ANALYSIS OF QUORUM SENSING INHIBITION AND PHYTOCHEMICALS IN DIFFERENT EXTRACTS OF GARLIC

ANITHA VADEKEETIL, SANJAY CHHIBBER AND KUSUM HARJAI *

Department of Microbiology, Panjab University, Chandigarh, India-160014

ABSTRACT

Usage of *Allium sativum* L. bulb (Amaryllidaceae) against the pathogenesis of *Pseudomonas aeruginosa* is an emphasized approach. However, biological response and safety of herbal extracts depend upon the processing methods and solvents used in preparation. In this study, various garlic bulb extracts (ethanol, ethyl acetate, methanol, acetone, chloroform, toluene, raw and heated) were prepared and examined for their quorum sensing inhibitory (QSI) potential in terms of reduction in quorum sensing signal molecules (QSSMs) production, biofilm formation and motility exhibited by *P. aeruginosa*. Out of all these, toluene extract of garlic (TGE) in its sub-MIC showed highly significant reduction in production of QSSMs (80%), biofilm formation (5 folds, p<0.01) and three forms of motilities (p<0.05) as compared to *P. aeruginosa* grown in the absence of any of the extracts. Further, phytochemical profiling of extracts indicated a maximum abundance of thiosulfinates (THS) in TGE and possibility of relevance of saponins in motility.

KEYWORDS: Garlic extracts; *Pseudomonas aeruginosa*; quorum sensing inhibitory potential; thiosulfinates; phytochemicals.

KUSUM HARJAI
Department of Microbiology, Panjab University, Chandigarh, India-160014

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INTRODUCTION

Pseudomonas aeruginosa is the third most common nosocomial pathogen. Formation of biofilms which are resistant to antimicrobials and host innate immune mechanisms makes Pseudomonas formidable in such infections. Quorum sensing (QS) plays an important role in pathogenicity of this organism by monitoring cell density, expression of virulence factors and biofilm differentiation. In this context, usage of quorum sensing inhibitors found to be promising to control the pathogenesis. Furanones, patulin, penicillic acid are some of the QSIs identified from natural sources. However, toxicity of such compounds limit their clinical applications. Hence, there has been immense search on dietary phytochemicals having therapeutic potential. Variety of herbs has been used as QSIs of P. aeruginosa which includes clove oil, honey, extracts of various South Florida plants, ellagitannin natural products, p-coumaric acid from plants, extracts of Combretum albiflorum bark, flavan-3-ol catechin etc. Allium sativum L. (Garlic), a dietary medicinal herb has also been exploited for its QSI potential. Its constituents include oil soluble and water soluble organosulphur (thiosulphinates) compounds, vitamins, minerals and proteins. Different extracts of garlic are known to exhibit hypolipidemic, antiplatelet, procirculatory, hepatoprotective, immune-enhancing, anticancerous and chemopreventive effects. This indicates the biological response of these extracts varies with the nature of extract. Much of the studies on QSI potential of garlic are focused on pulmonary infections. However, virulence of P. aeruginosa may vary depending on the site and type of infection. The main focus of the present study was to compare the effect of sub-MIC of various garlic extracts (aqueous as well as organic) on the quorum sensing signal molecule (QSSMs) production, biofilm formation and three forms of motilities exhibited by five clinical isolates of P. aeruginosa isolated from catheter associated urinary tract infections and standard strain. In addition, the QSI potential of garlic was correlated with major and minor phytochemicals present in these extracts.

MATERIALS AND METHODS

(i) Bacterial strains and chemicals
Standard strain of Pseudomonas aeruginosa PA01 and uro isolates (P1, P2, P14, P3 and P15) from patients having catheter associated urinary tract infections and attending Government medical college and hospital, sector-32, Chandigarh were identified by gram staining and biochemical tests, maintained on nutrient agar slants and stored at 4°C. Reporter strain E.coli MG410 was maintained on luria bertani agar slant containing ampicillin (100 µg/ml). Glycerol stocks of the strains were prepared and stored at -70°C. Fresh stocks were subcultured for every new experiment. All the bacterial culture media and the analytical grade solvents were procured from Himedia Laboratories Pvt. Ltd. India.

