International Journal of Biology Research
ISSN: 2455-6548; Impact Factor: RJIF 5.22
Received: 12-08-2019; Accepted: 16-09-2019
www.biologyjournal.in
Volume 4; Issue 4; October 2019; Page No. 36-43

Study on the antibacterial activity against pseudomonas aeruginosa of lemon seed extract

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Abstract
The present paper deals with, the antimicrobial efficacy of lemon seed extract against the growth and development of *Pseudomonas aeruginosa (P. aeruginosa)* was thoroughly investigated. Different concentrations of 5% to 15% and 20%, and then cultured in two separate characteristics of media as liquid and solid for testing the colony formation as well as to determine the minimum inhibitory concentration (MIC) against *P. aeruginosa*. The obtained results of this study have indicated that the extract of lemon seeds showed not only the statistically significant reduction of of P. aeruginosa colony formation in order of 5% to 15% and 20%, but also pointed out the statistically significant reduction of *P. aeruginosa* colony formation at 10% and 13%, with agar test and broth test in the ratio of 1:4 (v/v) respectively. Beside that this project also contributes to useful information for improving medicinal from solution extracts of lemon seed usage against the growth and development of *P. aeruginosa.*
In summary, the lemon seed extract could effectively inhibit the growth of Pseudomonas aeruginosa at the selectively applied concentrations.

Keywords: lemon seed extract, *Pseudomonas aeruginosa*, antimicrobial

1. Introduction
In the recent decades, infectious disease has been receiving a great deal of attention, worldwide. The incidence of infection has enhanced by population explosion and environmental pollution (Maria Cristina Schneidera *et al*., 2018) [1]. The hospital environment is especially, where to hide a lot of high risk of infections in hospital-acquired infections occur in patients under medical care. Hospital infections occur worldwide both in developed and developing countries, with 7% and 10% respectively. These infections occur during hospital stay, they cause long-drawn stay, disability, and economic burden. Frequently prevalent hospital infections include bloodstream infections-associated central line, urinary tract infections-associated catheter, surgical site infections, pneumonia-associated ventilator (Hassan Ahmed Khan *et al*., 2017) [2].
Hospital infection is commonly occurred as during hospitalization, the patient is exposed to pathogens such as bacteria, viruses and fungal parasites that through different sources environment, healthcare staff, and other infected patients [2]. Whereas, the burden of infection in hospitalized patients caused by *Pseudomonas aeruginosa (P. aeruginosa*) is common and emerging as an important pathogen during the past two decades. It causes between 10% and 20% of infections in most hospitals (Bodey GP *et al*., 1983) [3], in which *P. aeruginosa* to be the second most common organism isolated with 17% of cases in nosocomial pneumonia, the third most common organism isolated in both urinary tract infection (UTI) and surgical site infection with 11% of cases, and the fifth most common organism isolated with 9% of cases from all sites of nosocomial infection by The National Nosocomial Infections Surveillance (NNIS) System reports (Richards MJ *et al*., 1999) [4]. *P. aeruginosa* infection may occur at sites where a break in normal barrier defenses occurs through puncture wounds, skin trauma, or burns. More commonly, *P. aeruginosa* acts as an opportunist, infecting patients who have defective mucosal immunity, impaired pulmonary clearance as in cystic fibrosis, or disease-induced or iatrogenic neutropenia (Paul J. Planet, 2018) [5].
P. aeruginosa resides in the intestinal tract in about 10% of healthy individuals and it is found in most areas of human skin (axilla, groin), saliva and fresh water environments (streams, lakes, and rivers) (Dasantila Golemi-Kotra, 2008) [6], as well as sinks, showers, respiratory equipment, even contaminating distilled water (Favero MS *et al*., 1971) [7]. They are difficult organisms to eradicate from areas that become contaminated, such as operating rooms, hospital rooms, clinics, and medical equipment (Engleberg NC dV *et al*., 2007) [8]. *P. aeruginosa* is a member of the genus P*seudomonas*, an aerobic gram-negative bacterium, non-spore forming, an actively motile bacterium, rod shape (bacillus) bacterium, measuring 0.5–0.8 µm x 1.5–3.0 µm, ability to form biofilm, and positive oxidase, catalase test, negative coagulation test [6]. In the culture medium, 4 main types of pigment of *P. aeruginosa* produced and diffused out into the surrounding medium and imparts a characteristic color to the medium indicating the suspected growth of *P. aeruginosa* include: Pyocyanin (Bluish-Green), Fluorescein (Greenish-Yellow), Pyorubicin (Reddish-Brown), Pyomelanin (Brown to black). While culturing form colonies with a fluorescein greenish color, sweet odor, some of the strains show beta hemolysis in Blood Agar medium. In addition, *P. aeruginosa* remains among the top

five bacterial species most commonly found in nosocomial infections; furthermore, incidence of hospital-acquired *P. aeruginosa* infections is on the rise. The reported cases hospital acquired pneumonia caused by *P. aeruginosa* has increased from 9.6% to 18.1% from 1975 to 2003 (Annette E. LaBauve and Matthew J. Wargo Gaynes, 2012, Gaynes and Edwards, 2005) [9, 10]

