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Investigating the Antimicrobial Efficiency of Staple Food Against Enteric Pathogens

Heyuan Xu[1], Sayyida Aiza Bukhari[2], Nawab Ali[3], Muhammad Ilyas[4], Muhammad Abubakar[5], Muhammad Haidar Zaman[6*]

[1]Department of Biology, College of Arts and Sciences, New York University, USA. (b) IBD, Nan Shi Fu Zhang, Nanjing Normal University, Nanjing, China.
[2]Department of Agricultural Science and Engineering, Hohai University, Nanjing, China.
[3]Associate Professor, Department of Physiotherapy, Sahara University Narowal, Pakistan.
[4]Postgraduate Trainee, Department of Cardiology, Pakistan Institute of Medical Sciences, Islamabad, Pakistan.
[5]Department of Medical Lab Technology, Iqra National University, Peshawar, Pakistan.
[6]Assistant Professor, Department of Health and Biological Sciences, Abasyn University, Peshawar, Pakistan. (b) IBD, Nan Shi Fu Zhang, Nanjing Normal University, Nanjing, China.

**Corresponding author's E-mail: dr.mhaidarzaman@gmail.com*

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Abstract

Background: *The advent of antibiotics has significantly decreased human death rates. However, the problem of antibiotic overuse and the number of individuals who maintain a suspicious and defensive stance towards medicines remain unresolved concerns. Hence, it is important to investigate the antibacterial efficacy of commonly consumed dietary items.* ***Results and Discussion:*** *This research examined the antibacterial activity of thirteen different daily vegetables and fruits. Among them, the extract from Citrus limon (Eureka) had the highest efficacy as an antibacterial agent against Gram-positive bacteria. The study involved quantifying the antibacterial efficacy of Citrus limon (Eureka) extract by measuring the diameter of the antibacterial zone and determining the cell concentration in the sample using an Ultraviolet Spectrometer. The results revealed a positive correlation between the antibacterial ability of the extract and its quantity, as well as a negative correlation between the inhibition degree of S. aureus and the quantity of Citrus limon (Eureka) extract.* ***Conclusion:*** *The study revealed that the extract derived from Citrus limon (Eureka) has robust antibacterial properties against gram-positive bacteria while demonstrating no discernible effect on gram-negative bacteria.*

Keywords: *Antibiotics, Enteric Pathogens, Staple Food, Efficiency*

1. Introduction

Background Information

Symptom of gastrointestinal discomforts, such as bloating, is common among otherwise healthy individuals, but it causes a significant effect on the quality of life [1]. Specific individual intestine pathogens, often transmitted in food or water under normal conditions, can cause severe discomfort [2]. Usually, the doctor will give the patients some drugs to inhibit microbial activities and relieve discomfort. However, with the high intake of antibiotics, the composition of the gut microorganisms could be significantly affected, reducing its biodiversity and delaying colonization for an extended period after administration [3]. In addition, patients stop taking antibiotics once their health reappearances after relatively short courses of treatment. Combined, all of this cause gastrointestinal discomfort, which is more challenging to treat, and antibiotic therapy failure in patients could be as high as 15%, according to the medical professionals working in the hospitals [4]. This might lead to using more drugs or different combinations of antibiotics, which may cause a vicious cycle. At the same time, even the safest drug may have some unknown side effects on the human body. So, finding something to substitute the drugs is necessary and significant.

In recent years, some researchers have already started the investigation of antibiotically peptides [5] and polymers [6]. However, these researches are still ongoing and may take a long time before launching the products into the markets. Also, their products might be expensive and cannot satisfy the public. So, the aid substitute objects should typically be unharmful to human health and typical in daily life, so the economic cost will not be too high. To satisfy these requirements, fruits, and vegetables might be perfect objects. They are convenient to get and easy to ingest. In addition, the nutrients within fruits and vegetables benefit the human body [7], and more people are eager to find a natural way to improve their physical body condition. However, fruits and vegetables can be used to substitute drugs depending on whether they have antimicrobial activity.

To investigate the antimicrobial efficiency of different fruits and vegetables, it is implausible to conduct experiments on the human body because it takes time and money and requires many subjects. However, we can simulate an *in-vitro* environment with conditions similar to the human gut, including temperature, moisture, and pH. Also, the microorganisms within the human intestine are vital parts of the entire intestinal environment. The human gut microbiota represents a highly complex microbial community, consisting mainly of Bacteroides and Firmicutes as the majority, and involves some microorganisms of Proteobacteria.

The general population is looking for a fast way to get health back and trim down costs in daily life by not using or reducing the usage of antibiotics, but how? If fruits and vegetables work as antibiotics then people might have more choices for treating gastrointestinal discomfort. It also means that fruits and vegetables might potentially substitute parts of the antibiotics.

Variables

Independent variable

There are two independent variables during the experiments: the type of extracts of fruits and vegetables and the volume of extracts of fruits and vegetables. The previous one is for the qualitative experiment, and the latter is for the quantitative experiment.