(ii) Preparation of garlic extracts
Garlic bulbs (Allium sativum L., desi variety) were procured from agricultural fields near Kharar, Panjab, India. Voucher specimens were identified by Dr. Sunita Garg, chief scientist, RHMD, CSIR-NISCAIR, Delhi, India and deposited in the herbarium of raw material herbarium and museum (RHMD), Delhi, India with Ref No. NISCAIR/RHMD/Consult/2014/2430/9. Aqueous extracts such as raw garlic extract (RGE) and heated garlic extract (HGE) were prepared by soaking peeled and preweighed garlic bulbs which were homogenized aseptically in sterile chilled and boiled water respectively for 1 h. It was then filtered and centrifuged. Filtrate was stored at -20°C. Concentration of the extract was determined by dividing the weight of garlic cloves (g) with a volume of extract (ml). Organic extracts were prepared by overnight soaking of homogenized garlic bulbs in respective organic solvents [ethanol (EGE), methanol (MGE), ethyl acetate (EaGE), acetone (AGE), chloroform (CGE) and...
toluene (TGE)] under shaking conditions followed by filtration and concentration. All the extracts were stored at -20°C.

(iii) Selection of garlic extracts
Garlic extracts (raw, heated, ethanol, methanol, ethyl acetate, acetone, chloroform and toluene) of equal concentrations were spotted on Whatmann No.1 filter paper discs. The discs were dried and placed on luria agar plates spread with X-gal, concentrated AHL and overnight culture of reporter strain *E. coli* MG4. The plates were incubated at 37°C for 24 h. The diameter of colorless zone was measured for checking QSI potential of the extracts. Extracts showing maximum zone diameter were selected for further experiments.

(iv) Evaluation of antibacterial potential of garlic extracts
MIC of garlic extracts (RGE, HGE and TGE) was determined against all the isolates and standard strain by tube dilution method. The minimum dilution which showed visible inhibition of growth was taken as MIC of extract. Experiment was repeated thrice to confirm the concentration. Positive and negative controls were also used to avoid errors. For determining sub-MIC, growth profiling of *P. aeruginosa* PAO1 was done by growing the organism in absence and presence of concentrations below MIC of garlic extracts (20-40 mg/ml for RGE, HGE and 2-8 mg/ml for TGE) and OD\textsubscript{600nm} was measured at 2, 4, 6, 8, 16, 24 h time intervals. The concentration which showed no significant effect (p>0.05) on growth of organism with respect to control was taken as sub-MIC.

(v) Quantitative estimation of quorum sensing molecules
Ethyl acetate extracted cell free supernatant was prepared for overnight grown culture grown in absence and presence of sub-MIC of garlic extracts (RGE, HGE and TGE). Reporter culture, *E.coli* MG4 was diluted 1:1 in Z buffer, treated with culture supernatant and assayed for β-galactosidase activity by using o-nitrophenyl-D-galactopyranoside (ONPG) as a substrate.

(vi) Effect of garlic extracts on biofilm formation
Sterile Foley catheter pieces (Rusch) of 1 cm were cut and a biofilm was allowed to develop under static conditions for 7 days in the presence and absence of sub- MIC of garlic extracts (RGE, HGE and TGE). The catheter pieces were transferred to fresh medium (with or without sub-MIC of garlic extracts) every 24 h. The catheter pieces in duplicate were removed, rinsed with PBS and cells were removed by scraping the surface under sterile conditions. Cells were sonicated using a low-level sonication cycle. Samples were then centrifuged and cells were suspended in PBS. Serial dilutions were plated on MacConkey agar plates.

(vii) In situ visualization of biofilm cells
1 cm\textsuperscript{2} coverslips were placed in flasks containing media with or without sub-MIC of garlic extracts (RGE, HGE and TGE) and biofilms were allowed to develop on coverslip surface by incubating under static conditions at 37°C for four days by changing respective media daily. On the peak day, coverslips with attached biofilms were rinsed thrice with distilled water and then stained with 0.2 % crystal violet solution. Stained coverslips were placed on slides with the biofilm pointing up and biofilms were visualized by light microscope at magnifications of 40X.

(viii) Motility assays
Swimming, swarming and twitching motility assays were performed by inoculating overnight culture of *P. aeruginosa* grown in the absence and presence of sub-MICs of garlic extracts into respective media plates using a sterile toothpick following the standard protocol.

(ix) Profiling of minor phytochemicals and quantification of THS
Qualitative screening for tannins, phlobatannins, saponins, flavanoids, terpenoids, cardiac glycosides and alkaloids in garlic extracts was carried out using standard protocols. For quantification of THS in garlic extracts, excess of cysteine was allowed to react with extracts and then it was treated with 5, 5'-dithio-bis-(2-
nitrobenzoic acid) [DTNB reagent]. The amount of 2-nitro-5-thiobenzoate (NTB) formed was calculated by measuring the absorbance of reaction mixture at 412 nm. Residual cysteine reacting with DTNB was calculated from this absorbance. Concentration of total thiosulfimates was calculated by taking half of mean decrease in cysteine concentration.