Lemon is an herbal important plant from the Rutaceae family and originated in Southeast Asia. It is a rich source of vitamin C and it is cultivated mainly for its alkaloids, which are having the antibacterial potential and anticancer activities in crude extracts of different parts such as leaves, stem, root, juice, peel, seed and flower of Lemon. As a result, it against clinically significant bacterial strains has been reported (Kawaii *et al*., 2000) [11]. Citrus flavonoids have a broad biological activity spectrum that have shown to display numerous pharmacological activities including antibacterial, anticancer, antifungal, anti-diabetic, and antiviral activities (Burt, 2004; Ortuño *et al*., 2006) [12, 13]. Citrus species have reported that can be considered responsible for activity against many clinically, isolated bacterial strains by the presence of limonoids (Giuseppe *et al*., 2007) [14]. Limonoids showed good antibacterial and antifungal activity that obtained from Citrus Limon. In addition, extracts of citrus fruit (e.g. lemon, orange and grapefruit) have reported are among the most studied natural antimicrobials, and it has shown to be effectively go downward the growth of bacteria (Corbo *et al*., 2008) [15]. Limonoids have three forms in citrus seeds: monolactone, dilactone and glucosides by 36 different variations of limonoid skeleton. The first limonoids was identified mainly limonin as the bitter constituent of citrus seed which was following by accumulated nomilin, obacunone, and deacetylnomilin as well as dilactones, nomilinic acid, deacetylnomilinic acid and their glucosides (Nguyen Van Toan, 2018) [16]. Being good source for certain limonoids, seeds will be chosen as raw material for isolation and purification. Seeds contain relatively higher concentration of limonoids, speculated to be as much as 1 % of dry weight. Therefore, seeds, instead of the fruits, will be chosen for purification of limonoids (Amit Vikram, 2011) [17].

According to World Health Organization, the medicinal plants is the best source to obtain a variety of drugs. About 80% of individuals from developed countries relies on traditional medicine for their primary healthcare needs. Therefore, plants have been valuable and indispensable sources of natural products for the health of human beings and they have a great potential for producing new drugs (Nascimento *et al*., 2000). On the other hand, the side effect and adverse effect of the secondary metabolites may be considered as cytotoxicity compounds to a human body. Thus, the trend of studying the correlation of attracted therapeutic agents is becoming more and more essential and investigated to better understand their properties, safety and efficiency (Nascimento *et al*., 2000) [18] including the use of lemon seed. However, how to utilize and apply the lemon seed extracts for studying and evaluating the antimicrobial activity on the Pseudomonas aeruginosa is no clear information. In addition, the result of choosing the appropriate concentration of the extracts from lemon seeds should be controlled in order to meet the proposed works that need to be determined accurately.

In summary, this study has been investigated in a search for a better understanding of the lemon seed extract against the bacterial activity in general, determining as well as evaluating the possibly suitable concentrations of lemon seeds extracts against the growth of Pseudomonas aeruginosa.

2. Materials and methods

Materials

Lemon fruits were collected from Ben Tre province in the Southwest of Viet Nam.

The lemon seeds were dried at 105oC by using the drying oven, leading to get the consistent moisture content of 5% and then kept in Desiccator to ready for further study and analysis.

Pseudomonas aeruginosa ATCC 15442 with freeze-dried format was purchased from Lan Oanh Company, Ho Chi Minh City.

Brain Heart Infusion Broth were purchased from Doviet Joint Stock Company, Ho Chi Minh City. Brain Hear Infusion Agar was purchased from Gen Lab Company, Ho Chi Minh City.

Methods

Research location

All the research experiments were conducted and completed at the IU Laboratories on the 6th floor of School of Biotechnology, International University, Vietnam National University, HCMC.

Preparation of Lemon Seed Extract

The well-prepared lemon seeds were well grounded into a fine powder by using a blender.

The lemon seed powder and deionized water were well mixed with the ratio of 1:10 w/v, and then incubated in the water bath at 90°C for 2-3 hours and cool down to 50^{O}C-55^{O}C.

The extract was filtered by standard funnel, filter paper and then the filtrate was centrifuged at 1500 rpm for 15 minutes at 4°C to remove insoluble debris to collect the aqueous phase that was a purified lemon seed extract at a certain degree.

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

$$\text{W/v (\%)} = \frac{mass\ of\ solute(g)}{volume\ of\ solution(mL)}\ x\ 100$$

Each different final concentration was diluted by using deionized water. Each finally prepared solution was separated, labeled and autoclaved at 121^{O}C and 15lbs pressure before using for further antimicrobial tests.

