methanol; Merck), followed by incubation in dark at room temperature for 30 min. This was followed by centrifugation at 10,600 g for 10 min at 4°C. Prodigiosin was obtained in the resulting supernatant; absorbance was taken at 535 nm. Prodigiosin Unit was calculated as A_{535}/OD_{764}.

2.5. N- acyl-homoserinelactone (AHL) Extraction

OD of overnight grown bacterial culture was standardized to 1.00 at 764 nm. It was centrifuged at 5000 g for 5 min. Cell-free supernatant was filter-sterilized using 0.45 µm syringe filter (Axiva, Haryana), and was mixed with an equal volume of acidified ethyl acetate [0.1% formic acid (Merck) in Ethyl acetate (Merck)]. The ethyl acetate layer was collected, and evaporated at 55˚C, followed by the reconstitution of the dried crystals in 100µL phosphate buffer saline (pH 6.8). The identity of extracted AHL was confirmed by thin-layer chromatography (TLC). TLC of purified AHL was performed using Methanol (60): Water (40) solvent system on TLC Silica gel 60 F_{254} plates (Merck, Mumbai) [18, 19]. R_f value of purified AHL from *S. marcescens* was found to be 0.83, near to that (0.80) reported for C12-HSL [20].

2.6. Hemolysis Assay

OD_{764} of overnight grown (in presence or absence of PGPE) bacterial 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 (collected in a heparinized vial) was incubated with this cell-free supernatant for 2 h at 37 ˚C, followed by centrifugation at 800 g for 15 min. Phosphate buffer saline (pH 7) was used as a negative control. The absorbance of the supernatant was read at 540 nm, to quantify the amount of haemoglobin released (1% Triton X-100 (CDH, New Delhi) was used as a positive control) [21].

2.7. Catalase Assay

OD_{764} of overnight grown (in the presence or absence of PGPE) bacterial culture was adjusted to 1.00. Four hundred µL of phosphate buffer (pH 7) was added into a 2 mL vial followed by 400 µL H_2O_2. To this 200 µL of the bacterial culture was added and the mixture was incubated for 10 min. Then, 10 µM of sodium azide (Sigma-Aldrich, Mumbai) was added to stop the reaction [22], followed by centrifugation at 12,000 rpm for 10 min. The absorbance of the supernatant was measured at 240 nm to quantify the remaining H_2O_2[23] with phosphate buffer as blank.

2.8. Determination of the Effect of PGPE on Antibiotic Susceptibility of the Test Organism

The *S. marcescens* culture grown for 24 h in the presence or absence of PGPE was centrifuged (REMI CPR-24 Plus) at 10,600 g for 10 min at 4˚C. The resulting supernatant was discarded, and the remaining cell pellet was resuspended in 1 mL of sterile PBS (pH 7), and then again centrifuged for 10 min. This was followed by a second wash of the cell pellet with PBS. These bacterial cells (with or without PGPE pretreatment) were used to prepare inoculum for a subsequent challenge with a sub-MIC concentration of different antibiotics. All the antibiotics were procured from HiMedia, Mumbai.

2.9. *In vivo* Assay

2.9.1. Anti-infective Assay

In vivo anti-infective efficacy of the PGPE was evaluated using the nematode worm *Caenorhabditis elegans* as the model host, using the method described by Eng and Nathan [24] with some modification. This worm was maintained on Nematode Growing Medium (NGM; 3 g/L Nacl, 2.5 g/L peptone, 1 M $Cacl_2$, 1 M $MgSO_4$, 5 mg/mL cholesterol, 1 M phosphate buffer of pH 6, 17 g/L agar-agar) with *Escherichia coli* OP50 (procured from LabTIE B.V., JR Rosmalen, the Netherlands) as the feed. Worm population to be used for the *in vivo* assay was kept on NGM plates not seeded with *E. coli* OP50 for three days, before being challenged with the test pathogen.

The test bacterium was incubated with the PGPE for 22-24h. Following incubation, OD_{764} (1.50 - 2) of the culture suspension was equalized to that of the DMSO control. 100 µL of this bacterial suspension was mixed with 900 µL of the M9 buffer containing 10 worms (L3-L4 stage). This experiment was performed in 24-well (sterile, non-treated) polystyrene plates (HiMedia), and incubation was carried out at 22˚C. The number of live vs. dead worms was counted every day until five days by putting the plate (with lid) under a light microscope (4X). Ofloxacin (0.1 µg/mL; HiMedia, Mumbai) and catechin treated bacterial suspension was used as a positive control. Straight (non-moving) worms were considered to be dead. 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.

2.9.2. Prophylactic Assay

PGPE-fed worms were challenged with *S. marcescens* (previously not exposed to PGPE), and their ability to survive in face of pathogen challenge was compared with their PGPE-unfed counterparts. *C. elegans* worms maintained on NGM were kept unfed for 24 h before being used for exper-

iments. These worms were then fed with PGPE by mixing the 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) containing 10 worms per well. Duration of exposure of worms to PGPE was kept to 96 h, followed by the addition of pathogenic bacteria (100 µL of bacterial suspension with OD_{764}= 1.50 measured with Agilent Cary 60 UV-Vis spectrophotometer). Before mixing with the pathogenic bacteria, worms were washed twice with phosphate buffer saline (pH 7) to remove any traces of plant extract. Appropriate controls *i.e.* worms previously not exposed to PGPE, but exposed to pathogenic bacteria; worms exposed neither to PGPE nor bacteria; and worms exposed to PGPE, but not to bacterial pathogens, were also included in the experiment. Incubation was carried out at 22°C. Numbers of dead vs. live worms were counted every day for 5 days as described in the preceding paragraph.

2.10. Whole Transcriptome Analysis

PGPE (10 µg/mL)-treated culture of *S. marcescens* (along with control culture) was subjected to whole transcriptome analysis to get a holistic picture regarding the mode of action of this formulation.

