Antibacterial activities of rhubarb extract and the Bioactive compounds against Salmonella

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Abstract
Salmonella is one of the primary causes of food borne illnesses worldwide. In this study, antibacterial properties of rhubarb against Salmonella were investigated. Initial screening showed that rhubarb root ethanol extract strongly inhibited the growth of Salmonella serotype typhimurium, and the chloroform fraction was found to be the most active fraction. Five major Anthracaquinone derivatives were identified from the chloroform fraction by UPLC-MS/MS, namely emodin, aloe-emodin, rhein, physcion and chysophanol. Of these five compounds, rhein showed the greatest antibacterial activities against S. typhimurium. Time kill curve assay suggested that rhein killed the bacteria in a relatively fast rate. Further investigations on the mechanisms revealed that rhein significantly altered the integrity of the cell membrane, resulting in the loss of barrier function and leakage of the nucleotide. The morphological changes of S. typhimurium treated with rhein were also observed by scanning electron micrographs.

Key words: Anthracaquinone, Antibacterial, Rhein, Rhubarb, Salmonella

Introduction:
Salmonella is one of the primary causes of food borne diseases worldwide. In recent years, it was responsible for several worst food borne illness outbreak in the U.S. history, affecting millions of people. The United States Center for Disease Control and Prevention (CDC) estimated that approximately 1.4 million cases/year in US with ~40,000 confirmed cases and 1,000 deaths in the US alone (http://www.cdc.gov/foodsafety/outbreaks). Salmonella bacteria are zoonotic in nature, not only do they impede the food quality severely, they are also hazardous to human society [4]. Salmonellosis is an infection caused by the Salmonella bacteria. It is characterized by diarrhea, fever and cramps, and the symptoms usually last four to seven days. Severe illness and death may occur among very young, old and immunocompromised patients [10]. Various foods have been involved in the outbreaks of salmonellosis, including meat products [15], dairy foods [10], and vegetables [11]. Large outbreaks may also associate with un-pasteurized juice or raw fruits. Half the confirmed cases were due to Salmonella serotype typhimurium and Salmonella serotype enteritidis.

One key strategy to reduce food borne illnesses is to prevent growth of spoilage and pathogenic microorganisms in foods. A number of synthetic chemical preservatives were developed for this purpose. However, with the increasing consumer awareness and concern regarding synthetic chemical additives, foods preserved with natural additives have become popular in recent years. But the studies on natural antibacterial agents, especially their mechanisms are still limited. There is a continuing interest to search for the new antibacterial compounds, especially those from medicinal/edible plants [22-23]. Several medicinal plants have been shown to possess antibacterial potentials against Salmonella [17]. Rhubarb is an edible medical plant. Its fresh stems and petioles are consumed as vegetable and its roots and stems are used for medicinal purposes. [21] Rhubarb root is one of the...
petroleum ether, chloroform, ethyl acetate, dehyde and isosolym acetate were purchased from Sinopharm Chemical Reagent Corporation (Shanghai, China). Tryptone Soy Agar (TSA), Trypticase Soy Broth (TSB) was purchased from Hangzhou Tianhe Microorganism Reagent Corporation (Zhejiang, China). Standards of emodin, aloe-emodin, rhein, chrysophanol and physcion were purchased from Chengdu Must Biotechnology Corporation (Sichuan, China).

A. Plant material, chemicals and reagents.

B. Microbial strains.

Salmonella typhimurium CMCC 50041 were purchased from Institute of Microbiology, Chinese Academy of Science (Beijing, China). The bacteria were cultured at 37°C on Tryptone Soy Agar (TSA) and Trypticase Soy Broth (TSB) mediums.

C. Extraction and fractionation of rhubarb.

The dried rhubarb were ground to coarse powder using a grinder (Jin Sui, JSP-1000A). 10g of powder was extracted thrsee times with 500 mL absolute ethanol under reflux for 4 hrs. The extract solution was separated from residue by filtration. The ethanol extract was then concentrated in a rotary evaporator under vacum to obtain the rhubarb crude extract (ECE). For fractionation, 10g of ECE was dispersed in distilled water, followed by extraction with petroleum ether (PEF), chloroform (CF), ethyl acetate (EAF) and n-butanol (BF), successively. The solvent of these four fractions was removed in a rotary evaporator under vacum to yield gel like concentrates. The concentrates were further dried under N2. All dried extracts were stored at -20°C until testing.

