Hydnophytum formicarum Jack ethanol extract modulates quorum sensing-controlled pathogenicity in *Pseudomonas aeruginosa*

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Abstract: The discovery of new mechanism to control microbial pathogenicity by quorum sensing modulation has generated the search for quorum sensing inhibitor from natural resources. The objective of this research was to evaluate the ability of *Hydnophytum formicarum* Jack (Rubiaceae) ethanol extract to antagonize cell-to cell communication. Pulverized *H. formicarum* tuber was macerated in ethyl alcohol 96% and evaporated to yield ethanol extract. A dillution technique using Luria-Bertani (LB) medium was used to observe the capability of the extract to reduce the violacein production in *Chromobacterium violaceum*. Samples in two-fold dilution were prepared to obtain 2 - 0.0625 mg/mL concentration. The effects on swimming, swarming and twitching motility as well as the formation of biofilm towards *Pseudomonas aeruginosa* PAO1 were recorded over control. All experiments were done in triplicate. The architecture of *Ps. aeruginosa* biofilm treated with samples was examined by CLSM (Confocal Laser Scanning Microscopy). Our results suggested that the ethanol extract of *H. formicarum* caused violacein production inhibition. Furthermore, inhibition of *Ps. aeruginosa* motility and biofilm formation were recorded to be significant over control in a concentration dependant manner. *H. formicarum* serves as a potential source for new QS-based antibacterial drugs towards *Ps. aeruginosa*.

Keywords: *Hydnophytum formicarum* Jack, quorum sensing inhibitor, anti-biofilm, antimotility, *Pseudomonas aeruginosa.*

INTRODUCTION

Bacterial communication is mediated by a diffusible, small chemical signal molecule called auto-inducers. Oligopeptides and N-acyl homoserine lactone (AHL) are usually found as auto-inducers in Gram positive and Gram-negative bacteria, respectively. These auto inducers diffuse out from bacterial cell and its concentration increases along with the bacterial population until it reaches a quorum. Once a quorum level is reached, the auto-inducers then diffuse back into the bacteria and modulate genes-related quorum sensing transcription that contribute to bacterial pathogenicity (Waters and Bassler, 2005; Choo *et al.,* 2006). Cellular processes regulated by quorum sensing system include toxin production and formation of biofilm (McClean *et al*., 1997), bioluminescence (Nealson and Hastings, 2006) and swarming motility (Eberl *et al*., 1999).

Compounds causing a quorum sensing inhibition rather than direct killing of bacteria is expected to serve as anti-pathogenics which may emerge less resistance (Hentzer and Givskov, 2003). Therefore, compounds with anti-quorum sensing activity might be of interest to counter the microbial infections (Fuqua *et al*., 2001; Rice *et al*., 2005).

Several virulence functions in bacteria, including motility and biofilm formation, are under the regulation of quorum sensing related gene expression. Bacterial motility itself plays different role in biofilm formation. They can promote adhesion to the surface for the biofilm formation, for biofilm maturation and/or in dispersal processes (Marchal *et al*., 2010). However, motility is not critical for biofilm formation as could be observed in *Ps. aeruginosa* PAO1 mutant strains, which lacks flagella and type IV pili (Chow *et al*., 2011).

Six different forms of bacterial movements have been described including swimming, swarming, twitching, gliding, sliding and darting (Henrichsen, 1972). These varied types of bacterial motility on a surface do facilitate bacteria to increase the nutrient uptake, to deter toxic substances, infect preferred host during transmission and optimizing the colonization sites (Rashid and Kornberg, 2000). In the presence of a quorum sensing inhibitor compound, the motility may be limited and the bacterial pathogenicity may also be affected.

Anti-quorum sensing molecules have been found in garlic extract and compounds isolated from Penicillium species. From marine environment, red algae *Delisea pulchra* was reported to produce halogenated furanone compounds which are known to be the prominent anti-quorum sensing substances (Manefield *et al*., 1999). Unfortunately, these furanones have limited or no therapeutic application due to their toxicity and high reactivity of the host. Researches focusing on non-toxic, broad-spectrum quorum sensing inhibitors are still far from a successful drug lead discovery (Choo *et al*., 2006).

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Plants have always been a rich source of medicines and have enormously contributed to the development of important pharmaceuticals. Nevertheless, recent anti-infective studies have mainly based on a perspective of bacteriostatic/cidal mode of action. Changing the focus of study from finding antibacterial to anti-quorum sensing could reveal new anti infective substances (Adonizio *et al*., 2006). Therefore, further research in determining the anti-quorum sensing inhibitors from natural resources is worth exploring.

