Study on Antibacterial Activity against *Pseudomonas Aeruginosa* of Carica papaya Seed Extract

Nguyen Van Toan$^{1,2,*}$, Nguyen Thi Truc Linh$^1$

$^1$ School of Biotechnology, International University - Viet Nam National University in HCMC
$^2$ Vietnam National University, Ho Chi Minh City, Vietnam

*Corresponding Author: Nguyen Van Toan, School of Biotechnology, International University - Viet Nam National University in HCMC, Vietnam.

**ABSTRACT**

In this study, the antimicrobial efficacy of Carica papaya seed extract against the growth and development of *Pseudomonas Aeruginosa* was thoroughly investigated. Different concentrations of 0.1% to 0.2% and 0.25% of C. papaya seed extract cultured in two separated characteristics of medium (agar and broth) for testing the colony formation and minimum inhibitory concentration (MIC) against *P. aeruginosa*. The results of this study have shown that the extract of C. papaya seed significantly reduced the growth of *P. aeruginosa* at 0.15% and 0.21% with agar test and broth test in the ratio of 1:4 (v/v) respectively. On the other hand, this study gave the useful information for improving medical from extract of C. papaya seeds usage against the development of *P. aeruginosa*. In conclusion, the C. papaya seed extract could effectively prevent the growth of *P. aeruginosa* at the selectively applied concentrations.

**Keywords:** C. papaya seed extract, *Pseudomonas aeruginosa*, antimicrobial activity.

**INTRODUCTION**

Infectious diseases are disorders that are triggered by microorganisms, such as viruses, fungi, parasite and bacterium that are directly or indirectly passed from one person to another. Infectious disease is the leading cause of death worldwide, especially in young children, particularly in low income countries[1]. Hospital-acquired infections (HAI) is an infection that is acquired in a hospital or other health care facility. HAI can be acquired in hospital, nursing home, rehabilitation facilities, outpatient clinic, diagnostic laboratory or other clinical settings. These infections consist of central line-associated bloodstream infections, catheter-associated urinary tract infections, surgical site infections, hospital-acquired pneumonia, ventilator-associated pneumonia, and *Clostridium difficile* infections[2]. A survey carried out in 183 United State hospitals with 11,282 patients reported that 4% of patients had at least one HAI with the most common microorganism being *Clostridium difficile*. Most infections were surgical site infections, pneumonia, and gastrointestinal infections. A study 2 years earlier by the same group found that 6% (51) of patients had suffered from HAI with the top 75.8% acquiring surgical site infections, urinary tract infections, pneumonia, and bloodstream infections [3]

*P. aeruginosa* is a type of bacteria that is found mostly in the environment, such as soil and water. It can also be found in large numbers on fresh fruits and vegetables. *P. aeruginosa* is an aerobic gram-negative bacteria and *P. aeruginosa* is specified by motile, non-spore forming rods that are oxidase positive and lactose non-fermenters. The water-soluble pigments, pyocyanin and pyoverdine, donate *P. aeruginosa* its distinctive blue-green color on solid media. Like many environmental bacteria, *P. aeruginosa* live in slime-enclosed biofilms which allow for survival and replication within human tissues and medical devices. Related to the production of a biofilm protects *P. aeruginosa* from host-produced antibodies and phagocytes contributing to antibiotic resistance of this bacteria[4]. *Pseudomonas aeruginosa* can cause infections in the blood, lungs (pneumonia), or other parts of the body after surgery. Infection caused by *P. aeruginosa* is common, with the load of infection in hospitalized patients. The National Nosocomial Infections Surveillance (NNIS) System records *P. aeruginosa* to be the second most common organism isolated in nosocomial pneumonia (17% of cases), the third most common organism isolated in both urinary tract infection (UTI) and surgical site infection (11% of cases), and the fifth most common organism isolated
from all sites of nosocomial infection (9% of cases). *P. aeruginosa* is an opportunistic pathogen that rarely causes disease in healthy persons. This organism is mostly considered in the differential diagnosis of a number of gram-negative infections. It is related to the nosocomial infections, often severe and life-threatening, especially in immunocompromised hosts [4]

