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Medicinal plants from Indian Subcontinent decrease quorum sensing dependent virulence in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa (PAO1), an opportunistic pathogen in immune compromised patients is under the regulation of the *LasR-RhlR* system for quorum sensing (QS) mediated development of virulence. Natural products have recently become a promising source for deriving molecules that can potentially inhibit QS. Medicinal plants from Indian subcontinent used in traditional medicine are well known for their antimicrobial effects on a variety of human pathogens. However, few studies have investigated its QS related antivirulent activities. Our objective was to screen for the QS inhibitory properties of 16 high quality ayurvedic medicinal plants derived from Indian sub-continent, understand their mechanism of action and investigate their effect on the expression of QS regulated virulence factors in PAO1. QS inhibition of sub-lethal concentrations (SLC) of plant extracts was measured in violacein producing *Chromobacterium violaceum* bioassay model. Effect of these extracts on PAO1 virulent factors pyocyanin, elastase and total protease were quantified by standard protocols. Results indicated that all extracts reduced violacein production significantly. The results from modified screening assay suggested the primary mechanism of QSI was a combination of (1) reduction in AI formation and (2) inhibition of AI activity. The extracts reduced pyocyanin synthesis, inhibited the activity of elastase and other proteolytic enzymes.

Key words: Quorum sensing, acylated homoserine lactones (AHL), phytochemicals, antimicrobial activity, medicinal plants from India, virulence factors and *Pseudomonas aeruginosa.*

1. Introduction

Control of bacterial infections by inhibiting microbial growth has been a primary approach of antimicrobial chemotherapy. An emerging problem associated with continual indiscriminant use of this therapeutic strategy is the selection of resistant bacteria with higher level tolerance against these broad-spectrum antibiotics. Development of novel antibiotics that interfere with metabolism coupled with continued indiscriminant use of antibiotics will only lead

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to evolution of new resistance mechanisms and pathways by bacteria [1, 2]. Therefore, technology is required to block very basic mechanisms of bacterial communication that appear to control toxin production, adherence, motility, proteolysis and other virulence factors leading to pathogenicity [3-5]. Emerging research has suggested that functions including swarming, biofilm formation, secretion of virulence factors and acquiring competency play an important role in successful and recurrent establishment of bacterial infections in living systems. These processes common to several bacterial diseases are now shown to be related to a cell density-mediated regulation of gene expression mediated by the recognition of signaling molecules called autoinducers (AI). This process sometimes referred to as 'quorum sensing' (QS) has now been shown to modulate the expression of genes involved in processes related to survival, virulence and pathogenicity [6-8] and is mediated by small signaling molecules called autoinducers, such as the acetylated-homoserine lactones (AHLs) [9]. Opportunistic Gram negative bacteria such as *Pseudomonas* species have been shown to employ this type of AHL-mediated quorum sensing to regulate the expression of genes related to, survival, virulence and competency [9, 10]. Over the years several QS inhibitors have been developed including some from natural sources such as Australian macroalga, *Delisea pulchra* and green alga, *Ulva lactua* that produce QSI halogenated furanones and furanone-like pigments [11, 12]. These halogenated furanones have also been synthesized recently and show a high degree of AHL inhibition activity and were able to repress a number of genes controlled by QS. However, the application of current synthetic and natural halogenated furanones for therapeutic applications is limited as they are highly reactive and toxic to the host. Analogs of S-adenosylmethionine (SAM), such as S-adenosylcysteine and sinefungin can inhibit LuxI mediated synthesis of AHL but can interfere with eukaryotic pathways that use SAM [3]. Recent studies have shown that interference with *P.aeruginosa* quorum signaling by metabolites from *Penicillium* species can increase susceptibility to these agents [13]. Unfortunately, these metabolites from *Penicillium* and other fungal species were also found to be toxic to human cells, thereby limiting their potential usage *in vivo*. The current quest for new antimicrobials is therefore aimed at discovering nontoxic inhibitors of QS that can be used for treatment of bacterial infections in humans [3].

Natural products especially plants used in traditional medicines in different cultures are a promising source for deriving molecules that can potentially inhibit quorum sensing. These plants can offer a large and attractive repertoire for the discovery of QS inhibitors [14, 15]. These are of particular importance as these phytochemicals have been used for thousands of years for the treatment and management of diseases and may have few side-effects and toxicity issues as with many antibiotic regimens and currently known QS inhibitors [5].

Medicinal plants used in traditional Indian Medicine (Ayurveda) used for several centuries to treat common ailments [16], are well known for their antimicrobial effects on a variety of human pathogens [17]. However, few studies have investigated its QS related antivirulent activities of the extracts of these medicinal plants. The body of literature discussing medicinal plants from Indian subcontinent as sources of QSI is very limited. We believe that some of the antimicrobial properties of medicinal plants may be contributed by the QS inhibiting phytochemicals present in it.

