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Article

The *In vitro* Antibacterial Activity of Dried Flowers Used in Guangdong Traditional Soup

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Abstract: Objective: To research the antibacterial effect of deionized water and ethanol-water extracts from the dried flowers used in Guangdong traditional soup on 7 kinds of familiar intestinal pathogenic bacteria. **Methods:** We selected 8 types of dried flowers most commonly used in making Guangdong traditional soup, including *Rafflesia arnoldii, Bombax ceiba, Dolichos lablab, Pueraria lobata, Flos buddlejae, Lonicera japonica, Flos sophorae and Cordyceps militaris*. Seven target bacteria were chosen from usual intestinal pathogenic agents, which included *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Shigella flexneri, Bacillus cereus, Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. Bacteriostasis rate, MIC and MBC were examined to evaluate the dried flowers' antibacterial activity on these pathogens. **Results:** Our findings showed that all the samples exhibited potent antibacterial activity. Gram-positive bacteria were generally more sensitive to the tested extracts than Gram-negative ones. *Staphylococcus aureus* was the most sensitive, while *Escherichia coli* was the most resistant. The antibacterial activity of *Cordyceps militaris* was the strongest, and *Flos sophorae* and *Lonicera japonica* followed. At the same time, the antibacterial effect of ethanol-water extract was higher than that of deionized water extract. **Conclusions:** This was the first time to study the antibacterial property of principal material used in Guangdong traditional soup. These results might underline the theoretical foundation for the dried flowers related Guangdong Traditional Soup's function of adjusting human body's healthy condition which we thought was in relationship in some extent with mild inflammation of upper respiratory tract and tonsils. The more explicit mechanism of the antibacterial effect should be further studied.

Keywords: flower; *Cordyceps militaris*; *Flos sophorae*; antibacterial activity.

1. Introduction

In recent years, there is an increasing interest in investigating the medicinal properties of various plants because of their potent pharmacological activities, convenience to users, economic viability and low toxicity (Prashant et al., 2008). More and more studies found that synthetic drugs not only had adverse effects but also easily induced multidrug resistance of many antibiotics (Jigna and Sumitra, 2006). Nowadays, it is advocated that people should prefer to take diet to achieve therapeutic effect instead of medicine as far as possible.

Guangdong province is located in the south coastal areas of China. It belongs to subtropical climate, hot and humid. The local people have the traditional habit of drinking soup, with known as long as over one thousand years, which has gained its popularity in Southeast Asia, Canada and USA with the migration of Guangdong residents. Drinking Guangdong Traditional Soup (GTS) can complement the loss of moisture, mineral and other useful ingredients without intaking too much heat. According to the Guangdong folk saying, drinking the GTS with dried flowers as main materials can clear damp, decrease inner heat, and moisten dryness. Its essence is supposed to play some role in preventing and alleviating inflammation in the upper respiratory tract, tonsil and even the whole body (Fu et al., 2011). We selected the 8 kinds of dried flowers mostly commonly used in GTS, and studied their antimicrobial properties, targeting 7 kinds of familiar bacteria. This is the first attempt to investigate the antibacterial effect of such stuff. Study of the traditional food by modem scientific methods that reveals scientific basis of the traditional health care function is a hotspot of current research in this area and also is one of the important directions in future (Panichayupakaranant et al., 2010). The experimental results can also provide a basis for the study of new natural antibacterial agents (Popova et al., 2005; Ahmet Ayaz et al., 2008), and help develop natural antibacterial sources in food industry for the public health. Since GTS is popular throughout the world, this study is of great significant.

2. Materials and Methods

2.1. Materials and Sample Preparation

Muller-Hinton Broth and Nutrient Agar were purchased from Guangdong Huankai Microbial Sci. & Tech. Co. Ltd. (Guangdong, China). The 96-well culture cluster was obtained from Corning (USA).

The eight kinds of dried flowers were bought from Chinese herb store named Er Tian Tang in Guangzhou. The dried flowers were ground into fine particles with a special grinder for herbal medicines. Then, a weighed amount (about 10.0 g) of these particles was ultrasonic extracted with deionized water (100 mL) and ethanol-water (50:50, v/v), respectively, at room temperature for 45 min. The extract was filtered by filter paper and the collected fluid was concentrated to 1.0 g/mL. All the samples were sterilized at 121 ºC for 30 min, and stored at 4 ºC until being used.

