Inhibitory Effects of *Prunus mume*, *Coptis chinensis*, and *Crataegus pinnatifida* on *Vibrio parahaemolyticus* and Its Biofilm *in Vitro*

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Abstract In order to explore the inhibitory effects of *Prunus mume*, *Crataegus pinnatifida* and *Coptis chinensis* on *Vibrio parahaemolyticus* and its biofilm *in vitro*, the agar diffusion method was applied. These three Chinese herbal medicines had different inhibitory effects on *V. parahaemolyticus*. The inhibition zone of *C. pinnatifida* to *V. parahaemolyticus* was (15.25 ± 0.53) mm, and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *C. pinnatifida* on *V. parahaemolyticus* were both 31.25 mg/mL; the inhibition zone of *C. chinensis* to *V. parahaemolyticus* was (18.08 ± 0.10) mm, and the MIC and MBC of *C. chinensis* on *V. parahaemolyticus* were both 15.63 mg/mL; the inhibition zone of *P. mume* to *V. parahaemolyticus* was (28.99 ± 0.47) mm, and the MIC and MBC of *P. mume* on *V. parahaemolyticus* were both 7.81 mg/mL. The effects of three traditional Chinese medicines on the biofilm formation of *V. parahaemolyticus* were tested by MTT colorimetric method using methylthiazolyl tetrazolium (MTT). *P. mume*, *C. pinnatifida* and *C. chinensis* have significant inhibitory effects on *V. parahaemolyticus* biofilm and their MIC are 7.81 mg/mL, 3.125 mg/mL, and 62.5 mg/mL, respectively ($P < 0.01$). The experimental results are expected to provide certain references for the development of new fishery drugs.

Key words *Prunus mume*, *Crataegus pinnatifida*, *Coptis chinensis*, *Vibrio parahaemolyticus*, Biofilm, Inhibitory effects

1 Introduction

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium mainly distributed in seawater and aquatic products. It is reported that in the seawater along the coast of East China, the detection rate of *V. parahaemolyticus* is 47.5% - 66.5%, the average detection rate of marine fish and shrimp is 45.6% - 48.7%, in summer, the detection rate is up to 90%[1]. *V. parahaemolyticus* may cause vibriosis in aquatic economic animals, and the disease is rapidly transmitted and often causes death of large areas of fishes, shrimps and crabs, leading to huge economic losses[2-3]. Besides, this kind of bacteria can cause symptoms such as food poisoning in humans, so it is zoonotic bacteria[4].

At present, the main control of vibriosis is antibiotics, but the abuse of antibiotics often leads to an increase in bacterial resistance, especially after bacteria form biofilm. Biofilm is a group structure formed by bacteria in the natural world to resist external unfavorable factors[5]. Bacteria are more resistant to antibiotics after they form biofilms[6], and they are more likely to evade the host's immune defenses, making prevention and treatment difficult. How to inhibit biofilm has become one of the hottest spots.

Traditional Chinese medicines are characterized by natural, strong antibacterial activity and low toxicity. *Prunus mume* belongs to the Rosaceae family. *P. mume* is rich in organic acids, including citric acid, malic acid, oxalic acid, succinic acid and fumaric acid. Kernel of *P. mume* contains high amygdalin (4.3%) and the pulp contains about 0.5% amygdalin. The active ingredients of *P. mume* are 5-hydroxy-2-furfural and picric acid[7]. As a traditional Chinese medicine, *P. mume* has a long history; as long as in *Divine Farmer's Classic of Materia Medica*, *P. mume* has been described having functions of constraining the lung, astringing intestine, and promoting the secretion of saliva or body fluid, quieting roundworm. *P. mume* has inhibitory effects on *Escherichia coli*, *Shigella bacillus* and *Salmonella typhimurium*, and its decoction has strong acidity and obvious inhibitory effects. According to the study of Wang Yu'e *et al.*[8], *P. mume* has a strong inhibitory effect on five kinds of Vibrio. *Coptis chinensis* Franch. is a perennial herb of the genus *Coptis Salisb*, and its rhizome is important traditional Chinese medicine. The main effective fraction of *C. chinensis* is alkaloids, in addition to berberine, it also contains pamlatine, coptisine, epiberberine, jatrorrhizine and worenine. *C. chinensis* is a good antipyretic and anti-inflammatory drug with broad-spectrum antibiotics, and it has a certain inhibitory effect on Gram-positive and negative bacteria, protozoa, various influenza viruses and fungi[9]. *Crataegus pinnatifida* Bge., also known as mountain hawthorn, Chinese hawthorn or Chinese hawberry, is the fruit of the tree, and it belongs to *Rosaceae*. *C. pinnatifida* contains chlorogenic acid, caffeic acid,

