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Study on the Antimicrobial Activity of Rhizoma Homalomenae on Staphylococcus Aureus

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ABSTRACT

The antimicrobial activity of Rhizoma Homalomenae on the pathogenic Staphylococcus aureus was thoroughly investigated. It has been found that Rhizoma Homalomenae has good antimicrobial activity against the growth of Staphylococcus aureus at certain degrees of culturing conditions. It is, however, worth noting that with the presence of 7% up, of total prepared ingredients for a culture medium, Rhizoma Homalomenae shows its good potency against Staphylococcus aureus, after 24 hours of incubation at 37^{o} C.

Keywords: *Antimicrobial activity, Rhizoma Homalomenae, Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus (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. Staphylococcus aureus (S. aureus) causes most skin and soft tissue infections (SSTIs) in humans. S. aureus has become increasingly resistant to antibiotics and there is an urgent need for new strategies to tackle S. aureus infections. Vaccines offer a potential solution to this epidemic of antimicrobial resistance [1,2].

Each single cell has spherical shape of 0.5 to 1.0µm in diameter. S.aureus appears in pairs, short chains, or grapelike clusters under microscope. Typical colonies are yellow to golden yellow in color, smooth, entire, slightly raised, and hemolytic on blood agar [3]

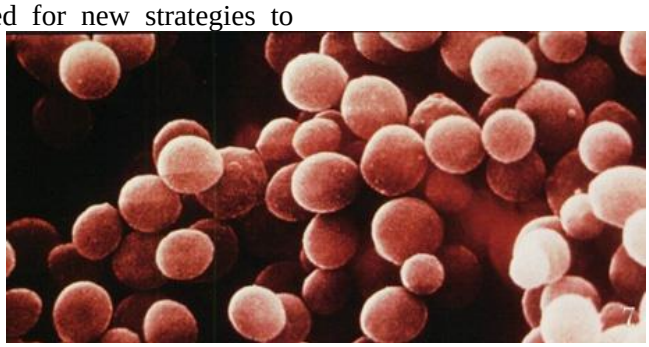

Figure1: *Electron micrograph of Staphylococcus aureus*

Staphylococcus aureus occurs as human normal flora, it is also considered as virulent since it can produce many toxins, of which producing enterotoxin is its capability. Enterotoxin is one of the four leading causes of food-borne illness, a leading cause of pus-forming skin and soft tissue infections as well as infectious heart disease. Additionally, Staphylococcus aureus presents asone of the most important and widespread hospital pathogens. It is the most common cause of pneumonia and the third most cause of blood infections [4].

Nevertheless the treatment for Staphylococcus aureus's associated diseases by using antibiotics was found from years ago, Staphylococcus aureus is always concerned as serious pathogen in human since it is resistant to many common antibiotics, such as penicillin, which lead to difficulties in treatment. Many antibiotic-resistant strains of S. aureus – such as Methicillin-resistant Staphylococcus aureus (MRSA) – are on the rise in hospitals and communities[2]. Staphylococcus aureus has been known as a significant threat to global public

health. It is therefore necessary to pay special attention to finding a new antimicrobial substance to Staphylococcus aureus to help relieving the burden treatment of infectious and related diseases.

Rhizoma Homalomenae is the dry rhizome of a plant called Homalomenae occulta (Lour.) Schott. It has been used for hundred years as a famous traditional Chinese medicine (called as Qiannianjian in Chinese). The Chinese name means thousand years healthy medicine. Rhizoma Homalomenae is a bitter slightly sweet warm herb. It has many medical functions like invigoration of the kidney and liver, strengthening of the muscles and bones, relieving stomachache, and relief from rheumatoid arthritis, healing pain and swelling due to traumatic injury (Zhong Hua Ben Cao Editorial Committee, 1999)' [5]. Traditionally, it is mostly used as an aqueous decoction, alcoholic beverage or for external application. Some of Rhizoma Biological activity compounds of Rhizoma Homalomenae have shown potential in antivirus and antibiotic activities and other perspective characteristics in medication [6].