Preparation of Microorganisms

Pseudomonas aeruginosa ATCC 15442 in a freezing-dried form was cultured in Blood Agar (BA) medium, then incubated for 16–24 hours at 37°C right after having checked without contamination, picked randomly one colony and sub-cultured on Brain-Heart infusion broth (BHI) at 37°C for 16–24 hours and used for antimicrobial activity testing.

To ensure only having Pseudomonas aeruginosa appeared in BHI broth medium, the taken bacteria in BHI was then cultured again in BA from 24-36 hours at 370C for gram stain and catalase test. To safely keep the bacterial for a

long time, this process needed to be weekly performed in order to check the bacterial.

Testing Antibacterial Activity

The test based on culturing of Pseudomonas aeruginosa on the medium containing the lemon seed extracts of which followed agar test and broth test methods (Nguyen Van Toan, 2018).

Agar Plate Test

The inoculum was cultured on BHI agar containing a specific concentration of the lemon seed extract. There were three ratios as 1:4 v/v, 1:2 v/v, 1:1 v/v and each different concentration repeated 5 times for each ratio.

BHI Agar was autoclaved at 121oC for 15 minutes and cooled down to about 600C and then the lemon seed extract is transferred into medium with a specific concentration and vortex well before pouring into the petri dish. 20µl of inoculum was taken and spread on the surface of the agar with a sterile cotton swab and then having it incubated at 37°C for 24 hours. There were also several treatments which distinct in added the sample into medium (Nguyen Van Toan, 2018).

Table 1: Experimental design for culturing the Pseudomonas aeruginosa for each treatment.

	No. of replicated	Added materials		
		Medium	Inoculum	Lemon seed extract
Negative control	1	√	X	x
Negative control 2	1	√	X	√
Positive control1	1	√	√	x
Sample	5	√	√	√

(√: with; x: without)

Table 2: Experimental design for culturing Pseudomonas aeruginosa on agar plate test.

Treatment No.	Concentration of sample	No. of replicates
1	20%	5
2	15%	5
3	14%	5
4	13%	5
5	12%	5
6	11%	5
7	10%	5
8	9%	5
9	8%	5
10	7%	5
11	6%	5
12	5%	5

Colony counter used to determine the number of colony growth and the Minimal Inhibitory Concentration of lemon seed extract.

Broth Test

Three different ratios as 1:4 v/v, 1:2 v/v, 1:1 v/v (lemon seed solution/medium) were performed to determine the Minimal Inhibitory Concentration (MIC) of lemon seed extract against P. aeruginosa. The five replicates were applied to this kind of tests.

Broth medium was autoclaved at 121oC for 15 minutes and cooled were down to about 600C, then transferred the sample to analyze into medium with a specific concentration, 20 µl of inoculum was added into the solution for incubated at 37°C for 24 hours (Nguyen Van Toan, 2018).

Table 3: Experimental design for culturing Pseudomonas aeruginosa on broth test.

Treatment No.	Concentration of sample	No. of replicates
1	20%	5
2	15%	5
3	14%	5
4	13%	5
5	12%	5
6	11%	5
7	10%	5
8	9%	5
9	8%	5
10	7%	5
11	6%	5
12	5%	5

The result of this test was collected by counting the colony with "liquid serial dilution" method (David R. Caprette, 2016). The effect of a lemon seed to the pathogen was determined by bacterial counting and calculated by the following the formula (Microchem Laboratory, 2015):

$$\textbf{\% Reduction (D-value)} = \frac{[(Mean\ CFU)control - (Mean\ CFU)sample]x\ 100}{(Mean\ CFU)control}$$

$$\textbf{Log (Reduction)} = \text{Log10}(Mean\ CFU)control - \text{Log10}(Mean\ CFU)sample$$

This method was intentionally used to determine the minimum concentration of the extract solution that completely inhibits the growth of visible bacterial as minimum inhibitory concentration (MIC).

Statistical analysis

All data were presented and treated as means ± standard deviation of representation of similar test carried out in 5 times of replication. Statistical differences in colony forming unit (CFU) were determined by two-way ANOVA

in which, P-values less than 0.05 was considered statistically significant.