2.10.1. Preparing Bacterial Sample for RNA Isolation

The *S. marcescens* culture grown for 48 h in the presence or absence of PGPE was separated into two parts under aseptic condition. The first part was subjected to quantification of cell density at OD_{764} followed by pigment extraction and estimation. Another part containing 2 mL of the culture broth was centrifuged (REMI CPR-24 Plus) at 5,500 g for 10 min at 4˚C. The resulting supernatant was discarded, and the remaining cell pellet was resuspended in 1 mL of sterile ice-cold PBS (pH 7), and then again centrifuged for 10 min. This process was repeated for three times. After completion of the washing, 500 µL RNA*later*® solution (Sigma- Aldrich, Mumbai) was added to the pellet. Control and experimental tubes containing cell pellets were stored at 4˚C till initiation of RNA isolation. Total RNA was isolated from the samples by Trizol method [https://health.ucdavis.edu/cancer/research/sharedresources/documents/totalRnaIsolationUsingTrizol.pdf]. The quality of the isolated RNA was checked on 1% formaldehyde denaturing agarose gel and quantified using Qubit® 2.0 fluorometer.

2.10.2. Illumina 2 x 150 PE Library Preparation:

The libraries were prepared with input total RNA ~4µg using MICROBExpress Bacterial mRNA Enrichment Kit and IlluminaTruSeq Stranded mRNA Library Preparation Kit as per the manufacturer's protocol. Briefly, total RNA was Ribo depleted using MICROBExpress Bacterial mRNA Enrichment Kit, then subjected to purification, fragmentation and priming for cDNA synthesis. The fragmented mRNA was converted into the first-strand cDNA, followed by second-strand cDNA synthesis, A-tailing, adapter-index ligation and finally amplified by the recommended number of PCR cycles. Library quality and quantity checks were performed using Agilent DNA High Sensitivity Assay Kit. The amplified libraries were analyzed on Bioanalyzer 2100 (Agilent Technologies) using high sensitivity DNA chip as per the manufacturer's instructions.

2.10.3. Cluster Generation and Sequencing

After obtaining the Qubit concentration for the library and the mean peak size from the Bioanalyzer profile, the library was loaded into the Illumina platform for cluster generation and sequencing. Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions. The library molecules bind to complementary adapter oligos on the paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment. All the raw sequence data was submitted to the Sequence Read Archive (SRA). Relevant accession no. are SRX5996223 and SRX5996224 (https://www.ncbi.nlm.nih.gov/sra/?term=SRX5996224).

2.11. 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 (two-tailed; equal variance) using Microsoft Excel®. P values ≤ 0.05 were considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. *In vitro* and *in vivo* Assays

S. marcescens was challenged with PGPE at 0.5-500 µg/mL. Growth of this organism was not much affected even at the highest test concentration, whereas production of the QS-regulated pigment, prodigiosin was affected in a dose-dependent fashion from 5 µg/mL onwards (Fig. **1A**). When AHL was exogenously supplied to the quorum-inhibited culture, the inhibitory effect on pigment production was reversed (Fig. **1B**), indicating that PGPE exerts its quorum modulatory effect against *S. marcescens* by acting as a *signal supply inhibitor*. *In vitro* prodigiosin production in *S. marcescens* was inhibited 3-fold more at 100 µg/mL of PGPE than that at 10 µg/mL (Fig. **1A**); but there was a mere 7% difference in *in vivo* efficacy of these two concentrations (Fig. **1D**). Enhancement in pigment production by *S. marcescens* challenged with an anti-infective quorum-modulatory polyherbal *Panchvalkal*formulation was reported by us earlier [13], and the formulation was effective *in vivo*. The effect of PGPE on *S. marcescens* pigment production seemed to follow the threshold model, wherein the threshold concentration can be said to be >2.5 µg/mL (Fig. **1A**).

PGPE was able to induce catalase activity and curb the haemolytic activity of *S. marcescens* (Table **1**). When PGPE-treated bacterial cells were subsequently challenged with sub-MIC concentrations of different antibiotics, there was a marginal decrease in the bacterial susceptibility towards tetracycline, ampicillin and chloramphenicol (Fig. **1C**). Such a reduction in antibiotic susceptibility of plant extract-treated *S. marcescens* was previously also observed by us [25, 26]. This suggests that complementary use of plant products along with conventional antibiotics needs careful consideration of the (a) possible effect of phytocompounds on bacterial antibiotic susceptibility, as well as (b) possible