Dependent variable

Once the juice of any fruits or vegetables works, an inhibition zone will exist. The diameter of the inhibition zones varies according to the antimicrobial efficiency of the extracts better the efficiency, the longer the diameter. Also, the absorption of the bacteria suspension varies after culturing under different conditions. This provides a quantitative result for the experiment.

Controlled variable

Temperature

Since the experiment aims to investigate the antimicrobial efficiency of samples against microorganisms within the intestine, creating a similar environment to the natural intestine environment is essential to stimulate the interaction between samples and microorganisms. Also, the temperature could significantly affect the growth rate of any organisms, so the temperature of all the experiments must be kept at a constant temperature of 37-celsius degrees.

Incubation time

The reproduction and metabolic reactions of bacteria are closely related to the cultivation time. To ensure that the same number of bacteria will exist and only reveal the difference in antibiotic efficiency of samples, the cultivation time must be limited to the same length.

Bacteria population in each Petri dish

The experiment aims to identify the antimicrobial ability of selected samples. To get accurate results, the initial population of bacteria in each Petri dish must be equal to avoid a systematic error.

2. Materials And Methods

Material

Table 1. *Materials used during the experiment*

Mangifera indica	*Pyrus spp*
Vaccinium spp	*Solanum lycopersicum*
Malus pumila	*Citrus limon*
Zingiber officinale	*Actinidia chinensis*
Plantago depressa	*Codonopsis pilosula*

Astragalus membranaceus	*Taraxacum mongolicum*
Ciprofloxacin susceptibility paper	Gentamycin susceptibility paper
Panax quiquefolium	75% alcohol
Escherichia coli	Ofloxacin susceptibility paper
Staphylococcus aureus	Amoxicillin susceptibility paper
Phosphate-Buffered Saline (PBS)	

Apparatus and Equipment

Table 2. *Apparatus and Equipment used during the experiment*

Pipette (100µl-1000µl, 10.0µl-100.0µl)	Ultraviolet Spectrometer
Test tubes (5ml)	Beaker (50ml)
Columbia Sheep-Blood agar	Alcohol burner
Glass spreading rod	Soybean agar
Thermostat	Iron support, filter paper
Vortex	Pulverizer

Methods

Sample extraction

Peel the fresh fruits and vegetables and cut them into pieces separately. Squeeze the pieces to get the juice of samples. Sterilize the test tube with 75% alcohol and filtrate the juice using iron support and filter paper. Transfer the solution after filtration into sterilized test tubes and mark them. Put the dried Traditional Chinese Medicine (TCM) into the pulverizer and crush them into powders. Sift the powders and put the eligible powders into a sealing bag, then mark the name on the bags.

Choosing bacteria

The gram-negative bacteria *Escherichia coli* and gram-positive bacteria *Staphylococcus aureus* are chosen to represent two groups of bacteria and used in the experiment.

Pilot experiment

Weigh 2g of each type of medicine powder and add 5 ml to 95-celsius degree purified water. Shake the test tubes to mix the solution adequately. Use the pipette to add 200µl purified water into the sterilized test tube and add 20µl *E.coli* suspension into the water. Shake the test tube to make diluted *E.coli* suspension. Use the pipette to add 200µl purified water into the sterilized test. Extract the proper amount of *S. aureus* and mix it into 20µl purified water to form the suspension. Mix the suspension with the previous 200µl water on the Vortex to form diluted *S. aureus* suspension.

Transfer 220µl *E.coli* suspension onto one soybean agar plate and use the glass spreading rod to smear the suspension among the entire surface of the plate. Repeat the procedure four times to make four same plates. Drop 10µl of each sample extract on the plate. Each plate involves four types of extract. Transfer 220µl *S. aureus* suspension onto one Columbia Sheep-Blood agar plate and use the glass spreading rod to smear the suspension among the entire surface of the plate. Repeat the procedure four times to make four same plates. Drop 10µl of each sample extract on the plate. Each plate should involve four types of extracts at most.

Determine the antibiotic efficiency of *Citrus limon*

Use a dissecting needle to extract one column and two columns of *S. aureus* and *E.coli* into 220µl and 240µl, respectively. Repeat the procedure twice to get 220µl×2 and 240µl×2 of *S. aureus* suspension and 220µl×2 and 240µl×2 of *E.coli* suspension. Use the Vortex to mix the suspension evenly. Take four soybean agar plates and four Columbia sheep blood agar plates. Then drop the extract of *Citrus limon (Eureka)* in the center of the Petri dish. This part's detailed permutation and combination are shown in the table below.

Table 3. *The detailed permutation and combination of suspension*

	Soybean agar plate		**Columbia sheep blood agar plate**	
E.coli	No.1 220µl	No.3 240µl	No.2 220µl	No.4 240µl
S. aureus	No.5 220µl	No.7 240µl	No.6 220µl	No.8 240µl

Confirmation experiment using Ultraviolet Spectrometer

Use a dissecting needle to extract a column of *S. aureus* and add it into 20μl purified water, which means there is 1×10^6 bacteria cell per 20μl water. Prepare six *S. aureus* suspensions with different compositions, shown in the table below.

