Research article

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Inhibiting foodborne pathogens *Vibrio parahaemolyticus* and *Listeria monocytogenes* using extracts from traditional medicine: Chinese gallnut, pomegranate peel, Baikal skullcap root and forsythia fruit

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Abstract: Foodborne illnesses have been a heavy burden in the United States and globally. Many medicinal herbs have been cultivated in the US and many of which contain antimicrobial compounds with the potential to be used for food preservation. Methanol/water extracts of pomegranate peel ("PP", *Punica Granatum* L.), Chinese gallnut ("CG", *Galla chinensis*), Forsythia fruit ("FF", *Forsythia suspensa*) and Baikal skullcap root ("BS", *Scutellaria baicalensis*) were tested for antimicrobial activity using the agar diffusion assay on tryptic soy agar (TSA) and microdilution assay in tryptic soy broth (TSB). CG and PP extracts showed good to excellent inhibitory effect against *Vibrio parahaemolyticus* and *Listeria monocytogenes* in both assays, with a minimum inhibitory concentration (MIC) range from 0.04 to 5 mg/mL. BS had moderate inhibitory effects against *V. parahaemolyticus* with an MIC of 5 mg/mL in TSB, and against *L. monocytogenes* with an MIC of 20 mg/mL on TSA. CG was analyzed using LC-MS and fractionated using HPLC. The major components were identified as gallic acid, digallic acid, methyl gallate, and gallotannins (oligo-galloyl-D-glucose, nGG, n = 1~10). Six fractions (I - VI) were collected and their antibacterial activities were tested against *L. monocytogenes*, and *V. parahaemolyticus* both on TSA and in TSB. On TSA, fraction III, IV and V inhibited *V. parahaemolyticus* but no fraction inhibited *L. monocytogenes*. In TSB, all fractions inhibited *V. parahaemolyticus* and fractions II – V inhibited *L. monocytogenes*. Future studies are needed to investigate the effects of medicinal plants on food products.

Keywords: Chinese gallnut, pomegranate peel, *Vibrio, Listeria*. Gallotannins, LC-MS

1 Introduction

Foodborne illnesses have been a major global health concern. In the US alone it is estimated that 48 million cases of food-borne illnesses occur annually. Among all food-borne illnesses, 9.4 million cases occur due to 31 major pathogens, including *Listeria monocytogenes* and *Vibrio parahaemolyticus* (Scallan et al. 2011). *L. monocytogenes* can cause life-threatening illnesses with high frequencies of hospitalization and death rates (Farber and Peterkin 1991). Compared with other pathogens, *L. monocytogenes* can survive and grow at chilling temperatures and is a major safety concern in ready-to-eat (RTE) foods. *V. parahaemolyticus* is a major human pathogen and is associated with the consumption of raw fish and shellfish, which has caused increased incidences of illness since 2007 (CDC 2014).

Traditional Chinese medicine (TCM) is a different diagnosis and treatment system which includes more than 2,500 herbal materials that possess medicinal functionalities (Chinese Pharmacopoeia Commission

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2015). Historically, while TCM has been used in China and other Asian countries for thousands of years, modern research has found more than 23,000 functional compounds being isolated from plant materials used in TCM (Zhou et al. 2011). Chinese gallnut (CG, *Galla chinensis*), pomegranate peel (PP, *Punica granatum* L.), forsythia fruit (FF, *Forsythia suspensa* (Thunb.) Vahl) and Baikal skullcap root (BS, *Scutellaria baicalensis* Georgi.) are good examples that are used in TCM for their antimicrobial activity (Kang et al. 2008; Al-Zoreky 2009; Fawole et al. 2012; Feng et al. 2012; Qu et al. 2012). Recent studies have shown these plants extracts, which are still widely cultivated in China and the US, are generally considered non-toxic (Akhtar et al. 2015; Lu et al. 2010; Xiang et al. 2015; Yimam et al. 2010). We have published results on evaluation of the antibacterial properties of extracts from selected herbal medicines on seafood matrices (Wu et al. 2016). The purpose of the current study is to evaluate the inhibitory effect of extracts of CG, PP, FF and BS on *L. monocytogenes* and *V. parahaemolyticus* on universal matrices (nutritional media). Based on results of antibacterial activity test, the CG extract was fractionated and each individual fraction was identified and tested for antibacterial activity.

