Phytochemical screening and anti-quorum sensing activity of *Eichhornia crassipes* methanolic extract against the virulence factors of *Pseudomonas aeruginosa*

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Abstract: *Pseudomonas aeruginosa* is classified under critical priority in the World Health Organization (WHO) priority pathogens list for research and development of new antibiotics due to its reported resistance to multiple antibiotics. Pyocyanin production, proteolytic activity, biofilm formation, and swarming motility, all under Quorum sensing (QS) activity greatly contribute to the virulence of *P. aeruginosa*. Several studies suggest that flavonoid-rich plants can attenuate virulence factors under QS activity. In this study, the secondary metabolites, functional groups, and Total Flavonoid Content (TFC) of *Eichhornia crassipes* (Mart) Solms methanolic extract were identified using Thin-Layer Chromatography (TLC), Fourier Transformed Infrared Spectrometer (FTIR), and Aluminum chloride colorimetric assay, respectively. Secondary metabolites were detected including flavonoids and polyphenols. Functional groups present showed similar bonds to that of quercetin. Assay for TFC showed 468.9120 ±2.39mg QE per gram sample. The Minimum Inhibitory Concentration (MIC) of the methanolic extract was determined at 0.6µg/mL using the Liquid Dilution Method. The extract at MIC, 50% sub-MIC, and 25% sub-MIC were tested against negative and positive controls for each virulence factor. Inhibition of pyocyanin production, proteolytic activity, and biofilm formation was tested using a UV/Vis Spectrophotometer while swarming motility was tested via motility determination on a butt-slant culture. The methanolic extract exhibited a significant reduction in the QS activity of *P. aeruginosa* at a concentration of 0.6µg/mL. The extract showed percent inhibition of 80.66% in pyocyanin production, 79.11% in swarming motility, 30.34% in proteolytic activity, and 79.78% in biofilm formation. Based on the given results, the methanolic extract of *E. crassipes* has the potential to inhibit QS-controlled virulence factors of *P. aeruginosa*. This may be used as a basis for antibiotic development not just for *P. aeruginosa* but also for other resistant pathogens in the WHO priority list.

Keywords: *Eichhornia crassipes*; *Pseudomonas aeruginosa*; Quorum sensing; Virulence factors; Water hyacinth

1. BACKGROUND

Pseudomonas aeruginosa has been known to use quorum sensing (QS) activity in expressing its virulence factors and has a major impact in medical practice (Miyoshi-Akiyama et. al., 2017). According to the World Health Organization (WHO) Priority Pathogen List for Research and Development for New Antibiotics (2017), *P. aeruginosa* is now categorized as Priority 1: Critical pathogen. It contributes to 11% of all nosocomial infections and can evenly distribute to the entire body sites, resulting in high mortality and morbidity rates (Hassan, Aftab, & Riffat, 2015).

QS is bacteria's way of communicating with each other by releasing, sensing, and responding to small diffusible signal molecules, all contributing to overall bacterial pathogenicity (Reen et. al., 2018). Inhibition of QS has been one of the major foci for the discovery of new anti-microbials. Several studies suggest that many traditional plants rich in flavonoids possess potent anti-QS activity. Flavanones like naringenin, eriodictol, and taxifolin (Vandeputte et. al., 2011), theaflavin found in black tea extract (Bell, 2014), and the flavonoid-rich fraction of *Centella Asiatica* L. (Sharma et. al., 2016) all exhibited significant inhibitions in the QS activity of *P. aeruginosa*.

Many plant species evolved to produce metabolites that can control the growth of microbes to survive in their environment (Sharma et. al., 2016). *Eichhornia crassipes* (Mart) Solms, also known as water hyacinth, is considered the world's worst invasive weed due to its rapid proliferation rate and its tendency to cause harm to the environment, economic development, and ecological adaptability (Vasavi, Arun, & Rekha, 2016). In the town of Cardona Rizal in the Philippines, the local government was forced to declare a state of calamity and released 4 million pesos to support 8,000 fishermen who have been affected by the invasiveness of *E. crassipes* (Cinco, 2017).

There are records of the use of *E. crassipes* as a folkloric and traditional medicine to ease swelling, burning, hemorrhage, and goiters (Jiyanthi et. al., 2013). Recent studies on *E. crassipes* extracts reveal the presence of polyphenol and flavonoids which contribute to its anti-microbial properties against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis,* and many other pathogenic microorganisms (Sunitha et. al., 2018). However, there is still no literature to support if water hyacinth possesses anti-QS activity against *P. aeruginosa*. This study initially evaluates the anti-QS property of the methanolic extract of *E. crassipes* based on its ability to inhibit pyocyanin production, proteolytic activity, swarming motility, and biofilm formation in *P. aeruginosa*.

