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Evaluation of the effect of some medicinal plants against quorum sensing regulated virulence factors in *Pseudomonas aeruginosa*

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Abstract
The effect of some commonly used medicinal plants in Southeast Nigeria namely *Carica papaya*, *Psidium gujava* and *Citrus cinensis* against quorum sensing regulated virulence factor such as twitching motility, pyocyanin production, proteolytic activity and cell adhesion in *Pseudomonas aeruginosa* was evaluated in this study using spectrophotometric methods. Result showed that *Carica papaya* and *Psidium gujava* plant extract revealed complete inhibition of *Pseudomonas aeruginosa* for twitching motility. *Carica papaya* plant extract showed greater inhibition on cell adhesion and proteolytic activity while the inhibition of pyocyanin production was lowest in *Citrus cinensis* and highest in *Carica papaya*. Overall, the plant extract showed variable effects with *Carica papaya* plant extract showing the best effect against quorum sensing regulated virulence factors in *Pseudomonas aeruginosa*. This study shows the potential use of these plant extracts in the treatment of microbial infections by inhibiting bacterial virulent factors and it's associated antibiotics resistance capabilities.

Keywords: anti-qourom sensing, bacterial infections, biofilm, *Pesudomonas aeruginosa*, plant extracts

Introduction
It has become evident that bacterial function and growth within a population is a fundamental aspect of bacterial survival and atypical life style of microorganisms (Davey, 2000).This typical lifestyle of survival has been highlighted in more recent studies (George and Muir, 2007; Clatworthy *et. al.*, 2007; Rasko *et. al.*, 2010; Kobayashi *et. al.*, 2011; Daly *et. al.*, 2015) [15, 4, 16, 13]. Therefore, some organisms are resistant to all approved antibiotics, because the environmental antibiotics pressure activates the evolutionary mechanisms that select for resistant strains (Ali *et. al.*, 2018) [1]. Bacterial populations coordinate communal behavior through a process of cell-to-cell signaling mediated by diffusible signal molecules (Schauder and Bassler, 2001) [18]. This process, termed quorum sensing (QS). Quorum sensing is a recently discovered chemical communication system that enhances survival of bacteria, as a group allowing resident bacteria to assume specialized roles vital for intra- and inter bacterial gene regulation, and for keeping bacterial colonies intact (Chan *et. al.*, 2011) [2]. These involve several processes, such as specific signaling molecules that bind to and activate receptors that transduce the quorum-sensing signal into intracellular second messenger responses, in a similar fashion to ligand-receptor interaction (Stanley *et. al.*, 2018) [21]. This similarity opens a novel alternative that should be looked for in combating drug resistance by the infectious bacteria, with inhibitor drugs that could be designed using current standard pharmacologic principles (Kumer *et. al.*, 2016). Therefore, quorum-sensing inhibition offers new hope in combating resistant bacteria with inhibitor drugs that might have novel mechanisms of action, and could, therefore, be more effective against antibiotic resistant strains of bacteria (Fuqua *et. al.*, 2001; Rasko and Sperandio, 2010) [16].
In the past few years, inhibition of QS has become an intense area of research because of its applications in medicine, industry, and biotechnology (Okhee *et. al.*, 2018) [15]. In the quest for QS inhibitors, studies have demonstrated that many eukaryotes, particularly plants, and even bacteria themselves produce anti-QS substances (Kalia, 2013) [11]. Ajoene from garlic, catechin from Combretumal biflorum, and iberin from horseradish specifically inhibit QS in reporter strains (Vandeputte *et. al.*, 2010) [22]. As an adaptive evolution, many plant species produce metabolites that can control the growth of microbes and have traditionally been used to treat human diseases, particularly microbial infections (Hayek *et. al.*, 2013) [6]. Traditional medicine is increasingly been recognized as an accepted alternate regimen to orthodox health-care system (Vasavi *et. al.*, 2016) [23]. This work focuses on evaluating selected medicinal plant extract against expression of quorum sensing regulated virulence factors in *Pseudomonas aeruginosa*.

Methodology
Collection of plant materials
Leaves of *Carica papaya* (Paw Paw), *Psidium gujava* (Guava), and *Citrus cinenesis* (Orange) were collected from Owerri, Imo state, Nigeria and used in the study.