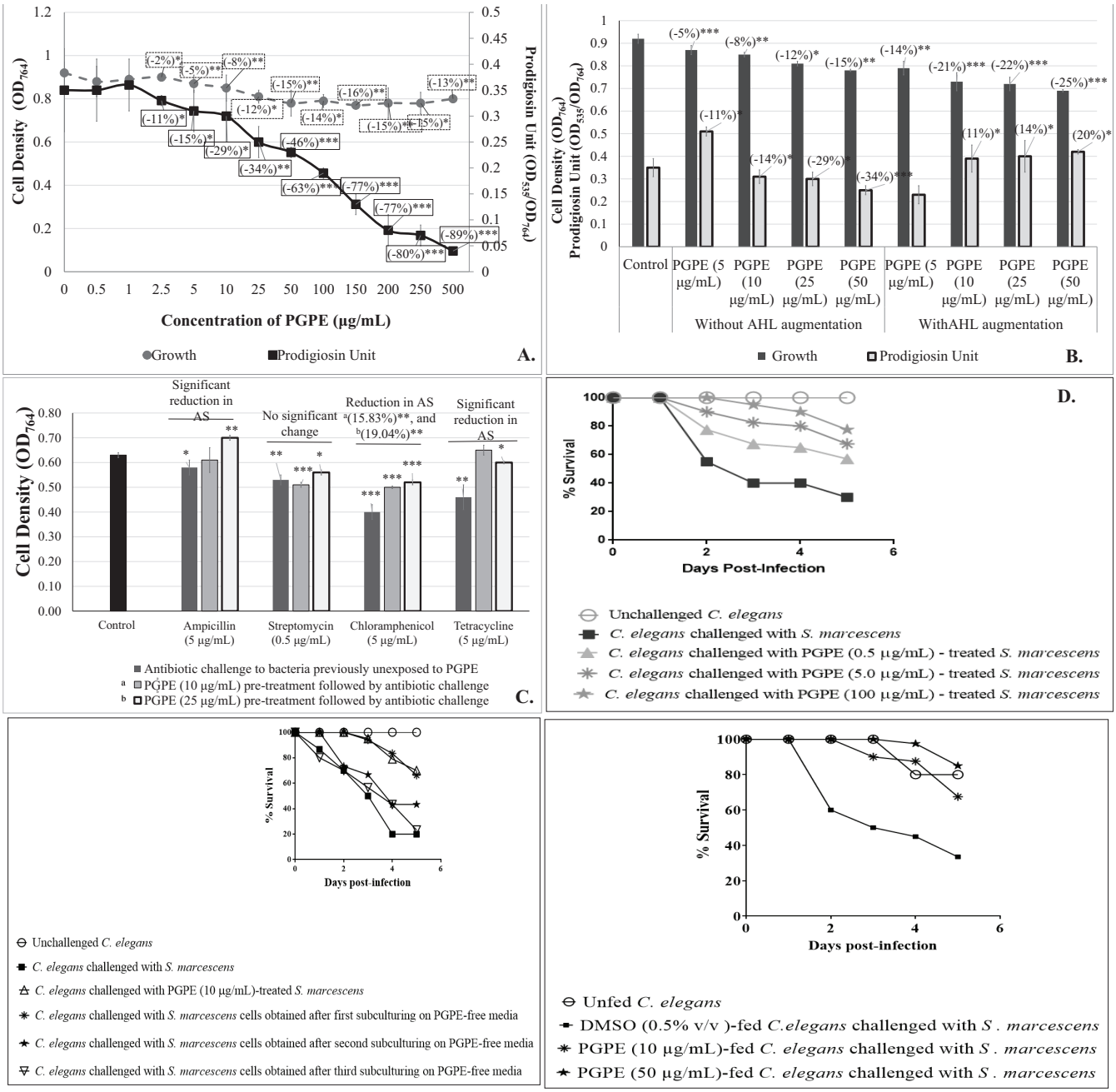

Fig. (1). Effect of PGPE against *S. marcescens*.

*p<0.05, **p<0.01, ***p<0.001; AS: Antibiotic susceptibility; QS: Quorum sensing; PGPE: *Punicagranatum*peel extract; 'Control' in this figure is the vehicle control (0.5%v/v DMSO), which did not exert any effect on growth and prodigiosin production of *S. marcescens*.

(A) Effect of PGPE on growth and QS-regulated prodigiosin production in *S. marcescens:* Bacterial cell density was quantified as OD_{764}; Absorbance of prodigiosin was measured at 535 nm, and Prodigiosin Unit was calculated as the ratio A_{535}/OD_{764} (an indication of prodigiosin production per unit of growth); Catechin (50 µg/mL) inhibited prodigiosin production by 10%* ± 0.05 without affecting bacterial growth; Chloramphenicol (5 µg/mL) inhibited the growth of *S. marcescens* by 33.36%*** ± 3.13, and prodigiosin production by 49.67%*** ± 2.61. **(B)** PGPE acts as a *signal-supply inhibitor* against *S. marcescens*. **(C)** PGPE -pre-treatment modulates susceptibility of *S. marcescens* to different antibiotics. **(D)** PGPE-treatment reduces the virulence of *S. marcescens* towards *C. elegans:* Worms challenged with catechin (50 µg/mL) - orofloxacin (0.1 µg/mL)- treated bacteria, employed as positive controls, registered 100% and 80% survival respectively. Pre-treatment of bacteria with PGPE at concentrations (0.5, 1, 2.5, 5, 10, 25, 50, and 100 µg/mL) conferred 22.5**± 6.76, 27.5***± 2.29, 30***± 2.64, 32.5***± 2.29, 35***± 7.21, 37.5***± 2.28, 40***± 2.64, and 42.5***± 0.5 % survival benefit, respectively; Survival benefit refers to the difference between number of worms surviving in experimental and control (worms infected with non-treated bacteria) wells; 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 PGPE at tested concentrations showed no toxicity towards the worm. **(E)** PGPE exerts a post-extract effect on *S. marcescens*: PGPE-exposed *S. marcescens* could not restore its virulence fully even after subsequent subculturing in PGPE-free media twice. **(F)** Prophylactic activity of PGPE against *S. marcescens*: In face of subsequent bacterial challenge, *C. elegans* pre-fed with PGPE (10 or 50 µg/mL) for 96 h registered 67.5% ±0.85 and 85% ±0.82 survival respectively at the end of the fifth day; Gentamicin (0.1 µg/mL)-exposed worms, employed as a positive control, registered 100%±0.00 survival, as against 33.5%±0.44 in vehicle control.

Table 1. Effect of PGPE on catalase and haemolytic activity of *S. marcescens*.

Conc. of extract (µg/mL)	(A_{240}) Mean±SD		% Enhancement in Catalase Activity (Mean±SD)	(A_{540}) Mean±SD		% Inhibition in Haemolytic Activity
	Control	Experimental		Control	Experimental	
5	0.88±0.01	0.87 ±0.01	1.31±0.85	0.62±0.01	0.52±0.01	16.12**±2.45
10		0.86±0.005	2.27*±1.31		0.50±0.01	19.35*±2.87
25		0.81±0.005	7.95*±1.08		0.45±0.02	27.41***±5.79
50		0.80±0.01	9.09**±0.73		0.40±0.01	35.48***±2.54

*$p<0.05$, **$p<0.01$, ***$p<0.001$; Catalase assay was done by monitoring disappearance of H_2O_2 at 240 nm; DMSO in 'vehicle control' tube had no effect on catalase and haemolytic activity of this bacterium; Chloramphenicol (5 µg/mL) enhanced catalase activity of *S. marcescens* by 14.20%* ± 1.02; 1% triton served as the positive control (A_{540} = 1.5), and PBS served as the negative control (A_{540} = 0.03) for haemolysis assay.

herb-drug interaction. Though this study did not try PGPE + antibiotic combination, one of our previous studies [13] involved combined use of a polyherbal *Panchvalkal* formulation with antibiotics, and among other bacteria, *S. marcescens*'s response to antibiotics was also found to be altered in the simultaneous presence of plant formulation.

