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ORIGINAL RESEARCH ARTICLE

Network analysis for identifying potential anti-virulence targets from whole transcriptome of *Pseudomonas aeruginosa* and *Staphylococcus aureus* exposed to certain anti-pathogenic polyherbal formulations

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ABSTRACT

Introduction: Antimicrobial resistance (AMR) is a serious global threat. Identification of novel antibacterial targets is urgently warranted to help antimicrobial drug discovery programs. This study attempted identification of potential targets in two important pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

Methods: Transcriptomes of *P. aeruginosa* and *S. aureus* exposed to two different quorum-modulatory polyherbal formulations were subjected to network analysis to identify the most highly networked differentially expressed genes (hubs) as potential anti-virulence targets.

Results: Genes associated with denitrification and sulfur metabolism emerged as the most important targets in *P. aeruginosa*. Increased buildup of nitrite (NO_2) in *P. aeruginosa* culture exposed to the polyherbal formulation *Panchvalkal* was confirmed through *in vitro* assay too. Generation of nitrosative stress and inducing sulfur starvation seemed to be effective anti-pathogenic strategies against this notorious gram-negative pathogen. Important targets identified in *S. aureus* were the transcriptional regulator *sarA*, immunoglobulin-binding protein Sbi, serine protease *SplA*, the *saeR/S* response regulator system, and gamma-hemolysin components *hlgB* and *hlgC*.

Conclusion: Further validation of the potential targets identified in this study is warranted through appropriate *in vitro* and *in vivo* assays in model hosts. Such validated targets can prove vital to many antibacterial drug discovery programs globally.

Keywords: AMR (antimicrobial resistance), Anti-virulence, Network Analysis, Novel antibacterial targets, Polyherbal, Protein-Protein Interaction (PPI)

Introduction

Despite wide recognition of antimicrobial resistance (AMR) as a major global health threat, the progress on discovery and development of new antibiotics in the last three to four decades clearly has fallen short from being satisfactory. For a variety of reasons, for example, lack of interest among major pharmaceutical firms, rapid emergence and spread of resistance among pathogenic bacterial populations, dearth of new validated cellular and molecular targets, the list of effective antimicrobials available for treatment of resistant infections remains short. The status of antibiotic discovery research has been reviewed thoroughly (1-4). Since most currently available antibiotics target a narrow range of bacterial traits, that is, cell envelope synthesis, protein or nucleic acid synthesis, or folic acid synthesis, a truly new class of antibiotics will be discovered only if we have a longer list of validated targets. Development of new bactericidal antibiotics is not the only way of tackling the slow pandemic of AMR infections; discovery of resistance modifiers and non-antibiotic virulence-attenuating agents can also be of great

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value (5,6). Hence identification of new potential targets for both bactericidal antibiotics as well as antibiotic adjuvants is useful. There is a clear need for antibiotics with previously unexploited new targets and wide target diversity in the discovery pipeline. One of the major challenges in antibacterial discovery is associated with the proper target selection, for example, the requirement of pursuing molecular targets that are not prone to rapid resistance development (7).

Various public health agencies like CDC (Centers for Disease Control and Prevention, USA), WHO (World Health Organization), and DBT (Department of Biotechnology, India) have published lists of priority pathogens against which novel antimicrobials need to be discovered urgently. Antibiotic-resistant strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus* commonly appear on all such lists. As per CDC's Vital Signs report (https://www.cdc.gov/vitalsigns/index.html) more than 33% of the bloodstream infections in patients on dialysis in the United States in 2020 were caused by *S. aureus.* This gram-positive human commensal has been recognized as an important opportunistic pathogen responsible for a wide range of infections (8). *P. aeruginosa* is the primary cause of gram-negative nosocomial infections. Its ability to adapt to a wide range of environmental niches combined with its nutritional versatility and genome plasticity, along with a multitude of intrinsic and acquired resistance mechanisms make it one of the most notorious pathogens of critical clinical importance. Efforts for finding perturbants capable of targeting the *P. aeruginosa* pathogenicity and antibiotic resistance are highly desired (9).

We had previously studied the anti-virulence effect of certain polyherbal formulations against *S. aureus* or *P. aeruginosa* at the whole transcriptome level of the target pathogen, wherein we gained some insight into the molecular mechanisms associated with the virulence-attenuating potential of the test formulations, which was largely independent of any growth-inhibitory effect. Pathogens exposed to the test formulations were compromised in their ability to kill the model host *Caenorhabditis elegans*. The current study attempted network analysis of the differentially expressed genes (DEG) of *P. aeruginosa* and *S. aureus* exposed to the anti-pathogenic polyherbal formulations *Panchvalkal* (10) and Herboheal (11), respectively, reported in the previous studies, with an aim to identify highly networked genes as potential anti-virulence targets. *Panchvalkal* is a mixture of bark extracts of five different plants – *Ficus benghalensis, Ficus religiosa, Ficus racemosa, Ficus lacor*, and *Albizia lebbeck.* Herboheal comprised of extracts of six different plants. Its full composition can be seen at: https://downloads.hindawi.com/journals/aps/2019/1739868.f1.pdf

Methods

Network analysis

We accessed the list of DEG for *Panchvalkal* (Pentaphyte-P-5®)-exposed *P. aeruginosa* (NCBI Bioproject ID 386078) and Herboheal-exposed *S. aureus* (NCBI Bioproject ID 427073). The *P. aeruginosa* used was a multidrug-resistant strain. Network analysis for both the studies was carried out independently, wherein only the DEG fulfilling the dual filter criteria of log fold change ≥2 and False Discovery Rate (FDR) ≤0.01 were selected for further analyses. The list of such DEG was fed into the database STRING (v. 11.5) (12) for generating the PPI (Protein-Protein Interaction) network. Then the genes were arranged in decreasing order of 'node degree' (a measure of connectivity with other genes or proteins), and those above a certain threshold value were subjected to ranking by cytoHubba (v. 3.9.1) (13). Since cytoHubba uses 12 different ranking methods, we considered the DEG being top-ranked by more than six 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 which were part of multiple clusters were considered 'hubs' which can be taken up for further validation 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 the host). This sequence of analysis allowed us to end with a limited number of proteins which satisfied various statistical and biological significance criteria simultaneously, that is, (i) log fold change ≥2; (ii) FDR ≤0.01; (iii) relatively higher node degree; (iv) top-ranking by at least six cytoHubba methods; (v) (preferably) member of more than one local network cluster; and (vi) high probability of the target being absent from the host. A schematic presentation of the methodology employed for network analysis is presented in Figure 1.