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crataegolic acid, quercetin, ursolic acid, oleanolic acid, hyperin, epicatechin, and tannins, *etc.* Tannin is the main bacteriostatic ingredient. The strong acidity of *C. pinnatifida* decoction also has a certain bacteriostatic effect[10]. At present, there are few reports about the inhibitory effects of *P. mume*, *C. chinensis*, and *C. pinnatifida* on *V. parahaemolyticus* and its biofilm *in vitro*. Through studying the inhibitory effects of *P. mume*, *C. chinensis*, and *C. pinnatifida* on *V. parahaemolyticus* and its biofilm *in vitro*, this experiment is intended to explore the bacteriostatic and anti-biofilm functions of these three Chinese herbal medicines, so as to provide theoretical basis for prevention and control of *V. parahaemolyticus* induced diseases.

2 Materials and methods

2.1 Experimental materials

2.1.1 Strain. *V. parahaemolyticus* GZ03 was stored in -80℃ refrigerator of this laboratory. *C. pinnatifida*, *C. chinensis*, and *P. mume* were bought from Zhanjiang Chuntian Pharmacy.

2.1.2 Instrument. The thermostatic incubator was bought from Shanghai Boxun Industrial Co., Ltd. The -80℃ cryogenic refrigerator was bought from Qingdao Haier Group. Autoclave (HVE-50) was bought from Japanese Hirayama. Super clean workbench was bought from Shanghai Huilong Instrument Electronics Co., Ltd. The shaker was bought from Shanghai Boxun Industrial Co., Ltd. The microplate reader was bought from American Bio-Rad. SZ-93 automatic double pure water distiller was bought from Shanghai Yarong Bio-chemical Instrument Co., Ltd.

2.1.3 Main experimental medicines and reagent preparation. Tryptone soy agar (TSA): 5 g of soy peptone, 15 g of tryptone, 15 g of agar powder, 20 g of NaCl, volume fixed with distilled water to 1 000 mL (pH adjusted to 7.2). Tryptone soy broth (TSB): 5 g of soy peptone, 15 g of tryptone, 20 g of NaCl, volume fixed with distilled water to 1 000 mL (pH adjusted to 7.2), sterilized in an autoclave, and stored at 4℃. MTT preparation: 0.5 g of 5 mg/mL MTT, dissolved in 100 mL of phosphate buffer solution (PBS), filtered through a 0.22-μm filter to remove bacteria from the solution, and stored at 4℃ in the dark. The phosphate buffer saline (PBS): 1.44 of disodium hydrogen phosphate (Na_2HPO_4), 8 g of sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 0.24 g of potassium dihydrogen phosphate (KH_2PO_4), pH adjusted to 7.4, volume fixed to 1 L, autoclaved, and stored at room temperature. The physiological saline (0.85%): 0.85g of NaCl, volume fixed with distilled water to 1 000 mL, sterilized in an autoclave for experiment.

2.2 Experimental methods

2.2.1 Culture of *V. alginolyticus*. The strain was inoculated in 2% NaCl tryptone soy broth (TSB), and cultured at 28℃ and 120 r/min shaker for 18 h. The density of the bacterial suspension was adjusted with sterile TSB to about OD_{600} = 0.8 (about 10^8 cfu/mL)[11].

