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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 Anthrsaquinone 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: Anthrsaquinone, 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

best-known traditional Chinese herbal medicines. It was traditionally used as a laxative, to treat constipation, jaundice, gastro-intestinal hemorrhage, and ulcers [14]. Modern studies have revealed its diverse biological activities, including antibacterial [19], anticancer [14], anti-inflammatory [8] and antioxidant [21] effects. Evidence accumulated in the past showed that rhubarb and its bioactive components had strong antimicrobial activities against a number of pathogenic microorganisms, such as *Bacteroidesfragilis*[9], Staphylococcus *aureus* [25], *Escherichia coli* [13], *Bifidobacterium adolescentis* [24], Candida *albicans*, Cryptococcus *neoformans and Trichophyton mentagrophytes* [1].

To our knowledge, there is only one recent published study focusing on the antibacterial activity of rhubarb extract against S. *enteritidis* [12]. But only crude extracts was screened in that study. No bioactive compounds were identified and no mechanisms were explored. The aim of this study was to assess the antibacterial activity of rhubarb root against *S. typhimurium*, and to further identify the bioactive components. The possible mechanisms of action of the major bioactive component were also investigated if such compounds were discovered.

Materials and Methods

A. Plant material, chemicals and reagents.

The root of rhubarb (*Rheum palmatu*m L.) was purchased from a local market in Shanghai, China. Dimethyl sulfoxide (DMSO), petroleum ether, chloroform, ethyl acetate, n-butanol, glutaraldehyde 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*, chrsysophanol 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 cultivated 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). 100g 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 vacuum to obtain the rhubarb crude extract (ECE). For fractionation, 10g of ECE was dispersed in distilled water, followed by extracting 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.