Nitrite estimation

Nitrite estimation in *P. aeruginosa* culture supernatant was done through Griess assay (14). *P. aeruginosa* strain studied by us is a multidrug-resistant strain, which is resistant to ampicillin (10 µg), augmentin (30 µg), nitrofurantoin (300 µg), clindamycin (2 µg), chloramphenicol (30 µg), cefixime (5 µg), and vancomycin (30 µg). This bacterium was grown in *Pseudomonas* broth (HiMedia, Mumbai) with or without *Panchvalkal* (547 µg/mL; dried extract powder without any bulking agent was procured from Dr. Palep's Medical Education and Research Foundation Pvt. Ltd., Mumbai, India, and dissolved in dimethylsulfoxide (DMSO) for assay purpose) at 35°C for 21±1 hour. Following incubation, cell density was quantified at 764 nm (15), and then the bacterial culture suspension was centrifuged at 13,600 *g* for 10 minutes. Resulting supernatant was mixed with Griess reagent (Sigma-Aldrich) in 1:1 ratio and incubated for 15 minutes in the dark at room temperature. Absorbance of the resulting pink color was quantified at 540 nm (Agilent Technology Cary 60 UV-Vis). These optical density (OD) values were plotted on standard curve prepared using $NaNO_2$ to calculate the nitrite concentration. To nullify any effect of variation in cell density between control and experimental culture, nitrite unit (i.e., nitrite produced per unit of growth) was calculated by dividing the nitrite concentration values by cell density. Sodium nitroprusside (Astron chemicals, Ahmedabad) being

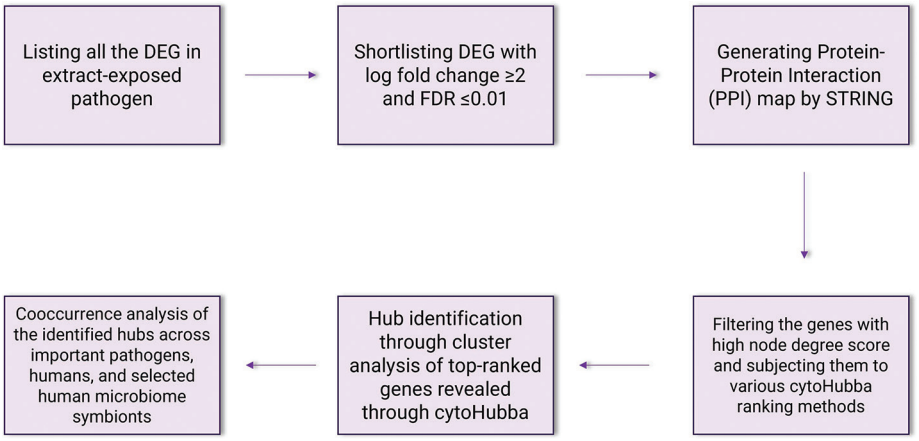


Fig. 1 - A schematic of methodology for network analysis and hub identification.

a chemical known to be capable of generating nitrosative stress in bacteria (16-18) was used as a positive control. Appropriate vehicle control (i.e., bacteria grown in the presence of 0.5% v/v DMSO (Merck)), negative control (deionized water), and abiotic control (*Panchvalkal*-supplemented *Pseudomonas* broth) were included in the experiment. Griess reagent was added in all these controls in the same proportion as that in extract-exposed or not-exposed bacterial culture samples.

Results

Network analysis of DEG in Panchvalkal-exposed P. aeruginosa

Our original experimental study exposed *P. aeruginosa* to *Panchvalkal* at 567 µg/mL, wherein the extract-exposed pathogen could kill 90% lesser host worms than its extract-not-exposed counterpart. Whole transcriptome study revealed that approximately 14% of the *P. aeruginosa* genome was expressed differently under the influence of *Panchvalkal*. The total number of DEG satisfying the dual criteria of log fold change ≥2 and FDR ≤0.01 was 228, of which 105 were downregulated (Tab. S1) and 123 were upregulated (Tab. S4). We created PPI network for up- and downregulated genes separately (Figs. 5 and 2, respectively). PPI network for downregulated genes generated through STRING is presented in Figure 2, which shows 101 nodes connected (105 genes were fed to string, out of which 101 were shown in the PPI network) through 86 edges with an average node degree of 1.7. Since the number of edges (86) in this PPI network is 3.18-fold higher than expected (27) 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 identical sample size and degree distribution. Such an enrichment can be taken as an indication of the member proteins being at least partially biologically connected. When we arranged the 105 downregulated genes in decreasing order of node degree, 52 nodes were found to have a nonzero score (Tab. S2), and we selected top 13 genes with a node degree ≥6 for further ranking by different cytoHubba methods. Then we looked for genes which appeared among the top-10 ranked candidates by ≥6 cytoHubba methods, and 10 such shortlisted genes (Tab. S3) were further checked for interactions among themselves followed by cluster analysis (Fig. 3), which showed them to be strongly networked as the average node degree score was 8. This network possessed 40 edges as against expected (zero) for any such random set of proteins (PPI enrichment *p* value <1.0e-16). The PPI network generated through STRING showed these 10 important genes to be distributed among three different local network clusters. Five (norB, norC, norD, nirS, and nirQ) of the predicted hubs were part of each of the three clusters, and they have a role in denitrification (19). Of the remaining five predicted hub proteins, one more (norE) is also associated with nitrogen metabolism, and two (nosL and nosY) have a role in denitrification as well as copper homeostasis. These three proteins were members of two out of three clusters. The eight proteins (Tab. I) found to be members of minimum two clusters can be said to be potential hubs, whose downregulation can be hypothesized to attenuate *P. aeruginosa* virulence.