2 Materials and Methods

2.1 Plant material preparation

Plant extracts were prepared as outlined in our previous publication (See Wu et al. 2016). To summarize, dry pomegranate peel (PP, *Punica granatum* L.), Chinese gallnut (CG, *Rhus chinensis* Mill.), forsythia fruit (FF, *Forsythia suspensa* (Thunb.) Vahl) and Baikal skullcap root (BS, *Scutellaria baicalensis* Georgi.) were purchased from a domestic herb provider (Mayway Inc., California, USA) and broken into 1 cm diameter pieces followed by grinding using a high-speed food processor (Osterizer™) into fine powder. The crude ground powder of each plant was sifted through a 40-mesh sieve and to obtain fine powder. Five grams of sifted powder was mixed with 100 mL methanol (HPLC, Fisher Scientific) water solution (70%, v/v) and stirred under slight heating (30-40°C) for 3 hours. The mixture was separated by gravity filtration using #1 filter paper (Whatman®, Fisher St. Louis, MO). The residue on filter paper was washed with 2 mL of 70% methanol and the filtrate was combined and brought up to 100 mL with 70% methanol and stored at -20°C as stock solutions for further use.

Concentrations (c) of plant extracts were calculated as:

c (mg/mL) = Mass of dry powdered plant (mg) / Volume of solution in 70% methanol (mL)

2.2 Preparation of bacterial cultures

The *Listeria monocytogenes* stock strains (LCDC 86-861 and Scott A) were obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and transferred into tryptic soy broth (TSB, BD Bacto™) and then incubated at 37°C for 24 h. The culture was T-streaked on Modified Oxford Agar (MOX, BD Difco™) and incubated for 24 h at 37°C. A single clear colony with dark media halo was taken from the MOX plate and transferred into TSB tube and incubated for 24 h at 37°C. The bacterial population was approximately 10^9 CFU/ml. Equal volume of cultures of two strains were mixed to create a *L. monocytogenes* cocktail. *Vibrio parahaemolyticus* stock strains (VP13 and VP16) were obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and transferred into TSB with 2% NaCl, and then incubated at 37°C for 24 hours. The culture was T-streaked on thiosulfate-citrate-bile salts-sucrose agar (TCBS, BD Difco™) and incubated for 24 hours at 37°C. A single colony with a dark green center on the TCBS plate was transferred into a TSB+NaCl tube and incubated for 24 hours at 37°C to reach a population of approximately 10^9 CFU/mL. Equal volume of cultures of two strains were mixed to create a *V. parahaemolyticus* cocktail.

2.3 Antimicrobial assays of plant extracts

2.3.1 Agar diffusion assay

Tryptic Soy Agar (TSA) plates were used for agar diffusion assay. Each TSA plate (10 cm * 15 mm) was marked into seven sections. Bacteria culture cocktails from Section 2.1 were inoculated onto the TSA plates using a sterile swab. The entire TSA plate was covered by swabbing in one direction, then covered again by swabbing in a direction perpendicular to the first. After swab inoculation, seven sterile paper disks were placed on the agar with one disk located in each marked section. The stock solutions of plant extracts were diluted to 1, 2, 5, 10, and 20 mg/mL. Ten microliters of each extract, 70% methanol, and diluted commercial bleach (5% solution of Clorox®, 6.0% sodium hypochlorite, where NaClO equivalent to approx. 3 mg/mL) were then pipetted onto one paper disks,

respectively. The TSA plates with bacterial inoculation and extracts added were incubated at 37°C for 24 hours. The diameter of inhibitory zones was measured using a ruler and the average of two perpendicular measurements was recorded.