H. formicarum (Rubiaceae) is a medicinal plant which is widely used in West Papua as a herbal tea for a broad range of therapeutic values. This plant lives as an ephyphyte on big trees and develops mutualistic symbiosis with ants. Therefore, in Indonesia, this plant is named "Sarang Semut" or ant nest. Reports on the pharmacological activity of this plant are limited to closely related plants, i.e *Myrmecodia pendens* and *M. tuberosa*. Those plants are locally known as "Sarang semut" as well. *M. pendens* was reported to possess antiproliferative agents against HeLa cells as well as mice Balb/c lymphocyte cells, macrophage phagocytosis enhancer and antioxidant (Simanjuntak *et al*., 2010; Soeksmanto *et al.*, 2010; Bustanussalam 2010; Hertiani *et al.,* 2010). Prachayasittikul and collaborators (2008) have reported the activity of *H. formicarum* as antimicrobial and antioxidant. In this study, *H. formicarum* ethanol extract potency for anti-quorum sensing activity using quorum sensing biosensor *Chromobacterium violaceum* 31532 wild type (WT) strain, *C. violaceum* CV026 mutant strain, and *Pseudomonas aeruginosa* PAO1 strain was investigated. The correlation of the quorum sensing inhibition in reducing the quorum sensing related motility of *Ps. aeruginosa* PAO1, i.e. swimming, swarming and twitching was evaluated.

MATERIALS AND METHODS

Bacterial strains and culture conditions

C.violaceum wild type (WT), *C. violaceum* CV026 and *P. aeruginosa* PAO1strains were strains used in this study. The C6-homoserine lactone (HHL) (Sigma Aldrich, Germany) was dissolved in dimethylsulfoxide (DMSO) and used as autoinducer for violacein production in CV026. Luria- Bertani (LB) Agar was used for culture stock. After incubated for 24 h at 30°C, the colonies were transferred onto LB broth and further incubated for another 24 h at 30°C with continuous shaking. Cells were suspended to achieve 10^8CFU/mL as observed as optical density of 0.1 at 600 nm (equivalent to 0.5 McFarland scale).

Plant material

Plant material of *H. formicarum* Jack (Rubiaceae) was obtained from Babo, Bintuni Bay Region, West Papua, Indonesia in March 2011. Plant taxonomy was studied at the Plant Taxonomy Laboratory, Faculty of Biology, Universitas Gadjah Mada (UGM), Yogyakarta. The plant specimen was kept in the Green House, Faculty of Pharmacy, UGM.

Hypocotyls (the tuberous stem of the plants) were cut and washed, and then were dried in oven (50°C). Dried samples were sorted out and pulverized. Dried powders were extracted by maceration method with 95% ethanol. Maceration was performed up to 5 times with stirring. The residue was then discarded. All macerates were mixed, filtered and the residue was discarded. The filtrate was concentrated by rotary evaporator to obtain ethanol extract.

Anti-quorum sensing assay using diffusion method

A standard diffusion method was used to observe anti-quorum sensing activity. *C. violaceum* wild type broth culture (100µl) was adjusted at OD_{600} for 0.1 (approx. 1 x 10^8 CFU/mL) and was spread on plates containing LB Agar. A 6 mm in diameter cork-borer was used to dig wells on the agar plates where the plant extract (1mg/mL) in DMSO was loaded. Antibiotic streptomycin (100µg/mL) was used as growth inhibition control. Quorum sensing inhibition was measured according to Zahin *et al*., (2010). Radius (r1) in mm represented bacterial growth inhibition, while r2 (mm) represented growth and pigment inhibition together. The quorum sensing inhibition thus was determined by substracting r1 from the total radius (r2) in mm.

Determination of growth inhibitory concentration (MIC)

Ps. aeruginosa PAO1 and *C. violaceum* CV026 were grown on LB agar plates at 28°C for 24h. A single colony was inoculated into LB broth. After incubation for overnight the OD_{600} was set to 0.01 (10^8CFU/mL). Cells were incubated for two hours and finally the OD_{600} suspension was diluted to 0.005 (equal to 5 x 10^5 CFU/mL). Inhibition concentrations of extracts were determined in sterile flat-bottom 96-well polystyrene plates containing Mueller-Hinton broth media (Difco) based on guidelines of the Clinical and Laboratory Standards Institute [(CSLI) (NCCLS, 2003)]. The sample concentration ranged from 0.0625 to 1 mg/mL. Controls description were: medium-control (not incubated: 0% growth), control of untreated bacteria (100% growth or growth control), vehicle control, and antibiotic-control. All tests were repeated twice (n=2). Culture plates were incubated overnight. Optical density readings were obtained by using plate read outs at 595 nm. Growth reduction was calculated as % of inhibition by using the formula (1), of which the MIC_{50} value was calculated. The extract concentration at which the bacteria growth was inhibited by at least 50% was determined as the MIC_{50}.

