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MICROBIOLOGY

Antibacterial metabolites from the beetle-associated fungus *Penicillium chrysogenum*

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Abstract: The antibacterial secondary metabolites of the fungus *Penicillium chrysogenum* associated with the beetle *Aspongopus chinensis* were investigated through chromatographic fractionation methods of ethyl acetate extracts of the fungal cultures. Five compounds were isolated, and their structures were determined as emodin, 4-(methoxymethyl)benzoic acid, isoochracinic acid, secalonic acid D, and dicerandrol A using mass spectroscopy and nuclear magnetic resonance spectroscopic analyses. Emodin exhibited strong antimicrobial activity, especially against *Staphylococcus aureus* even when growing on cooked pork, with a minimal inhibitory concentration (MIC) of 6.3 µg/mL. Dimeric tetrahydroxanthones, such as secalonic acid D and dicerandrol A, also exhibited potent activity, with MIC values ranging from 9.5 to 28.5 µg/mL. In summary, *P. chrysogenum* was isolated as a symbiotic fungus of the beetle *A. chinensis* for the first time and this strain could generate antibacterial secondary metabolites, which could potently inhibit gram-positive bacteria growth *in vitro*.

Key words: insect-associated fungus, *Penicillium chrysogenum*, antibacterial activity, *Staphylococcus aureus*.

INTRODUCTION

Bacteria, particularly drug-resistant bacteria, are considered as a huge obstacle to human health, and can cause serious respiratory, gastrointestinal tract, and bloodstream infections (Poirel et al. 2010, Robert & Moellering 2010). Among the drug resistant bacteria, *Staphylococcus aureus* is a well-known species, and many drug-resistant strains have been reported, such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) (Magiorakos et al. 2012, Senok et al. 2020). *S. aureus* also leads to food spoilage and poisoning, causing health risks for consumers and economic losses for the food industry (Ifesan et al. 2009). Since the discovery of penicillin, fungal secondary metabolites have become one of the hot spots for antibacterial drug development. Many fungal metabolites with diverse chemical structures that could inhibit the growth of human pathogenic microbes have been reported, such as anthraquinones (Qiu et al. 2021), dimeric tetrahydroxanthones (Li et al. 2016), and alkaloids (Li et al. 2021, Pinheiro et al. 2013).

In our ongoing search for potential antibacterial fungal metabolites, the fungus *Penicillium chrysogenum* that is present in the gut of *Aspongopus chinensis* attracted our attention, since its ethyl acetate (EtOAc) extract exhibited potent antimicrobial activity against *S. aureus* [MIC = 170.5 µg/mL]. Using a combination of chromatographic fractionation methods, five metabolites (Figure 1) were isolated and identified as emodin (1) (Liu et al. 2006), 4-(methoxymethyl)benzoic acid (2) (Strazzolini & Runcio 2003), isoochracinic acid

