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Research Article
Investigation on Inhibitory Potential of *Myrmecodia tuberosa* on Quorum Sensing-related Pathogenicity in *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* Cowan I Strains

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Abstract

Background and Objectives: Tuber of *Myrmecodia tuberosa* Jack (Rubiaceae) has been widely used as herbal remedy in Indonesia. This research aimed to evaluate the effects of *M. tuberosa* Jack on quorum-sensing related pathogenicity of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. **Material and Methods:** Following delipidation with petroleum ether, pulverized tuber was macerated in methanol. After evaporation to obtain methanol extract, liquid-liquid partition was perform on the extract to yield hexane, ethyl acetate and water fractions. The extract and fractions were screened for the effects on the planktonic growth of *P. aeruginosa* and *S. aureus*. Inhibition of pigment production was observed on cetrimide Agar following sample treatment on *Pseudomonas aeruginosa*. Samples were prepared as 2-0.0625 mg mL^{-1} concentration. The effects on swimming, swarming and twitching motility of *Pseudomonas aeruginosa* PAO1 following sample application were observed. All experiments were done in triplicate. **Results:** Results showed that the ethyl acetate fraction caused a prominent effect on quorum sensing inhibition which might explain its biofilm inhibition effect on *P. aeruginosa*. Significant inhibitory effect in a concentration dependent manner towards pigment production inhibitor and motilities were observed over control. **Conclusion:** Despite being active as planktonic growth inhibitor towards *S. aureus* and *P. aeruginosa*, *M. tuberosa* ethyl acetate fraction is recommended to be investigated further as anti-infective against *P. aeruginosa*.

Key words: *Myrmecodia tuberosa* Jack, quorum sensing inhibitor, anti-motility, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Plants have been widely accepted as a rich sources of valuable medicines. Anti-infective is one of the most explored bioactivity. Recent anti-infective studies have mainly based on a perspective of planktonic growth inhibition/killing action. Progress on knowledge on cell-to cell communication between bacteria and how this affect bacterial pathogenicity has given a new wave in finding of anti-infective[1].

Compounds causing inhibition in the quorum sensing system rather than direct killing of bacteria is expected to serve as an anti-pathogenic effect with less extent of antibiotics resistance[2]. Therefore, compounds having anti-quorum sensing activity might be of interest to battle microbial infections[3,4].

Several virulence functions in bacteria, including motility and biofilm formation, are under regulation of quorum sensing related gene expression. Role of bacterial motility on biofilm can be in form of promotion of surface adhesion, biofilm maturation and/or in dispersal processes[5]. Interestingly, its role is not critical at formation stage as was reported in *P. aeruginosa* PAO1 mutant strains having no flagella and type IV pili[6]. Nevertheless, the motility capabilities of *P. aeruginosa* in aqueous and dry environments are associated with its virulence.

There are six bacterial movements described which may involve individual movement and in groups, i.e. swimming, swarming, twitching, gliding, sliding and darting[7]. These motility on a surface facilitate bacteria to increase the nutrient uptake, avoid toxic substances, infect preferred host by dispersing to the surroundings during transmission and optimizing the colonization sites[8]. A quorum sensing inhibitor compound may limit the motility and further is expected to modulate the bacterial pathogenicity.

M. tuberosa (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 epiphyte on big trees and has mutualistic symbiosis with ants. Therefore in Indonesia, this plant is named "Sarangsemut" or Ant-Nest. Report on the pharmacological activity is limited to close related plants, *Myrmecodia pendens* and *H. formicarum*. Those plants are known by the local people as "Sarangsemut" as well. *M. pendens* was reported to possess anti-proliferative agents against HeLa cell as well as mice Balb/c lymphocyte cells, macrophage phagocytosis enhancer and antioxidant[9-12]. It is interesting to explore this plant potency as anti-infective, especially towards *P. aeruginosa*. This pathogenic opportunity microbe has mainly correlated to the infection of immune-suppressed patient. At the same time, *M. tuberosa* has been reported as a potential immunomodulator[12,13].