2.2. Microorganisms and Culture

Staphylococcus aureus CMCC(B)26003, *Escherichia coli* ATCC25922, *Salmonella typhimurium* CMCC(B)50115, *Shigella flexneri* CMCC(B)51572, *Bacillus cereus* CMCC(B)63301, *Pseudomonas aeruginosa* ATCC27853 *and Staphylococcus epidermidis* CMCC(B)26069 were obtained from Guangdong Huankai Microbial Sci. & Tech. Co. Ltd. The bacterial strains were cultured at 37 ºC in Muller-Hinton Broth.

2.3. Screening of the Dried Flowers via Inhibition Ratio of Bacteria

Sterile 96-well microplates were used for the assay. The bacterial suspension was adjusted by a turbidity of a 0.5 McFarland standard (approximately 1.5×10^8 colony-forming units per mL), then a further 1:10 dilution was made twice, so that each well contained approximate 5×10^5 colony forming units (CFU)/mL of test bacteria (Zhao et al., 2010). Seventy microliters of each sample was transferred into 96-well microplates, followed by adding 70 µL of MHB, and inoculating 70 µL of bacterial suspensions, in total 210 µL each well. Normal saline was used as the growth control and the experiments were performed in triplicate. Then, these 96-well microplates were mixed by the vibrator and put in the ELIAS reader for measuring absorbance at 630 nm (Oroojalian et al., 2010). After being incubated at 37 ºC for about 24 h in an ambient air incubator, the 96-well microplates were checked with the ELISA reader again to detect the growth inhibition of the bacteria. The percentage inhibition ratio was calculated using the equation (Viuda-Martos et al., 2010):

$$\% \text{ inhibition} = (A_{Control} - A_{Sample})/A_{Control} \times 100\%$$

2.4. Determination of Minimum Inhibitory Concentration (MIC)

All the samples were two-fold diluted by deionized water. Two-fold microdilution broth method (NCCLS, 2003) was used to determine the minimum inhibitory concentration (MIC). Then, 70 µL of each sample solution was added into the sterile 96 wells that contained 70 µL Muller-Hinton Broth and 70 µL bacterial suspensions. The experiments were performed in triplicate. The absorbance was checked after the samples being vibrated and mixed. After that, the samples were cultivated in 37 ºC incubator for 24 h, and their absorbance was checked again. The microdilution trays were inspected from the minimum concentration with naked eyes to detect the growth inhibition of the bacteria. MIC refers to the lowest concentration of an antimicrobial effect that can inhibit the visible growth of the microorganism (Baron et al., 1994).

2.5. Determination of Minimum Bactericidal Concentration (MBC)

The 200 µL of sample solution was transferred from each well that inhibited the visible growth of a microorganism to Muller-Hinton agar plates and incubated at 37 ºC for 24 h. The lowest concentration of sample that no viable bacteria were identified was the MBC (Patrick, 1996; Zhao et al., 2010).

2.6. Statistical Analysis

All the experiments were performed in triplicate, and the results were expressed as mean ± SD (standard deviation). Statistical analysis was performed using Excel 2007 and SPSS 13.0.

3. Results

3.1. Scientific and Chinese Names of Eight Flowers

The names of these flowers are given in Table 1.

Table 1. Names of eight flowers

No.	Scientific name	Name in Chinese
1	*Rafflesia arnoldii*	霸王花
2	*Bombax ceiba*	木棉花
3	*Dolichos lablab*	扁豆花
4	*Pueraria lobata*	葛花
5	*Flos buddlejae*	密蒙花
6	*Lonicera japonica*	金银花
7	*Flos sophorae*	槐花
8	*Cordyceps militaris*	虫草花

3.2. Antibacterial Activity of Eight Flowers

From Table 2, we could see that all the samples had strong antibacterial activity except *Pueraria lobata* to *E. coli, S. typhimurium, S. flexneri and P. aeruginosa* as well as *Flos buddlejae* to *S. aureus*, *E. coli*, *S. typhimurium, S. flexneri and P. aeruginosa* when the samples extracted by deionized water. When they were extracted by ethanol-water (50:50, v/v), all samples had strong antibacterial effect except that *Flos sophorae* had no or very low antibacterial to *B. cereus*, *E. coli*, *S. flexneri*. In this study, we found that, after incubation some bacterial suspensions became clearer than those before incubation. Except the instrumental error, maybe some dried flowers not only have the function of antibacterial effect but also could create something that made the suspensions clearer. Therefore some of the results were higher than 100%. But we still consider those results as approximate 100% (Ma et al., 2000; Liao, 2010). Overall, most of the selected samples had strong antibacterial power to the bacteria.