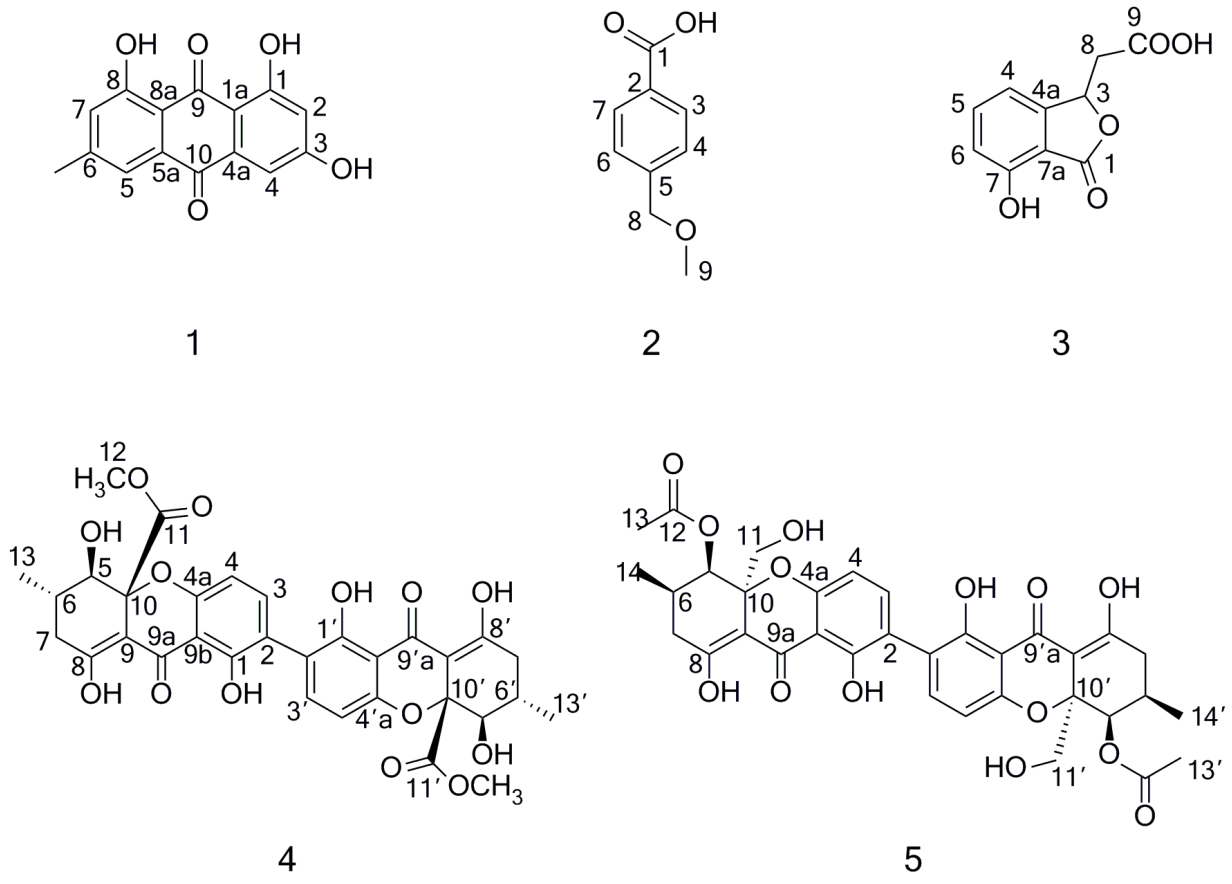

Figure 1. Structures of compounds 1-5.

(3) (Trost et al. 1980), secalonic acid D (4) (Steyn 1970), and dicerandrol A (5) (Wagenaar & Clardy 2001). Compounds 1, 4, and 5 showed strong antibacterial effects against four strains of gram-positive bacteria, with MIC values ranging from 6.3 to 65.8 μg/mL. In addition, emodin (1) also significantly inhibited *S. aureus* growth on cooked pork. This study mainly describes the fungal fermentation, compound isolation, bioactive studies, and elucidation of the structures of these metabolites.

MATERIALS AND METHODS

General experimental procedures

NMR data were measured on Bruker AVIII-500 and AVIII-600 NMR instrument with tetramethyl silane (TMS) as the internal standard. ESIMS was determined on a LTQ XL mass equipment (Thermofisher Technologies, USA). For the compound purification, silica gel (Qingdao Hailang Chemical Co. Ltd., China) and Sephadex LH-20 (Pharmacia, Sweden) column chromatographies (CCs) were used, which was further carried out through semi-preparative HPLC (Waters 1525-2998 instrument, Waters, USA; YMC-ODS-A, 250 × 10 mm, 5 μm, YMC, Japan). The optical density (OD) values were measured on an Infinite-F50 microplate reader (Tecan, Switzerland).