Our previous report on the extract antibacterial activity resulted potential inhibition towards *S. aureus, E. coli* and *C. albicans*[14]. Setyani *et al.*[15] reported the active constituent responsible for antibacterial activity are phenolics. A close related plant *H. formicarum* ethanol extract's showed an anti-quorum sensing activity using quorum sensing biosensor *Chromobacterium violaceum* 31532 wild type (WT) strain, *C. violaceum* CV026 mutant strain and *Pseudomonas aeruginosa* PAO1[16]. In order to evaluate the potency of *M. tuberosa* as a source of new anti-infective, current study investigated the extract and fractions on quorum-sensing related pathogenicity of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

MATERIALS AND METHODS

The research has taken place on April-October, 2012 at the Laboratory of Phytochemistry for plant sample preparation, while the microbiological assay was performed at the Laboratory of Microbiology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. Plant samples were collected from West Papua on May, 2011. The taxonomy was kindly determined by Mr. Joko Santosa (Pharmaceutical Biology Department, Faculty of Pharmacy, UGM).

Bacterial suspension preparation: The bacterial strain used in this study was *P. aeruginosa* PAO1 strain and *Staphylococcus aureus* PCA which were cultured in Luria-Bertani (LB) Agar. After incubated for 24 h at 30°C, the colonies were transferred to LB broth and incubated further in a shaker for another 24 h. Cell density was by adjusted to the optical density of 0.01 at 600 nm to obtain 10^7 CFU mL^{-1} bacterial suspension.

Plant material: Hypocotyl (tuberous stem of the plants) were cut and washed and then were dried in oven (50°C). Dried samples were sorted and pulverized. Dried powders were extracted by maceration method. There were two method performed as follows:

- For screening purpose: Maceration was performed in70% ethanol to yield ethanol extract after solvent evaporation
- For exploring quorum sensing active ingredients, maceration was initiated with petroleum ether as a delipidation process. Maceration was performed up to 5 times with stirring. All macerates were mixed, filtered and was concentrated by rotary evaporator to obtain petroleum ether extract. The residue was further extracted with methanol and the extraction was

performed as described previously to obtain methanol extract. The extract was further fractionated by using hexane-90% methanol with liquid-liquid partition method and the result was evaporated to yield hexane and non hexane fractions. The non hexane fraction was further partitioned by using ethyl acetate-water, followed by solvent evaporation to yield ethyl acetate and water fractions

Determination of growth inhibitory concentration (MIC)[17]: After incubated for 2 h the cell suspension was diluted to 5×10^5 CFU mL^{-1}. Growth inhibitory concentrations were determined on sterile flat-bottom 96-well polystyrene plates containing Mueller-Hinton broth medium. Controls used were: medium-control (not incubated: 0% growth), infected untreated control (100% growth), vehicle control and reference controls (Streptomycin 100 mg mL^{-1}). Culture plates were incubated overnight. Optical density were read by using plate reader at 595 nm. All tests were performed in triplicate.

Growth inhibition was calculated according to the formula mentioned below[18]:

$$\text{Inhibition (\%)} = \left(1-\left(\frac{\text{ODt24}-\text{ODt0}}{\text{ODgc24}-\text{ODgc0}}\right)\right)\times100$$

Where:
ODt24 = Optical density of the test well at 24 h
ODt0 = Optical of the test well at 0 h
ODgc24 = Optical density of the growth control well at 18 h
ODgc0 = Optical density of the growth control well at 0 h[18]

Determination of biofilm formation inhibition[18]: PVC flexible U bottom 96 wells plates were used to investigate the biofilm formation inhibition following sample application. Negative controls (cells+media: M63 supplemented with 20% casamino acid, 20% glucose and 1 mM $MgSO_4$ for *P. aeruginosa* PAO1), positive control (cells+media+streptomycin), vehicle controls (cells+media+DMSO) and media controls were included. Antibiotic as the positive control was prepared at 100 µg mL^{-1}. Blanks were prepared as for samples, only without incubation.

After 24 h incubation at 28°C, the suspension were aspired from each wells. Following three times rinsing with distilled water, fixation was done for 10 min. Staining with 125 µL of 1% crystal violet was stand for 15 min. After rinsing off excess stain with water, ethanol (200 µL) was added to each wells and the solution was transferred to flat-bottom 96-well plates. Optical density (OD) were measured at 595 nm. ODs for each concentration tested were averaged and the average of the media control (media+extract) was subtracted. This value was then divided by the mean absorbance of the vehicle control and multiplied by 100 to determine the biofilm inhibition concentration[18]. All test was conducted in triplicate.