2.2.2 Preparation of Chinese herbal medicine decoction. According to the method of Li Jiaqin *et al.*[12], the preparation of Chinese herbal medicine decoction underwent soaking, boiling, filtering and concentrating processes: precisely weighed 20 g of Chinese herbal medicines, cut into pieces and placed into a ceramic pot with 400 mL of distilled water to soak for 4 h, heated on electric stove to boiling, then changed to slow fire to decoct 40 min; the decocted Chinese herbal medicines were first cooled and immediately filtered with 8 layers of gauze to another beaker, and the dregs of the decoction were boiled with distilled water again, and then cooled and filtered with gauze, the filtrate was combined twice. The filtrate was heated and concentrated to 20 mL, cooled and placed in a 50-mL vial. The Chinese herbal decoction with final concentration of 1 g/mL was prepared and placed in a refrigerator at 4℃ for use.

2.2.3 Determination of bacteriostatic effects by the agar diffusion method. With reference to the method of Feng Ruiji[13], set three parallel groups for each medicine. First, prepared 10 TSA plates, added 100 μL of *V. parahaemolyticus* solution to 10 plates with a pipette, and evenly spread the bacterial solution with a coating rod. Then, punched three holes on each plate, each hole had a diameter of 6.00 mm and a distance of more than 2 cm between two holes. Added 30 μL of the medicine solution with a pipette to the holes of 9 plates, and took the remaining plate coated with the bacteria solution as the control. Placed all plates in a 28℃ incubator for 24 h, to observe the bacteriostatic effects and measure the inhibition zone with a vernier caliper. Determination of bacteriostatic effects: strong bacteriostatic effects when the inhibition zone diameter is ≥ 20 mm, expressed by "+++"; moderate bacteriostatic effects when the inhibition zone diameter is 15-20 mm, expressed by "++"; weak bacteriostatic effects when the inhibition zone diameter is 10-15 mm, expressed by "+"; no bacteriostatic effect when the inhibition zone diameter is ≤10 mm, expressed by "-".

2.2.4 Determination of minimum inhibitory concentration (MIC). The double dilution method was used to determine the MIC of three kinds of traditional Chinese medicines with reference to method of Huang[14]. Specific steps: Experimental group: set 13 pieces of 5-mL sterile centrifuge tubes (No. 1 to No. 13). Added 2 mL of TSB medium to the No. 1 to No. 13 tubes; added 2 mL of the medicine solution to the No. 1 tube, shook up, used a pipette to take 2 mL of the liquid from the No. 1 tube, added it to No. 2 tube, and so on, till No. 11 tube and then absorbed 2 mL of the mixture from No. 11 tube and discarded. Added 20 μL of the bacterial solution to each of the 1-12 tubes. Took No. 12 tube as the negative control and No. 13 tube as the blank control. Control group: except that no bacterial solution was added, the contents were the same as those in the experimental groups. Placed all centrifuge tubes in a 28℃ incubator for 24 h, and the experimental group and the control group were compared to observe the growth conditions of the bacteria. The drug concentration of the centrifuge tube without bacterial growth is the minimal inhibitory concentration (MIC) of the medicine.

2.2.5 Determination of minimum bactericidal concentration (MBC). In the test tube with the minimum inhibitory concentra-

tion, took test tubes without obvious bacterial growth, spread 100 μL of solution on the TSA plate, and placed in a constant temperature incubator at 28℃ for 24 h for observation. The lowest concentration of the medicine solution of the tube on the TSA plate without bacterial growth is the minimum bactericidal concentration (MBC) of the medicine against *V. parahaemolyticus*.