2.3.2 Microdilution assay

Sterile 96-well plates (BD Biosciences) were inoculated with 125 µL of TSB in each well. Bacterial cocktails from Section 2.2 were diluted accordingly and 10 µL (approx. 10^5 CFU) was inoculated into each well. Fifteen microliters of a plant extract at 50, 10, 2, 0.4, or 0.08 mg/µL were added to assigned wells with final extract concentrations equivalent to 5, 1, 0.2, 0.04, or 0.008 mg/mL, respectively. Diluted commercial bleach (15 µL, 5% in water, 3 mg/mL hypochlorite equivalent), 15 µL 70% methanol, or 15 µL sterile water was added into the assigned wells, respectively, as controls.

The 96-well plate with lid was further sealed with Parafilm M® to reduce moisture loss through evaporation and then incubated at 37°C. The inoculum was enumerated on TSA at time zero. Samples were collected at 8, 24 and 96 h intervals and spread plated using a spiral plater (Spiral Biotech Autoplate® 4000). The plates were incubated at 37°C for 24 h before enumerated.

2.4 Composition analysis and collection of Chinese Gallnut

The composition of Chinese Gallnut (CG) was analyzed using LC-MS method on a Waters™ Acquity H-class separation module. An Agilent™ Eclipse XDB-C18 reversed-phase column was used. The elution performed was described as follows: Solution A: 0.1% formic acid in acetonitrile; Solvent B: 0.1% formic acid in water, 0 - 30 min, 0% - 20% A; 30 - 45 min, 20% - 90% A; 45 - 50 min, 90% A; 50 - 51 min, 90% - 0% A; 51 - 56 min, 0% A. MS analysis of column effluent was performed by (–)-electrospray ionization (ESI) on a Waters Acquity TQD (triple quadrupole) mass spectrometer equipped with a Z-spray electrospray interface. The ESI capillary voltage was –4.00 kV. The source and desolvation temperatures were 150°C and 400°C, respectively. The desolvation gas and cone gasses were N_2 at flow rates of 480 L/min and 20 L/min, respectively, while the scanned m/z was 150-1500 (parent ion scan). Data acquisition was carried out with MassLynx software (version 4.1, Waters™).

Fractionation of CG was performed on an HP Agilent™ 1100 series with Diode-Array Detector and an Agilent Eclipse XDB-C18 reversed-phase column with a guard column was used for collecting fractions (I – VI) based on retention time as follows: I: 6.0 – 12.0 min; II: 16.0 – 22.0 min; III: 22.0 – 28.0 min; IV: 28.0 – 32.5 min; V: 32.5 – 34.0 min; VI: 34.0–40.0 min. The fractionation process was repeated 50 times, then the same fraction from each collecting cycle was combined. The fractions (I – VI) were condensed at 45°C using a rotary evaporator and then were freeze dried at -40°C for 72 h until all moisture was removed. The residues of the fractions were collected and re-dissolved in 70% methanol and standardized to 10 mg/mL. The fractions of CG were tested for antimicrobial activities using methods described in Section 2.3.

2.5 Statistical Analysis

Results were analyzed using a two-way analysis of variance (ANOVA) with Tukey's multiple comparisons in GraphPad Prism® Version 6.01. Significant differences were determined by use of a P-value of less than 0.05 (Zar 2010).

Ethical approval: The conducted research is not related to either human or animals use.

3 Results and Discussion

3.1 Antimicrobial activity of plant extracts

Against both bacteria species, Chinese gall (CG) had the greatest inhibitory effect among all the four plant extracts. *V. parahaemolyticus* was inhibited by CG at 1 mg/mL on tryptic soy agar (TSA) and 0.04 mg/mL in TSB, while *L. monocytogenes* was inhibited by 10 mg/mL CG on TSA and 1 mg/mL in TSB (Table 1 and Figure 1 and 2). The minimum inhibitory concentration (MIC) range was comparable with previous studies on a variety of bacteria (Tian et al. 2009; Feng et al. 2012). Pomegranate peel (PP) extract inhibited *V. parahaemolyticus* at 5 mg/mL on TSA and 1 mg/mL in tryptic soy broth (TSB), and inhibited *L. monocytogenes* at 5 mg/mL only in TSB. Baikal skullcap (BS) only showed inhibition agains *V. parahaemolyticus* in TSB at 5 mg/mL, or against *L. monocytogenes* on TSA at 20 mg/mL (Table 1, Figure 1 and 2).

