Comparing The Antibacterial Properties of TCM Herbal Extracts

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Abstract

As Traditional Chinese Medicine (TCM) becomes increasingly popular amongst Singaporeans, and often used as a form of Complementary and Alternative Medicine (CAM) to Western medicine, many studies have been done to prove the efficacy of TCM both as a substitute for Western medicine and as a CAM. This study aims to investigate the antibacterial properties of TCM on Gram-Positive (Gram+) and Gram-Negative (Gram-) bacteria. In this study, 3 variables were independently investigated to gain insight into its impact on the antibacterial properties of TCM Herbal Extracts - types of herb used, preparatory method, and concentration of Herbal Extract. The pathogenic bacteria selected were *Escherichia coli* as the Gram- bacteria, and *Staphylococcus epidermidis* as the Gram+ bacteria, while the herbs used were *Lonicera japonica* (Jin Yin Hua) and *Houttuynia cordata* (Yu Xing Cao) plants. The herbs were prepared as a dissolved powder solution and boiled decoction solution, and were subject to a well-diffusion test to measure the zone of inhibition of the herbal extracts, as well as MIC test to determine the minimum inhibitory concentration of the herbal extracts and MBC test to determine the minimum bactericidal concentration of the herbal extracts to determine whether the herbs were bactericidal or bacteriostatic. The results showed that TCM Herbal Extracts had the same effect on Gram+ bacteria and the Gram- bacteria. Furthermore, it was found that while the preparatory methods had no effect on *H. cordata*, the *L. japonica* decoction showed significantly greater antibacterial activity than its dissolved powder counterpart. Lastly, the study showed that as the concentration of herbal extract increased, its antibacterial activity increased as well, with the MIC tests showing that both herbs contained bacteriostatic properties. The bactericidal properties of the extracts could not be determined using the MBC due to contamination and insufficient time.

Introduction

Recently, there has been growing interests in the use of TCM as a form of CAM in countries outside of Mainland China, such as in Hong Kong or the US, which experienced a growth, from 34% in 1989 to 42% in 1997, as reported by Eisenberg (as cited by Wong, Lam, Sham & Fong, 2012). Reported by Fisher and van Haselen, many of these patients who used TCM as a CAM method reported improvements with their illnesses which Western Medicine had been unable to help (as cited by Wong et al., 2012).

Acute Respiratory Tract Infections (ARTI) are more widespread now, as reported by WHO. For instance, Williams, Gouws, Bosichi-Pinto, Bryce and Dye (2002) have reported that approximately 21% of child deaths are due to pneumonia (as cited by Scott, Brooks, Peiris, Holtzman & Mulhollan, 2008). Regarding Pneumonia, hospital-acquired (HAP) and

healthcare-associated (HCAP) types are mainly transmitted and caused by Pseudomonas Infections and Staphylococcus Infections (Bin Abdul Sattar & Sharma, 2019).

There has been research to prove the efficacy of TCM on the treatment of ARTIs, SARS, bronchitis, pneumonia and the common flu. According to Wei et al. (2005), patients treated with Chinese herbs had a shorter duration of signs and symptoms for ARTIs and were beneficial in terms of relief of signs and symptoms. The conclusion made was that TCM Herbal Medicine was more effective than routine treatment. Apart from which, studies have been conducted to investigate the efficacy of TCM Herbal Medicines in comparison to antibiotics or placebo. The studies showed that TCM Herbal Medicines may shorten the symptomatic phase in patients with the common cold, granting them relief from its symptoms and aiding in recovery (Zhang et al., 2007). Studies carried out by Sun et al. also proved the efficacy of TCM Herbs, notably the Ji Shi Granule, to treat certain ARTIs such as bronchitis or coughs (as cited by Jiang, Li, Wu (2012)).

In a more recent study carried out by Cai et al. (2017), the effectiveness of Chinese Herbal Medicine was tested by combining it with antibiotics, such as carbapenems and aminoglycosides, with a sample size ove 766 patients, 547 in the Chinese Herbal Medicine and antibiotic group, and 219 patients in the control group, accepting antibiotic monotherapy. The patients were administered treatment for 14 days, and observed for another 14 days, after which the survival rate of the patients were calculated and recorded. From the experimental group who received both Chinese Herbal Medicine and antibiotics, the survival rate was over 86%, while that in the control group was approximately 82%, noted to be an insignificant difference. The study also found that while different types of herbal extracts were administered to different patients who suffered different conditions, it was found that the *L. japonica* herb was administered to most patients who had developed ARTIs, with the study noting the presence of over 140 active components and a strong capability of *L. japonica* to inhibit bacterial growth.