Anti-quorum sensing assay using diffusion method[19]: A standard diffusion method was used to observe anti-quorum sensing activity. *P. aeruginosa* culture (100 µL) was adjusted to 0.5 McFarland were spread on cetrimide Agar. A 6 mm cork-borer was used on the Agar plates to create wells to load the plant extract (1-6 mg mL^{-1}) in DMSO. Antibiotic streptomycin in 10 mg mL^{-1} was used as growth inhibition control. Bacterial growth inhibition was measured as radius (r1) in mm while r2 (mm) represented growth and pigment inhibition together. The pigment inhibition zone was calculated as r2-r1 referring to quorum sensing inhibition[19].

***P. aeruginosa* PAO1 motility test:** The extracts and fractions was further tested to explore their effects on *P. aeruginosa* motilities, i.e. swarming and swimming according to Rashid and Kornberg[8]. Assay for swimming motility was performed on 0.3% LB agar plates while a concentration of 0.5% LB agar plates was used for swarming motility assay. The Agar containing sub-inhibitory concentration of ethanol extract was prepared and allowed to solidify for 3-4 h at 30°C. Plates were inoculated by pointing a blunt ended sterile toothpick containing a freshly grown bacteria culture. After 24 h incubation at 30°C, the motility was observed by a measurement of the bacteria colony formed in square millimeter.

Statistical analysis: Data obtained were analyzed using Kolmogorov-Smirnov for homogeneity and distribution test. Normal distribution was analyzed further by using one-way ANOVA (significance level 95%), followed by Tuckey HSD and LSD using SPSS 19 software. p-value of 0.05 or less was considered to be statistically significant.

RESULT AND DISCUSSION

Antibacterial and antibiofilm screening of the extract and fractions towards *Staphylococcus aureus* and *Pseudomonas aeruginosa*: The ethanol extract showed planktonic growth and biofilm formation inhibition in a concentration dependent manner against *P. aeruginosa* and *S. aureus* (Fig. 1 and 2). In general, the effect against *S. aureus* was slightly higher than that against *P. aeruginosa*.

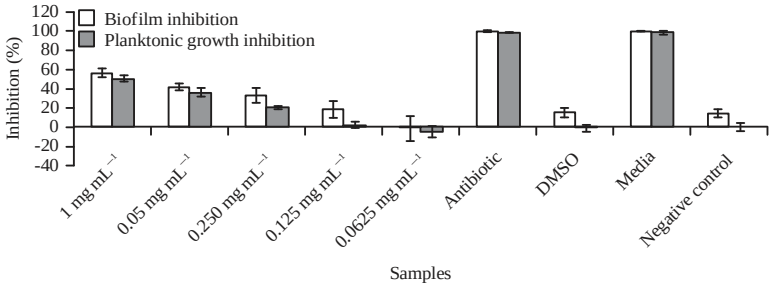

Fig. 1: Inhibitory effects of *M. tuberosa* ethanol extract against biofilm and planktonic growth of *S. aureus* PAO1 (n = 3). Antibiotic used as control was chloramphenicol 500 mg mL^{-1}

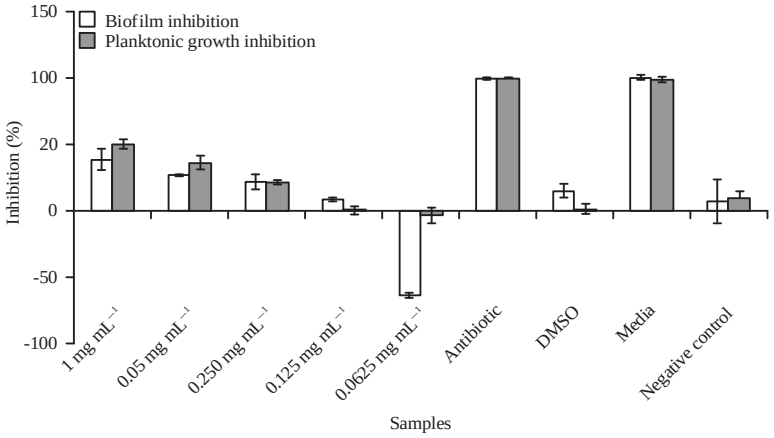

Fig. 2: Inhibitory effects of *M. tuberosa* ethanol extract against biofilm and planktonic growth of *P. aeruginosa* PAO1 (n = 3). Antibiotic used as control was chloramphenicol 500 mg mL^{-1}