3. Results and discussion

Preparation of Lemon Seed Extract

After the lemon seed powder and deionized water were well mixed with the ratio of 1:10 w/v, the mixture was sonicated in the water bath at 90°C for 2-3 hours and followed by filtering, using standard funnel, filter paper and then centrifuging the filtrate to obtain the aqueous phase. The lemon seed extract had a dark yellowish-brown colored solution. The obtained yield was calculated by applying the formula:

$$\text{Yield of extraction (w/v \%)} = \frac{\textbf{dissolved powder in solution x 100}}{\textbf{intitial biomass of powder}}$$

The initial biomass of a lemon seed powder = 30 grams
The insoluble powder into the solution = 15 grams
The dissolved powder into the solution = 15 grams

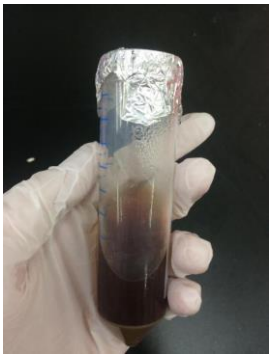


Fig 1: The solution of lemon seed extract.

Lemon seed is a significant medical resource containing the high level of bioactive compounds which have been found to have antimicrobial properties (Mabhiza *et al*., 2016)[19] and exhibits anti-oxidative, anti-inflammatory, anti-cancer, and anti-allergy effects (Zhao *et al*., 2015. And Chaudhari *et al*., 2016) [20, 21]. The difficulty in comparison in-between research results was due to analytical differences. The difficulty in comparison in-between research results was due to analytical differences. The material used, manufacturers, and the extraction technique implied during the experiment were factors, leading to alter the obtained results. In addition, the concentration of bioactive components might be different in terms of the geographical location, seasonal, and cultivation processes and all these factors affect the efficacy of lemon seed extract, or some or all the active extraction ingredients might be inactivated during the extraction preparation processes (Betz *et al*., 2011)[22]

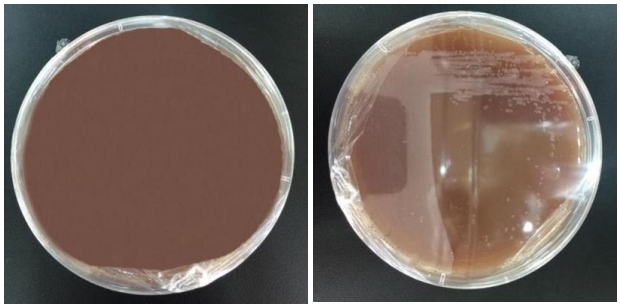
Preparation of Microorganisms

Identification of the cultured Pseudomonas *aeruginosa*

Aeruginosa is a member's belongings to Pseudomonadaceae family (a member of the Gammaproteobacteria). On the blood agar plate, the colony formation has exposed white color, sweet odor and formed weak beta hemolysis due to secrete phospholipase enzymes (Ostroff *et al*., 1990) [23] which directly lyse sheep erythrocytes after an overnight (Figure 2). Beside that could be clearly observed a bluish green pigment because of Pyocyanin (Reyes *et al*., 1981) [24] produced and diffused through the medium on BHI agar (Figure 3). The colony of P. aeruginosa was formed on Blood Agar and BHI agar after incubating at 37oC for 24 hours as completely opposed to the negative control was indicated in the figure 2 and figure 3 respectively.

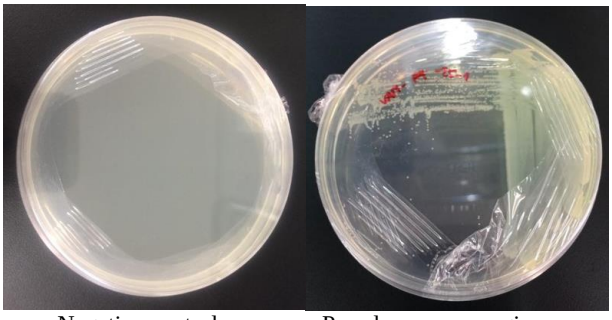
Figure 2 Pseudomonas aeruginosa grown on blood agar medium (on the right).

Figure 3 Pseudomonas aeruginosa grown on a BHI agar medium (on the right).



Negative control | Pseudomonas aeruginosa Grown on blood agar

Fig 2: Pseudomonas aeruginosa grown on blood agar medium (on the right).



Negative control | Pseudomonas aeruginosa Grown on BHI agar

Fig 3: Pseudomonas aeruginosa grown on a BHI agar medium (on the right).

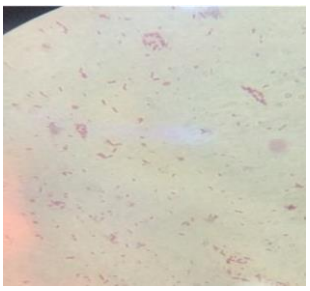


Fig 4: Gram stain of Pseudomonas aeruginosa.

Fig 5: Catalase test of Pseudomonas aeruginosa.