2. METHODOLOGY

2.1 Plant sample and methanolic crude extract preparation

Plant samples of *Eichhornia crassipes* (Mart.) Solms were collected from the municipality of Cardona, Rizal, and authenticated by the Research Center for Natural and Applied Science Herbarium Department of the University of Santo Tomas, Manila, Philippines. Leaves and petioles were

cleaned, air-dried under a shade at room temperature until a constant weight is achieved. The plant samples were crushed into a fine powder using Oster® Osterizer 10 Speed Blender, then subjected to further reduction using Marathon Electric 5KH39QN5529X Wiley® Mill until it passed through sieve no. 40. Fifty (50) grams of the powdered sample was extracted by Soxhlet extraction method using 1.5 liters of methanol as the solvent. The extract was concentrated by EYELA® N-1110 series vacuum rotary evaporator and Thermo Scientific® Digital Water Bath 2350 at 50°C until a viscous consistency was achieved.

The crude extract was then tested for the presence of methanol using the Iodoform test. The iodoform odor was tested by wafting the air towards the nose and observed for the formation of yellow precipitate. The formation of a yellow precipitate indicates the presence of methanol and acetone (Lancashire, 2005). After ensuring that there is no solvent left in the crude extract, computation of the percentage yield and organoleptic test were performed.

2.2 Phytochemical screening of the methanolic crude extract

The secondary metabolites present in the methanolic crude extract were identified using Thin-Layer Chromatography (Guevarra, 2005). Merck Millipore® Aluminum Thin-layer Chromatography plates, aluminum oxide coated with fluorescent indicator F254 was used as adsorbent. Various freshly prepared solvent systems were used as mobile phases.

The developed chromatogram was visualized under ultraviolet (UV) light, short wave UV (240 nm), and longwave UV (365 nm) using UVG® UVGL-58 Handheld UV Lamp. Suitable visualizing reagents for the desired constituents were applied using a glass sprayer. A suitable solvent should give an *hRf* value of 30 to 70 for the components of analytical interest in the plant sample.

2.3 Identification of Functional Groups and Total Flavonoid Content (TFC)

The NICOLET 6700 Fourier – Transform Infrared Spectrometer Thermo Scientific® was used for elucidating the functional groups in the crude extract. The total flavonoid content of the crude extract was measured by the aluminum chloride colorimetric method (Tyagi, et. al., 2017). Findings were compared to that of functional groups and the absorbance measured against blank at 420nm using BIOBASE® UV1100 Spectrophotometer of quercetin.

2.4 Determination of Minimum Inhibitory Concentration (MIC)

The liquid dilution method was used in the determination of the Minimum Inhibitory Concentration of the crude extracts. 24-hour culture of *Pseudomonas aeruginosa* ATCC 27853 grown on Cetrimide agar was collected and suspended in Cetrimide broth. It was then compared with 0.5 McFarland standard to achieve a concentration of $1.5x10^8$ CFU/mL. Various concentrations of crude extracts were introduced to different tubes containing

1mL of the bacterial inoculum and were further subjected to tests for inhibitory activity (Andrews, 2001). MIC was determined using BIOBASE® UV1100 Spectrophotometer.

2.5 Inhibition of QS-controlled virulence factors in Pseudomonas aeruginosa

Three (3) concentrations of the plant extract: MIC, Sub-MIC at 50%, and Sub-MIC at 25%, were used for the quorum-sensing – controlled virulence factor inhibition in *Pseudomonas aeruginosa* ATCC 27853. Absorbance was measured spectrophotometrically. All inhibitory effects against the different virulence factors were calculated using the following equation:

$$\%\ inhibition\ activity = \frac{\text{absorbance of control}}{\text{absorbance of sample}} x100$$

Procedures conducted for the testing of anti-QS activity were also done separately with 6mg/mL Aspirin as a positive control (El-Mowafy et. al., 2014) and Type 1 sterile water as a negative control.