Since all the targets mentioned in Table I are known to play an important role in *P. aeruginosa* with respect to detoxification of reactive nitrogen species, we hypothesized that *Panchvalkal*-treated *P. aeruginosa*'s ability to detoxify reactive nitrogen species is compromised. To check this hypothesis, we quantified nitrite concentration in extract-treated *P. aeruginosa* culture, wherein it was found to have 31% higher nitrite concentration in supernatant as compared to control (Fig. 4). This higher accumulation of nitrite can be taken as an indication of compromised denitrification efficiency as nitrite is an intermediate of denitrification pathway (22).

PPI network for upregulated genes in *Panchvalkal*-exposed *P. aeruginosa* generated through STRING is presented in Figure 5, which shows 121 nodes connected through 70 edges with an average node degree of 1.16. Though empirically the centrality of the upregulated genes appeared to be lesser than those downregulated in *Panchvalkal*-exposed *P. aeruginosa*, since the number of edges (70) in this PPI network is 1.89-fold higher than expected (37) with a PPI enrichment *p* value of 1.27e-06, 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 this much sample size and degree distribution. Such an enrichment can be

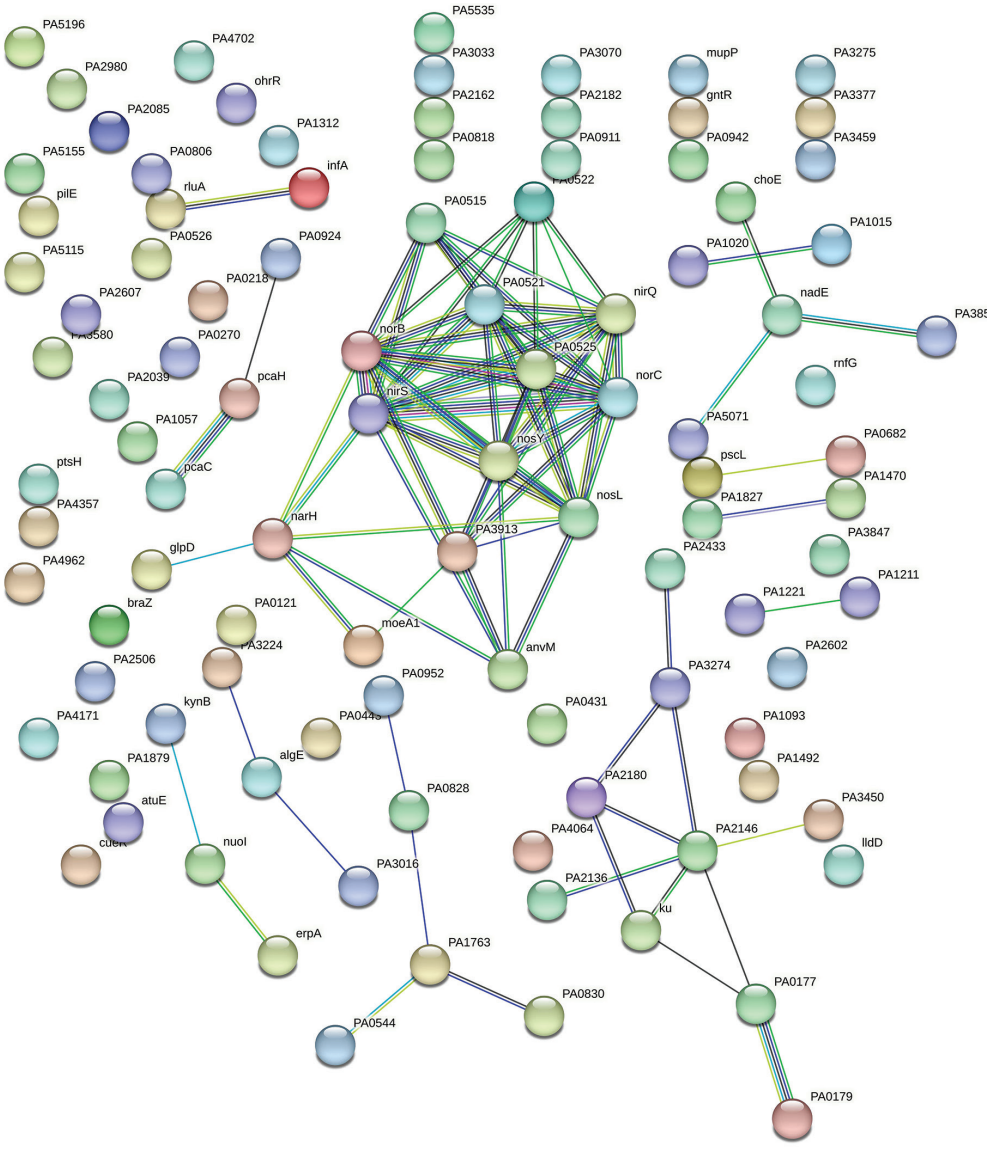
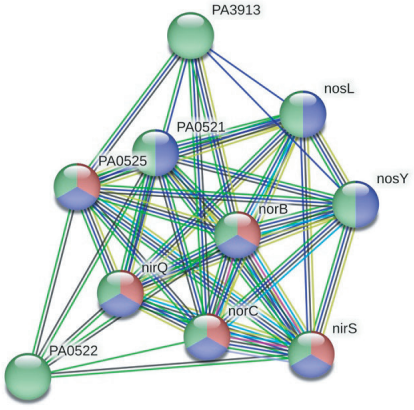


Fig. 2 - Protein-Protein Interaction (PPI) network of downregulated genes in *Panchvalkal*-exposed *Pseudomonas aeruginosa.* Edges represent protein-protein associations that are meant to be specific and meaningful, that is, proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding to each other. Network nodes represent proteins. Splice isoforms or post-translational modifications are collapsed, that is, each node represents all the proteins produced by a single, protein-coding gene locus.



