



Study of antibacterial activity against staphylococcus aureus of lemon seed extract

Nguyen Van Toan^{1*}, Ngo Hong Loan²

¹ Department of Food Technology, School of Biotechnology, International University - Viet Nam National University in HCM, Vietnam

² Study field of applied biomedicine, Department of Biotechnology, School of Biotechnology, International University, Vietnam National University Ho Chi Minh City, Vietnam

Abstract

Staphylococcus aureus (*S. aureus*) is the almost-universal cause of furuncles, carbuncles, and skin abscesses which is the most commonly identified agent responsible for skin infections. Most strains of *S. aureus* are now resistant to antibiotics, forcing the patients treated with high dosage drug, of which must be observed closely for potentially severe adverse effects. Efforts, therefore, have been increasingly made and paid attention to antimicrobials derived from the plants either as pure compounds or as standardized plant extracts. Among a variety of natural sources, seeds from citrus plant are reported to have antioxidant, anti-mutagenic, anti-inflammatory and antibacterial properties. Accordingly, successful isolation and extraction of phytochemicals from lemon seed would be medicinally advantageous.

In this study, the antimicrobial efficacy of lemon seed extract against the growth and development of *S. aureus* was thoroughly investigated. The mixture of lemon seed extract with different concentrations and ratios, and a certain amount of medium and inoculum, of which ranging from 5% to over 20% to determine the Minimum Inhibitory Concentration (MIC) against *S. aureus*. The obtained results of this study have indicated that the extract of lemon seed showed not only the statistically significant reduction of *S. aureus* colony formation at 15% in agar test and 22% at broth test with the ratio of 1:4 v/v but also the improved medicinal advantages of aqueous extracts of lemon seed usage against the growth and development of *S. aureus*. In summary, the lemon seed extract could effectively inhibit the growth of *S. aureus* at the selectively applied concentration.

Keywords: Lemon seed extracts, *streptococcus aureus*, antibacterial activity

1. Introduction

Infectious disease is the most common problem that receiving the deserved attention of many countries around the world, especially from developing countries. The explosion of population and environmental pollution have increased the incidence of infection ^[1]. Among many different types of mentioned problem, skin infection is the most susceptible one with approximately 300 million peoples being infected with various types yearly all over the world, and it was listed in the top 10 most prevalent diseases in 2010 by the Global Burden of Disease (GBD) ^[2]. Skin infection is commonly occurred at all ages, but 90% of the cases are in kindergarten age ^[3]. The factors such as climate, low socioeconomic status, overcrowding, poor skin condition, low hygienic level facilitate the bacterial growth and development in the skin ^[4]. The skin infection is caused by microbial invasion of the layers of skin and underlying soft tissues, it may occur at sites where the skin surface has been scratched, such as a wound or surgical site infection or it may also appear without apparent breach of the skin barrier, such as folliculitis occurring at hair follicles, furuncles and carbuncles forming at pores ^[5]. *S. aureus* is considered to be a major pathogen that colonizes and infects both hospitalized patients with decreased immunity, and healthy immuno-competent people in the community.

S. aureus is one of the well-known parts of normal microbial flora in the upper respiratory tract or on the skin. It is facultative, non-motile, non-spore forming Gram-positive bacteria. Every single cell has the spherical shape,

0.5 to 1.0µm in diameter. *S. aureus* appears in pairs, short chains, or grape-like clusters under the microscope. Typical colonies are yellow to golden yellow in color, smooth, entire, slightly raised, and hemolytic on blood agar. However, many strains of *S. aureus* may appear dirty white and non-hemolytic ^[6]. *S. aureus* grows rapidly in aerobic conditions, the range for the growth is between 10 and 47°C, with an optimum of 30 to 37°C; pH of 4.2 to 9.3. *S. aureus* can produce catalase enzyme to react with H₂O₂ from WBCs in human plasma or serum, it is also oxidase-negative, hemolytic bacteria. Their ability to ferment glucose and mannitol can be used to distinguish other staphylococci ^[7].

The pathogens from *S. aureus* often have high antibiotic resistance, so the use of antibiotics for treatment is significantly hampered because patients may be allergic to antibiotics with high doses ^[5]. Historically, plants have been valuable and indispensable sources of therapeutic agents for the health of human beings and they have a great potential for producing new drugs. In today's global market, more than 50 major drugs originated from tropical plants, therefore; such plants should be investigated to better understand their properties, safety, and efficiency ^[8]. In addition, the antimicrobial effects of compounds derived from plants have especially been paid to the roles of biologically active compounds in human health and disease treatment instead of nutritional value only. It has been reported that the phenolic compounds might significantly decrease the risk of some health problems due to

their antioxidant, antimutagenic, anti-inflammatory and antibacterial properties^[9, 10].