2.2.6 Formation and quantitative detection method of Vibrio biofilm. Detection of biofilm activity: methylthiazolyl tetrazolium (MTT) method[15] was used to detect the biofilm activity of *V. parahaemolyticus*. The principle of MTT method: the mitochondrial dehydrogenase of living cells can decompose the tetrazolium ring of MTT, change the yellow MTT into purple MTT-Fonmazan. Spectrophotometer was used to measure the absorbance of MTT-Fonmazan, to reflect the cell viability. Specific experimental steps were as follows. (i) Biofilm culture: The bacterial suspension was diluted 1∶100 into fresh TSB medium; the diluted mixture was added to a 96-well plate at 200 μL per well, and 8 replicate wells were set in each group, and sterile TSB control wells were placed; the cells were incubated at 28℃ in a wet box at a constant temperature. (ii) Pipetted the culture solution, discarded it, gently washed the bacteria on the plate wall with PSB buffer, added 20 μL of 5 mg/mL MTT and continued to culture for 4 h. Carefully absorbed the culture medium , added 150 μL of dimethyl sulfoxide (DMSO) to each well, and placed on the shaker and shook 10 min at a low speed. (iii) Quantitative detection: measured the absorbance of 96-well plate at the wavelength of 540 nm (OD_{540}) with the microplate reader. The difference between the *OD* value of each well and the mean value of the control represents the metabolic activity of the biofilm of each well.

2.2.7 Effects of different culture time on the film formation of Vibrio. After inoculation into a 96-well plate, placed *V. parahaemolyticus* in a 28℃ wet box for static culture, took samples every 4 – 6 h, and measured the film formation according to the method in Section **2.2.6**.

2.2.8 Effects of Chinese herbal medicine solution on the formation of Vibrio biofilm. Under sterile conditions, mixed *V. parahaemolyticus* suspension with fresh tryptone soy broth (TSB) medium at a ratio of 1∶100, added Chinese herbal decoction, and diluted the mixture, then loaded 100 μL of samples into a 96-well plate, set 8 replicate wells for each group, set a positive control group (containing 0.1 mol/L EDTA), a negative control group (containing medium and bacterial solution), and a blank control group (containing only medium), cultured in a wet box at 28℃ for 24 h, and measured the film formation according to the method in Section **2.2.6**.

2.2.9 Data processing. With the aid of paired sample *T* test in SPSS Statistics17.0 software, we carried out statistical analysis. In the significance test, $P < 0.05$ means significant difference, and $P < 0.01$ means extremely significant difference.

3 Results and analysis

3.1 Bacteriostatic effects of three Chinese herbal medicines

The *in vitro* bacteriostatic experimental results of three kinds of Chinese herbal medicines showed that *V. parahaemolyticus* had high sensitivity to three kinds of Chinese herbal medicines, among which *P. mume* had strong inhibitory effects on *V. parahaemolyticus*, and *C. pinnatifida* and *C. chinensis* had weak inhibitory effects (Table 1).

Table 1 *In vitro* bacteriostatic experiment of three Chinese herbal medicines against *Vibrio parahaemolyticus*

Chinese herbal medicine	Inhibition zone diameter//mm	Bacteriostatic effects
Crataegus pinnatifida	18.08 ±0.10	+ +
Coptis chinensis	15.25 ±0.53	+ +
Prunus mume	28.99 ±0.47	+ + +

3.2 Determination results of MIC and MBC

The double dilution method was used to determine the MIC and MBC of three Chinese herbal medicines on *V. parahaemolyticus* (Table 2). The bacteriostatic effect of *P. mume* was the strongest, the MIC and MBC were both 7.81 mg/mL; the bacteriostatic effect of *C. chinensis* was weak, the MIC and MBC were both 15.63 mg/mL; the bacteriostatic effect of *C. pinnatifida* was the weakest, and the MIC and MBC were both 31.25 mg/mL.

Table 2 MIC and MBC of three Chinese herbal medicines against *Vibrio parahaemolyticus*

Chinese herbal medicine	MIC//mg/mL	MBC//mg/mL
Crataegus pinnatifida	31.25	31.25
Coptis chinensis	15.63	15.63
Prunus mume	7.81	7.81

3.3 Growth cure of *V. parahaemolyticus* biofilm

The *V. parahaemolyticus* biofilm was quantitatively detected by MTT method, and the growth curve of *V. parahaemolyticus* biofilm over time was plotted (Fig.1). As shown in Fig.1, the growth cycle of *V. parahaemolyticus* biofilm: 0 – 2 h was the initial colonization stage; 4 – 8 h was the adhesion stage; the biofilm formation reached the maximum at 8 h, and depolymerization started after 8 h.