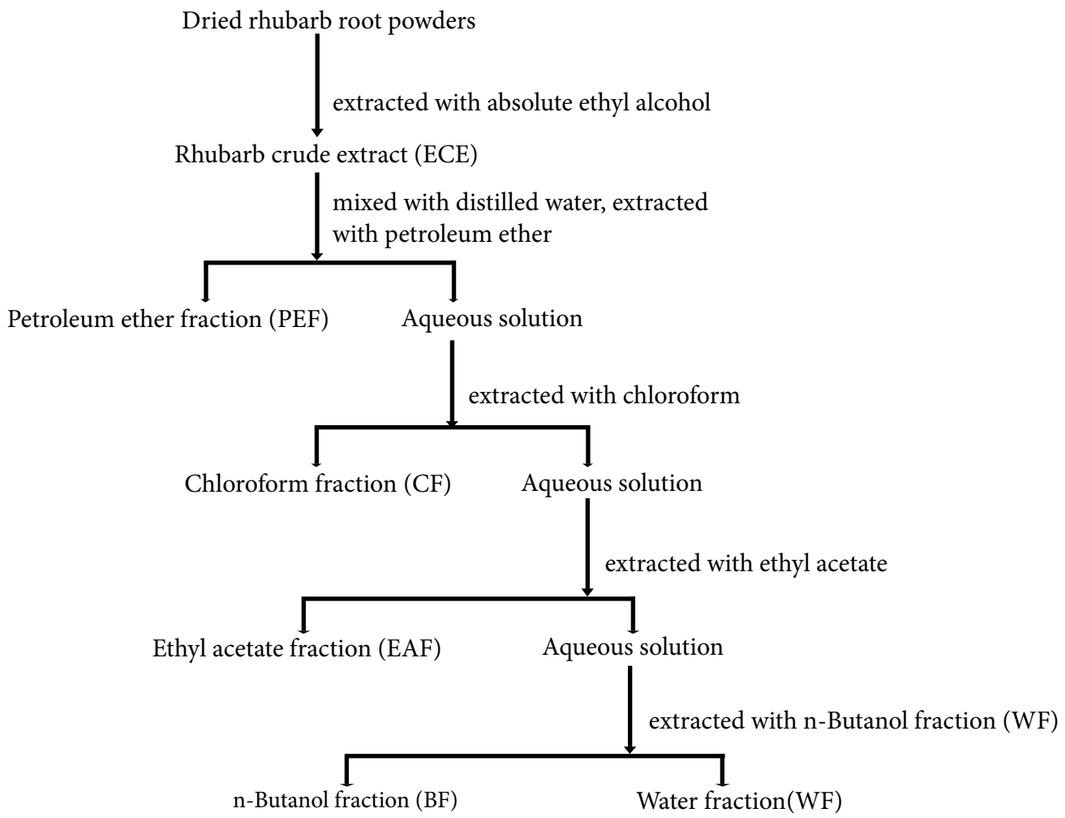

Figure 1. The extraction process of rhubarb root by ethanol and further fractionation to five fractions

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 modifications. In brief, 50 µL of *S. typhimurium* was injected into 5 mL TSB, and cultured under condition of 37 °C, 150 r/min, for 6 hrs in a bed 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 onto the surface of TSA with a SS-Spreader, then the *inoculum* on the plate 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, at a column 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 wave length 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/hrs; desolvation temperature, 450 □; capillary voltage, 2.5 kV; cone voltage, 35 V; cone gas flow, 50 L/hrs. 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 antibacterial 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 NCCLS 96-well plate micro dilution broth method (NCCLS, 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 1000, 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 ºC for 24 hrs, covered with a parafilm paper. Afterwards, 10 µL of INT (Iodonitrotetrazolium) was added to each well. Half an hour later, the color changes were observed with the naked eye, color changes from colorless to purple were noted as positive. The MIC was defined as the lowest concentration that color change from purple to colorless occurred. To measure MBC, 50 µL of each well which no color change occurred, the mixture of samples and the strain was isolated on sterile TSA poured in Petri dishes, then cultured at 37 ºC for 24 hrs. The MBC was defined as the lowest concentration of sample which no viable bacteria occurred on the agar culture surface. All analysis was carried out thrsee times.

G. The time kill curve assay.

The time kill curve assay was conducted according to a recent paper (Vivek K. Bajpai, Sharma, & Baek, 2013). Briefly,1 mL bacteria solution of *S. typhimurium* were inoculated with 4 mL of TSB broth. Then cultured in 37 ºC 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) *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 ºC with shaking. The number of viable cells was detected as followed: 100 µL sample of each treatment tube was diluted with 0.1M PBS, 10-fold serial dilutions. Then spread on the surface of TSA. The plates cultured for 24 hrs at 37 ºC 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 thrsee 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 incubated with *rhein* at the concentration of 2× MICs, cultured with shaking at 37 ºC. 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 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*.

I. 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 Baipai 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 incubated 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

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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).

J. Statistical analysis.

One-way analysis of variance (ANOVA) and Duncan's multiple range tests were performed to determine significant differences ($p < 0.05$) between the means by Statistical Product and Service Solutions (SPSS v.19.0, IBM, Armonk, NY).

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.