Fungal material

Beetles (*A. chinensis*) were caught at Tiande Lake Park (Zhengzhou, Henan, People's Republic of China) at the end of August 2017. After surface sterilization by 1.5% NaClO and 75.0% ethanol

solution, the guts of the beetles were taken out and homogenized with sterile saline to generate a tissue fluid. Then the tissue fluid was diluted with saline and spread on potato dextrose agar (PDA, AOBOX Biotechnology Co. Ltd., China). After approximately 4 days, the title strain was isolated. The fungus was identified by its morphological and microscopic characteristics as well as internal transcribed spacer (ITS) and 18S rDNA sequence analyses.

After activation on PDA for a week at 28°C, the spores of *P. chrysogenum* were collected by washing with sterile water. Potato dextrose liquid medium was used as seed and fermentation cultures. Six Erlenmeyer flasks (500 mL, containing 200 mL of liquid medium) were prepared and 1 mL of spore suspension was pipetted into each flask. The seed cultures were incubated at 28°C and 120 rpm for a week. Twenty liters of liquid fermentation were prepared in Erlenmeyer flasks (1000 mL), inoculated with 10% seed cultures, and cultivated under the abovementioned conditions for two weeks.

Extraction and isolation

After liquid fermentation, the fungal cultures were filtered, and the supernatant was treated with EtOAc thrice. The mycelium was extracted with EtOAc with ultrasonication twice, and the crude extracts of the two parts were combined (4.9 g). Interestingly, the crude extract showed antibacterial activity against *S. aureus* ATCC 25923 (MIC = 170.5 μg/mL). Preliminary silica gel CC was carried out, using CH_2Cl_2–MeOH as the eluent (from 20:1 to 1:1). The eluent was combined to obtain fractions A–E. Fraction B (0.8 g) were further purified by Sephadex LH-20 CC and semi-preparative HPLC (70% MeOH + 0.1% HCOOH) to obtain compounds 1 (82.0 mg, t_R 18.0 min) and 2 (12.3 mg, t_R 26.2 min). Compounds 4 (360.8 mg, t_R 32.7 min) and 5 (25.1 mg, t_R 22.9 min) were isolated from fraction C in the same manner by semi-preparative HPLC (65% MeOH + 0.1% HCOOH). And compound 3 (54.5 mg, t_R 23.6 min, 25% MeOH + 0.1% HCOOH) was obtained from fraction D.

Antimicrobial assays

The bacteria *Pseudomonas aeruginosa* ATCC 27853 (gram-negative), *Escherichia coli* ATCC 25922 (gram-negative), *Bacillus altitudinis* (gram-positive), *S. aureus* ATCC 25923 (gram-positive), *B. licheniformis* (gram-positive), *B. subtilis* ATCC 6633 (gram-positive) were used for the antibacterial assays on the basis of broth microdilution method (Li et al. 2016). In brief, the bacteria were cultivated at 35°C and 120 rpm in Mueller-Hinton (MH) liquid broth for around 5-7 h and diluted to the final concentration of 1.0×10^4–1.0×10^5 CFU/mL. Subsequently, positive controls (penicillin G and streptomycin) were prepared in 0.1–10.0 μg/mL and compound solutions were prepared in 1.0–200.0 μg/mL with the above-mentioned broth. In a bacteria-free work bench, 100 μL of compound solution and equal volume of bacterial suspensions were added into the 96-well plates, which were further cultivated in a 37°C incubator for 24 h. OD values were recorded at 530 nm and the tests were carried out in triplicate. The compound concentrations that inhibited 50% of the bacteria growth were considered as MIC values, which were calculated by GraphPad Prism 5.