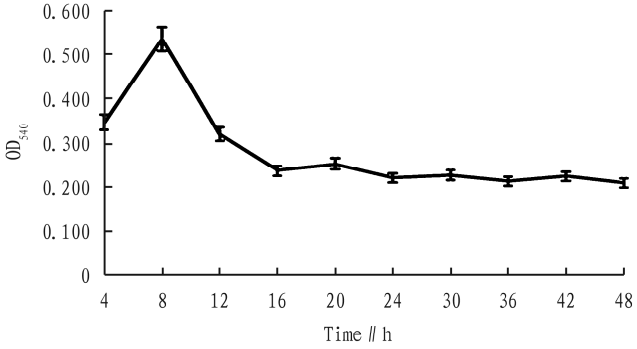
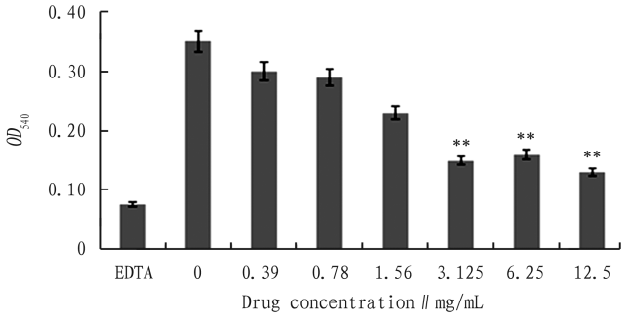


Fig.1 Growth cure of *Vibrio parahaemolyticus* biofilm

3.4 Effects of different drug concentrations on the formation of *V. parahaemolyticus* biofilm

3.4.1 Inhibitory effects of *C. pinnatifida* on *V. parahaemolyticus* biofilm. Compared with the negative control, when the concentration of *C. pinnatifida* was 1.56 mg/mL or lower, there was no significant difference in the inhibitory effects on *V. parahaemolyticus*; when the concentration was 3.125 mg/mL or higher, it had

significant inhibitory effects on the biofilm formation of *V. parahaemolyticus* ($P<0.01$), as shown in Fig. 2.



Note: ** denotes $P<0.01$, the same below.

Fig. 2 Effects of different drug concentrations of *Crataegus pinnatifida* on the formation of *Vibrio parahaemolyticus* biofilm

3.4.2 Inhibitory effects of *C. chinensis* on *V. parahaemolyticus* biofilm. Compared with the negative control, when the concentration of *C. chinensis* was 31.25 mg/mL or lower, there was no significant difference in the inhibitory effects on *V. parahaemolyticus*; when the concentration was 62.5 mg/mL or higher, it had significant inhibitory effects on the biofilm formation of *V. parahaemolyticus* ($P<0.01$), as shown in Fig. 3.

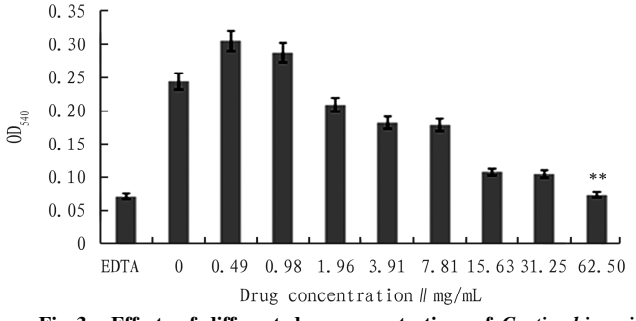


Fig. 3 Effects of different drug concentrations of *Coptis chinensis* on the formation of *Vibrio parahaemolyticus* biofilm

3.4.3 Inhibitory effects of *P. mume* on *V. parahaemolyticus* biofilm. Compared with the negative control, when the concentration of *P. mume* was 3.91 mg/mL or lower, there was no significant difference in the inhibitory effects on *V. parahaemolyticus*; when the concentration was 7.81 mg/mL or higher, it had extremely significant inhibitory effects on the biofilm formation of *V. parahaemolyticus* ($P<0.01$), as shown in Fig. 4.

