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Antibacterial Activity of *Polygonum Orientale* Extracts Against *Clavibacter Michiganensis* subsp. *michiganensis*, the Agent of Bacterial Canker of Tomato Disease

Jin Cai[1*]
https://orcid.org/0000-0003-1597-8143

Yichen Gao[2]
https://orcid.org/0000-0002-8363-1232

Mengliang Wang[1]
https://orcid.org/0000-0003-1888-6893

Jing Zhang[1]
https://orcid.org/0000-0001-5966-5208

Yongbin Zhang[1]
https://orcid.org/0000-0001-7119-9721

Qi Wang[2]
https://orcid.org/0000-0001-7665-0024

Jiquan Liu[3]
https://orcid.org/0000-0002-4681-6705

[1] Shanxi University, Institute of Applied Chemistry, Taiyuan, Shanxi Province, China; [2] Shanxi University, School of Life Science, Taiyuan, Shanxi Province, China; [3] Shanxi University of Chinese Medicine, College of Traditional Chinese Medicine, Yuci, Shanxi Province, China.

*Correspondence: caijin@sxu.edu.cn; Tel.: +86-351-3586672 (J.C.)

HIGHLIGHTS

- *P. orientale* extracts had a strong antibacterial activity to inhibit Cmm.

- Optimized extraction obtained high levels of antimicrobial substance production.

- Living Cmm cells from survival rate test had almost no resistance or adaptability.

- Cell membrane and cell wall of Cmm were the targets of antibacterial action.

Abstract: *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), which is a Gram positive bacterium, causes the bacterial canker of tomato disease. The purpose of the study was to evaluate the antibacterial activity of *Polygonum orientale* extracts against Cmm. In this study, firstly, effects of three extracting parameters (extractive time, extractive temperature, and solid to liquid ratio) of orthogonal experiment design L_{27} (3^{13}) were conducted. Secondly, survival rate was determined and inhibition zone of Cmm rescued post-stress was monitored. Finally, extracellular OD_{260nm} value, extracellular protein content, conformational structure of membrane protein, extracellular alkaline phosphatase (AKP) activity, and ATPase activity were measured to investigate the antibacterial mechanism. Results of orthogonal experiment revealed that extractive time and extractive temperature had highly significant ($P<0.01$) effects on the antibacterial activity of *P. orientale* extracts. The optimum conditions were as follows: 10h of extractive time, 60°C of extractive temperature, and 1:20 (g:mL) of solid to liquid ratio. This study also demonstrated that the living cells of each sample from survival rate test had almost no resistance or adaptability, and rescued Cmm cells were much easier to be inhibited by *P. orientale* extracts. The results of antibacterial mechanism indicated that cell membrane and cell wall of Cmm were seriously damaged by *P. orientale* extracts, and *P. orientale* extracts reduced the intracellular ATPase activity dramatically. All these findings suggested that *P. orientale* extracts had a strong antibacterial activity to inhibit Cmm, and could be used for the ecological management of the bacterial canker of tomato disease.

Keywords: plant bioactive compounds; antibacterial agent; plant pathogenic bacterium; orthogonal experiment design; biological control.

INTRODUCTION

Clavibacter michiganensis subsp*. michiganensis* (Cmm) is the causative agent of bacterial canker of tomato disease [1], an important disease which causes substantial losses in global tomato production areas [2]. Once Cmm infects tomato, entering through natural openings and wounds, it causes a lot of wilting of leaves, canker of tomato, bird's-eye spots in fruit, and finally death [3,4]. It is difficult to control the bacterial canker of tomato disease. Until now, the main method of controlling Cmm is using chemical bactericides, such as copper compounds and streptomycin [5,6]. However, chemical controls have some serious problems, such as toxic residues, environmental pollution, affecting people's health, and so on [7]. Recently, biological control including using plant extracts has become a useful method for plant disease controlling [8-13].

Polygonum orientale L. is one kind of herbaceous weeds, which is widely distributed in China [14]. As a folk medicine, *P. orientale* has been used for treating coronary heart disease, edema, and diarrhea [14]. Previous studies also showed that the extracts from *P. orientale* had significant effects against *Plutella xylostella* L., *Helicoverpa armigera* H., *Mythimna separate* W., and *Mamestra brassicae* L. [15,16]. To date, there was no report of *P. orientale* extracts against Cmm.

In this paper, we first conducted the orthogonal experiment design L_{27} (3^{13}) at three controlling factors to optimize the extractive parameters of antibacterial substances from *P. orientale*. Then, we determined the survival rate and monitored the existing time of inhibition zone of Cmm rescued post-stress, to verify if there is occurrence of Cmm cells adapted to *P. orientale* extract stress. Thirdly, we investigated the antibacterial mechanism of *P. orientale* extracts against Cmm by analyzing extracellular OD_{260nm} value, extracellular protein content, conformational structure of membrane protein, extracellular alkaline phosphatase (AKP) activity, and ATPase activity.

MATERIALS AND METHODS

Orthogonal experiment design

The whole herbs of *P. orientale* were obtained from Fenhe River (Shanxi Province, China) in August 2016. Collected *P. orientale* were air dried and grounded in a blender [17]. The *P. orientale* powder (5g) was mixed with ethanol of corresponding volume and was extracted according to the design of L_{27} (3^{13}) (Table 1 and Table 2). After filtration, each extract was evaporated, and then each dried sample was dissolved in 40% DMSO to 15mg/mL.

Table 1. Optimization of antibacterial substance extraction.

