Journal of Applied Botany and Food Quality 90, 183 - 190 (2017), DOI:10.5073/JABFQ.2017.090.023

[1]Institute of Applied Chemistry, Shanxi University, Taiyuan, Shanxi Province, People's Republic of China
[2]Institute of Biotechnology, Shanxi University, Taiyuan, Shanxi Province, People's Republic of China

Antimicrobial activity of *Laminaria japonica* extracts against bacterial canker of tomato disease agent *Clavibacter michiganensis* subsp. *michiganensis*

Jin Cai[1], Mengliang Wang[1*], Xiaoxia Zhao[1], Yanyan Zhang[1,2], Xin Yuan[1,2]

(Received December 23, 2016)

Summary

Laminaria japonica was extracted with ethanol, and tested for antimicrobial activity against *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). This gram-positive bacterium is the agent of a serious tomato disease called bacterial canker. On *in vitro* antimicrobial assays, *L. japonica* extracts showed intensive antimicrobial activity to inhibit Cmm. Extracting parameters (time, temperature, and solid/liquid ratio) were investigated and further optimized integratedly with $L_{27}(3^{13})$ orthogonal array design (OAD). Our data implied that the optimum extraction conditions were $A_3B_3C_3$, that were extracting time 12 h, extracting temperature 80 °C, and solid/liquid ratio 1:30 (g:mL). Extracting temperature was the significant factor affecting extracting condition. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *L. japonica* extracts against Cmm were 2.5 mg/mL and 5 mg/mL. Scanning electron microscopy (SEM) observation indicated *L. japonica* extracts caused considerable morphological alterations in Cmm cells, such as protoplast leakage, cell membrane disruption, cell clumped, and cell fragmented. Reactive oxygen species (ROS) level determination and ATPase activity assay revealed that *L. japonica* extracts did cause significant accumulation in ROS and reduce the intracellular ATPase activity rapidly. Accordingly, these results indicated that *L. japonica* extracts could open a new promising opportunity for this tomato disease control.

Keywords: Bacterial canker of tomato, *Clavibacter michiganensis* subsp. *michiganensis*, *Laminaria japonica*, Antimicrobial activity, Orthogonal array design, Antimicrobial mechanism

Introduction

Because of causing great losses in yields, crop diseases infected by bacterial pathogens are the major problem in world agriculture (Frenkel et al., 2016). *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is a gram-positive bacterium. It can cause a serious tomato disease called bacterial canker. In recent years, the incidence of bacterial canker has increased, and Cmm is now present in the world's main tomato-production areas (Yogev et al., 2009). Infection of tomato by this pathogen leads to the whole plant infection with the typical feature of "bird's-eye spots" lesions, and the yield losses can be severe (Flügel et al., 2012). Chemical control, such as copper hydroxide, remains the main measure to inhibit Cmm (Lalacci et al., 2016). However, chemical controls have two drawbacks: the highly toxicity and residue in crops, and the adverse effects on environment and human health (Cardoso et al., 2010). Therefore, it is urgent to find alternative antimicrobial substances to control bacterial canker of tomato disease.

Plants possess a range of secondary metabolites, which contribute to resistance against a variety of pathogens (Asano et al., 2013). More and more plant extracts, as substitutes for chemical control, are used in plant disease treatment (Morales-Soto et al., 2015). *Laminaria japonica* is the economic-seaweed in China (Li et al., 2005). It has been used as a source of traditional Chinese medicine to treat various diseases, such as edema, encephalitis B, and tumor (Go et al., 2010). It has been reported that *L. japonica* contains various bioactive compounds for numerous biological activities including antibacterial, anti-inflammatory, and anti-complement activities (Choi et al., 2012). As far as we know, there is no previous study on antimicrobial activity of *L. japonica* extracts for inhibiting Cmm.

Therefore, this study aimed to primarily test the antimicrobial activity of *L. japonica* extracts against Cmm *in vitro* for the first time. Furthermore, we evaluated the optimization of antimicrobial substance production by using orthogonal experimental design. Then we tested the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Finally, we investigated the possible mechanism by scanning electron microscopy (SEM) observation, reactive oxygen species (ROS) level determination, and ATPase activity analyses.

Materials and methods

Plant material

L. japonica was obtained from a local market in China. The sample was finely ground down by a blender to provide a powder (sieved 130-200 mesh size), and was stored in an airtight container (Akter et al., 2016).

Bacterial strain and culture medium

Cmm ATCC 492 was obtained from China General Microbiological Culture Collection Center. The strain was routinely grown at 28 °C in the culture medium, which contained 20 g/L agar, 10 g/L peptone, 5 g/L yeast extracts, 5 g/L malt extracts, 5 g/L caseinate, 2 g/L beef extracts, 2 g/L glycerol, 50 mg/L twain-80, and 1 g/L $MgSO_47H_2O$. The pH of the culture medium was 7.2.

Orthogonal array design (OAD)

The OAD experiments were performed according to Huang et al. (2009). Three parameters affecting antimicrobial substance extraction were investigated by $L_{27}(3^{13})$ orthogonal design as shown in Tab. 1. They were A: extracting time (h), B: extracting temperature (°C), and C: solid/liquid ratio (g:mL). For each experiment, 8 g sample powder and ethanol (corresponding volume) were packed into a flask and extracted successively as described in Tab. 2. The supernatants of each extraction (sterilized by filteration technique) were evaporated, and each dried mass was individually dissolved in dimethyl sulfoxide (DMSO) for giving concentration of 8mg/mL. Then, each sample was stored at -4 °C until antimicrobial determination.

* Corresponding author

Antimicrobial determination was performed by the agar-dilution method (Wang et al., 2016). A hole (1cm diameter) was perforated in the culture medium, and 150 μL sample was added in it. Because DMSO does not have antimicrobial activity, it was used as the control in our study (Smânia et al., 1999). Then, the test plates were incubated at 28±2 °C, and the inhibition zones of Cmm were measured after 24 h. Each test was performed in six replications.

MIC and MBC assays

To test the MIC of *L. japonica* extracts against Cmm, micro-well dilution method was carried out in a 96-well plate (Chen et al., 2016). *L. japonica* extracts (final concentration ranging from 20 mg/mL to 0.07 mg/mL) were prepared with sterile culture medium by two-fold dilution (final volume of each test well was 200 μL) (Sun et al., 2016). Then 10 μL of Cmm (10^6 CFU/mL) was added into each test well (Bouaziz et al., 2016). The sterility controls were that the test wells only contained culture medium. The positive controls were that the test wells contained culture medium, Cmm, and streptomycin (final concentration, 10 mg/mL) (Ahila et al., 2016; Lalacci et al., 2016). The negative controls were that the test wells only contained culture medium and Cmm. Each test was performed in three replications. The 96-well plate was incubated at 28±2 °C for 24 h. After incubation, we observed the turbidity of culture medium in the well. The MIC was considered as the lowest concentration of *L. japonica* extracts with no visible bacterial growth (Raffaella et al., 2017). To test the MBC of *L. japonica* extracts against Cmm, 50 μL sample from each MIC test well was spread uniformly on the nutrient agar surface (Jalali et al., 2016). Then all the samples were incubated at 28±2 °C for 24 h. Each test was performed in three replications. The MBC was considered as the lowest concentration of *L. japonica* extracts that ceased the growth of Cmm (Raffaella et al., 2017).