Our objective was to screen for the QS inhibitory properties of 16 high quality herbs, spices & medicinal plants (HSMP) from Indian sub-continent, understand their mechanism of

action and investigate their effect on the expression of QS regulated virulence factors in *P.aeruginosa*.

2. Materials and Methods

2.1. Samples and Extraction

Sixteen different Indian herbs and medicinal plants were obtained directly from Raja Foods (Houston, TX). 0.5 g of different samples (Table 1) were suspended in 10 ml of distilled water and extracted on a rotary shaker for 15 min. The samples were then centrifuged (3000 rpm, 10°C, 10 min) and supernatant was sterile filtered and stored at 4°C (18).

Microorganisms: Pseudomonas aeruginosa (ATCC 15692), Chromobacterium violaceum (ATCC 12472) were purchased from American Type Culture Collection (ATCC, Bethesda, MD) and were subcultured and maintained in LB. *Chromobacterium violaceum* O26 and *Chromobacterium violaceum 31532* kind gifts from Dr. Bob McLean (Texas State University, San Marcos, TX) were also subcultured and maintained in LB

2.2. AHL Bioassays

Determination of Minimal Inhibitory Concentrations (MIC): MIC values were determined by the broth microdilution test on *Pseudomonas aeruginosa* (PAO1) by modifying a previously described method [19]. The MIC value was recorded as the lowest treatment concentration at which there was no visible growth of the organism. All the quorum sensing assays and anti-virulence assays were performed at concentrations lower than the MIC values known as sub-inhibitory concentration.

2.3. Qualitative Agar Diffusion assay

The effect of 75 µl of extract on (1) modulation of AHL activity (Set-A) and (2) Inhibition of AHL synthesis (Set-B) was determined using the agar diffusion double ring assay at sub-lethal concentrations as described previously (18). Briefly, two strains an AHL overproducer strain *Chromobacterium violaceum* 31532 (CV31532), and an AHL biosensor *C.violaceum* O26 (CVO26) are used (20). Extracts are placed in an absorbent, sterile filter paper disk and the AHL overproducer and biosensor strains inoculated in varying concentric proximity to the disk. To test for potential LuxI inhibition, the AHL overproducer is placed in close proximity to the test substance and the AHL biosensor placed distal. To test for LuxR inhibition, the location of the AHL overproducer and biosensor strains is reversed. In either case, potential QSI activity results in a lowered signal from the AHL biosensor and therefore decrease in violacein pigment formation.

2.4. Quantitative AHL bioassay [18]

Total inhibition in quorum sensing which was a combination of modulation of AHL activity and modulation of AHL synthesis was determined by measuring the violacein pigment formation in the wild type bacteria *C. violaceum* CV 12472. The extracts that showed the best results in the agar diffusion assay (described above) were chosen. CV 12472 bacteria was grown in the presence of 1% extract in LB for 24 hr at 32°C. The bacterial cultures were centrifuged (14,000 rpm, 10°C, 15 min) and pelleted cells were lysed with 0.1% SDS and pigment was extracted in DMSO and read at A595nm using a microplate reader [18].

2.5 Modulation of AHL activity

AHL overproducers CV31532 were grown for 48 h at 32°C. The cultures were then centrifuged (8000 rpm, 4°C, 10 min) and the supernatant was sterile filtered. The filtrate containing AHLs was inoculated with AHL biosensor CVO26 and the LB was supplemented to contain 1% extract and incubated for 24 h at 32°C. The cultures were subsequently assayed for violacein production as discussed above.

2.6. Modulation of AHL synthesis

AHL over producers CV31532 were grown in LB was supplemented to contain 1% extract for 48 h at 32°C. The cultures were then centrifuged (8000 rpm, 4°C, 10 min) and the supernatant was sterile filtered. The filtrate containing AHLs was extracted in AcOEt with 0.1% acetic acid and evaporated in vacuum at 45°C. The isolated AHLs were suspended in sterile LB and inoculated with CVO26 and incubated for 24 h at 32°C. The cultures were subsequently assayed for violacein production as described above.

2.7. Measurement of QS dependent virulence factors in Pseudomonas aeruginosa (PAO1)

PAO1 was grown in LB supplemented to contain 1% extract for 24 hrs at 30°C, then centrifuged (8000 rpm, 4°C, 10min) the virulence factors were determined in cell free supernatants using standard protocols.

2.8. Total proteolytic activity

The protein concentrations of the cell supernatants were determined by Lowry's method [21]. Total proteolytic activity of the culture supernatants was estimated according to a method described earlier [22]. Briefly, to 100 µl of culture supernatants 900 µl of 0.5% azocasein (Sigma Chemical Co., St. Louis, MO) prepared in 50 mM tris buffer containing 2 mM $CaCl_2$. Samples were incubated at 37°C for 30 min. After this, 15% TCA (100 ml) was added to stop the reaction and centrifuged (8000 rpm, 4°C, 10min). The absorbance of the supernatants from both controls and treatments was measured at 440 nm. The % change in absorbance was then calculated from the absorbance values.