Citrus fruits originated in South East Asia and belong to family Rutaceae, subfamily Aurantoideae and tribe Citreae. This widely grown fruits on the planet consisting of 40 species that are distributed around the world^[11]. Bioactive compounds such as phenolics, flavonoids, and vitamins in citrus fruits are believed to prevent antimicrobial activities and protect human health^[11, 12]. Lemon fruit is an inexpensive, easily available citrus fruit, popular for its culinary and medicinal uses, contain many biological active compounds such as polyphenolic compounds of flavonoids and some limonoids. The crude extracts of different parts (leaves, stem, root, juice, peel, seed and flower) of lemon are having different potential in against clinically significant bacterial strains has been reported^[13].

Different part of lemon fruit contains different biologically active compounds, and the seeds are being the primary stage of the life cycle of plant, containing phytoconstituents which have been proven to contribute to antimicrobial activity by owing a strong defense mechanism^[14, 15]. Lemon seeds contain many secondary metabolites^[16] and prominently high concentration of limonoids^[17]. There are three forms of limonoids in citrus seeds: monolactone, dilactone, and glucosides by 36 different variations of limonoid skeleton. The first limonoids were 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^[18]. These compounds also contributed to plant defense by demonstrating anti-infection pathogenic bacteria.

Although lemon seed extracts are applying for testing the antimicrobial activity for a numerous strain of bacterial, there is no information about the how to utilize and apply the extracts from lemon seeds for evaluating and testing the antimicrobial activity on the *S. aureus*. It is, therefore, scientifically and economically important to know whether the lemon seed extract can be used to inhibit the activity of the *S. aureus*. Also, the consequence of choosing a suitable concentration of the lemon seeds extracts should be controlled in order to meet the proposed works need to be determined as well.

In summary, this study has been conducted to determine as well as to evaluate the possible and highly suitable concentration of lemon seeds extracts against the growth of *S. aureus*. The aim of this study was to evaluate the effect of the lemon seeds extracts against the *S. aureus*.

Materials and Methods

Materials

Lemon fruits were collected from the South Western province of Ben Tre province, Vietnam. The lemon seeds were dried in drying oven at 105°C to get the consistent moisture content of 5% and then store in Desiccator ready for further use and analysis.

S. aureus ATCC 25923 with freeze-dried format was purchased from Lan Oanh Company, Ho Chi Minh, Vietnam.

Brain Heart Infusion Broth was 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

Preparation of Lemon seed extract

The dried lemon seeds were well ground into fine powder by using a blender.

To prepare the aqueous extract, 10g of lemon seed powder was added to 100 ml deionized water (ratio of 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 at room temperature for 15 minutes to remove the insoluble and debris in order to have a purified lemon seed extract at a certain degree.

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

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

Each different final concentration was diluted using deionized water. Each concentration was labeled and stored at 4°C. The solution should be autoclaved at 121°C and 15lbs pressure before using for further antimicrobial tests.

Microorganisms preparation

The freezing-dried form of *S. aureus* ATCC 25923 was cultured in Blood Agar (BA) medium and then were incubated for 16–24 hours at 37°C before culturing in Brain-Heart infusion broth (BHI). After checking without contamination on Agar Base, randomly take one colony on the BA plate to culture in BHI broth medium at 37°C for 24-36 hours for further antimicrobial activity testing.

To ensure the bacteria appeared in BHI broth medium was *Staphylococcus aureus* only, the taken bacteria in BHI was then cultured again in BA from 24-36 hours at 37°C for gram stain and catalase test. This process was weekly performed in order to check the bacterial as well as to safely keep the bacterial for such a long time.

Agar plate test

This test was performed by culturing the inoculum on BHI agar containing a specific concentration of lemon seed extract. Three ratios of 1:4 v/v, 1:2 v/v, 1:1 v/v were repeated 5 times, for each concentration.

In details, 10 ml BHI agar medium was autoclaved at 121°C, 15 minutes and cooled down to about 60°C before transferring the lemon seed extract into the medium, vortex well and pour into the petri dish. A sterile cotton swab was used for spreading out 20 µm inoculum on the surface plates and incubated at 37°C, 24 hours. There were several treatments which distinct in added the sample into the medium.

Table 1: Experimental design for culturing the *Staphylococcus aureus* 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 *Staphylococcus aureus*.