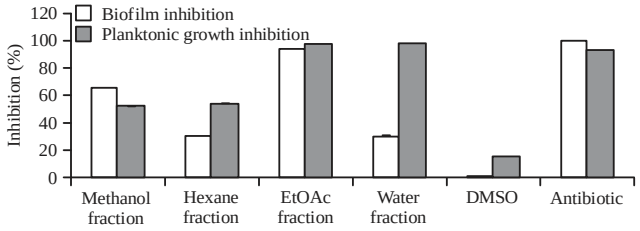

Fig. 3: Biofilm and planktonic growth inhibitions of the fractions of *M. tuberosa* against *S. aureus* PCA at 1 mg mL^{-1} concentration (n = 3)

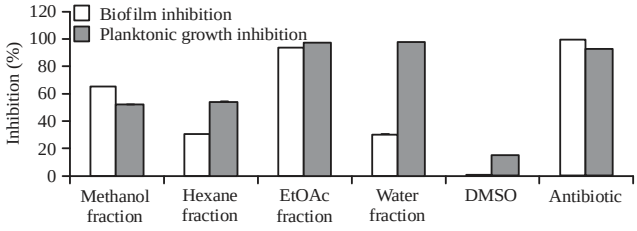

Fig. 4: Biofilm and planktonic growth inhibitions of the fractions of *M. tuberosa* against *P. aeruginosa* PAO1 at 1 mg mL^{-1} concentration (n = 3)

Both activities were not significantly different suggested that the biofilm inhibition activity might be resulted from inhibition in the planktonic growth.

Antibacterial and antibiofilm activities of the extract and fractions against *Pseudomonas aeruginosa* and *Staphylococcus aureus*: Following delipidation with petroleum ether, the residue was dissolved in methanol and fractionated. Fractionation was performed in order to explore the possibility of finding new anti-quorum sensing substance from *M. tuberosa*. After fractionation, each extract and fractions were screened for planktonic growth and biofilm formation inhibition against *P. aeruginosa* and *S. aureus*.

Results as described in Fig. 3 and 4 showed that the ethyl acetate fraction was the most active as biofilm inhibitor against *P. aeruginosa* at concentration 1 mg mL^{-1}. The effect on the planktonic growth was observed to be similar suggesting that the biofilm inhibition might be resulted from

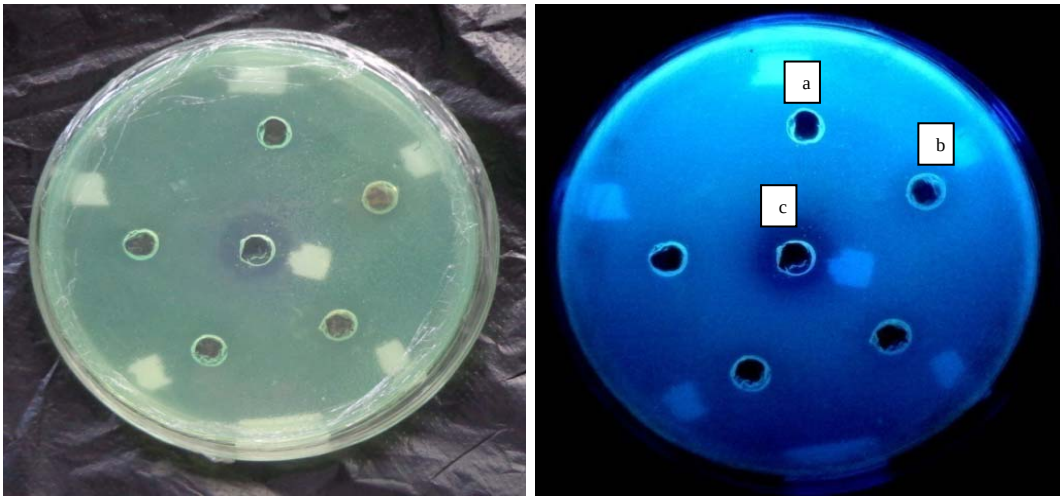

Fig. 5: Anti-quorum sensing activity vs antibacterial activity. (a) No anti-quorum sensing nor antibacterial activity of DMSO, (b) Anti-quorum sensing activity of ethyl acetate fraction and (c) Antibacterial activity of Streptomycin