During *in vivo* assay, PGPE was able to confer notable survival benefit on *C. elegans*, when this worm was challenged with *S. marcescens*. Against 35% of worm survival in the control set, survival percentage ranged from 57.5 -77.5% in the experimental sets, wherein PGPE-treated bacteria were allowed to attack *C. elegans* (Fig. **1D**). Interestingly, PGPE showed no effect on *S. marcescens* till 1 µg/mL *in vitro* (Fig. **1A**), but these concentrations were able to offer significant survival benefit to *C. elegans* during *in vivo* assay. Though at 2.5 µg/mL PGPE, there was no *in vitro* effect on prodigiosin production by *S. marcescens*, and only a minor (2.17%) inhibitory effect on its growth; this concentration could attenuate *S. marcescens*'s virulence towards *C. elegans* notably. Despite a huge difference (34.29%; p=0.001) in the magnitude of *in vitro* quorum modulation by PGPE at 5 and 100 µg/mL, there was relatively much lesser difference (10%; p =0.002) in the magnitude of *in vivo* effect of these concentrations. The onset of death in the worm population challenged with bacteria (treated with 100 µg/mL PGPE) was delayed by 1 day (*i.e.* death started 3 days post-infection, as against on the second day in other wells).

When PGPE-exposed *S. marcescens* was subsequently transferred on PGPE-free media, *S. marcescens* obtained after such growth on PGPE-free media till second subculturing was still able to kill lesser worms as compared to control (never exposed to PGPE) *S. marcescens* (Fig. **1E**). Even after third subculturing on PGPE-free media, survival in worm population challenged with experimental bacterial culture was ~20% (p=0.004) higher till the fourth day, than the worm population challenged with control (*S. marcescens* with no PGPE exposure). Thus PGPE can be said to exert post-extract effect (PEE) on this bacterial pathogen. PEE refers to the long-lasting effect of the extract on bacteria, after single-time exposure to the plant extract (https://doi.org/10.32388/359873). It refers to the persistent suppression of one or more bacterial traits (*e.g.* growth/ virulence, *etc*.) after one-time-exposure to antimicrobial agents, and may last for many hours depending on the concentration of test agent and the susceptibility of the target bacterium [27-29]. During actual animal/human infections, such a phenomenon can be of high significance, as though the infectious bacteria may multiply inside the host system, their progenies may not have the virulence at par with the parent cells.

Hitherto described results confirmed PGPE's anti-virulence effect on *S. marcescens*. We conducted an additional experiment to see whether PGPE can offer any prophylactic benefit to worms in face of subsequent bacterial challenge; wherein PGPE-fed worms were challenged with *S. marcescens* (receiving no PGPE treatment). PGPE-pre-fed worms registered 34-51.50% better survival in the face of a bacterial challenge than control worms (Fig. **1F**). Thus, PGPE can be said to be capable of exerting an anti-pathogenic effect on a susceptible pathogen, as well as a protective effect on the host. Latter needs further investigation to elucidate the possible immunomodulatory effect of PGPE on the nematode host.

3.2. Whole Transcriptome Analysis

After confirming the anti-infective activity of PGPE, we attempted to understand the molecular basis underlining the anti-pathogenic potential of this extract. This was achieved by subjecting the PGPE-treated *S. marcescens* (experimental culture) to whole transcriptome analysis (WTA) and comparing its gene expression profile to that of control (*S. marcescens* not exposed to PGPE). This analysis revealed a total of 73 genes (2.41% of the total 3,022) expressed differently ($p \leq 0.05$) in PGPE-treated *S. marcescens*. However, to make our interpretation more robust, for analytical purpose, we focused only on those differentially expressed genes which passed the dual filter of $p \leq 0.05$ and fold-change values of ≥1.5 [30,31]. The number of genes passing these dual criteria turned out to be 66.Of these, expression of 3 genes was altered ≥10 fold, and of 10 genes ≥5 fold. Fold change value of the up-regulated (Table **2**) and down-regulatedd (Table **3**) genes ranged till 10.63 and 14.36 fold, respectively. Function-wise categorization of the differently expressed genes is presented in Fig. (**2**). Maximum number (28) of differentially expressed genes belonged to those associated with basic cellular processes and metabolism, followed by those associated with efflux/ transport (7 genes) and stress-response (6 genes).

Table 2. List of up-regulated genes in PGPE-exposed *S. marcescens*.

Sr. No.	Feature ID	Coding for/ Function	Fold Change	P value
1.	MSTRG.2322.2	Cell wall-associated hydrolase	10.63	2.15E-24
2.	MSTRG.2903.2	Hypothetical protein	9.87	5.84E-26
3.	MSTRG.2977.5	Hypothetical protein TI10_22595, partial	7.86	4.25E-06
4.	Gene2365	Flagellarflis	4.77	0.0003
5.	Gene3137	DUF2778 domain-containing	4.25	2.94E-10
6.	Gene3111	Multidrug transporter	3.91	0.01
7.	Gene3790	DUF1656 domain-containing	3.84	0.02
8.	Gene4283	Probable transport	3.77	0.02
9.	Gene2352	Iron uptake system	3.69	0.03
10.	Gene4621	Non-ribosomal peptide synthetase	3.69	0.03
11.	Gene2746	Pseudo-hpr	3.62	0.04
12.	Gene1578	Transporter of cystinetcyp	3.15	0.02
13.	Gene4322	Signal transduction histidine- kinase phosphatase	2.84	0.05
14.	Gene3179	DUF1007 family	2.77	0.01
15.	Gene479	Hypothetical protein	2.77	0.02
16.	MSTRG.2325.3	Hypothetical protein CS370_21105 (plasmid)	2.70	0.02
17.	Gene4629	2,3-dihydroxybenzoate-AMP ligase	2.69	0.017
18.	Gene2923	Fatty acid oxidation complex subunit alpha	2.65	0.05
19.	Gene1199	Paraquat-inducible B	2.60	0.01
20.	Gene3269	Glutaredoxin-like protein nrdH	2.48	0.0004
21.	Gene4628	Isochorismate synthase	2.40	0.03
22.	Gene2036	Hypothetical protein	2.31	0.04
23.	Gene3540	Daunorubicin doxorubicin resistance ATP-binding	2.15	0.03
24.	Gene202	DNA polymerase III subunit epsilon	2.08	0.007
25.	Gene26	Glutamine-hydrolyzing carbamoyl-phosphate synthase small subunit	2.05	0.006
26.	Gene1635	Uncharacterised	2.03	0.05
27.	Gene1415	DNA polymerase III subunit theta	1.97	0.03
28.	Gene817	Crown gall tumor 1	1.86	0.009
29.	Gene139	5 3 -nucleotidase	1.78	0.03
30.	Gene3888	Malate synthase A	1.72	0.01
31.	Gene4905	3,4-dihydroxyphenylacetate 2,3-dioxygenase	1.63	0.02
32.	Gene158	Alanine--tRNA ligase	1.61	0.002