The bacteria species used in this study showed different sensitivities to plant extracts. *L. monocytogenes* showed higher resistance to the extracts than did *V. parahaemolyticus*. The difference in sensitivities may be due to many factors, including different cell wall

Table 1: Diameters of inhibitory zones (mm) formed by plant extracts against *V. parahaemolyticus* on salted tryptic soy agar (TSA + 1.5% NaCl), and *L. monocytogenes* on TSA

Plant Extract	Tested concentration of extract						
	70% MeOH	1mg/mL	2mg/mL	5mg/mL	10mg/mL	20mg/mL	Diluted Bleach
Against *Vibrio parahaemolyticus*							
CG	7.8 ± 0.4^{a}	9.7 ± 0.8^{b}	12.3 ± 0.8^{c}	16.1 ± 0.3^{d}	$17.8\pm0.7^{d,e}$	$19.5\pm0.8^{e,f}$	$8.2\pm0.2^{a,b}$
PP	8.4 ± 0.1^{a}	8.3 ± 0.2^{a}	9.5 ± 0.5^{a}	13.3 ± 0.4^{b}	15.7 ± 0.4^{c}	17.1 ± 0.2^{c}	9.1 ± 0.4^{a}
BS	8.5 ± 0.3^{a}	7.9 ± 0.5^{a}	8.3 ± 0.3^{a}	8.2 ± 0.1^{a}	7.8 ± 0.3^{a}	8.1 ± 0.1^{a}	9.5 ± 0.4^{a}
FF	8.4 ± 0.2^{a}	8.7 ± 0.2^{a}	7.6 ± 0.4^{a}	7.9 ± 0.4^{a}	7.5 ± 0.2^{a}	7.5 ± 0.2^{a}	8.3 ± 0.2^{a}
Against *Listeria monocytogenes*							
CG	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	8.3 ± 0.6^{b}	$9.2\pm0.4^{b,c}$	9.0 ± 0.3^{c}
PP	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.3 ± 0.1^{a}	9.1 ± 0.3^{b}
BS	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	8.3 ± 0.3^{b}	10.4 ± 0.6^{c}
FF	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.2 ± 0.0^{a}	7.6 ± 0.2^{a}	7.2 ± 0.0^{a}	10.8 ± 0.7^{b}

Results were obtained based on triplicate experiments and expressed as mean ± standard deviation. Within each row, significantly different ($P < 0.05$) values are noted with different superscripts, based on one-way analysis of variance and Tukey's multiple comparison. The paper disks used had a diameter of 7.2 mm; diameter equal to 7.2 mm indicates no inhibition. CG, PP, BS, and FF stand for Chinese gallnut, Pomegranate peel; Baikal skullcap root and Forsythia fruit, respectively.

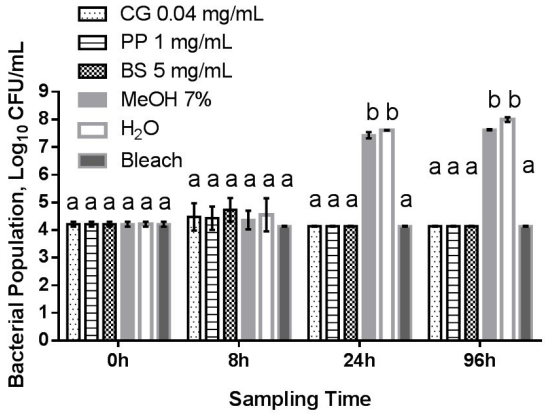

Figure 1: Logarithmic population ($\log_{10}$ CFU/mL) of *V. parahaemolyticus* in tryptic soy broth (TSB) with plant extract treatments, after 0, 8, 24 and 96 h incubation at 37 ± 1°C. The initial populations (0 h) of bacteria cultures were calculated from the plate count of the inoculum. At both 24h and 96h, Chinese gall (CG), pomegranate peel (PP), and Baikal skullcap root (BS) treatments prevented (inhibited) the bacterial growth ($P < 0.001$). Error bar represents standard deviation. At each sampling time, populations marked with the same lowercase letter are not significantly different ($P > 0.05$) from each other