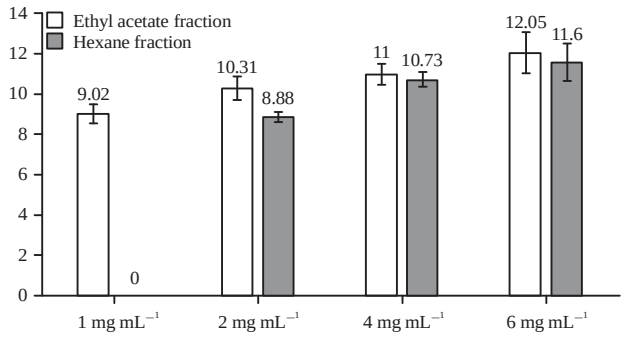

Fig. 6: Diameter zones showing *Pseudomomas aeruginosa* quorum sensing inhibition on cetrimide agar after the application of ethyl acetate and hexane fractions (n = 3)
DMSO was used as negative control

the planktonic growth inhibition. This was supported by the result of the negative inhibition effect on biofilm formation of *S. aureus*. On the other hand, the water fraction showed a promising effect as anti-quorum sensing as the antibiofilm was found much higher than toward the planktonic growth of *P. aeruginosa*.

Quorum sensing inhibition activity: The inhibition in *P. aeruginosa* quorum sensing activity was observed only after application of ethyl acetate and hexane fractions (Streptomycin showed 14.23±2.06 mm). The activities of *M. tuberosa* ethyl acetate (Fig. 5-6) and hexane fractions (Fig. 6) were both concentration dependent. Statistical analyses resulted in a significant difference between the effects of different sample concentration (p-value<0.05). Interestingly, the water fraction which is expected to content quorum sensing effect due to result on the biofilm inhibition, exhibited no quorum sensing inhibition (not displayed). Extract and petroleum ether fraction were also inactive.

The pigment production inhibition was exhibited by a disappearance of green color growth observed in ultraviolet light at 366 nm (Fig. 5). A zone of growth inhibition (antibacterial activity) was observed in Streptomycin, whereas no inhibition was apparent with DMSO. The presence of pigment inhibition of ethyl acetate and hexane fractions as shown by turbid halo of colorless cells of *P. aeruginosa* was a strong evidence of quorum sensing inhibition.

According to Adonizio *et al.*[1], 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 a disruption in cell to cell communication caused by the plant extract did occurs, further assays in quorum sensing manifestation inhibition, i.e. motility inhibition was conducted.

Motility assay: The significant potency of the ethyl acetate fraction in decreasing the swimming and swarming motilities of *P. aeruginosa* at concentration of 1 mg mL^{-1} by 84.14±5.55 and 76.80±4.48% reduction was described in Fig. 7-9, respectively. Ethyl acetate fraction at lower concentration i.e., 0.5, 0.25, 0.125 and 0.0625 mg mL^{-1} towards swimming and swarming motilities was further investigated. Figure 10 showed the ethyl acetate fraction inhibition on the swimming motility on all concentration, however, 0.5 mg mL^{-1} was the lowest concentration which still showed inhibition of swarming activity.