Inhibition of *S. aureus* growth on cooked pork

The antibacterial effects of compound 1 were further evaluated by determining its ability to inhibit the growth of *S. aureus* ATCC 25923 on cooked pork (Ifesan et al. 2009). Lean pork was cut into small pieces (1.5 g) and sterilized at 121°C for 20 min to obtain cooked pork (approximately 1.0 g per patch). *S. aureus* was cultivated in MH for 4–5 h and diluted to a final concentration of 1.0×10^4 CFU/mL. Emodin (1) was prepared with

MH at concentrations of 1×MIC (6.3 μg/mL) and 2×MIC (12.6 μg/mL). The cooked pork was first soaked into solutions containing the compound for 10 s, followed by an incubation in a sterile culture dish for 30 min. Afterward, the pork was inoculated with *S. aureus* suspensions and incubated in a sterile culture dish at 4°C. The numbers of bacteria were examined at 0, 3, 6, and 9 d. The control groups were treated with sterilized water in the same manner, and the tests were performed in triplicate.

Statistical analysis

The above tests were carried out in triplicate. All the data were handled with ANOVA (one-way analysis of variance) using GraphPad Prism 5. ± SD values were determined according to three individual measurements.

RESULTS

Fungal identification

The fungus *P. chrysogenum* was cultivated on PDA for 5 days, generating a blue-green felt colony with an orange-yellow color on the back (Figure 2). Under the microscope (10 × 40), the broom-shaped stipes were clearly observed with several linear round spores on top (Figure 2). This strain was further confirmed as *P. chrysogenum* by its 18S rDNA sequences and ITS analysis (100% similar to the *P. chrysogenum* isolate No. JF777507.1).

Structure elucidation

Emodin (1): orange solid; ESIMS m/z 268.9 $[M-H]^-$, $C_{15}H_{10}O_5$; ^{1}H NMR (DMSO-d_6, 500 MHz) δ_H 12.08 (1H, s, OH-1), 12.01 (1H, s, OH-8), 11.38 (1H, br s, OH-6), 7.50 (1H, s, H-4), 7.17 (1H, s, H-2), 7.12 (1H, d, J = 2.0 Hz, H-5), 6.59 (1H, d, J = 2.2 Hz, H-7), 2.41 (3H, s, CH_3-11); ^{13}C NMR (DMSO-d_6, 125 MHz) δ_C 189.7 (C-9), 181.4 (C-10), 165.5 (C-3), 164.4 (C-1), 161.4 (C-8), 148.2 (C-6), 135.1 (C-4a), 132.8 (C-5a), 124.1 (C-7), 120.4 (C-5), 113.4 (C-8a), 108.9 (C-1a), 108.7 (C-4), 107.9 (C-2), 21.4 (C-11).

4-(Methoxymethyl)benzoic acid (2): colorless oil; ESIMS m/z 164.9 $[M-H]^-$, $C_9H_{10}O_3$; ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H 7.12 (2H, d, J = 8.5 Hz, H-3 and H-7), 6.75 (2H, d, J = 8.5 Hz, H-4 and H-6), 3.70 (3H, s, OCH_3-9), 3.56 (2H, s, H-8); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 172.9 (C-1), 154.9 (C-5), 130.4 (C-3), 130.4 (C-7), 125.9 (C-2), 115.5 (C-4), 115.5 (C-6), 52.1 (C-8), 40.3 (C-9).

Isoochracinic acid (3): colorless oil; ESI-MS m/z 209.1 $[M+H]^+$, $C_{10}H_8O_5$; ^{1}H-NMR (Acetone-d_6,