Table 4. *Composition of suspension in six test tubes*

No.	Composition	No.	Composition
S1	200μl PBS + 20μl ($1x10^6$) *S. aureus* suspension	**S2**	200μl PBS + 40μl ($2x10^6$) *S. aureus*
S3	200μl PBS + 20μl ($1x10^6$) *S. aureus* suspension + 10μl lemon extract	**S4**	200μl PBS + 20μl ($1x10^6$) *S. aureus* + 20μl lemon extract
S5	200μl PBS + 40μl ($2x10^6$) *S. aureus* suspension + 10μl lemon extract	**S6**	200μl PBS + 40μl ($2x10^6$) *S. aureus* + 20μl lemon extract

Seal the suspension in test tubes and incubate them within the Thermostat at 37-celsius degrees for 24 hours.

After 24-hour incubation, take the test tubes out of the Thermostat to determine the absorption of the suspension. Use solvent, which is purified water, to calibrate the UV Spectrometer. Then put, the suspensions into the machine respectively to determine the absorption at 600nm. Record the data for later concentration determination and analysis (Appendix-2).

Comparison between antibiotic efficiency of *Citrus limon (Eureka)* extract and antibiotics

Mix 200μl PBS and 20μl ($1x10^6$) *S. aureus* suspension. Put the suspension on the agar and spread it evenly using the glass spreading rod. Locate one of each antibiotic susceptibility paper on the agar in four corners. Keep the petri dish in the Thermostat at constant 37-celsius degrees for 24 hours for incubation. Compare the diameter of the inhibition zone of each antibiotic with that of *Citrus limon (Eureka)* extract.

Equipment usage

Thermostat

It is used to create an environment with constant temperature for bacteria incubation. The metabolic reactions of bacteria require a constant temperature for hours because the temperature is an essential factor of growth. Without a thermostat, it is impossible to simulate the intestine environment and stimulate the growth of bacteria. For the experiments, the temperature should be 37-celsius degrees constantly for at least 12 hours.

Vortex

This equipment is designed to mix the solution or suspension evenly. The plate of the Vortex can vibrate at high speed in the circle when pressed. So the test tube can be pressed on the plate, and then the solution can be mixed properly. The solute could not spread in the entire solvent if the solution or suspension were not pressed on the Vortex. Thus, when the bacteria suspension is transferred into Petri dishes, the bacteria may not grow among the entire plate but only grow in a specific area because the suspension is not even.

Ultraviolet Spectrometer

The Ultraviolet Spectrometer is good quantitative equipment. It can emit UV light and determine the absorption of the target solution at a specific wavelength. With the help of a calibration curve, the concentration of the solution can be deduced. Since the spectrometer can detect tiny amounts of solute, it helps me perform the quantitative investigation better.

Pulverizer

During the experiment, five types of Traditional Chinese Medicine were used. However, all of them were dried in their original form. It is impossible to smash them into powders because they are too complicated. Therefore, a pulverizer is necessary since it can crack the dried medicine into minimal powder, allowing me to prepare the suspensions.

3. Results and Discussion

Antibiotic efficiency verification by a pilot test

To avoid the waste of materials and reduce the amount of failed experiments, the pilot experiment is essential to determine the availability of antibiotic efficiency of each substance. However, it is not essential to strictly control the concentration or amount of extracts. The purpose of the pilot test is only to justify the property. After 24-hour incubation within the Thermostat at constant 37-celsius degrees, all the Petri dishes were taken out to verify whether inhibition circles existed around the sample extractions. The appearance of the inhibition zone indicates the availability of antibiotic efficiency in certain substances. The qualitative results are shown in Table 6. All the dishes were photographed within 10 minutes as long as they were removed from the thermostat.

Table 5. *Determination in the availability of inhibition zone*

Testing species	*E.coli*	*S. aureus*	Testing species	*E.coli*	*S. aureus*
Mangifera indica	-	-	*Citrus limon*	+	+
Vaccinium spp	-	-	*Actinidia chinensis*	-	-
Malus pumila	-	-	*Codonopsis pilosula*	-	-
Zingiber officinale	-	-	*Taraxacum mongolicum*	-	-
Plantago depressa	-	-	*Panax quiquefolium*	-	-
Astragalus membranaceus	-	-	*Solanum lycopersicum*	-	-
Pyrus spp	-	-			

According to the results listed in table 6, it can be seen that the inhibition zone only appeared around the extract of *Citrus limon*, which demonstrates that *Citrus limon (Eureka)* contains antibacterial activity. Thus, only the extract of *Citrus limon (Eureka)* will be further investigated in the later quantitative experiment.

