Available online at www.ijpsdr.com

International Journal of Pharmaceutical Sciences and Drug Research 2015; 7(4): 349-353

Research Article

ISSN: 0975-248X
CODEN (USA): IJPSPP

Controlling Biofilm Formation by Inhibiting the Quorum-Sensing Activity of *Pseudomonas aeruginosa* using the Ethanolic Extracts of *Piper nigrum* (Piperaceae) Fruit, *Punica granatum* (Lythraceae) Pericarp, and *Pisum sativum* (Fabaceae) Seed

M.V. Dazal[1], E. Gonzales[1], A. K. Sabino[1], R. G. Salazar[1], B. M. Samar[1], M. Y. San Juan[1*], A. Castillo[2]

[1]*Department of Pharmacy, Faculty of Pharmacy, University of Santo Tomas, España, Manila, 1015, Philippines*
[2]*The Graduate School, University of Santo Tomas, España, Manila, 1015, Philippines*

ABSTRACT

Bacterial biofilm formation can cause serious problems in clinical and industrial settings, which drives the development or screening of biofilm inhibitors. *Pseudomonas aeruginosa* is a well-known pathogen that exhibit biofilm formation through quorum-sensing, which is a bacterial cell-to-cell communication that regulates the production of many virulence factors. The inhibition of biofilm formation is a viable option for bacterial eradication. The antibacterial effect of *Piper nigrum* is related to the presence of phenolic and flavonoid components. *Punica granatum* has been reported to possess a wide range of biological actions, with tannins and alkaloids stated to be the reason of its antibacterial property. *Pisum sativum,* on the other hand, contains various constituents, but the tannins and phenolic compounds stated as responsible for its antibacterial property. The minimum inhibitory concentration using the susceptibility testing of *P. nigrum, P. granatum, P. sativum* ethanolic extracts were 6.67×10^{-4} g/mL, 2.1978×10^{-5} g/mL, and 6.25×10^{-4} g/mL, respectively. On the swarming assay, *P. granatum* and *P. sativum* inhibits swarming motility at concentrations of 2.1978×10^{-2} up to 2.1978×10^{-4} g/mL, and 6.25×10^{-2} to 6.25×10^{-3} g/mL, respectively. The *P. nigrum* extract did not inhibit the motility.

Keywords: Biofilm formation, *Pseudomonas aeruginosa, Piper nigrum, Punica granatum, Pisum sativum,* quorum-sensing.

INTRODUCTION

Gram negative bacteria play a major role in causing superficial cutaneous infections which includes extensive folliculitis, hot tub rashes as well as infectious conditions ranging from localized skin infections to life threatening systemic diseases. [1]

***Corresponding author: Ms. M. Y. San Juan,**
Department of Pharmacy, Faculty of Pharmacy, University of Santo Tomas, España, Manila, 1015, Philippines; **E-mail:** ma.ysabelsj@yahoo.com.ph
Received: 03 June, 2015; **Accepted:** 30 June, 2015

P. aeruginosa is an ubiquitous environmental gram-negative soil bacterium that is also an opportunistic human pathogen causing a variety of different nosocomial infections including pneumonia, catheter and urinary tract infections, as well as sepsis in burn wound and immunocompromised patients. In addition, *P. aeruginosa* is the most prevalent and significant pulmonary pathogen in patients with cystic fibrosis eventually causing fatal lung disease. It demonstrates high intrinsic resistance to antibiotics and has the ability to develop even higher resistance through mutation, acquisition of genetic elements, and

adaptation to environmental conditions, for example through biofilm formation on surfaces. [2] The factors that attributed to resistance in biofilm forming bacteria include the slow growth rate, decreased diffusion of antimicrobials and accumulation of enzymes that are involved in the resistance. Quorum-sensing (QS) or bacterial cell-to-cell communication regulates the production of many virulence factors including biofilm formation in *P. aeruginosa.* [3]