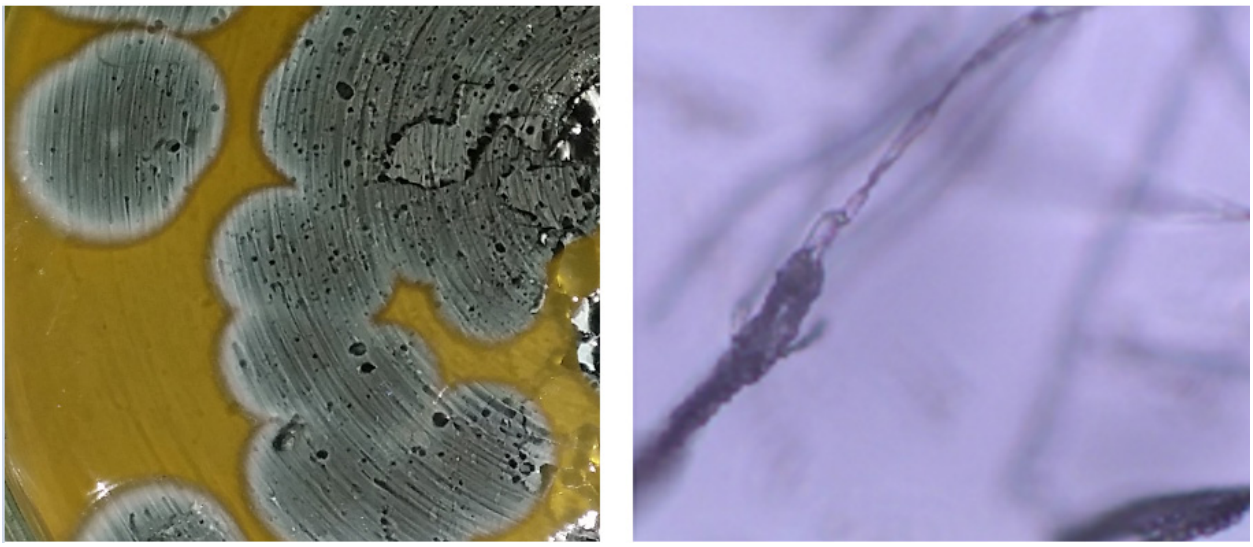

Figure 2. Morphological and microscopic (10 × 40) characteristics of *P. chrysogenum*.

500 MHz) δ_H 7.60 (1H, t, *J* = 7.8 Hz, H-5), 7.14 (1H, d, *J* = 7.5 Hz, H-4), 6.95 (1H, d, *J* = 8.1 Hz, H-6), 5.87 (1H, dd, *J* = 7.5, 5.0 Hz, H-3), 3.11 (1H, dd, *J* = 16.8, 4.8 Hz, H-8α), 2.87 (1H, dd, *J* = 16.8, 7.8 Hz, H-8β); ^{13}C-NMR (Acetone-d_6, 125 MHz) δ_C 171.2 (C-9), 170.9 (C-1), 157.6 (C-7), 151.8 (C-4a), 137.8 (C-5), 116.9 (C-6), 114.7 (C-4), 112.7 (C-7a), 78.8 (C-3), 39.8 (C-9).

Secalonic acid D (4): yellow powder; ESI-MS *m/z* 639.1 [M+H]$^+$, $C_{32}H_{30}O_{14}$; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H 13.78 (1H, s, 8-OH and 8'-OH), 11.74 (1H, s, 1-OH and 1'-OH), 7.46 (1H, d, *J* = 8.5 Hz, H-3 and H-3'), 6.63 (1H, d, *J* = 8.5 Hz, H-4 and H-4'), 3.93 (1H, d, *J* = 11.0 Hz, H-5 and H-5'), 3.73 (3H, s, OCH_3-12 and OCH_3-12'), 2.74 (1H, dd, *J* = 19.1, 6.2 Hz, H-7a and H-7'a), 2.42 (1H, m, H-6 and H-6'), 2.32 (1H, dd, *J* = 19.1, 10.6 Hz, H-7b and H-7'b), 1.18 (3H, d, *J* = 6.4 Hz, CH_3-13 and CH_3-13'); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ_C 187.2 (C-9a and C-9'a), 177.6 (C-8 and C-8'), 170.3 (C-11 and C-11'), 159.4 (C-1 and C-1'), 158.3 (C-4a and C-4'a), 140.2 (C-3 and C-3'), 115.3 (C-2 and C-2'), 107.6 (C-4 and C-4'), 106.9 (C-9b and C-9'b), 101.6 (C-9 and C-9'), 84.8 (C-10 and C-10'), 77.0 (C-5 and C-5'), 53.2 (C-12 and C-12'), 36.3 (C-7 and C-7'), 29.3 (C-6 and C-6'), 18.0 (C-13 and C-13').