$$\% \text{ inhibition} = \left(1 - \left(\frac{OD_{s24} - OD_{s0}}{(OD_{gc24} - OD_{gc0})}\right)\right) \times 100\% \quad ...(1)$$

OD_{s24}=optical density at 595 nm of the sample at 24 h post-inoculation; OD_{s0}: optical density at 595nm of the sample measured directly after inoculation; OD_{gc24}: optical density at 595 nm of the growth control at 18h post-inoculation; OD_{gc0}: optical density at 595nm of the growth control measured directly after inoculation (Quave *et al*., 2008).

Quantification of violacein production

To measure the production of violacein pigment of *C. violaceum* CV026 in the present or absent of plant extract tested, test tubes containing LB broth and HHL; LB broth, HHL and DMSO; LB broth, HHL and different dilutions of plant extract were applied (table 1). 100µl volume of bacterial suspension equal to McFarland 0.5 standard was placed in each tube that were then incubated for 24 h at 30° C. Following the incubation, centrifugation at 18894.2 g was done for 10 minutes to separate the insoluble violacein. After supernatant was being discarded, 1mL of DMSO was added and the solution was vortexed for 30 seconds to dissolve the violacein completely, and centrifuged at 18894.2 g for 10 min in order to remove the remaining cells. As much as 200µl of the violacein (containing supernatants) was added into 96-well flat bottom microplates, and the absorbance was measured at 595 nm (Choo *et al*., 2006)

Determination of the biofilm formation inhibition of Ps. aeruginosa

To test for biofilm formation inhibition, PVC (polyvinyl chloride) flexible U bottom 96 wells plates (Falcon 3911, Becton Dickinson, Franklin Lakes, NY) were used. The extracts with concentrations ranging from 2 - 0.5mg/mL were used. Negative controls contained cells + media (M63 supplemented with 20% casamino acid, 20% glucose and 1mM $MgSO_4$ for *Ps. aeruginosa* PAO1); antibiotic controls were consisted of cells + media + antibiotic streptomycin 1000µg/mL; vehicle controls comprised of cells + media +DMSO. Media controls were also included in the experiment. Antibiotic as the positive control was prepared at 100µg/mL via serial dilution. Blanks were prepared by performing the same treatment as for the samples, without incubation. All tests were performed three times. After being incubated for 24 h at 28° C, the supernatants were aspired. Following three times rinsing with distilled water, fixation was done for 10 min. Furthermore, 125µL of 1% crystal violet stain was added to the wells and left to stain for 15 min. Afterwards, the plates were rinsed with tap water to discard the excess stain. Ethyl alcohol (200 µL) was added to the wells and transferred to 96-well microtiter plates (flat bottom). Optical density (OD) was observed at 595nm by plate reader. The average values were substracted with the optical density of the media control (media + extract). After dividing by the optical density of the vehicle control, the result was multiplied by 100 (Quave *et al*., 2008).

Ps. aeruginosa PAO1 biofilm morphology

Biofilm was grown in a tube (50mL volume) which contained 76 x 26 mm glass slides. Overnight culture of *Ps. aeruginosa* PAO1 cells (20mL) which was diluted 1,000 times in M63 medium were inoculated into the tube and incubated at 30°C for biofilm formation on the slides. Biofilms were grown in the presence or absence of the extract. After 24h, the slides were gently rinsed by using sterile PBS. Biofilm staining was performed for 30 min by using 25 µl of the LIVE/DEAD BacLight (Molecular Probes Europe BV, Leiden, The Netherlands) containing 3.34mM of SYTO9 dye and 20mM of propidium iodide. Biofilm formation was then visualized by a Confocal Lasser Scanning Microscope (CLSM) (Carl Zeiss, Jena, Germany) with 63×/1.3 water objective. The 3-D images were generated by a software packed with Zeiss LSM 510. Five images per slide were obtained randomly on the glass surface.