Treatment No.	Concentration of sample	No. of replicates
1	5%	5
2	6%	5
3	6.5%	5
4	7%	5
5	8%	5
6	9%	5
7	10%	5
8	15%	5
9	20%	5

Colony counter was used to determine the number of colonies growing on the agar medium after incubated.

Broth test

S. aureus was cultured in BHI broth medium containing specific lemon seed extract concentration, for each treatment the number of replicated was five times. Three different ratios of 1:4 v/v, 1:2 v/v, 1:1 v/v (lemon seed solution/medium) were performed to determine the minimal inhibitory concentration of lemon seed extract against *Staphylococcus aureus*.

Broth medium was autoclaved at 121°C, 15 minutes and cooled down to approximately 6°C, then transferred the sample to medium which was followed by adding 20µl of the inoculum before incubated at 37°C for 24 hours.

Table 3: Experimental design for culturing *Staphylococcus aureus*.

Treatment No.	Concentrate of sample	No. of replicates
1	5%	5
2	6%	5
3	6.5%	5
4	7%	5
5	8%	5
6	9%	5
7	10%	5
8	15%	5
9	20%	5
10	21%	5
11	22%	5
12	25%	5

The result of this test was collected by counting the colony with “liquid serial dilution” method. Bacterial counting was determined to the effect of lemon seed to the pathogen and calculated by the following the formula:

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

$$\text{Log (Reduction)} = \text{Log}_{10} [(\text{Mean CFU})_{\text{control}} - \text{Log}_{10} (\text{Mean CFU})_{\text{sample}}]$$

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

Statistical 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 one-way ANOVA in which, P-values less than 0.05 was considered statistically significant.

Results and Discussions

After the dried powder of lemon seed was added to deionized water, then sonicated in water bath at 90°C for 2-3 hours followed by filtering using filter paper, the aqueous phase of lemon seed was obtained. It had a yellowish-brown colored solution.

The formula below was used to calculate the obtained yield:

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

As a consequence, the concentration of lemon seed extract was of 15% (w/v) which was followed by adding deionized water to have it diluted into different concentrations of lemon seed solution (5%, 6%, 7%, 8%, 9%, 10%) by applying the formula C1V1= C2V2 (Where C1 and C2 are the concentration (%) of the initial and final solution, respectively, V1 and V2 are the volume (ml) of the initial and final solution).

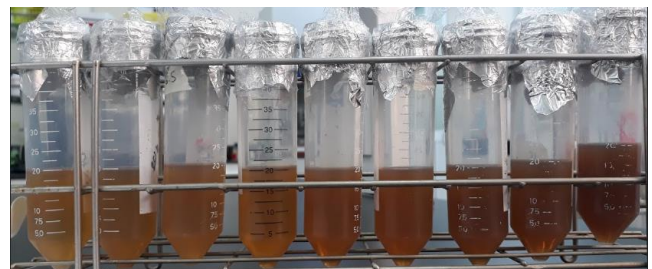


Fig 1: Lemon seed extract with difference concentration

Lemon seeds are commonly herbal medicine in traditional medicine systems which contains the high level of bioactive compounds, it used to be potent analgesic, anti-inflammatory, antibacterial, antifungal agent [19].

- **Microorganism preparation**

- **Staphylococcus aureus culture' identification**

Staphylococcus aureus belongs to the family Staphylococcaceae, the colony formation on Blood Agar of *S. aureus* usually displays a light to golden yellow pigment

and frequently surrounded by zones of clear beta-hemolysis which is sphingomyelinase that is highly active against sheep erythrocytes²⁰. The formation of colony on blood agar after incubated at 37°C, 24 hours, as opposed to the negative control, is shown in figure 2:

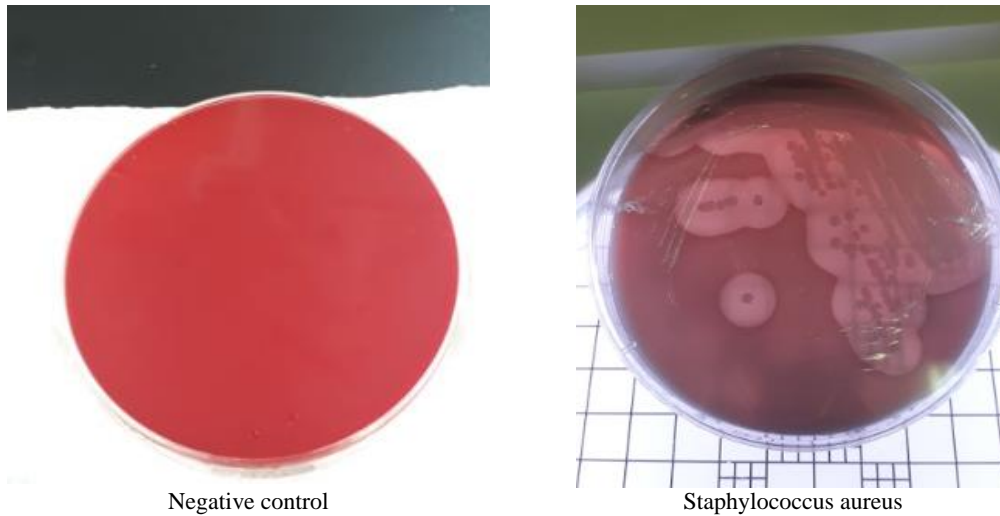


Fig 2: *S. aureus* colonies on blood agar

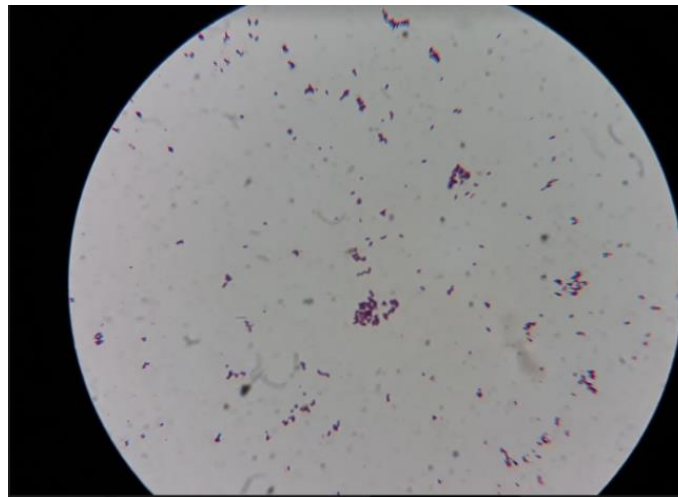


Fig 3: Gram stain of *S. aureus* showing typical gram-positive cocci in pairs, tetrads and grape-like clusters



Fig 4: Catalase test of *S. aureus*

From figure 2 and 3, *S. aureus* could be differentiated from others since it is a gram-positive bacterium and the color of gram stain was a purple and in cocci shape. In addition, the identified Staphylococcus aureus from another aerobic gram-positive cocci catalase test was used²¹. The test was performed by adding bacterial cells from a colony to a drop of 3% by using hydrogen peroxide. The appearance of bubbles (right) indicates the enzyme catalase while catalase-

negative bacteria give no reaction (left) was shown in figure 4.

In the laboratory, many strains were cultured at the same time, so opportunistic bacteria contamination could not be avoidable. Therefore, each individual test should be performed singularly and separately in order to prevent and avoid any possible contaminants.

• **Determination of antimicrobial activity**

➤ **Agar plate test**

In this test, the experiment was performed with 2 controls (positive and negative control) and three tasting sample ratios of is 1:4 v/v, 1:2 v/v and 1:1 v/v (ml of medium : ml of lemon seed extract), nine different concentrations of

lemon seed extract including 5%, 6%, 6.5%, 7%, 8%, 9%, 10%,15% and 20% (w/v). Each ratio was named test 1, test 2 and test 3, respectively. The experiment was repeated five times. The data was recorded after adding lemon seed solution and incubated 37°C, 24hours.

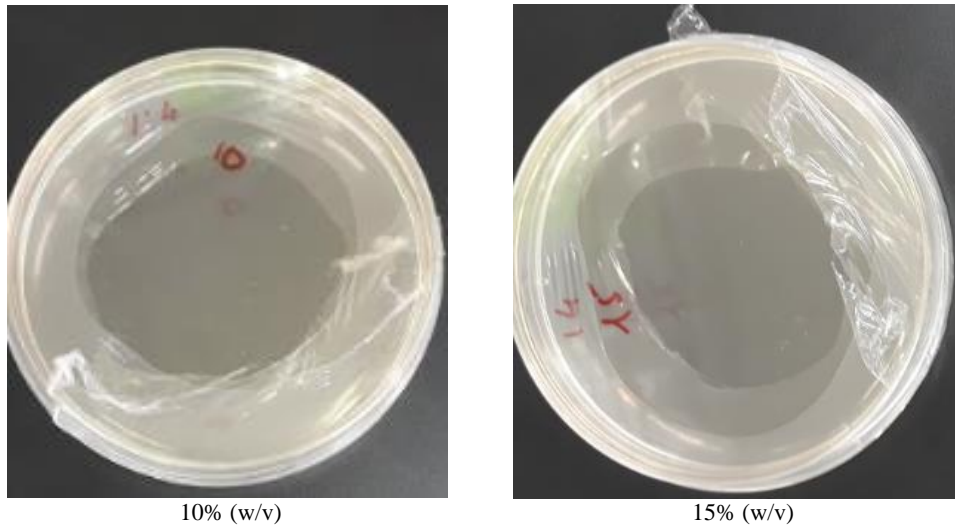


Fig 5: Sample concentration testing on agar plate with the ratio of 1:4 (v/v) with the minimal inhibitory concentration (on the right) in comparison to the 10% of sample (on the left).