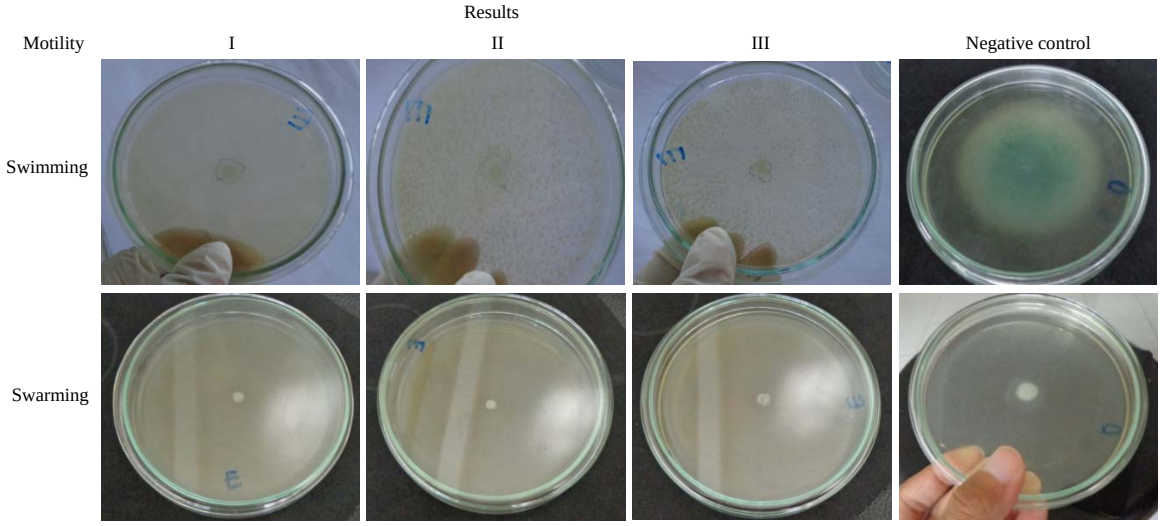

Fig. 7: Effect of *M. tuberosa* ethyl acetate fraction on swarming and swarming of *P. aeruginosa* PAO1

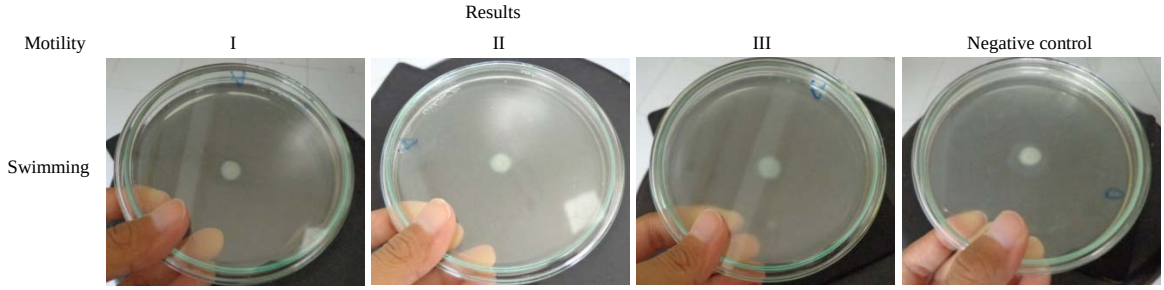

Fig. 8: Effect of *M. tuberosa* water fraction on swarming motility of *P. aeruginosa* PAO1

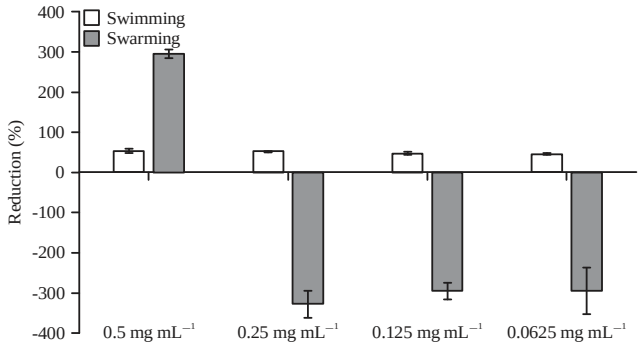

Fig. 9: Effects of *M. tuberosa* extracts and fractions on swimming and swarming motilities of *P. aeruginosa* PAO1 at concentration of 1 mg mL^{-1} (n = 3)

The water fraction decreased the swarming motility of *P. aeruginosa* of 1 mg mL^{-1} by 52.27±3.02% reduction, while the hexane fraction and petroleum ether extract showed negative results, suggesting a stimulation to motility. The fact that the hexane fraction caused the stimulatory effect explains low reduction rate showed by the methanol extract. This suggest that compounds responsible for stimulatory activity were relatively non polar compounds.

Considering that the hexane fraction showed inhibition of pigment production and the fact that solubility seems to be the problem in diluting the fraction in the media, negative result of the hexane fraction might be due to poor solubility. It is interesting to find out that other fractions showed inhibitions in swarming but only the ethyl acetate inhibited the swimming behavior.