Ps. aeruginosa PAO1 motility test

The extract was further tested to explore the effects on quorum sensing related motility of *Ps. aeruginosa* PAO1, i.e. swarming, swimming and twitching. Motility assays were performed according to Rashid and Kornberg (2000). Briefly, 0.3% LB agar plates (for swimming motility), 0.5% LB agar plates (for swarming motility), and 1% LB agar plates (for twitching motility) contained sub-inhibitory concentration of plant ethanol extract that was prepared and allowed to dry for 3-4 h at 30° C. Plates were point inoculated with freshly grown culture of bacteria using blunt ended sterile toothpick. For twitching motility, bacterial inoculation was performed by stabbing a toothpick at its sharp end to reach the bottom of the Petri dish. After 24 hours of incubation at 30°C, the bacterial motility was determined by measuring bacterial colony formed in square millimeter.

RESULTS

The inhibition in quorum sensing activity of *H. formicarum* ethanol extract is shown in fig. 1-2. The violacein production inhibition was exhibited by a disappearance of violet color growth of *C. violaceum*. A zone of growth inhibition (antibacterial activity) was observed in Streptomycin (1000 µg/mL), whereas no

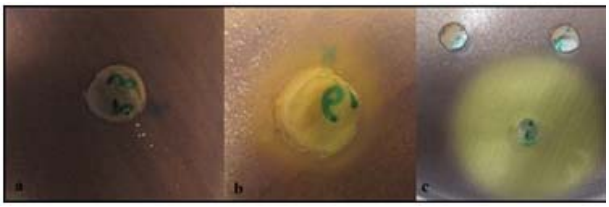

Fig. 1: Anti-quorum sensing activity vs antibacterial activity. (**a**) no anti-quorum sensing nor antibacterial activity of DMSO, (**b**) anti-quorum sensing activity of *H. formicarum* ethanol extract, (**c**) antibacterial activity of Streptomycin.

inhibition was observed with DMSO. The presence of violacein inhibition from *H. formicarum* ethanol extract tested shown by turbid halo of colorless cells of *C. violaceum* was a strong evidence of quorum sensing inhibition. Zone of inhibition observed was 13.17±1.26 mm. (fig 1).

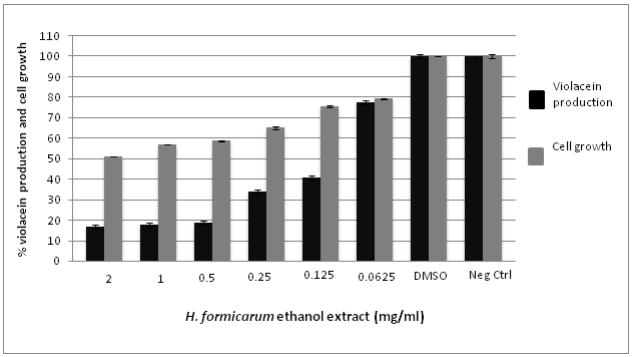

Fig. 2: Quantitative assessment of violacein inhibition and influence on the growth of CV026 by ethanol extract of *H. formicarum*. Data are represented as percentage of violacein inhibition and growth inhibition. Cell density was quantified by measuring at A_{495nm}. Mean values of triplicate independent experiments and SDs are shown.

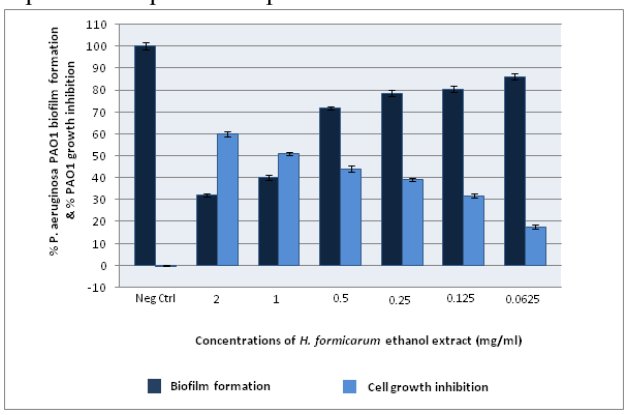

Fig. 3: Effect of *H. formicarum* ethanol extract on biofilm formation and cell growth of *P. aeruginosa* PAO1. Biofilm formation was quantified by crystal violet staining followed by measurement at A_{495nm}. Data are represented as the percentage of biofilm formation/cell growth in the presence of the extract tested. Mean values of triplicate independent experiments and SD are shown.