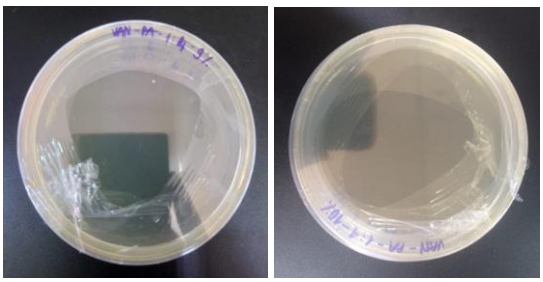
From the figure 2, 3 and 4, Pseudomonas aeruginosa could be differentiated from others since it was a gram-negative bacterium and had a pink color and in rod shape (bacillus) bacterium after performing gram staining. In addition, the identified Pseudomonas aeruginosa from other aerobic gram negative, catalase test was used (Adams *et al*., 2000) [25] and the catalase test showed a positive result from the figure 5.

To avoid any possible contaminants, each individual test should be performed singularly and separately since many strains were cultured at the same time in the laboratory, thus opportunistic bacteria contamination could not be avoided.

Determination of antimicrobial activity

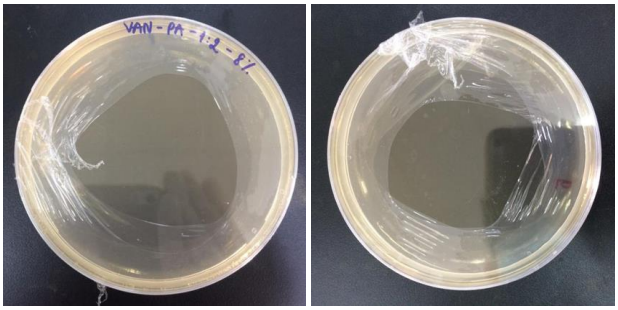
Agar plate test

In the agar plate test, the experiment was conducted 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 medium: ml of lemon seed extract) as well as with twelve different concentrations of lemon seed extraction (5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15% and 20% (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 lemon seed solutions and incubating at 37oC for 24 hours, the data will be recorded.



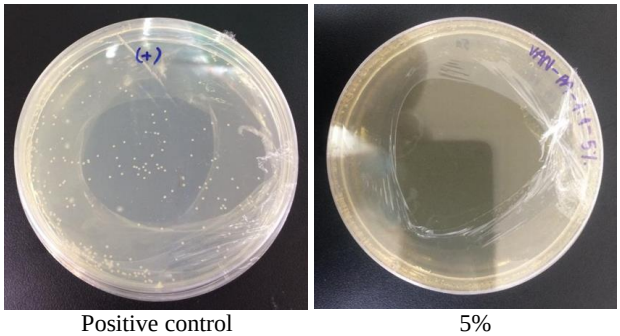
9% 10%

Fig 6: Sample concentration testing on agar plate with ratio 1:4 (v/v) with the minimal inhibitory concentration at 10% (on the right) in comparison with 9% of sample (on the left).



8% 9%

Fig 7: Sample concentration testing on agar plate with ratio 1:2 (v/v) with the minimal inhibitory concentration at 9% (on the right) in comparison with 8% of sample (on the left).



Positive control 5%

Fig 8: Sample concentration testing on agar plate with ratio 1:1(v/v) with the minimal inhibitory concentration at 5% (on the right) in comparison with control (on the left).

Table 4: The antimicrobial activity result of lemon seed extract testing on agar plate.

		Concentration (%)	Mean	Standard deviation	Reduction (%)	Log reduction
Test 1 (1:4)	Control		292.8	23.435		
		5%	88	8.689	69.95	0.5221
		6%	49.4	4.722	83.13	0.7728
		7%	35.6	2.702	87.84	0.9151
		8%	3.4	0.548	98.84	1.9351
		9%	1.2	0.447	99.59	2.3874
Test 2 (1:2)	Control		290.6	20.995		
		5%	48.6	2.881	83.28	0.7767
		6%	24.4	3.507	91.60	1.0759
		7%	15.2	0.837	94.77	1.2815
		8%	0.6	0.548	99.79	2.6851
Test 3(1:1)	Control		293.2	17.138		

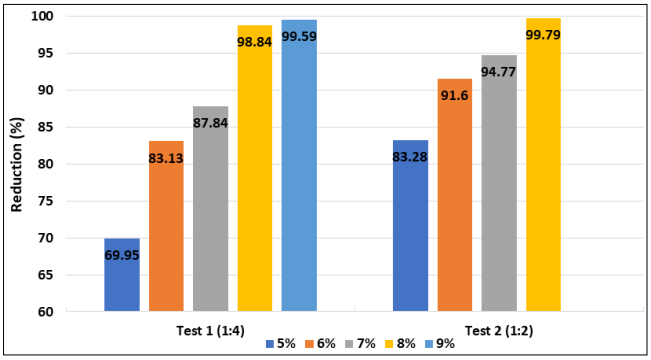


Fig 9: The percentage of bacterial reduction (%) at different concentration lemon seed extract with different ratios in agar media.