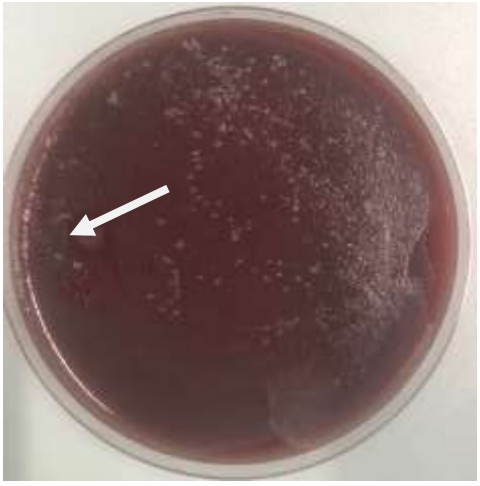


Figure 1. The antimicrobial sensitivity zone of *Citrus limon (Eureka)* on enriched Sheep-blood agar against the combined culture of gram-positive and gram-negative bacteria (*S. aureus* and *E.coli*)

The inhibition zone's low obviousness is due to the large number of bacteria on the agar. Therefore, the ability of antibacterial activity is reduced. In order to increase the obviousness and better determine the efficiency of the antibacterial activity of *Citrus limon*, the *S. aureus* suspension has to be diluted in the later experiments.

Antibiotic efficiency of *Citrus limon (Eureka)* test

Keep the Petri dishes in the Thermostat for 24 hours at constant 37-celsius degrees. Then take them out of the Thermostat and determine the inhibition zone's availability. The camera records the status of each sample.

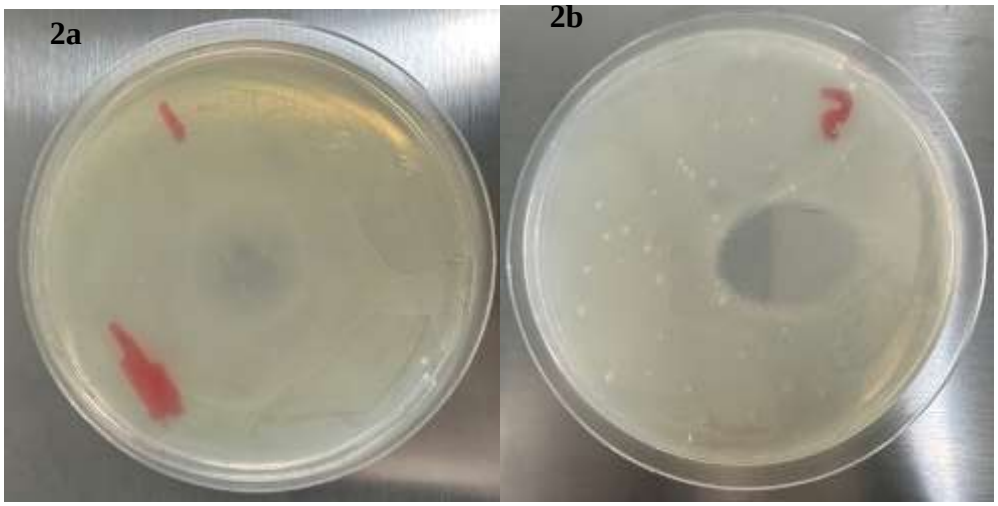


Figure 2. Antibacterial activity of *Citrus limon*: a) Antibacterial activity of *Citrus limon (Eureka)* against gram-negative bacteria (*E.coli*). **b)** Antibacterial activity of *Citrus limon (Eureka)* against gram-positive bacteria (*S. aureus*)

Accordingly, the observation results are listed in the table.

Table 6. *The status of the inhibition circle*

	Inhibition zone existence		**Inhibition zone existence**
No. 1	No	**No. 5**	Yes
No. 2	No	**No. 6**	Yes
No. 3	No	**No. 7**	Yes
No. 4	No	**No. 8**	Yes

Figures 2a and 2b show the feedback from *E.coli* after adding *Citrus limon (Eureka)* extract, and figure 3 shows the feedback from *S. aureus* after adding *Citrus limon (Eureka)* extract. Combining the information in the figure with the results shown in table 6 reveals that the *Citrus limon (Eureka)* extract does not have a noticeable impact on *E.coli*, but an apparent inhibition zone exists in every Petri dish grown with *S. aureus*. The phenomena indicate that *Citrus limon (Eureka)* extract only has antibiotic efficiency against *S. aureus* but not against *E.coli*. Since *E.coli* represents gram-negative bacteria while S. aureus represents gram-positive bacteria, *Citrus limon (Eureka)* extract might be effective in inhibiting other gram-positive bacteria. The figurative data about the pilot test has been shown in Appendix 1.

Hence, each inhibition zone's diameter is measured using a ruler. The camera recorded all the measurements and finished in 10 minutes after the Petri dishes were removed from the Thermostat.