Table 2. Antibacterial activity (%) of eight flowers

No.	Gram-positive bacteria			Gram-negative bacteria			
	S. aureus	***B. cereus***	***S. epidermidis***	***E. coli***	***S. typhimurium***	***S. flexneri***	***P. aeruginosa***
1	100.00±1.39	100.00±4.74	100.00±10.97	100.00±1.37	100.00±3.95	100.00±2.64	100.00±0.38
2	95.72±0.70	100.00±2.27	100.00±0.52	100.00±0.68	100.00±3.32	100.00±1.68	100.00±1.41
3	96.36±0.37	100.00±0.10	100.00±1.05	100.00±0.22	100.00±2.78	100.00±0.55	100.00±0.84
4	93.44±0.45	100.00±0.73	100.00±2.04	/	/	/	/
5	/	99.52±0.69	99.40±1.08	/	/	/	/
6	97.22±0.21	98.30±0.28	96.61±1.75	97.17±0.34	97.84±0.16	97.33±0.16	98.15±0.13
7	94.08±0.69	95.46±2.58	93.12±1.67	10.88±2.69	90.99±1.69	92.82±2.26	96.30±0.46
8	97.29±0.65	98.00±0.48	95.31±1.65	96.87±0.59	95.87±0.86	96.13±0.48	97.11±0.89
1 *	100.00±17.91	100.00±0.55	100.00±1.80	100.00±1.27	100.00±0.59	100.00±1.12	100.00±0.77
2 *	84.17±11.56	86.07±4.75	85.14±6.53	72.73±8.06	72.86±5.56	83.33±10.61	88.81±3.68
3 *	96.22±2.08	100.00±1.64	100.00±4.50	100.00±12.52	100.00±3.32	100.00±1.99	100.00±9.49
4 *	86.80±1.99	90.98±2.55	81.06±1.48	14.16±5.06	84.60±1.81	84.90±4.25	90.59±1.51
5 *	100.00±3.52	100.00±1.79	100.00±1.87	49.70±9.72	45.63±8.18	42.82±7.43	100.00±2.74
6 *	100.00±2.67	100.00±2.28	100.00±2.42	100.00±6.46	99.10±1.46	94.95±3.83	100.00±0.77
7 *	92.98±0.26	/	94.73±3.25	/	63.38±1.59	31.46±9.36	92.6±0.15
8 *	100.00±6.41	98.36±0.83	100.00±5.22	100.00±2.35	100.00±1.83	100.00±1.84	100.00±4.35

Note: No. 1-8 were the results of deionized water extracts; No. 1*-8* were the results of ethanol-water extracts; "/" means the well changed into turbidity after 24 h incubation in 37 ºC.

3.3. Minimum Inhibitory Concentration (MIC) of Eight Flowers

According to the result of MIC (Table 3), it was observed that the inhibitory ratio of Gram-positive bacteria was higher than Gram-negative bacteria when extracted by deionized water. *Cordyceps militaris* had the highest inhibitory ability, followed by *Flos sophorae*, *Lonicera japonica* and *Pueraria lobata*, but *Flos buddlejae* showed the lowest power.

