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Antibacterial Activities and Mechanism of Action of Acetone Extracts from *Rabdosia rubescens*

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Abstract - The antibacterial activities and mechanism of action of acetone extracts from *R. rubescens* were reported in this paper. The results showed that 80% acetone extracts had both the highest contents of total phenolics and flavonoids. Acetone extracts showed better antibacterial activities against Gram-positive bacterial strains and there were no inhibitory effects found on tested Gram-negative bacteria. In addition, 80% acetone extracts from *R. rubescens* had relatively higher antibacterial activities with the lowest values of MIC and MBC at 2.5 mg/mL and 5 mg/mL against *B. subtilis*. The antibacterial mechanism of 80% acetone extracts against *Bacillus subtilis* might be described as disrupting cell wall, increasing cell membrane permeability, and finally leading to the leakage of cell constituents.
Keywords: *Rabdosia rubescens*; Extracts; Antibacterial activity; Mechanism of action

Introduction

Food poisoning and spoilage caused by microorganisms are the most important issues facing the food industry and consumers (Shan *et al.*, 2007). For many years, different chemical and synthetic compounds have been used to control microbial growth in food factories. However, much attention has been paid to use natural antibacterial products for food preservation because of the safety of synthetic chemicals. Plants can be an excellent source of natural antibacterial agents and can be effectively used in the food industry to improve the shelf-life of food products (Tiwari *et al.*, 2009).

Rabdosia rubescens is an herb found in China. Traditionally, the whole plant of *R. rubescens* has been used as a medicine supplement by cooking in water and widely drank to treat inflammatory and pain related diseases (Lu *et al.*, 2007; Bai *et al.*, 2010). The *R. rubescens* contained abundant bioactive compounds such as diterpenoids, flavonoids, phenolic compounds (Liu *et al.*, 2000; Tang *et al.*, 2011). These compounds showed various biological activities including anti-inflammatory, antitumor actions, antibacterial and antioxidant activities (Hsieh *et al.*, 2005; Bai *et al.*, 2010; Feng and Xu, 2014). In addition, little work has been reported on the antibacterial activities of acetone extracts from *R. rubescens* as well as action mechanism on the growth of food-borne pathogens (Bai *et al.*, 2010; Feng and Xu, 2014). Therefore, the objective of the present study was to investigate the antibacterial activities of acetone extracts from *R. rubescens* against several food-borne pathogens, and to further evaluate the possible action mechanism responsible for the antibacterial activity against sensitive strains.

Materials and Methods

Microbial strains and preparation of extracts

The dried herb of *R. rubescens* was collected in Jiyuan City, Henan Province of China in the Fall 2012. The antimicrobial activity was tested against seven different microorganisms. Three Gram-positive bacterial strains were *Staphylococcus aureus* ATCC 25923, *Staphylococcus albus* ATCC 8799, *Bacillus subtilis* ATCC 6051. Four Gram-negative bacteria were *Salmonella typhimurium* ATCC 19430, *Pseudomonas aeruginosa* ATCC 9027, *Shigella dysenteriae* CMCC (B) 51252, and *Escherichia coli*

ATCC 25922. The strains were provided by the College of Life Science, Shanxi Normal University, and cultured at 37 °C on NA or NB mediums.

The dried *R. rubescens* are finely ground and blended with different concentrations of acetone (40%-100%) at 150 rpm for 8 h at 25 °C, and then the homogenates were centrifuged for 15 min at 4 °C and 5000 g. The supernatants are pooled, and vacuum-evaporated to dryness at 40 °C.

Determination of total phenolics and flavonoid content

Total phenolic content was determined using the Folin-Ciocalteu colorimetric method as described by Rebey *et al.* (2012). Gallic acid was used for calibration of the standard curve and total phenolic content was expressed as milligram gallic acid equivalent per gram dried weight (mg GAE/g DW). The level of flavonoid was measured as described by Liu *et al.* (2008). Rutin was used for calibration of the standard curve and the content of flavonoids was expressed as milligram rutin equivalent per gram dried weight (mg RE/g DW).