Preparation of plant extract
The plant extract were prepared according to method described by (Ibe, 2017). The collected plant material will be air-dried under shade at room temperature, finely ground into powder using domestic mixture and will be stored in airtight labeled plastic sampling bags for further studies.

Extraction of plant samples
The grounded plant samples were extracted using three solvents i.e cold water, hot water and ethanol.

Cold water
10 g of the grounded plant samples were soaked in 100 ml cold distilled water for 72 hours with occasional agitation. The extract of each plant were filtered using (What man No.1) filter paper.

Hot water
10 g of the grounded plant samples were soaked in 100 ml hot distilled water for 72 hours with occasional agitation. The extract of each plant were filtered using (What man No.1) filter paper.

Ethanol extracts
Extraction was carried out by the modified method of Hussaini and Mahasneh. The plant material were extracted at room temperature with ethanol 95% (100 mL/10 g of plant material). The extract of each plant was filtered using (What man No.1) filter paper and evaporated under vacuum at 40 °C using a rotary vacuum evaporator, the concentrated extract thus obtained was collected in screw cap vial and was used for further studies.

Test organism and growth condition
Isolate of the test organism i.e *Pseudomonas aeruginosa* was obtained from the department of microbiology, Federal Medical Centre, Owerri. The isolate was propagated on nutrient agar plates and maintained on the plates at 4 ºC. The isolates were sub-cultured in nutrient agar at 37 ºC for 24 hours prior to further studies.

Quorum sensing mediated virulence factors
The following quorum sensing mediated virulence factors such as twitching motility, pyocyanin production and proteolytic activity of the test organism were conducted.

Twitching motility
Twitching Motility was determined according to methods described by Karthick and Vivek (2016) [12]. 500µl of respective plant extracts and 250µl of bacterial inoculums was prepared in LB (Lysogeny broth) broth and were mixed in sterile eppendorf tube, kept in room temperature for 30minutes.The respective plant extracts treated cultures were stab inoculated through LB agar plates. The plates were incubated at 32°C for 24hours. Bacterial grew at the interface between the plastic surface and the agar, which is indicative of twitching motility. To visualize the bacterial growth on the plastic surface, the agar was removed and the plate was stained with a 1% solution of crystal violet. Twitching motility was determined by measuring the diameter of the stained growth.

Pyocyanin production
Effect of plant extracts on pyocyanine production was done by the modified method described by Karthick and Vivek (2016) [12]. Pyocyanine was extracted from the supernatant fraction of test organism grown in trypticase soy broth medium with 500µl of plant extracts for 24hours. 5 ml sample of the supernatant was mixed with 5ml chloroform and the lower organic layer was separated. To this layer 1.5ml of 0.2 M of HCl will be added and the pyocyanine rich organic layer was separated. The amount of the pyocyanine within the extracted layer was determined by measuring the absorbance at 520nm.

Cell adhesion
Cell adhesion was studied by using 96 well flat bottom micro well plate was previously coated with bovine serum albumin (BSA). Wells was coated with 150µl of freshly prepared 1.0% BSA, incubated at 30 ºC for 30 minutes. After the incubation period, wells was washed thrice with sterile phosphate buffered saline (PBS). Fifty micro litre of bacterial inocula thus prepared was transferred to the well followed by the addition of 50µl of the respective plant extracts. Seeded micro titre plate was incubated at 37°C for 24 hours. Cells were allowed to adhere and the non-adhered cells were washed 5 times with PBS at room temperature. Adhered cells were detected by adding 50µl of 0.1% crystal violet per well, incubated at room temperature for 30 minutes. Wells was washed with sterile distilled water to remove excess stain. 10µl of ethanol was added to fix the adhered cells.50µl of 0.2 % Triton X was then be added to the wells for lyse of cells and the absorbance was read at 570nm.

Proteolytic activity
Proteolytic activity was carried out by modified method of Karthick and Vivek (2016) [12]. Crude enzyme preparation 0.1 ml of tryptic soy broth bacterial culture was inoculated into 100 ml of protease production media (Yeast extract5mg/l, Peptone-10mg/l, Glucose-10mg/l, Caesin-15mg/L) supplemented with 200µl of respective plant extracts. Flasks were incubated at 37ºC for 48 hours. Broth was centrifuged after the incubation period at 10,000rpm for 10 minutes; the collected supernatant was used as the source of protease enzyme.