For the testing with agar plate, the bacterial reduction of different concentration as 5 %, 6%, 7% of test 1 and 2 were significant differences. Comparisons of Pseudomonas aeruginosa reduction showed no significant differences between the 8%, 9% in test 1 and 8% in test 2 of lemon seed extract concentrations. Because of the differences between the other concentrations of the extract between 5% to 7% in each test were significant ($p<0.005$), and from 8% to 15% and 20% 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 5%, 6%, 7% concentrations and almost no colony from the concentration of 8% to 15% and 20%. So, the proportion of bacterial reduction underwent a rise as well as the increasing concentration of lemon seed extract (Figure 9). Beside that to have the influence of ratios on the formation of colony with different concentrations and three

ratios (1:4 v/v, 1:2 v/v and 1:1 v/v) were significant different ($p<0.005$). When the volume of the lemon seed solution was increased in the agar medium, leading to the concentration of lemon seed extract can be decreased but still inhibited the bacteria growth or kill them.

In addition, there were no colony formation in the 5 replicates at the concentration of 10%, 9% and 5% of the sample, with test 1, test 2 and test 3 respectively after incubating for 24 hours. At the concentration of 8% in test 1, 6% in test 2, the lemon seed extract starts their antimicrobial activity with the percentage of Pseudomonas aeruginosa reduction was higher 90% (Figure 9) due to the steadily low colony formation. Although Pseudomonas aeruginosa was found to be effective at a higher volume and concentration, which showed a marked antimicrobial activity and should be considered to replace the synthetic medicine for treating. In conclusion, the lemon seed solution given their antimicrobial activity for Pseudomonas aeruginosa when it contributed at least 10% (MIC) at a one-fourth per total

Broth test

The broth experiment was conducted with 3 control samples and 3 ratios testing samples as 1:4 v/v, 1:2 v/v and 1:1 v/v (ml of medium: ml of lemon seed extract) with different concentrations of lemon seed extract and each experiment was repeated five times. The turbidity of the liquid medium was used to test the bacterial growth after incubating for 24 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 distill water. From this suspension, 12-fold-dilution factor was done, and 0.1 ml of final dilution was cultured on agar plated at 37oC for 24 hours.

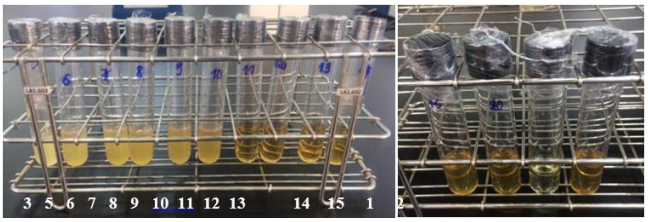


Fig 10: Concentration broth with the ratio 1:4(v/v) in test 1.

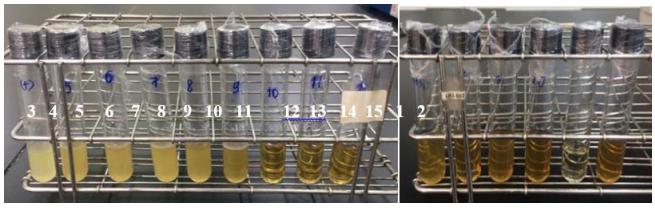


Fig 11: Concentration broth with the ratio 1:2(v/v) in test 2.

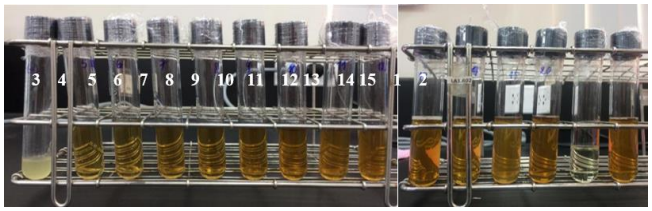


Fig 12: Concentration broth with the ratio 1:1(v/v) in test 3.

Test tube 1: Negative control 1 with 3ml BHI broth medium only.

Test tube 2: Negative control 2 with 3ml BHI broth medium and the lemon seed extract.

Test tube 3: Positive control with 3ml BHI broth medium and 20ul Pseudomonas aeruginosa inoculum.

Test tube from 4 to 15: contains 20µl bacterial inoculum with three ratios of the lemon seed extract containing different concentration 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, and 20%.

In a broth test experiment, the solution in the test tube with concentrations of 11% to 15% and 20% was clear and slightly transparent, which illustrated no bacteria growth in the ratio 1:4 (v/v) in figure 10. As shown in figure 11, the liquid inside test tube with concentrations from 10% to 15% and 20% indicated the clear and transparent liquid that verify no bacteria growth. Similarly, at the ratio 1:1 (v/v), the concentration of 5% to 15% and 20% showed no bacteria growth in the test tube with the clear and transparent liquid in figure 12.