Due to rapid development of microbial resistance, herbal plant extract has become essential currently to screen effective, safe, cheap, and available therapeutics for their potential antimicrobial effect. According to the report of the World Health Organization, about 80% people used traditional medicine for primary health care treatment. In Asia, plants as medicine indicate long history with human involvement in the environment. Herbal medicines contain different types of novel and unique substances to treat infectious diseases[5] According to pharmaceutical studies, approaching 10 to 20% of plants are used in a positive way in health care to treat harmful diseases[6]

*Carica papaya* (Carica papaya L.), a kind of tropical evergreen fruit tree originated from Mexico and Central America, is mainly found distributed in South Asia. In Vietnam, it is mostly found in South Region. It is one of the herbal remedies, which has recently become a subject of research focus. It is used in traditional medicine for variety of purposes in treating infectious diseases. It is a rich source of three powerful antioxidant vitamin C, vitamin A and vitamin E; the minerals, magnesium and potassium; the B vitamin pantothenic acid and folate and fiber[7] . C. papaya seeds are edible and have spicy taste. C. papaya seeds are grinded and used as a substitute for black pepper. A small pin of C. papaya seeds over a meal is a way to add extra enzymes to the diet and improve digestive appetite.

In summary, this study has been conducted to determine as well as evaluate the possible and highly suitable concentration papaya seed extracts against the growth of *P. aeruginosa*. The aim of this study was to evaluate the effect of papaya seed extracts against the *P. aeruginosa*.

**Materials and Methods**

**Materials**

*C. papaya* fruits were collected from NinhThuan province in the South-Central Coast region of Vietnam. The *C. papaya* seeds were dried overnight at 60°C by using drying oven (Memmert Universal Oven UN75 plus), and then kept in Desiccator for further study and analysis.

*Pseudomonas aeruginosa* ATCC 9027 with deep freeze format (-80°C) was purchased from ATCC (American Type Culture Collection) organization, United States.

Brain Heart Infusion Broth (BHI broth) and Brain Heart Infusion Agar (BHI agar) were purchased from Ensure company, Ho Chi Minh City.

**Methods**

**Preparation of C. Papaya Seed Extract**

The well-prepared C. Papaya seeds were well ground into fine powder by using electric grinder.

To prepare the aqueous extract, 10g of C. Papaya seed powder was added to 100 ml deionized water (ratio 1:10 w/v) at 90°C for 2-3 hours and cool down to 50°C-55°C. The collected mixture was filtered using standard funnel and filtering paper to collect the filtrate (aqueous phase). The filtrate was then centrifuged at the speed of 1500rpm and at room temperature for 15 minutes to remove the insoluble and debris in order to have a purified C. Papaya seed extract at a certain degree.

The following process of antimicrobial activity tests were performed with different concentrations of dilution from the total C. Papaya seed extract; computed by the formula:

\[
\text{w/v} (\%) = \frac{\text{mass of solute (g)}}{\text{volume of solution (mL)}} \times 100
\]

Each different final concentration was diluted using deionized water. The finalized prepared solution was autoclaved at 121°C and 15lbs pressure. After that, each solution was labeled and stored at 4°C for further antimicrobial tests. [8]

**Preparation of the Testing Microorganisms**

*Pseudomonas aeruginosa* ATCC 9027 with deep freeze format (-80°C) was cultured in Mueller-Hinton agar (MHA) medium, then kept in an incubator (Daihan Thermostable If, Incubator, General PurposeThermostable If-50/105/155) for 16-20 hours at 37°C right after checking without contamination, picked randomly one colony and sub-cultured on BHI broth at 37°C for 16-20 hours and used for anti-
bacterial activity testing. To make sure only having *P. aeruginosa* appeared in BHI broth, the taken bacteria in BHI broth was then cultured again in MHA for 16-20 hours at 37°C for gram stain and catalase test.

Before testing anti-bacterial activity, concentration of *Pseudomonas aeruginosa* needed to be known in order to count colony on the agar plate by making serial dilution method to make sure the number of colonies on agar plate wasn’t too many or few. A plate having 30-300 colonies was chosen due to this range is considered statistically significant.