MATERIALS AND METHODS

❖ **Plant material:** Rhizoma Homalomenae powder was Obtained from a Herbal Medicine Shop in Ho Chi Minh City

Figure 2: *Rhizoma Homalomenae powder*

❖ **Inoculum: Staphylococcus aureus**

Figure3: *The purchased Staphylococcus aureus from Pasteur Institute Ho Chi Minh City*

❖ **Culture media: for culturing the S. aureus**

➢ **Luria Broth:** also called as Lysogeny Broth (LB)[7]

It is a nutritionally rich medium used for the growth of bacteria.

Table1: *The LB medium (composition per liter)*

Bacto-Tryptone	10 gram (g)
Bacto-yeast extract	5 gram (g)
NaCl	10 gram (g)
ddH_2O	to 1 liter
pH=7.0	

Bacto-tryptone is used to provide essential amino acids to the growing bacteria, while the bacto- yeast extract is used to provide organic compounds like Vitamins (including B vitamins), helpful for bacterial growth.

- **Blood agar:**

A rich, non-selective, differential medium supports the growth of a wide variety of microbes. It is made by adding 5% sheep's blood to a complex medium containing peptone just before the plates are poured. The red blood cells remain intact, and the medium resembles fresh blood. Some bacteria grow on blood agar without disturbing the red cells; others hemolyze the cells to various degrees. Bacteria that lyse the red cells with a complete clearing of the medium around the colonies are referred to as beta hemolytic. Some bacteria lyse the cells but do not break down the hemoglobin completely leaving greenish methemoglobin in the medium. This is called alpha-hemolysis. Non-hemolytic bacteria have no visible effect on the red blood cells and are said to be gamma-hemolytic[8].

- Blood agar base was purchased from Hoa Nam Ltd. Company.
- Defibrinated Sheep Blood: was purchased from Nam Khoa Bioteck Company.

- **Mannitol Salt Agar (MSA):** MSA plates were purchased from Nam Khoa Bioteck Company.

MSA is a selective medium that favors the growth of Staphylococci. MSA contains 7.5% NaCl and staphylococci are characteristically able to tolerate this high salt concentration. MSA also contains the sugar-alcohol mannitol and the pH indicator phenol red. Generally pathogenic staphylococci can ferment the mannitol, lowering the pH, and thereby turning the indicator yellow. The non-pathogenic staphylococci do not ferment mannitol and the medium remains pink in their vicinity [6]

Figure4: *A positive (yellow) Mannitol fermentation of S.aureus (right)*

Equipments:

- ✓ Petri dishes
- ✓ Test tube
- ✓ Erlen-Meyer flask, beaker, cylinder
- ✓ Duran bottle
- ✓ Eppendorf tube
- ✓ Inoculating loop, glass spreaders

Machine:

- ✓ Digital Balance
- ✓ pH Measure
- ✓ Microwave
- ✓ Water bath
- ✓ uto-clave
- ✓ Drying oven
- ✓ Laminar flow hood
- ✓ Spectrophotometer
- ✓ Incubator
- ✓ Colonies counter

Preparation of Sample Materials

The sieved powder was added to deionized water at about 900C for 2-3 h with the ratio 1:10w/v. The aqueous phase, after filtered by filter paper, was store at 40C for further antimicrobial tests. The solution is clear with slight-brown color.

Figure5: *Rhizoma Homalomenae solution after extracted with distilled water.*

Preparation of Medium

- ❖ *LB medium: Take 10g tryptone, 5g yeast extract and 10g NaCl in 1 liter of distilled water. Autoclave at 121 °C. After cooling, LB is ready for use.*
- ❖ *Blood agar medium*
- ✓ *Suspend 4.0 g of dehydrated base medium (BK055) in 100 mL of deionized water.*
- ✓ *Slowly bring to boiling, stirring with constant agitation until complete dissolution.*
- ✓ *Sterilize in an autoclave at 121°C for 15 minutes.*
- ✓ *After sterilized, cool and maintain at 50-60°C.*
- ✓ *Aseptically add 5 mL of defibrinated sheep blood to 95 mL of base, and*
- ✓ *For the Petri dishes used for testing antimicrobial activity of sample, add a specific amount of sample solution into base.*
- ✓ *Mix rapidly and thoroughly.*
- ✓ *Pour into sterile Petri dishes*
- ✓ *Let solidify on a cool surface.*
- ✓ *Dry the plates in an incubator with the covers partially removed.*