SEM

Logarithmic phase cells of Cmm (treated with *L. japonica* extracts at 0.03 mg/mL, 0.04 mg/mL, and 0.05 mg/mL for 8 h respectively) were washed and resuspended in phosphate buffer (pH 7.2) to 10^8 CFU/mL. Cells without *L. japonica* extracts were used as the control. All the samples were centrifuged at 10000 rpm for 5 min, and the cell pellets were infiltrated into 2.5% glutaraldehyde at 4 °C overnight (Devi et al., 2010). After rinsing, the pellets were post-fixed for 2 h in 1% OsO_4 solution. Then, the samples were dehydrated in an ethanol series (ranging from 30% to 100%) for 20 min in each alcohol dilution. After critical point drying, the samples were subjected to gold covering. The observation was performed under scanning electron microscope (JSM-35C, JEOL, Japan).

Detection of ROS

Cmm cells (logarithmic phase) were treated with *L. japonica* extracts at 0.02 mg/mL, 0.03 mg/mL, and 0.04 mg/mL respectively. Cells without *L. japonica* extracts were used as the control. After incubating 8 h, all the samples were centrifuged and then washed twice with phosphate buffer (pH 7.2). Cells were resuspended in phosphate buffer containing 20 μmol/L 2,7-dichlorofluorescin diacetate (DCFH-DA). Then, each sample was shaken (in dark) at 28±2 °C for 20 min in order for the probes to enter Cmm cells (Wang et al., 2015). Cells were collected by centrifugation at 3000 rpm for 5 min and washed three times with phosphate buffer to remove free DCFH-DA. The data were evaluated by FACS Calibur flow cytometer (Becton Dickinson, USA), at excitation wavelength of 488 nm and emission wavelength of 535 nm.

Measurement of intracellular ATPase activity

The assay was performed by mixing Cmm (logarithmic phase) with increasing amounts of *L. japonica* extracts (final concentrations were 0.02 mg/mL, 0.03 mg/mL, 0.04 mg/mL, and 0.05 mg/mL respectively). Cells without *L. japonica* extracts were used as the control. After incubating 8 h, each sample (8 mL) was centrifuged at 5000 rpm, and then mixed in sodium chloride (1.0%). A total of 1mL sample was sonicated by a probe sonicator at 200 W for 20 min. The mode was pulsing 2 s followed by 4 s stop. Subsequently, each suspension was centrifuged (12000 rpm, 10 min, 4 °C). Then ATPase activity in the supernatant was tested by a kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Each test was performed in seven replications.

Statistical analyses

One-way analysis of variance (ANOVA) was carried out to determine significant differences between the means (SPSS 17.0).

Results

Optimization of the extraction condition with OAD

According to OAD $L_{27}(3^{13})$, twenty-seven experimental trials were performed to optimize the extracting time, extracting temperature and solid/liquid ratio. Tab. 2 shows twenty-seven rows corresponding to the number of tests with thirteen columns at three levels. The first column is assigned to extracting time (A), the second column is assigned to extracting temperature (B), and the fifth column is assigned to solid/liquid ratio (C). Meanwhile, column three and column four are used to study the interactions of factors A and B. Column six and column seven are used to study the interactions of factors A and C. Column eight and column eleven are used to study the interactions of factors B and C. From Tab. 2, we would see the results of antimicrobial activity tested under the experimental conditions (Adnani et al., 2010). For example, in the first row of Tab. 2, levels of factors A, B, and C are 1, 1, and 1. Thus the experiment, which was performed firstly, was carried under extracting time 4 h, temperature 40 °C, and solid/liquid ratio 1:10 (g:mL), and the inhibition zone of the first experiment was 10.17 mm. Fig. 1 indicated that the best extracting time level was A_3, the best extracting temperature was B_3, and the best solid/liquid ratio level was C_3. Therefore, the best combination to obtain antimicrobial substance from *L. japonica* was $A_3B_3C_3$, that were extracting time 12 h, extracting temperature 80 °C, and solid/liquid ratio 1:30. The factors influencing extraction were listed as follow: extracting temperature (B)>solid/liquid ratio (C)>extracting time (A) according to the *R* value (Tab. 2).

Data analysis by ANOVA is much more scientific than the direct observation analysis (Adnani et al., 2010). In Tab. 3, SS is defined as the sum of deviation squares; *df* is defined as degree of freedom; and MS is defined as mean square. *F* shows the significance of factors' or interactions' influence on the results. The analysis (Tab. 3) showed that extracting temperature (B) had significant effect on antimicrobial activity, while the other two factors and three interactions, i.e.

Tab. 1: Extracting parameters and levels for orthogonal array design

Levels	Factors		
	Extracting time (A)	Extracting temperature (B)	Solid/liquid ratio (C)
1	4 h	40 °C	1:10 (g:mL)
2	8 h	60 °C	1:20 (g:mL)
3	12 h	80 °C	1:30 (g:mL)