**Testing Antibacterial Activity**

The test based on culturing of *Pseudomonas aeruginosa* on the medium containing the *C. papaya* seed extracts of which followed agar test and broth test methods[8]

**Agar Plate Test**

The inoculum was cultured on BHI agar having a specific concentration of the *C. papaya* seed extract. There were three ratios as 1:4 v/v, 1:2 v/v (mL of *C. papaya* seed extract : mL of medium) with the name test 1, test 2 and test 3 respectively. In each test, 12 different concentration of *C. papaya* seed extract was diluted by using this formula:

\[
C1V1 = C2V2
\]

Where

- \(C1\): concentration of the stock (%)
- \(V1\): volume of the stock (mL)
- \(C2\): concentration of the final solution (%)
- \(V2\): volume of the final solution (mL)

Each different concentration repeated five times. BHI agar was autoclaved at 121°C for 15 minutes and let cool down to 60°C and then the *C. papaya* seed extract was transferred into medium and vortex well before pouring in the petri dish. 10µl of inoculum was taken and spread on the surface of the agar with a L-shaped spreader. The agar plate was incubated at 37°C for 16-20 hours. There were also several treatments which distinct in added the sample into medium [8]

**Broth Test**

Three different ratios as 1: v/v, 1:2 v/v, 1:1 v/v (*C. papaya* seed solution/medium) were done to determine the MIC of the extract against *P. aeruginosa*. Five replicates were applied to this test. BHI broth was autoclaved at 121°C for 15 minutes and let cool down to 60°C, then transferred the sample to analyze into medium with a specific concentration, 10µl of inoculum was added into the solution for incubated at 37°C for 16-20 hours.

| Treatment No. | Concentrate of sample | No. of replicates |
|---------------|-----------------------|------------------|
| 1             | 0.25%                 | 5                |
| 2             | 0.2%                  | 5                |
| 3             | 0.19%                 | 5                |
| 4             | 0.18%                 | 5                |
| 5             | 0.17%                 | 5                |
| 6             | 0.16%                 | 5                |
| 7             | 0.15%                 | 5                |
| 8             | 0.14%                 | 5                |
| 9             | 0.13%                 | 5                |
| 10            | 0.12%                 | 5                |
| 11            | 0.11%                 | 5                |
| 12            | 0.1%                  | 5                |

Colony counter (Galaxy 330’s Colony Counter) was used to determine the number of colony growth and the Minimal Inhibitory Concentration of *C. papaya* seed extract.

**Table1. Experimental design for culturing the *Pseudomonas aeruginosa* for each treatment**

|                             | No. of replicated | Added materials     |        |
|-----------------------------|-------------------|---------------------|--------|
|                             |                   | Agar plate          | Inoculum | *C. Papaya* Seed Extract |
| Negative control            | 1                 | √                   | x       | x                       |
| Negative control 2          | 1                 | √                   | x       | √                       |
| Positive control 1          | 1                 | √                   | √       | x                       |
| Sample                      | 5                 | √                   | √       | √                       |

**Table2. Experimental design for culturing *Pseudomonas aeruginosa* on agar plate test**

| Treatment No. | Concentrate of sample | No. of replicates |
|---------------|-----------------------|------------------|
| 1             | 0.25%                 | 5                |
| 2             | 0.2%                  | 5                |

**Table3. Experimental design for culturing *Pseudomonas aeruginosa* on broth test**
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| Concentration | Reduction (%) |
|---------------|--------------|
| 3             | 0.19%        |
| 4             | 0.18%        |
| 5             | 0.17%        |
| 6             | 0.16%        |
| 7             | 0.15%        |
| 8             | 0.14%        |
| 9             | 0.13%        |
| 10            | 0.12%        |
| 11            | 0.11%        |
| 12            | 0.1%         |

The result of this test was taken by counting the colony with “liquid serial dilution”. The effect of *C. papaya* seed to the *P. aeruginosa* was determined by bacterial counting and calculated by the following formula:

\[
\text{% Reduction (D-value)} = \frac{(\text{Mean CFU } \text{control} - \text{Mean CFU } \text{sample}) \times 100}{\text{Mean CFU } \text{control}}
\]

\[
\log(\text{Reduction}) = \log_{10}(\frac{\text{Mean CFU } \text{control}}{\text{Mean CFU } \text{sample}})
\]

This method was intentionally used to define the minimum concentration of the extract solution that completely forbids the growth of visible bacteria as MIC value.