Table 3. List of down-regulated genes in PGPE-exposed *S. marcescens*.

Sr. No.	Feature ID	Coding for/Function	Fold Change	P-value
1.	MSTRG.3451.4	Hypothetical protein o7e_02948, partial	14.36	2.83E-10
2.	MSTRG.3452.4	Putative orf 58e	13.71	2.14E-08
3.	Gene4792	LPS export ABC transporter permease	6.40	4.54E-08
4.	Gene3717	DUF1090 domain-containing	6.07	1.25E-06
5.	Gene4735	t-RNA pseudouridine(55) synthase	5.46	3.75E-14
6.	Gene71	Phospho-n-acetylmuramoyl-pentapeptide-transferase	5.30	5.94E-07
7.	Gene2729	Uncharacterised	4.59	0.01
8.	Gene4489	Bifunctionalphosphopantothenoylcysteine decarbox-ylase/phosphopantothenate synthase (pathway: pantothenate and coa biosynthesis)	4.55	5.00E-07
9.	Gene2379	Flagellar motor switch	3.59	0.02
10.	Gene4008	Heat shock 15	3.54	0.001
11.	Gene3570	YggT family protein	3.32	0.01
12.	Gene4663	Epoxyqueuosinereductase	3.30	0.05
13.	Gene4355	Formate dehydrogenase cytochrome b556 subunit	3.28	0.01
14.	Gene4754	Multispecies: hypothetical protein	3.24	0.01
15.	Gene4523	Envelope stress response regulator transcription factor	3.00	2.26E-05
16.	Gene4271	Diaminopimelateepimerase	2.83	0.02
17.	Gene3433	Flavinator of succinate dehydrogenase	2.76	0.001
18.	Gene3325	Lipid-a-disaccharide synthase (lpxB)	2.65	0.02
19.	Gene1350	MFS transporter	2.54	0.02
20.	Gene1399	Rrf2 family transcriptional regulator (repressor of oqxAB)	2.29	0.005
21.	Gene4004	Penicillin-binding 1a (mrcA)	2.27	0.0005
22.	Gene2025	Murein peptide amidase a (mpaA)	2.23	0.04
23.	Gene1668	Extracellular lipase	2.11	0.01
24.	Gene3545	Spermidine synthase	1.99	0.05
25.	Gene618	FMN-dependent NADH-azoreductase	1.89	0.03
26.	Gene2496	Cold-shock	1.89	0.005
27.	Gene1180	DUF882 domain-containing	1.87	0.003
28.	Gene3525	Sugar fermentation stimulation a	1.87	0.0004
29.	Gene2928	C-type cytochrome biogenesis	1.84	0.01
30.	Gene3632	Cell division precursor	1.77	0.03
31.	Gene3945	Trna 2-thiouridine synthesizing b	1.76	0.001
32.	Gene4515	Glutaredoxin-3	1.71	0.03
33.	Gene920	Glutamine abc transporter atp-binding	1.60	0.02
34.	Gene3118	U32 family peptidase	1.53	0.04

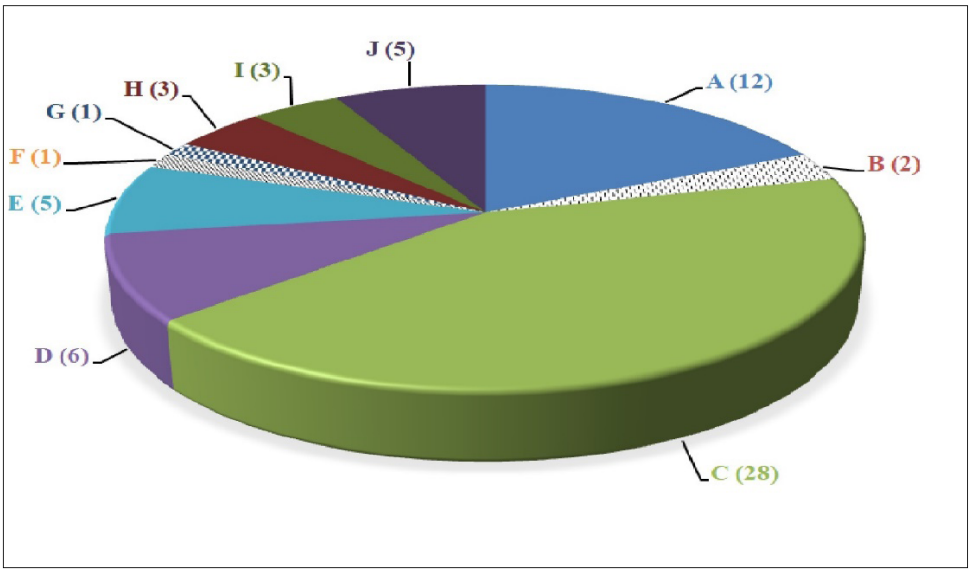

Fig. (2). Function-wise categorization of the significantly differentially expressed genes in PGPE-treated *S. marcescens*.