2.9. Elastase Activity

Elastase activity was measured by the modifying methods described before in literature [23, 24]. Briefly, to 200 µl of culture supernatant 1 mL of 0.5% elastin-congo red solution (in 10 mM PBS) (Sigma Chemical Co., St. Louis, MO) and incubated at 37°C for 3 hours in a water bath. The samples were then vortexed and centrifuged at 1200g for 10 minutes at 10°C to remove insoluble elastin congo red. The absorbance of the supernatants from both controls and treatments was measured at 494 nm. The % change in absorbance was then calculated from the absorbance values.

2.10. Pyocyanin Assay

Pyocyanin was extracted from culture supernatants and measured using the method described previously [25, 26]. Briefly, 3 ml of chloroform was mixed with 5 ml culture supernatant. The chloroform layer was transferred to a fresh tube and mixed with 1 ml 0.2 M HCl. After centrifugation, the top layer (0.2 M HCl) was removed and its absorption measured at 520 nm.

3. Results

3.1. Qualitative Agar Diffusion Assay

The qualitative determination of QS modulation in agar diffusion assay as described in Mihalik *et al*., 2007 (18) was performed on all the HSMP from Indian subcontinent (Table 1, Figure 1) at sub-lethal concentrations (SLC). Our results in the CV31532/CV026 system indicated that qualitatively *Harde* had the highest ability to interfere with the activity of the AHL's produced by CV 31532 (Set 1, Table 2, Figure 1A). Slightly lower ability to interfere with AHL activity was seen with *Amala, Aritha, Brahmi* and *Karithu* extracts (Table 2). Nagakesar, had the lowest most observable reduction in violacein pigment formation due to interference with AHL activity. All other extracts *(Akalargo, Ardusi, Aswagandha, Kadu, Kalijiri, Shankh Pushpi, Shikakai, Vavding)* had no significant observable reduction in violacein pigment formation compared to the control (Table 2). When we quantitatively tried

to determine if any of the extracts had ability to reduce the formation of AHL molecules from CV31532 (Set 2, Table 2, Figure 1B), or results were significantly different. *Harde, Aritha* and *Vavding* appeared to have the highest ability to decrease the AHL synthesis by CV31532 (Table 2). *Shankh Pushpi* and *Arjun Chal* also appeared to have a minor effect in reducing the AHL synthesis dependent violacein pigment formation in CV O26. All other extracts did not have any apparent effect on the AHL synthesis of CV31532 and were not significantly different from the control (Table 2).

3.2. Quantitative AHL bioassay

We suspected that several herbs which did not show any effect in the qualitative diffusion agar assay was due to the limitations of the agar diffusion assay such as poor and uneven diffusion. In order to test if the extracts had effect on over all inhibition of quorum sensing we investigated if it can reduce the quorum sensing related violacein production in the wild type bacteria CV 12472. Our results indicated that *Harde, Karaithu* and *Vavding* had the strongest inhibition and decreased the QS related violacein production by 55%, 48% and 52% respectively (Figure 2). *Amala* and *Aritha* decreased the violacein pigment formation by 33% and 39% respectively. All other samples exhibited lower QS inhibition properties and inhibited the QS mediated violacein formation by only 10-25% (Figure 2).

Table 1. List of herbs and medicinal plants used (Alphabetical)

	Common Name	Scientific Name
1.	Akalgaro	*Anacyclus pyrethrum*
2.	Amala	*Emblica officinalis*
3.	Ardusi	*Ailanthus excelsa*
4.	Arjun Chal	*Terminalia arjuna*
5.	Aritha	*Sapindus laurifolius*
6.	Aswagandha	*Withania somnifera*
7.	Brahmi	*Centella asiatica*
8.	Harde	*Terminalia chebula*
9.	Kadu	*Swertia chirayata*
10.	Kalijiri	*Centratherum anthelminticum*
11.	Kamarkas	*Pterocarpus marsupium*
12.	Karaithu	*Enicostemma littorale*
13.	Nagkesar	*Mesua ferrea*
14.	Shankh Pushpi	*Evolvulus alsinoides*
15.	Shikakai	*Acacia concinna*
16.	Vavding	*Embelia ribes*

Table 2. Effect of sub-lethal concentrations of HSMP from Indian subcontinent on quorum sensing inhibition as determined by decrease in violacein pigment formation in qualitative agar diffusion assay. Set 1: . Quorum sensing inhibition by modulation of AHL activity in CV31532/CVO26 bioassay; Set 2: . Quorum sensing inhibition by modulation of AHL synthesis in CV31532/CVO26 bioassay.