Herbs and spices have been used for thousands of years in traditional medicine to enhance the flavor, color and aroma of food, and also known for their preservative, antioxidative, and antimicrobial roles. [4] These are attributed to the presence of the secondary metabolites, such as proteins, flavonoids, alkaloids, steroids, and phenolic substances, which are used to regain health status. [5]

Piper nigrum (black pepper) is used extensively in Ayurvedic system of medicine. It contains a number of piperidine and pyrrolidine alkamides. [6] The ethanolic extract of *P. nigrum* displayed a broad antimicrobial spectrum and exerted significant antibacterial effect against both gram-positive and gram-negative bacteria. [6] Its antibacterial activity could be related to the presence of phenolic and flavonoid components. [7]

Punica granatum, also known as pomegranate, has a fruit that can be recognized by its round shape, hard and shiny yellow or red colored skin and by its unusual flesh that contains around small edible seeds. [8] Pomegranate peel extracts have been reported to possess wide range of biological actions including anticancer, anti-inflammatory, and antimicrobial activity. Polyphenol compounds such as ellagic tannins, flavanols, anthocyanins, catechins, procyanidins, ellagic acid, and gallic acid have been implicated in various pharmacological activities in the fruit peel. [9] It contains tannins and alkaloids which contribute to its antimicrobial activity. [10]

Common or garden pea, scientifically known as *Pisum sativum*, is an herbaceous annual in the Fabaceae family. [11] It is used as herbal plasters, beauty packs, and cures for fungus infection. [12] It also decreases incidence of cancer, aging, and cardiovascular diseases. [13] The different health benefits of garden pea are due to the presence of various components such as protein, complex carbohydrates, vitamins, minerals, fibers, and antioxidant compound. [14] The *P. sativum* also contains proanthocyanidine, phytoalexins, hydroxybenzoic acid and hydroxycinnamic acid. [15] The antimicrobial activity of *P.* sativum is due to the presence of tannins and phenolic compounds. [16]

Antimicrobials are very important drug category because these drugs are prescribed for a wide range of illnesses from a simple infection to life threatening infections. Because of its wide spectrum, this category of drugs is very important in the medical field. However, microbial resistance towards these drugs is a very serious problem in the medical world. Many drugs nowadays develop resistance even if they were once the most effective in the past. [17]

P. aeruginosa has the capacity to develop multi-drug resistance when they are not given attention especially, when they start to form a biofilm. This phenomenon can cause more complex *P. aeruginosa* infections as time passes. [18] Blocking its QS property is one way of stopping the transmission of signals which causes its biofilm formation.

Nature offers a wide range of plants, algae, and bacteria capable of inhibiting QS mechanisms. This fact has allowed the development of various studies aimed in finding new options for treatment of infections caused by pathogenic bacteria. Accordingly, QS systems are molecular targets with applications in various fields, including medicine, veterinary medicine, agriculture and aquaculture.

P. aeruginosa is one of the most difficult organisms to eradicate therefore modifying the mechanism of action, such as inhibiting its QS activity, will provide a more efficient and more effective means of eradication than targeting the entire organism to be killed. Several studies show that inhibiting the QS would lessen the biofilm formation of *P. aeruginosa* thereby, minimizing its virulence factor, multi-drug resistant incidents will be prevented, and severe health problem cases will be avoided.

MATERIALS AND METHODS

All materials, including Petri-dishes, serological pipettes, test tubes, culture tubes, medium, inoculating loops, cotton swabs, and forceps, were sterilized by autoclaving at 160°C to 170°C for 1 ½ hours.

Collection and Preparation of Plant Material

The plants were collected from various places; the unripe fruit of *P. nigrum* from Polomolok, South Cotabato, Philippines; the fruit of *P. granatum* from San Jose City, Nueva Ecija, Philippines; and the dried seeds of *P. sativum* from Ramgo International Corporation, Quezon City, Philippines. The plants were then transported and authenticated at the Thomas Aquinas Research Complex – Herbarium Department (ISO: S037-00-F047).