Tab. 2: Intuitive analysis of orthogonal array design

Test	1	2	3	4	5	6	7	8	9	10	11	12	13	Inhibition zone (mm)[a]
	A	B	A×B	A×B	C	A×C	A×C	B×C			B×C			
1	1 (4 h)	1 (40 °C)	1	1	1 (1:10)	1	1	1	1	1	1	1	1	10.17
2	1 (4 h)	1 (40 °C)	1	1	2 (1:20)	2	2	2	2	2	2	2	2	12.33
3	1 (4 h)	1 (40 °C)	1	1	3 (1:30)	3	3	3	3	3	3	3	3	13.08
4	1 (4 h)	2 (60 °C)	2	2	1 (1:10)	1	1	2	2	2	3	3	3	10.16
5	1 (4 h)	2 (60 °C)	2	2	2 (1:20)	2	2	3	3	3	1	1	1	14.32
6	1 (4 h)	2 (60 °C)	2	2	3 (1:30)	3	3	1	1	1	2	2	2	18.97
7	1 (4 h)	3 (80 °C)	3	3	1 (1:10)	1	1	3	3	3	2	2	2	10.00
8	1 (4 h)	3 (80 °C)	3	3	2 (1:20)	2	2	1	1	1	3	3	3	17.62
9	1 (4 h)	3 (80 °C)	3	3	3 (1:30)	3	3	2	2	2	1	1	1	21.75
10	2 (8 h)	1 (40 °C)	2	3	1 (1:10)	2	3	1	2	3	1	2	3	12.08
11	2 (8 h)	1 (40 °C)	2	3	2 (1:20)	3	1	2	3	1	2	3	1	12.00
12	2 (8 h)	1 (40 °C)	2	3	3 (1:30)	1	2	3	1	2	3	1	2	10.83
13	2 (8 h)	2 (60 °C)	3	1	1 (1:10)	2	3	2	3	1	3	1	2	11.89
14	2 (8 h)	2 (60 °C)	3	1	2 (1:20)	3	1	3	1	2	1	2	3	18.98
15	2 (8 h)	2 (60 °C)	3	1	3 (1:30)	1	2	1	2	3	2	3	1	18.38
16	2 (8 h)	3 (80 °C)	1	2	1 (1:10)	2	3	3	1	2	2	3	1	18.58
17	2 (8 h)	3 (80 °C)	1	2	2 (1:20)	3	1	1	2	3	3	1	2	15.68
18	2 (8 h)	3 (80 °C)	1	2	3 (1:30)	1	2	2	3	1	1	2	3	16.15
19	3 (12 h)	1 (40 °C)	3	2	1 (1:10)	3	2	1	3	2	1	3	2	10.00
20	3 (12 h)	1 (40 °C)	3	2	2 (1:20)	1	3	2	1	3	2	1	3	16.61
21	3 (12 h)	1 (40 °C)	3	2	3 (1:30)	2	1	3	2	1	3	2	1	14.83
22	3 (12 h)	2 (60 °C)	1	3	1 (1:10)	3	2	2	1	3	3	2	1	13.33
23	3 (12 h)	2 (60 °C)	1	3	2 (1:20)	1	3	3	2	1	1	3	2	10.00
24	3 (12 h)	2 (60 °C)	1	3	3 (1:30)	2	1	1	3	2	2	1	3	20.97
25	3 (12 h)	3 (80 °C)	2	1	1 (1:10)	3	2	3	2	1	2	1	3	22.57
26	3 (12 h)	3 (80 °C)	2	1	2 (1:20)	1	3	1	3	2	3	2	1	27.33
27	3 (12 h)	3 (80 °C)	2	1	3 (1:30)	2	1	2	1	3	1	3	2	22.92
K_{1j}[b]	14.27	12.44			13.20									∑421.53
K_{2j}	14.95	15.22			16.10									
K_{3j}	17.61	19.18			17.54									
R[c]	3.34	6.74			4.34									

A represented extracting time (h), B represented extracting temperature (°C), and C represented solid/liquid ratio (g : mL).
[a] Each value was the mean of six replications.
[b] K_{ij}=(1/9) ∑ mean inhibition zone of *Laminaria japonica* extracts at factor j (j=A, B, C).
[c] R= max {K_{ij}}−min {K_{ij}}, j and i mean factor and level respectively.

extracting time (A), solid/liquid ratio (C), interaction of A and B, interaction of B and C, and interaction of A and C were not identified as significant factors or interactions.

MIC and MBC of *L. japonica* extracts against Cmm

The MIC test was determined by micro-well dilution method. As shown in Tab. 4 and Fig. 2A, the samples were clear when concentration of *L. japonica* extracts ranged from 20 mg/mL to 2.5 mg/mL. However, when concentration was less than 2.5 mg/mL, the samples in test wells became turbid. Therefore, the MIC value of *L. japonica* extracts against Cmm was 2.5 mg/mL. In MBC test (Tab. 4 and Fig. 2B), Cmm were completely killed when concentration of *L. japonica* extracts ranged from 20 mg/mL to 5 mg/mL. However, when concentration was less than 5 mg/mL, Cmm were increased gradually. Therefore, the MBC value of *L. japonica* extracts against Cmm was 5 mg/mL.

SEM image

In the absence of *L. japonica* extracts, Cmm cells were generally intact, smooth, and separated from each other (Fig. 3A). After

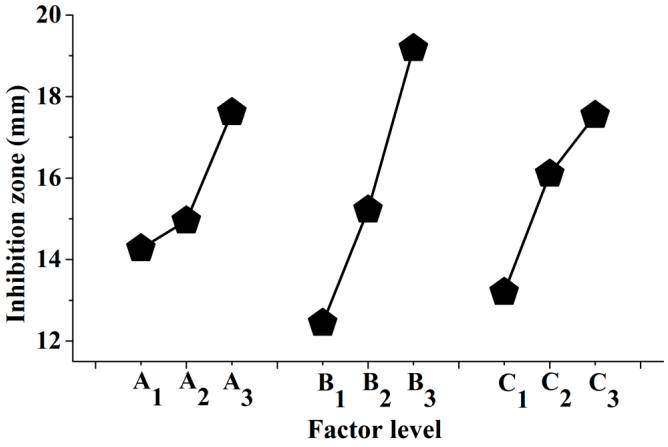

Fig. 1: Effect of parameters on extraction: A, extracting time: A_1: 4 h, A_2: 8 h, A_3: 12 h; B, extracting temperature: B_1: 40 °C, B_2: 60 °C, B_3: 80 °C; C, solid/liquid ratio (g:mL): C_1: 1:10, C_2: 1:20, C_3: 1:30.

treating with *L. japonica* extracts at 0.03 mg/mL, cell shape was still intact, while the protoplast outflowed from cell membrane (Fig. 3B, 1). The damaged cells treated with *L. japonica* extracts at 0.04 mg/mL were significantly greater than 0.03 mg/mL treatment group. Fig 3C showed that the cell surface appeared to be rough (Fig. 3C, 3), compared to control. Some damaged cells were to clump (Fig. 3C, 2), while others showed misshapen (Fig. 3C, 4). However, treated cells exhibited fragment and were completely lysed (Fig. 3D, 5), when concentration reached to 0.05 mg/mL.

Effect of *L. japonica* extracts on ROS production

As shown in Fig. 4, the fluorescence intensity was 10.15 in control treatment (Fig. 4A), whereas it was 10.49 for 0.02 mg/mL treatment group (Fig. 4B), 11.86 for 0.03 mg/mL treatment group (Fig. 4C), and 55.75 for 0.04 mg/mL treatment group (Fig. 4D), respectively. The results indicated that intracellular ROS level was increased after treating with *L. japonica* extracts.

Effect of *L. japonica* extracts on ATPase activity

As shown in Fig. 5, there was no significant difference between control and treatment at 0.02 mg/mL. However, when concentration of *L. japonica* extracts increased to 0.03 mg/mL, 0.04 mg/mL, and 0.05 mg/mL. ATPase activities were all significantly (P<0.05) lower than the control.