Symbolic representation	Cluster	Description	Count in network	Strength of the enrichment effect	FDR
	CL: 3517	Mixed, incl. oxidoreductase activity, acting on other nitrogenous compounds as donors, cytochrome as acceptor and quinone metabolic process	10 of 27	2.31	5.65e-20
	CL: 3520	Mixed, incl. oxidoreductase activity, acting on other nitrogenous compounds as donors, cytochrome as acceptor and signal anchor	8 of 13	2.54	1.70e-16
	CL: 3523	oxidoreductase activity,acting on other nitrogenous compounds as donors, cytochrome as acceptor and signal anchor	5 of 6	2.67	4.18e-10

Fig. 3 - Protein-Protein Interaction (PPI) network of top-ranked genes revealed through cytoHubba among downregulated differentially expressed genes (DEG) in *Panchvalkal*-exposed *Pseudomonas aeruginosa*.

Table I - Hubs identified as potential targets from among the downregulated genes in *Panchvalkal*-exposed *Pseudomonas aeruginosa*

No.	Gene ID	Gene name	Functional role
1	PA0520	*nirQ*	Denitrification regulatory protein NirQ
2	PA0519	*nirS*	Heme d1 biosynthesis protein, which is important for denitrification (20)
3	PA0524	*norB*	Nitric oxide reductase subunit B
4	PA0523	*norC*	Nitric oxide reductase subunit C
5	PA0525	*NorD*	Nitric oxide reductase NorD protein
6	PA0521	*NorE*	Nitric oxide reductase NorE protein
7	PA3395	*nosY*	Nitrous oxide reductase; a Cu-processing system permease protein having role in denitrification pathway (21)
8	PA3396	*nosL*	A lipoprotein attached to the outer membrane described as a copper-binding protein. Regulator of *nos* operon, NosR also associates with NosL. This protein is probably responsible for the insertion and coordination of the multicopper center within NosZ (22).

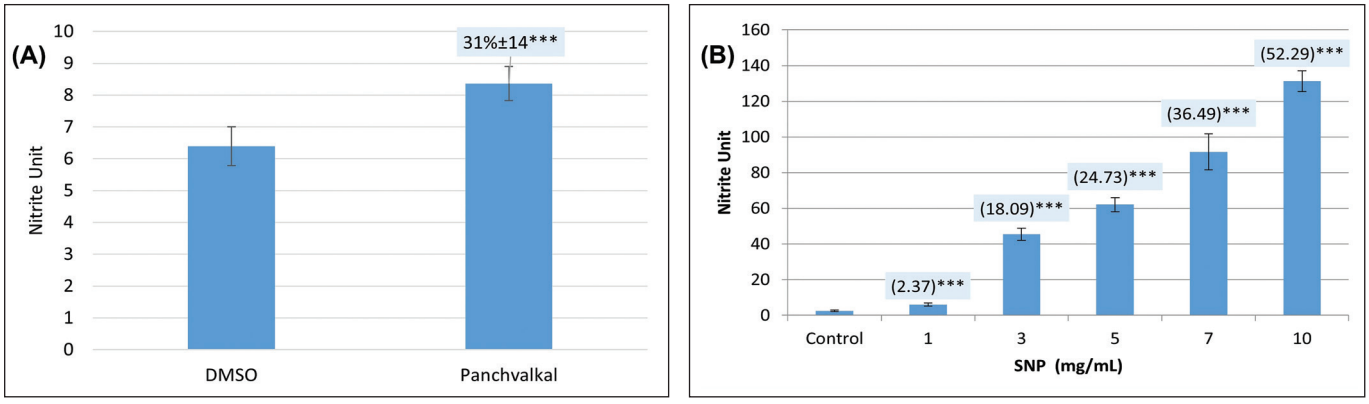


Fig. 4 - *Panchvalkal*-treated *Pseudomonas aeruginosa* culture has higher extracellular accumulation of nitrite. While nitrite concentration in vehicle control (*P. aeruginosa* incubated in media supplemented with 0.5% v/v dimethylsulfoxide (DMSO)) was at par to that without DMSO, *Panchvalkal* caused nitrite concentration in *P. aeruginosa* culture supernatant to rise (A). Sodium nitroprusside used as positive control caused a dose-dependent 2.37 to 52.29-fold higher nitrite buildup in *P. aeruginosa* culture (B). Nitrite unit (i.e., nitrite concentration:cell density ratio) was calculated to nullify any effect of cell density on nitrite production. ***p<0.001.

taken as an indication of the member proteins being at least partially biologically connected. When we arranged the 121 upregulated genes in decreasing order of node degree, 62 nodes were found to have a nonzero score, and we selected the top 26 genes with a node degree ≥3 (Tab. S5) for further ranking by different cytoHubba methods. Then we looked for genes which appeared among top-ranked candidates by ≥6 cytoHubba methods, and 14 such genes (Tab. S6) were identified for further cluster analysis. Interaction map of these 14 important genes (Fig. 6) showed them to be networked with the average node degree score of 2.29. Number of edges possessed by this network was 16 as against expected 1 for any such random set of proteins. These 14 genes were found to be distributed among five different local network clusters. Strength score for each of these clusters was >1.5. While three of the proteins (*atsB, msuE,* and *ssuB1*) were common members of three different clusters, one gene (*tauA*) appeared in two clusters**.** All these four highly networked upregulated genes (Tab. II) are involved in sulfur metabolism in *P. aeruginosa* (23). Hence it may be speculated that *Panchvalkal* has induced sulfur starvation in *P. aeruginosa*, to overcome which the pathogen is forced to upregulate genes involved in sulfur transport and metabolism.