Extraction of Plant Material

The three plants were air-dried at room temperature and were ground into smaller particles using the Wiley Mill. The ground dried unripe fruit of *P. nigrum,* dried pericarp of *P. granatum,* and dried seed of *P. sativum* were then weighed using a Mettler balance and percolated with 80% ethanol until exhaustion. The extracts were combined and concentrated using a rotary evaporator until a viscous extract was obtained.

Collection of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa ATCC 27853 strain was requested and purchased at the Thomas Aquinas Research Complex – Microbiology Department.

Identification Test of *Pseudomonas aeruginosa*

P. aeruginosa was inoculated directly on Cetrimide agar using the streak method. Colonies were examined

under a short wavelength (254 nm) ultraviolet light to test the presence of fluorescein. Visual examination revealed the typical yellow-green to blue color indicating the production of pyocyanin. Both pyocyanin and fluorescein are typically produced by strains of *P. aeruginosa* (Figure 1).

Subculturing of *Psuedomonas aeruginosa*

About five milliliters of Nutrient Broth (NB) were dispensed to five screw-capped test tubes and a loopful of bacteria, from the culture plate, was inoculated into the nutrient broth. The culture broth was incubated for 18-24 hours at 35 ± 2°C. The observed turbidity indicates microbial growth.

The turbidity of the incubated bacteria was adjusted with sterile normal saline solution (NSS) to obtain turbidity optically comparable to that of the 0.5 McFarland standard. The absorbance of tubes was read under 625 nm and visually compared against a card with white background and contrasting black lines.

Preparation of Stock Solution

One gram of each plant extract was dissolved in a suitable amount of sterile NSS. Tween 80 was used as a solubilizer. Stock solutions of *P. nigrum, P. granatum,* and *P. sativum* extracts were prepared as 1g/150mL, 1g/45.5mL, and 1g/16mL respectively.

Susceptibility Testing of the Stock Solutions

Standardized inoculum was smeared across Mueller Hinton Agar (MHA) plate in a zigzag pattern four times. After two to three minutes, the sterile blank discs soaked in plant stock solutions were placed on the center of the plates. The plates were then incubated at 35 ± 2°C for 18-24 hours. The zone of inhibition was measured using a vernier caliper. This procedure was done in triplicates and NSS served as the negative control.

Determination of Minimum Inhibitory Concentration

Nine milliliters of Mueller Hinton Broth (MHB) were placed on twelve screw-capped test tubes. In the first test tube, one milliliter of *P. nigrum* stock solution was placed and mixed well. Next, one milliliter of the previous dilution was added onto the succeeding test tubes until one milliliter from the eighth tube was transferred to the ninth test tube. One milliliter from the ninth test tube was removed and discarded. Each step resulted in a further 10-fold decrease in each concentration. Then, 0.5 mL of subculture was placed on the first, 11^{th}, and 12^{th} test tubes, leaving the 10^{th} test tube without subculture. One milliliter of NSS was added on the 12^{th} tube. All tubes were incubated at 35 ± 2°C for 18-24 hours. The presence of turbidity indicates bacterial growth. This procedure was repeated using the *P. sativum* and *P. granatum* stock solutions.

For counterchecking, the contents of all 12 test tubes were smeared on MHA plate in a zigzag pattern.

Disk Diffusion Test for the Serial Dilutions of the Stock Solutions

On the MHA, standardized inoculum was smeared in a zigzag pattern four times. After two to three minutes, the sterile blank discs soaked in the nine serial dilutions of the stock solutions were placed on each plate, and were then incubated at 35 ± 2°C for 18-24 hours. The zone of inhibition was measured using a vernier caliper. This procedure was repeated using the *P. sativum* and *P. granatum* extracts, and done in triplicates with NSS serving as the negative control.