Discussion

With improved awareness of environmental conservation, chemical control of crop disease has been banned in many countries. Plants are frequently subjected to pathogens and pests. As defensiveness, they synthesize secondary metabolites to protect themselves, such as alkaloids, flavonoids, tannins, coumarins, glucosides, and terpenes (Park et al., 2016). Therefore, the secondary metabolites produced by plants might be used as the control of crop disease.

Bacterial diseases are often resulting in great reductions in crop yields (Choi et al., 2014). Bacterial canker caused by Cmm is a worldwide problem affecting tomato, and drastically reduces the yields of tomato recently (Tancos et al., 2013). It has been reported that some plant extracts have antimicrobial activities against Cmm.

Tab. 3: Results of one-way analysis of variance analysis

Source	SS	*df*	MS	F^a	Significance[b]
A (Extracting time)	56.41	2	28.21	2.49	
B (Extracting temperature)	206.54	2	103.27	9.07	**
C (Solid/liquid ratio)	88.10	2	44.05	3.87	
A×B (Interaction of extracting time and extracting temperature)	75.75	4	18.94	1.66	
A×C (Interaction of extracting time and solid/liquid ratio)	35.71	4	8.93	0.78	
B×C (Interaction of extracting temperature and solid/liquid ratio)	36.39	4	9.09	0.80	
Error	91.08	8	11.39		
Total	589.98	26			

[a] Significant parameter, $F_{0.01}(2,8)=8.65$, $F_{0.01}(4,8)=7.01$.
[b] ** indicated more significant different.

Tab. 4: Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *Laminaria japonica* extracts against *Clavibacter michiganensis* subsp. *michiganensis* (Cmm).

Test	*Clavibacter michiganensis* subsp. *michiganensis*												Efficacy	Result (mg/mL)
	Concentration of *Laminaria japonica* (mg/mL)									SC	PC	NC		
	20	10	5	2.5	1.25	0.62	0.31	0.15	0.07					
MIC test	-	-	-	-	+	++	++	+++	+++	-	-	+++	MIC	2.5
MBC test	-	-	-	+	++	+++	+++	+++	+++	-	-	+++	MBC	5

SC: sterility control (only culture medium); PC: positive control (culture medium, Cmm, and 10 mg/mL streptomycin); NP: negative control (culture medium and Cmm);
- indicated no growth of Cmm; + indicated weak growth of Cmm; ++ indicated moderate growth of Cmm; +++ indicated extreme growth of Cmm.

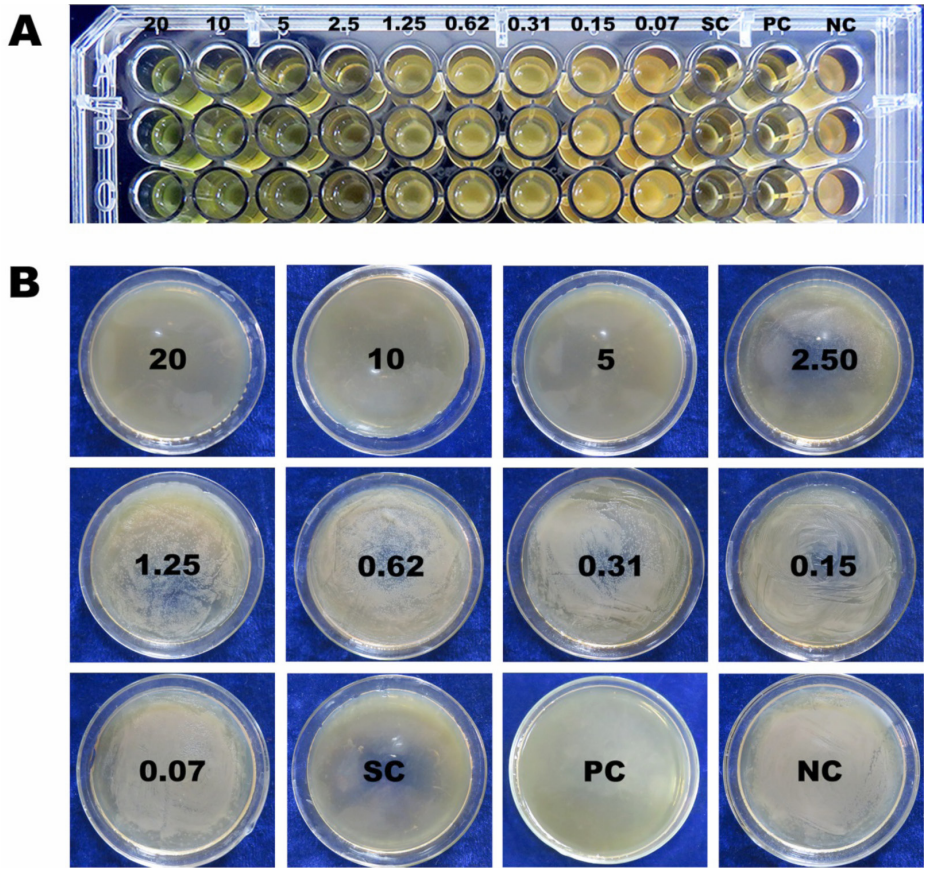

Fig. 2: Photographs of the minimal inhibitory concentration (MIC) (A) and minimal bactericidal concentration (MBC) (B) tests against *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). In the photographs of MIC and MBC tests, concentrations of *Laminaria japonica* extracts (mg/mL) were marked as the black numbers. SC: sterility control (only culture medium); PC: positive control (culture medium, Cmm, and 10 mg/mL streptomycin); NP: negative control (culture medium and Cmm).