D. Disc diffusion assay.

The disc diffusion assay was performed according to a published method (V. K. Bajpai, Al-Reza, Choi, Lee, & Kang, 2009) with minor modifications. In brief, 50 μL of S. typhimurium was injected into 5 mL TSB and cultured under condition 37°C, 150 min, for 6 hrs in a bed temperature incubator. The inoculum was adjusted with 0.1 M PBS (pH 7.2) to 10.0 CFU/mL. 1 mL prepared suspension was streaked onto the surface of TSA with a SS-Spreader, then the inoculum on the plates was allowed to dry for 10 min in drying oven at 37°C. 6 mm diameter sterile paper discs were placed on the surface of agar culture. Afterwards, 5 μL of sample was injected onto the disc. The plates were then cultured under 37°C for 22 hrs in a temperature incubator (37°C). Finally, the diameters of inhibition zones against the tested bacteria of each paper disc were measured. DMSO was used as negative control. Tests were performed in triplicate.

E. UPLC-MS/MS Analysis.

Dried CF (1 mg) was reconstituted in 10 mL methanol to make a sample concentration of 100 μg/mL. The sample solution was sonicated in an ultrasonic bath at room temperature for 5 min, and was filtered with a 0.22 μm syringe filter for UPLC-MS/MS analysis.

UPLC was performed using a Waters ACQUITY UPLC™ system, equipped with a binary solvent delivery system, an autosampler, a thermostat column compartment and a diode array detector (DAD). A Waters UPLC BEH C18 column, 150 mm × 4.6 mm, 5 μm particle size with temperature of 40°C, was used for separation. The mobile phase consisted of 0.05% acetic acid in water (A) and acetonitrile (B) using a gradient program of 30→60% (B) in 0→4.5 min, 4.5→5.0 min, 5.0→6.0 min, 6.0→7.0 min. The flow rate was 0.4 mL/min. The detection wavelength was set at 268 nm and the UV spectrum was recorded from 190 to 400 nm.

The mass spectrometric analysis was performed in a Waters Q-TOF Micro TM mass spectrometer (Milford, MA, USA) connected to the UPLC via ESI interface. Nitrogen was used as desolvation gas and ultra-high pure helium was used as the colision gas. The optimized parameters in the negative ion mode were as follows: the rate of nitrogen (N2), 800 L/hrs; desolvation temperature, 450°C; capillary voltage, 2.5 kV; cone voltage, 35 V; cone gas flow, 50 L/hr. The full-scan MS data were recorded in the range of m/z 100→1000. A data-dependet program was used in the UPLC-MS/MS analysis, so that the protonated or deprotonated ions in MS spectra could be selected for further MS/MS analysis.

F. Minimum inhibitory and minimum bactericidal concentrations.

The minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of the five compounds identified from CF were further evaluated by measuring their minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC). The measurement was performed on a NCCS 96-well plate micro dilution broth method (NCCS, 2008) using thrsee times. The tubes were inoculated with S. typhimurium at a concentration of MIC and were cultured for 24 hrs at 37°C. The OD260 of the supernate was measured by Pharma Spec UV-3600 (Shimadzu, Kyoto, Japan) at room temperature. The controls were tested without adding rhein.

1. Scanning electron microscopic analysis.

To further confirm the effect of rhein affecting the morphology of S. typhimurium, a scanning electron microscopic (SEM) assay was performed according to the method published by Bajpai et al (Vivek K. Bajpai, et al., 2013). Logarithmic stage S. typhimurium were inoculated with rhein at 2× MICs in TSB medium for 12 hrs at 37°C with shaking. Strains incubated with TSB without rhein were used as control. Samples with different time treatment were centrifuged at 4000 rpm for 10 min and then the supernatant was collected. The OD260 of the supernate was measured by Pharma Spec UV-3600 (Shimadzu, Kyoto, Japan) at room temperature. The controls were tested similarly as mentioned above. Each assay was carried out thrree times.