	Extract	Set 1	Set2
1.	Akalargo	(+,-)	(+,+)
2.	Ardusi	(+,-)	(+,+)
3.	Arjun Chal	(+,+)	(-)
4.	Aritha	(-,-)	(-,-)
5.	Amala	(-,-)	(+-)
6.	Aswagandha	(+,+)	(+-)
7.	Brahmi	(-,-)	(+-)
8.	Harde	(-,-,-)	(-,-)
9.	Kadu	(+,-)	(+-)
10.	Kalijiri	(+,+)	(++)
11.	Kamarkas	(+,-)	(+-)
12.	Karaithu	(-,-)	(+,+)
13.	Nagkasar	(-)	(+-)
14.	Shankh Pushpi	(-,+)	(-)
15.	Shikakai	(+,+)	(-,+)
16.	Vavding	(+,+)	(-,-)
	Control	(+,+)	(+,+)

(+) = Violacein pigment

(-) = absence Violacein pigment

(+,-) = Not confirmatory

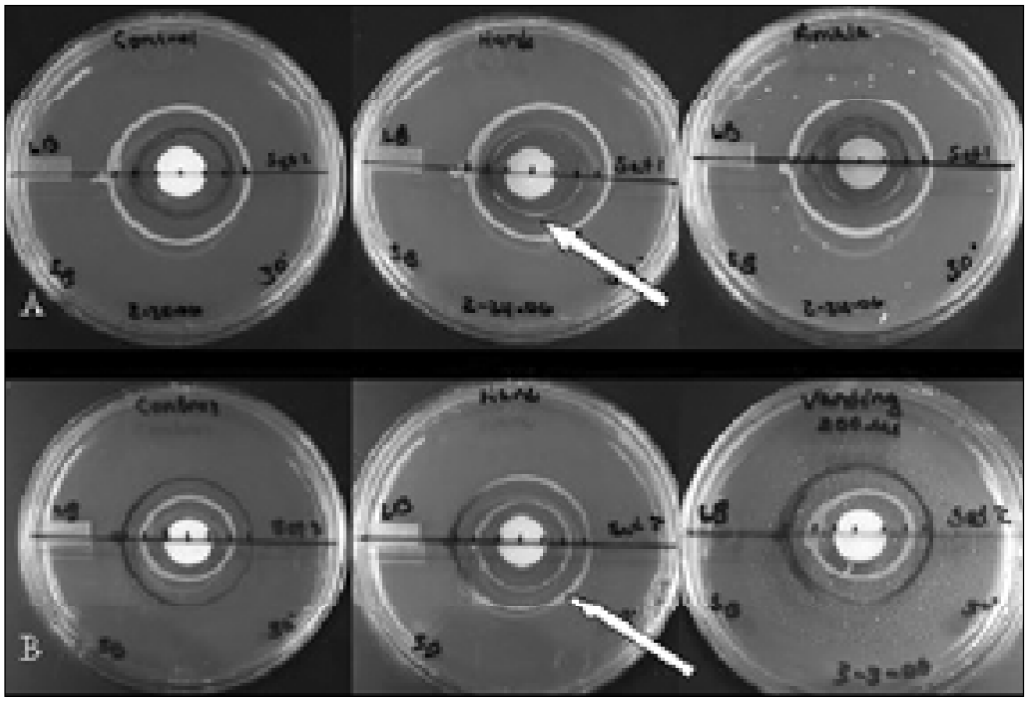

Fig. 1. Effect of sub-lethal concentrations of HSMP from Indian subcontinent on quorum sensing inhibition as determined by decrease in violacein pigment formation in qualitative agar diffusion assay. Set 1 (A): Quorum sensing inhibition by modulation of AHL activity in CV31532/CVO26 bioassay; Set 2 (B): Quorum sensing inhibition by modulation of AHL synthesis in CV31532/CVO26 bioassay.

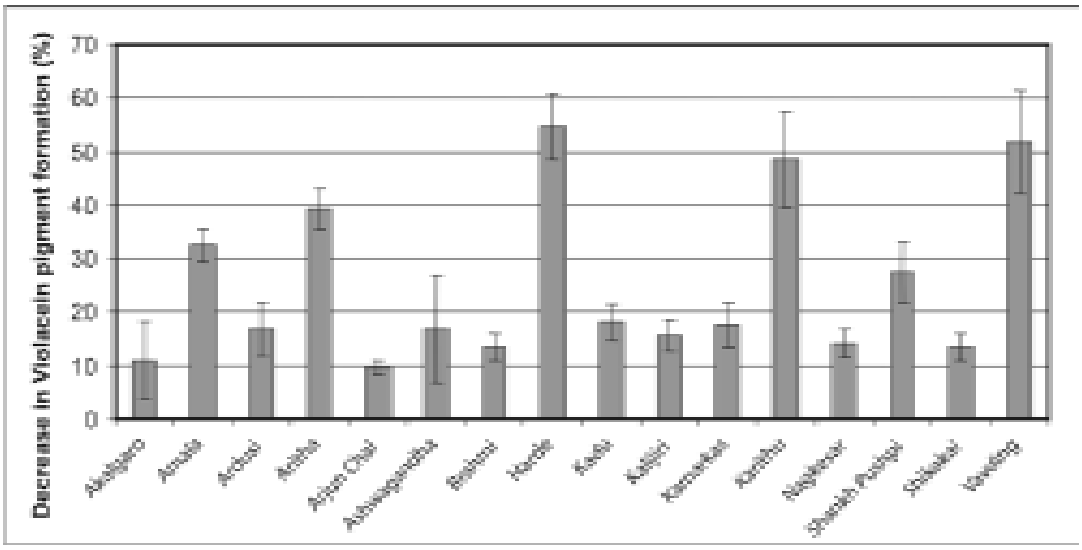

Fig. 2. Effect of sub-lethal concentrations of HSMP from Indian subcontinent (at 1% level) on quorum sensing inhibition as determined by reduction in violacein pigment formation in wild type Chromobacterium violaceum (CV 12472).