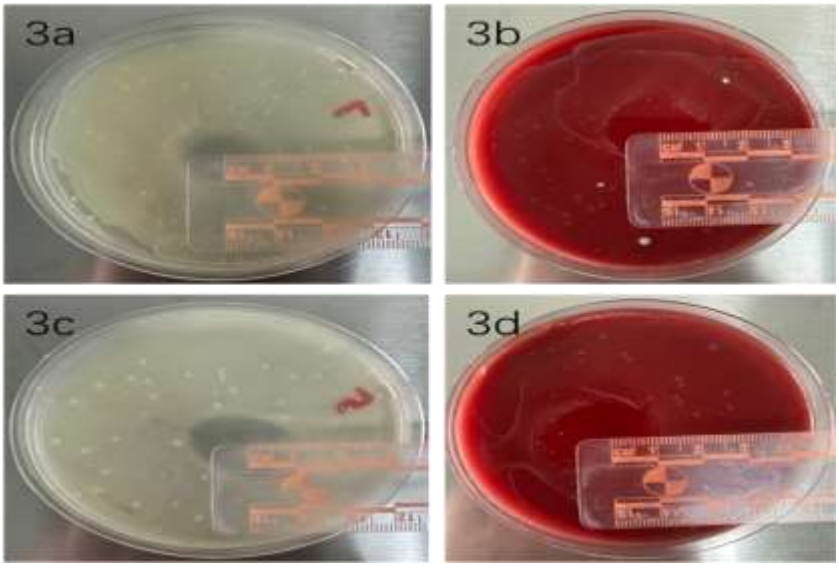


Figure 3. Diameters of the inhibition zone by *Citrus limon*: a) 1×10^6 *S. aureus* on soybean agar **b)** 1×10^6 *S. aureus* on Sheep-blood agar **c)** 1×10^6 *S. aureus* on soybean agar **d)** 1×10^6 *S. aureus* on Sheep-blood agar

Table 7. *Diameter of inhibition zone in S. aureus*

	No.5	No.6	No.7	No.8
Diameter of inhibition zone(mm)	22	23	17	19

According to the procedure of preparation of bacteria suspension in 3.4, the density of the suspension can be calculated from the following equation:

$$Cell\ Density\ \left(\frac{CFU}{mL}\right) = \frac{n_{colonies} \times 10^6}{V_{H_2O}}$$

The percentage difference between two data based on the first one can be calculated in this way:

$$\%\ difference = \frac{data_2 - data_1}{data_1} \times 100$$

Therefore, the density and the percentage differences can be evaluated.

Table 8. *Calculated data of density and percentage difference*

Cell Density (CFU/ml)	**No.5&No.6**	**No.7&No.8**
	4.54×10^6	8.33×10^6
% difference in cell density	+83.5%	
% difference in diameter	**No.5 → No.7**	**No.6 → No.8**
	−29.4%	−21.1%
Average % difference in diameter	−25.3%	

According to the content in table 8, as the cell density of the bacteria suspension increases, the length of the diameter of the inhibition zone is reduced. This indicates that a negative correlation exists between the cell density of bacteria suspension and the diameter of the inhibition zone. Based on the data collected from the experiment, as the cell density of bacteria suspension ascends 83.5%, the diameter of the corresponding inhibition zone descends 25.3% on average.

Determining the change in concentration and confirming the results by Ultraviolet Spectrometer

Keep all the S. aureus suspension samples in a Thermostat for 24 hours at a constant 37-celsius degree for incubation. Use the Ultraviolet Spectrometer to determine the absorption of the suspension at 600nm. Since the absorption of one substance has a positive correlation with the concentration of this substance, the concentration of *S. aureus* suspension can therefore be deduced.

Table 9. *Absorption of each S. aureus suspension sample after 24h incubation at 600nm*

Number of samples	OD (600nm)
S1	0.230
S2	0.238
S3	0.109
S4	0.037
S5	0.224
S6	0.148

S1 and S2 are considered negative controls within six samples under the different initial amounts of bacteria conditions since solvent and bacteria exist in the test tubes. For S3, S4, S5, and S6, the *Citrus limon (Eureka)* extract is added, which means bacteria inhibition should exist within the suspension.

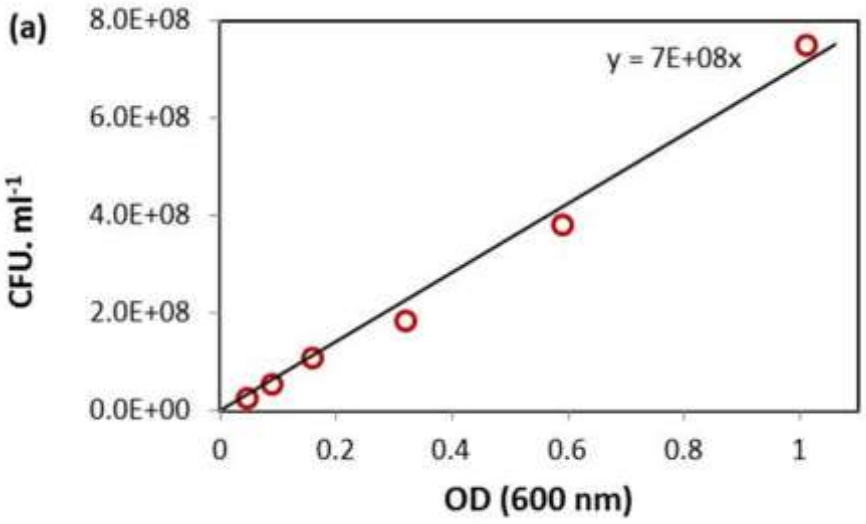


Figure 4. Calibration curve of *S. aureus* at 600nm [8]

Plug the data in table 9. into the function of the calibration curve of *S. aureus* shown in figure 4, and the cell density of each *S. aureus* suspension can be calculated.