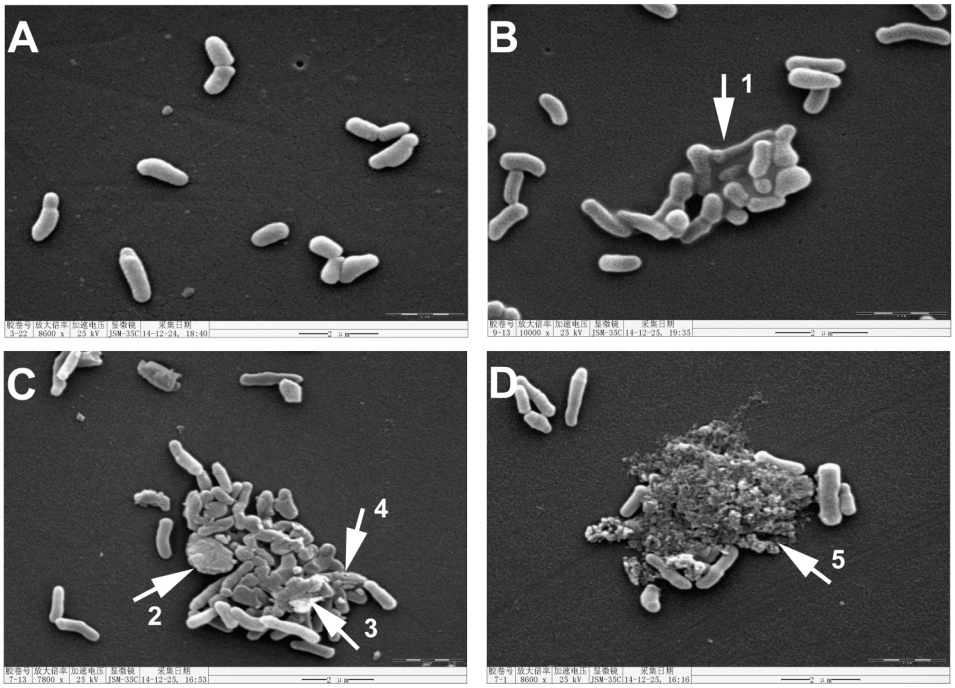

Fig. 3: Scanning electron microscopy of *Clavibacter michiganensis* subsp. *michiganensis* cells exposed to *Laminaria japonica* extracts. (A)-Untreated cells (8600x); (B)-Cells after exposure to *L. japonica* extracts at 0.03 mg/mL (10000x); (C)-Cells after exposure to *L. japonica* extracts at 0.04 mg/mL (7800x); (D)-Cells after exposure to *L. japonica* extracts at 0.05 mg/mL (8600x). Arrows indicated: 1, protoplast leakage; 2, clumped cells; 3, collapsed surfaces; 4, misshapen cells; 5, cell fragmented.

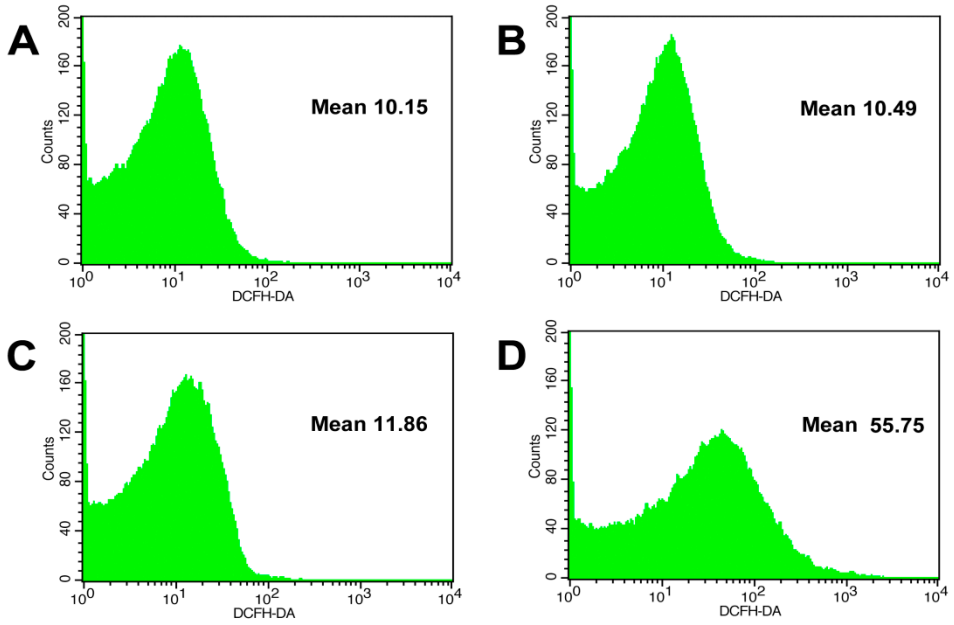

Fig. 4: Effect of *Laminaria japonica* extracts on reactive oxygen species (ROS) production by 2,7-dichlorofluorescin diacetate (DCFH-DA) staining. DCFH-DA stained cells after treatment with 0 mg/mL *L. japonica* extracts (control; A); DCFH-DA stained cells after treatment with 0.02 mg/mL *L. japonica* extracts (B); DCFH-DA stained cells after treatment with 0.03 mg/mL *L. japonica* extracts (C); DCFH-DA stained cells after treatment with 0.04 mg/mL *L. japonica* extracts (D).

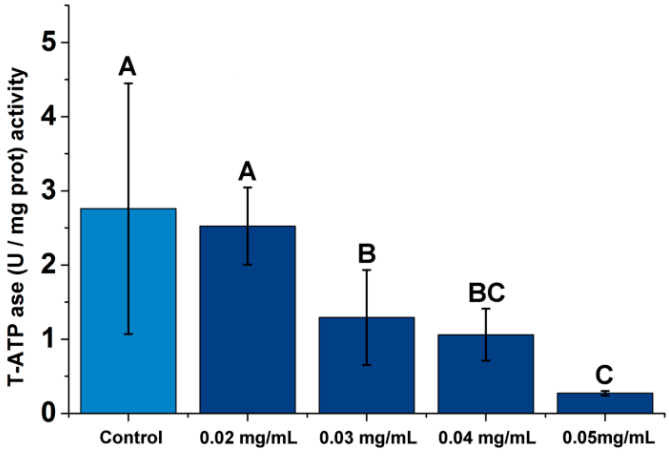

Fig. 5: Effect of *Laminaria japonica* extracts on ATPase activity in *Clavibacter michiganensis* subsp. *michiganensis*. Cells without *L. japonica* extracts were used as the control. The results represented the means of tests (standard deviations for seven independent experiments). Different letters indicated significant differences ($P<0.05$).

Allium sativum and *Ficus carica* extracts were demonstrated to have antimicrobial activity for inhibiting Cmm (Balestra et al., 2009). Govindappa et al. (2011) indicated that the water extracts of *Wedelia trilobata* moderately inhibited the growth of Cmm. Kotan et al. (2013) reported that the hexane and acetone extracts of *Satureja hortensis* had antimicrobial effects on Cmm. Kotan et al. (2014) also reported that the hexane, chloroform, and acetone extracts of *Origanum onites* had antimicrobial effects on Cmm, but the methanol extracts of *O. onites* did not show inhibitory activity. In this study, *L. japonica* were extracted with ethanol, and tested for its antimicrobial activity against Cmm. The results showed that *L. japonica* extracts were effective for inhibiting Cmm. As far as we know, this is the first report on antimicrobial activity of *L. japonica* extracts for inhibiting this epidemic tomato pathogen.