Determination of Anti-Quorum-Sensing Property Swarming Assay

P. aeruginosa swarming plates were prepared by adding 100 g of glucose, 50 g Bacto agar, 50 g of Bacto peptone, and 20 g of yeast extract in one liter of distilled water. An overlay of four milliliters swarming agar was made prior to adding an additional layer of four milliliters swarming agar seeded with 250µL of plant extract solution. After solidification, one loopful of standardized inoculum was streaked on the agar, and was incubated at 35 ± 2°C for 16 hours. Impaired swarming motility of *P. aeruginosa* indicated an anti-QS property of the plant extract.

Statistical Analysis

The statistical tool used for the Disk Diffusion Testing of the serial dilutions of the stock solutions is the One-way ANOVA test. For the determination of the significant difference among the concentrations of each plant extract on the Swarming Assay, the Generalized Fischer test was used.

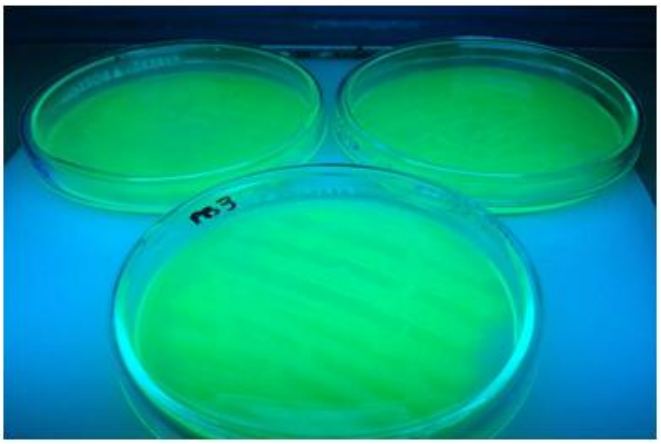

Fig. 1: *P. aeruginosa* colonies

RESULTS

Identification Test of *Pseudomonas aeruginosa*

Visual examination revealed yellow-green colonies, which indicate the presence of pyocyanin. Both pyocyanin and fluorescein are produced by strains of *P. aeruginosa* as shown in Figure 1.

Minimum Inhibitory Concentration and Disk Diffusion

Table 1 shows the presence or absence of microbial growth and the zones of inhibition of the *P. aeruginosa* using the different concentrations of the *P. nigrum* plant extract.

P. nigrum extract concentrations of 6.67×10^{-3} to 6.67×10^{-4} g/mL showed an absence of turbidity, indicating the absence of microbial growth. The stock solution of 6.67×10^{-3} g/mL presented a mean zone of inhibition of 0.825 cm.

There is a significant difference [F= 173.570, p<0.001] in the mean zone of inhibition using 10 concentrations. Post hoc analysis indicates that the mean zone of inhibition using the concentrations 6.67×10^{-12} to 6.67×10^{-6} do not differ (p=1.000). The minimum inhibitory concentration of *P. nigrum* is 6.67×0^{-4} g/mL.

Table 2 shows the presence or absence of microbial growth using different concentrations of the *P. granatum* plant extract against *P. aeruginosa*, as well as its zone of inhibition.

The stock solution of the *P. granatum* extract with a concentration of 2.1978×10^{-2} g/mL has a mean zone of inhibition of 1.546 cm, with a clear solution indicating that the plant extract inhibited the growth of *P. aeruginosa.* There is a significant difference [F= 173.570, p<0.001] in the mean zone of inhibition using the 10 concentrations. Post hoc analysis indicates that the extract concentrations of 2.1978×10^{-2} and 2.1978×10^{-3} exhibit the same inhibitory activity against *Pseudomonas* (p=0.205). The minimum inhibitory concentration of *P. granatum* is 2.1978×10^{-5}g/mL.