Microbial Preparation

Staphylococcus aureus is culture in LB medium as well as in BA and MSA. After checking no contaminants in agar base, one colony is culture in LB medium at 370C for 24-36hrs. Then, bacteria are culture again in BA and MSA for at 370C for 24-36hrs and stored in refrigerator at 40C. Each week this process is repeated to obtain Staphylococcus aureus for testing the antimicrobial activity.

In the case if there is something wrong during sub-culturing Staphylococcus aureus, from the beginning, Staphylococcus aureus is store in Glycerol 80% at -200C in Eppendorf and can be used to culture. Note that every time before inoculums is cultured, it is measure for fix OD at approximately 0.5.

Staphylococcus Aureus Culture Contaminating Test

To identify if there were contaminants of other bacterial strain in the laboratory, besides making control each time culturing the bacteria with Rhizoma Homalomenae, there are some tests done for identifying exactly Staphylococcus aureus strain.

Gram-stain

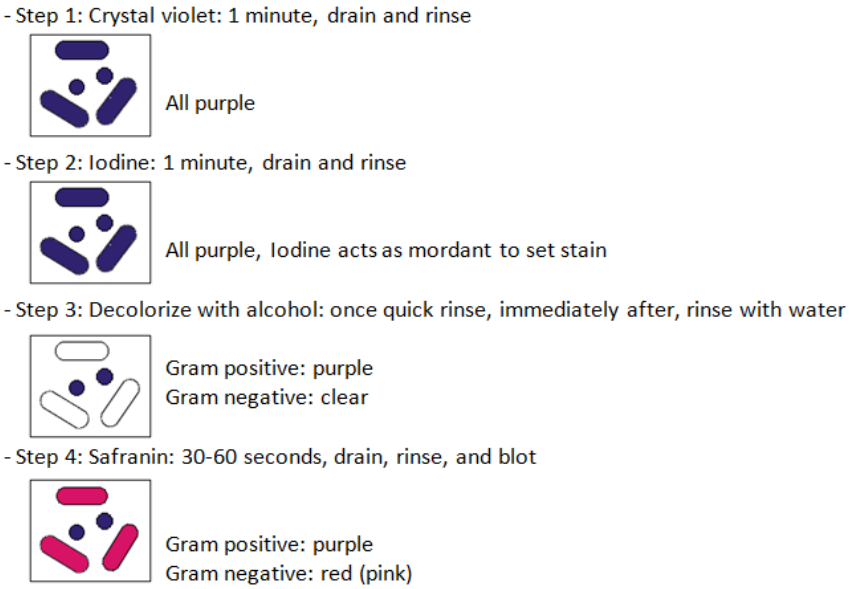

Figure6: *Gram stain procedure*[7,8]

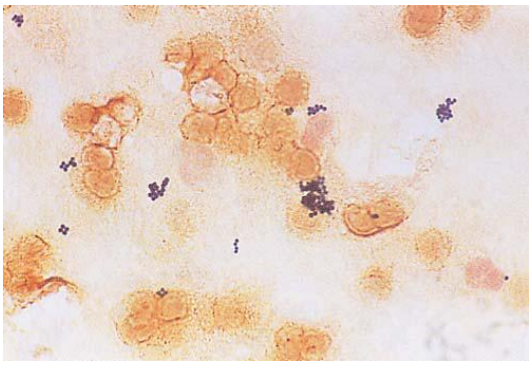

Figure7: *Gram stain of Staphylococcus aureus showing typical gram positive cocci in pairs, tetrads and grape-like clusters*

Culturing bacterial strain in MSA

Growth on Mannitol-Salt agar differentiates catalase positive gram-positive cocci as shown here on an agar medium containing 7.5% NaCl which inhibits the growth of many other organisms. S. aureus also can ferment mannitol into acid detected here by the change in pH indicator from red to yellow.