In this study, we performed a series of trials for antimicrobial substance extraction by OAD. OAD can provide an efficient method to find an optimal combination of factor levels (Ma et al., 2011). The advantage of OAD is that the optimal combination can be achieved with minimizing assay numbers and keeping the cost at a minimum level. The results obtained from OAD experiments can be utilized for larger scale of production (Liu et al., 2008). Results of OAD can be treated by direct observation and ANOVA analysis. ANOVA is a statistical technique, which is used for revealing the significance of factor or interaction of factors on a particular response (Sahoo, 2009). The effects of different factors or interactions were evaluated by calculating F value. We identified the importance of the factor or interaction in extraction whenever the F value exceeded the critical points F ($F_{critical}$). Tab. 3 showed that temperature was the significant factor affecting antimicrobial substance extraction. Therefore, we concluded that the extracting temperature played a critical role in extraction of antimicrobial substance from *L. japonica*. Tab. 2 and Fig. 1 revealed that the formula $A_3B_3C_3$ was used as optimal extracting condition to extract antimicrobial substances from *L. japonica*. Compared with conventional extraction, this optimal extracting condition is more efficient, and it can also meet industrial applications in the future (Li et al., 2010).

MIC was defined as the lowest concentration of *L. japonica* extracts that could prevent Cmm from obvious growth. MBC was defined as the lowest concentration of *L. japonica* extracts that could lead to kill Cmm (Raffaella et al., 2017). There are only a few reports about MIC and MBC of plant extracts against Cmm. Against Cmm, Talibi et al. (2011) screened forty plants in Moroccan and reported that the MIC values were in the range of 3.125 mg/mL-6.25 mg/mL, and the MBC values were in the range of 6.25 mg/mL-25 mg/mL. Kotan et al. (2013) reported that the hexane and acetone extracts of *S. hortensis* had the same MIC value of 80 mg/mL. Kotan et al. (2014) also reported that the MIC values of hexane, chloroform, and acetone extracts from *O. onites* were in the range of 40 mg/mL-60 mg/mL. In this study, we first determined the MIC and MBC values of *L. japonica* extracts against Cmm. The *L. japonica* extracts had the lower MIC and MBC values (2.5 mg/ml and 5 mg/ml respectively) (Tab. 4 and Fig. 2) than those plant extracts studied by Talibi et al. (2011),

KOTAN et al. (2013) and KOTAN et al. (2014). The results clearly demonstrated that *L. japonica* extracts had a strong inhibitory effect on Cmm, and should be an effective bacterial inhibitor against Cmm.

SEM is very useful for investigating the microstructure of bacteria surface. Fig 3A showed a representative SEM image of Cmm. They were all smooth and intact. It showed that Cmm cells were healthy before treating with *L. japonica* extracts. However, after exposure to *L. japonica* extracts, Cmm cells were more severely damaged (Fig. 3B, C, and D). At the experimental concentration (0.03 mg/mL and 0.04 mg/mL), the treated cells lost their cytoplasm. They were deformed and clumped together (polysaccharides leaking from bacteria cells should cause adhesions), and deep wrinkles were also observed in the cells (Fig. 3B and C). When the concentration reached to 0.05 mg/mL, Cmm cells became completely fragmented and lysed (Fig. 3D). SEM observations indicated that antimicrobial substances of *L. japonica* extracts might assist in penetration of cell membrane and lead to cell death finally. It is speculated that the disruption and dysfunction of Cmm cell membrane caused inhibition of proton motive force, inhibition of electron flux, and breakdown of active transport imbalance (KHAN and AHMAD, 2011).

It was reported that ROS accumulation was one of the mechanisms of several antifungal agents (XU et al., 2009). ROS have strong oxidant activities and attack organism to cause oxidative damage (YE et al., 2013). DCFH-DA is an oxidation-sensitive fluorescent probe. In this study, it was used to detect the level of ROS generated in Cmm cells. We observed that ROS production could be strongly increased, when concentration of *L. japonica* extracts reached to 0.04 mg/mL (Fig. 4). It is speculated that antimicrobial substances from *L. japonica* extracts accumulated and adhered to the surface of Cmm cell membrane, which would lead to membrane permeability changed, membrane structure destroyed, and ROS accumulation. What's more, ROS accumulation disrupted the bacterial cell membrane more severely, causing the leakage of intercellular contents.

The plasma membrane ATPase plays a critical role in energy generation in bacteria (TURGIS et al., 2009). Results of Fig. 5 indicated that antimicrobial substances from *L. japonica* extracts had an inhibitory effect on ATPase activity in Cmm, and the effect was dose-dependent. The data therefore indicated that ATPase inhibition could be one of the possible antimicrobial mechanisms of *L. japonica* extracts. We speculated that the affection of *L. japonica* extracts enhanced ROS accumulation in Cmm, resulting in cell membrane disruption. Furthermore, cell membrane disruption would directly inhibit the plasma membrane ATPase activity.

Conclusion

To the best of our knowledge, this study is the first report on the antimicrobial activity of *L. japonica* extracts against Cmm. OAD results revealed that the optimum extraction condition was $A_3B_3C_3$: extracting time 12 h, extracting temperature 80 °C, and solid/liquid ratio 1:30. ANOVA results indicated that the most significant parameter was temperature, which affected the optimization of extraction. The efficacy of *L. japonica* extracts against Cmm was also determined by MIC and MBC tests. The MIC value was 2.5 mg/mL, and the MBC value was 5 mg/mL. SEM images directly provided the information that *L. japonica* extracts had drastic effects on Cmm. The results of ROS and ATPase activity assays implied that ROS accumulation, interfering with energy generation system, might lead to the death of Cmm. These results indicated that *L. japonica* extracts could be a safer alternative to chemical bactericide for inhibiting Cmm.

Acknowledgements

This study was supported by Scientific and Technologial Innovation Programs of Higher Education Institutions in Shanxi (STIP; No.2016117), and National Natural Science Foundation of China (No.31601677).