Table 10. *Cell Density of S. aureus suspensions*

Number of samples	Cell Density / CFU•ml^{-1}
S1	1.61×10^{8}
S2	1.67×10^{8}
S3	7.63×10^{7}
S4	2.59×10^{7}
S5	1.57×10^{8}
S6	1.04×10^{8}

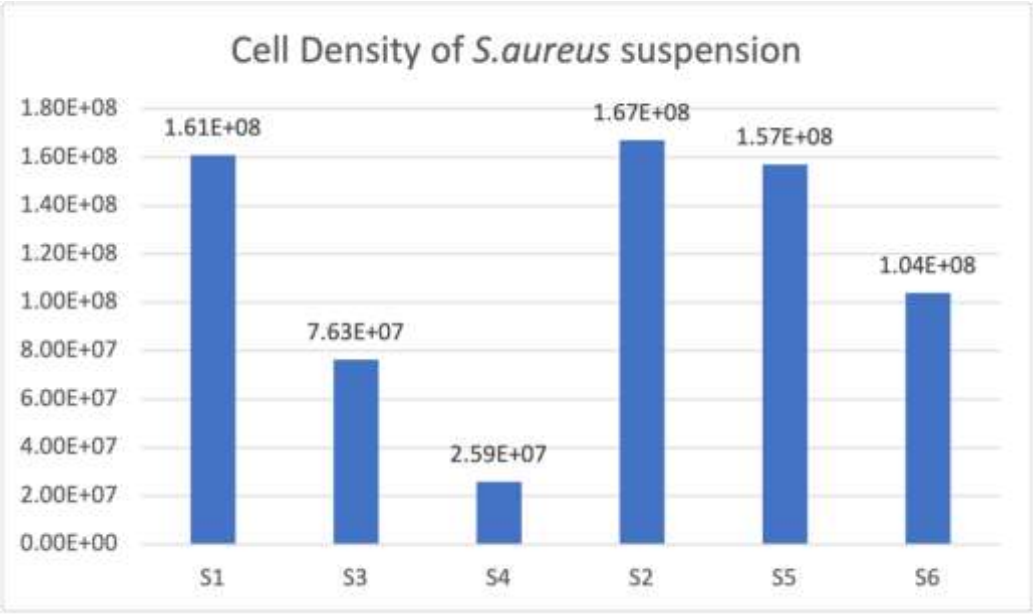


Figure 5. Bar chart of Cell Density

Figure 5. exhibits the Cell Density of *S. aureus* suspensions more directly. It clearly shows the negative correlation between cell density and the amount of available *Citrus limon (Eureka)* extract. More *Citrus limon (Eureka)* extract is added, and more bacteria growth is inhibited.

The following equation can measure the percentage change in cell density between each sample:

$$\%\ change = \frac{CD_a - CD_b}{CD_a} \times 100$$

Therefore, the changes in cell density in the percentage form are shown in the table below.

Table 11. *Changes in cell density under different conditions*

Origin Cell Density (CFU/ml)		The volume of *Citrus limon (Eureka)* added to the sample				Percentage change of CD between conditional samples	
		10ml		20ml			
S1	1.61×10^8	S1→S3	-52.6%	S1→S4	-83.9%	S3→S4	-66.1%
S2	1.67×10^8	S2→S5	-5.99%	S2→S6	-37.7%	S5→S6	-33.8%

In addition, by comparing the percentage change of S1→S3 with S3→S4, and S2→S5 with S5→S6, it can be revealed that the absolute value of percentage changes of the latter one in both comparisons' groups are more significant than that of the previous one. This data indicates that the antibiotic efficiency of a specific *Citrus limon (Eureka)* ascends as more of the specific volume exists. For example, suppose the antibiotic efficiency of 1ml extract is 1, the efficiency will increase to 1.5 per 1ml when there exist 2ml extracts. The reason for such a phenomenon might be that as the amount of *Citrus limon (Eureka)* extract increases, the number of inhibitions factors each bacteria cell faces increases too. Hence, the inhibition degree is intensified.