Agar disc diffusion assay

The antibacterial activity was determined as described by Rammanee and Hongpattarakere (2011) with some modifications. Briefly, a suspension (0.1 mL of 10^7 CFU/mL) of each bacterium was spread on the solid medium plates. Whatman no.1 sterile filter paper discs (6 mm diameter) were impregnated with the extracts and then placed on the inoculated plates, and these plates were incubated at 37 °C for 24 h. The antibacterial activity was evaluated by measuring the diameter of zones of inhibition (ZOI).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

Two fold serial dilutions of samples were prepared in sterile NB medium. To each tube 100 μL of the exponentially growing bacterial cells was added to give a cell concentration of approximately 1×10^7 CFU/mL. The tubes were incubated at 37 °C for 24 h and then examined for evidence of the growth. The MIC and MBC were determined according to the method described by Diao *et al.* (2013).

Cell membrane permeability

The permeability of bacteria membrane is determined according to the method described by Diao *et al.* (2014). After incubated at 37 °C for 10 h, *B. subtilis* strains were separated by centrifugation at 5000 rpm for 10min. Then the bacteria were washed with 5% of glucose until their electric conductivities were near to that of 5% glucose, and they were the case for isotonic bacteria. The essential oils at two different concentrations (MIC, and 2×MIC) were added to 5% glucose and the electric conductivities of the mixtures were marked as L_1. Then different concentrations of essential oils were added into the isotonic bacteria solution. After completely mixed, the samples were incubated at 37 °C for 8 h, and then the conductivities were measured and marked as L_2. The conductivity of bacteria in 5% glucose treated in boiling water for 5 min was served as the control and marked as L_0. The permeability of bacteria membrane is calculated according to the formula, the relative electric conductivity (%) $=100\times (L_2 - L_1)/L_0$.

Integrity of cell membrane

The cell integrity of tested bacteria is examined by determining the release of cell constituents into supernatant according to the method described by Du *et al.* (2012) with slight modifications. Cells from the 100 mL working culture of tested microorganisms were collected by centrifuged for 15 min at 5000 rpm, washed three times, and resuspended in 0.1 M phosphate buffer solution (PBS, pH 7.4). One hundred milliliters of cell suspension were incubated at 37 °C under agitation for 4 h in the presence of extracts at three different concentrations (control, MIC and 2×MIC). Then, 25 mL of samples were collected and centrifuged at 11,000 g for 5 min. And then the concentrations of proteins and reducing sugars in supernatant were determined according to the method described by Xu *et al.* (2010). In addition, to determine the concentration of the constituents released, 3 mL supernatant was used to measure UV absorption at 260 nm.

Scanning electron microscope (SEM)

The bacteria cells were incubated in nutrient broth at 37 °C for 10 h. The suspensions were added 0×, 1×MIC of extracts, respectively; control culture was left untreated. Next the suspensions were incubated at 37 °C for 4 h respectively, and then the suspensions were centrifuged. The precipitated cells were washed twice with 0.1 M PBS (pH 7.4) and fixed with 2.5% (v/v) glutaraldehyde in 0.1 M PBS overnight at 4°C. After this, the cells were dehydrated using sequential exposure per ethanol concentrations ranging from 30-100% and the ethanol was then replaced by tertiary butyl alcohol. The cells after centrifugation were dried at "critical point" in liquid CO_2 under pressure at 95 bar, and samples were gold-covered by cathodic spraying. Finally, morphology of the bacterial cells was observed on a scanning electron microscope.

Results and Discussion

Contents of total phenolics and flavonoids

The levels of total phenolics and flavonoids in different extracts from *R. rubescens* were shown in Table 1. Results showed that the contents of total phenolics and flavonoids increased significantly with the increase of acetone concentrations between 40% ~ 80% (V/V), and total phenolics and flavonoids increased from 2.52 to 8.16 mg GAE /g DW and from 2.03 to 5.74 mg RE/g DW respectively. However, total phenolics and flavonoids in 100% acetone extracts only had 1.20 mg GAE /g DW and 1.13 mg RE/g DW. These results indicated that acetone concentrations had great influences on total phenolics and flavonoids content in extracts from *R. rubescens*.