DUF: Domain of unknown function; PGPE: *Punicagranatum* peel extract.

Among the stress-response associated genes expressed differently, five (gene4735, gene4008, gene4523, gene3545, and gene2496) were down-regulated, indicating that PGPE put the bacterium under stress and/or compromised its ability to deal with stress. These genes were down-regulated by 1.87-5.46 fold. Gene 4735 coding for t-RNA pseudouridine synthase was down-regulated by 5.46 fold. Downregulation of this gene may cause insufficient post-transcriptional modification, and such hypomodification may lead to a translational slowdown, which in turn can lead to disruption of protein homeostasis culminating into an overall reduced cellular fitness [32]. Proper expression of t-RNA modifying genes like this is crucial to proper t-RNA modification. Their under-expression may compromise cellular function and viability during stress. Gene 4008 downregulated by 3.54 fold codes for a heat shock protein (HSP). HSP production is enhanced by cells facing environmental stress [33], and PGPE seems to have interfered with the bacterial strategy of confronting stress through HSP. Since the induction of HSPs is a ubiquitous cellular response to cope with the accumulation of misfolded and unfolded proteins following stress exposure [34], their downregulation may render the HSP-deficient bacteria handicapped with respect to stress-response. HSP 15 in *Escherichia coli* (a member of *Enterobacteriaceae*, to which *S. marcescens* also belongs) is involved in the recycling of free 50S ribosomal subunits carrying a nascent chain and is a useful component of the bacterial stress response machinery. Another gene (feature ID 4523) was downregulated by 3-fold codes for an envelope stress response (ESR) regulator transcription factor. ESR system has an important role in the maintenance of envelope integrity and virulence-related functions [35]. Anti-virulence preparations like PGPE can compromise bacteria's ability to survive inside the host by disrupting their ESR system.A further indication of PGPE-exposed *S. marcescens* struggling to maintain its envelope-integrity is differential expression of multiple genes associated with cell wall/ membrane synthesis: gene 71 coding for a phosphor-n-acetylmuramoyl-pentapeptidetransfersase (5.30 fold ↓); gene 3570 (3.32 fold ↓) coding for a YggTfamility protein; gene 4663 (3.30 fold ↓) coding for epoxyqueuosinereductase; MSTRG.2322.2 (10.63 fold ↑) coding for a cell wall-associated hydrolase; gene 4004 (2.27 fold ↓) coding for a penicillin-binding protein; and gene 1199 (2.60 fold ↑) coding for a paraquat-inducible B protein. Gene 3545 coding for spermidine synthase was downregulated by 1.99 fold. This is a key enzyme for polyamine biosynthesis. Owing to their involvement in multiple cellular processes such as gene expression, survival, stress response, and proliferation, cells have evolved strict regulation of intracellular polyamine concentration. Polyamine depletion can have negative effects on cell survival. Protein products of genes involved in polyamine biosynthesis are considered to be drug-targets against pathogenic protozoa [36]. Gene coding for a cold-shock protein (CSP; bearing feature ID 2496) was down-regulated by 1.89 fold in PGPE-treated *S. marcescens.* Some CSPs are non-cold inducible, and they are involved in stress adaptation responses. They contribute towards bacterial adaption to oxidative stress,

osmotic stress, starvation, and also help the bacteria for host cell invasion. Owing to the wider role of CSPs in determining stress tolerance of bacteria, plant extracts capable of down-regulating them can be expected to compromise bacterial stress tolerance notably. Gene 3269 coding for a glutaredoxin-like protein (nrdH) with a thioredoxin-like activity profile was up-regulated by 2.48 fold. Glutaredoxin and thioredoxin systems are important antioxidants [37], and bacteria might be trying to overexpress such antioxidant mechanisms to counteract the oxidative stress induced by PGPE. Elevation of NrdHredoxin activity can be considered as a response from the bacterial population against the damaging effects of reactive oxygen species (ROS) induced by various exogenous oxidative stresses (*e.g.*, PGPE-induced, in this study) by acting as a peroxidase cofactor [38]. This corroborates well with the marginal increase in catalase activity of PGPE-exposed *S. marcescens* (Table **1**).

A total of 5 efflux-associated genes (gene 4792↓, gene 1350↓, gene 1399↓, gene 3111↑, and gene 3540↑) were differentially expressed in the PGPE-exposed *S. marcescens*. Since bacterial multidrug efflux pumps have crucial roles in bacterial physiology besides acting as an important mechanism of AMR development, interfering with their functionality can be an effective anti-virulence strategy [39]. Gene 4792 coding for an LPS export ABC transporter permease was down-regulated by 6.40 fold. This downregulation is considered important because LPS has been shown to be an important virulence factor of *S. marcescens*. This bacterium kills immune cells *via* an lipopolysaccharide- and flagella-dependent mechanism [40]. LPS is among the virulence factors necessary for full *in vivo* virulence expression of *S. marcescens* in *C. elegans* model [41].Such proteins are responsible for the export of a wide variety of oligo- and polysaccharidess playing critical roles in the biology of microbes [42]. Gene 3111 coding for a multidrug transporter was up-regulated by 3.91 fold. Gene 1350 coding for an MFS transporter was down-regulated by 2.54 fold. Gene 1399 coding for an Rrf2 family transcriptional regulator (repressor of oqxAB) was down-regulated by 2.29 fold. OqxAB efflux pump has become increasingly prevalent among members of *Enterobacteriaceae* over the recent past. Different Rrf2 family regulators are known to repress genes associated with nitrite/ nitric oxide/ iron metabolism. PGPE-exposed *S. marcescens* was found to experience the up-regulation of 4 genes (gene 2352, gene 4905, gene 4628, and gene 4322) associated with iron metabolism. Gene 3540 coding for daunorubicin and doxorubicin resistance was up-regulated by 2.15 fold, which is also responsible for coupling energy to the transport system. Of the five efflux-associated genes listed above, 3 were down-regulated, and 2 were up-regulated. Abnormal regulation of efflux activity, whether up or down, can compromise bacterial fitness. While efflux pump inhibition is considered a promising anti-drug resistance intervention; improper overexpression of efflux pumps may lead to unwanted export of metabolites or signaling molecules culminating in deleterious impact on cell physiology [39]. Two of the differentially expressed efflux-associated genes belonged to the ABC (ATP-binding cassette) superfamily of efflux transporters. Since the ABC efflux system is one of the widely distributed systems among gram-positive as well as gram-negative bacteria, extracts capable of affecting their expression may be expected to be effective against a broad range of pathogens. In fact, we have found PGPE to be effective as an anti-virulence preparation against multiple gram-positive and gram-negative bacteria such as *Staphylococcus aureus* [43], *Pseudomonas aeruginosa*, and *Chromobacterium violaceum* [44]. Multidrug efflux pumps may be important for the intrinsic antibiotic resistance of *S. marcescens*, whose antibiotic resistance profile is roughly comparable to that of *P. aeruginosa* [45].