2. H. Nucleotide leakage.

The experiment was implemented according to a published method (Lou, Wang, Zhu, Ma, & Wang, 2011) with minor modification. Exponential phase S. typhimurium were washed with 0.1 M PBS, then re-suspended in PBS. Bacteria were incubated with rhein at the concentration of 2× MICs, cultured with shaking at 37°C. At the time intervals of 0, 2, 4, 6 and 8 hrs, strains incubated with 0.1 M PBS without rhein were used as control. Samples with different time treatment were centrifuged at 8000 rpm for 10 min and then the supernatant was collected. The OD260 of the supernate was measured by Pharma Spec UV-3600 (Shimadzu, Kyoto, Japan) at room temperature. The controls were tested similarly as mentioned above. Each assay was carried out thrree times.
were washed with 0.1 M PBS for 3 times, then fixed with 2.5% glutaraldehyde for 6 hrs, followed by fixing with 1% osmic acid solution for 6 hrs. The samples were dehydrated for 15 mins with ethanol of different concentrations for as followed: 30%, 50%, 70%, 85%, 95% and 100%. Then the ethanol was replaced by isoamyl acetate. The samples were dried with carbon dioxide (CO2). Lastly, the samples were sputter coated with gold for 2 min, then were observed with scanning electron microscopic (S-4800; Hitachi, Hitachi City, Japan).

### Results

#### Antibacterial activity of fractions of rhubarb extract.

Antibacterial activities of the crude ethanol extract as well as the five fractions against *S. typhimurium* were measured by the disc diffusion assay. The five fractions showed different antibacterial activities with the order CF > PEF > EAF > BF = WF. CF appeared to be the most effective fractions among all fractions, with diameters of inhibition zones 15.4 ± 0.40 mm.

#### UPLC-MS/MS analysis of CF

Figure 3. UPLC chromatogram of the five major compounds identified from chloroform fraction of rhubarb crude extract (DAD at 268 nm). 1. Aloe-emodin; 2. Rhein; 3. Emodin; 4. Chrysophanol; 5. Physcion

Five major components were identified by comparing their retention time and MS data with the standards.

Table 1. Chemical composition of chloroform extraction of rhubarb

| Compound | tR(min) | UVλmax(nm) | [M-H(m/z)] | MS2          | Name                  |
|----------|---------|------------|------------|--------------|-----------------------|
| 1        | 3.02    | 256.5      | 269        | 269,225,183  | Aloe-emodin           |
| 2        | 3.27    | 258.5      | 283        | 269,225      | Rhein                 |
| 3        | 4.33    | 287.5      | 269        | 253,225,152  | Emodin                |
| 4        | 5.16    | 256.5      | 253        | 253,225,152  | Chrysophanol          |
| 5        | 5.76    | 266.5      | 283        | 283,253,225  | Physcion              |

They were aloe-emodin, rhein, emodin, chrysophanol and physcion, all of which are Anthraquinone derivatives.

#### Antibacterial activity of compounds identified from rhubarb.

Antibacterial activities of the five compounds identified from CF of the rhubarb crude extract were tested again by the disc diffusion assay. The concentrations of the five compounds used in this assay were their relevant concentrations in CF. ECE and CF were included for comparative purpose. Rhein showed the greatest inhibitory effects for *S. typhimurium* (15.8 ± 0.42 mm).
Table 2. The MIC and MBC of five components from chloroform extraction against *S. typhimurium*

| Samples       | Strains (MIC 1) µg/mL | Strains (MBC 2) µg/mL |
|---------------|---------------------|---------------------|
| Aloe-emodin   | >1000               | >1000               |
| Rhein         | 250                 | 500                 |
| Emodin        | 500                 | 1000                |
| Chrysophanol  | >1000               | >1000               |
| Physcion      | >1000               | >1000               |

Rhein showed the lowest MIC (250 µg/mL) and MBC values (500 µg/mL) comparing to the other four compounds. The values of MIC and MBC of emodin were two times higher than that of rhein, while the MIC and MBC values of aloe-emodin, chrysophanol and physcion were all greater than 1000 µg/mL.

The time kill curve assay:
The effect of rhein on the number of viable cells of *S. typhimurium* were evaluated by the time kill curve assay.