Table 1: Minimum inhibitory concentration *P. nigrum* extract

Dilution (g/mL)	Microbial Growth	Mean Zone of Inhibition (cm)
6.67×10^{-3}	(–)	0.825
6.67×10^{-4}	(–)	0.726
6.67×10^{-5}	(+)	0.661
6.67×10^{-6}	(+)	0.502
6.67×10^{-7}	(+)	0.501
6.67×10^{-8}	(+)	0.501
6.67×10^{-9}	(+)	0.500
6.67×10^{-10}	(+)	0.500
6.67×10^{-11}	(+)	0.500
6.67×10^{-12}	(+)	0.500

(+) Presence of microbial growth; (–) Absence of microbial growth

Table 2: Minimum inhibitory concentration *P. granatum* extract

Dilution (g/mL)	Microbial Growth	Mean Zone of Inhibition (cm)
2.1978×10^{-2}	(–)	1.546
2.1978×10^{-3}	(–)	1.434
2.1978×10^{-4}	(–)	1.277
2.1978×10^{-5}	(–)	1.101
2.1978×10^{-6}	(+)	1.004
2.1978×10^{-7}	(+)	0.901
2.1978×10^{-8}	(+)	0.806
2.1978×10^{-9}	(+)	0.676
2.1978×10^{-10}	(+)	0.503
2.1978×10^{-11}	(+)	0.503

(+) Presence of microbial growth; (–) Absence of microbial growth

Table 3: Minimum inhibitory concentration *P. sativum* extract

Dilution (g/mL)	Microbial Growth	Mean Zone of Inhibition (cm)
6.25×10^{-2}	(–)	1.751
6.25×10^{-3}	(–)	1.604
6.25×10^{-4}	(–)	1.485
6.25×10^{-5}	(+)	1.333
6.25×10^{-6}	(+)	1.213
6.25×10^{-7}	(+)	1.058
6.25×10^{-8}	(+)	0.896
6.25×10^{-9}	(+)	0.723
6.25×10^{-10}	(+)	0.637
6.25×10^{-11}	(+)	0.503

(+) Presence of microbial growth; (–) Absence of microbial growth

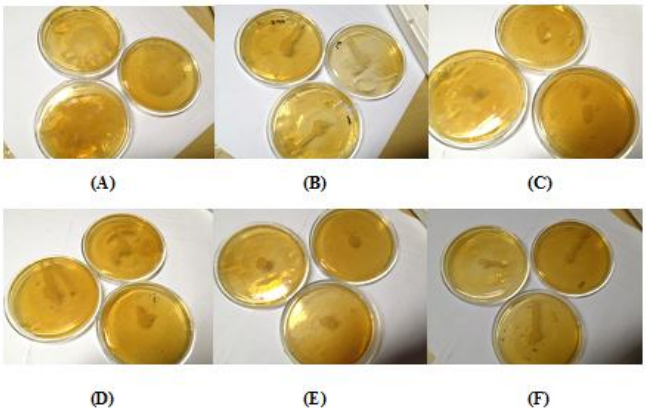

Fig. 2: Swarming assay result. (A) NSS [negative control], motile; (B) *P. nigrum* 6.67×10^{-3} g/mL, motile; (C) *P. granatum* 2.1978×10^{-4} g/mL, non-motile; (D) *P. granatum* 2.1978×10^{-5} g/mL, motile; (E) *P. sativum* 6.25×10^{-3} g/mL, non-motile; (F) *P. sativum* 6.25×10^{-4} g/mL, motile

Table 3 shows the presence or absence of microbial growth and the zones of inhibition of the *P. aeruginosa* using the different concentrations of the *P. sativum* plant extract.

The *P. sativum* concentrations of 6.25×10^{-2} to 6.25×10^{-4} g/mL showed an absence of turbidity indicating the inhibition of the growth of *P. aeruginosa.* The stock solution of the *P. sativum* extract with the concentration of 6.25×10^{-2} g/mL has a mean zone of inhibition of 1.751 centimeters indicating that the plant has an antibiotic property against *P. aeruginosa,* higher than the pomegranate extract. There is a significant difference [F= 359.082, p<0.001] in the mean zone of inhibition using 10 concentrations. Post hoc analysis indicates that the greatest mean zone of inhibition is exhibited by the concentration 6.25×10^{-2} (p=1.000), followed by 6.25×10^{-3}. The mean zone of inhibition of concentrations 6.25×10^{-10} and 6.25×10^{-9} are comparable with each other (p=0.240). The minimum inhibitory concentration of *P. sativum* is 6.25×10^{-4} g/mL.