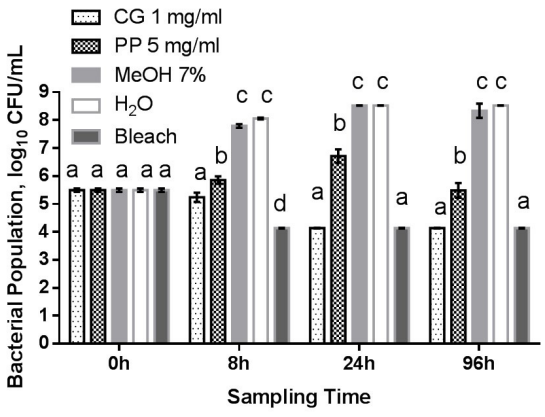

Figure 2: Logarithmic population ($\log_{10}$ CFU/mL) of *L. monocytogenes* in broth with plant extract treatments, after 0, 8, 24 and 96 h incubation at 37±1°C. The initial populations (0 h) of bacteria cultures were calculated from the plate count of the inoculum. Starting from 8 hours, Chinese gall (CG) and pomegranate peel (PP) treatments significantly reduced the bacterial growth ($P < 0.001$). Error bar represents standard deviation. At each sampling time, populations marked with the same lowercase letter are not significantly different ($P > 0.05$) from each other

structures between Gram-positive and Gram-negative organisms (Montville and Matthews 2005: Ch.2) and nutrient deprivation (Engels et al. 2009). Previously, CG has been reported to inhibit human and plant pathogenic bacteria (Tian et al. 2009; Feng et al. 2012). Gallotannins, a series of hydrolysable tannins, the major composition of CG have been reported to inhibit *L. monocytogenes* (Engels et al. 2011); however, inhibitory effect of gallotannins against *V. parahaemolyticus* has seldom been reported. Our study showed that *V. parahaemolyticus*, as a Gram-negative bacterium, is even more sensitive to tannins than *Listeria*, which does not happen to typical Gram-negative bacteria which has different cell wall and membrane (Proestos et al. 2006; Tian et al. 2009). The pH of plant extracts varied from 5.3-5.6, which may have formed a different pH zone on TSA but they had little effect on the pH of TSB when added. Typically, *V. parahaemolyticus* and *L. monocytogenes* prefer higher pH to achieve optimal growth, while the lowest pH for *V. parahaemolyticus* and *L. monocytogenes* in nutrient-rich culture, otherwise, were

reported to be 4.5 and 4.4, respectively (Adams and Moss 2008; Doyle and Buchanan 2011). Thus, we believe that the inhibitory effect was not solely from acidity.

During our preliminary study many extraction solvents were tested, including water, methanol, ethanol and ethyl acetate. Finally we found methanol/water and ethanol/water were the most efficient solvents with highest yield of extracts. 70% methanol was finally chosen over ethanol as the solvent in this study because of its minimal antimicrobial activity among the alcohols (Tilley and Schaffer 1926), although both methanol and ethanol as solvents have been reported in other studies (Cai et al. 2004; Engels et al. 2009; Lu et al. 2011; Fawole et al. 2012). The weak antimicrobial activity was demonstrated in microdilution assay, where bacterial growths with or without methanol added were not significantly different ($P > 0.05$). The weak antibacterial activity of methanol facilitated antibacterial assays by minimizing the contribution from solvent.

3.2 Fractionation and LC-MS analysis of CG composition

The composition of CG was analyzed by LC-MS and major compounds are listed in Table 2. Fractionation of CG was conducted using the same conditions except for a larger injection volume, 25 mL instead of 5 mL in identification process, in order to reduce the number of cycles that were required to obtain enough fractions for antibacterial assay, yet ensure resolution of peaks. The collection time period of fractions was determined based on the retention of major peaks at 280 nm. We employed the method from Tian et al. (2009) during preliminary research, and later we developed a modified gradient elution for better resolution. Since the elution time was not the primary consideration, we extended it as needed. The fractionation and collection of gallotannins from plant sources can also be achieved using different chromatographic methods, including column chromatography (Tian et al. 2009) and high-speed counter-current chromatography (HSCCC) (Engels et al. 2010). However, we found the HPLC fractionation method used in this study as simple, flexible and consistent among batches.