Enzyme activity
Enzyme activity was assayed using casein as the substrate as described (Karthick and Vivek, 2016) [12]. The reaction mixture consisted of 0.25 ml of 50mM sodium phosphate buffer (pH 7.0) containing 2.0% (w/v) of azocasein and 0.15 ml of enzyme solution. After incubating at 25ºC for 15 min, the reaction was stopped by adding 1.2 ml of 10.0% (w/v) TCA, incubating at room temperature for an additional 15 min, and then the precipitate was removed by centrifugation at 8,000 g for 5min. 1.4ml of 1.0 M NaOH was added to 1.2 ml of the supernatant, and its absorbance was measured at 440nm.

Statistical analysis
The One-Way analysis of variance (ANOVA) was employed in determining if there is significance in the difference that exists between test samples and also with control sample.

Result
Effects on twitching motility
The results of extracts on twitching motility of *Pseudomonas aeruginosa* is presented in Figure 1.

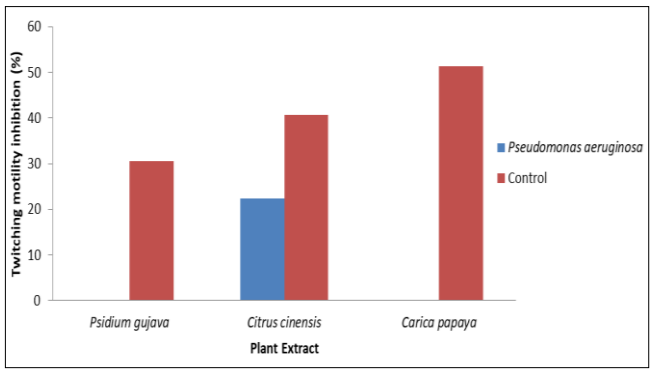

Fig 1: Effect (cm) of plant extract against twitching motility of *Pseudomonas aeruginosa*

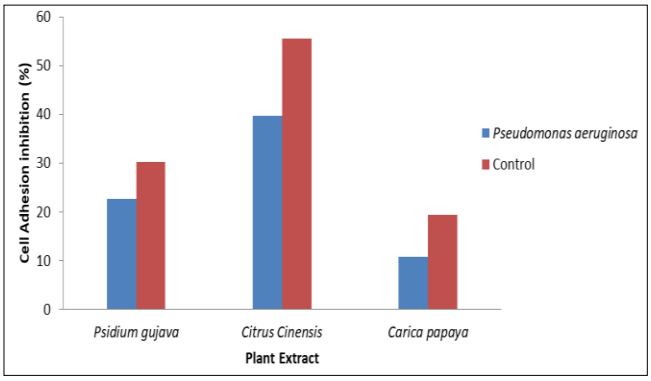

Fig 2: Inhibitory effect (%) of plant extract on cell adhesion of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Effects on cell adhesion

Cell adhesion initiates the biofilm formation and pathogenicity in the host. The distribution for the plant extracts is presented in Figure 2.

Effects on proteolytic activity

The distribution for the plant extracts against proteolytic activity is presented in Figure 3.

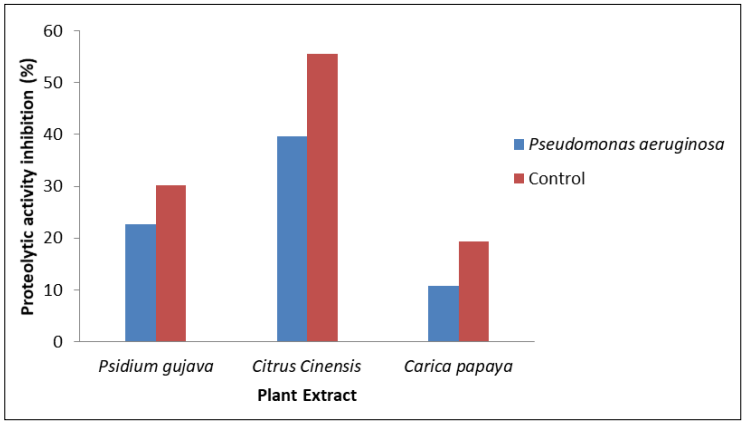

Fig 3: Inhibitory effect (%) of plant extract on proteolytic activity of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Effects on pyocyanin production

Table 1: The result for pyocyanin production is presented in Table 1.