Levels	Optimization		
	Extractive time (A)	Extractive temperature (B)	Solid to liquid ratio (C)
1	6h	40°C	1:10 (g:mL)
2	10h	60°C	1:15 (g:mL)
3	14h	80°C	1:20 (g:mL)

Antibacterial activity was determined by agar-dilution assay with slightly modified [18-20]. The pathogen Cmm (cgmcc 1.1909) was obtained from the China General Microbiological Culture Collection Center (Beijing, China). The medium contained 5g/L malt extract, 20g/L agar, 50mg/L twain-80, 10g/L peptone, 2g/L beef extract, 1g/L $MgSO_4 7H_2O$, 5g/L caseinate, 2g/L glycerol, and 5g/L yeast extract, and the pH was 7.2. 200μL of Cmm suspension (10^6CFU/mL) was spread uniformly on the medium surface. Holes (10mm diameter) were perforated in the medium followed by the addition of 200μL of prepared samples respectively. The control was 40% DMSO, because 40% DMSO does not affect Cmm growth [21]. Samples were cultivated at 28°C for 24h to allow the diffusions of *P. orientale* extracts into the medium. Then the diameter of inhibition zone was measured to evaluate antibacterial activity. All the experiments were performed in triplicate.

Determination of survival rate and monitoring of inhibition zone of Cmm rescued post-stress

Survival rate of Cmm was determined according to Wu et al. with several modifications [22]. Each liquid medium was added 50μL of Cmm suspension (10^6CFU/mL), and then was added different concentrations of *P. orientale* extracts with two-fold dilution method. For this test, *P. orientale* extracts were dissolved in DMSO, and were added into the medium to obtain the concentration of 0.02mg/mL, 0.04mg/mL, 0.08mg/mL, 0.16mg/mL, 0.31mg/mL, 0.63mg/mL, 1.25mg/mL, 2.5mg/mL, 5mg/mL, 10mg/mL or 20mg/mL respectively. In this assay, the total volume of each sample was 50mL, and the final DMSO concentration was 7% (v/v). The sample, which was treated with 7% DMSO only, was determined as the control. Each sample was incubated in a shaker at 28°C for 8h, and then was stained by methylene-blue. The number of living Cmm cells (C_L) and dead Cmm cells (C_D) were quantified by blood cell counting board under an optical microscope. All the experiments were performed in triplicate. The survival rate was calculated as follow formula:

$$Survival\ rate = \frac{CL}{CL + CD} \times 100\% \tag{1}$$

C_L=the number of cells, which were colorless.
C_D=the number of cells, which presented blue color.

In order to verify if there is the occurrence of Cmm cells adapted to *P. orientale* extract stress, following experiments were carried out. The living (remaining) cells of each sample from survival rate test were adjusted to the same concentration and spread on the medium surface. Holes (10mm diameter) were perforated in the medium followed by the addition of 200μL of prepared *P. orientale* extracts (15mg/mL). The DMSO (40%) was determined as the control. Samples were cultivated at 28°C for 24h, and the diameter of inhibition zone was measured to evaluate antibacterial activity. Then, the existing time of inhibition zone of Cmm rescued post-stress were recorded from the date of antimicrobial test to the 14th day. When the inhibition zone of Cmm still existed on the 14th day, we record 14 day in the test. All the experiments were performed in nine times.

Determination of extracellular OD_{260nm} value and extracellular protein content

Each liquid medium was added 200μL of Cmm suspension (10^6CFU/mL), and then was added different concentrations of *P. orientale* extracts. For this test, *P. orientale* extracts were dissolved in DMSO, and were added into the medium to obtain concentration of 0.06mg/mL, 0.08mg/mL, 0.10mg/mL or 0.12mg/mL respectively. In this assay, the total volume of each sample was 200mL, and the final DMSO concentration was 0.4% (v/v). The sample, which was treated with 0.4% DMSO only, was determined as the control. Then each sample was incubated in a shaker at 28°C for 8h, and the supernatant was obtained by centrifugation at 12000rpm for 5 minutes. The OD_{260nm} value of the supernatant was determined by the UV absorption spectrophotometer. The extracellular protein content of the supernatant was determined according to the soluble protein kit (Nanjing Jiancheng Bioengineering Institute; China). All the experiments were performed in triplicate.

Determination of conformational structure of membrane protein

Each liquid medium was added 200μL of Cmm suspension (10^6CFU/mL), and then was mixed with different concentrations (0.08mg/mL, 0.10mg/mL or 0.12mg/mL) of *P. orientale* extracts. For this test, *P. orientale* extracts were dissolved in DMSO, and were added into the medium to obtain concentration of 0.08mg/mL, 0.10mg/mL or 0.12mg/mL respectively. In this assay, the total volume of each sample was 200mL, and the final DMSO concentration was 0.4% (v/v). The sample, which was treated with 0.4% DMSO only, was determined as the control. The suspensions were incubated at 28°C for 8h, and then were centrifuged at 5000rpm for 5 minutes. The deposits of each sample were resuspended in sodium chloride (0.9%) to adjusting the OD_{630nm} to 0.3. The emission spectra of these samples were scanned from 300nm to 500nm with a fixed excitation wavelength of 280nm [23]. The widths of both excitation and emission slit were set to 5nm on the F-280 fluorescence spectrophotometer (Tianjin Gangdong Sci & Tech Development Co. LTD, China). This assay was used to determine the effects of *P. orientale* extracts on the conformational structure of Cmm membrane protein [24,25].