2.5.1 Pyocyanin production

Cultures of *Pseudomonas aeruginosa* ATCC 27853 with the crude extracts of varying MICs and controls were grown in cetrimide broth medium and incubated at 37°C for 24 hours. The supernatant liquid with 5mL of volume from pyocyanin was extracted with 3mL chloroform before the acidification of the chloroform layer with 1mL of 0.2M HCl. UV/VIS Spectrophotometer was used to quantify the recording of OD_{520} after the separation of the acid layer containing the pyocyanin**.**

2.5.2 Swarming motility

Cultures of *Pseudomonas aeruginosa* ATCC 27853 with the crude extracts of varying MICs and controls were grown in 1.5% Cetrimide agar at 37°C for 18-20 hrs. The diameter of the twitching zone at the plastic-agar interface was measured using Vernier caliper and observed for the reduction or complete inhibition of *P. aeruginosa* ATCC 27853 (Murray, Ledizet, & Kazmierczak, 2010).

2.5.3 Proteolytic activity

Modified skim milk assay was performed in the testing proteolytic activity. *Pseudomonas aeruginosa* ATCC 27853 culture supernatant (0.5 mL) with 1mL of 1.25% skim milk and 1mL of the plant extract of different concentrations were incubated at 37°C for 30min. IKA® UV-1100 UV/VIS Spectrophotometer with OD_{600} was used in measuring the turbidity of the samples.

2.5.4 Biofilm formation

Biofilm formation assay was conducted as described by Agarwala et. al., (2014) in the presence and absence of the crude extract. *Pseudomonas aeruginosa* ATCC 27853 grown with three MIC and a series of sub-MIC concentrations of the crude extract were incubated for 18 hours without shaking. The incubated mixture was discarded and washed three times with phosphate-buffered saline and was set in 10% formaldehyde for 10 minutes. The solution was removed and air-dried at room temperature. The biofilm was stained with Crystal violet (0.1% in ethanol) for 15 minutes. The unbound dye was washed three times with deionized water while the absorbed dye was eluted with ethanol. The OD_{650} was recorded using IKA® UV-1100 UV/VIS Spectrophotometer.

2.6 Data Analysis

All tests that were performed are in triplicates (3 three samples). One-way ANOVA was used to analyze the variations between the treatments using the SPSS v.22. It was considered significant if the p-value is equal to or less than 0.05. Post – hoc analysis was followed using Scheffe Test by SPSS v.22 statistical software. For the total flavonoid content determination, all results were expressed as Mean ± Standard Error Mean (SEM).

3. RESULTS AND DISCUSSION

3.1 Plant sample and methanolic crude extract assessment result

Fifty grams (50g) of the plant sample of *E. crassipes* yielded 8.9558g after extraction (equivalent to 17.91%). The obtained percentage yield was higher compared to the 15% yield from a study that used the leaves only (Joshi & Kaur, 2013). *E. crassipes* (Mart.) Solms extract was observed to be green in color with an aromatic odor, viscous consistency, and mucilaginous texture. No yellow precipitate was observed with Lieberman's Iodoform test, indicating the absence of methanol in the crude extract.

3.2 Secondary metabolites

Eichhornia crassipes (Mart.) Solms was found to contain flavonoids, phenols, tannins, alkaloids, anthraquinones, anthrones, indoles, higher alcohols, phenols, steroids, and essential oils.

3.3 Functional groups present in the Methanolic Extract.

Figure 1 and Table 1 show the FTIR spectrum and absorption bands of the functional groups in *Eichhornia crassipes (Mart.) Solms*, respectively. Results show that the functional groups in *E. crassipes (Mart.) Solms* extract has similarities with the functional groups present in quercetin shown in Table 2. They contain similar functional groups such as like alkanes, alkenes, alkynes, alcohols, amides, ethers, and aromatic compounds.

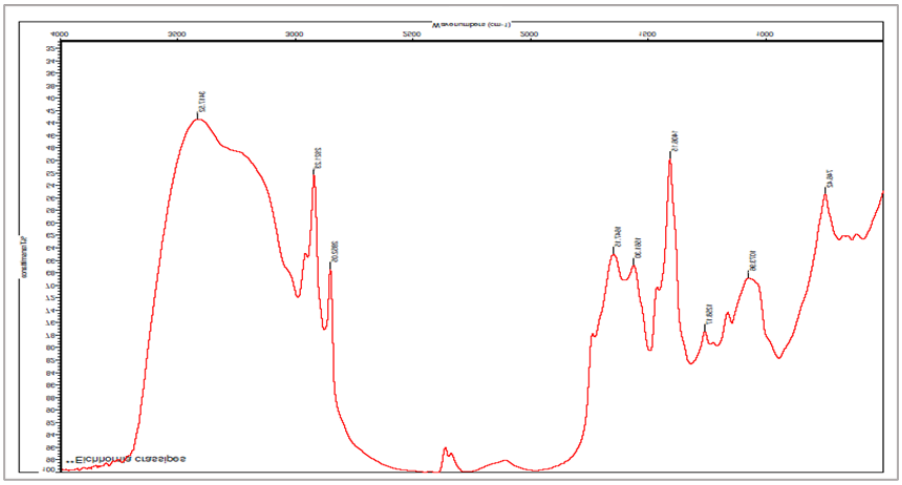


Figure 1. FTIR spectrum of *Eichhornia crassipes* (Mart.) Solms.