Table 1: Composition of the tubes for determining cell growth inhibition and anti-quorum sensing activity

	A	B	C	Final Concentration
LB	945µl	895µl	895µl	
CV026	50µl	50µl	50µl	$1x10^8$CFU/mL
HHL	5µl	5µl	5µl	15µmol/mL
Ethanol extract	-	-	50µl	½MIC
DMSO	-	50µl	-	≤1% v/v
Total Volume	1000µl	1000µl	1000µl	

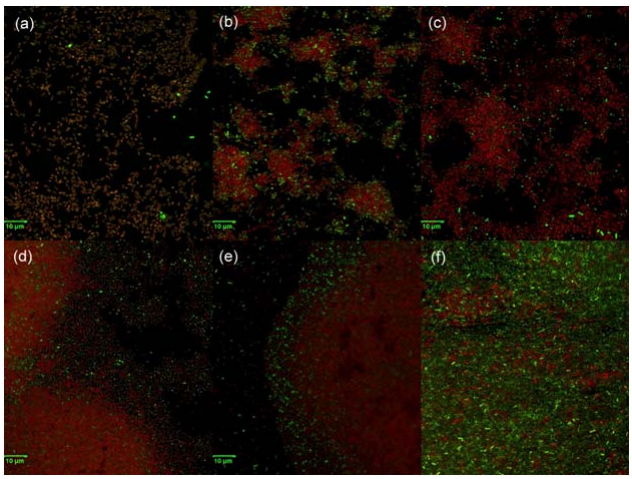

Fig. 4: Three-dimensonal images of PAO1 biofilms grown in the presence or absence of *H. formicarum* extract. (a-e) concentration of 1 - 0.0625 mg/mL of *H. formicarum* ethanol extract; (**f**) PAO1 biofilm Negative control. Bacterial viability was assessed with the Backlight viability stain: green (live)/red (dead), taken by employing a Leica *63× water immersion* lens (use Zeiss 518F immersion oil).

Ps. aeruginosa PAO1 biofilm formed on slides was monitored by CLSM (fig. 4) following the determination of biofilm and plaktonic growth inhibitions (fig. 3). Staining did allow to distinguish live cells from the dead ones. The viable bacteria stained as fluorescent green while the non viable stained fluorescent red. The background remained non-fluorescent.

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HHL	5µl	5µl	5µl	15µmol/mL
Ethanol extract	-	-	50µl	½MIC
DMSO	-	50µl	-	≤1% v/v
Total Volume	1000µl	1000µl	1000µl	

DISCUSSION

H. formicarum extract exhibited concentration dependent inhibition of *C. violaceum* (CV026) violacein production. IC_{50} value was observed at 1.68%. A maximum of 83% violacein production inhibition was observed at 2 mg/mL (fig. 1). According to Adonizio *et al*. (2006), a decrease in cell count to a number below the quorum can cause inhibition in quorum, not necessarily related to a specific inhibition on the quorum sensing itself. Therefore, in

order to determine whether the disruption in cell to cell communication (caused by the plant extract) did occur, further assays in quorum sensing inhibition manifestation were conducted. The results of a micro broth dilution assay of the extract against *C. violaceum* CV026 showed no growth inhibition at all the tested concentrations (Fig. 2). This finding indicates that the anti-quorum sensing of *H. formicarum* extract was not derived from its growth inhibition property.

The anti-quorum sensing activity was further tested on biofilm formation as well as on swimming, swarming and twitching motility of *Ps. aeruginosa* PAO1. A decrease in this particular bacterial biofilm formation was observed in the presence of *H. formicarum* ethanol extract. At 2 and 1 mg/mL concentrations, the extract showed a maximum of 70 and 60% biofilm reduction, respectively (fig. 3). However at lower concentration, the ethanol extract failed to inhibit *Ps. aeruginosa* PAO1 biofilm formation as shown by a significant increase in the biofilm density. Moreover, the extract showed growth inhibition activity as could be seen by the IC_{50} value at 0.96%. It was only slightly higher as compared to IC_{50} observed for biofilm formation inhibition (0.85%).

Results from CLSM observation of formed biofilm revealed that concentration of 1 - 0.5 mg/mL caused a significant reduction of *Ps. aeruginosa* PAO1 biofilm formation (fig.4), whereas untreated biofilms exhibited normal thickness and fully developed (fig. 4f). Accordingly, the viability of the cells was decreased, while the slime density increased significantly. At lower concentrations (0.25 - 0.0625mg/mL) the extract failed to inhibit the formation of biofilm as shown by a thick cell growth of biofilm.