Determination of extracellular AKP activity

Each liquid medium was added 200μL of Cmm suspension (10^6CFU/mL), and then was added different concentrations of *P. orientale* extracts. For this test, *P. orientale* extracts were dissolved in DMSO, and were added into the medium to obtain concentration of 0.06mg/mL, 0.08mg/mL, 0.10mg/mL or 0.12mg/mL respectively. In this assay, the total volume of each sample was 200mL, and the final DMSO concentration was 0.4% (v/v). The sample, which was treated with 0.4% DMSO only, was determined as the control. Then each sample was incubated in a shaker at 28°C for 8h, and the supernatant was obtained by centrifugation at 12000rpm for 5 minutes. The extracellular AKP activity of the supernatant was determined according to the AKP assay kit (Nanjing Jiancheng Bioengineering Institute; China). All the experiments were performed in triplicate.

Determination of intracellular ATPase activity

Intracellular ATPase activity was measured according to Li et al. with several modifications [26]. For this test, *P. orientale* extracts were dissolved in DMSO, and were added into the medium to obtain concentration of 0.06mg/mL, 0.08mg/mL, 0.10mg/mL or 0.12mg/mL respectively. In this assay, the total volume of each sample was 200mL, and the final DMSO concentration was 0.4% (v/v). The sample, which was treated with 0.4% DMSO only, was determined as the control. The suspensions were incubated at 28°C for 8h. Two-milliliter of each sample was centrifuged at 5000rpm for 5 minutes after incubating 8h. The deposits of each sample were resuspended in 1mL sodium chloride (0.9%), and then were broken by the sonication method (200w, 15 minutes, 2s pulsing, 4s intervals). After centrifugation (4°C, 12000rpm, 8 minutes), the supernatant of each sample was determined as kit instructions (Nanjing Jiancheng Bioengineering Institute, China). All the experiments were performed in triplicate.

Statistics

Significance (at the 5% level or at the 1% level) was analyzed by one-way analysis of variance (ANOVA) using the software (SPSS 17.0).

RESULTS AND DISCUSSION

Optimization of antibacterial substance extraction

Orthogonal experimental design is a main method to find out the optimal extractive conditions of antibacterial substance, including extractive time (h), extractive temperature (°C), and solid to liquid ratio (g:mL) (Table 1) [27]. The advantage of this design is that it can optimize extraction conditions within a limited number of tests [28]. According to L_{27} (3^{13}), twenty-seven extraction experiments were carried out (Table 2). The results of Table 2 showed that the highest *R* value was 6.97, and the factor of extractive time was the most dominant effect on antibacterial substance extraction. The levels within three factors were ranked as: A: 2>3>1; B: 2>3>1; C: 3>2>1 (Figure 1). Analysis of variance (ANOVA) is an objective and statistical decision-making tool for analyzing the differences in experiments [29]. In Table 3, ANOVA was used to analyze the significant factor(s) or interaction(s). Based on ANOVA, extractive time and extractive temperature exert the significant influences on antibacterial substance extraction (Table 3). Solid to liquid ratio, interaction of extractive time and extractive temperature, interaction of extractive temperature and solid to liquid ratio, and interaction of extractive time and solid to liquid ratio do not exert significant influences (Table 3). Accordingly, extractive time and extractive temperature should be paid more attention in industrial extraction. Table 2 showed that the optimal levels of extraction factors were $A_2B_2C_3$. Thus, 10h of extractive time, 60°C of extractive temperature, and 1:20 (g:mL) of solid to liquid ratio were selected as the optimum extraction conditions. The optimum extraction conditions should satisfy the efficiency of industrial applications [27].

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Table 2. Orthogonal experiment L$_{27}$(3^{13}) and intuitive analysis.

Test	1	2	3	4	5	6	7	8	9	10	11	12	13	Inhibition zone (mm)
	A	B	A×B	A×B	C	A×C	A×C	B×C			B×C			
1	1 (6h)	1 (40°C)	1	1	1 (1:10)	1	1	1	1	1	1	1	1	10.00
2	1 (6h)	1 (40°C)	1	1	2 (1:15)	2	2	2	2	2	2	2	2	10.00
3	1 (6h)	1 (40°C)	1	1	3 (1:20)	3	3	3	3	3	3	3	3	10.00
4	1 (6h)	2 (60°C)	2	2	1 (1:10)	1	1	2	2	2	3	3	3	17.99
5	1 (6h)	2 (60°C)	2	2	2 (1:15)	2	2	3	3	3	1	1	1	20.85
6	1 (6h)	2 (60°C)	2	2	3 (1:20)	3	3	1	1	1	2	2	2	16.33
7	1 (6h)	3 (80°C)	3	3	1 (1:10)	1	1	3	3	3	2	2	2	13.42
8	1 (6h)	3 (80°C)	3	3	2 (1:15)	2	2	1	1	1	3	3	3	15.34
9	1 (6h)	3 (80°C)	3	3	3 (1:20)	3	3	2	2	2	1	1	1	21.81
10	2 (10h)	1 (40°C)	2	3	1 (1:10)	2	3	1	2	3	1	2	3	20.35
11	2 (10h)	1 (40°C)	2	3	2 (1:15)	3	1	2	3	1	2	3	1	20.48
12	2 (10h)	1 (40°C)	2	3	3 (1:20)	1	2	3	1	2	3	1	2	22.92
13	2 (10h)	2 (60°C)	3	1	1 (1:10)	2	3	2	3	1	3	1	2	21.54
14	2 (10h)	2 (60°C)	3	1	2 (1:15)	3	1	3	1	2	1	2	3	22.48
15	2 (10h)	2 (60°C)	3	1	3 (1:20)	1	2	1	2	3	2	3	1	24.72
16	2 (10h)	3 (80°C)	1	2	1 (1:10)	2	3	3	1	2	2	3	1	20.50
17	2 (10h)	3 (80°C)	1	2	2 (1:15)	3	1	1	2	3	3	1	2	22.67