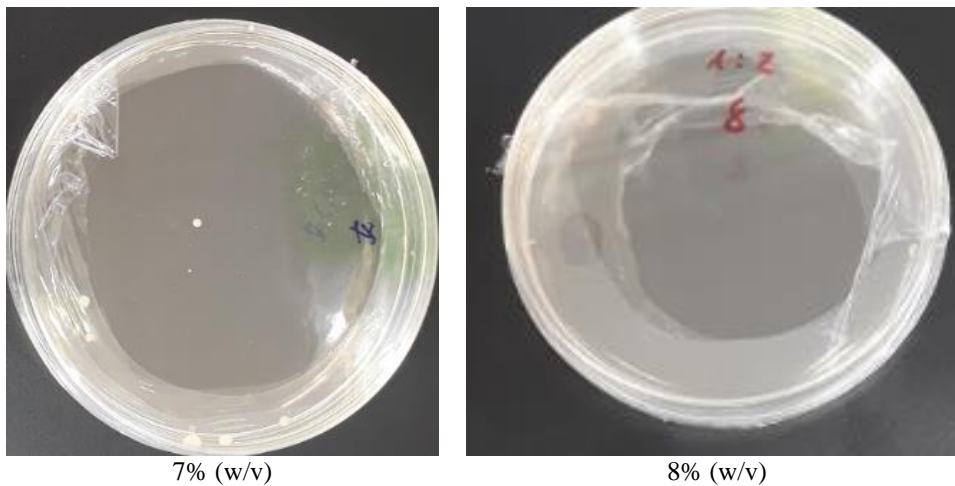


Fig 6: Sample concentration testing on agar plate, ratio of 1:2 (v/v) with the minimal inhibitory concentration (on the right) in comparison to 7% of sample (on the left).

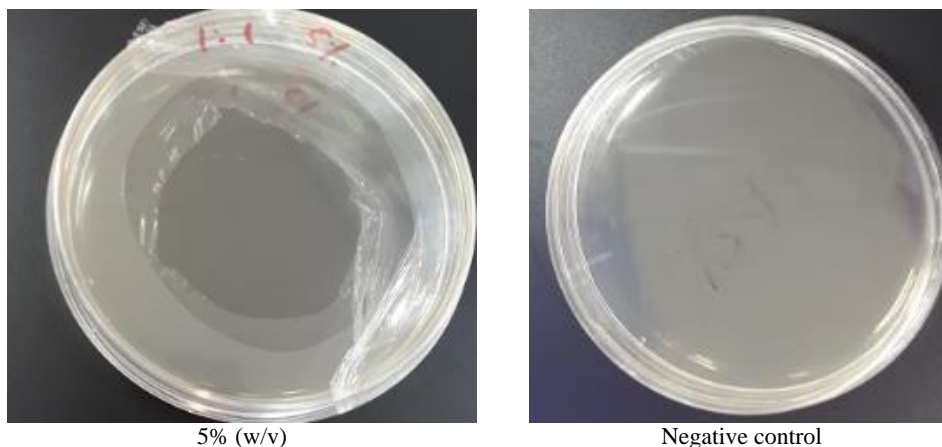


Fig 7: Sample concentration testing on agar plate, ratio of 1:1(v/v) with the minimal inhibitory concentration (on the right) at 5% in comparison to control (on the left).