Network analysis of DEG in Herboheal-exposed S. aureus

Herboheal is a folk-inspired wound-healing formulation, and we had earlier demonstrated its anti-virulence potential against multiple bacterial pathogens including *S. aureus*. Pretreatment of *S. aureus* with Herboheal (0.1% v/v) could attenuate its virulence toward the surrogate host *C. elegans* by 55%. This concentration had a moderate growth-inhibitory effect (32%) on *S. aureus*, while heavily inhibiting staphyloxanthin production (79%). Whole transcriptome study revealed that approximately 17% of the *S. aureus* genome was expressed differently under the influence of Herboheal. The total number of DEG satisfying the dual criteria of log fold change ≥2 and FDR ≤0.01 was 113, of which 57 were upregulated and 56 were downregulated (Tab. S7). Since the number of genes amenable to mapping by STRING turned out to be

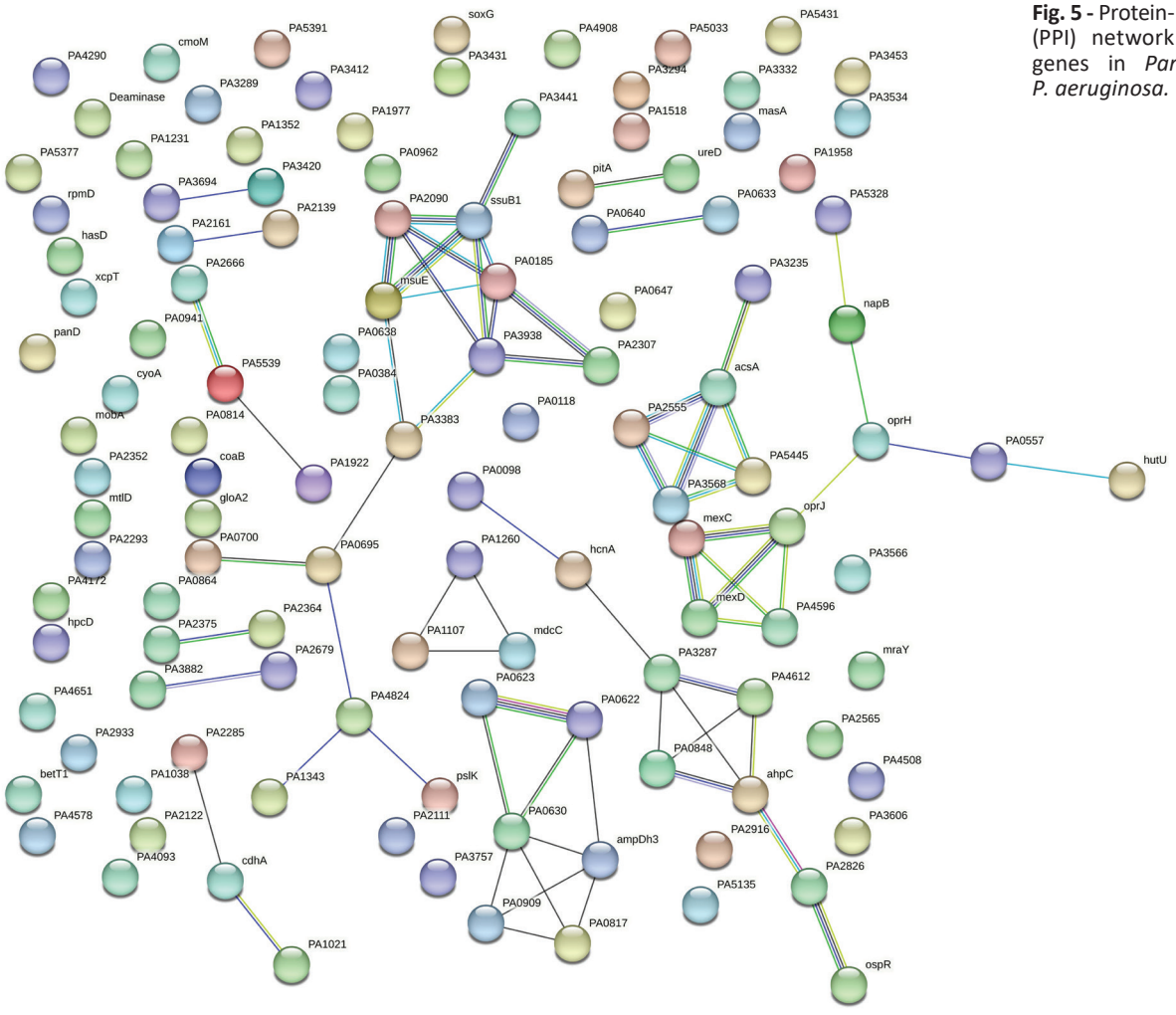


Fig. 5 - Protein-Protein Interaction (PPI) network of up-regulated genes in *Panchvalkal*-exposed *P. aeruginosa.*

Symbolic representation	Cluster	Description	Count in network	Strength of the enrichment effect	False discovery rate
	CL:9698	Beta-lactamase resistance	3 of 12	2.0	0.0012
	CL:2228	Acetyl-CoA metabolic process and, acylphosphatase activity	2 of 10	1.9	0.0380
	CL:8737	Alkanesulphonate transport, and sulphur metabolism	4 of 26	1.79	0.00055
	CL: 8738	Mixed, incl. alkanesulfonate transmembrane transporter activity, and alkane sulfonate monooxygenase activity	3 of 21	1.75	0.0036
	CL: 8733	Mixed, incl. Sulfur metabolism, and siderophore uptake transmembrane transporter activity	5 of 63	1.5	0.00055

Fig. 6 - PPI network of top-ranked genes revealed through cytoHubba among up-regulated DEG in *Panchvalkal*-exposed *P. aeruginosa*.

Table II - Hubs identified as potential targets from among the upregulated genes in *Panchvalkal*-exposed *Pseudomonas aeruginosa*

No.	Gene ID/name	Codes for	Remarks
1	PA2357/*msuE* (*slfA*)	FMN reductase	Involved in riboflavin metabolism and sulfur metabolism pathways
2	PA3442/*ssub1*	Aliphatic sulfonates import ATP-binding protein SsuB 1	Aliphatic sulfonates import ATP-binding protein SsuB 1; part of the ABC transporter complex SsuABC involved in aliphatic sulfonate import. Responsible for energy coupling to the transport system
3	PA0185/*atsB*	Serine-modifying enzyme (24); probable permease of ABC transporter	*atsB* is a member of a *cys* regulon in *P. aeruginosa*, which constitutes a general sulfate ester transport system (25)
4	PA3938/*tauA*	TauA (sulfonate transport system ATP-binding protein)	This probable periplasmic taurine-binding protein precursor is part of *tau* operon involved in sulfur metabolism