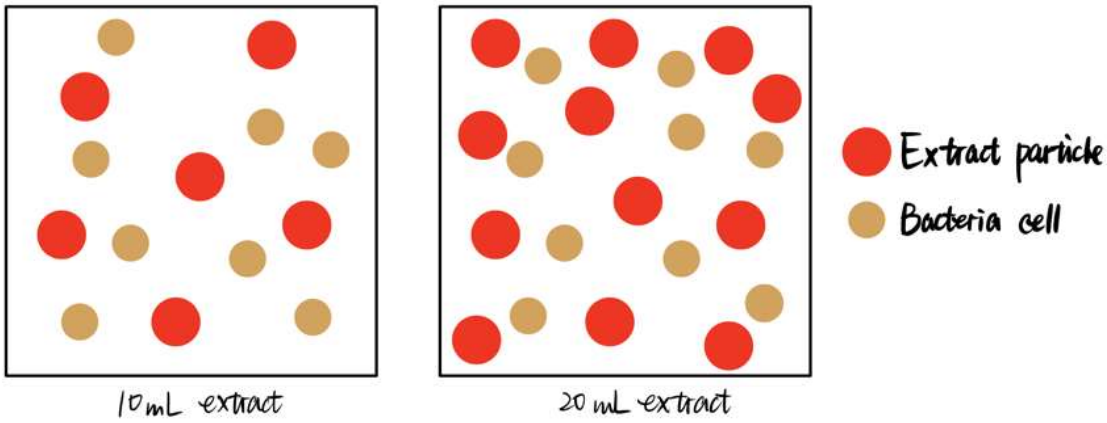


Figure 6. Sketch map of the posited reason for the phenomena

Comparing the antibiotic efficiency of *Citrus limon (Eureka)* extract with antibiotics

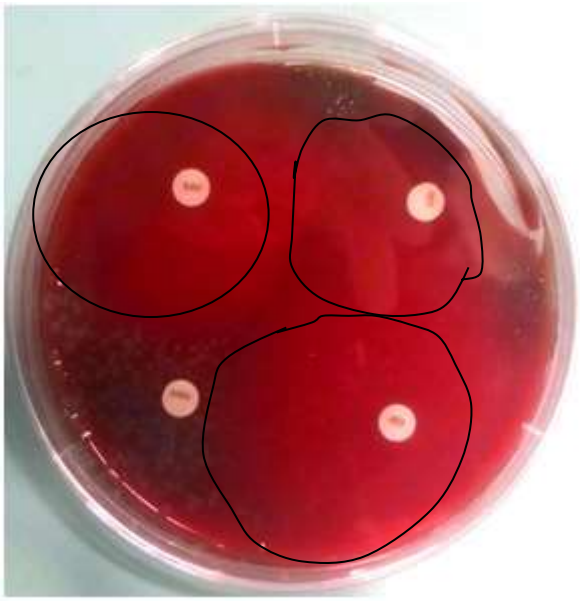
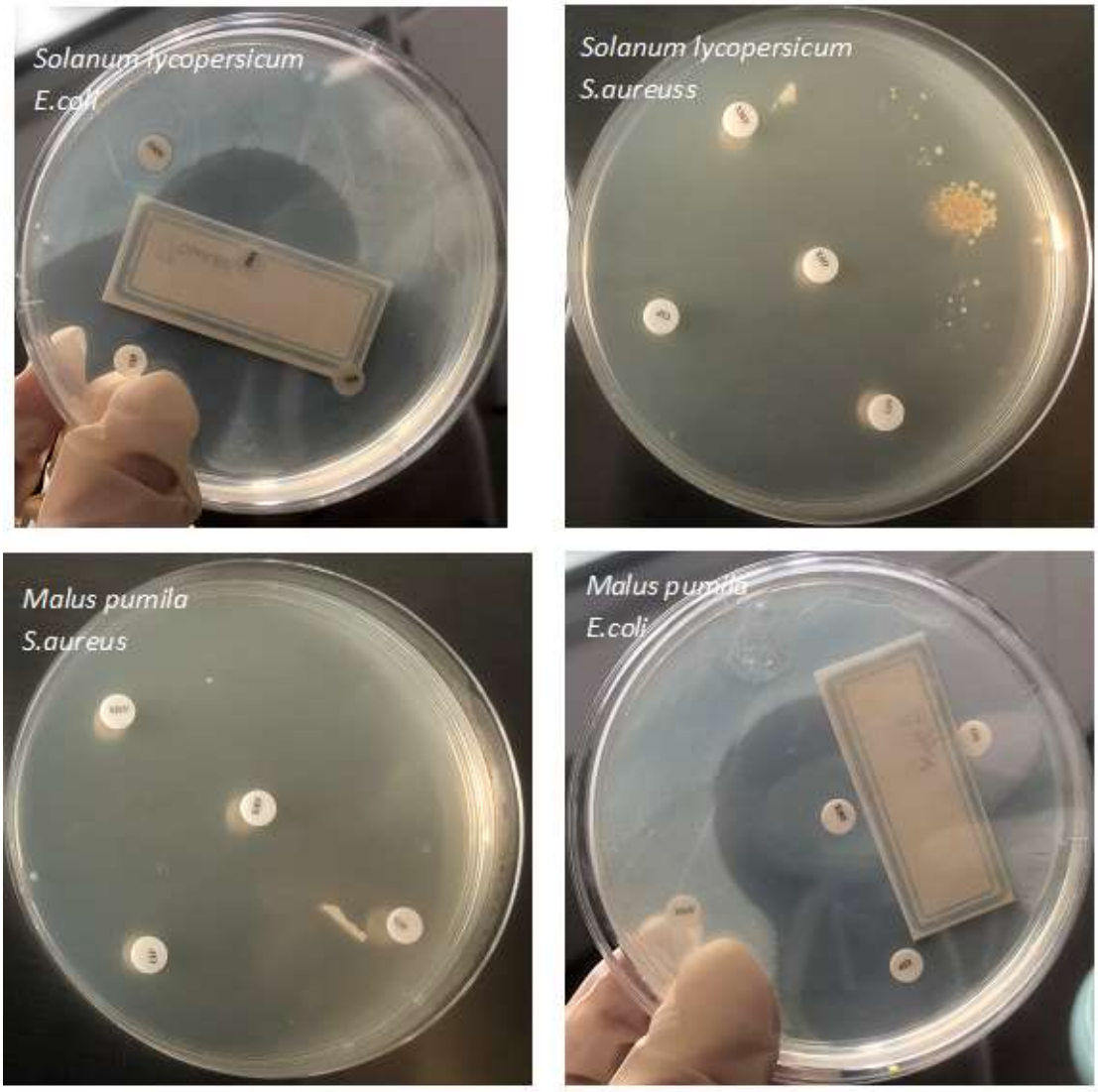