Catalase test

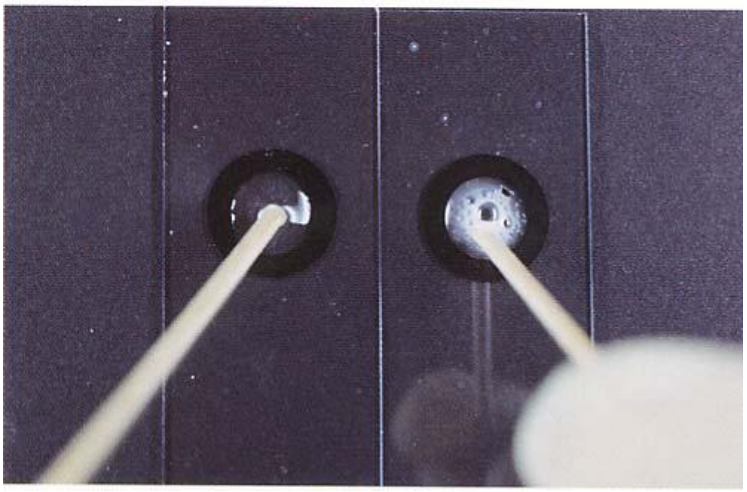

Figure8: *Staphylococcus aureus catalase test*

Staphylococcus aureus can be differentiated from other aerobic gram positive cocci by a positive catalase test. The test is performed by adding bacterial cells from a colony to a drop of 3% hydrogen peroxide. The appearance of bubbles (right) indicates the enzyme catalase while catalase negative bacteria give no reaction (left).

Testing antimicrobial activity: by culturing of Staphylococcus aureus on medium containing Rhizoma Homalomenae.

Agar Plate Test

- ✓ Agar medium was autoclaved and cooled down to about 60^0C.
- ✓ Transfer the sample to analyze into medium with the specific concentration.
- ✓ Spread the 20 µl of inoculum on the surface of the agar with a sterile triangle spreader.
- ✓ Incubate at 37°C for 24 hours.

Table2: *Experimental design for culturing Staphylococcus aureus for each treatment*

	No. of replicated	Added materials		
		Medium	Inoculum	Rhizoma Homalomenae
Negative control	1	√	X	x
Negative control 2	1	√	X	√
Positive control1	1	√	√	x
Sample	15	√	√	√

(√: with; x: without)

For treatment, the concentration of sample in medium will alter and be optimized later in other to test the antimicrobial activity of Rhizoma Homalomenae.

Table 3: *Experimental design for culturing Staphylococcus aureus*

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

The status of each agar plate was observed after 24hrs and 36hrs of incubation.

Broth testing

Staphylococcus aureus is culture on broth containing different concentration of sample solution and then incubated at 37^0C in 24 hours. There were triplicates for each treatment with different concentration of Rhizoma Homalomenae solution.

- Broth medium was autoclaved and cooled down to about 60^0C.

- Transfer the sample to analyze into medium with the specific concentration.

- Transfer the 100 µl of inoculum into 10ml of medium.

- Incubate at 37°C for 24 hours.

Table 4: *Experimental design for culturing Staphylococcus aureus*

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

After that, each sample test tubes was done with serial dilution for bacterial count.

Bacterial count

The bacteria from original sample test is diluted in serial number and then added on agar plate in order to count for the colony forming. Since Staphylococcus aureus occur in clumps, the CFU was better used instead of cells per ml.