Among the differentially expressed genes in the PGPE-treated *S. marcescens*, two genes (gene 2365↑, gene2379↓) were associated with flagellar motility. Gene 2365 coding for the FliS chaperone was up-regulated by 4.77 fold; whereas gene 2379 coding for the flagellar motor switch was down-regulated by 3.59 fold. This becomes important considering that flagellar motor switching is necessary for chemotaxis, which in turn is essential for the initial stages of infection [46]. Paralyzing the flagellar motor functioning can also cripple biofilm formation, another important virulence trait [47]. Flagellar variation in *Serratiamarcescens* is associated with color variation, and PGPE-treated *S. marcescens* indeed was found to have reduced pigmentation (Fig. **1A**). Variation of surface proteins often provides the bacteria with alternate offense-defense strategies for survival in a challenging environment. The pigment, in association with flagella, may offer such a function for *S. marcescens* [48]. Since the function of FliS is to prevent premature polymerization of newly synthesized flagellin molecules [49, 50], its over-expression in PGPE-exposed *S. marcescens* may perhaps be taken as an indirect indication of premature flagellin polymerization happening under the influence of this plant extract.

PGPE-exposed *S. marcescens* was found to experience up-regulation (1.63 - 3.69 fold) of 4 genes (gene 2352, gene 4905, gene 4628, and gene 4629) associated with iron metabolism. Among them, gene 2352 has a role in iron uptake. Gene 4629 codes for 2,3-dihydroxybenzoate-AMP ligase, which is involved in siderophore biosynthesis. Gene 4628 coding for isochorismate synthase involved in the biosynthesis of siderophore group non-ribosomal peptides was upregulated by 2.40 fold. Bacteria may be forced to lift their siderophore production and iron uptake machinery when made to face iron-deficient conditions. Since PGPE curbed haemolytic activity of *S. marcescens* (Table **1**), which is a strategy used by many haemolytic pathogens to get access to otherwise bound-iron, to compensate for this, the bacterium might have been forced to upregulate the siderophore-dependent iron acquisition machinery. Thus, PGPE seems to have disturbed iron homeostasis in *S. marcescens*. Disturbance of iron-homeostasis in susceptible bacterial pathogens (*S. aureus* and *P. aeruginosa*) exposed to anti-virulence herbal formulations has been demonstrated by us in recent past [51, 52].

Three more virulence-associated genes down-regulated under the influence of PGPE were those bearing feature ID 3433, 1668, and 3118. Gene 3433 coding for flavinator of succinate dehydrogenase was down-regulated by 2.76 fold, and it is required for the flavinylation and activation of fumeratereductase and succinate dehydrogenase. These enzymes play an important role in cellular energetics and required for virulence of many pathogenic bacteria. SdhE is a conserved protein required for flavinylation of succinate dehydrogenase