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		444.4	4.037		
		5%	255.4	26.633		0.2406
		6%	214.2	11.367	51.80	0.3170
		6.50%	199.8	9.445	55.04	0.3472
		7%	171.4	6.914	61.43	0.4138
		8%	90.2	6.496	79.70	0.6926
		9%	28.6	9.044	93.56	1.1914
Test 2 (1:2)	Control		493	3.933		
		5%	48.4	3.109	90.18	1.0080
		6%	29	5.888	94.12	1.2304
		6.50%	28.6	4.031	94.20	1.2365
		7%	3.8	0.957	99.23	2.1131
Test 3 (1:1)	Control		489	2.828		

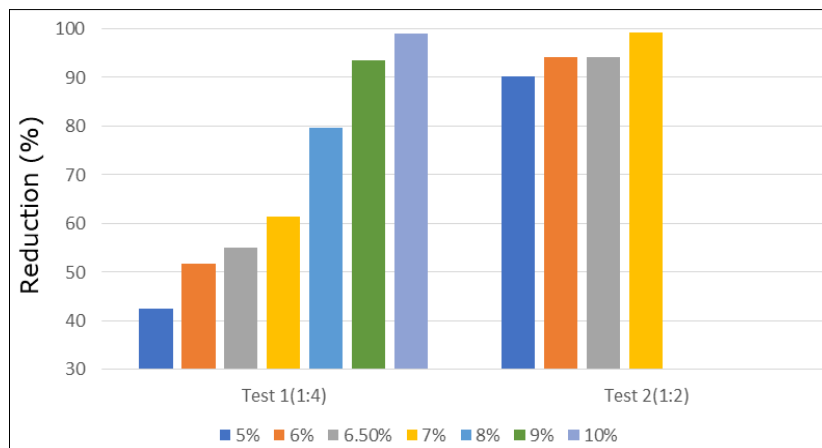


Fig 8: The percentage of bacterial reduction (%) at different concentration lemon seed extract with different ratios in agar media.

In the agar plate testing, there were significant differences in the bacterial reduction at different concentration of 5%, 6%, 6.5%, 7% of test 1 and 2. Comparisons of the *S. aureus* reduction at different concentrations of the lemon seed extract showed no significant differences between 10% and 7%, 9% and 6%, 6.5% in test 1 and in test 2 respectively. However, the differences between the other concentrations of the extract in each test were significant ($p < 0.005$). Therefore, the probability of bacterial growth would have reduced when increased the sample concentration. Also, the increased volume of lemon seed extract in the agar medium lead to the inhibition of bacterial growth at the lower concentration of lemon seed extract. Hence, the proportion of bacterial reduction underwent a rise as well as the increasing concentration of lemon seed extract (figure 8).

In addition, at the concentration of 15%, 8% and 5% of the sample solution in test 1, test 2 and test 3, respectively, showed 5 of 5 replicates had no colony formation after incubation 24 hours. There was demonstrated that lemon

seed was able to inhibit the growth of *S. aureus* though it was found to be effective at a higher concentration and volume, it indicated a marked antimicrobial activity and has an ability to replace the synthetic medicine for skin infectious treatment. To sum up, the lemon seed solution given their antimicrobial activity when it contributed at least 15% (MIC) at a quarter per total solution.

▪ **Broth test**

The broth experiment was conducted with 3 control samples and 9 testing samples with different concentrations of lemon seed extract and was repeated five consecutive times. The turbidity was used to test the bacterial growth in the liquid medium. After 24 hours of incubation, the dilution method was used to count the colony from broth medium into agar plates. Taking 0.1 ml of the mentioned suspension and added with 0.9 ml of sterile distilled water. From this suspension, 8 dilution factors were done and 100 µl of lasted dilution is cultured on agar plated for 37°C, 24hours.



Fig 9: Concentration broth with the ratio of 1:4(v/v) in test 1

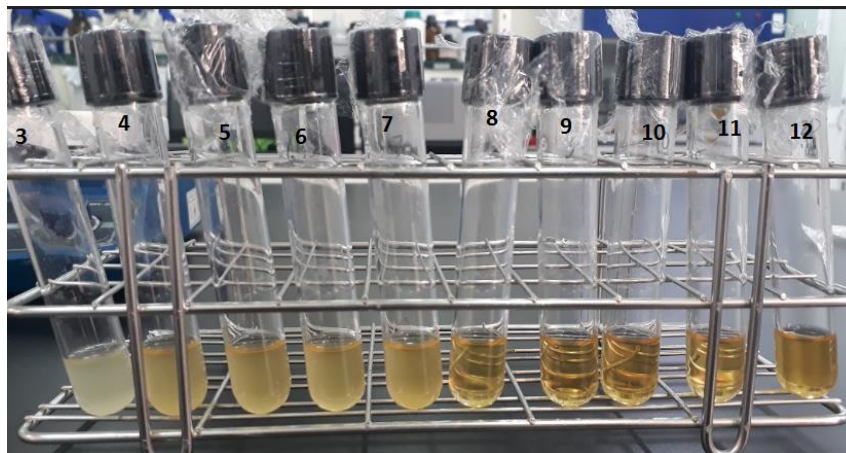


Fig 10: Concentration broth with the ratio of 1:2(v/v) in test 2



Fig 11: Concentration broth with the ratio of 1:1(v/v) in test 3

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

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

Test tube 3: Positive control with BHI broth medium and 20µl *Staphylococcus aureus* inoculum.