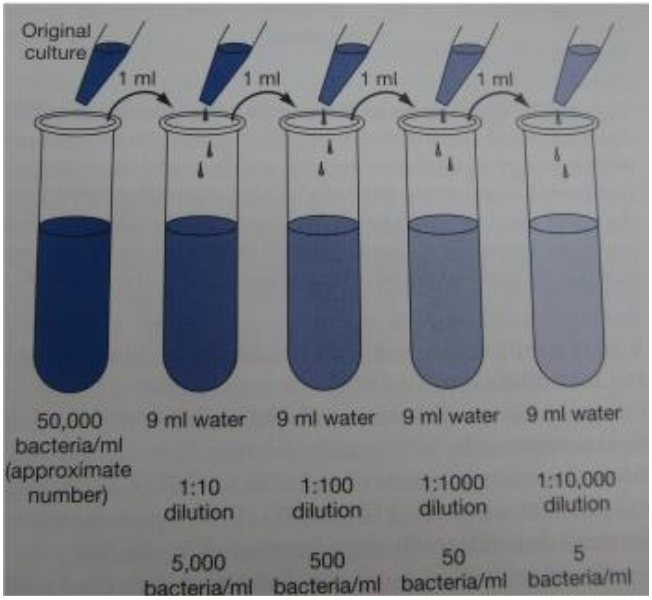

Figure 9: *Serial dilution: one milliliter is taken form culture and added to 9ml of sterile water, there by diluting culture by a factor of 10. This procedure is repeated until 10-8 dilution.*

The CFU number then was used to determine the antimicrobial performance category of Rhizoma Homalomenae based on the performance metrics calculated as follows:

$$\%\ \text{Reduction} = \frac{(Meancfu)control - (Meancfu)sample}{(Meancfu)control} \times 100$$

$$Logreduction = Log_{10}(Meancfu)control - Log_{10}(Meancfu)sample$$

Data Analysis

All data are expressed as means ± standard deviation of representative of similar test carried out in triplicate. Statistical differences in colony forming unit (CFU) were determined by student's t-test in which, $p<0.005$ was considered statistically significant.

RESULTS

Preparation of sample materials

After sieved powder was added to deionized water at about 900C for 2-3 h with the ratio 1:10w/v and filtered by filter paper, the sample solution was obtained. It is clear with slight-brown colored solution with pH=7±0.2

Figure 10: *Rhizoma Homalomenae solution*

❖ Staphylococcus aureus culture contamination test

These tests were carried out during each test batch to identify contamination beside

Staphylococcus aureus.

Macro morphology:

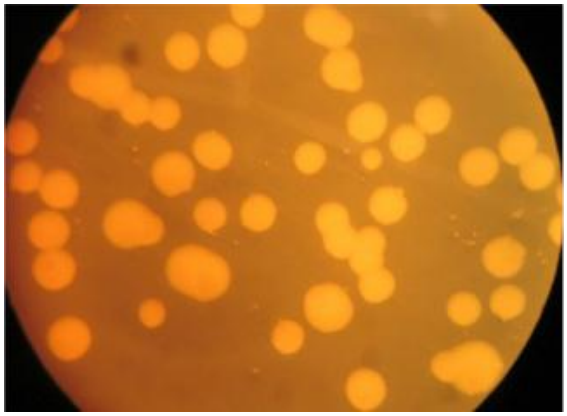

Figure 11: *Staphylococcus aureus's macro morphology: opaque, white to yellow round shaped colony under microscope*

❖ **Gram-stain:** Since Staphylococcus aureus is Gram positive cocci, it appears as grape-like clusters in slightly purple.