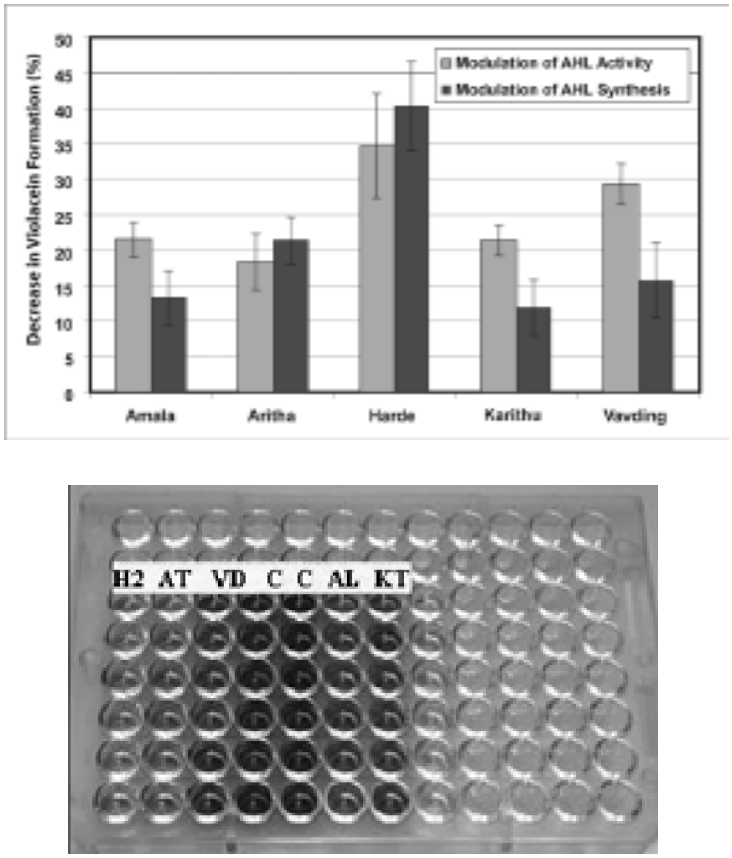

Fig. 3. Quantitative determination of quorum sensing inhibition in CV31532/CVO26 Bioassay by modulation of AHL activity and modulation of AHL synthesis along with microtitre plate with the extracted violacein pigment for Set 2. H2=Harde; AT=Aritha; VD=Vavding; C=Control; AL=Amala; KT=Karaitu.

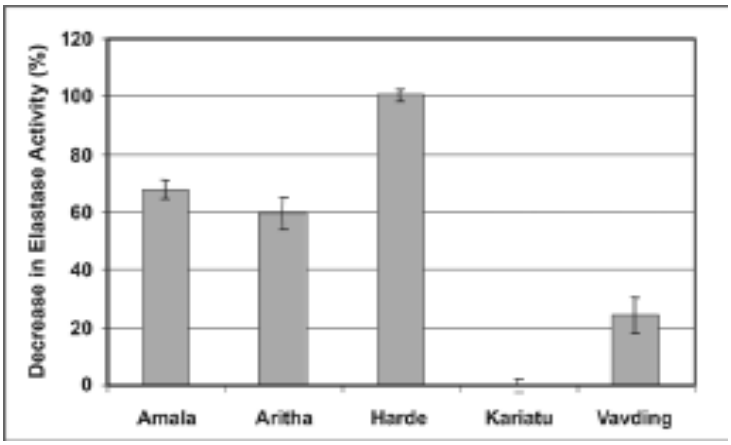

Fig. 4. Effect of sub-lethal concentrations of HSMP from Indian subcontinent on quorum sensing dependent elastase activity in P. aeruginosa.