Only ethyl acetate fraction was tested further for twitching inhibition assay. Twitching motility of *P. aeuginosa* was significantly reduced by the fraction at concentration of 1 mg mL^{-1} by 70.90±0.05%. Twitching is a critical behavior for the *P. aeruginosa* host infections. The morphological pattern of twitching can be observed to be less organized than that of swarming[8]. Twitching requires type IV pili and is important for the surface adherence and the development of biofilm[20]. Unlike, twitching and swarming, swimming motility of bacteria formed on the medium having lower Agar concentration.

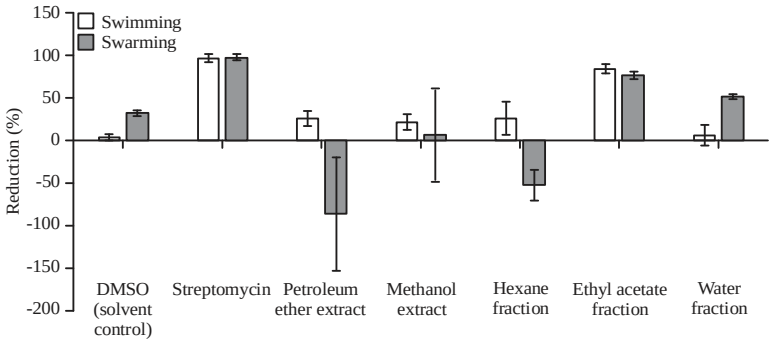

Fig. 10: Effects of *M. tuberosa* ethyl acetate fraction on swimming and swarming motilities of *P. aeruginosa* PAO1 at concentration of 0.5-0.0625 mg mL^{-1} (n = 3)

Swimming represents individual cell movement of which the cells move independently, unorganized and involving no differentiation into polar hyper flagellated cells[8,21]. On the other hand, even though swarming also depends on flagella, this motility is cell density dependent. It is formed on more solidified media. It is interesting to note that the fraction at the same concentration have higher inhibition on the swarming motility than the swimming motility of *P. aeruginosa*. Active compounds having capabilities in reducing the amount of the quorum sensing signal in *P. aeruginosa* PAO1 may explain the modulation of bacterial behaviors. This condition was supported by the extract capability on reducing the pigment production. 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 less effect on the swimming motility modulation.

Production of several virulence factors are quorum sensing-dependent[22,23]. In order to explore for quorum sensing active constituent of the active fraction, the ethyl acetate fraction was further tested for bioautography. After loading application of the fraction up to 500 mg, no sign of inhibition observed. Considering that the same amount of sample application for bioautography showed pigment formation inhibition in diffusion assay, suggests that the activity might be resulted from collaborative work and not a single constituent action. Our previous result showed that phenolics might contribute in the antibacterial activity against *Staphylococcus aureus*. There were at least two phenolic components showed planktonic growth inhibition[14]. On the other hand, Hanh *et al.*[24] reported that two iridoids have been isolated from *M. tuberosa* which are active against *Staphylococcus aureus*.

CONCLUSION

It has widely accepted that microbial biofilms cause elevated resistance to most anti-microbial drugs, which further may result in persistent and difficult to treat infections. The discovery of anti-infective agents which are effective against planktonic and biofilm micro organism are therefore urgently required to deal with these biofilm-mediated infections.

While immune-compromised patients may suffer from biofim-related infection, anti-infective agent which at the same time can stimulate host immune system such as *M. tuberosa* extract is therefore worth exploring. This research find out that the ethyl acetate fraction showed potential anti-planktonic growth towards *S. aureus* and *P. aeruginosa* while anti-pathogenic activity was observed towards *P. aeruginosa*. Quorum sensing inhibition of the ethyl acetate fraction might be resulted from a collaborative works among several chemical constituents. Phenolic content is expected to play role in the activity. This study has discovered the potential of ethyl acetate fraction of *M. tuberosa* in immune compromised related infection involving *P. aeruginosa*. Further study on ethyl acetate fraction to isolate and identify the compound responsible for the anti-pathogenic activity of *M. tuberosa* is necessary.

SIGNIFICANCE STATEMENT

This research was performed to explore the effects of *M. tuberosa* Jack extract and fractions on quorum-sensing related pathogenicity of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The plant extract has been reported before as a potential immunomodulator, therefore its

inhibitory effect towards biofilm related microbes will beneficial to support further research on the plant utilization as an effective anti-biofilm in immune compromised condition.

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