No.	A	B			C									
18	2 (10h)	3 (80°C)	1	2	3 (1:20)	1	2	2	3	1	1	2	3	22.75
19	3 (14h)	1 (40°C)	3	2	1 (1:10)	3	2	1	3	2	1	3	2	13.58
20	3 (14h)	1 (40°C)	3	2	2 (1:15)	1	3	2	1	3	2	1	3	10.33
21	3 (14h)	1 (40°C)	3	2	3 (1:20)	2	1	3	2	1	3	2	1	20.85
22	3 (14h)	2 (60°C)	1	3	1 (1:10)	3	2	2	1	3	3	2	1	23.75
23	3 (14h)	2 (60°C)	1	3	2 (1:15)	1	3	3	2	1	1	3	2	27.55
24	3 (14h)	2 (60°C)	1	3	3 (1:20)	2	1	1	3	2	2	1	3	20.97
25	3 (14h)	3 (80°C)	2	1	1 (1:10)	3	2	3	2	1	2	1	3	23.00
26	3 (14h)	3 (80°C)	2	1	2 (1:15)	1	3	1	3	2	3	2	1	27.75
27	3 (14h)	3 (80°C)	2	1	3 (1:20)	2	1	2	1	3	1	3	2	27.67
K_{1j}[a]	15.08	15.39			18.24									∑529.60
K_{2j}	22.05	21.80			19.72									
K_{3j}	21.72	21.66			20.89									
R[b]	6.97	6.41			2.65									

A, extractive time (h), B, extractive temperature (°C), C, solid to liquid ratio (g:mL), and "×", interaction.

[a] K_{ij}=(1/9) ∑ indicated antibacterial zone at factor. *j* indicated factor.

[b] R= max $\{K_{ij}\}$−min $\{K_{ij}\}$, *j* indicated factor and *i* indicated level.
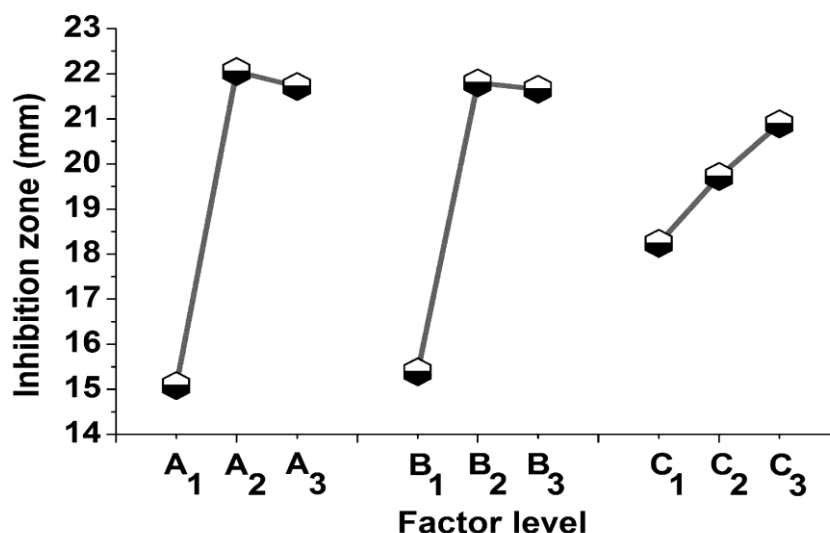
The result shown is the mean of three replicates.

Figure 1. Effect of each factor on antibacterial substance extraction. A, extractive time: A_1: 6h, A_2: 10h, A_3: 14h; B, extractive temperature: B_1: 40°C, B_2: 60°C, B_3: 80°C; C, solid to liquid ratio: C_1: 1:10 (g:mL), C_2: 1:15 (g:mL), C_3: 1:20 (g:mL).

Table 3. Analysis of variance (ANOVA).

Source	SS	*df*	MS	F^a	Significance[b]
A	277.83	2	139.92	15.92	**
B	241.05	2	120.53	13.71	**
C	31.84	2	15.92	1.81	
A×B	97.05	4	24.26	2.76	
A×C	1.03	4	0.26	0.03	
B×C	53.34	4	13.34	1.52	
Error	70.35	8	8.79		
Total	772.49	26			

A, extractive time (h), B, extractive temperature (°C), C, solid to liquid ratio (g:mL), and "×", interaction. SS = sum of squares; *df* = degree of freedom; MS = mean square; F = MSF/MSE (MSF represented the mean square of factors or interactions, and MSE represented the mean square of errors).
[a] Significant parameter, $F_{0.01}(2, 8)=8.65$, $F_{0.01}(4, 8)=7.01$.
[b] ** indicated more significant different.