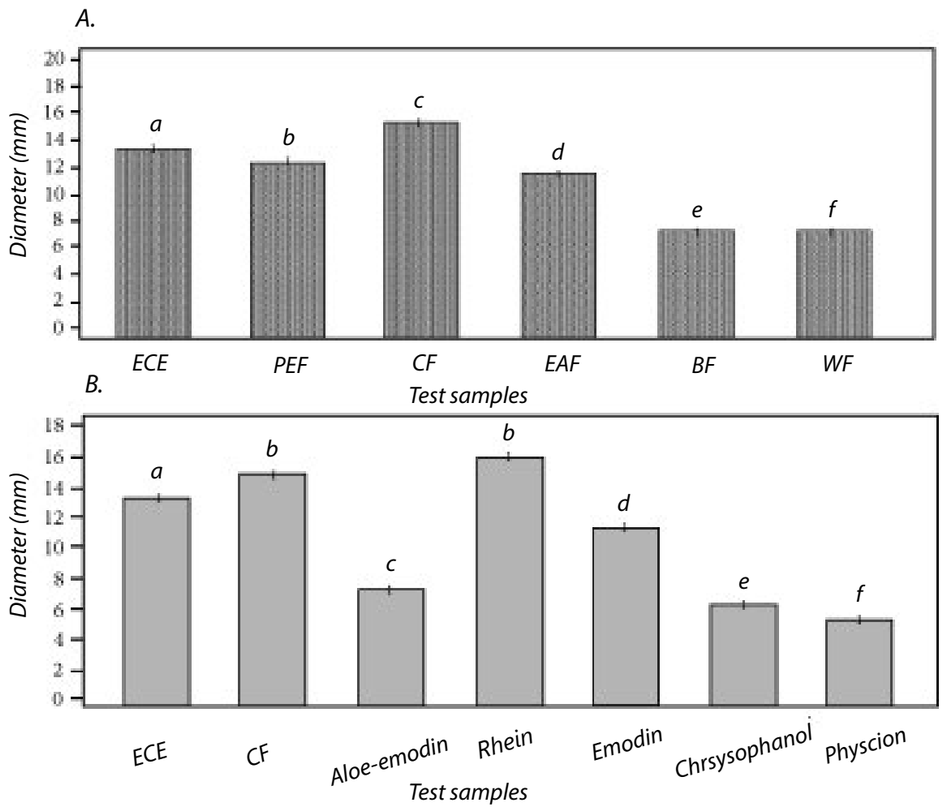

Figure 2. Diameters of inhibition zone of rhubarb ethanol crude extract (10 mg/mL) and five fractions (10 mg/mL) (A); and of crude extract (10 mg/mL), chloroform fraction and five compounds (concentrations used in assay were the concentrations of their relevant concentrations in 10 mg/mL CF) (B). (ECE: 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

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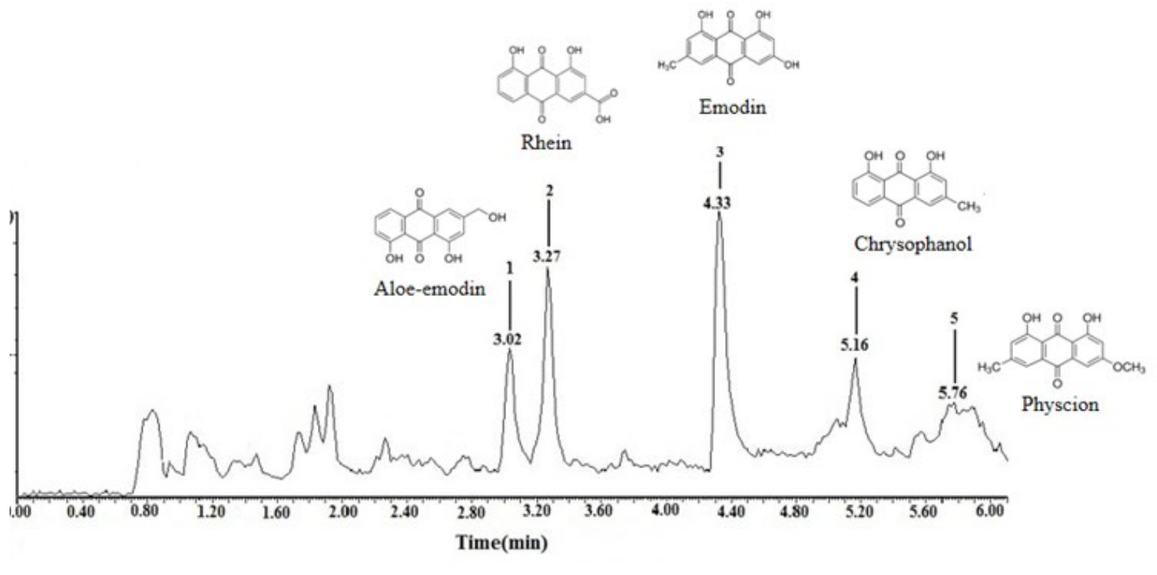

Figure 3. UPLC chrsomatogram of the five major compounds identified from chloroform fraction of rhubarb crude extract (DAD at 268 nm). 1. Aole-emodin; 2. *Rhein*; 3.Emodin; 4.Chrsysophanol; 5.*Physcion*

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

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	269,225	Emodin
4	5.16	256.5	253	253,225,152	Chrsysophanol
5	5.76	266.5	283	283,253,225	Physcion

Table 1. Chemical composition of chloroform extraction of rhubarb

They were aloe-emodin, *rhein*, emodin, chrsysophanol and *physcion*, all of which are Anthrsaquinone 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), almost the same as CF.