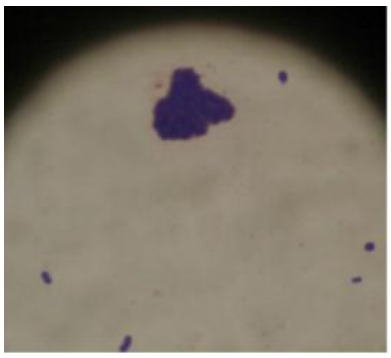

Figure 12: *Gram stain test of Staphylococcus aureus*

❖ **Catalase test:**

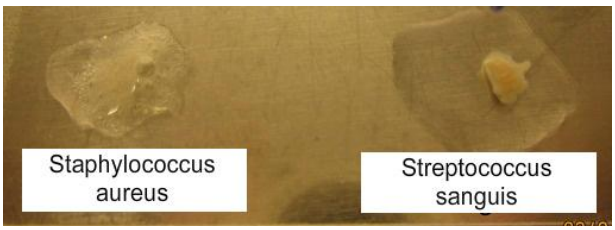

Figure 13: *Staphylococcus aureus catalase test*

The appearance of bubbles (right) on Staphylococcus aureus when adding a colony into Peroxide solution indicates the enzyme catalase while catalase negative bacteria, Streptococcus sanguis, gave no reaction (left).

❖ **Culturing bacterial strain in different media:**

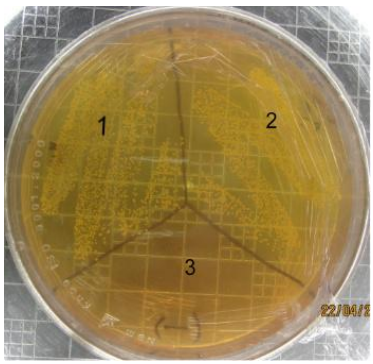

Figure 14: *Staphylococcus aureus on MSA plate: the agar color changed from red into yellow color where Staphylococcus aureus colonies appear (1, 2) and remained slightly red color without Staphylococcus aureus (3).*

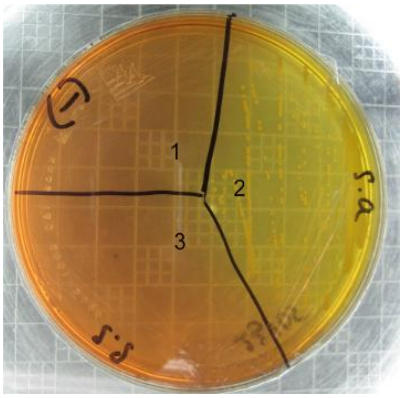

Figure 15: *Culturing Staphylococcus aureus and Streptococcus sanguis on MSA: although both strain formed colonies on MSA (2, 3), only part where Staphylococcus aureus colonies formed changed color from red to yellow (2). These two-remaining part, one was without any strain (1) and one was with Streptococcus sanguis was (3) remained original red color of MSA.*

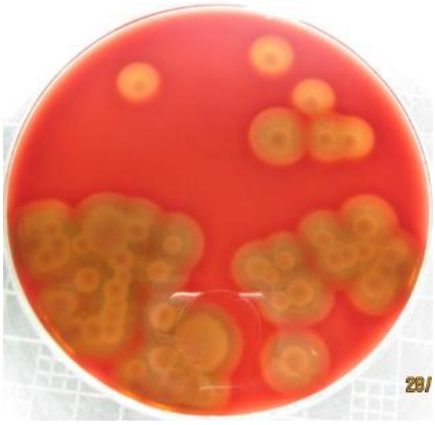

Figure 16: *Staphylococcus aureus cultured on BA: Staphylococcus aureus appeared as yellow colonies with clearly hemolysis on BA plate.*

Figure 17: *Staphylococcus aureus on BA: Staphylococci appear as opaque, white to gold-yellow colonies with clearly hemolysis on BA plate (2) while Streptococcus sanguis appeared unclearly hemolysis (3).*

❖ Antimicrobial test:

Table 5: *results for agar testing*

Concentration (%)	No. of replicate	No. of replicate without colony forming after 24hrs
5	15	0
6	15	0
6.5	15	0
7	15	14
8	15	10
9	15	10
10	15	10