Effect of *P. orientale* extracts on survival rate

Methylene-blue was used to distinguish living and dead bacteria. The viable bacterial had strong reducing power, which could transform methylene-blue from blue to no color. Therefore, the living Cmm cells were colorless and the dead Cmm cells presented blue color. Figure 2 b showed that the numbers of dead bacteria of all treatments were significantly ($P<0.05$) higher than control. The number of living bacteria (Figure 2 a) and survival rate (Figure 2 c) showed that when the concentration of *P. orientale* extracts was 0.02mg/mL, there were no significant differences between control and test group. However when concentration was higher than 0.02mg/mL, the number of living Cmm cells and survival rate were significantly

(*P*<0.05) decreased. When concentration of *P. orientale* extracts was 20mg/mL, the survival rate of Cmm was only 17.10%. These results revealed that *P. orientale* extracts had a strong antibacterial activity against Cmm, and the inhibitory activity had significant concentration effects.
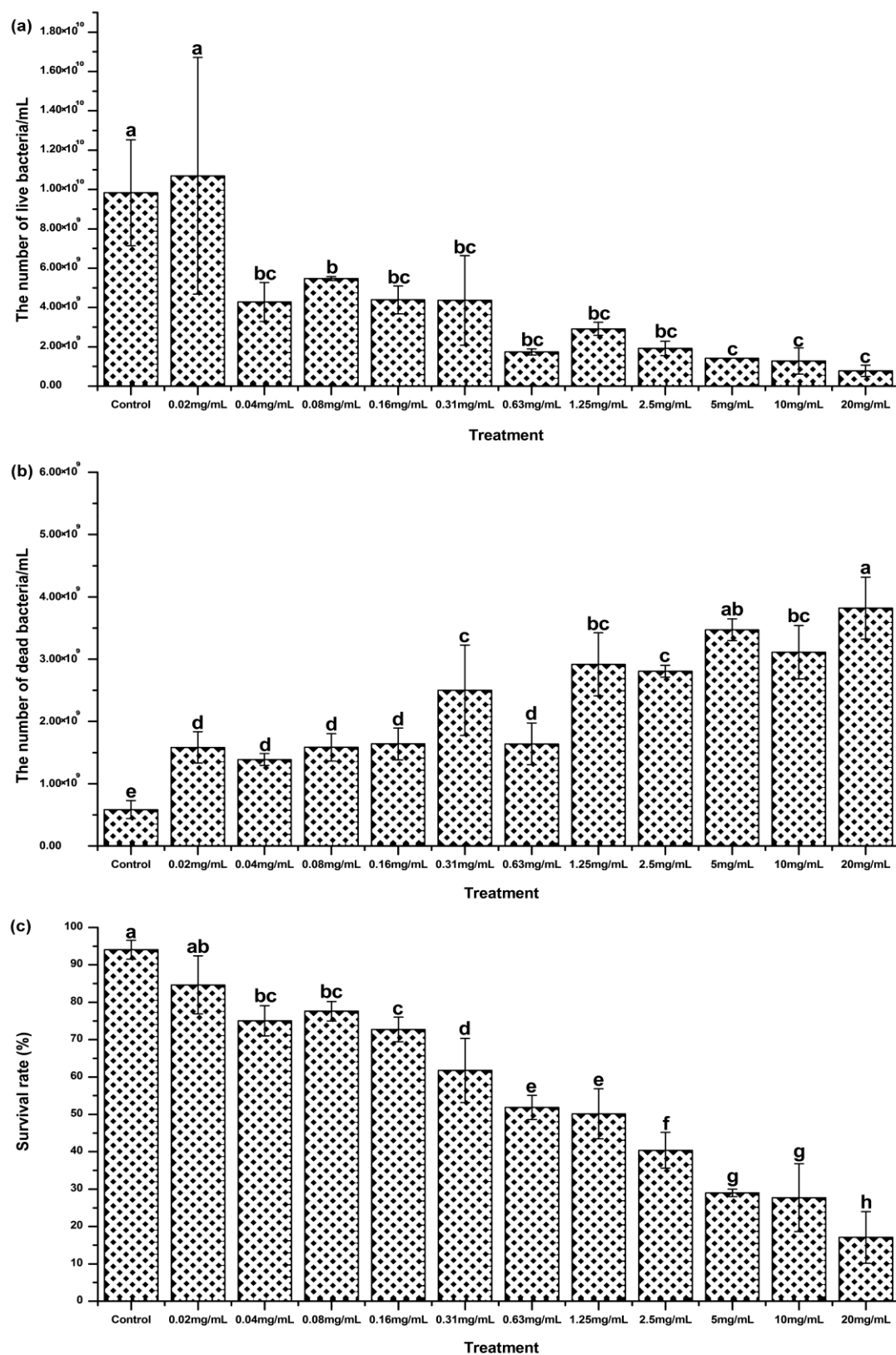
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Figure 2. Effect of *Polygonum orientale* extracts on survival rate of *Clavibacter michiganensis* subsp*. michiganensis* (Cmm). (**a**), the number of living Cmm cells; (**b**), the number of dead Cmm cells; (**c**), survival rate of Cmm cells. Cmm cells treated with 7% DMSO were used as the control. Different letters represented significant differences (*P*<0.05). The result shown is the mean of three replicates.