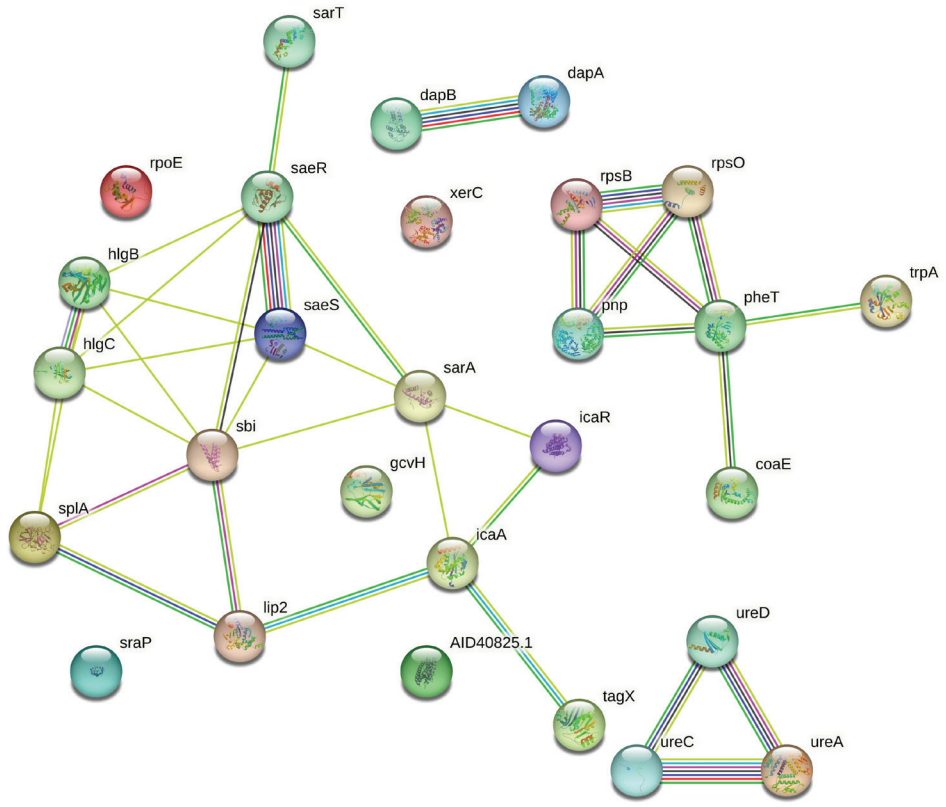
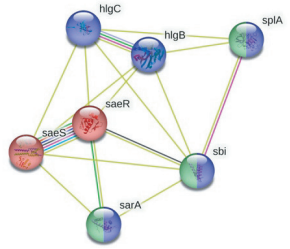


Fig. 7 - Protein-Protein Interaction (PPI) network of upregulated and downregulated genes in Herboheal-exposed *Staphylococcus aureus*.

only 28 of these 113, we went for a combined PPI network (Fig. 7) of all these DEG instead of preparing separate PPI map of upregulated or downregulated genes. The said PPI network had 28 nodes connected through 36 edges with an average node degree of 2.57. Since the number of edges (36) in this PPI network is threefold higher than expected (12) with a PPI enrichment *p* value of 1.02e-08, 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 having identical sample size and degree distribution. Such an enrichment is suggestive of the member proteins being at least partially biologically connected.

When we arranged all the 28 nodes in decreasing order of node degree, 23 nodes were found to have a nonzero score, and we selected the top 13 genes with a node degree ≥3 (Tab. S8) for further ranking by different cytoHubba methods. Then we looked for genes which appeared among top-ranked candidates by ≥6 cytoHubba methods. Of such 12 genes, 8 (Tab. S9) which were ranked among top 10 by ≥11 cytoHubba methods were taken for further cluster analysis. Interaction map of these eight important genes (Fig. 8) showed them to be networked with the average node degree score of 4. Number of edges possessed by this network was 16 as against expected 1 for any such random set of proteins. These eight genes were found to be distributed among three different local network clusters. Strength score for each of these clusters was >1.46. While three of the proteins (*sarA, sbi*, and *splA*) were common members of two different clusters, four proteins were part of any one cluster, while *pnp* was not shown to be connected to the remaining seven genes.



Symbolic representation	Cluster	Description	Count in network	Strength of the enrichment effect	False discovery rate
	CL: 4867	Two component regulatory system, and DM13 domain	2 of 7	2.09	0.0188
	CL: 2906	Secreted, and LPXTG cell wall anchor motif	5 of 45	1.68	1.46e-05
	CL: 2908	Secreted, and LPXTG cell wall anchor motif	3 of 39	1.52	0.0116

Fig. 8 - Protein-Protein Interaction (PPI) network of top-ranked genes revealed through cytoHubba among differentially expressed genes (DEG) in Herboheal-exposed *Staphylococcus aureus*.

Table III - Hubs identified as potential targets from among the up- and down-regulated genes in Herboheal-exposed *Staphylococcus aureus*

No.	Gene ID	Gene name	Codes for	Function
1	SAXN108_0683	*sarA*	Transcriptional regulator SarA	Probably activates the development of biofilm by both enhancing the *ica* operon transcription and suppressing the transcription of either a protein involved in the turnover of PIA/PNAG or a repressor of its synthesis, whose expression would be sigma-B-dependent
2	SAXN108_2673	*sbi*	Immunoglobulin-binding protein Sbi	Plays a role in the inhibition of both the innate and adaptive immune responses
3	SAXN108_1846	*splA*	Serine protease SplA	Poorly characterized secreted protein probably involved in virulence
4	SAXN108_0774	*saeR*	Response regulator transcription factor SaeR	The saeR/S system plays a role in regulating such virulence factors which decrease neutrophil hydrogen peroxide and hypochlorous acid production following *S. aureus* phagocytosis
5	SAXN108_0773	*saeS*	Histidine kinase	
6	SAXN108_2677	*hlgB*	Gamma-hemolysin component B precursor	Toxins that seem to act by forming pores in the membrane of the cell; has a hemolytic and a leukotoxic activity
7	SAXN108_2676	*hlgC*	Gamma-hemolysin component C precursor	

PIA = polysaccharide intercellular adhesin; PNAG = poly-*N*-acetyl-β-(1-6)-glucosamine.