**RESULTS**

**Preparation of *C. papaya* seed extract**

After the *C. papaya* seed powder and deionized water were well mixed with the ratio of 1:10 w/v, the mixture was sonicated in the water bath (WiseClean WUC Digital Ultrasonic Cleaner) at 90°C for 3 hours and followed by filtering, using standard funnel, filter paper. The *C. papaya* seed mixture had a pungent smell and produced a layer of oil while filtering. Next, the mixture was centrifuged (Universal 320 | 320R Centrifuge) to obtain the aqueous phase.

The obtained yield was calculated by applying the formula:

\[
\text{Yield of extraction (\%)} = \frac{\text{dissolved powder in solution} \times 100}{\text{initial biomass of powder}}
\]

The initial biomass of *C. papaya* powder = 23 g
The insoluble powder into the solution = 21.6 g
The dissolved powder into the solution = 1.4 g
Thus, the yield of extraction is 6.09%.

**Data Analysis**

All data were treated and presented as means ± standard deviation of representatives of similar test carried out in 5 times of replication. Statistical differences in colony forming unit (CFU) were determined by student’s t-test in which, P-values less than 0.05 was considered statistically significant.

The *C. papaya* seed extract had a pale-yellow color after the extraction. From the dried materials of *C. payaya* seeds (23 g), by extraction method 21.6 g the insoluble powder was obtained. After applying the yield of extraction process formula, the extraction yield of *C. papaya* seed extract was 6.09%.

The total *C. papaya* seed extract was diluted into different concentrations and used in the process of antimicrobial activity tests, computed using the formula:

\[
\text{w/v\%} = \frac{\text{mass of solute (g)} \times 100}{\text{volume of solution (mL)}}
\]

\[
\text{w/v\%} = \frac{1.4 (g) \times 100}{140 (mL)} = 1\% 
\]
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The stock solution had the concentration as 1% (w/v%). From the stock solution, 12 concentration of *C. papaya* seed were made (0.1%, 0.11%, 0.12%, 0.13%, 0.14%, 0.15%, 0.16%, 0.17%, 0.18%, 0.19%, 0.2%, 0.25%) in order to test antimicrobial activity.

**Preparation of Microorganisms**

**Identification of the Cultured Streptococcus mutans**

**Identification of the Cultured *P. aeruginosa***

*P. aeruginosa* is a member’s belongings to Pseudomonadaceae family (a member of the Gammaproteobacteria). On the MHA plate, the colony formation has exposed green color due to Pyocyanin produced and diffused through the medium. The colony of *P. aeruginosa* was formed on MHA and BHI agar after incubating at 37°C for 16-20 hours as completely opposed to the negative control was indicated in the Figure 2 and Figure 3 respectively.

![Negative Control](image1.png) ![P. aeruginosa grow on MHA](image2.png) **Figure 2.** *Pseudomonas aeruginosa* grown on MHA

![Negative Control](image3.png) ![P. aeruginosa grow on BHI agar](image4.png) **Figure 3.** *Pseudomonas aeruginosa* grown on BHI agar

![Figure 4. Gram stain of P. aeruginosa](image5.png)
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**Figure 5. Catalase test of P. aeruginosa**

**Serial Dilution Method on P. aeruginosa**

Picked up colonies on BHI agar plate and incubated in BHI broth overnight at 37°C. Diluted the suspension by taking 1 mL from the stock solution to 9 mL of BHI broth. After first tube, each tube was the dilution (1:9) of the previous tube. Repeated this step until 6 test tubes with different concentration was done, then measured OD for each tube. 10µl of each test tube was spread on BHI agar plate and incubated overnight at 37°C.

**Figure 6. Serial dilution of P. aeruginosa performed on BHI agar**

**Figure 6** presented the $10^{-4}$ (CFU/mL) was relevant with 184 colonies formed on BHI agar. According to the result shown on **Figure 3.3.2.1**, the stock solution colony formation unit was $2 \times 10^6$. The OD recorded on $10^{-4}$ (CFU/mL) was at 0.008.