Figure 7. Inhibition zones of antibiotics against *S. aureus*

Table 12. *Diameter of inhibition zones of antibiotics*

	Ofloxacin	Ciprofloxacin	Gentamycin	Amoxicillin	*Citrus limon*
Diameter(mm)	45	60	36	-	23

Appendix-1: Figurative data about the pilot test



Appendix-2: Ultraviolet Spectrometer

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14-3月-2023 11:17 上午 仪器序列号: 9A3Z315016

方法名称: 固定测量 22-2月-2023 仪器型号: GENESYS 50

方法创建时间: 22-2月-2023 10:55 上午 软件包版本: 2.6 签名:

方法更新时间: 22-2月-2023 10:55 上午

固定测量方法参数

方程：结果 = ABS(600)x1

λ1：600nm 波长2

F_1：1.000 系数2

样品	ABS(600)	结果 (-)
空白		
diluent	0.000	0.000
200+20+20	0.037	0.037
200+20+10	0.109	0.109
200+40+20	0.148	0.148
200+40+10	0.224	0.224
200+20	0.230	0.230
200+40	0.238	0.238

According to the data in table 12, the inhibition zones of Ofloxacin, Ciprofloxacin, and Gentamycin are more significant than that of *Citrus limon*. No inhibition zone exists around Amoxicillin, meaning it does not have antibiotic efficiency against *S. aureus*. Hence, it can be concluded that the antibiotic efficiency of *Citrus limon (Eureka)* is better than Amoxicillin but worse than Ofloxacin, Ciprofloxacin, and Gentamycin. However, it cannot be denied that *Citrus limon (Eureka)* extract still has good antibacterial ability against *S. aureus*

4. Conclusion

In conclusion, the pilot test result demonstrates the non-antibiotic efficiency of *Mangifera indica*, *Vaccinium spp*, *Malus pumila*, *Zingiber officinale*, *Plantago depressa*, *Astragalus membranaceus*, *Pyrus spp*, *Actinidia chinensis*, *Codonopsis pilosula*, *Taraxacum mongolicum*, *Panax quiquefolium*, *Solanum lycopersicum*, and the antibiotic efficiency of *Citrus limon*. The bacteria incubation in Petri dishes indicates that *Citrus limon (Eureka)* extract contains vigorous antibacterial activity against gram-positive bacteria but no evident impact against gram-negative bacteria. To confirm the finding from the incubation test, the Ultraviolet Spectrometer test is used to determine the cell density of the suspensions to get quantitative results. Both tests reveal the negative correlation between the cell density of bacteria and the amount of *Citrus limon (Eureka)* extract. In addition, the UV Spectrometer test shows an increase in antibiotic ability per 10ml *Citrus limon (Eureka)* extract due to the cumulative amount of 10ml.

However, the experiment still has limitations that can be improved. Firstly, during the incubation test, many colonies exist on the agar after 24 hours of incubation. The phenomenon indicates that too many bacteria (more than 1×10^6cells) were put onto the agar the previous day. Overpopulation of bacteria could reduce the antibiotic efficiency of *Citrus limon (Eureka)* extract in the form of a shorter inhibition zone diameter. Therefore, the result will contain systematic errors. Secondly, the quantity of experiment groups is too small, which results in a limitation of data. Without enough valid data, random uncertainty could significantly influence the results and lead to the unreliability of the conclusion. To reduce the impact of random uncertainty, the experiment should be repeated. Thirdly, the period of the incubation test should be extended. In the actual situation, the patients are typically asked to take Traditional Chinese Medicine for months continuously. This requirement indicates that the impact of TCM often takes time to be revealed. Thus, the antibiotic efficiency of TCM might be discovered after prolonged incubation.

Acknowledgment

Thanks to Prof Dr. Muhammad Sohail (Nanjing Normal University) for providing the materials and supporting our research.

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