**(x) Statistical analysis**

All experiments were carried out in triplicate to validate the reproducibility of the experiments. The results were analyzed statistically using Student’s t-test with GraphPad Prism software to calculate p values. p<0.05 was taken as statistically significant.

**RESULTS**

1. **Determination of antibacterial potential of extracts**

   Different extracts of garlic bulb were prepared. Selection of garlic extracts which showed maximum QSI effect was selected (data not shown). MIC of selected extracts against all the clinical isolates and standard strain PAO1 was determined by the tube dilution method (supplementary material).

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Clinical isolates</th>
<th>MIC of Garlic Extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw Extract</td>
<td>Garlic Extract</td>
</tr>
<tr>
<td>1</td>
<td>P1</td>
<td>50 mg/ml</td>
</tr>
<tr>
<td>2</td>
<td>P2</td>
<td>50 mg/ml</td>
</tr>
<tr>
<td>3</td>
<td>P3</td>
<td>50 mg/ml</td>
</tr>
<tr>
<td>4</td>
<td>P4</td>
<td>50 mg/ml</td>
</tr>
<tr>
<td>5</td>
<td>P5</td>
<td>50 mg/ml</td>
</tr>
<tr>
<td>6</td>
<td>PAO1</td>
<td>50 mg/ml</td>
</tr>
</tbody>
</table>

Further, growth profile of standard strain PAO1 was studied in subinhibitory concentrations of RGE, HGE and TGE. It was observed that in presence of all garlic extracts, there was elongation of lag phase in growth profile of *P. aeruginosa*. In presence of raw and heated garlic extract, concentration of 40 mg/ml showed no significant decrease (p>0.05) in growth as compared to control (Fig 1A & B). The same observed with 2 mg/ml of TGE and with same concentration of toluene (Fig 1C). Hence these concentrations were selected for further studies.
2. Effect of garlic extracts on quorum sensing molecules

Effect of sub-MIC of all the three garlic extracts (RGE, HGE and TGE) on production of quorum sensing signal molecules by clinical isolates and standard strain PAO1 was studied. All the three extracts showed significant reduction (p<0.05) in production of QSSMs as compared with the control (QSSMs produced in absence of any of the extracts). Around 30-40 % reduction with RGE, 40-50 % with HGE and 70-80 % reduction with TGE in production of QSSMs was observed with respect to the control for all the isolates and confirmed the highest reducing potential of QSSMs production by TGE (Fig. 2).
3. Effect of garlic extracts on biofilm formation

Effect of sub-MIC of garlic extracts (RGE, HGE and TGE) on biofilm formation by all the isolates and standard strain PAO1 was studied for 7 days. The pattern of results was same in all the isolates. It was observed that in the presence of garlic extracts, biofilm growth declined from day 1. On the peak day (day 4), around 5 folds (p<0.01) reduction in biofilm formation with TGE, 2 fold reduction with HGE and 1-2 folds reduction with RGE (Fig. 3) with respect to control (biofilms formed in the absence of any of the extracts) was observed in standard strain PAO1. Microscopic images of 4th day (peak day) biofilm cells of PAO1 grown in the absence and presence of sub-MICs of garlic extracts on coverslips showed correlation with colony forming units (c.f.u) of biofilms. Mature and thick biofilm was observed for the control (PAO1 alone) (Fig. 4A) while in the presence of the raw garlic extract, biofilm architecture was very thin (Fig. 4B). In the presence of HGE, loose aggregates of biofilm cells were observed (Fig. 4C) while with TGE, highly dispersed cells with no sign of biofilm was observed (Fig. 4D). This indicated that TGE was having high potential to attenuate biofilm formation by P. aeruginosa.
Figure 3

*Estimation of biofilm cells of PAO1 generated in absence and presence of RGE, HGE and TGE*

Logarithmic count of biofilm cells (log c.f.u/ml) of PAO1 generated on Foley catheter in absence and presence of sub-MIC of RGE (R), HGE (H) and TGE (T) from day 1 to day 7. The results are expressed as mean ± SD obtained from three independent experiments. *p values were calculated and represented as *p<0.05 (significant) and **p<0.01 (highly significant).*