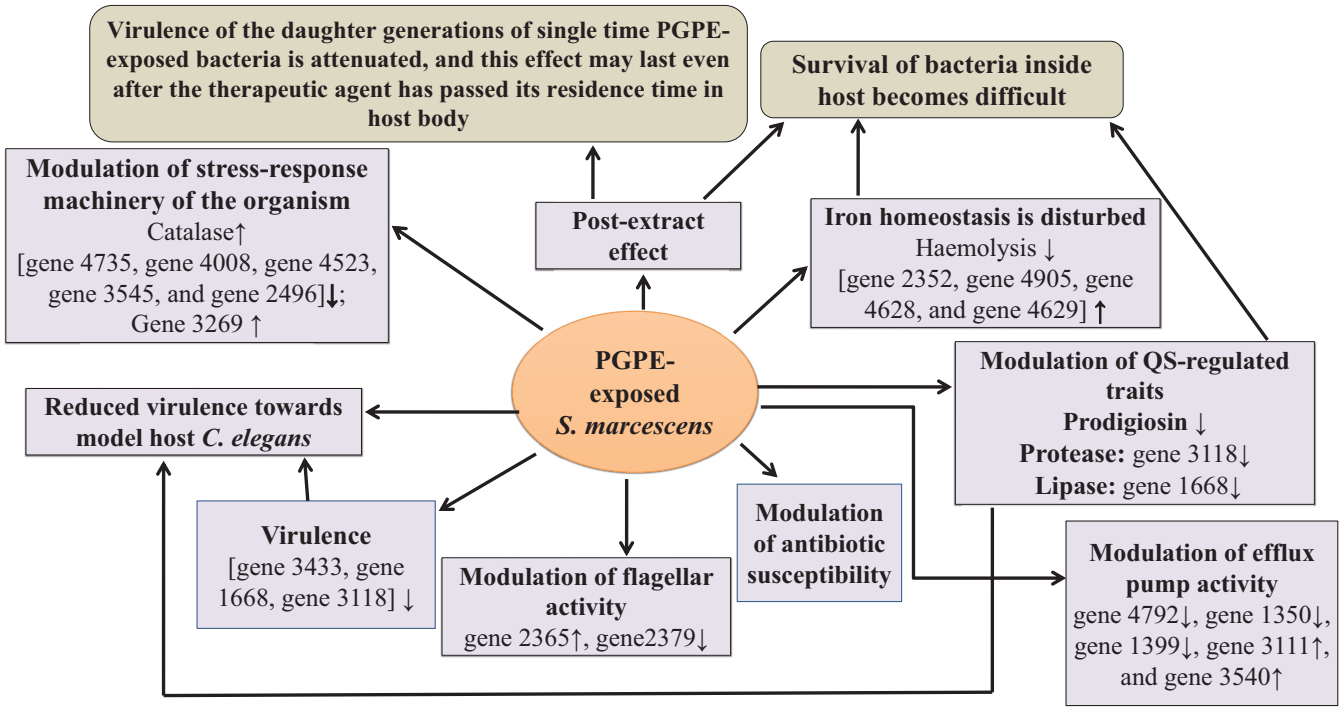

Fig. (3). Overall schematic representation explaining the anti-infective effect of PGPE on *S. marcescens*.
PGPE: *Punica granatum* peel extract.

in bacteria, and its deletion was demonstrated to have pleiotropic effects, using *Serratia*as a model system [53], suggesting that SdhE might flavinylate multiple flavoproteins. Gene 1668 coding for an extracellular lipase and gene 3118 coding for a U32 family peptidase (protease) were down-regulated by 2.11 and 1.53 fold, respectively. Extracellular lipase and protease in *S. marcescens* are believed to be virulence factors controlled by QS [54]. Among the traits which are believed to be QS-regulated in *S. marcescens*, PGPE was able to modulate prodigiosin production, haemolytic activity, lipase and protease gene expression, and siderophore-associated gene expression. Though the SmaI/SmaR genes were not found to be expressed differently in PGPE-exposed culture since the prodigiosin inhibition was reversed upon exogenous AHL augmentation (Fig. **1b**), it can be speculated that components of PGPE might be having AHL-scavenging activity, or some of them may be AHL analogues which may compete with AHLs for binding to SmaR. QS is a mechanism of intercellular communication among bacteria, which seems to have been influenced by PGPE. Another indication of PGPE's ability to affect bacterial communication is the up-regulation (2.84 fold) of gene 4322 coding for a signal transduction histidine-kinase phosphatase. Such two-component signal-transducing systems are ubiquitously distributed communication interfaces in bacteria. They sense a specific environmental stimulus, and mediate the cellular response, mostly through differential expression of target genes [55].

Maximum number (28) of differently expressed genes belonged to those associated with t-RNA/ basic cellular processes and metabolism. Some of them whose expression was altered with a relatively high fold-change value are discussed here. Gene 4489 coding for a bifunctional phosphopantothenoylcysteine decarboxylase/ phosphopantothenate synthase was down-regulated by 4.55 fold. This is a key enzyme participating in the production of coenzyme-A. Coenzyme A biosynthesis is considered as an antimicrobial drug target [56]. Gene (feature ID 4271) coding for diaminopimelate (DAP) epimerase was down-regulated by 2.83 fold. DAP epimerase owing to its involvement in the biosynthesis of *meso*-DAP and lysine, which are important precursors for the synthesis of peptidoglycan, housekeeping proteins, and virulence factors in bacteria, is viewed as a promising antimicrobial target [57].

This study has found *Punica granatum* peel extract to possess notable anti-virulence activity against the multidrug-resistant beta-lactamase producing member of *Enterobacteriaceae*: *Serratia marcescens*. PGPE could exert its anti-virulence effect on this pathogen without inhibiting its growth much, and thus can be expected to exert lesser selection pressure. This extract seemed to be capable of modulating QS by acting as a signal-supply inhibitor. Besides curbing the haemolytic activity of *S. marcescens*, PGPE was also able to exert PEE on this pathogen. Major modes of PGPE's action against *S. marcescens* (Fig. **3**) revealed from whole transcriptome analysis are: stress induction and compromising bacterial stress-response capacity; disturbance of bacterial efflux activity, cellular energetics, flagellar motility, quorum sensing, and iron homeostasis. Despite the human opportunistic pathogen *Serratia marcescens* is a bacterium with a broad host range representing a growing public health problem, its pathogenicity has remained under-investigated [41]. Besides validating the anti-infective potential of pomegranate peel extract indicated in traditional medicine, this study has also provided insights into molecular details of PGPE's modes of action, as well as *S. marcescens* pathogenicity. Such studies can prove useful in identifying novel anti-pathogenic mechanisms as well as new bacterial targets for next-generation 'pathoblockers'. Potent targets indicated in

this study are: cell wall-associated hydrolase, bifunctional phosphopantothenoylcysteine decarboxylase/ phosphopantothenate synthase, diaminopimelate (DAP) epimerase, flagellarFliS, flagellar motor switch, cystine transporter, non-ribosomal peptide synthetase, LPS export ABC transport permease, pseudouridine synthase, phospho-n-acetylmura moyl-pentapeptidetransferase, ERS regulator, and epoxy queuosinereductase. Taking the results of this study along with our previous reports on PGPE [43, 44], this extract can be considered a broad-spectrum anti-virulence preparation. Further efforts towards developing it into an effective anti-virulence phytopharmaceutical are warranted, which may include detailed phytochemical characterization of this extract, investigating its effect on host transcriptome/ immune system activity, *etc*.

LIST OF ABBREVIATIONS

AHL	=	N-acyl-homoserine Lactones
AMR	=	Antimicrobial Resistance
C12-HSL	=	N-(3-oxododecanoyl)-l-homoserine Lactone (3O-C12-HSL)
MAE	=	Microwave-Assisted Extraction
PEE	=	Post Extract Effect
QS	=	Quorum Sensing
PGPE	=	Hydroalcoholic Extract of *Punicagranatum* peel

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The use of this blood was approved by the Institutional Ethics Committee of the Institute of Science, Nirma University, India (Approval no: EC/NU/18/IS/4).

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

A small volume of human blood used in this study was sourced from the authors themselves, who each gave their written informed consent for it.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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