Dicerandrol A (5): yellow powder; ESIMS *m/z* 667.4 [M+H]$^+$, $C_{34}H_{34}O_{14}$; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H 13.98 (1H, s, 8-OH and 8'-OH), 11.91 (1H, s, 1-OH and 1'-OH), 7.40 (1H, d, *J* = 8.2 Hz, H-3 and H-3'), 6.51 (1H, d, *J* = 8.2 Hz, H-4 and H-4'), 5.75 (1H, s, H-5 and H-5'), 4.10 (1H, d, *J* = 13.1 Hz, H-11a and H-11'a), 3.54 (1H, d, *J* = 12.9 Hz, H-11b and H-11'b), 2.47 (1H, m, CH_3-13 and CH_3-13'), 2.42 (1H, m, H-6 and H-6'), 2.39 (1H, m, H-7b and H-7'b), 2.10 (3H, s, H-5 and H-5'), 1.08 (3H, d, *J* = 5.3 Hz, CH_3-14 and CH_3-14'); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ_C 187.2 (C-9a and C-9'a), 178.0 (C-8 and C-8'), 170.6 (C-12 and C-12'), 159.5 (C-1 and C-1'), 157.2 (C-4a and C-4'a), 140.1 (C-3 and C-3'), 118.0 (C-2 and C-2'), 107.9 (C-4 and C-4'), 106.5 (C-9b and C-9'b), 100.9 (C-9 and C-9'), 82.5 (C-10 and C-10'), 70.3 (C-5 and C-5'), 65.6 (C-11 and C-11'), 33.4 (C-7 and C-7'), 27.7 (C-6 and C-6'), 20.9 (C-13 and C-13'), 17.6 (C-14 and C-14').

Antibacterial activity

As shown in Table I and Figure 3, compound 1 exhibited strong antibacterial activity against all four gram-positive bacteria with the best effect against *S. aureus* (MIC = 6.3 μg/mL). Dimeric tetrahydroxanthones (4 and 5) also showed strong activity (MIC = 9.5 to 28.5 μg/mL). However, they were all inactive against gram-negative bacteria.

Table I. The MIC values of compounds 1-5 (μg/mL)[a].

Compounds	Sa	Bl	Ba	Bs	Pa	Ec
1	6.3 ± 0.5	18.3 ± 1.4	27.9 ± 1.8	65.8 ± 3.9	> 100.0	> 100.0
2	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0
3	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0
4	9.5 ± 0.8	16.9 ± 1.0	24.8 ± 1.7	15.7 ± 1.4	> 100.0	> 100.0
5	12.4 ± 1.3	18.6 ± 1.9	28.5 ± 2.4	22.5 ± 2.6	> 100.0	> 100.0
Penicillin G[b]	0.4 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1		
Streptomycin[b]					1.0 ± 0.2	0.4 ± 0.1

[a]Compounds were considered as inactive when MICs > 100.0 μg/mL, ± SD values were calculated based on three individual experiments, Sa (*S. aureus* ATCC 25923), Bl (*B. licheniformis*), Ba (*B. altitudinis*), Bs (*B. subtilis* ATCC 6633), Ec (*E. coli* ATCC 25922), Pa (*P. aeruginosa* ATCC 27853). [b]Positive control.

Antibacterial effects on *S. aureus* inoculated in cooked pork

The antibacterial effects of emodin (1) on *S. aureus* growth in cooked pork were subsequently tested. At both concentrations (1×MIC and 2×MIC), *S. aureus* growth was noticeably reduced (Figure 4). During the early stage (3 d), 6.3 μg/mL (1×MIC) of emodin inhibited *S. aureus* growth, which was similar to the potency of 2×MIC. For longer periods of time, the concentration of 2×MIC showed better effects.