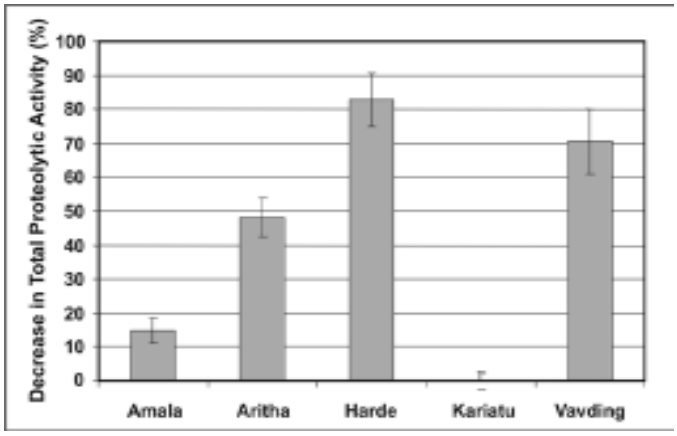

Fig. 5. Effect of sub-lethal concentrations of HSMP from Indian subcontinent on quorum sensing dependent total proteolytic enzyme acticity in P. aeruginosa.

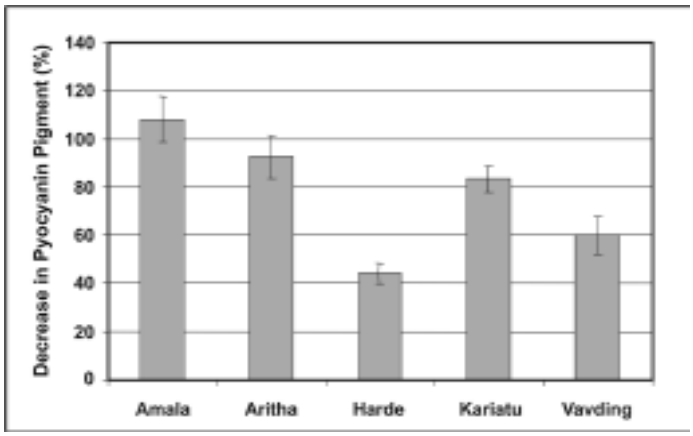

Fig. 6. Effect of sub-lethal concentrations of HSMP from Indian subcontinent on formation quorum sensing dependent pyocyanin pigment formation in P. aeruginosa.

For the extracts that decreased the formation of violacein pigment by 30% or more we wanted to see if the inhibition was due to the ability of the extracts to modulate AHL activity or modulate the AHL synthesis. We conducted this quantitative investigation in the CV31532/CV026 bioassay as described in the methods section.

3.3. Modulation of AHL activity

We selected *Amala, Aritha, Harde, Karaithu* and *Vavding* and tested for their effect on modulating AHL activity. We incubated the AHL's purified from CV 31532 and the extracts with CV O26 for 24 hrs. After 24 hrs we extracted the violacein pigment and quantified it spectrophotometrically to determine if the extracts had interfered with the ability of the AHL's to produce violacein pigment in CV O26. Our results indicated that *Harde* had highest ability to interfere with the activity of the AHLs produced by CV 31532 and reduced the AHL mediated violacein production in CV O26

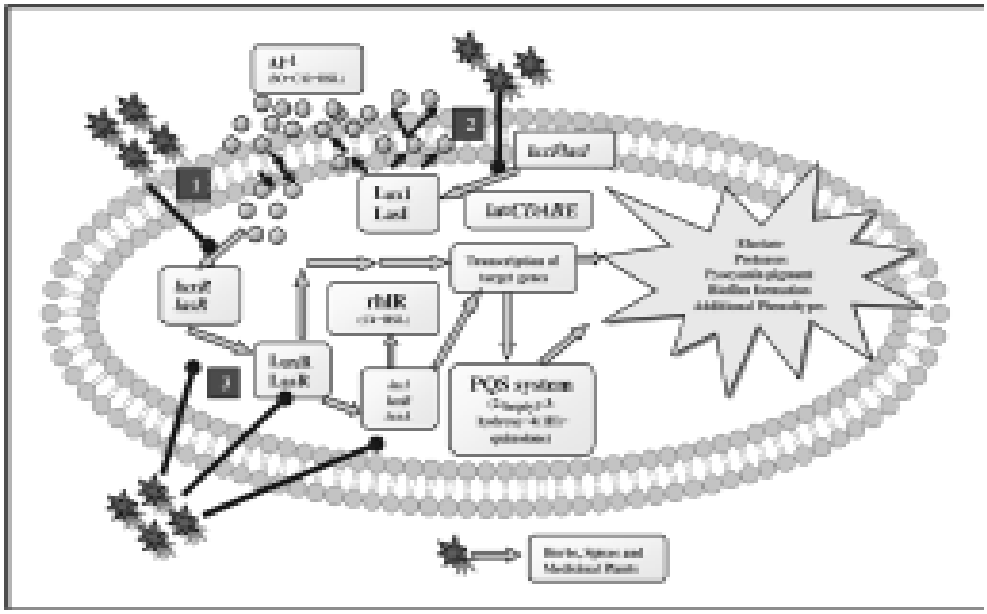

Fig. 7. A model for the quorum sensing inhibitory effects of herbs, spices and medicinal plants.

biosensor by 34% (Figure 3). The second highest activity was found in *Vavding* extracts, which interefered with AHL activity and decreased AHL mediated violacein pigment production by 28% (Figure 3). *Amala, Karaithu* and *Aritha* lower ability to interfere with AHL activity and inhibited violacein production by 23%, 22% and 17% respectively (Figure 3).