Table 5: The antimicrobial activity result of lemon seed extract testing on broth media.

		Concentration (%)	Mean	Standard deviation	Reduction (%)	Log reduction
Test 1 (1:4)	Control		292.8	23.435		
		5%	121.2	5.404	58.61	0.3831
		6%	100.4	5.727	65.71	0.4648
		7%	71.8	6.301	75.48	0.6104
		8%	54.6	4.037	81.35	0.7294
		9%	45.2	2.775	84.56	0.8114

		10%	22.6	2.408	92.28	1.1125
		11%	7	1.581	97.61	1.6215
		12%	2.4	1.140	99.18	2.0864
Test 2 (1:2)	Control		290.6	20.995		
		5%	89.4	1.342	69.24	0.5120
		6%	73.8	2.775	74.60	0.5952
		7%	27.2	3.114	90.64	1.0287
		8%	5.4	1.673	98.14	1.7309
		9%	2	0.707	99.31	2.1623
Test 3 (1:1)	Control		293.2	17.138		
		5%	0.6	0.548	99.80	2.6851

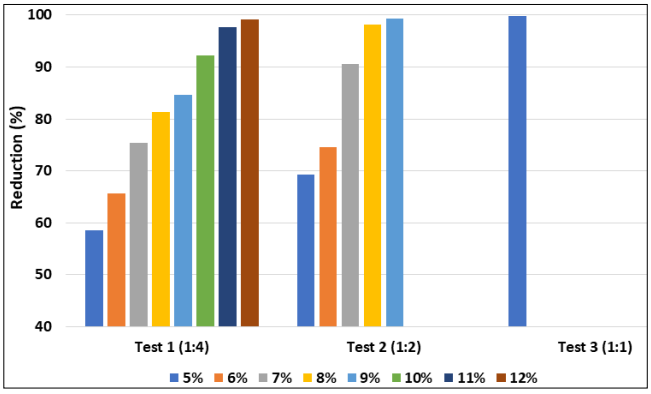


Fig 13: The proportion of bacterial reduction (%) at different concentration lemon seed extract with different ratios in broth media.

In table 5 showed the percentage of bacterial reduction performing in broth medium, after the broth culture was sub-cultured agar plate, and incubated in 24 hours of counting the colony formation. The CFU was calculated for the reduction's proportion to determine whether sample had antimicrobial activity or not. Beside that minimal inhibitory concentration (MIC) from extraction of lemon seed was applied in broth testing that indicated the difference and higher concentration compared to agar plate testing due to Pseudomonas aeruginosa can increasingly grow in culture medium with high moisture contents. Firstly, the comparison between bacterial reduction of the difference in concentration and ratios showed significant difference between the three tests ($p<0.05$). On the other hand, a comparison between the same extract concentrations on the reduction of Pseudomonas aeruginosa determined a significant difference at 5% from the three tests and at 6%, 7% in test 1 and test 2. On the other hand, the extract concentration on bacteria reduction in the concentration of 8%, 9% did not show a significant difference in test 1 as well as in test 2. Furthermore, there was no significant difference at 10%, 11%, 12% in test 1, and also from 11% to 15% and 20% in three tests. Secondly, the reduction bacteria of three ratios of lemon seed extract (1:4 v/v, 1:2 v/v and 1:1 v/v) were significant difference in each experiment ($p<0.05$). When the increased amount of lemon seed extract in the medium could inhibit the growth of P. aeruginosa at a wider range (Table 5).

The experiment has proven that the sample concentrations of 13%, 10% and 6% represented for minimal inhibitory concentration (MIC) in each test, with test 1, test 2, and test 3 respectively. At the concentration of 10% in test 1, 7% in test 2 and 5% in test 3, the lemon seed extract starts their antimicrobial activity with the percentage of P. aeruginosa reduction was higher 90% (Figure 13) because of the steadily low colony formation. This concludes the growth of P. aeruginosa was able to be inhibited by the lemon seed extract for at least 24 hours at the concentration of 13% for a quarter per total solution.

4. Conclusion

In this investigation, the effects of the lemon seed extract against the growth and development of Pseudomonas aeruginosa were successful and thoroughly investigated.

The extracted concentration of lemon seed was clearly found that at least 11% (w/v) of one-fourth per total medium preparation brought the reduced proportion of Pseudomonas aeruginosa over 95%.

The extracted concentration of the lemon seed at 12% was found of the most suitable concentration, which indicated a minimal and strongest effect on the inhibition of Pseudomonas aeruginosa.

5. Acknowledgement

The authors are very much thankful to Co-Ordinator's of the Food microbiology lab of the Department of Food Technology, International University as well as for providing needed laboratory facilities. Thanks, are also given to the Lab technicians of the Department of Food Technology for their hearty supporting in arrangement of clean and ready lab work daily.