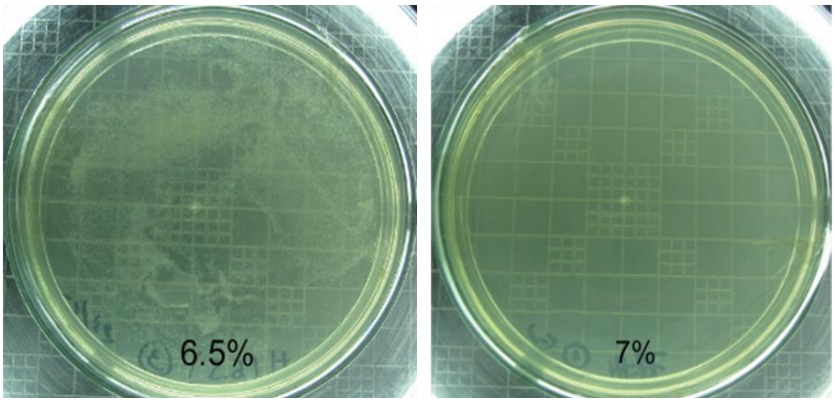

Figure18: *Sample concentration testing on agar plate: with 6.5% of sample (left picture), there were colony forming but not at 7% of sample (right picture).*

Broth Testing

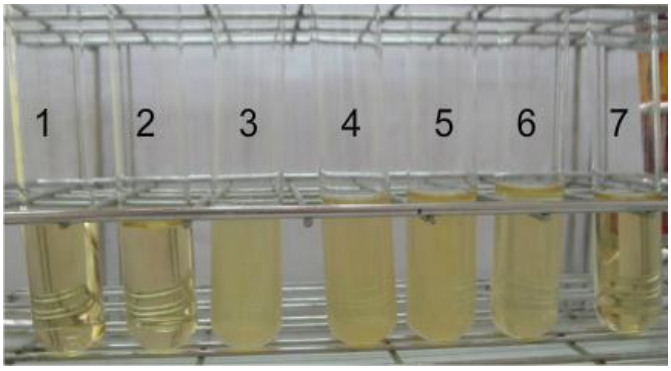

Figure 19a: *Concentration broth testing 1*

- ✓ Test tube 1: Negative control 1 with LB broth only.
- ✓ Test tube 2: Negative control 2 with LB broth and Rhizoma Homalomenae solution.
- ✓ Test tube 3: Positive control with S.aureus inoculum added in LB only. The liquid indicated the growth of Staphylococcus aureus.
- ✓ Test tube 4: contains Rhizoma Homalomenae solution added with ratio 6v/v.
- ✓ Test tube 5: contains Rhizoma Homalomenae solution added with ratio 6.5v/v.
- ✓ Test tube 6 contains Rhizoma Homalomenae solution added with ratio 7 v/v. The liquid inside the tube was clear and slightly transparent which might indicate no bacterial growth.
- ✓ Test tube 7 contains Rhizoma Homalomenae solution added with ratio 8 v/v. The liquid in this tube was clear and transparent which indicate that no bacterial growth inside.