Effect of *P. orientale* extracts on inhibition zone of Cmm rescued post-stress

The living cells of each sample from survival rate test were used for antibacterial assay to verify if there is the occurrence of Cmm cells adapted to *P. orientale* extract stress. After 24 hours of cultivation, we tested antibacterial activity with measuring inhibition zone (Figure 3 a). When the concentration of *P. orientale* extracts in survival rate test ranged from 0.02mg/mL to 1.25 mg/mL, there were no significant differences between control and test group. When concentration in survival rate test was higher than 1.25mg/mL, the antibacterial activity of sample was increased significantly ($P<0.05$). Then, we observed the inhibition zone from the date of antibacterial test to the 14th day, and recorded the existing time of inhibition zone (Figure 3 b). When concentration of *P. orientale* extracts in survival rate test ranged from 0.02mg/mL to 5mg/mL, there were no significant differences between control and test group. When the concentration in survival rate test is higher than 5mg/mL, the existing time of inhibition zone was increased significantly ($P<0.05$). These results demonstrated that living cells from survival rate test had almost no resistance or adaptability to *P. orientale* extracts. We speculated that the bioactive compounds of *P. orientale* extracts synergistically inhibited Cmm from multi-sites, which led to nearly no occurrence of Cmm cells adapted to *P. orientale* extract stress. Furthermore, the result (Figure 3) revealed that when *P. orientale* extract concentration in survival rate test was higher, the antibacterial zone was bigger, and the existing time of inhibition zone was longer. It was proved that the Cmm cells rescued from *P. orientale* extracts of high concentration were much easier to be inhibited by *P. orientale* extracts. It is probably because that *P. orientale* extracts of high concentration might damage the genetic material of Cmm dramatically.
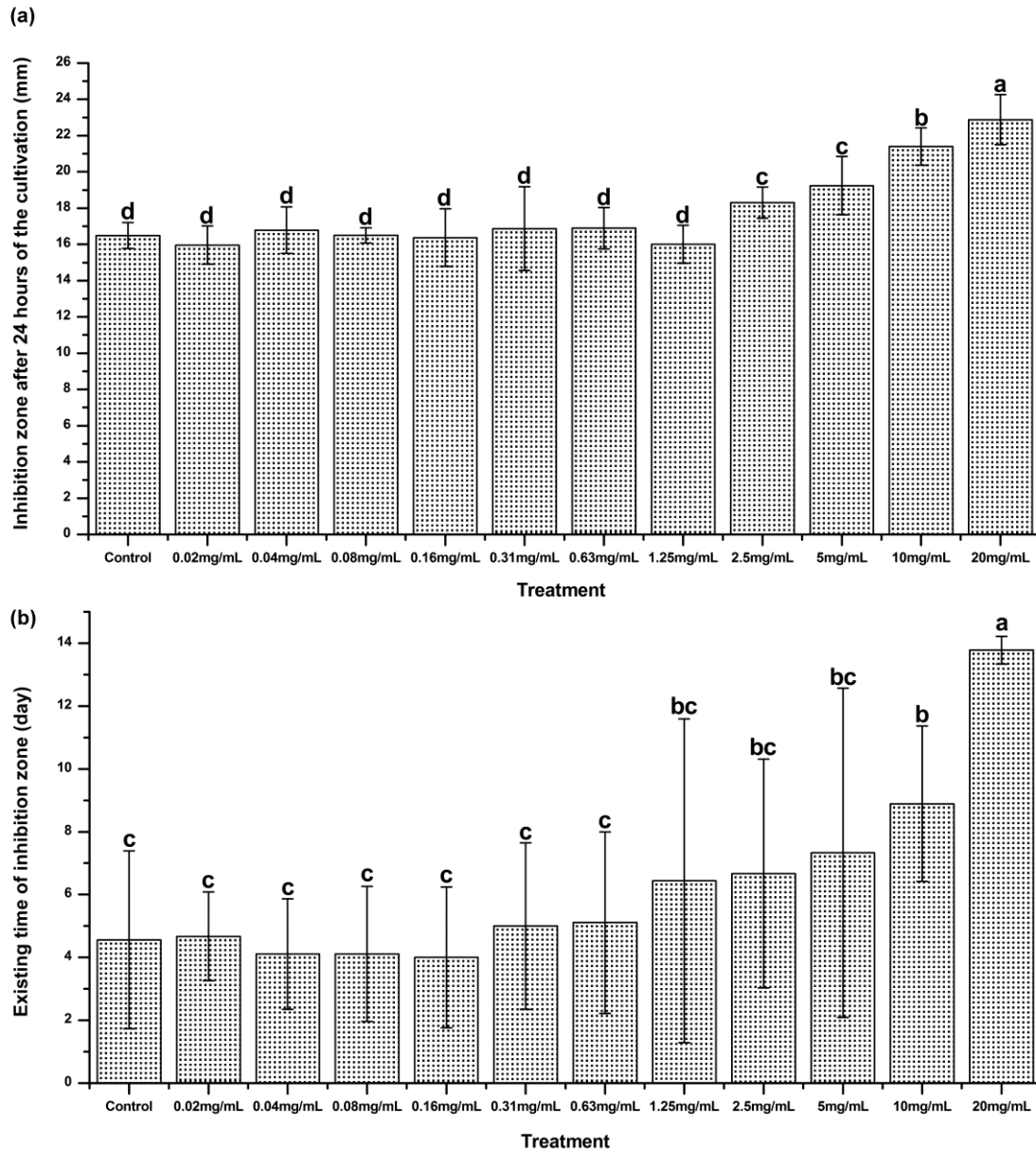
(a)

(b)

Figure 3. Effect of *Polygonum orientale* extracts on inhibition zone of *Clavibacter michiganensis* subsp*. michiganensis* (Cmm) cells rescued post-stress. (**a**), the inhibition zone of Cmm after cultivation for 24 hour; (**b**), the existing time of inhibition zone of Cmm rescued post-stress. The DMSO (40%) was used as the control. When the inhibition zone of Cmm still existed on the 14th day, we record 14day in the test. Different letters represented significant differences (*P*<0.05). The result shown is the mean of nine replicates.