6. References

1. Maria CS, Gustavo M. Environmental and socioeconomic drivers in infectious disease, The Lancet Planetary Health. 2018; 2(5):198-199.
2. Khan HA, Baig FK, Mehboob R. Nosocomial infections: Epidemiology, prevention, control and surveillance. Asian Pacific Journal of Tropical Biomedicine. 2017; 7(5):478-482.
3. Bodey GP, Bolivar R, Fainstein V, Jadeja L. Infections caused by Pseudomonas aeruginosa. Reviews of infectious diseases. 1983; 5(2):279-313
4. Richards MJ, Edwards JR, Culver DH, Gaynes RP. Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. Crit Care Med. 1999; 27(5):887-92.
5. Paul J Planet. Pseudomonas aeruginosa in Principles and Practice of Pediatric Infectious Diseases (Fifth Edition), 2018, 866-870.
6. Dasantila Golemi-Kotra. Reference Module in Biomedical SciencesxPharm: The Comprehensive Pharmacology Reference, 2008, 1-8.
7. Favero MS, Carson LA, Bond WW, Petersen NJ. Pseudomonas aeruginosa: growth in distilled water from hospitals. Science. 1971; 173(999):836-8.
8. Engleberg NC, DV Dermondy TS. Fourth ed: Lippincott Williams & Wilkins, 2007.

9. Annette E LaBauve, Matthew J Wargo. Growth and Laboratory Maintenance of Pseudomonas aeruginosa. Curr Protoc Microbiol, 2012.
10. Gaynes R, Edwards JR. Overview of nosocomial infections caused by gram-negative bacilli. National Nosocomial Infections Surveillance System. Clin Infect Dis. 2005; 41(6):848-54.
11. Kawaii S, Yasuhiko T, Eriko K, Kazunori O, Masamichi Y, Meisaku K, *et al*. Quantitative study of flavonoids in leaves of Citrus plants. J Agric Food Chem, 2000; 48:3865-3871
12. Burt SA. Essential oils: Their antibacterial properties and potential applications in foods: A review. Inter. J. Food Microbiol, 2004; 94:223-253
13. Ortuno AM, Báidez A, Del Río JA. Citrus paradisi and Citrus sinensis flavonoids: Their influence in the defence mechanism against Penicillium digitatum
14. Giuseppe G, Davide B, Claudia G, Ugo L, Corrado C. Flavonoid Composition of Citrus Juices. Molecules, 2007; 12:1641-1673.
15. Corbo MR, Speranza B, Filippone A, Granatiero S, Conte A, Sinigaglia M. *et al*. Study on the synergic effect of natural compounds on the microbial quality decay of packed fish hamburger. Inter J Food Microb, 2008, 127:261-267.
16. Nguyen Van Toan, Le Thi Kim Dung. Study of Antibacterial Activity against Streptococcus Mutans of Lemon Seed Extract. International Journal of Research Studies in Medical and Health Sciences. 2018; 3(9):06-17.
17. Amit Vikram, Citrus Bioactive Compounds: Isolation, Characterization and Modulation of Bacterial Intercellular Communication and Virulence. Biology, Doctoral Dessertation, 2011.
18. Nascimento GG, Locatelli J, Freitas PC, Silva GL. Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. Braz J Microbiol, 2000; 31:247-256
19. Donald Mabhiza, Tariro Chitemerere, Stanley Mukanganyama. Antibacterial Properties of Alkaloid Extracts from Callistemon citrinus and Vernonia adoensis against Staphylococcus aureus and Pseudomonas aeruginosa. International Journal of Medicinal Chemistry, 2007.
20. Lv X, Zhao S, Ning Z, Zeng H, Shu Y, Tao O. *et al*. Citrus fruits as a treasure trove of active natural metabolites that potentially provide benefits for human health. Chemistry Central Journal. 2015; 9(1):68.
21. Chaudhari SY, Ruknuddin G, Prajapati P. Ethno medicinal values of Citrus genus: A review. Medical Journal of Dr. DY Patil University. 2016; 9(5):560.
22. Betz JM, Brown PN, Roman MC. Accuracy, precision, and reliability of chemical measurements in natural products research. Fitoterapia. 2011; 82(1):44-52.
23. Ostroff RM, Vasil AI, Vasil ML. Molecular comparison of a nonhemolytic and a hemolytic phospholipase C from Pseudomonas aeruginosa. J Bacteriol, 1990; 172:5915-5923.
24. Reyes EA, Bale MJ, Cannon WH, Amp Matsen JM. Identification of Pseudomonas aeruginosa by pyocyanin production on Tech agar. Journal of clinical microbiology. 1981; 13(3):456-458.
25. Adams MR, Moss MO. Food microbiology-2nd ed, Univeristy of Surrey, Guildford, UK, 2000; 17:253