References

ADNANI, A., BASRI, M., MALEK, E.A., SALLEH, A.B., RAHMAN, M.B.A., CHAIBAKHSH, N., RAHMAN, R.N.Z.R.A., 2010: Optimization of lipase-catalyzed synthesis of xylitol ester by Taguchi robust design method. Ind. Crop. Prod. 31, 350-356. DOI: 10.1016/j.indcrop.2009.12.001

AHILA, N.K., RAMKUMAR, V.S., PRAKASH, S., MANIKANDAN, B., RAVINDRAN, J., DHANALAKSHMI, P.K., KANNAPIRAN, E., 2016: Synthesis of stable nanosilver particles (AgNPs) by the proteins of seagrass *Syringodium isoetifolium* and its biomedicinal properties. Biomed. Pharmacother. 84, 60-70. DOI: 10.1016/j.biopha.2016.09.004

AKTER, K., BARNES, E.C., LOA-KUM-CHEUNG, W.L., YIN, P., KICHU, M., BROPHY, J.J., BARROW, R.A., IMCHEN, I., VEMULPAD, S.R., JAMIE, J.F., 2016: Antimicrobial and antioxidant activity and chemical characterization of *Erythrina stricta* Roxb. (Fabaceae). J. Ethnopharmacol. 185, 171-181. DOI: 10.1016/j.jep.2016.03.011

ASANO, T., MIWA, A., MAEDA, K., KIMURA, M., NISHIUCHI, T., 2013: The secreted antifungal protein thionin 2.4 in *Arabidopsis thaliana* suppresses the toxicity of a fungal fruit body lectin from *Fusarium graminearum*. Plos Pathog. 9, e1003581. DOI: 10.1371/journal.ppat.1003581

BALESTRA, G.M., HEYDARI, A., CECCARELLI, D., OVIDI, E., QUATTRUCCI, A., 2009: Antibacterial effect of *Allium sativum* and *Ficus carica* extracts on tomato bacterial pathogens. Crop Prot. 28, 807-811. DOI: 10.1016/j.cropro.2009.06.004

BOUAZIZ, A., MHALLA, D., ZOUARI, I., JLAIEL, L., TOUNSI, S., JARRAYA, R., TRIGUI, M., 2016: Antibacterial and antioxidant activities of *Hammada scoparia* extracts and its major purified alkaloids. S. Afr. J. Bot. 105, 89-96. DOI: 10.1016/j.sajb.2016.03.012

CARDOSO, R.A., PIRES, L.T.A., ZUCCHI, T.D., ZUCCHI, F.D., ZUCCHI, T.M.A.D., 2010: Mitotic crossing-over induced by two commercial herbicides in diploid strains of the fungus *Aspergillus nidulans*. Genet. Mol. Res. 9, 231-238. DOI: 10.4238/vol9-1gmr688

CHEN, Z.F., HE, B., ZHOU, J., HE, D.H., DENG, J.D., ZENG, R.H., 2016: Chemical compositions and antibacterial activities of essential oils extracted from *Alpinia guilinensis* against selected foodborne pathogens. Ind. Crop. Prod. 83, 607-613. DOI: 10.1016/j.indcrop.2015.12.063

CHOI, J., BAEK, K.H., MOON, E., 2014: Antimicrobial effects of a hexapetide KCM21 against *Pseudomonas syringae* pv. *tomato* DC3000 and *Clavibacter michiganensis* subsp. *michiganensis*. Plant Pathol. J. 30, 245-253. DOI: 10.5423/PPJ.OA.02.2014.0011

CHOI, Y.S., CHOI, J.H., HAN, D.J., KIM, H.Y., KIM, H.W., LEE, M.A., CHUNG, H.J., KIM, C.J., 2012: Effects of *Laminaria japonica* on the physicchemical and sensory characteristics of reduced-fat pork patties. Meat Sci. 91, 1-7. DOI: 10.1016/j.meatsci.2011.11.011

DEVI, K.P., NISHA, S.A., SAKTHIVEL, R., PANDIAN, S.K., 2010: Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. J. Ethnopharmacol. 130, 107-115. DOI: 10.1016/j.jep.2010.04.025

FLÜGEL, M., BECKER, A., GARTEMANN, K.H., EICHENLAUB, R., 2012: Analysis of the interaction of *Clavibacter michiganensis* subsp. *michiganensis* with its host plant tomato by genome-wide expression profiling. J. Biotechnol. 160, 42-54. DOI: 10.1016/j.jbiotec.2012.01.023

FRENKEL, O., BORNESTEIN, M., SHULHANI, R., SHARABANI, G., SOFER, M., ABO-MOCH, F., LOFTHOUSE, M., MANULIS-SASSON, S., SHTIENBERG, D., 2016: Secondary spread of *Clavibacter michiganensis* subsp. *michiganensis* in nurseries and the conditions leading to infection of tomato seedlings. Eur. J. Plant Pathol. 144, 569-579. DOI: 10.1007/s10658-015-0795-4

GO, H., HWANG, H.J., NAM, T.J., 2010: A glycoprotein from *Laminaria japonica* induces apoptosis in HT-29 colon cancer cells. Toxicol. in vitro 24, 1546-1553. DOI: 10.1016/j.tiv.2010.06.018

GOVINDAPPA, M., SRAVYA, S.N., POOJASHRI, M.N., SADANANDA, T.S.,

Chandrappa, C.P., Santoyo, G., Sharanappa, P., Kumar, N.V.A., 2011: Antimicrobial, antioxidant and *in vitro* anti-inflammatory activity and phytochemical screening of water extract of *Wedelia trilobata* (L.) Hitchc. J. Med. Plants Res. 5, 5718-5729.

Huang, G.H., Chen, G., Chen, F., 2009: Rapid screening method for lipid production in alga based on Nile red fluorescence. Biomass Bioenerg. 33, 1386-1392. DOI: 10.1016/j.biombioe.2009.05.022

Jalali, S.A.H., Allafchian, A.R., Banifatemi, S.S., Tamai, I.A., 2016: The antibacterial properties of Ag/TiO_2 nanoparticles embedded in silane sol-gel matrix. J. Taiwan Inst. Chem. E. 66, 357-362. DOI: 10.1016/j.jtice.2016.06.011

Khan, M.S.A., Ahamd, I., 2011: *In vitro* antifungal, anti-elastase and anti-keratinase activity of essential oils of *Cinnamomum*-, *Syzygium*- and *Cymbopogon*-species against *Aspergillus fumigatus* and *Trichophyton rubrum*. Phytomedicine 19, 48-55. DOI: 10.1016/j.phymed.2011.07.005

Kotan, R., Dadasoğlu, F., Karagoz, K., Cakir, A., Ozer, H., Kordali, S., Cakmakci, R., Dikbas, N., 2013: Antibacterial activity of the essential oil and extracts of *Satureja hortensis* against plant pathogenic bacteria and their potential use as seed disinfectants. Sci. Hortic. 153, 34-41. DOI: 10.1016/j.scienta.2013.01.027

Kotan, R., Cakir, A., Ozer, H., Kordali, S., Cakmakci, R., Dadasoglu, F., Dikbas, N., Aydin, T., Kazaz, C., 2014: Antibacterial effects of *Origanum onites* against phytopathogenic bacteria: Possible use of the extracts from protection of disease caused by some phytopathogenic bacteria. Sci. Hortic. 172, 210-220. DOI: 10.1016/j.scienta.2014.03.016