Figure 4

*In situ visualization of peak day biofilm cells of PAO1 grown in absence and presence of RGE, HGE and TGE*

Microscopic visualization of peak day biofilm cells of PAO1 which was grown in absence (A) and presence of sub-MICs of RGE (B), HGE (C) and TGE (D) under 40X magnification.
4. Effect of garlic extracts on motility of *P. aeruginosa*

Effect of sub-MIC of garlic extracts on motility of *P. aeruginosa* was assessed. It was found that swimming motility zone of PAO1 in absence of any extract was 1.7 ± 0.141 cm whereas the zone diameter of swimming got significantly reduced to 0.765 ± 0.049 cm in presence of RGE and 0.615 ± 0.021 cm in the presence of TGE. In case of swarming, PAO1 alone shown a diameter of 1.335 ± 0.049 cm and in presence of RGE and TGE, the diameter significantly reduced to 0.86 ± 0.056 cm and 0.615 ± 0.02 cm respectively. However, both the motilities remain unaffected in the presence of HGE. In case of twitching, significant reduction in zone diameter was observed only in presence of TGE (0.19 ± 0.014 cm) compared to control (1.23 ± 0.042 cm) (Fig. 5).

![Demonstration of motilities of P. aeruginosa grown in absence and presence of RGE, HGE and TGE](image)

**Figure 5**

*Demonstration of motilities of P. aeruginosa grown in absence and presence of RGE, HGE and TGE*

5. Phytochemical analysis of garlic extracts

Phytochemical analysis was done for all the organic extracts (ethanol, ethyl acetate, methanol, acetone, chloroform and toluene) and aqueous extracts (raw and heated) of garlic bulb. Tannins and phlobatannins was absent in all the extracts. Other than saponins, RGE and HGE (aqueous extracts) showed similar profile of phytochemicals. It was observed that saponins were absent in HGE. In TGE, besides saponins, terpenoids and cardiac glycosides were present. Further, thiosulfinates (the major PCs in garlic bulbs) in garlic extracts were quantified. THS content was found to be maximum in TGE followed by EaGE, HGE, MGE, CGE, EGE and minimum in AGE (Table 2). Among aqueous extracts, HGE showed higher amounts of THS as compared to RGE.
**Table 2**

**Phytochemical screening of aqueous and organic garlic extracts**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>EXTRACTS</th>
<th>Tannins</th>
<th>Flavonoids</th>
<th>Terpenoids</th>
<th>Cardiac glycosides</th>
<th>Alkaloids</th>
<th>Thiosulfonates (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>RGE</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>3.48</td>
</tr>
<tr>
<td>2.</td>
<td>HGE</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>3.72</td>
</tr>
<tr>
<td>3.</td>
<td>TGE</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>4.005</td>
</tr>
<tr>
<td>4.</td>
<td>EGE</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>3.13</td>
</tr>
<tr>
<td>5.</td>
<td>MGE</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>3.7</td>
</tr>
<tr>
<td>6.</td>
<td>AGE</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>2.64</td>
</tr>
<tr>
<td>7.</td>
<td>EaGE</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>3.8</td>
</tr>
<tr>
<td>8.</td>
<td>CGE</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Biochemical testing for minor phytochemicals and quantification of thiosulfonates in aqueous (RGE-Raw Garlic Extract, HGE-Heated Garlic Extract) and organic garlic extracts (TGE- Toluene garlic extract, EGE – Ethanolic extract of garlic, MGE- Methanolic extract of garlic, AGE – Acetone extract of garlic, EaGE – Ethyl acetate extract of garlic, CGE – Chloroform extract of garlic) was done. + indicates presence of phytochemical and – indicates absent of phytochemical in the corresponding extract