Since in case of *S. aureus*, we analyzed up- and downregulated genes together, instead of considering only the multicluster proteins as hubs, we took all of those which appeared to be part of PPI network as shown in Figure 7. Functions of these seven potential hubs are listed in Table III.

Discussion

Panchvalkal-exposed *P. aeruginosa* appears to suffer from sulfur starvation and nitrosative stress. Compromised nitric oxide (NO) detoxification can render bacteria more susceptible to the NO produced by the host immune system (19). Mutant *P. aeruginosa* deficient in NO reductase was shown to register a reduced survival rate in NO-producing macrophages (26). NO has a strategic role in the metabolism of microorganisms in natural environments and also during host-pathogen interactions. NO as a signaling molecule is able to influence group behavior in microorganisms. Downregulation of the denitrification pathway can disturb the homeostasis of the bacterial biofilms. NO levels can also affect motility, attachment, and group behavior in bacteria by affecting various signaling pathways involved in the metabolism of 3′,5′-cyclic diguanylic acid (c-di-GMP). Suppressing bacterial detoxification of NO can be an effective anti-pathogenic strategy, as NO is known to modulate several aspects of bacterial physiology, including protection from oxidative stress and antimicrobials, homeostasis of the bacterial biofilm, etc. (27-29). From this *in silico* exercise, nitric oxide reductase (NOR) has emerged as the most important target of *Panchvalkal* in *P. aeruginosa*. NOR is one of the important detoxifying enzymes of this pathogen, which is crucial to its ability to withstand nitrosative stress, and has also been reported to be important for virulence expression of this pathogen, and thus can be a plausible potential target for novel anti-virulence agents (19). NOR inhibitors can be expected to compromise the pathogen's ability to detoxify nitric oxide (NO), not allowing its virulence traits (e.g., biofilm formation, as NO has been indicated to act as a biofilm-dispersal signal) to be expressed fully. NOR inhibitors can be expected to be effective not only against *P. aeruginosa* but against multiple other pathogens too, as NO is reported to be perceived as a dispersal signal by

various gram-negative and gram-positive bacteria (30). This is to say, NOR inhibitors may be expected to have broad-spectrum activity against multiple pathogens. Major function of NOR is to detoxify NO generated by nitrite reductase (NIR). NO is a toxic byproduct of anaerobic respiration in *P. aeruginosa*. NO-derived nitrosative species can damage DNA and compromise protein function. Intracellular accumulation of NO is likely to be lethal for the pathogen. It can be logically anticipated that *P. aeruginosa*'s ability to detoxify NO will be compromised under the influence of potent NOR inhibitors like *Panchvalkal.* Since NO seems to have a broad-spectrum anti-biofilm effect, NOR activity is essential for effective biofilm formation by the pathogens. NOR activity and NO concentration can modulate cellular levels of c-di-GMP, which is a secondary messenger molecule recognized as a key bacterial regulator of multiple processes such as virulence, differentiation, and biofilm formation (31). In the mammalian pathogens, the host's macrophages are a likely source of NO. NOR expressed by the pathogen provides protection against the host defense mechanism (26). Since NOR activity is known to be important in multiple pathogenic bacteria (e.g., *P. aeruginosa*, *S. aureus*, *Serratia marcescens*) for biofilm formation, virulence expression, combating nitrosative stress, and evading hose defense, NOR seems to be an important target for novel broad-spectrum anti-pathogenic agents. A potential NOR inhibitor besides troubling the pathogen directly may also boost its clearance by the host macrophages (32).

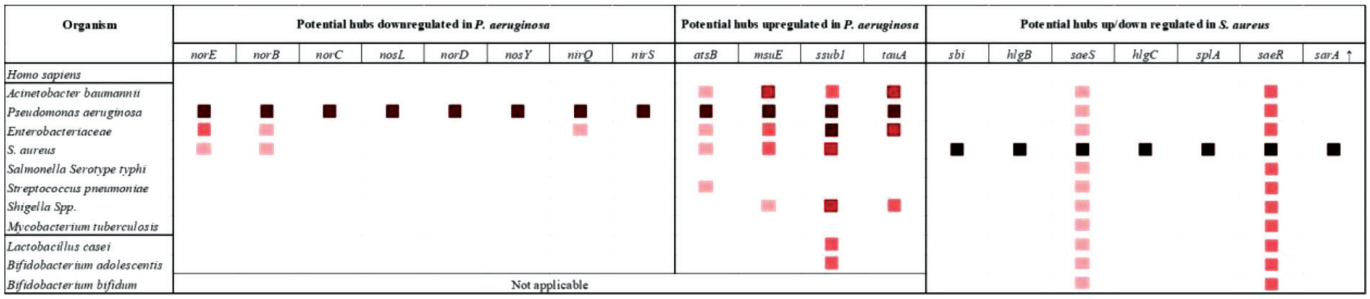
Based on the analysis of differently expressed upregulated genes, sulfur-starved culture of *P. aeruginosa* can be expected to experience compromised virulence. Upregulation of organic sulfur transport and metabolism genes has been reported in *P. aeruginosa* facing sodium hypochlorite-induced oxidative stress (33). Two of the upregulated hubs mentioned in Table II are part of *tau* or *ssu* gene clusters, which are reported in gram-negative bacteria like *Escherichia coli* too for being necessary for the utilization of taurine and alkane sulfonates as sulfur sources. Since these genes are exclusively expressed under conditions of sulfate or cysteine starvation (34), one of the multiple effects exerted by *Panchvalkal* on *P. aeruginosa* can be said to be sulfur starvation. Upregulation of n-alkane sulfonates or taurine (sources of carbon and organic sulfur) utilization genes in *P. aeruginosa* suggests that the sulfur in these compounds was used to counter *Panchvalkal*-induced sulfur starvation, and that the neutrophilic amines and alpha-amino acids formed by catabolization of n-alkane sulfonates may guard the cell against oxidative stress (35). Thus, depriving *P. aeruginosa* of sulfur can be viewed as a potential anti-virulence strategy.