Table 1. The contents of total phenolics, flavonoids of extracts from *R. rubescens*. Values are represented as mean ± standard deviation of triplicates. Different letters within a column indicate significant differences ($p < 0.05$)

Extracts	Total phenolics (mg GAE/g DW)	Total flavonoid (mg RE/g DW)
40% Acetone	2.52±0.13 c	2.03±0.16 c
60% Acetone	5.34±0.14 b	3.45±0.21 b
80% Acetone	8.16±0.17 a	5.74±0.11 a
100%Acetone	1.20±0.09 d	1.13±0.12 d

ZOI, MIC and MBC of 80% acetone extracts

The DIZ, MIC, and MBC values of 80% acetone extracts from *R. rubescens* are presented in Table 2. The results showed that 80% acetone extracts had a satisfactory antibacterial activity on tested Gram-positive bacteria, while no inhibitory effect on tested Gram-negative bacteria was found. The DIZ values for all tested Gram-positive bacterial strains were in the range of 22.1-28.2 mm. The DIZ was the maximum for *B. subtilis*, followed by *S. aureus* and *S. albus*. Some studies reported that the Gram-positive bacteria were more sensitive than the Gram-negative ones to some plant extracts (Diao *et al.*, 2013; Adámez *et al.*, 2012). To some extent, these reports were basically consistent with the present study, which was likely due to the structural differences in the outer layers of Gram-negative and Gram-positive bacteria (Diao *et al.*, 2013).

The MIC and MBC values for Gram-positive bacterial strains were in the range of 2.5-5 mg/mL and 5-10 mg/mL respectively (Table 2). The 80% acetone extracts had the lowest MIC of 2.5 mg/mL and MBC of 5 mg/mL against *B. subtilis*, which indicated it was the most effective bacterial inhibitor and bactericide against *B. subtilis*. Therefore, the antibacterial properties and action mechanism of 80% acetone extracts from *R. rubescens* against *B. subtilis* will be further investigated in this study.

Table 2. DIZ, MIC, and MBC of 80% acetone extracts. Values are represented as mean ± standard deviation of triplicates. Different small letters within a column indicate significant differences ($p < 0.05$).

Microorganisms	DIZ (mm)	MIC(mg/mL)	MBC(mg/mL)
Gram-positive			
S. aureus	23.9±1.1 b	5	10
S. albus	22.1±0.9 b	5	10
B. subtilis	28.2±1.3 a	2.5	5
Gram-negative			
S. typhimurium	NI	NT	NT
P. aeruginosa	NI	NT	NT
S. dysenteriae	NI	NT	NT
E. coli	NI	NT	NT

NI= No inhibition; NT= Not tested.

The integrity of cell membrane

The integrity of cell membrane were determined by the measurement of the release of cell constituents including protein, reducing sugar and the absorbance at 260 nm of the supernatant on tested bacteria. Table 3 showed the results when *B. subtilis* were treated with different concentrations of 80% acetone extracts from *R. rubescens* for 4 h. The results indicated that after adding the corresponding extracts to strains, the cell constituents' release increased significantly with the increased concentration of the extracts. Compared to control, the concentration of proteins, reducing sugars and cell constituents (OD_{260nm}) in suspensions treated with 1×MIC extracts increased by 8.06, 1.99, 9.18 times respectively, while they increased by 16.67, 4.28, 14.73 times respectively when treatment at 2×MIC. These results were agreement with previous reports studied on other plants (Diao *et al.*, 2013; Diao *et al.*, 2014), and indicated that the irreversible damage to the cytoplasmic membranes might occur, which could lead to the losses of cell constituents such as protein and some essential molecules and to cell death.

Table 3. The effect of the extracts on cell constituents' release of tested *B. subtilis*. Values represent means of three independent replicates ± SD. Different letters within a column indicate statistically significant differences between the means ($p < 0.05$).

Concentrations	Cell constituents' release		
	Protein (μg/mL)	Reducing sugar (μg/mL)	Cell constituents (OD_{260nm})
Control	8.2± 1.5 c	14.3±1.2 c	0.022±0.009 c
1×MIC	74.3±10.4 b	42.8±6.5 b	0.224±0.013 b
2×MIC	144.8±12.5 a	75.5±9.1 a	0.346±0.028 a

Cell membrane permeability

Figure 1 showed the effect of 80% acetone extracts from *R. rubescens* on the membrane permeability of *B. subtilis*. There was little change in the relative electric conductivity of the control during the first 13 h period of the test, and then an increase in the relative electric conductivity was found, which may be due to normal lysis and death of bacteria, resulting in the increase in the relative electric conductivity. Compared to the control, the relative electric conductivity of the suspensions increased immediately after the addition of extracts at greater than or equal to MIC concentration and it also increased rapidly with the increasing treatment time and concentration of extracts. The results meant that the permeability of bacteria membrane would be increased correspondingly, which caused the leakage of intracellular ingredient, especially the loss of

electrolytes including K^+, Ca^{2+}, Na^+. Similar to our findings, some authors suggested that the distortion of the cell wall and cytoplasmic membrane would cause the expansion and destabilization of the membrane and increase membrane permeability, resulting in a leakage of various vital intracellular constituents, which lead to cell death (Denyer, 1990 ; Diao *et al.*, 2013).