3.4. Modulation of AHL synthesis

We assessed the ability of the extracts to reduce the AHL synthesis by the overproducer, by incubating the overproducer overnight with 1% of the extract, and then extracting the AHLs. The AHL's were then used to produce violacein in the biosensor CV O26, the violacein was extracted and quantified spectrophotometrically using a microplate reader (Figure 3). Our results indicated that again *Harde* extract had the highest effect on AHL synthesis and decreased the violacein pigment formation by 41% (Figure 3). This was followed by *Aritha* extract which had a 22% activity (Figure 3). *Vavding* and *Amala* appeared to decrease AHL synthesis in CV 31532, which lowered the AHL mediated violacein production in CV O26 by 13% and 16 % respectively. *Karaithu* had the lowest ability to modulate AHL synthesis in CV 31532 and was able to decrease violacein production only by 12% (Figure 3).

3.5. Effect on QS mediated expression of virulence factors.

We wanted to test if the modulation of quorum sensing in the *Chrmobacterium CV 31532/CV O26* system had any affect on the expression of virulence factors in an opportunistic pathogen *P. aeruginosa* (PA O1).

3.6. Elastase Activity

Along with other proteases, elastase is an important proteolytic enzyme that allows the bacteria to invade and colonize host tissues. We investigated the effect of our extracts on

reducing the total elastase related proteolytic activity in PA O1. Our results show that *Harde* had the highest ability to reduce the activity of elastase and completely (100%) able to decrease elastase activity (Figure 4). This was followed by *Amala* and *Aritha*, which decreased elastase activity by 68% and 60% respectively (Figure 4). *Vavding*, which had one of the highest ability to decrease violacein formation in the wild type CV O26, decreased elastase activity only by 22%. *Karaithu*, when tested at 1% level had no effect on the elastase activity compared with the control which did not contain any extract (Figure 4).

3.7. Effect on Total proteolytic activity

We measured the ability of the extracts to decrease the total proteolytic activity in PA O1. The highest activity was again observed by *Harde* extracts which decreased the proteolytic activity in PA O1 cultures by 82% (Figure 5). The second highest activity was observed by *Vavding* which decreased the proteolytic activity by 70% compared to the control (Figure 5). *Aritha* was also effective in decreasing the overall proteolytic activity of PA O1, it inhibited the total proteolytic activity by 48% (Figure 5). *Amala*, which was quite effective in decreasing elastase activity was not very potent and reducing the total proteolytic activity by only 14% (Figure 5). *Karaithu* again was completely ineffective in reducing the total proteolytic activity compared to the control (Figure 5).

3.8. Pyocyanin synthesis

Pyocyanin (1-hydroxy-5-methylphenazine) is an antibiotic pigment secreted by *P.aeruginosa* which allows the bacteria to preferentially grow in an environment and is regulated by genes controlled by quorum sensing. Pyocyanin is a secondary metabolite and has antimicrobial activity against several species of bacteria, fungi and protozoa, due to its ability to interfere with the redox metabolism in the target organism (Sorenson *et al.*, 1983). We investigated the affect of our medicinal plant extracts on the ability of the bacteria to synthesize and secrete this virulent pigment. Our results indicate that the all the extracts significantly decreased the formation of the pigment in the bacteria (Figure 6). *Amala* had the maximum activity and reduced the formation of pyocyanin by over 100% (Figure 6). This was followed by *Aritha*, *Karaithu* and *Vavding* which decreased the pyocyanin formation in PA O1 by 94 %, 82%, and 60% respectively. The lowest activity among the samples tested was observed in *Harde*, which decreased the pigment formation in the bacteria by 41% compared to the control (Figure 6).

4. Discussion

Many gram negative bacteria use small signaling molecules called acyl homoserine lactones (AHL) to mediate quorum sensing related bacterial functions. These molecules are made from a category of LuxI synthases (Figure 7) (or their homologues) which use S-adenosylmethionine (SAM) and differ in their varying acyl chain length and oxidative substitutions and are specific for each species of bacteria. At high cell density the concentration of AHL increases and as it diffuses across the cell membrane of the bacteria where it binds and activates LuxR protein (Figure 7). LuxR functions as a transcription factor and induces the transcription of the *luxCDABEGH* genes involved in many bacterial processes [6-8]. In *Chromobacterium sp*. this QS is mediated predominantly by the N-hexanoyl-l-homoserine lactone and other minor C4 AHL's [20]. Inhibition QS in *Chromobacterium sp*. would therefore indicate modulation of a QS signaling cascade mediated by this AHL.