Among the potential targets identified in *S. aureus* in this study, first we discuss two such downregulated genes which are common members of two different clusters. Of them, *splA* is a serine protease, exclusively specific to *S. aureus*, and thought to have a role in the second invasive stage of the infection (36). Another potential hub *sbi* is an IgG-binding protein, which has a role in the inhibition of the innate as well as adaptive immune responses. Its secreted form acts as a potent complement inhibitor of the alternative pathway-mediated lysis. *sbi* helps mediate bacterial evasion of complement via a mechanism called futile fluid-phase consumption (37). Among the remaining potential hubs listed in Table III, *SaeR/S* two-component system is recognized as a major contributor to *S. aureus* pathogenesis and neutrophil evasion. *SaeR/S* also plays a role in regulating such virulence factors which decrease neutrophil hydrogen peroxide and hypochlorous acid production following *S. aureus* phagocytosis (38). *S. aureus* escapes from the antimicrobial protein's neutrophil extracellular traps (NETs), which is dependent on its secreting nuclease (*nuc*), and the latter in turn is regulated by *SaeR/S*. The *SaeR/S* system also modulates neutrophil fate by inhibiting interleukin (IL)-8 production and nuclear factor (NF)-κB activation. *SaeR/S* deletion mutant of *S. aureus* was shown to be inferior than its wild-type counterpart in causing programmed neutrophil death (39). The *SaeR/S* system regulates expression of many important virulence factors in *S. aureus*, and some of them do appear in our list of important targets such as *sbi, hlgB*, and *hlgC*. Thus, inhibiting *SaeR/S* from sensing its environment can be expected to prevent expression of a multitude of *S. aureus* virulence factors in response to host signals. *hlgB* and *hlgC* are hemolytic proteins, and such proteins are used by many pathogens to fulfill their iron requirement as the concentration of free iron in human serum is much lesser than that required by the bacteria (40). Downregulation of bacterial hemolytic machinery may push them toward iron starvation, thus compromising their fitness for in-host survival. This corroborates well with our earlier report (11) describing reduced hemolytic potential of *S. aureus* under the influence of Herboheal. Among all the potential hubs identified in Herboheal-exposed *S. aureus*, only one (*sarA*) was upregulated, and its upregulation seems to be a response from *S. aureus* to compensate the Herboheal-induced downregulation of many important virulence traits. For example, *sarA* regulates expression of *ica* operon, which is required for biofilm formation in *S. aureus*. It can be said that *S. aureus*'s ability to adhere to surfaces and biofilm formation was compromised in the presence of Herboheal as suggested by downregulation of adhesion/biofilm-relevant genes (*SaeR/S* and *sarA*), and as an adaptation to such challenge the pathogen is trying to upregulate *SarA*. This corroborates well with our previous report describing 56% reduced biofilm formation by *S. aureus* in the presence of Herboheal (11).

This study has identified certain potential hubs in *P. aeruginosa* (Tabs. I and II) and *S. aureus* (Tab. III) which should further be investigated for their candidature as potential anti-pathogenic targets. The most suitable targets in bacterial pathogens would be the ones which are absent from their host, as this will allow the criteria of selective toxicity to be satisfied for a newly discovered drug. We did a gene co-occurrence pattern analysis of gene families across genomes (through STRING) with respect to the major hubs identified in each of the pathogens (Tab. IV). Of the 19 hubs identified in either of the pathogen, none was shown to be present in *Homo sapiens*, and hence drugs causing dysregulation of one or more of these genes in pathogens are less likely to be toxic to humans.

If any target gene is present among multiple pathogens, then it can be considered suitable for a broad-spectrum

Table IV - Co-occurrence analysis of genes coding for potential targets in *Pseudomonas aeruginosa* and *Staphylococcus aureus*



The darker the shade of the squares, higher is the homology between the genes being compared.

antibacterial. We analyzed the co-occurrence of identified hubs among some of the important pathogens listed by CDC and WHO. From among those listed in Table IV, *atsB, msuE, ssub1, norE,* and *norB* seemed to be present in multiple gram-negative as well as gram-positive pathogens, and thus suitable to be targeted by a broad-spectrum anti-pathogenic discovery program. On the other hand, *tauA* and *nirQ* seemed to be present only among gram-negative pathogens. They can prove to be important targets in light of the fact that discovery of novel antimicrobials against gram-negative bacteria is relatively more challenging (41).

One of the issues with conventional antibiotics is that they cannot differentiate between the 'good' (symbionts in human microbiome) and 'bad' (pathogens) bacteria, and hence their consumption may lead to gut dysbiosis. An ideal antimicrobial agent should target pathogens exclusively without causing gut dysbiosis. In this respect, a target in pathogenic bacteria absent from symbionts of human microbiome will be the most suitable candidate for antibiotic discovery programs. To gain some insight on this front regarding the targets identified by us, we run a gene co-occurrence analysis with some representative 'good' bacteria reported to be part of healthy human microbiome. *Bifidobacterium* species showed presence of no other target except *SaeR/S. SaeR/S* being widely distributed among bacteria can be considered a valid target; however, an antibacterial agent targeting it may lead to gut dysbiosis too. All downregulated targets in *P. aeruginosa* were absent from the selected symbionts, which further adds value to their potential candidature as anti-virulence targets. However, *atsB* and *ssub1* appeared to be present in *Lactobacillus casei*.

Conclusion

This study has identified certain potential targets in two important pathogens. Such *in silico* studies being predictive in nature, further work is warranted on wet-lab validation of the identified targets. Deletion mutants of the identified hub genes should be assessed for their expected attenuated virulence in appropriate host models. Next-generation patho-blockers targeting any one of these genes may not always be effective as stand-alone therapeutic, and simultaneous targeting of more than one of these genes may be required for an effective therapy. They can also prove to be useful adjuvants to conventional antibiotics allowing use of bactericidal antibiotics at lower concentrations.

Besides indicating generation of nitrosative stress, inducing sulfur starvation, and disturbing regulation of bacterial virulence as potentially effective anti-pathogenic strategies, this study also demonstrates the relevance of the polyherbalism concept of the Traditional Medicine systems, and utility of the network analysis approach in elucidating the multiple modes of anti-pathogenic action exerted by the multicomponent natural extracts.

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Abbreviations

AMR = antimicrobial resistance; DEG = differentially expressed genes; NO = nitric oxide; NOR = nitric oxide reductase; PPI = protein-protein interaction

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