Test tube from 4 to 12: contains 20µl bacterial inoculum with three different ratios between the BHI medium and the concentration of the lemon seed extract from 5%, 6%, 6.5%, 7%, 8%, 9%, 10%, 15%, and 20%. There are three more tubes were used for the concentration of the extract 21%,

22% and 25% in test 1.

As showed in figure 9, the liquid inside the tube with the concentration of 22% and 25% was clear and slightly transparent which indicated no bacteria growth in the ratio 1:4(v/v). Similarly, with the ratio of 1:2(v/v) and 1:1(v/v), the tube arrangement from 10 to 12 and from 8 to 12 indicated the clear and transparent liquid that verified no bacteria growth in figure 10 and figure 11 in respectively. In a short summary, the increased amount of lemon seed extract in the medium could inhibit the growth of *S. aureus* at a wider range.

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		500.6	10.807		
		5%	309.4	85.981	38.19	0.2090
		6%	215.4	19.074	56.97	0.3662
		6.50%	136.2	20.825	72.79	0.5653
		7%	133.8	8.556	73.27	0.5730
		8%	31.6	16.288	93.69	1.1998
		9%	6	1.000	98.80	1.9213
		10%	5.2	2.387	98.96	1.9835
		15%	5.2	1.483	98.96	1.9835
		20%	4	1.414	99.20	2.0974
	21%	1.6	2.074	99.68	2.4954	
Test 2 (1:2)	Control		501	9.950		
		5%	137.4	12.502	72.57	0.5619
		6%	73.4	32.478	85.35	0.8341
		6.50%	65.6	28.290	86.91	0.8829
		7%	19.8	7.328	96.05	1.4032
		8%	4	4.637	99.20	2.0978
		9%	3.2	4.604	99.36	2.1947
Test 3 (1:1)	Control		486.6	36.672		
		5%	17.2	26.442	96.47	1.4516
		6%	10.6	15.010	97.82	1.6619
		6.50%	7.6	10.550	98.44	1.8064
		7%	3.8	6.870	99.22	2.1074

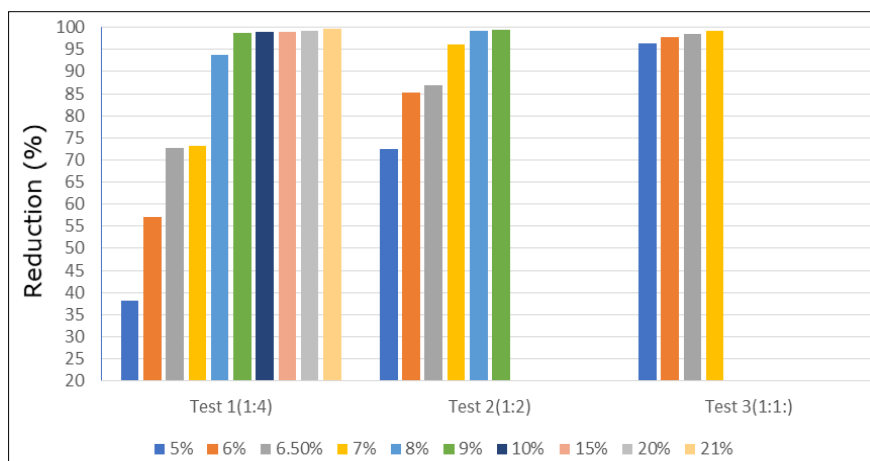


Fig 12: The proportion of bacterial reduction (%) at different concentrations of lemon seed extract with different ratios in broth media

Table 5 showed the percentage of bacterial reduction performing in broth medium after the broth culture was put on the agar plate, and then incubated in 24 hours for counting the colony formation. The CFU was calculated for the reduction's proportion to determine the antibacterial activity of sample²². Since *S. aureus* can increasingly grow in the high moisture content medium, the MIC of lemon seed extract in broth medium was different and higher from agar medium. As presented in this table, the comparison between bacterial reduction of different concentrations showed a significant difference between test 1, 2 and 3. On the other hand, the comparison between the same extract concentration on *S. aureus* reduction determined a significant difference between three tests in broth medium. The difference of reduction for *S. aureus* at 21% (test 1), 9% (test 2) and 7% (test 3) was no significant.