Table 1

Characteristic Infrared Absorption Bands of Functional Groups in Eichhornia crassipes (Mart.) Solms

Frequency (cm^{-1})	*Functional Group*	*Bond Vibration*	*Intensity*
3417.52	Amides	W – M	N – H stretch
2921.23	Alkanes and Alkynes	S	C – H stretch
2852.05	Alkanes and Alkynes	S	C – H stretch
1647.15	Alkenes	VW – M	C = C stretch
1561.30	Amides	M – S	N – H bend
1406.15	Nitriles / Nitro group	S	N = O stretch
1258.17	Ethers	M – S	= C – O – C stretch
1073.96	Alcohols	M – S	C – O stretch
746.42	Aromatic Compounds	S	C – H bend

Legend: Characteristic bands; VW = very weak, W = weak, M = medium, S = strong VS = very strong

Note: Adapted from "Characteristic Infrared Absorption Bands of Functional Groups," by the University of Mexico, n.d.

Table 2
Characteristic Infrared Absorption Bands of Functional Groups in Quercetin

Frequency (cm^{-1})	*Functional Group*	*Bond Vibration*	*Intensity*
3366	Alcohols	S, broad	O – H stretch
3273	Alkynes	S, sharp	☰ C – H stretch
2924	Alkanes and Alkynes	S	C – H stretch
2883	Alkanes and Alkynes	S	C – H stretch
2130	Alkynes	M	C ☰ H stretch
1640	Amides	M – S	C = O stretch
1457	Aromatic Compounds	M – S	ring C = C stretch
1252	Ethers	M – S	= C – O – C stretch
1086	Alkyl Halides	VS	C – F stretch
945	Alkenes	M + S	= C – H stretch
878	Arenes / Aromatic compounds	S – M	C – C stretch

Legend: Characteristic bands; VW = very weak, W = weak, M = medium, S = strong VS = very strong
Note Ädapted from "Characteristic Infrared Absorption Bands of Functional Groups," by the University of Mexico, n.d.

3.4 Total Flavonoid Content

The crude extract produced a maximum amount of 468.9120±2.39 mg QE per g^{-1} dry weight sample as shown in Table 3. The total flavonoid content obtained in the study was higher compared to what was obtained in previous studies. This may be due to the inclusion of other plant parts aside from the leaves.

Table 3
Quantitative Analysis of Total Flavonoid Content in the Crude Extract

Wt. (mg)	Vol. (mL)	Mean Absorbance	QE conc. C (mg/mL)	TFC (µg/mL)	SEM	TFC mg QE/ g^{-1} Sample)
500	0.5mL	0.301	37.51298	37.51298	2.39	468.9120±2.39

Note: QE = Quercetin equivalent; TFC = Total Flavonoid Content; SEM = Standard Error Mean

3.5 Minimum Inhibitory Concentration (MIC)

The MIC of *Eichhornia crassipes* (Mart.) Solms were determined to be at $6x10^{-4}$ mg/mL (0.0006 mg/mL) using the McFarland standard. Sub-MICs at 50% and 25% were computed from the obtained MIC.