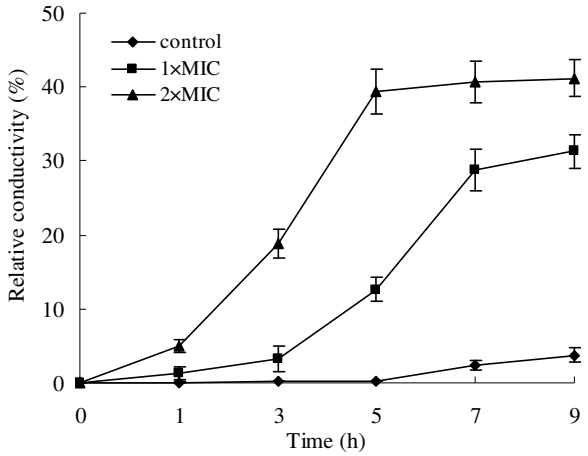

Figure 1. Effect of the extracts on the impermeability of cell membrane of tested *B. subtilis*

Scanning electron microscope (SEM) observation

Figure 2 showed the SEM images of the treated and untreated bacteria. These images directly illustrated the destructive effects of the extracts on the tested bacteria. The surfaces of the treated strains underwent obvious morphological changes compared with the untreated controls. Untreated cells were rod shaped, regular, and intact (Figure 2a), while some bacterial cells treated with the extracts became deformed, pitted, shriveled and parts of the cell were broken (Figure 2b), which may give rise to the leaching out of nutrient and genetic materials. This supported the results of the permeability and integrity of cell membrane assays, and indicated that the 80% acetone extracts from *R. rubescens* had severe effects on the cell wall and cytoplasmic membrane, which have also been observed for various kinds of tested organisms when treated with different extracts (Du *et al.*, 2012; Diao *et al.*, 2013; Diao *et al.*, 2014).

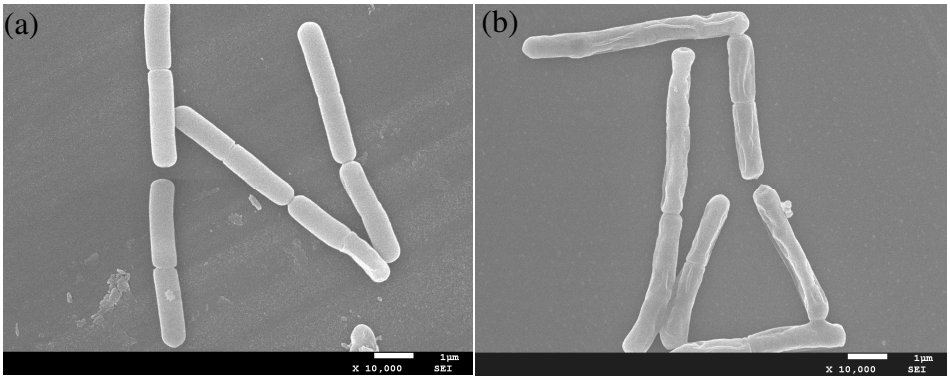

Figure 2. The photography of SEM of *B. subtilis* for **a** (untreated bacteria) and **b** (bacteria treated with the extracts at 1×MIC)

Conclusions

The concentrations of acetone significantly affected total polyphenols, flavonoids, and antibacterial activities of *R. rubescens* extracts. In our study, the 80% acetone extracts from *R. rubescens* had both the highest content of total polyphenols and flavonoids. The different concentrations of acetone extracts from *R. rubescens* exhibited better antibacterial activity against

tested Gram-positive bacteria while no inhibitory effects on all of the tested Gram-negative bacteria were found. Especially, 80% acetone had relatively higher antibacterial activities with the lowest MIC and MBC values of 2.5 mg/mL and 5 mg/mL against *B. subtilis*. The antibacterial mechanism of 80% acetone extracts against *B. subtilis* might be described as it disrupting cell wall, increasing cell membrane permeability, and then leading to the leakage of cell constituents.

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