Table 2 and fig. 5 described the ethanol extract of *H. formicarum* efficacy in decreasing the swarming motility of *Ps. aeruginosa* at concentration of 2 and 1mg/mL by 62% and 59% reduction, respectively ($P \leq 0.05$). Twitching motility of *Ps. aeuginosa* PAO1 was also significantly reduced by the *H. formicarum* extract at concentrations of 2 and 1mg/mL by 60% and 55% respectively. However, the same concentrations of the extract (2 - 0.0625 mg/mL) have less effective in decreasing swimming motility of the bacteria tested. This results are in accordance with the previously mentioned result showing that *H. formicarum* ethanol extract have limited inhibitory activity on the biofilm formation of *Ps.aeruginosa* PAO1 at the concentrations <1mg/mL (fig. 3).

It is interesting to note that *H. formicarum* extract at the same concentration exerts higher inhibition on the swarming motility than the swimming motility of *Ps. aeruginosa.* Active compounds contained in *H. formicarum* extract having capabilities in reducing the amount of the quorum sensing signal in *Ps. aeruginosa* PAO1 may explain the modulation of bacterial behaviors. This condition was supported by the extract capability on reducing the violacein production by*C. violaceum* 026 impregnated with HHL (fig. 2). Even though the inhibition in quorum sensing signal could block cell-to-cell communication, the extract failed to interfere with surface colonization as could be seen by a lesser effect on the swimming motility modulation.

Swimming and swarming are types of motility which depend on flagella (Harshey, 2003). Swimming was observed on the medium having low agar concentration. It is noteworthy, that swimming is not a social event as occurs in swarming motility; swimming represents individual cell movement. The cells move separately in an unorganized manner and pattern, and do not involve differentiation into polar hyper flagellated cells (Harshey, 2003; Rashid and Kornberg, 2000). Swarming motility depends on cell density; therefore, it plays as one of models used in a study of social behavior in bacteria. This motility is formed on more solidified media than required for swimming. With the relatively thin fluid layer on the surface, the cells are formed in hyper flagellated with coordinated movement. In favor of surface friction reduction and smooth migration of cells, an extra cellular components known as wetting agents are also needed by the cells on viscous surfaces (Inoue *et al*., 2008). On the other hand, twitching motility is generated by type IV pili as being extended and retracted from the cell poles. This behavior is important for the host infection process of *Ps. aeruginosa*. The morphological pattern of twitching is less organized than that of swarming (Rashid and Kornberg, 2000).

The blocking in the bacterial cell-to-cell communication may reduce the production of several virulence factors and cytotoxic compounds such as elastase, rhamnolipids, and pycocyanine. Further, this condition will strongly reduce the swarming motility, which requires not only flagella but also rhamnolipids (RLs) and 3- (3-hydoxylalkanoyloxy) alkanoic acids (HAAs) (Reimmann *et al*., 2002; Tremblay and Déziel, 2010). In order to have a better understanding on the compounds responsible for the antibacterial and anti-quorum sensing effects of this particular extract, further studies focusing on the anti-quorum sensing compounds isolation as well as the structure identification.

CONCLUSION

H. formicarum ethanol extract exhibited quorum sensing inhibition as shown by violacein production, biofilm formation, swimming and twitching motility inhibition of the tested strains in a concentration dependant manner. These results suggested *H. formicarum* potency to serve as a source in the development of new QS-based antiinfective drugs from natural resources.

Table 2: Effect of *H. formicarum* ethanol extract on motilities of *P. aeruginosa* PAO1. Values are means of ±SD of 3 experiments

Extract concentration (mg/mL)	Rate of motility diamater (mm)			% Reduction		
	Swimming	Swarming	Twitching	Swimming	Swarming	Twitching
2	35.33±0.57	7.00±0.00	7.77±0.57	7.03	62.51	60.34
1	36.00±0.00	7.66±0.57	8.66±0.57	5.26	58.97	55.17
0.5	37.33±0.57	10.00±1.00	11.33±0.57	1.76	46.44	41.38
0.25	41.33±1.15	13.00±1.00	14.33±0.57	-8.76	30.37	25.86
0.125	45.00±0.00	15.00±1.00	14.83±2.56	-18.42	19.66	23.28
0.0625	46.00±0.86	17.67±0.57	17.00±2.00	-21.05	5.36	12.069
Negative Control	38.00±0.00	18.67±0.57	19.33±1.54	0.00	0.00	3.45

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