Our results suggest that several medicinal plants had significant quorum sensing inhibition activity at sub-inhibitory concentrations in the CV 12472 system in which the QS is mediated by C-6 AHL (Figure 2). There are several natural extracts that are believed to inhibit quorum sensing by either interfering with the AHL activity by competing with them due to their structural similarity and/or accelerate the degradation of the LuxR/LasR receptors of the AHL molecules [3, 27]. While all the extracts inhibited the violacein pigment formation in the wild type bacteria CV 12472, the differential effect on quorum sensing could probably suggest different concentration and/or different profile type of QS inhibitors in different medicinal plant extracts (Figure 2). The results suggest that *Amala, Aritha, Harde, Karaithu* and *Vavding*, had the most potent activity in inhibiting the QS sensing cascade in *chromobacterium sp*. mediated by C6-AHL's. Further investigation into mechanism of QS inhibition activity in the CV 31532/CV O26 system revealed that, the mechanism of quorum sensing inhibition activity appeared to be a net effect/combination of [1] the ability of phytochemicals to interfere with the activity of AHL produced by CV 31532 and [2] modulate the synthesis of AHL's by the bacteria as detected by the extent of violacein production by the AHL biosensor CV O26, which could suggest that these medicinal plants harbor phytochemicals that either interfere with the AHL activity and transcriptional regulation of LuxR as described above, or are somehow, reducing the luxI mediated synthesis of the AHL molecules in the AHL over producer CV 31532 (Figure 7). Our results for the first time showed that phytochemical extracts from medicinal plants from Indian subcontinent can not only interfere with the AHL activity but also affect quorum sensing by modulating AHL synthesis.

Our results also suggest these extracts also decreased the production of virulence factors regulated by quorum sensing in *Pseudomonas aeruginosa* albeit with different potency. Our results suggested that while *Harde* was most effective in inhibiting QS related total proteolytic activity (Figure 4) and elastase activity (Figure 5), it was not very effective in decreasing *pyocyanin* synthesis (Figure 6). Whereas *Karaithu* which did not have any effect on elastase and total protease activity was quite effective in reducing pyocyanin synthesis. In *P.aeruginosa* the QS cascade is also mediated AHL's that are synthesized by the products of the *lasI* and *rhlI* genes. The products of the *lasR* and *rhlR* genes monitor the concentration of these AHL's and serve as transcriptional activators of a variety of products important in *P. aeruginosa* virulence and pathogenesis [28]. Some important virulent factors regulated this way are elastase, alkaline protease, hemolysin, pyocyanin, and rhamnolipids (Figure 7) There are two AHL systems in *P.aeruginosa*-N-(3-oxododecanoyl) homoserine lactone (3O–C12–HSL), (*las* system) and N-butyryl homoserine lactone (C4–HSL) (Rhamnolipid *rhl* system) which mediate QS in a hierarchical fashion (28, 29). Where *LasR*-C12-HSL activates the transcription of *rhlR* which then induces the transcription of *rhlI* to synthesize C4-HSL. The differential effects of the extracts on modulating different virulence factors in *Pseudomonas* might suggest that the extracts are acting at different hierarchical positions in the over all QS cascade again either by interfering with the activity of the AHL molecules or their synthesis by *Pseudomonas* as depicted in our model (Figure 7). More importantly, all the extracts which inhibited quorum sensing in the Chromobacterium system also inhibited the QS related virulence factors in *Pseudomonas*. This might suggest that these medicinal plants

actually contain not a single compound but a group (profile) of compounds which give them a broad spectrum capability to inhibit multiple QS systems in different species of bacteria mediated by different categories of AHL molecules. In this case C6 AHL in the Chromobacterium system and the 3O–C12–HSL [20] (McLean *et al*., 1997) and C4–HSL in *P. aeruginosa* [28, 29] (Pesci, & Iglewski, 1997; Pesci *et al*., 1997). Therefore, based on our results it appears that these phytochemicals can have multiple modes of action in controlling microbial virulence. It is possible that in addition to their well documented antimicrobial activity they could affect microbial virulence by modulating QS related genes. As represented in Figure 7, we believe that these phytochemicals can inhibit and/or modulate quorum sensing act multiple levels. Phytochemicals could either be 1). Binding directly to the LuxR/LasR receptor by competing with the AHL molecules and/or prevent the binding of the AHL molecules to these receptors and accelerating their degradation as discussed in previous studies [3, 13, 30]. These phytochemicals can also affect the ability of the bacteria to synthesize AHL molecules as discussed above. This could either be due to the ability of the phytochemicals to decrease the expression of the LuxI/LasI synthase which synthesizes the AHL molecules. Decreased AHL synthesis could also be due to the ability of these phytochemicals to inhibit the LuxI/LasI enzyme activity directly [6-8]. The phytochemicals from these medicinal plants can also directly inhibit the *Pseudomonas* Quinolone Signal (PQS) which is mediated by 2-heptyl-3-hydroxy-4(1H)-quinolone 4-quinolone which induces expression of lasB (coding for elastase), rhlI and rhlR [28, 29, 31]. Isolating the bioactive QS inhibitory compounds and elucidating the possible modes of action will be the objective of our future investigations.

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