Damage to cell membrane of Cmm

Cell membranes are important functional barriers of bacteria [30]. They play the important roles in information exchange, energy transmission and maintaining intracellular homeostasis [31,32]. When cell membranes of Cmm are intact, intracellular nucleic acids and intracellular soluble proteins cannot overflow from cells. Only when cell membranes get incomplete can we detect extracellular nucleotides and extracellular proteins [33]. The absorption peak of nucleotide is 260nm and therefore we can detect extracellular OD_{260nm} values to detect extracellular nucleotides. The results of OD_{260nm} analysis (Figure 4 a) showed that when the

concentration of *P. orientale* extracts was 0.06mg/mL, there were no significant differences between control and test group. When the concentration is higher than 0.06mg/mL, the OD_{260nm} value of each test group was significantly ($P<0.05$) higher than control. The results (Figure 4 a) indicated that when *P. orientale* extracts (greater than or equal to 0.08mg/mL) acted on Cmm membrane, the nucleotides would leak from cells. In addition, this phenomenon had obvious concentration effects. Proteins are biological macromolecules, which play important roles in bacterial cells. They are involved in almost all of metabolic activities in the cells [34]. The leakage of intracellular proteins can destroy normal physiological activities of bacterial cells. We can also deduce the damaged condition of Cmm cell membrane from Figure 4 b. When the concentration of *P. orientale* extracts was 0.06mg/mL or 0.08mg/mL, there were no significant differences between control and treatments. When the concentration of *P. orientale* extracts was 0.10mg/mL or 0.12mg/mL, the extracellular protein content of each treatment was significantly ($P<0.05$) higher than control. The results (Figure 4 b) indicated that when *P. orientale* extracts (greater than or equal to 0.10mg/mL) acted on Cmm cells, the intracellular proteins would leak from Cmm cells, and this phenomenon also had obvious concentration effects.

The phenylalanine, tryptophan, and tyrosine residues in protein could emit fluorescence [35]. In most cases, the fluorescence of membrane protein is from tryptophan residues, and changes in fluorescence reflect changes of cell membrane and its surroundings [36]. The effect of *P. orientale* extracts on the fluorescence of membrane protein was showed in Figure 4 c. With increase of *P. orientale* extracts, the fluorescence intensity would be increased correspondingly. The results (Figure 4 c) indicated that *P. orientale* extracts could disrupt the native structure of cell membrane or the outside peptidoglycan layer, resulting in the exposure of tryptophan residues in the inner membrane and an increase in fluorescence [37].
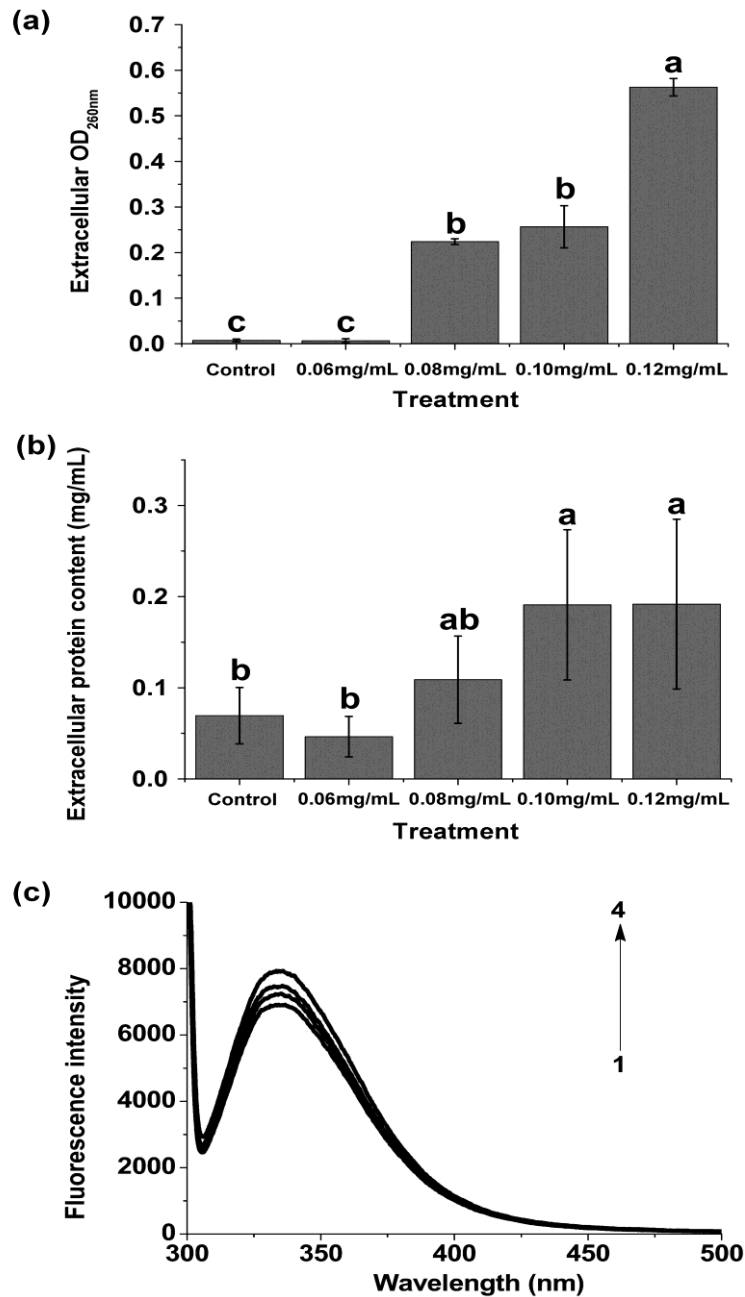
(a)

(b)

(c)

Figure 4. Effect of *Polygonum orientale* extracts on cell membrane of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). (**a**), effect of *P. orientale* extracts on extracellular OD_{260nm} value; (**b**), effect of *P. orientale* extracts on extracellular protein content; (**c**), effect of *P. orientale* extracts on the fluorescence intensity of Cmm membrane protein. Cmm cells treated with 0.4% DMSO were used as the control. In Figure 4 (a) and Figure 4 (b), different letters represented significant differences ($P<0.05$). The result shown is the mean of three replicates. In Figure 4 (c), "1" represented the control; "2" represented Cmm cells treated with *P. orientale* extracts at 0.08mg/mL; "3" represented Cmm cells treated with *P. orientale* extracts at 0.10mg/mL; "4" represented Cmm cells treated with *P. orientale* extracts at 0.12mg/mL.