TCM is, therefore commonly used as a form of complementary medicine, and is coupled with regular intake of antibiotics. TCM herbal medicines are able to shorten the symptomatic phase for patients, and are used to provide comfort to patients and improve their quality of life (Wei et al., 2012).

According to the studies of how TCM can be used in the treatment and improvement of quality of life of numerous ARTIs, and its comparisons of effects to placebo showing positive results, numerous limitations have been identified present in each experimental group (Wu, Yang, Zeng & Poole, 2008). Design-limitations influence the outcome of the studies, as patients used in sampling sizes for the control and experimental group were unequal in most of the reports, rendering them less reliable. In addition, some studies failed to mention how the participants were ‘randomly’ selected, and the selection of materials, participants and equipment as many of these researches were carried out by researchers and practitioners from different hospitals, introducing the possibility of biasness. Furthermore, many of the studies show that the antibiotics used and their efficacy was not named, instead using the term, ‘positive effect drugs’,

and the methods of studies were all rated Category C, low-quality. Another limitation is the use of different prescriptions to different people due to their different body constituents as well as the ARTI causative bacteria.

In this study the *L. japonica* and *H. cordata* plants were chosen for their use in many TCM herbal extracts to treat the patient with coughs, fevers, and colds, while the bacteria strains *E. coli* and *S. epidermidis* were chosen as they had a BSL Level of 1, making them safer to handle than usual ARTI-causing pathogenic bacteria. Furthermore, using these 2 strains, as they do cause certain ARTIs such as lower respiratory tract infections, results for these 2 bacteria can be extrapolated to other pathogenic strains..

Objectives and Hypotheses

Objective

The objective of this study is to investigate how the types of herb used affects the growth and reproduction rate of *E. coli* and *S. epidermidis* bacteria, as well as how the method of preparation of the herb and concentration of herb might inhibit its growth and possibly kill the bacteria.

Hypotheses

It was hypothesised that the Gram- bacteria, *E. coli* would be less affected by the herbs compared to the Gram+ bacteria, *S. epidermidis*. Furthermore, it was also hypothesised that while preparatory methods would have no effect on the efficacy of the herbal extracts to inhibit bacterial growth, increasing the concentration of the extract would increase antibacterial activity, with *L. japonica* having a greater effect than *H. cordata*.

Experimental Procedures

Preparing Herbal Decoctions

2 batches of herbal extracts were prepared per experimental set, one for the well-diffusion test, and the other for the MIC and MBC tests. For the well-diffusion test, a 10% herbal decoction by mass was prepared. 10g of *L. japonica* was added to a beaker, and 90g of water was added to the beaker. A piece of aluminium foil was used to cover the beaker to prevent loss of water to surroundings due to evaporation. The contents were then heated until it reached boiling point, before being heated at 100℃ for another 10min, and left to cool for 15min. The heating process was repeated once more, and the decoction was decanted into a bottle. The decoction was allowed to cool to room temperature before it was filtered by means of a syringe filter. This was repeated for a 20% decoction, in which 20g of herb and 80g of water was used, for the MIC and MBC tests. Both procedures were repeated for the *H. cordata* herb.

Preparation of Dissolved Powder Solution

For the well-diffusion test, a 10% Dissolved Powder Solution was required. The *L. japonica* herbs were first dry-blended into a fine powder, before 10g of the powder was dissolved in room temperature water. The mixture was stirred for 5-10min before it was left for 10min, to allow additional fine powder to sediment to the bottom. The solution was then decanted off and collected, before being filtered by means of a syringe filter. This was repeated for the 20% Dissolved Powder solution required for the MIC and MBC tests, with 20g fine powder and 80g water. Both procedures were repeated for *H. cordata* herb.

Inoculating Liquid Bacterial Culture

7mL of LB broth was poured into a falcon tube. Next, an inoculating loop was heated until red hot before it was shaken to cool, and used to extract an *E. coli* bacteria colony streak. The bacteria was put into the falcon tube with LB broth, and swirled. After loosely capping the tube, it was placed in a shaking incubator at 37℃ for 12 hours. This was repeated for *S. epidermidis* bacteria.