**Determination of Antimicrobial Activity**

**Agar Plate Test**

In the agar plate test, the experiment was carried out by using two controls (positive and negative controls) and three testing samples, including ratios as 1:4 v/v, 1:2 v/v and 1:1 v/v (mL of *C. papaya* seed extract : mL of medium) as well as with twelve different concentrations of *C. papaya* seed extraction (0.1%, 0.11%, 0.12%, 0.13%, 0.14%, 0.15%, 0.16%, 0.17%, 0.18%, 0.19%, 0.2% and 0.25% (w/v)) for each the ratios, with name test 1, test 2, test 3 respectively. The experiment was repeated five times with each ratio and concentration. After adding *C. papaya* seed solutions and incubating at 37°C for 16-20 hours, the data will be recorded.
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Table 4. The antimicrobial activity result of *C. papaya* seed extract testing against *P. aeruginosa* after incubation at 37°C for 16-20 hours on BHI agar plate

| No. of test | Concentration of *C. papaya* seed extract (% w/v) | Mean of *P. aeruginosa* colony formation per plate | Standard deviation | Reduction of *P. aeruginosa* colony formation per plate (%) | Log reduction |
|-------------|-----------------------------------------------|---------------------------------------------|------------------|-------------------------------------------------|--------------|
| Test 1 (1:4) | Control                                      | 207.5                                      | 81.51            | 63.47                                          | 0.44         |
|             | 0.1%                                         | 75.8                                       | 20.82            | 63.47                                          | 0.44         |
|             | 0.11%                                        | 58.2                                       | 16.58            | 71.95                                          | 0.55         |
|             | 0.12%                                        | 44.4                                       | 6.34             | 78.60                                          | 0.67         |
|             | 0.13%                                        | 5                                          | 1.26             | 97.60                                          | 1.62         |
|             | 0.14%                                        | 1.6                                        | 1.35             | 99.23                                          | 2.11         |
| Test 2 (1:2) | Control                                      | 133.5                                      | 68.86            | 76.93                                          | 0.64         |
|             | 0.1%                                         | 30.8                                       | 5.07             | 88.47                                          | 0.94         |
|             | 0.11%                                        | 15.4                                       | 3.9              | 88.47                                          | 0.94         |
|             | 0.12%                                        | 0.4                                        | 0.48             | 99.70                                          | 2.52         |
| Test 3 (1:1) | Control                                      | 227.6                                      | 67.22            |                                                 |              |

Figure 7. The percentage of bacterial reduction (%) at different concentration of *C. papaya* seed extract with different ratios in agar media

**Broth Test**

The broth experiment was conducted with three control samples and three ratios testing samples as 1:4 v/v, 1:2 v/v and 1:1 v/v (ml of medium: ml of *C. papaya* seed extract) with different concentrations of the extract and each experiment was repeated five times. The turbidity of the solution was used to test the bacterial growth after incubating for 16-20 hours, and dilution method was used to count colony from a broth medium that sub-cultured on agar plates. Taking 1 mL of the inoculum suspension mixed with 9 mL of sterile distilled water. From this suspension, 12-fold dilution factor was made and 0.1 mL of final dilution was cultured on agar plated at 37°C for 16-20 hours.

Table 5. The antimicrobial activity result of *C. papaya* seed extract testing against *P. aeruginosa* after sub-culture and incubation at 37°C for 16-20 hours on BHI agar plate from broth test pieces

| No. of test | Concentration of *C. papaya* seed extract (% w/v) | Mean of *P. aeruginosa* colony formation per plate | Standard deviation | Reduction of *P. aeruginosa* colony formation per plate (%) | Log reduction |
|-------------|-----------------------------------------------|---------------------------------------------|------------------|-------------------------------------------------|--------------|
| Test 1 (1:4) | Control                                      | 207.5                                      | 81.51            | 31.2                                           | 0.16         |
|             | 0.12%                                        | 142.8                                      | 36.18            | 31.2                                           | 0.16         |
|             | 0.13%                                        | 128.6                                      | 24.71            | 38                                             | 0.2          |
|             | 0.14%                                        | 103.4                                      | 27.6             | 50                                             | 0.3          |
|             | 0.15%                                        | 101.8                                      | 38.2             | 51                                             | 0.309        |
|             | 0.16%                                        | 84.4                                       | 7.3              | 59.33                                          | 0.4          |
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| Test 2 (1:2) | Control | 0.17% | 83.2 | 26.16 | 60 | 0.4 |
|-------------|---------|-------|------|-------|-----|-----|
|             |         | 0.18% | 45.2 | 16.02 | 78.22 | 0.66 |
|             |         | 0.19% | 10.8 | 8.3   | 94.8 | 1.28 |
|             |         | 0.2%  | 0.6  | 0.8   | 99.77 | 2.54 |
| Test 3 (1:1) Control | 133.5 |
|             |         | 0.13% | 128.2 | 17.37 | 4.12 | 0.02 |
|             |         | 0.14% | 113.4 | 22.00 | 15.06 | 0.07 |
|             |         | 0.15% | 73.8 | 18.69 | 64.44 | 0.25 |
|             |         | 0.16% | 36.6 | 16.95 | 72.59 | 0.56 |
|             |         | 0.17% | 20.8 | 10.32 | 84.42 | 0.8 |
|             |         | 0.18% | 0.8 | 1.16 | 99.4 | 2.22 |
| Test 2 (1:2) Control | 227.6 | 67.22 |