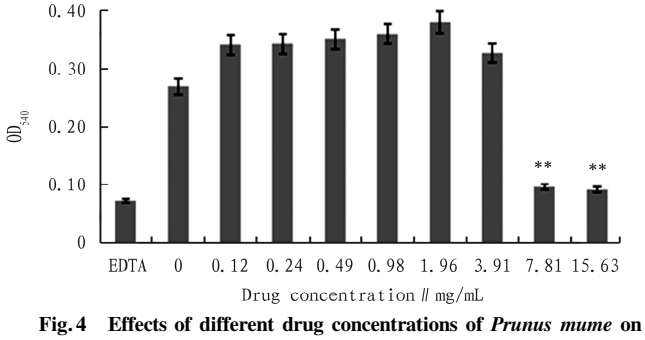


Fig. 4 Effects of different drug concentrations of *Prunus mume* on the formation of *Vibrio parahaemolyticus* biofilm

4 Conclusions and discussions

Vibrio disease has always been a great threat to the safe production of aquatic economic animals. At present, the main treatment methods for vibrio disease are immunization and antibiotic treatment, and a widely applied method is use of antibiotics. However, the disaster resulted from use of antibiotics can not be underestimated. Therefore, it is particularly important to find effective antibiotic alternatives or to improve antibiotic treatment. Studies have shown that the formation of biofilms increases drug resistance[6]. Thus, how to effectively inhibit the growth of biofilms will be greatly helpful for the prevention and control of vibrio disease. In view of the above situation, this experiment selected natural antibacterial Chinese herbal medicine, extracted the active ingredients, and carried out *in vitro* inhibition test on common *V. parahaemolyticus* screened out the best effective medicines.

The experimental results indicate that *P. mume*, *C. pinnatifida* and *C. chinensis* have certain inhibitory effects on *V. parahaemolyticus*, and also have certain effects on the growth of *V. parahaemolyticus* biofilm; specifically, *P. mume* had the highest inhibitory effects, followed by *C. pinnatifida* and *C. chinensis*, which are consistent with the experimental results of Wang Yu'e *et al.*[8]. In the inhibition experiment, the bacteriostatic concentration of *P. mume* was the same as that of the biofilm, while the bacteriostatic concentration of *C. pinnatifida* and *C. chinensis* was higher than that of the biofilm. According to the research of Geng Fei *et al.*[16], the extract of *P. mume* will act on the cell membrane of bacteria, destroy the symmetry of cell membrane and eventually leading to cell death. In other words, the extract of *P. mume* has destroyed the cell membrane when it acts on the biofilm, so its inhibitory concentration is similar to the concentration of the biofilm. In comparison, the data of *C. pinnatifida* and *C. chinensis* may indicate that the growth of biofilm is inhibited before sterilization, and the inhibitory concentration is greater than the inhibition of biofilm concentration. When the amount of biofilm is reduced, it can significantly reduce the drug resistance of bacteria and increase its sensitivity to drugs, suggesting that after using Chinese herbal medicines, it can improve the effect of antibiotics. In future, it is intended to research and develop new herbal medicines combining Chinese herbal medicines with antibiotics to achieve low-efficiency effects.

Using 96-well plate culture method, we successfully detected the *in vitro* antibacterial effect of three kinds of Chinese herbal medicines and the sensitivity and specific values of MIC and MBV of *V. parahaemolyticus*. These proved that these three Chinese herbal medicines can inhibit the growth of *V. parahaemolyticus* and its biofilm at a certain concentration. The results indicated that *P. mume* and *C. pinnatifida* could inhibit *V. parahaemolyticus* and its biofilm at a lower concentration, while the effect of *C. chinensis* was slightly weaker.