Lalacci, G.M., Bella, P., Licciardello, G., Strano, C.P., Eichenlaub, R., Gartemann, K.H., La Rosa, R., Catara, V., 2016: Clonal populations of *Clavibacter michiganensis* subsp. *michiganensis* are responsible for the outbreaks of bacterial canker in greenhouse tomatoes in Italy. Plant Pathol. 65, 484-495. DOI: 10.1111/ppa.12424

Li, N., Zhang, Q.B., Song, J.M., 2005: Toxicological evaluation of fucoidan extracted from *Laminaria japonica* in Wistar rats. Food Chem. Toxicol. 43, 421-426. DOI: 10.1016/j.fct.2004.12.001

Li, Z., Pan, Q.H., Cui, X.Y., Duan, C.Q., 2010: Optimization on anthocyanins extraction from wine grape skins using orthogonal test design. Food Sci. Biotechnol. 19, 1047-1053. DOI: 10.1007/s10068-010-0147-2

Liu, B., Shen, B., Guo, F., Chang, Y.L., 2008: Optimization of supercritical fluid extraction of *dl*-tetrahydropalmatine from rhizome of *Corydalis yanhusuo* W.T. Wang with orthogonal array design. Sep. Purif. Technol. 64, 242-246. DOI: 10.1016/j.seppur.2008.10.003

Ma, S.H., Wang, H., Wang, Y., Bu, H.Y., Bai, J.B., 2011: Bio-hydrogen production from cornstalk wastes by orthogonal design method. Renew. Energ. 36, 709-713. DOI: 10.1016/j.renene.2010.08.019

Morales-Soto, A., Oruna-Concha, M.J., Elmore, J.S., Barrajón-Catalán, E., Micol, V., Roldán, C., Segura-Carretero, A., 2015: Volatile profile of Spanish *Cistus* plants as sources of antimicrobials for industrial applications. Ind. Crop. Prod. 74, 425-433. DOI: 10.1016/j.indcrop.2015.04.034

Park, D., Shin, K., Choi, Y., Guo, H., Cha, Y., Kim, S.H., Han, N.S., Joo, S.S., Choi, J.K., Lee, Y.B., Choi, E.K., Kim, J.B., Kim, Y.B., 2016: Antimicrobial activities of ethanol and butanol fractions of white rose petal extract. Regul. Toxicol. Pharm. 76, 57-62. DOI: 10.1016/j.yrtph.2016.01.011

Raffaella, C., Casettari, L., Fagioli, L., Cespi, M., Bonacucina, G., Baffone, W., 2017: Activity of essential oil-based microemulsions against *Staphylococcus aureus* biofilms developed on stainless steel surface in different culture media and growth conditions. Int. J. Food Microbiol. 241, 132-140. DOI: 10.1016/j.ijfoodmicro.2016.10.021

Sahoo, P., 2009: Wear behaviour of electroless Ni-P coatings and optimization of process parameters using Taguchi method. Mater. Design 30, 1341-1349. DOI: 10.1016/j.matdes.2008.06.031

Smânia, A., Jr., Monache, F.D., Smânia, E.D.F.A., Cuneo, R.S., 1999: Antibacterial activity of steroidal compounds isolated from *Ganoderma applanatum* (Pers.) Pat. (Aphyllophoromycetideae) fruit body. Int. J. Med. Mushrooms 1, 325-330. DOI: 10.1615/IntJMedMushr.v1.i4.40

Sun, M., Zhou, Z., Dong, J., Zhang, J., Xia, Y., Shu, R., 2016: Antibacterial and antibiofilm activities of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) against periodontopathic bacteria. Microb. Pathogenesis 99, 196-203. DOI: 10.1016/j.micpath.2016.08.025

Talibi, I., Amkraz, N., Askarne, L., Msanda, F., Saadi, B., Boudyach, E.H., Boubaker, H., Bouizgarne, B., Ben Aoumar, A.A., 2011: Antibacterial activity of moroccan plants extracts against *Clavibacter michiganensis* subsp. *michiganensis,* the causal agent of tomatoes' bacterial canker. J. Med. Plants Res. 5, 4332-4338.

Tancos, M.A., Chalupowicz, L., Barash, I., Manulis-Sasson, S., Smart, C.D., 2013: Tomato fruit and seed colonization by *Clavibacter michiganensis* subsp. *michiganensis* through external and internal routes. Appl. Environ. Microbiol. 79, 6948-6957. DOI: 10.1128/AEM.02495-13

Turgis, M., Han, J., Caillet, S., Lacroix, M., 2009: Antimicrobial activity of mustard essential oil against *Escherichia coli* O157:H7 and *Salmonella typhi*. Food Control 20, 1073-1079. DOI: 10.1016/j.foodcont.2009.02.001

Wang, K.R., Dang, W., Xie, J.Q., Zhu, R.R., Sun, M.Y., Jia, F.J., Zhao, Y.Y., An, X.P., Qiu, S., Li, X.Y., Ma, Z.L., Yan, W.J., Wang, R., 2015: Antimicrobial peptide protonectin disturbs the membrane integrity and induces ROS production in yeast cells. BBA-Biomembranes 1848, 2365-2373. DOI: 10.1016/j.bbamem.2015.07.008

Wang, L., Qiu, P., Long, X.F., Zhang, S., Zeng, Z.G., Tian, Y.Q., 2016: Comparative analysis of chemical constituents, antimicrobial and antioxidant activities of ethylacetate extracts of *Polygonum cuspidatum* and its endophytic actinomycete, *Streptomyces* sp. A0916. Chin. J. Nat. Med. 14, 117-123. DOI: 10.1016/S1875-5364(16)60004-3

Xu, Y., Wang, Y., Yan, L., Liang, R.M., Dai, B.D., Tang, R.J., Gao, P.H., Jiang, Y.Y., 2009: Proteomic analysis reveals a synergistic mechanism of fluconazole and berberine against fluconazole-resistant *Candida albicans*: endogenous ROS augmentation. J. Proteome Res. 8, 5296-5304. DOI: 10.1021/pr9005074

Ye, R., Xu, H., Wan, C., Peng, S., Wang, L., Xu, H., Aguilar, Z.P., Xiong, Y., Zeng, Z., Wei, H., 2013: Antibacterial activity and mechanism of action of ε-poly-L-lysine. Biochem. Bioph. Res. Co. 439, 148-153. DOI: 10.1016/j.bbrc.2013.08.001

Yogev, A., Raviv, M., Kritzman, G., Hadar, Y., Cohen, R., Kirshner, B., Katan, J., 2009: Suppression of bacterial canker of tomato by composts. Crop Prot. 28, 97-103. DOI: 10.1016/j.cropro.2008.09.003

Address of the corresponding author:
Mengliang Wang, Institute of Applied Chemistry, Shanxi University, Taiyuan 030006, China
E-mail: mlwang1212@163.com; caijin6323@126.com