**Figure 8.** The percentage of bacterial reduction (%) at different concentration of *C. papaya* seed extract with different ratios in broth media

**DISCUSSION**

Carica papaya seed extracts inhibited majority of tested microorganisms[9]

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a highly drug resistant and opportunistic pathogen. Due to the permeability barrier in the outer membrane it is naturally resistant to many antibiotics. Infections caused by *P. aeruginosa* are increasing both in hospitals and in general community and it has been reported as one of the principal causes of nosocomial pathogen, particularly among immuno-compromised patients [10]. People with cystic fibrosis, burn victims, individuals with cancer, and persons infected with HIV are particularly at risk of disease resulting from *Pseudomonas aeruginosa*. Unlike many environmental bacteria, *Pseudomonas aeruginosa* has a remarkable capacity to cause disease in susceptible hosts [11].

From the all presented results, it can easily be seen that the identification tests were appropriately conducted and that was why the all collected data had shown a clear picture of inhibition of Carica papaya seed extract against the growth of *P. aeruginosa* at certain degrees.

For the testing with agar plate, the bacterial reduction of different concentration as 0.1% and 0.11% of test 1 and 2 were significant differences. Comparisons of *P. aeruginosa* reduction showed no significant differences between the 0.13%, 0.14% in test 1 of *C. papaya* seed extract concentrations. Because of the differences among the other concentrations of the extract between 0.1% to 0.13% in test 1 and 0.1% to 0.12% in test 2 were significant (p<0.05) and from 0.14% to 0.2% and 0.25% was no significant difference. When the concentration of sample increasing, the probability of colony formation on agar plates would be a clear reduction in order of 0.1%, and 0.11% concentrations in test 1, test 2, and approximate or no colony from the concentration of 0.13% to 0.2% and 0.25% in three tests. So, the proportion of bacterial reduction underwent a rise as well as the increasing concentration of *C. papaya* seed extract. As a result, the increased amount of *C. papaya* seed extract in the medium inhibited the growth of *P. aeruginosa* at a wider range.

In the case of broth testing, as shown in the Figure 7 and Figure 8, At the concentration of 0.19% in test 1, 0.18% in test 2, the *C. papaya*
seed extract started their antimicrobial activity with the percentage of *P. aeruginosa* reduction was higher than 90% due to the evenly low colony formation.

Also, from the Table 5, the percentage of bacterial reduction performing in broth medium, after the broth culture was put on agar plate, and then incubated in 24 hours for counting the colony formation units as CFU. The CFU was calculated for the reduction’s proportion to determine whether sample had antimicrobial activity or not. It was finally found that the growth of *P. aeruginosa* was about to be inhibited by the C. Papaya seed extract for at least 24 hours at the concentration of 0.20 % with one-fourth per total solution.

**CONCLUSIONS**

In this study, the effects of *C. papaya* seed extract against the growth of *P. aeruginosa* were successful investigated. The extracted concentration of *C. papaya* was established that at least 0.14% (w/v) of one-fourth per total medium preparation reduced the proportion of *P. aeruginosa* over 95%. The extracted concentration of the *C. papaya* seeds at 0.20 % of the most suitable concentration, which showed a minimal and most effective on the inhibition of *P. aeruginosa*.

Compare to other studies, water extract of *C. papaya* seed had lower effect than ethanol extract.

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