Moreover, the experiment has proven that the sample concentration of 22%, 10% and 8% in test 1, test 2, and test 3, respectively represented for MIC in each test. At the concentration of 22% in test 1, 10% in test 2 and 8% in test 3, the lemon seed extract started their antimicrobial activity

with the percentage of *S. aureus* reduction was higher 90% (figure 12) as the steadily low CFU was seen. This could conclude that the growth of *S. aureus* was able to be inhibited by the lemon seed extract for 24 hours at the concentration of 22% with one-fourth per total solution.

Conclusions

In this study, the effects of the lemon seeds extract against the growth and development of *S. aureus* was successfully and thoroughly investigated.

Lemon seed extract showed its antimicrobial activity when its extracted solution contributes to about 15% of a quarter per total prepared medium affected the reduction proportion of *S. aureus* over 95%

The suitable concentration of the extract was at about of 15% showed the strongest effect on the inhibition of *S. aureus*.

Acknowledgement

The authors would like are thankful to the A1-602 Lab of Department of Food Technology for providing necessary to

carry out this study

Conflict of interest

We declare that we have no conflict of interest.

References

1. Maria CS, Gustavo M. Environmental and socioeconomic drivers in infectious disease, *The Lancet Planetary Health*. 2018; 2(5):198-199.
2. Jayshalene B, Jamuna R, Rohit KV. A study on awareness of skin infection among adults in petaling district, Malaysia. *International Journal of Pharmacy and Pharmaceutical Sciences*. 2015; 2:136-139.
3. Marjo T, Parminder K. Most Common Infectious Diseases in Daycare. *Laurea University of Applied Sciences*, 2018. (www.theseus.fi/handle/10024/141742)
4. Cathryn O', Michael GB. Serious skin infections in children: a review of admissions to Gisborne Hospital (2006–2007). *The New Zealand Medical Journal*. 2012; 125:1351.
5. Keenan AL, Joan AG, Rachel MM. The role of *Staphylococcus aureus* virulence factors in skin infection and their potential as vaccine antigens. *Pathogens*. 2016; 5(1):22.
6. Kenneth T. *Staphylococcus aureus* - Textbook of Bacteriology.
7. Hiba SIM. *Staphylococcus aureus* Can Produce Catalase Enzyme When Adding to Human WBCs as a Source of H₂O₂ Productions in Human Plasma or Serum in the Laboratory. *Open Journal of Medical Microbiology*. 2014; 4(4):249-251.
8. Nascimento GGF *et al.* Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Braz. J. Microbiol.* 2000; 31(4):886-891.
9. Surh YJ. Anti- tumor promoting potential of selected spice ingredients with antioxidative and anti- inflammatory activities: a short review. *Food Chem Toxicol.* 2002; 40:1091-1097.
10. Albayrak S *et al.* Compositions, antioxidant and antimicrobial activities of *Helichrysum* (Asteraceae) species collected from Turkey. *Food Chem.* 2010; 119:114-122.
11. Karimi E, Oskoueian E, Hendra R, Oskoueian A, Jaafar HZE. Phenolic compounds characterization and biological activities of *Citrus aurantium* bloom. *Molecules*. 2012; 17:1203-1218.
12. Aruoma OI, Landes B, Ramful- Baboolall D, Bourdond E, Neergheen- Bhujune V, Wagnerf K *et al.* Functional benefits of citrus fruits in the management of diabetes. *Prev. Med.* 2012; 54:S12-S16.
13. 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.
14. Bennett RD, Hasegawa S, Herman Z. Glucosides of acidic limonoids in citrus. *Phytochemistry.* 1989; 28:2777-2781.
15. Bennett RN, Wallsgrove RM. Secondary Metabolites in Plant Defense Mechanisms. *New Phytology.* 1994; 127:617-633.
16. Bentley MD *et al.* Limonoid model insect antifeedants. *J Agric. Chem.* 1990; 38:1400-1403.
17. Hasegawa S, King AD. A Species of Bacterium-Producing Constitutive Enzymes for Limonoid Metabolism. *Journal of Agricultural and Food Chemistry.* 1983; 31(4):807-809.
18. 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.
19. Swapnil YC, Galib R, Pradeepkumar P. *Medical Journal of Dr. D.Y. Patil University*, 2016.
20. Bremer PJ, Fletcher GC, Osborne C. *Staphylococcus aureus*. New Zealand Institute for Crop & Food Research Limited, 2004.
21. Adams MR, Moss MO. *Food microbiology*, Royal Society of Chemistry; 2nd edition, 2000.
22. Andrews JM. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy.* 2001; 48(1):5-16.