Well-Diffusion Test

The well-diffusion test was carried out in a laminar fume hood with a lighted flame to ensure a sterile environment and prevent contamination. A cotton swab was heated slightly before it was placed in the falcon tube containing LB broth and *E. coli* bacteria. The bacteria was then spread over Muller-Hinton (MH) agar, before 4 wells of 0.8cm diameter were made in the agar. A pair of forceps were used to remove the agar in the wells. Then 80μL of *L. japonica* decoction and dissolved powder were pipetted into 2 of these wells separately with the use of a micropipette. 80μL of 10% bleach and water were pipetted into the other 2 wells separately, to act as a positive and negative control, respectively. The lid was then put on and the agar plate sealed with parafilm before being stored in an incubator at 37℃ overnight. After which, a ruler was used to measure the diameter of the zone of inhibition. This was repeated, using *S. epidermidis* instead of *E. coli* bacteria, and both were repeated for *H. cordata*. Then, all 4 plates were replicated again for 6 more sets of data

MIC Test

The MIC test was carried out in a laminar fume hood with a lighted flame to ensure a sterile environment and prevent contamination. Add 500μL of the sterile LB broth into 1 microfuge tube using a micropipette, and add sterile LB broth of concentration half of the first one, into 8 other tubes. Next, add 500μL of *L. japonica* decoction into the first microfuge tube. The contents were mixed by pipetting up and down 2-3 times. Then 2-fold serial dilution was performed by using a new pipette tip to transfer 500μL of the mixture into the next microfuge tube. This process is repeated for tubes 1 through 8, and discarding the additional 500μL, while the 9th tube will act as a control. Then, with a new pipette tip, 100μL of *E. coli* bacteria was added to each tube, except for the 9th tube. After overnight incubation at 37℃, the tubes were taken out and compared with one another to record the turbidity in each tube using the categories ‘0’ for no growth, ‘+’ for little growth, ‘++’ for medium growth and ‘+++’ for much growth. Repeat this

procedure with the *S. epidermidis bacteria*, and then both series' with *L. japonica* Dissolved Powder. Then repeat all 4 series' with *H. cordata*.