DISCUSSION

Insect-associated fungi are valuable and special microbial resources, and they can provide nutrition and antibiotics for the host insect (Baumann 2005, Li et al. 2016). The insect *A. chinensis* has been used as a kind of traditional Chinese medicine to treat gastritis and back pain (Yan et al. 2014). However, few symbiotic fungi have been isolated from this insect, and the strain *P. chrysogenum* was reported for the first time by our laboratory (Li et al. 2021).

A combination of semi-preparative HPLC and CC methods was used for the purification of antibacterial secondary metabolites produced by this fungus, and their structures were confirmed by analyses of the ^{1}H NMR, ^{13}C NMR, and MS data. Interestingly, emodin (1) was revealed as an anthraquinone derivative, and compounds 4 and 5 were tetrahydroxanthone dimers. Emodin, a valuable medicinal and chemical ingredient, is metabolized by many fungi, such as *Aspergillus* sp., *A. favipes*, *Hamigera avellanea*, and *Penicillium* sp. (Isaka et al. 2008, Liu et al. 2006, 2013, Qian et al. 2011, Qiu et al. 2021). The isolation of emodin from *P. chrysogenum* could expand its fungal sources, and the high yield rate (4.1 mg/L) suggested the promising application of this strain.

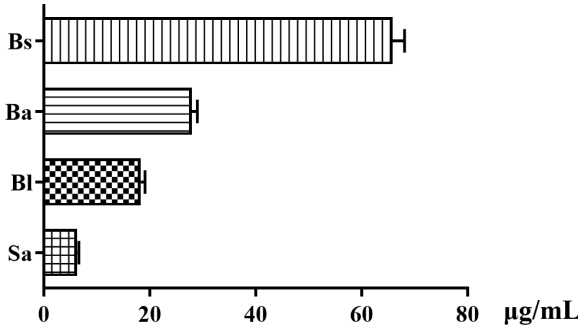

Figure 3. Antibacterial effects of emodin (1). ± SD values were calculated based on three individual experiments. Sa (*S. aureus* ATCC 25923), Bl (*B. licheniformis*), Ba (*B. altitudinis*), Bs (*B. subtilis* ATCC 6633).

Emodin has been known to possess antibacterial activities (Liu et al. 2006, Molee et al. 2018), which was also proven in this study because it inhibited the growth of four strains of gram-positive bacteria, especially *S. aureus* (Table I). Dimeric tetrahydroxanthones also showed potent activities (Ola et al. 2014). Interestingly, these metabolites showed selective antibacterial activities, since they were all inactive against gram-negative bacteria. The olefinic acid group might be one of the key active sites for antibacterial effects when investigating the structure-function relationship of these compounds (Chopra & Roberts 2001, Li et al. 2016, Ola et al. 2014). Moreover, emodin also potently inhibited *S. aureus* growth on pork, suggesting that it could be further developed as a food preservative.

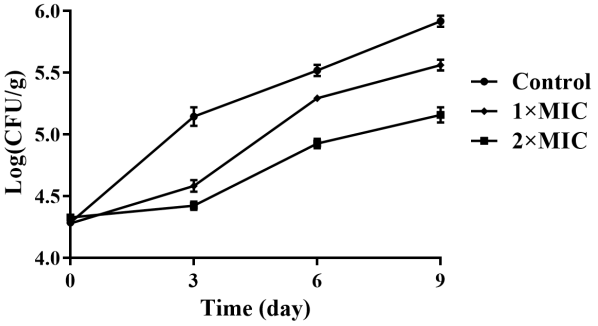

Figure 4. Antibacterial effects of emodin (1) on *S. aureus* inoculated in cooked pork.

Acknowledgments

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