**Figure 4. Effect of rhein on the viability of *S. typhimurium* (B) from the time kill curve assay.**

Nucleotide leakage:
The optical density at 260 nm of *S. typhimurium* treated with rhein increased with a period of 8 hrs comparing to that of the control. The first two hour saw the sharpest increase, over a 6-fold increase of the UV absorption comparing to the control.

**Figure 5. Total nucleotide leakage measured by UV absorption at 260 nm from *S. typhimurium* (B) treated with rhein.**

Scanning electron microscopy:
The Scanning electron microscopy (SEM) was utilized to check the cell morphology of *S. typhimurium* with and without treatment of rhein. Pictures taken from electron micrographs showed that non-treated cells had no changes in cell morphology, displaying a regular, intact and smooth surface. But the membrane of *S. typhimurium* cells treated with rhein showed obvious rupture.

**Figure 6. Scanning electron micrographs of *S. typhimurium* treated with rhein for 12 hours (A. *S. typhimurium* treated with control; B. *S. typhimurium* treated with rhein)**

Discussion:
The antibacterial properties of rhubarb have been known for a long time. Rhubarb extracts and compounds showed inhibitory effects against a number of microorganisms including both Gram-negative and Gram-positive bacteria. Nonetheless, very few attentions have been paid on its antibacterial activities against *Salmonella*. Therefore, a systematic approach was adopted in this study to examine the antibacterial effects of rhubarb against *Salmonella*, to identify the major bioactive compound(s) and to investigate the possible mechanisms. As the first step, rhubarb crude extract ECE and the five fractions made from ECE were screened by using disc diffusion assay against *S. typhimurium*. There are many different assays for screening antimicrobial activity. Disc diffusion assays was chosen because it is the most widely used method for screening antibacterial properties of natural extracts and compounds. The screening results showed, for the first time, that rhubarb ECE did significantly inhibit the growth of *S. typhimurium*. Among the five fractions from...
The possible mechanism of action suggested that rhein killed *Salmonella* of *rhein*. The effects of *rhein* on the morphological and physical changes which further led to significant leakage of DNA and/or RNA indicated that *rhein* nm absorption with *rhein* induced damage to the cell membranes, like protein, nucleotide. Since nucleotide including DNA and RNA showed strong UV absorption at 260 nm, the absorbance changing the cell morphology were examined. When the treatment, the number of bacteria actually increased. To understand why and how *S. typhimurium* treatment, the number of bacteria would leach out, including small ions like K+, large molecules including Anthraquinone, anthrsone, stilbene, flavonoids, acyl glucoside, and pyrone. Anthraquinones have been reported to have different antibacterial activities against different bacteria. So our next step was to look for the most effective compound(s) that specifically inhibited the growth of *S. typhimurium*. By conducting disc diffusion assays again on the five compounds, *rhein* was found to be the most effective antibacterial compounds. The effectiveness of *rhein* was further confirmed by the measurement of MIC and MBC values. Taking together, it is reasonable to believe that *rhein* is a major bioactive antibacterial compounds in rhubarb root against *S. typhimurium*. Despite many years of antibacterial studies on rhubarb, the mechanism of action, especially those associated with the specific bioactive compounds, are still largely unknown. In this study, we explored the possible mechanisms of antibacterial activities of *rhein* on *S. typhimurium*. Firstly, the time kill curve assay was performed to determine the rate of the bacteria being killed by *rhein*. After being treated with 2 M *rhein* for 5 hrs, almost no live bacteria can be visualized. Without *rhein* treatment, the number of bacteria actually increased. To understand why and how *rhein* kill *S. typhimurium*, the possible effects of *rhein* in altering the integrity of cell membrane and changing the cell morphology were examined. When the membrane integrity of bacteria is destroyed, cell constituents would leak out, including small ions like K+, large molecules like protein, nucleotide. Since nucleotide including DNA and RNA showed strong UV absorption at 260 nm, the absorbance at 260 nm has been used as detection index of membrane integrity. Our results showed significant increase of UV 260 nm absorption with *S. typhimurium* treated with *rhein*, clearly indicated that *rhein* induced damage to the cell membranes, which further led to significant leakage of DNA and/or RNA. The effects of *rhein* on the morphological and physical changes of *S. typhimurium* were checked by SEM. The membrane of *S. typhimurium* cells treated with *rhein* showed obvious rupture. This change resulted in cell decomposition and death eventually. 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