Minimum inhibitory and minimum bactericide concentration.

Antibacterial effects of the five major Anthrsaquinone compounds identified from CF were further checked by measuring their minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC)

Samples	Strains (MIC 1) μg/mL	Strains (MBC 2) μg/mL
	S. typhimurium	*S. typhimurium*
Aloe-emodin	>1000	>1000
Rhein	250	500
Emodin	500	1000
Chrsysophnol	>1000	>1000
Physcion	>1000	>1000

Table 2. The MIC and MBC of five components from chloroform extraction against *S. typhimurium*

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, chrsysophanol 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.

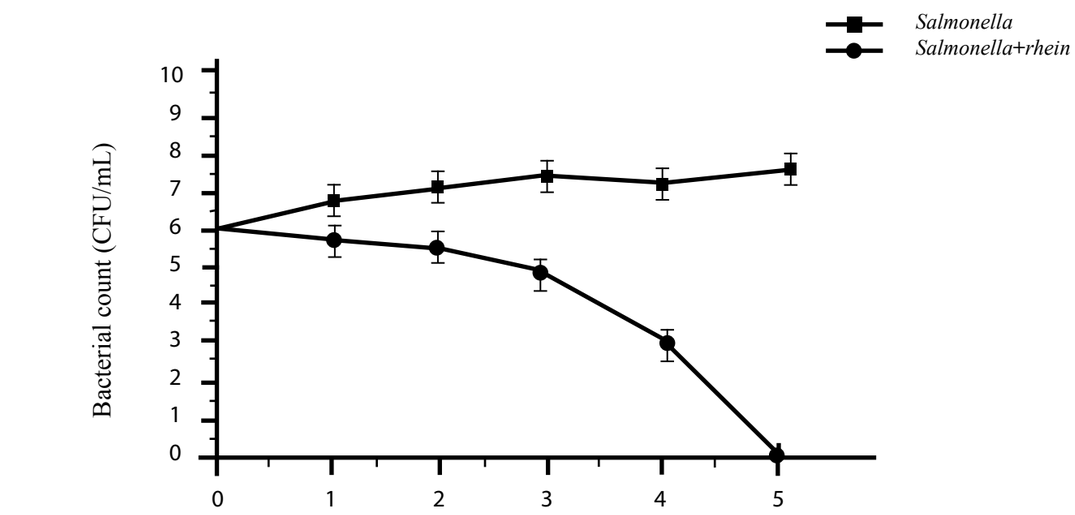

Figure 4. Effect of *rhein* on the viability of *S. typhimurium* (B) from the time kill curve assay.

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.

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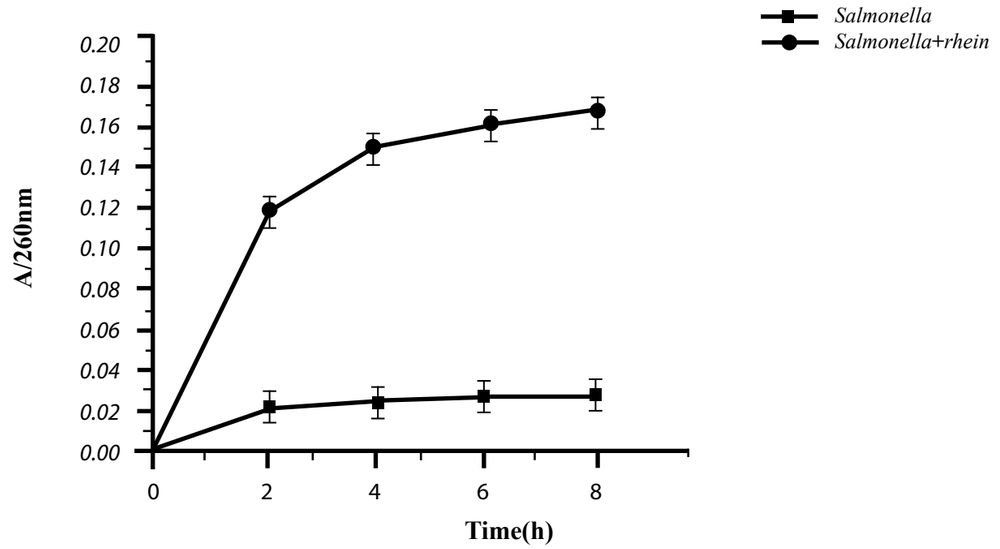