3.6 QS-controlled Virulence Factors

3.6.1 Pyocyanin Production Inhibition

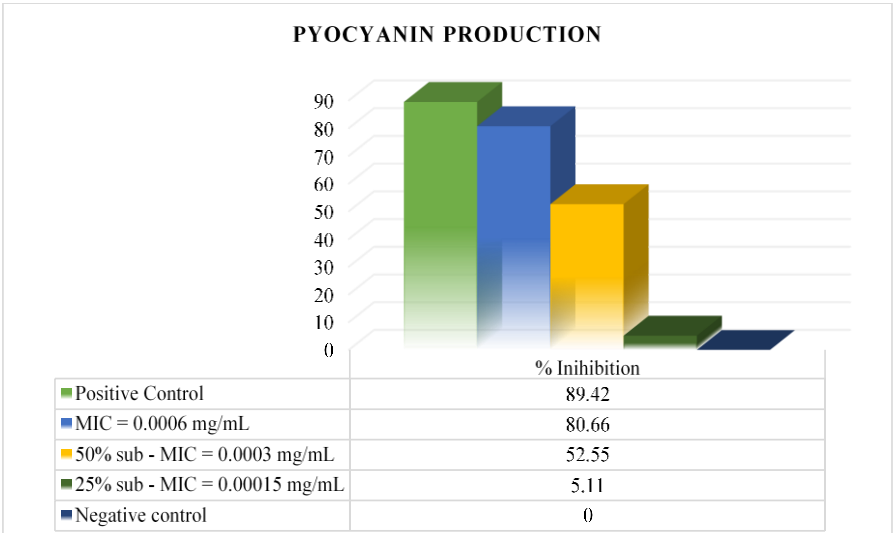


Figure 2. Inhibition of Pyocyanin Production. The average results of triple measurements for the inhibitory effect of Aspirin, Type 1 sterile water, MIC, 50% sub – MIC, and 25% sub-MIC against the pyocyanin production of *Pseudomonas aeruginosa* ATCC 27853.

The inhibition of pyocyanin production among the three concentrations, positive control, and negative controls were compared. Statistical analysis showed that two out of the three concentrations of the crude extract (0.0006 mg/mL and 0.0003 mg/ml) were able to significantly reduce pyocyanin production of *Pseudomonas aeruginosa* ATCC 27853. The MIC, however, is not comparable to the positive control.

Comparing the result with the study by Vasavi et. al. (2014) where the flavonoid (FL)-a fraction of *Psidium guajava* at 200 µg/mL completely inhibited the QS-controlled pyocyanin production and elastolytic activity in *Pseudomonas aeruginosa, E. crassipes* extract at 0.6 µg /mL was able to achieve 80.66% inhibition of pyocyanin production. A higher concentration of the extract may be tested in future studies to achieve a comparable result with the previous study. A better comparison may be done if the FL portion of *E. crassipes* will be tested.

3.6.2 Swarming motility

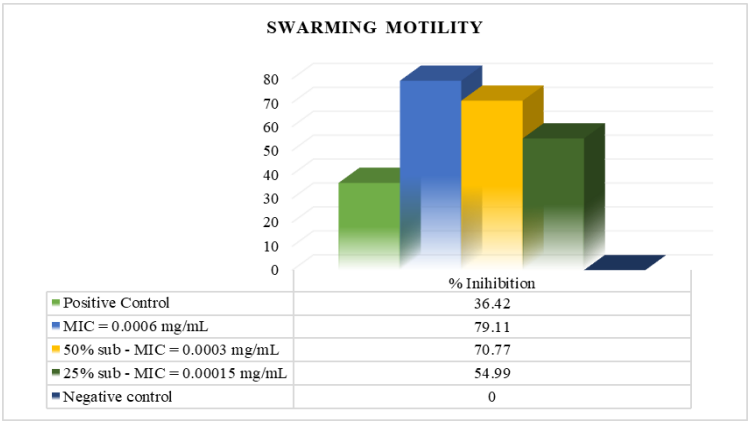


Figure 3. Inhibition of Swarming Motility. The average results of triple measurements for the inhibitory effect of Aspirin, Type 1 sterile water, MIC, 50% sub – MIC, and 25% sub-MIC against the swarming motility of *Pseudomonas aeruginosa.*

The percent inhibition for each concentration tested is shown in Figure 3. Post-hoc analysis revealed that all concentrations of the extract were able to inhibit swarming motility significantly at 0.05 level. All extract concentrations were also found to be comparable to the positive control in inhibiting swarming motility. In the previous study done by Vasavi, et. Al. (2014), the FL fraction of *Psidium guajava* was able to completely inhibit swarming motility at a minimum concentration of 25 µg /mL.