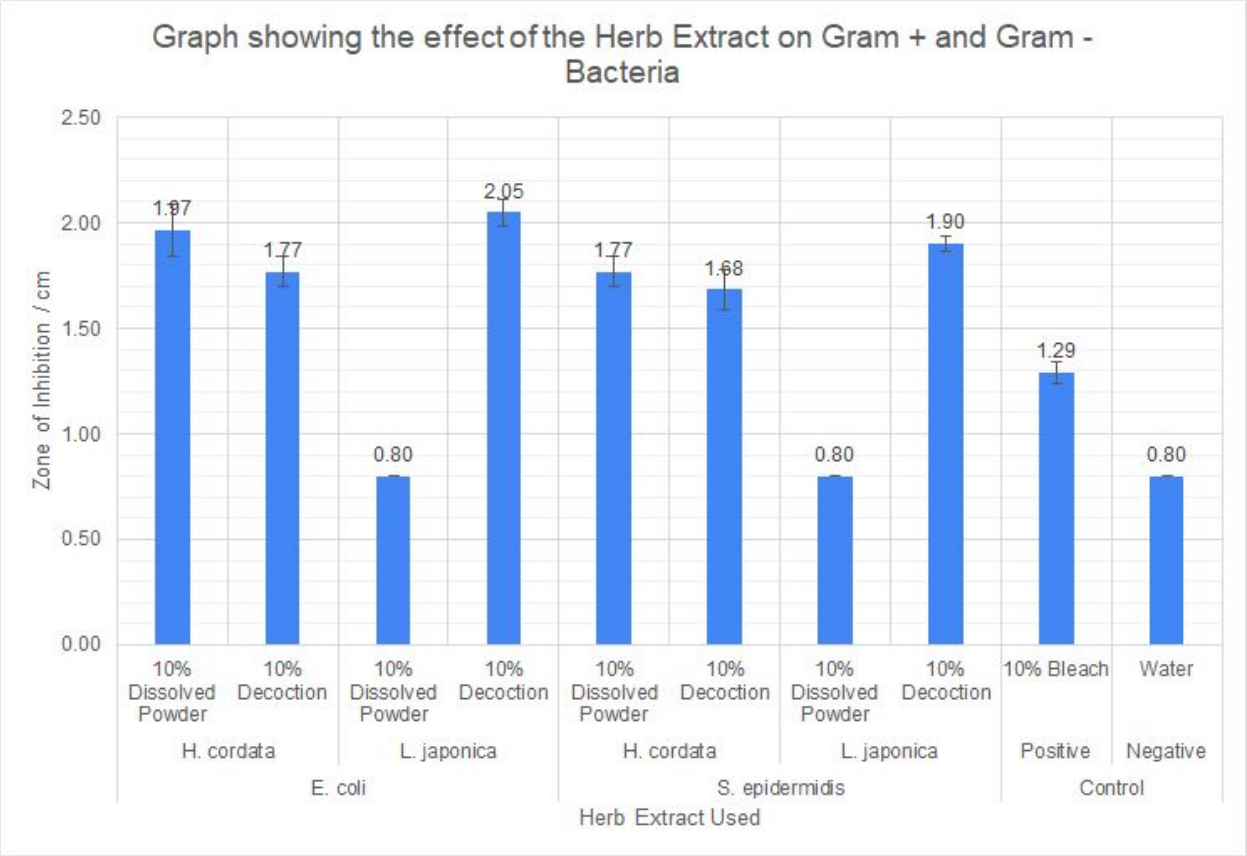
MBC Test

This test is carried out in a laminar fume hood with a lighted flame as well. Using the results from the *L. japonica* decoction with *E. coli* MIC test, the tubes with turbidity of '0', as well as the first concentration at which there is little bacterial growth, were selected. The nutrient agar was split into 4 quartiles. An inoculating loop was heated until red-hot, then cooled by cutting it into the sides of the agar. The inoculating loop was then dipped in a selected tube, and the sample was spread over one of the agar quartiles. This process is repeated until all selected samples have been spread. Finally, the agar was incubated overnight at 37℃, before it was removed and the results of bacterial growth were recorded using the same code of '0/+/++/+++'.

Results and Discussion

Well-Diffusion Test Results

Two sets of triplicates were deemed suitable for analysis. The results of these two sets of triplicates were combined and averaged. A graph of the results is found below.



Wells-Diffusion Test Discussions

The larger the zone of inhibition, the more effective the herbal extract in inhibiting bacteria.

The average zone of inhibition for *L. japonica* decoction was higher in *E. coli* (2.05cm) than in *S. epidermidis* (1.90cm). For the *H. cordata* decoction, the average zone of inhibition was also higher in *E. coli* (1.77cm) than in *S. epidermidis* (1.68cm).

The average zone of inhibition for *L. japonica* dissolved powder solution was the same in both *E. coli* and *S. epidermidis* (0.80cm). On the other hand, *H. cordata* showed a higher average zone of inhibition in *E. coli* (1.97cm) than in *S. epidermidis* (1.77cm).

The type of preparation for *H. cordata* had no observable difference in bactericidal properties against both *E. coli* and *S. epidermidis*. However, as for *L. japonica*, the decoction showed a much larger zone of inhibition against both *E. coli* and *S. epidermidis* than the dissolved powder solution. Possible reasons include the active components' ability to pass through selectively permeable cell surface membrane and a lot of overlap in data for *H. cordata*, with close results in Test 1.

MIC Test Results

The MIC Test was conducted twice. However, due to contamination and hence inaccurate results in the first test, only data from the second test will be presented here.

Bacteria used: *E. coli*					
Tube	Concentration (µg/ml)	*L. japonica* decoction	*L. japonica* dissolved powder solution	*H. cordata* decoction	*H. cordata* dissolved powder solution
1	200	0	0	0	0
2	100	0	0	0	0
3	50	0	0	0	0
4	25	+	0	0	+
5	12.5	++	+	+	+
6	6.25	++	++	+	++
7	3.125	++	+++	+++	++
8	1.5625	+++	+++	+++	+++

Bacteria used: *S. epidermidis*					
Tube	Concentration (µg/ml)	*L. japonica* decoction	*L. japonica* dissolved powder solution	*H. cordata* decoction	*H. cordata* dissolved powder solution
1	200	0	0	0	0
2	100	0	0	0	0
3	50	0	0	0	0
4	25	0	0	0	0
5	12.5	0	+	+	+
6	6.25	++	++	+	++
7	3.125	++	++	+	++
8	1.5625	++	++	++	+++

MIC Test Discussions

As the concentration of the TCM herbal extract solution increases, the effectiveness of the solution in inhibiting bacterial growth increases. For the purposes of measurement, we analysed the turbidity of the tubes.