Figure 5. Total nucleotide leakage measured by UV absorption at 260 nm from *S. typhimurium* (B) treated with *rhein*.

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 rapture

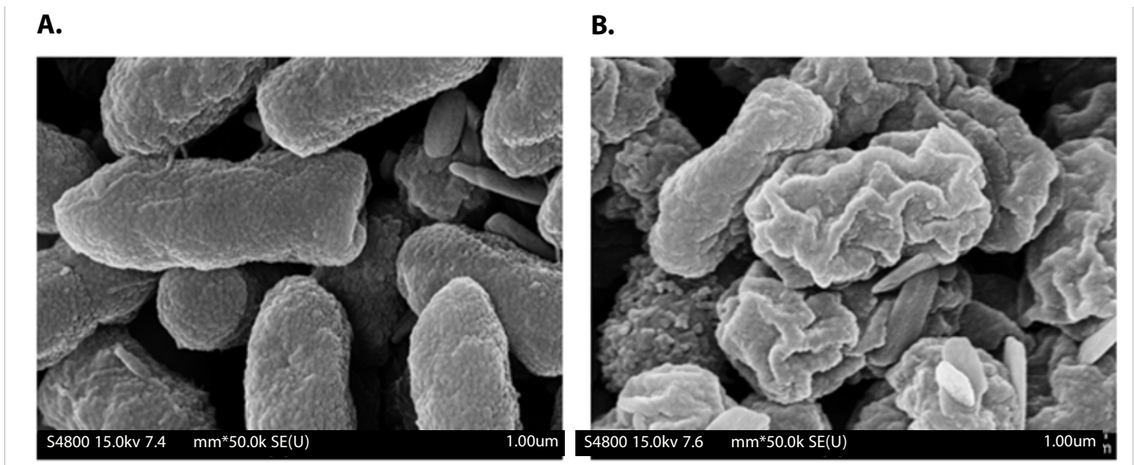

Figure 6. Scanning electron micrographs of *S. typhimurium* treated with *rhein* for 12 hours (A. *S. typhimurium* treated with control; B. *S. typhimurium* treated with *rhein*)

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

ECE,CF was found to be the most effective, thus contains the major antimicrobial compounds.

In order to search for major bioactive compounds, CF were analyzed by UPLC-MS/MS and five major Anthrsaquinone derivatives were identify. About 200 phytochemicals have been identified thus far from eighteen species of the genus Rheum L. They belong to several different groups of compounds including Anthrsaquinone, anthrsone, stilbene, flavonoids, acyl glucoside, and pyrone. Anthrsaquinones have been reported to be the major antibacterial compounds from several species of Rheum L. Different Anthrsaquinones, 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 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* against *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× MIC *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 leach 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. A similar study of morphological changes was observed for *Aeromonashydrophila* when treated with emodin. How these Anthrsaquinone compounds alter integrity of cell membrane remains an open question. Further research is warranted to fully understand the mechanisms of rhubarb and its bioactive compounds against *Salmonella* and other food-borne pathogens.

In conclusion, the results of this study showed that rhubarb root possessed strong antibacterial activity against *Salmonella*. *Rhein*, an Anthrsaquinones component identified from CF of rhubarb crude extract, was found to be the major bioactive compound. It killed *Salmonella* at a relatively fast rate. Investigation on the possible mechanism of action suggested that *rhein* could damage the integrality of cell membrane leading to nucleotide leakage, and changing the cell morphologies. Further research is warranted to fully understand the mechanisms in the molecular levels.

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