The parent ions ($[M–H]^-$) and most probable identities of compounds in the major peaks in chromatograph are listed in Table 2. Generally speaking, n-galloyl-D-glucose (n = 1-8), gallic acid, and digallic acid were the major compounds identified in CG. The mono-galloyl-D-glucose (or glucogallin, 1GG) and free gallic acid were the major compounds in the fraction I; Digallic acid, methyl gallate, and di-galloyl-D-glucose (2GG) were the major compounds found in fraction II. For fraction III - V, the major compounds were 3GG, 4GG and 5GG, respectively. The major compounds in fraction VI were 5GG, 6GG, 7GG and 8GG. The crude CG extract may also contain compounds with molecular weight higher than 1500, which was beyond the detection range of the MS analysis. However, gallotannins beyond fraction VI with higher molecular weight composed only a minimal portion (by weight collected) of the whole extract. It was also reported that the aqueous extract contained mostly free gallic acid and gallotannins with lower degree of galloylation (1GG-3GG) (Huang et al. 2012), while gallotannins with higher degrees of galloylation (4GG and higher) appeared in organic solvents such as methanol, ethanol, and ethyl acetate (Tian et al. 2009).

Table 2: Major compounds in CG extract in 70% methanol, according to LC-MS analysis

Observed Peaks	Fraction Collected	Retention time	Observed [M–H]– (m z-1)	Theoretical MW (g/mol)	Identity
1	I	7.88	331	332	1GG
2	I	8.45	169	170	Gallic acid
3	II	18.37.	183	184	Methyl gallate
4	II	19.27	483	484	2GG
5	II	20.17	321	322	Digallic acid
6	III	24.44	635	636	3GG
7	IV	28.98	787	788	4GG
8	IV	30.54	787	788	4GG
9	V	33.27	939	940	5GG
10	VI	34.17	939, 1091	940, 1092	5GG, 6GG
11	VI	34.61	939, 1091, 1243,	940, 1092, 1244	5GG, 6GG, 7GG
12	VI	35.05	1091, 1243, 1395	1092, 1244, 1396	6GG, 7GG, 8GG

I - VI refer to the six fractions of CG crude extract; nGG stands for n-galloyl-D-glucose (n = 1-8); the detection range of molecular weight was 150-1500.

3.3 Antibacterial activity of the fractions

Crude CG extract showed antibacterial activity across microorganisms and media (Table 3 and Figure 3). CG fraction III, IV, and V showed significant inhibition against *V. parahaemolyticus* in TSA (Table 3). In TSB, all fractions inhibited *V. parahaemolyticus* and II - V inhibited *L. monocytogenes* (Figure 3). In comparing inhibitions between two target bacterial strains, the results of the antibacterial assays confirmed with our previous results in Section 3.1 that *V. parahaemolyticus* is more susceptible than *L. monocytogenes*.

Fractions of the CG did not significantly inhibit *L. monocytogenes* on TSA like crude CG extract did; in TSB broth, the inhibition was compromised as well. This indicated the loss of the anti-listerial activity during the fractionation. The change might have been from the degradation of compounds in CG during the processing; however, it is more likely from the solubility – it was observed that some fractions of CG, once dried from freeze drying, cannot be totally re-dissolved in 70% methanol. As a result, the actual concentration of fractions may be lower than calculated in TSB, and the solubility may also have compromised agar diffusion. The antibacterial analysis of individual fraction coupled with LC-MS identification of compounds in fractions helped to determine the most promising antibacterial compounds in CG that may be used as natural food preservatives. In this study the most active antimicrobial fractions were III - V, while the LC-MS analysis showed that the major compositions in fraction III - V were 3GG – 5GG. Thus we can reasonably claim that 3GG, 4GG and 5GG have the strongest antibacterial activities among all gallotannins in CG extract.

