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 Anthraquinone derivatives were identified from the chloroform fraction by UPLC-MS/MS, namely emodin, aloe-emodin, rhein, physcion and chrysophanol. 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: Anthraquinone, 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
Rheum A. Plant material, chemicals and reagents.

Materials and Methods

A. Plant material, chemicals and reagents.

The root of rhubarb (Rheum palmatum L.) was purchased from a local market in Shanghai, China. Dimethyl sulfoxide (DMSO), petroleum ether, chloroform, ethyl acetate, n-butanol, gluta-naldehyde and isoamyl 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).

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 Jui, SJP-100A). 100g of powder was extracted three 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 vacuum 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 vacuum 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, 2011) with modifications. In brief, 50 μL of S. typhimurium was injected into 5 mL of TSA and cultured under condition of 37 °C for 150 min, for 6 hrs in a heat temperature incubator. The inoculum was adjusted with 0.1 M PBS (pH 7.2) to 10.6 CFU/mL. 1 mL prepared suspension was streaked unto the surface of TSA with a SS-Spreader, then the inoculum on the plate was allowed to dry to 10 min in dry 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, a diode array detector (DAD). A Waters BEH C18 column (50 mm × 2.1 mm, 1.7 mm, Waters Corporation, USA) was used at a 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, 60-80% (B) in 4.5-5.0 min, 80-30% (B) in 5.0-5.1 min, 30% (B) in 5.1-7.0 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 collision gas. The optimized parameters in the negative ion mode were as follows: the rate of nitrogen (N2), 800 L/hr; desolvation temperature, 450 ºC; 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-dependent 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 minimal inhibitory activities of the five compounds identified from CF were further evaluated by measuring their minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC). The measurement was followed a NCCCLS 96-plate micro dilution broth method (NCCCLS, 2008) using the plates purchased from Chengdu Must Biotechnology Corporation (Chengdu, China). The populations of S. typhimurium were adjusted to 10.5 CFU/mL. The sample was dissolved in DMSO and merged into TSB culture at a concentration of 2000 μg/mL. Serial dilution was then conducted to obtain concentrations of 100, 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 μg/mL. 50 μL inoculum of tested bacteria was added to each well. The negative control containing only bacteria suspension. The bacteria were incubated in 96-well plate at 37 °C for 24 hrs, covered with a parafilm plate and incubated at 37 °C for 24 hrs. The MIC was defined as the lowest concentration that color change from purple to colorless occurred. To measure MBC, 50 μL of each well no color change occurred, the mixture of samples and the strain was isolated on sterile TSA poured in Petri dishes, then cultured at 37 °C for 24 hrs. The MIC was defined as the lowest concentration of sample which no viable bacteria occurred on the agar culture surface. All analysis was carried out three times.

G. The time kill curve assay.

The time kill curve assay was conducted according to a recent paper (Vivek K. Bajpai, Sharma, & Bae, 2011). Bacteria and solution of S. typhimurium were inoculated with 4 mL of TSB broth. Then cultured in 37 °C for 4 hrs. The bacterial suspension was centrifuged at 8000 rpm for 10 min and the supernate was discarded. The precipitate bacterium was re-suspended with 1 mL 0.1 M PBS. Each tube that was used for the kill-time curve assay contained the re-suspended bacteria suspension (107 CFU/mL) of S. typhimurium in the TSB medium. The tubes were inoculated with rhein at a concentration of MIC in 5 mL TSB medium, and cultured at 37 °C with shaking. The number of viable cells was detected as followed: 100 μL sample of each treatment tube was dilute with 0.1% PBS, 10-fold serial dilutions. Then spread on the surface of TSA. The plates cultured for 24 hrs at 37 °C and then counted the colonies. The controls were inoculated without rhein and each test strain was tested similarly as mentioned above. Each assay was carried out three times.

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 inoculated 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 inoculated with 0.1 M PBS 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 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 phase S. typhimurium were inoculated with rhein at 2× MICs in TSB medium for 12 hrs at 37 ºC with shaking. Strains inoculated with TSB without rhein were used as control. The samples were centrifuged at 7500 rpm for 5 min, and the supernate was removed. Bacteria precipitate
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 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.

![Figure 2](source)

**Figure 2**. Diameters of inhibition zone of rhubarb ethanol crude extract (10 mg/mL) and five fractions (10 mg/mL) (A); and of crude extract (10 mg/mL), chloroform fraction and five compounds (concentrations used in assay were the concentrations of their relevant concentrations in 10 mg/mL CF) (B). (ECE: the rhubarb ethanol crude extract, PEF: petroleum ether; CF: chloroform; EAF: ethyl acetate; BF: n-butanol).

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. CF was analyzed by UPLC-MS/MS.

![Figure 3](source)

**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](source)

**Table 1.** Chemical composition of chloroform extraction of rhubarb

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

Antibacterial activity of compounds identified from rhubarb extract.

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), almost the same as CF. Minimum inhibitory and minimum bactericide concentration.

Antibacterial effects of the five major Anthraquinone compounds identified from CF were further checked by measuring their minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC).
Table 2. The MIC and MBC of five components from chloroform extraction against S. typhimurium

<table>
<thead>
<tr>
<th>Samples</th>
<th>Strains (MIC 1) µg/mL</th>
<th>Strains (MBC 2) µg/mL</th>
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<tbody>
<tr>
<td>Aloe-Emodin</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Rhein</td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td>Emodin</td>
<td>500</td>
<td>1000</td>
</tr>
<tr>
<td>Chrysophanol</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Physcion</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
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</table>

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.

After being treated with rhein at 2× MIC or without rhein, bacterial cells were counted every hour in a course of 5 hrs. The viable counts with rhein treatment showed a constant reduction for S. typhimurium. After 5 hrs, the viable counts with rhein treatment were almost zero, indicating complete inhibition against the two bacteria.

**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...
In order to see the major bioactive compounds, CF were analyzed by UPLC-MS/MS and five major Anthraquinone derivatives were identified. About 200 phytochemicals have been identified thus far from eighteen species of the genus Rheum L. They belong to different groups of compounds including Anthraquinone, anthrone, stilbene, flavonoids, acyl glucoside, and pyrone. Anthraquinones have been reported to be the major bioactive compounds from several species of Rheum L. Different Anthraquinones, due to their different chemical structure, appeared to have different antibacterial activities against different bacteria. So our next step was to look for the most effective compounds(s) that specifically inhibited the growth of S. typhimurium. Determining the disc diffusion assay on the five compounds, rhein was found to be the most effective antibacterial compound. 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 polyphenolic content of the crude extract, was found to be the major bioactive compound. The possible mechanism of action suggested that rhein could damage the integrality of cell membrane leading to nucleotide leakage, and changing the cell morphology. But further research is required to fully understand the mechanisms in the molecular levels.

References


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