For the *L. japonica* decoction samples, the minimum inhibitory concentration was higher in *E. coli* (50µg/ml) than in *S. epidermidis* (12.5µg/ml), showing that the samples were less effective on *E. coli*. However, for the *H. cordata* decoction samples, the minimum inhibitory concentration, and thus the inhibitory ability, was the same in both *E. coli* and *S. epidermidis* (25µg/ml). Such results are similar to those gathered in the Well-Diffusion Test for *H. cordata* but not for *L. japonica.*

For the *L. japonica* dissolved powder solution samples, the minimum inhibitory concentration was the same for both *E. coli* and *S. epidermidis* (25µg/ml). For the *H. cordata* dissolved powder samples, the minimum inhibitory concentration was higher in *E. coli* (50µg/ml) than in *S. epidermidis* (25µg/ml). Such results are slightly different from those gathered in the Well-Diffusion Test.

The type of preparation for both *L. japonica* and *H. cordata* did not affect the inhibitory effect on both *E. coli* and *S. epidermidis* by a significant amount, though there was some variation. This is in line with what we have gathered in the Well-Diffusion Test, further affirming the statement that the type of preparation does not affect the bactericidal properties of the herb extract solution.

MBC Test Results

The MBC Test was conducted twice as well, using the tubes obtained through the MIC Test. However, the first set of testing was heavily contaminated and only the second set will be presented here.

Bacteria used: *E. coli*				
Concentration (µg/ml)	*L. japonica* decoction	*L. japonica* dissolved powder solution	*H. cordata* decoction	*H. cordata* dissolved powder solution
200	+	0	0	0
100	+	0	++	0
50	+++	0	++	0
25	+++	+	++	+

Bacteria used: *S. epidermidis*				
Concentration (µg/ml)	*L. japonica* decoction	*L. japonica* dissolved powder solution	*H. cordata* decoction	*H. cordata* dissolved powder solution
200	Due to suspected contamination, the results will not be reported here.	++	0	0
100		++	+	0
50		++	+	+
25		+++	+	++

MBC Test Discussions

The minimum bactericidal concentration was measured here. A higher minimum bactericidal concentration signifies a lower bactericidal ability of the herb extract solution.

Comparison was unable to be made between *L. japonica* decoction in *E. coli* and *S. epidermidis* due to suspected contamination for the *L. japonica* decoction in *S. epidermidis*. However, it was ascertained that *H. cordata* decoction has an equal minimum bactericidal concentration, and hence an equal bactericidal ability, in both *E. coli* and *S. epidermidis* (200μg/ml). This is in line with both our Well-Diffusion test and MIC Test results.

The *L. japonica* dissolved powder solution showed a much lower minimum bactericidal concentration, and hence a much higher bactericidal ability, in *E. coli* (50μg/ml) than in *S. epidermidis* (>200μg/ml). The *H. cordata* dissolved powder solution showed a slightly lower minimum bactericidal concentration, and hence a slightly higher bactericidal ability, in *E. coli* (50μg/ml) than in *S. epidermidis* (100μg/ml). This follows the trend displayed in both our Well-Diffusion and MIC Test results for *H. cordata* but not for *L. japonica*.

Possible Sources of Error

Some of our data in the tests did not match with each other. For example, where the *L. japonica* decoction was more effective in *E. coli* than in *S. epidermidis* during the Well-Diffusion Test, the reverse was true (i.e. *L. japonica* decoction was less effective in *E. coli* than in *S. epidermidis*) during the MIC Test. Possible reasons for any discrepancies could include a slight contamination of certain results, or alternatively a varying volume of spread. Furthermore, a spectrophotometer was not used for measuring our data, hence the data could be subjective.

Kruskal-Wallis Test

The Kruskal-Wallis Test was conducted on our results for the Well-Diffusion test to achieve a statistical analysis of our results.

p-value of Decoction VS Dissolved Powder Preparatory Methods Zone of Inhibition in Well-Diffusion Test			
E. coli		*S. epidermidis*	
H. cordata Decoction VS *H. cordata* Dissolved Powder	*L. japonica* Decoction VS *L. japonica* Dissolved Powder	*H. cordata* Decoction VS *H. cordata* Dissolved Powder	*L. japonica* Decoction VS *L. japonica* Dissolved Powder
H. cordata dissolved powder has larger zone of inhibition than decoction, $p>0.05$	*L. japonica* decoction has larger zone of inhibition than dissolved powder, $p<0.05$	*H. cordata* dissolved powder has larger zone of inhibition than decoction, $p>0.05$	*L. japonica* decoction has greater zone of inhibition than dissolved powder, $p<0.05$