The mechanisms of inhibition were discussed in previous studies (Tian et al. 2009; Lu et al. 2011; Feng et al. 2012). Composed mostly of gallotannins, the extract of CG and its fractions are able to combine with protein and cause precipitation (Mueller-Harvey 2006). This precipitation may involve bacteria's protein and the protein in culture media. The protein-tannin complexation has also been reported by others (Zhao et al. 1997; He and Yao 2006; Engels et al. 2011). It can be implied that the

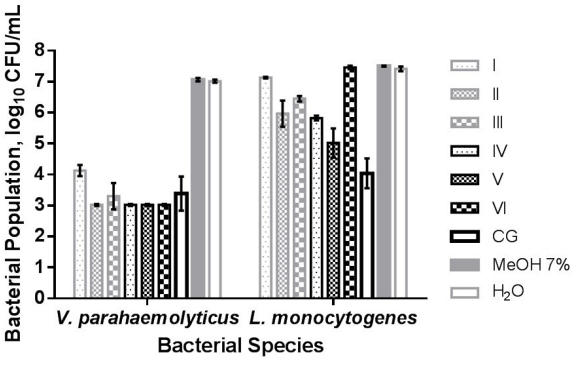

Figure 3: Logarithmic population (log_{10} CFU/mL) of *V. parahaemolyticus* and *L. monocytogenes* in tryptic soy broth (TSB) with treatments of CG crude extract, CG HPLC fractions, 70% methanol, and sterile water. Column shows bacterial growth in samples. Error bar represents standard deviation. CG: The crude Chinese gall extract; I – VI: the 6 HPLC fractions of CG

Table 3: Diameters of inhibitory zones (mm) formed by CG crude extract and fractions against *V. parahaemolyticus* and *L. monocytogenes* cultures on tryptic soy agar, after 24 h incubation at 37 ± 1°C

	Bacterium	
Treatment	*V. parahaemolyticus*	*L. monocytogenes*
MeOH, 70 %	7.9±0.4	7.1±0.0
Crude CG, 10 mg/mL	18.4±0.4	8.7±0.3
Fraction I, 10 mg/mL	8.6±0.4	7.1±0.0
Fraction II, 10 mg/mL	8.8±0.2	7.1±0.0
Fraction III, 10 mg/mL	17.3±0.4	7.2±0.0
Fraction IV, 10 mg/mL	15.1±0.3	7.2±0.0
Fraction V, 10 mg/mL	12.7±0.5	7.2±0.0
Fraction VI, 10 mg/mL	8.3±0.3	7.2±0.0
Diluted Bleach	9.6±0.2	8.5±0.2

The values (mm) of diameters of inhibitory zones were based on triplicated samples, and each sample with 2 perpendicular measurements. Crude extract of Chinese gallnut (CG) and its fractions (I – VI) were dissolved in 70% methanol with a concentration of 10 mg/mL. Within each column, the underlined values are significantly different ($P < 0.05$) from negative control (i.e. 70% methanol), according to one-way analysis of variance with Dunnett's comparisons.

gallotannins can deprive the protein components in the culture media which may starve the bacteria. Additionally, when interacted with gallotannins via hydrogen bonding, the proteins on bacterial membrane lose their biological functions (Konishi et al. 1993; Burt et al. 2004). It is also discussed that gallotannins chelate metal ions, especially iron (ferrous ion), which contributes to the total inhibition effect against microorganisms (Mila et al. 1996; Scalbert et al. 1999). The loss of iron may seriously impact the growth of most iron dependent bacteria. On the other hand, extra supplies of iron may neutralize the antibacterial effect of gallotannins (Engels et al. 2009).

In summary, several medicinal plants from traditional Chinese medicine have antibacterial activities against food-borne pathogens such as *L. monocytogenes* and *V. parahaemolyticus*. Composed mainly by a series of gallotannins, Chinese gall (CG) has the strongest inhibitory activity among all tested plants and deserves further study as a potential antimicrobial for food preserving applications.

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Conflict of interest: Authors state no conflict of interest.

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