Table 3. MIC and MBC (mg/mL) of eight flowers

No.	Gram-positive bacteria						Gram-negative bacteria							
	S. aureus		*B. cereus*		*S. epidermidis*		*E. coli*		*S. typhimurium*		*S. flexneri*		*P. aeruginosa*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	250	/	250	/	250	/	1000	/	1000	/	1000	/	250	1000
2	500	500	500	500	1000	1000	1000	/	1000	/	1000	/	500	1000
3	500	1000	500	500	500	/	1000	/	500	1000	1000	/	500	1000
4	/	/	1000	/	500	/	/	/	/	/	/	/	/	/
5	/	/	1000	/	500	/	/	/	/	/	/	/	/	/
6	250	1000	250	500	250	/	500	/	500	1000	500	1000	250	500
7	125	250	500	500	125	500	/	/	1000	1000	1000	1000	500	500
8	67.5	250	250	500	125	250	250	1000	250	/	250	/	250	1000
1 *	250	500	250	500	250	500	1000	/	500	1000	500	/	250	500
2 *	1000	1000	250	250	250	1000	1000	1000	1000	1000	1000	1000	250	500
3 *	250	500	250	250	250	500	500	/	500	1000	500	500	250	500
4 *	250	250	1000	1000	250	1000	/	/	1000	/	1000	/	1000	1000
5 *	125	250	1000	1000	250	1000	/	/	/	/	/	/	1000	1000
6 *	125	125	125	250	125	250	250	1000	250	500	250	500	125	250
7 *	250	250	1000	/	500	1000	/	/	/	/	/	/	1000	1000
8 *	125	125	500	/	125	500	250	/	250	1000	250	1000	500	1000

Note: No. 1-8 were the results of deionized water extracts; No. 1*-8* were the results of ethanol-water extracts; "/" of MIC imply turbidity by naked eye with the concentration of 1000 mg/mL; "/" of MBC show bacteria growing in culture dish after inoculate culturing with the concentration of 1000 mg/mL.

The result also showed that the inhibitory activity of ethanol-water extract was higher than that of deionized water extract, and ability of inhibiting Gram-positive bacteria was higher than that of

Gram-negative bacteria. *Flos buddlejae*, *Lonicera japonica* and *Cordyceps militaris* showed high inhibitory ability and their MIC could reach 125 mg/mL.

Among all the selected pathogens, *Staphylococcus aureus* was the most sensitive, while *Escherichia coli* was the most resistant.

3.4. Minimum Bactericidal Concentration (MBC) of Eight Flowers

According to result of MIC, all the wells that remained clear were selected to detect MBC. When extracted by deionized water, it showed that almost half of the samples had no MBC, suggesting they had no bactericidal effect. The MBC of Gram-positive bacteria was lower than Gram-negative bacteria, suggesting the dried flowers were more inclined to kill the Gram-positive bacteria. *Flos sophorae* and *Cordyceps militaris* had the lowest MBC and it could reach 250 mg/mL, suggesting the two flowers owned the strongest bactericidal activity.

When extracted by ethanol-water, the results implied that bactericidal activity was higher than by deionized water. The bactericidal effect on Gram-positive bacteria was also higher than Gram-negative bacteria. The MBC of *Lonicera japonica* and *Cordyceps militaris* was the lowest and could reach 125 mg/mL.

Also, *Staphylococcus aureus* was the most sensitive, while *Escherichia coli* was the most resistant.

4. Discussion

This study suggested that the selected 8 dried flowers had strong antibacterial activity not only to Gram-positive bacteria but also to Gram-negative bacteria. The results showed that the antibacterial effect of these dried flowers to Gram-positive bacteria was stronger than that to Gram-negative bacteria, which was in according with the antibacterial research of others (Ceylan and Fung, 2004; Lopez et al., 2005; Shan et al., 2005; Shan et al., 2007a; Al Momani et al., 2013; Rahman and Islam, 2013). The reason may be that there is a significant difference of the outer layer between Gram-positive and Gram-negative bacteria. In addition, Gram-negative bacteria had a unique periplasmic space which was not found in Gram-positive bacteria (Nikaido, 1994; Duffy and Power, 2001).The outer layer of Gram-negative bacteria has the resistance toward antibacterial substances. The hydrophilic surface of Gram-negative bacteria's outer membrane was abundant in lipopolysaccharide molecules, which not only supported a barrier to the penetration of numerous antibiotic molecules, but also were associated with the enzymes in the periplasmic space, which was capable of breaking down the molecules introduced from outside (Nikaido, 1994; Gao et al., 1999; Bouhdid et al., 2010). However, to Gram-positive bacteria, some antibacterial substances could easily destroy the bacterial cell and cytoplasmic membrane and led to the cytoplasm and its coagulation leakage loss because there was no such special outer membrane and cell wall structure of it (Kalemba and Kunicka, 2003).