3.6.3 Proteolytic activity

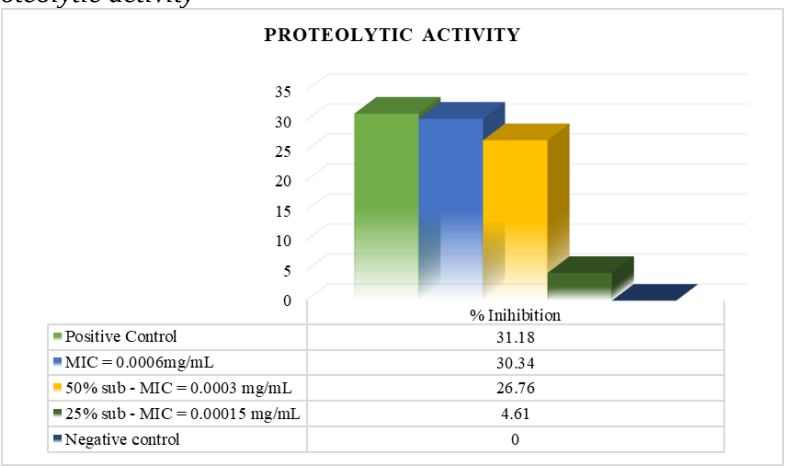
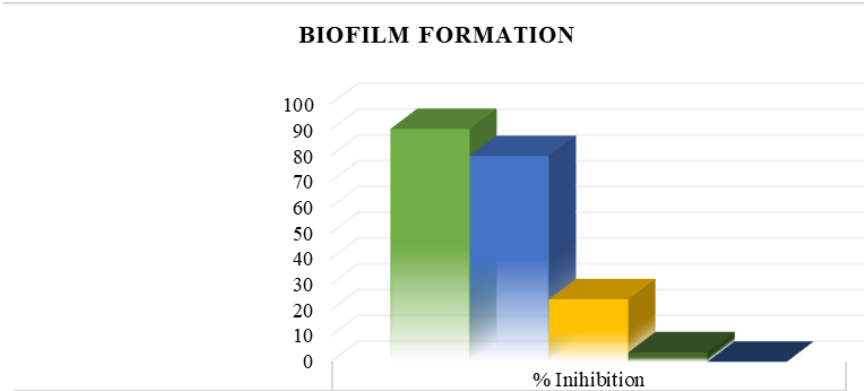


Figure 4. Inhibition of Proteolytic activity. The average results of triple measurements for the inhibitory effect of Aspirin, Type 1 sterile water, MIC, 50% sub – MIC, and 25% sub-MIC against the proteolytic activity of *Pseudomonas aeruginosa.*

The percent inhibition for each concentration tested is shown in Figure 4. Post-hoc analysis revealed that all concentrations of the extract were able to inhibit proteolytic activity significantly at 0.05 level. Of the three extract concentrations tested, however, only the extract at 0.0006 mg/mL was found to be comparable with the positive control. With the percent inhibition results at different concentrations, it appears that *E. crassipes* extract shows exerts less effect against proteolytic activity compared to pyocyanin production, swarming motility, and biofilm formation.

3.6.4 Biofilm formation



	% Inihibition
Positive Control	90.25
MIC = 0.0006mg/mL	79.78
50% sub - MIC = 0.0003 mg/mL	24.18
25% sub - MIC = 0.00015 mg/mL	3.61
Negative control	0

Figure 5. Inhibition of Biofilm Formation. The average results of triple measurements for the inhibitory effect of Aspirin, Type 1 sterile water, MIC, 50% sub – MIC, and 25% sub-MIC against the biofilm formation of *Pseudomonas aeruginosa.*

Figure 5 shows the percent inhibition results for each extract concentration, negative control, and positive control. Post-hoc analysis revealed that only the MIC at 0.0006 mg/mL was able to significantly inhibit biofilm formation. The said concentration is also found to be comparable with the positive control. In the study done by Vasavi, et.al. (2014), the FL fraction of *Psidium guajava* at 200 µg /mL produced 80% inhibition against biofilm formation of *P. aeruginosa*. This is almost close to the percent inhibition produced by *E. crassipes* at 0.0006 mg/mL.

This study tested the methanolic extract *E. crassipes* for anti-QS activity. Comparison of results with previous studies may be more significant if the FL fraction of *E. crassipes* will be specifically studied for its anti-QS activity.

4. CONCLUSION

Eichhornia crassipes (Mart) Solms has a very good potential in inhibiting QS-controlled virulence factors of the pathogenic microorganism *Pseudomonas aeruginosa* especially pyocyanin production, biofilm formation, and swarming motility where it showed high percent inhibition. Assay for the total flavonoid content also showed that *E. crassipes* (Mart.) Solms as a good source of flavonoids (468.9120±2.39mg QE per g^{-1} sample) which are known to have anti-microbial activity. This may have contributed to its anti-QS activity. Further studies may also be conducted to investigate the anti-QS activity of *E. crassipes* against other pathogenic and resistant microorganisms included in the WHO Priority Pathogens List for Research and Development. The flavonoid fraction may also be tested for future studies,

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