References

[1] LI Y, ZHU XQ. Research progress of *Vibrio parahaemolyticus* and its hemolytic toxin[J]. Chinses Journal of Health Laboratory Technology, 2008, 18(12): 2835 – 2839. (in Chinese).

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tion model of cucumber for nitrogen, phosphorus and manure application in greenhouse under drip irrigation[J]. Plant Nutrition and Fertilizer Science, 2009, 15(1): 177 -182. (in Chinese).
[18] XU FL, LIANG YL, DU SN, *et al.* Optimum rates of fertilizers and optimization of fertilization models for greenhouse cucumber in the Loess Plateau[J]. Agricultural Research in the Arid Areas, 2005, 23(1): 75 -80. (in Chinese).
[19] BAO SD. Soil agrochemical analysis[M]. Beijing: China Agriculture Press, 2008. (in Chinese).
[20] LU RK. Agricultural chemical analysis of soil[M]. Beijing: China Agricultural Science and Technology Press, 2000. (in Chinese).
[21] QIN QY, JIA CZ, QU D, *et al.* Advances and characters of fertilizer application of protected field agriculture in China[J]. Journal of Hubei Agricultural College, 2002, 22(4): 373 - 376. (in Chinese).
[22] ZHU JH, LI JL, LI XL, *et al.* Effects of compound fertilizers utilized on soil environmental quality in protected vegetable field[J]. Agro-Environmental Protection, 2002, 21(1): 5 -8. (in Chinese).
[23] LEI BK, CHEN Q, FAN MS, *et al.* Changes of soil carbon and nitrogen in Shouguang intensive vegetable production fields and their impacts on soil properties[J]. Plant Nutrition and Fertilizer Science, 2008, 14(5): 914 -922. (in Chinese).
[24] FAN ZB, LIU MJ, ZHANG XM, *et al.* Effect of dripper fertigation on tomato yield and apparent N balance in a greenhouse[J]. Plant Nutrition and Fertilizer Science, 2011, 17(4): 970 -976. (in Chinese).
[25] FAN ZB. Evaluation of tomato yield and water nitrogen utilization efficiency under drip and spread irrigation[D]. Beijing: China Agricultural University, 2014. (in Chinese).
[26] YU HY. Nutrient state and characteristics of accumulation and translocation of salt in greenhouse soil profiles[D]. Ya'an: Sichuan Agricultural University, 2006. (in Chinese).
[27] GUO JH. Life cycle assessment of water fertilizer, pesticide input and environmental impact in typical vegetable production system[D]. Beijing: China Agricultural University, 2016. (in Chinese).
[28] GUO PQ. The study on regularity of nitrate accumulation in greenhouse soil and vegetables[D]. Tai'an: Shandong Agricultural University, 2009. (in Chinese).
[29] LIU P, LI Y, JIANG LH, *et al.* Effects of fertilizer application on greenhouse vegetable yield: A case study of Shouguang City[J]. Chinese Journal of Applied Ecology, 2014, 25(6): 1752 -1758. (in Chinese).
[30] LIU ZH, JIANG LH, ZHANG WJ, *et al.* Evolution of fertilization rate and variation of soil nutrient contents in greenhouse vegetable cultivation in Shandong[J]. Acta Pedologica Sinica, 2008, 45(2): 296 -303. (in Chinese).
[31] ZHAO WY, ZHANG XM, SHI ZL, *et al.* Effects of application of nitrogen and potassium fertilizers on available nutrient, water-soluble salt in soil and the growth of tomato[J]. Journal of Soil and Water Conservation, 2011, 25(4): 100 -103, 109. (in Chinese).
[32] ZENG LS, GAO Y, LI JL, *et al.* Changes of acidification and nutrient accumulation in greenhouse vegetable soils in Shouguang[J]. Journal of Soil and Water Conservation, 2010, 24(4): 157 -161. (in Chinese).
[33] NIU JL, LI YM, CHEN Q. Solid organic waste fertilizer utilization technology[M]. Beijing: Chemical Industry Press, 2010: 136 -140. (in Chinese).