In this study, 7 kinds of bacteria were selected, among which *S. aureus* was the most sensitive, while *E. coli* was the most resistant. This result was similar to the study on dietary spice and medicinal herb by Shan et al. (2007a). It was found that, for the selected three Gram-positive bacteria, the dried flowers were more sensitive to *S. aureus* compared to *S. epidermidis* and *B. cereus*, which indicated that they could inhibit and kill *S. aureus* at a lower concentration. The result was similar to those reported in the literature (Shan et al., 2007a; Al Momani et al., 2013). The MIC and MBC to *S. aureus* was 67.5 mg/mL and 125 mg/mL, respectively. To the Gram-positive bacteria, *Cordyceps militaris* had the strongest antibacterial activity, followed by *Flos sophorae* and *Lonicera japonica*.

Among the selected four Gram-negative bacteria, it was found that the dried flowers were more sensitive to *P. aeruginosa* compared to the other three. The sensitivity to *S. typhimurium* and *S. flexneri* was stronger than that to *E. coli*. The MIC and MBC to *P. aeruginosa* was 125 mg/mL and 250 mg/mL, respectively. In other studies, it was found that *P. aeruginosa* was the most sensitive (Rahman and Islam, 2013), while *E. coli* was the most resistant to Gram-negative bacteria (Shan et al., 2007a, 2008). To the Gram-negative bacteria, *Cordyceps militaris* had the strongest antibacterial activity, followed by *Lonicera japonica*, *Flos sophorae* and *Dolichos lablab*.

The difference of MIC results between the two pre-treatments was statistically significant （$F=8.415$, $P<0.05$, and the difference in MIC experimental results between two strains of Gram was statistically significant（$F=31.972$, $P<0.05$; while factors that pretreatment and Gram, different pretreatment on the MIC of different strains of Gram had no effect on measured values（$F=2.033$, $P>0.05$. The statistics showed that the results of MBC were the same as MIC.

The result of the study could support some experimental data about the antibacterial effect of GTS. Because *Lonicera japonica* belongs to medical herb, there were some researches about it. It showed that *Lonicera japonica* had significant antibacterial effect to *S. aureus* (Li et al., 2006). The study of Chen et al. (2005) found that *Lonicera japonica* at the concentration of 500 mg/mL had antibacterial effect to *E. coli* and *Bacillus subtilis*. Though the data of these studies were not really consistent completely due to the different extraction way, all the studies indicated that the plant had special antibacterial effect. However, the antibacterial effect of the other 7 dried flowers in this study has not been reported till now.

Although antibacterial activity of eight dried flowers was confirmed explicitly from this study, there was still no study on antimicrobial mechanism of these dried flowers. Some studies on spice and herb found that phenols and essential oil were responsible for the main biological activity for their antibacterial effect (Shan et al., 2007b, 2008; Oroojalian et al., 2010). The investigation of some essential oil and phenolic compounds found that these compounds could increase membrane permeability, which dissolved the membrane, caused swelling and reducing membrane function and led to cell death (Holly and Patel, 2005; Shan et al., 2008). Some phenolic compounds have certain hydroxyl groups which might bind to the active site of enzymes, form hydrogen bonds with enzymes and alter their metabolism. In addition, the lipid solubility and the degree of steric hindrance of the phenolic compounds might be the cause of their antimicrobial activity (Ceylan and Fung, 2004). Maybe these compounds were also the dried flowers' antibacterial ingredients, which should be studied in further research.

5. Conclusions

In summary, all the samples exhibited potent antibacterial activity. Gram-positive bacteria were generally more sensitive to the tested extracts than Gram-negative ones. *Staphylococcus aureus* was the most sensitive, while *Escherichia coli* was the most resistant. At the same time, the antibacterial effect of ethanol-water extract was higher than that of deionized water extract. Our results provided the medical evidence for the Guangdong folk saying that the Guangdong traditional soup could have the effect of clearing damp, decreasing inner heat, and moistening dryness, which we thought was in relationship in some extent with mild inflammation of upper respiratory tract and tonsils. The more explicit mechanism of the antibacterial effect should be further studied.

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