Swarming Assay Result

The swarming assay was performed to determine the anti-quorum-sensing ability of the plant extracts against *P. aeruginosa*. The motility of *P. aeruginosa* was expressed qualitatively by the spread of the colonies from the center of the plates.

Figure 2 shows the inhibition of the swarming activity of *P. aeruginosa* by the plant extracts wherein *P. granatum* at the concentration of 2.1978×10^{-4} g/mL inhibited the motility of the bacteria. The dilution of 6.25×10^{-3} g/mL was the concentration of *P. sativum* that inhibited the swarming activity of *P. aeruginosa* while *P. nigrum* extract did not inhibit any swarming activity.

DISCUSSION

The antibacterial property of the ethanolic extract of *P. nigrum* could be related to the presence of phenolic and flavonoid components. [7] Studies have reported that the phenolic and flavonoid components have positive effects on human nutrition and health for their antioxidant and antimicrobial activities. [7] The ellagitannins and alkaloids present in the pomegranate

plant contributed to its anti-pseudomonal activity. [10] The antimicrobial activity of *P. sativum* is due to the presence of tannins and phenolic compounds. [16]

Quorum-sensing (QS), the bacterial communication system of gene regulation, controls the basic bacterial processes, including bacterial physiology, adhesion, biofilm formation and virulence. [3] Studies provide increasing evidence that QS has a vital role in the pathogenesis of bacteria and is one of the major causes of persistent infections. Studies have demonstrated the bacteria lacking in QS produce less virulence factors resulting to milder infections. [3] Developing drugs specifically designed to interfere with QS in infecting bacteria could be of considerable medical value, in the event of the increasing antibiotic resistance of many pathogenic species, including *P. aeruginosa.*

There is an increasing trend in the studies concerning biofilm inhibitory properties of plants like ginger, *Zingiber officinale,* which has been used as a culinary and medicinal herb. The plant has antibacterial, anti-tumorigenic, anti-inflammatory, and anti-apoptotic activities. The study of Kim and Park (2013) showed that the ginger extract inhibits the PA14 biofilm formation. [19]

In addition, the ethanolic extracts of *P. granatum* and *P. sativum* are proven to have inhibited biofilm formation of *Pseudomonas.* The researchers concluded that the minimum inhibitory concentrations of *P. granatum* and *P. sativum* are 2.1978×10^{-5} and 6.25×10^{-4} g/mL, respectively. The concentrations of *P. granatum* and *P. sativum* that significantly showed inhibition of quorum-sensing activity of *P. aeruginosa* are 2.1978×10^{-2} to 2.1978×10^{-4} g/mL and 6.25×10^{-2} to 6.25×10^{-3} g/mL, respectively. The MIC of *P. nigrum* extract is 6.67×10^{-4} g/mL, however, it did not inhibit the QS activity of *P. aeruginosa.*

The researchers recommend a more intensive study about understanding the quorum-sensing mechanisms using DNA microarray, focusing on the QS inhibiting activity of *P. granatum.* The researchers also recommend formulating a suitable dosage form from the *P. granatum* pericarp ethanolic extract against *P. aeruginosa*-related diseases.

ACKNOWLEDGEMENT

The researchers would like to take this opportunity to express their profound gratitude to the following people and institutions who helped and inspired the group to finish this study: Manila Central University and University of Santo Tomas for providing facilities to conduct our study, Ms. Nimfa Gambalan, Ms. Joy Calayo and Dr. Jose Mallari for their guidance in the antimicrobial assay and Ms. Andrea Carigma and Ms. Bettina Maria Acuña for the support all throughout this study.

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Source of Support: Nil, Conflict of Interest: None declared.