Plants	Extract	Inhibitory effect (%)
Psidiumgujava	Cold water	15.17±2.08
	Hot water	14.07±0.51
	Ethanol	8.50±0.10
Citrus cinensis	Cold water	3.97±0.55
	Hot water	1.80±0.44
	Ethanol	2.17±0.06
Carica papaya	Cold water	26.60±0.10
	Hot water	24.87±0.55
	Ethanol	35.47±3.68

Discussion

The results of extracts on twitching motility of *Pseudomonas aeruginosa* are displayed in Figure 1. Twitching motility is associated with high cell densities and cell to cell communication, which are the hallmarks of quorum-sensing systems in bacteria. Recently, quorum sensing has been shown to be involved in initiating and controlling motility. Diameter of the stained growth (radius) of tested bacterial strain treated with the plant extracts was found to be less that of control. *Psidium gujava and Carica papaya* extracts, showed complete inhibition of twitching motility in *Pseudomonas aeruginosa*. The comparative plot for the effects of the plant extract is presented in Figure 1. Among the plant extracts tested, *Carica papaya* and *Psidium gujava* extract revealed complete inhibition with 0 cm of stained growth of *Pseudomonas aeruginosa*. In a related study, Karthick and Vivek (2016) [12] obtained similar results for *Aeglemarmelos* (1.0 cm) and *Cynodondactylon* (1.5cm).

Cell adhesion initiates the biofilm formation and pathogenicity in the host. The results for the tested plant extracts on cell

adhesion in figure 2. *Carica papaya* extracts, showed the highest inhibition of cell adhesion in *Pseudomonas aeruginosa*.

Total proteolytic activity of *Pseudomonas aeruginosa* treated with plant extracts were determined by measuring the reduction of azocasein as the substrate by the crude protease present in the supernatant.The result for the tested plant extracts on proteolytic activity is presented in figure 3. *Carica papaya* plant extracts, showed the best inhibition for *Pseudomonas aeruginosa*.

The result for pyocyanin production is presented in Table 1, with *Citrus cinensis* showing the best inhibition. A study by Karthick and Vivek (2016) [12] obtained similar result. Karthick and Vivek (2016) [12] reported pyocyanin production reduction was reduced to 51.85 % in *Aeglemarmelos*, *Azadirachtaindica* (25.46%), *Cynodondactylon* (19.35%), *Eucalyptus globules* (5.46%) and *Ocimum tenuiflorum* (5.25%) respectively. However, the order for reduction by the plant extract was *Carica papaya*>*Psidiumgujava*>*Citrus cinensis*.

Conclusion

The continuous emergence of multidrug-resistant bacteria caused increased need of anti-pathogenic and anti-infective strategy to combat bacterial infections. Natural products provide alternative medicine for treating emerging bacterial infections without leading to antibiotic resistance. *Carica papaya* and *Psidium gujava* plant extract revealed complete inhibition of *Pseudomonas aeruginosa* for twitching motility. *Carica papaya* plant extract showed greater inhibition on cell adhesion and proteolytic activity while the inhibition of pyocyanin production was lowest in *Citrus cinensis* and highest in *Carica papaya*. Overall, the plant extract showed variable effect with *Carica papaya* plant extract showing the best effect against quorum sensing regulated virulence factors in *Pseudomonas aeruginosa*. This study shows the potential use of these plant extracts in the treatment of microbial infections by inhibiting bacterial virulent factors and its associated antibiotics resistance capabilities. It is recommended for medicinal plant extracts such as *Carica papaya* is used as an alternative to existing drugs which will reduce the occurrence of resistance of antibiotics in target organisms

Conflict of interest

The authors declared that there is no conflict of interest regarding the publication of this manuscript.

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