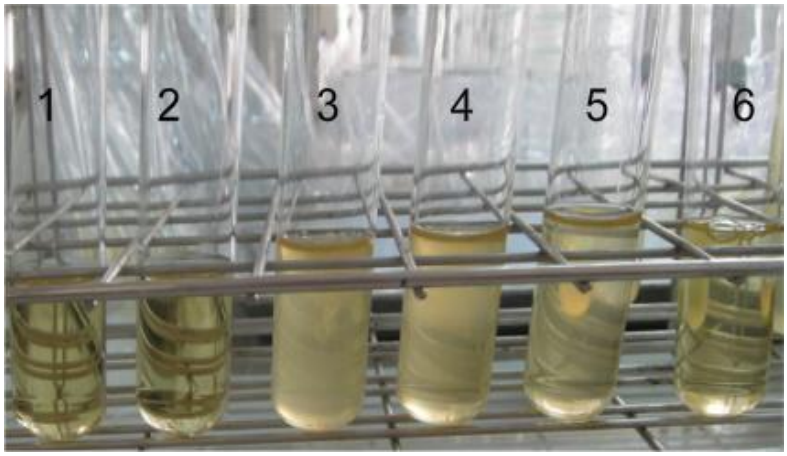

Figure 19b: *Concentration broth testing 2*

- ✓ Test tube 1: Negative control 1 with LB broth only.
- ✓ Test tube 2: Negative control 2 with LB broth and Rhizoma Homalomenae solution.
- ✓ Test tube 3: Positive control with S.aureus inoculum added in LB only.
- ✓ Test tube 4: contains Rhizoma Homalomenae solution added with ratio 6.5 v/v.
- ✓ Test tube 5 contains Rhizoma Homalomenae solution added with ratio 7v/v. The liquid inside the tube was clear and slightly transparent which might indicate no bacterial growth.
- ✓ Test tube 6 contains Rhizoma Homalomenae solution added with ratio 8 v/v. The liquid in this tube was clear and transparent which indicate that no bacterial growth inside.

Table 6: *Results for antimicrobial performance testing*

		Concentration (%)	Mean (CFU/ml)	SD	Reduction %	Log reduction	Performance
Test 1	Control (-)		4.0E+05				
		6	1.2E+07	1.1E+06	-	-	
		6.5	8.5E+06	6.0E+05	-	-	
		7	1.0E+04	5.8E+03	97.5	1.60	MEDIUM
Test 2	Control (-)	-	4.0E+05				
		6	9.8E+06	6.7E+05	-	-	
		6.5	7.5E+06	8.2E+05	-	-	
		7	3.0E+04	1.2E+04	92.5	1.13	MEDIUM
Test 3	Control (-)	-	4.0E+05				
		6	1.5E+07	2.3E+06	-	-	
		6.5	1.0E+07	1.0E+06	-	-	
		7	5.0E+04	1.2E+04	87.5	0.90	SLIGHT

DISCUSSION

For the testing with agar plate, at concentration of 7% of sample solution, 14 of 15 replicates had no colony formed after 24 hours of incubation, providing that the prepared sample solution of Rhizoma Homalomenae was able to inhibit the growth of Staphylococcus aureus in 24 hours. When the concentration of sample was increased, the probability for forming of colony on agar plates reduced. To conclude, the sample solution of Rhizoma Homalomenae showed their antimicrobial activity when it contributes to at least 7% per total solution.

For the testing with broth, after the broth culture was put on agar plate, and then inoculated for 24hrs, formed colony was counted as CFU and calculated for the percentage of reduction to determine whether sample had antimicrobial activity or not. The experiment showed that the sample concentration of 7% per total broth was especially significant for antimicrobial activity. At this concentration, Rhizoma Homalomenae starts their antimicrobial activity on Staphylococcus aureus while the CFU was found quite low. The performance of antimicrobial activity of the sample was determined as slight medium with the percentage of reduction was over 90%.

Through done tests, it would be highly convinced that there maybe one or some elements contained in Rhizoma Homalomenae have antimicrobial activity.

Since the antimicrobial activity of Rhizoma Homalomenae on Staphylococcus aureus was proved, it can be used to produce antimicrobial products against Staphylococcus aureus, which is more and more resistant to antibiotics nowadays. Producing antimicrobial agents form plant such as Rhizoma Homalomenae maybe a perspective way because Rhizoma Homalomenae is quite easy to get and treat since it has been used for years.

For Staphylococcus aureus identification test, although this study did not focus on identifying bacterial strain, all identification tests were necessary. The tests were done to test if our testing plates or broths were contaminated with other bacterial strains or not. In the laboratory, there were many strains cultured at the same time, so there was high probability of contamination. As this consequence, for each time test was carried out, many sample controls and identifying tests should be done to detect the contaminant.

CONCLUSIONS

From the obtained results above, it could be concluded that:

- ➢ Rhizome Homalomenae shows its good potency against Staphylococcus aureus.

- Rhizoma Homalomenae shows it antimicrobial activity when its solution contributes to about 7% of total prepared medium.

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