p-value of *H. cordata* VS *L. japonica* Zone of Inhibition in Wells-Diffusion Test			
E. coli		*S. epidermidis*	
H. cordata Decoction VS *L. japonica* Decoction	*H. cordata* Dissolved Powder VS *L. japonica* Dissolved Powder	*H. cordata* Decoction VS *L. japonica* Decoction	*H. cordata* Dissolved Powder VS *L. japonica* Dissolved Powder
L. japonica decoction has greater zone of inhibition than *H. cordata* decoction, $p<0.05$	*H. cordata* dissolved powder had greater zone of inhibition than *L. japonica* dissolved powder, $p<0.05$	*L. japonica* decoction has a greater Zone of Inhibition than *H. cordata* decoction, $p>0.5$	*H. cordata* dissolved powder had greater zone of inhibition than *L. japonica* dissolved powder, $p<0.05$

p-value of Positive Control (Bleach) VS Negative Control (Water)	
E. coli	*S. epidermidis*
Bleach solution has a greater zone of inhibition than water, $p>0.5$	Bleach solution has a greater zone of inhibition than water, $p>0.5$

For our data analysis, we carried out a Kruskal-Wallis Test. Blue is used to represent significant p-values, while red is used to represent insignificant ones.

The p-value of *H. cordata* Decoction vs *L. japonica* Decoction are statistically significant in *E. coli*, but not in *S. epidermidis*. This could be because the samples have different effectiveness on gram-positive and gram-negative bacteria. *L. japonica* Decoction could be more effective on gram-negative bacteria while *H. cordata* Decoction could be less effective in gram-negative bacteria.

The p-value of *H. cordata* Decoction vs *H. cordata* Dissolved Powder are statistically insignificant in both *E. coli* and *S. epidermidis*. This could be because the preparatory method used for *H. cordata* does not affect its antibacterial properties in any meaningful way. Hence the difference in effect between the 2 results remains small regardless of the bacteria used.

Another possible reason for these observations is that contamination in the samples when they were being prepared, or that not enough replicates of the results were taken, due to time constraints.

The same data analysis could not be performed on our MIC or MBC Test Results because the data was not taken in numerical form. This is because there is no need for such specific data due to our intention to do an objective comparison. More specific data can and should be taken in a future study.

Conclusions

From the Well-Diffusion tests, the zone of inhibition draws the conclusion that while the type of preparation of *H. cordata* did not have any effect on the zone of inhibition for both bacteria, the decoction form of *L. japonica* exhibited stronger antibacterial activity than its dissolved powder counterpart. This is likely due to the rate of diffusion of active components out of the cell wall of the *L. japonica* herb. By comparing the zones of inhibition, it was concluded that both herbs affect antibacterial growth to a similar extent in their decoction form. Finally, as zones of inhibition do not have any statistical significance between the *E. coli* bacteria and *S. epidermidis* bacteria, it is concluded that Gram+ and Gram- bacteria are affected by both herbs equally.

The MIC test also shows that as concentration of herbal extract increases, efficacy of herbal extracts in inhibiting bacterial growth increases. By comparing the MIC results to MBC results, it was concluded that *L. japonica* and *H. cordata* decoctions exhibit bactericidal effects on *S. epidermidis*. However, both decoctions exhibited bacteriostatic effects on *E. coli*, similar to both dissolved powders on both bacteria. Overall limitations of the MIC and MBC tests were that there could have been slight contamination of certain results, which could cause varying results from the Well-Diffusion Test.

Further Studies

Due to time constraints as a result of COVID-19, our results for the MIC and MBC test cannot be considered reliable as each was conducted twice. Thus, the MIC and MBC tests should be repeated in order to obtain more reliable results to establish a reliable relationship between concentration of herbal extract and its efficacy of inhibiting bacterial growth. Next, although there are already 6 replicates for the well-diffusion test, obtaining at least 15 results would be ideal in order to carry out more reliable statistical analyses such as the T-test. Next, the herbs should be mixed together, as TCM is often seen as a combination of the properties of many herbs in one decoction. By combining the extracts, an observation can be made on whether combining the herbs are more effective. Finally, in order to deduce what could be the active components that affect the antibacterial activity of TCM herbs, Multiple other herbs would have to be tested as well in order to compare present active components and investigate its effects. Testing could also be extended to find out under what conditions did the active components work best, for example, temperature, or even the medium in which it dissolved, such as using honey instead of water.

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