**DISCUSSION**

*Pseudomonas aeruginosa* emerged to be a notorious pathogen in nosocomial UTIs. Treatment approaches such as antibiotic therapy became less acceptable due to the emergence of multidrug resistant organisms, limited life span of antimicrobials and its side effects. Another approach is the usage of antimicrobial catheters which was found to have limited application in long term usage. In this scenario, application of quorum sensing inhibitors was found to be the most promising approach as quorum sensing regulates virulence and biofilm differentiation of this opportunistic pathogen. There have been a number of non-natural QS antagonists reported over the last decade. However, as most of them are AHL analogues, the conformational instability, high specificity of LasR, degradation by mammalian lactonases narrowed their usage. Hence, there has been immense interest of the therapeutical potential of dietary phytochemicals as they are safe for consumption and have antioxidant property. In our study, we have tried to exploit the QSI potential of garlic (*Allium sativum* L.) bulb extracts against *P. aeruginosa*. As the effect of plant extracts depend on solvent used for extraction and the extraction method, we have prepared various organic (ethanol, ethyl acetate, methanol, acetone, chloroform and toluene) and aqueous (RGE and HGE) extracts of garlic bulb. Among all the extracts, TGE was found to have highest QSI potential against *P. aeruginosa* (data not shown). Earlier, we had reported quantitative attenuation in QSSMs of *P. aeruginosa* by sub-MIC of raw garlic extract. In continuation, effect of sub-MIC of TGE, RGE and HGE was assessed in attenuation of QSSMs of *P. aeruginosa*. TGE was found to be superior among the aqueous extracts since reduction of QSSMs by TGE was found to be more by two folds. Biofilms of *P. aeruginosa* are the major threat in catheterized UTI patients. The characteristic outer exopolysaccharide matrix, high cell densities, coordinated cellular behavioural patterns, slow and variable growth rate accounts for biofilms’ survival advantages. There are reports on antibiofilm potential of...
natural products such as methyl extract of *Cuminum cyminum* and its secondary metabolite eugenol, salicylic acid, *Lagerstroemia speciosa* fruit extract active components from TCM plants etc. It has been shown that garlic extract and its active component (ajoene) increase susceptibility of biofilms formed by pulmonary isolates of *P. aeruginosa* to tobramycin. In our study, we have compared the effect of sub-MIC of garlic extracts (RGE, HGE and TGE) on biofilm formation with that of control. Significant reduction in number of viable biofilm cells and highly dispersed cells in microscopic images of biofilms grown on coverslips suggested TGE to be the most promising antibiofilm extract in comparison with other aqueous extracts. Clinical isolates of *P. aeruginosa* showed biofilm peak on different days (2nd-5th day), other than this, the pattern of biofilm formation in presence of garlic extracts were similar to standard strain PAO1 (data not shown).

Motility exhibited by *P. aeruginosa* is an inevitable factor associated with biofilm formation. *Pseudomonas aeruginosa* is capable of three forms of motility. The flagellum-mediated swimming motility, the surface-associated swarming, mediated by flagella and type IV pili and twitching motility, mediated by type-IV pili. Branched chain fatty acids, antibiotic azithromycin, cranberry proanthocyanidins are reported inhibitors of motility by *P. aeruginosa*. In the present study, we compared antimotility effects of sub-MIC of TGE and aqueous extracts with that of control. TGE reduced all the three forms of motility significantly exhibited by *P. aeruginosa* as compared with aqueous extracts. This further confirmed and strongly indicated TGE to be the most potent QSI extract of garlic. Presence of phytochemicals plays a major role in the therapeutic efficacy of herbal preparations. Hence, phytochemical profiling of organic and aqueous garlic bulb extracts was done to correlate the QSI effects with that of phytochemicals present in it. We observed absence of tannins and phlobatannins in all the extracts which correlated with earlier findings of phytochemical profiling. Other than saponins, RGE and HGE (aqueous extracts) showed similar profile of phytochemicals. It was observed that saponins were absent in HGE. Ineffectiveness of HGE in reducing motility might be related to the absence of saponins which point towards the relevance of saponins in motility. No reports on THS profile of garlic extracts are available in literature so far. Therefore, we have tried to quantify THS content in all the garlic preparations. Among aqueous extracts, HGE showed higher amount of THS as compared to RGE and it might be due to instability of sulfur compounds in water with room temperature. However, aqueous extracts contained lesser THS content compared to organic extracts which might be the reason for their reduced QSI potential. On the contrary, high amount of THS and limited minor PCs was observed in organic extracts and amongst all the extracts, TGE was found to have highest THS content. The property of toluene to dissolve sulfur might be the reason for its high THS content.

**CONCLUSION**

From our study, we concluded that as compared to aqueous extracts (RGE and HGE), toluene extract of garlic (TGE) exhibits highest QSI potential in terms of reduction in QSSMs production, biofilm formation and three forms of motility exhibited by *P. aeruginosa* associated with nosocomial UTIs. High amount of THS in TGE might be the reason for this. Although one of the QSI compound from TGE had been reported, we suggest that more than one component among THS may be associated with QSI potential. In addition, saponins in garlic extracts might have a role in motility. However, studies on identification and characterization of compound (s) in THS with QSI potential is required to validate the present results.
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CONFLICT OF INTEREST
Conflict of interest declared none.

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