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[2] MOU HJ, LI Y, BAO ZM, *et al.* Pathogenicity of extracellular products of *Vibrio parahaemolyticus* to *Penaeus chinensis*[J]. Oceanologia Et Limnologia Sinica, 2000, 31(3): 273 -280. (in Chinese).
[3] YAN QP, YI JB, CHEN Q, *et al.* Starvation responses of pathogenic *Vibrio parahaemolyticus* in natural seawater[J]. Acta Oceanologica Sinica, 2007, 29(3): 131 -136. (in Chinese).
[4] DANIELS NA, MACKINNON L, BISHOP R, *et al.* 1973 -1998, *Vibrio parahaemoly ticus* infections in the United States[J]. Journal of Infectious Diseases, 2000, 181(5): 1661 -1666.
[5] BACHMANN SP, VANDEWALLE K, RAMAGE G, *et al.* *In vitro* activity of caspofungin against *Candida albicans* Biofilms[J]. Antimicrob Agents, 2002, 46(11): 3591 -3596.
[6] HUANG SJ. Biofilm and antibiotic therapy: A new drug delivery system to counter bacterial resistance[J]. Foreign Medical Sciences (Section of Pharmarcy), 2006, 33(1): 31 -33. (in Chinese).
[7] CHEN L, CHEN HP, LIU YP, *et al.* Pharmacological action of different part of *Fructus mume*[J]. China Pharmacy, 2007, 18(27): 2089 -2090. (in Chinese).
[8] WANG YE, XING CG, WANG GL. Susceptibilities of five marine pathogenic vibrios to 34 Chinese herbs[J]. Fisheries Science, 2008, 27(5): 221 -225. (in Chinese).
[9] LI CH, ZHOU KY. Study advances on the effects and mechanisms of active constituents of Coptidis Rhizoma[J]. Lishizhen Medicine and Materia Medica Research, 2010, 21(2): 466 -468. (in Chinese).
[10] WANG CL, LU BZ, HOU GL. Chemical composition, pharmacological action and clinical application of *Crataegus pinnatifida* Bunge[J]. Strait Pharmaceutical Journal, 2010, 22(3): 75 -78. (in Chinese).
[11] CHEN H, ZHANG QZ. Antibacterial effect of 100 Chinese herbal medicines against *Vibrio alginolyticus in vitro*[J]. Chinese Journal of Preventive Veterinary Medicine, 2012, 34(6): 452 -455. (in Chinese).
[12] LI JQ, PANG HY, JIAN JC, *et al.* The inhibitory activity of *Flos caryophylli* and *Rheum officinale* on *Vibrio harveyi* and its biofilm[J]. Journal of Anhui Agricultural Sciences, 2014, 42(24): 8188 -8190, 8202. (in Chinese).
[13] FENG RJ, PANG HY, HUANG YC, *et al.* Inhibitory activity of *Rhus chinenis* and *Folium sennae* on *Vibrio alginolyticus* and its biofilm[J]. Journal of Anhui Agricultural Sciences, 2016, 44(11): 1 -3, 11. (in Chinese).
[14] HUANG ZF, PANG HY, HUANG Y, *et al.* *In vitro* inhibitory activity of *Shisandra chinensis* and *Polygonatum sibiricum* against *Vibrio harveyi* and its biofilms[J]. Agricultural Biotechnology, 2016, 5(4): 57 -60.
[15] JIA HL. Study on the preliminary analysis of the exocrine protein of *Cryptococcus neoformans*[D]. Shanghai: The Second Military Medical University, 2012. (in Chinese).
[16] GENG F, WANG W, ZHOU T. Antibacterial mechanisms of *Fructus mume* extract against *Listeria innocua*[J]. Food Science, 2011, 32(15): 88 -93. (in Chinese).