Damage to cell wall and intracellular ATPase activity

Bacterial cell walls play important roles in assisting cell movement, transporting materials, and maintaining cell shape. AKP is an enzyme, which is found between cell wall and cell membrane. When cell walls of Cmm are intact, AKP cannot overflow from cells. Only when cell walls get incomplete can we detect extracellular AKP

activity [38]. Figure 5 a showed that the extracellular AKP activities of all treatments were significantly ($P<0.05$) higher than control. This result revealed that *P. orientale* extracts could damage the cell walls of Cmm.

The ATPase is an ATP-driven transporter in bacteria, which can pump hydrogen, calcium, potassium, or sodium ions to produce energy [39,40]. When ATPase transports these ions, some important substances, such as sugars and amino acids, can also enter the cells. Therefore, ATPase plays important roles in a variety of vital biological processes. Figure 5 b showed that, when the concentrations of *P. orientale* extracts were 0.06mg/mL, 0.08mg/mL, 0.10mg/mL and 0.12mg/mL, the ATPase activity was significantly ($P<0.05$) lower than control. The results (Figure 5 b) revealed that *P. orientale* extracts could damage the intracellular ATPase. We speculated that *P. orientale* extracts damaged the cell membrane of Cmm, resulting in inhibiting ATPase activity in plasma membrane. Furthermore, the decreased ATPase activity should also cause intracellular energy reduction.
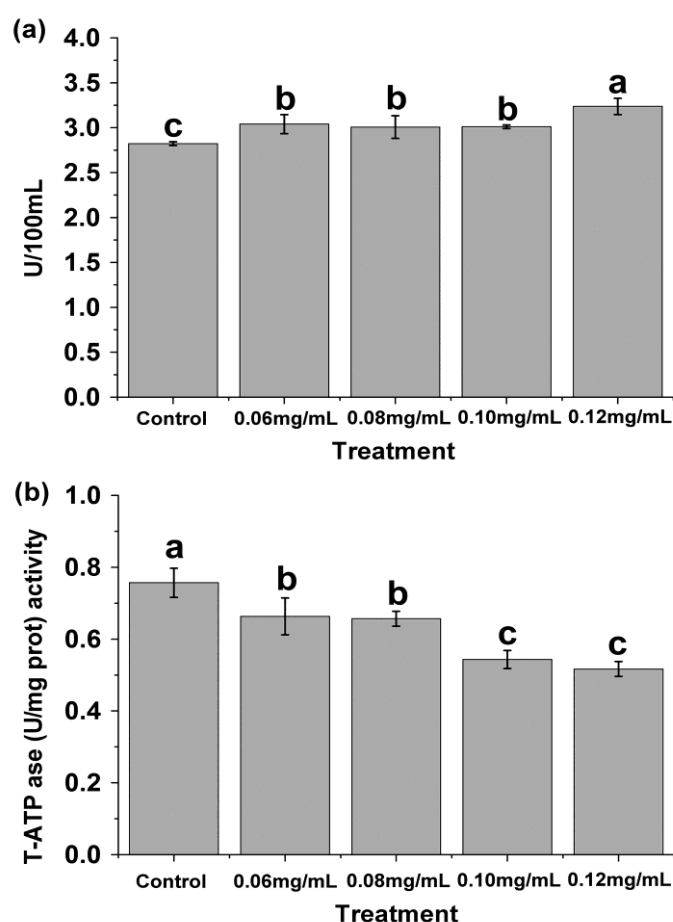

Figure 5. Effect of *Polygonum orientale* extracts on *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) cell physiology. (**a**), effect of *Polygonum orientale* extracts on extracellular alkaline phosphatase (AKP); (**b**), effect of *Polygonum orientale* extracts on ATPase activity. Cmm cells treated with 0.4% DMSO were used as the control. Different letters represented significant differences ($P<0.05$). The result shown is the mean of three replicates.

CONCLUSION

In conclusion, this work presented a study, which found out the optimized extracting condition of maximum antibacterial activity from *P. orientale* extracts by orthogonal experiment design L_{27} (3^{13}). The optimal extraction conditions were $A_2B_2C_3$ (extractive time: 10h; extractive temperature: 60°C; solid to liquid ratio: 1:20 (g:mL)).

This work also demonstrated that living cells of each sample from survival rate test had almost no resistance or adaptability, and rescued Cmm cells were much easier to be inhibited by *P. orientale* extracts. Cell membrane is a barrier of bacteria, which can prevent the outflow of internal substances. Hence, we detected extracellular OD_{260nm} value, extracellular protein content, and the conformational structure of cell membrane protein to indicate the obvious breakage of Cmm cell membrane. Finally, we demonstrated that *P. orientale* extracts could disrupt cell wall of Cmm and induce a decrease of intracellular ATPase activity. The results of these assays revealed that *P. orientale* extracts had a strong antibacterial activity against Cmm, and should be used as a botanical bactericide for the management of bacterial canker of tomato disease.

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