Pomegranate peel (*Punica granatum* L) extract and Chinese gall (*Galla chinensis*)
extract inhibit *Vibrio parahaemolyticus* and *Listeria monocytogenes* on cooked shrimp
and raw tuna

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Abstract

Vibrio parahaemolyticus and *Listeria monocytogenes* are bacterial pathogens associated with raw or ready-to-eat seafood products. Many compounds extracted from plant material have shown promise for inhibiting bacterial pathogens when applied to some foods. In this study, aqueous methanol extracts from pomegranate peel (*Punica granatum* L.) and Chinese gallnut (*Galla chinensis*) were tested against *Vibrio parahaemolyticus* and *Listeria monocytogenes* on cooked shrimp and raw homogenized tuna. The extracts were applied to the shrimp by soaking for 2 min (5 mg/ml). The extracts (1.7 mg/ml) were added to homogenized tuna and stirred. The antimicrobial assay on *V. parahaemolyticus* was conducted at 12°C, and the assay on tuna was conducted at both 4° and 12°C. Both Chinese gall and pomegranate peel extracts significantly inhibited the growth of *V. parahaemolyticus* in both shrimp and tuna. Only Chinese gall extract significantly inhibited growth of *L. monocytogenes*. Overall *V. parahaemolyticus* was more sensitive to both plant extracts compared with *L. monocytogenes*. Both plant extracts had stronger antimicrobial activity on shrimp compared with the tuna. Neither extract completely inhibited the growth of *V. parahaemolyticus* or *L. monocytogenes*.

Keywords: Chinese gallnut, pomegranate, *Vibrio*, *Listeria*, shrimp, tuna

1. Introduction

Vibrio parahaemolyticus and *Listeria monocytogenes* are important foodborne pathogenic bacteria. According to Centers for Disease Control and Prevention (CDC, 2011), the number of foodborne illnesses caused by *V. parahaemolyticus* is increasing. In 2013, the highest number of cases was observed since 1996. And, *L. monocytogenes* was responsible for the third largest number of foodborne deaths in the USA (CDC, 2010). According to CDC's foodborne outbreak online database, many outbreaks from *V. parahaemolyticus* are associated with consumption of raw shellfish. Many *L. monocytogenes* outbreaks are associated with ready-to-eat (RTE) food products. In both situations, pre-consumption heat treatments are either absent or insufficient.

Many plant-source materials have been used to combat infectious bacteria in Traditional Chinese Medicine (TCM) for many centuries in China and other Asian countries. Traditional Chinese Medicines have been gradually accepted by the western world. TCM has been studied and used as a Complementary and Alternative Medicine as well as a dietary supplement. More than 35,000 plant-source materials have been screened for potential medical use by the National Cancer Institute and the United States Department of Agriculture (Yuan. et al, 2000). More than 23,000 compounds have been isolated and identified from medicinal plants (Zhou et al., 2011). Chinese gallnut ("CG", *Galla chinensis*) and pomegranate ("PP", *Punica granatum* L.) peel are two commonly

used plant materials in TCM. Previous research discussed the antimicrobial activities of CG and PP extracts (Al-Zoreky, 2009; Tian et al., 2009; Feng et al., 2012). Other research addressed the potential application of plant-source polyphenols in food preservation (Taguri et al., 2004; Guillén and García, 2007; AL-Zoreky, 2009; González et al., 2010; Xi et al., 2012). However, there has been little research specifically addressing the application of CG or its major components, gallotannins' in seafood products. A previous study showed that CG and PP inhibited the growth of *V. parahaemolyticus* and *L. monocytogenes* both in broth and agar media assays (Wu, 2014). In this study, cooked, peeled shrimp and raw homogenized tuna fillets were used as the food matrices. Inoculation of *V. parahaemolyticus* and *L. monocytogenes* on the cooked shrimp were designed to simulate post-processing cross-contamination. The raw tuna study was used to simulate contamination of a raw ready-to-eat (RTE) sashimi or sushi product.

2. Materials and Methods

2.1. Plant materials

Pomegranate peel (PP, *Punica Granatum* L.) and Chinese gallnut (CG, *Galla chinensis*) were purchased from Mayway Inc. (CA, USA) and pharmacy of No. 263 Military Hospital (Beijing, China), respectively, in a dried whole form. Each dry, capsule-like CG was carefully cracked using a hammer. Aphid larvae and eggs found in

the central cavity of the galls were removed and the hard shell tissue was ground with a food processor (Osterizer™). The crude ground gall shells were sifted through a 40-mesh sieve and only the fine powder was collected. Five grams of sifted powder was mixed with 100 ml aqueous methanol solution (MeOH:H₂O = 70:30, v/v) and stirred with mild heating (30-40°C) for 3 h. The mixture was separated by gravity filtration using #1 filter paper (Whatman®) and the filtrate was collected. The aliquot was standardized with 70% methanol to 100 ml and stored at -20°C for further use.

Large pieces of dry PP were separated by hand into approximately 1 × 1 cm pieces or smaller, and then ground and sifted through a 40-mesh sieve. The fine powder was extracted and collected using the same protocol as for the CG.

The concentration “C” of the extract solutions were calculated as:

$$C \text{ (mg/ml)} = \text{Mass of dry powdered plant (mg)} / \text{Volume of solution in 70\% methanol (ml)}$$

Stock solutions (50 mg/ml) of the two plant extracts were stored at -20 °C for later dilutions and uses in antimicrobial tests in shrimp and tuna.

2.2. Preparation of bacterial cultures

The *Listeria monocytogenes* stock strain (LCDC 86-861, serotype 4b) was obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and transferred into 9.9 ml tryptic soy broth (TSB, BD Bacto™) and then incubated at 37°C for 24 h. After the first incubation, the cultures were transferred

into another TSB tube and incubated for another 24 h at 37°C. After the second incubation, 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 surrounded was taken from the MOX plate and transferred into TSB tube and incubated for 24 h at 37°C to reach the population of approximately 10^9 CFU/ml.

The *Vibrio parahaemolyticus* stock strain (VP16) was obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and transferred into 9.9 ml TSB+NaCl (1.5% sodium chloride, the total NaCl concentration of the medium was 2%) tube and then incubated at 37°C for 24 h. After the first incubation, the cultures were transferred into another TSB+NaCl tube and incubated for an additional 24 h at 37°C. After the second incubation, the culture was T-streaked on Thiosulfate-citrate-bile salts-sucrose agar (TCBS, BD Difco™) and incubated for 24 h at 37°C. A single colony with dark green center was taken from the TCBS plate and transferred into a TSB+NaCl tube and incubated for 24 h at 37°C to produce a population of approximately 10^9 CFU/ml.

2.3. Antimicrobial test on RTE food model – fully cooked shrimp

Frozen, cooked shrimp (tail-on, peeled and deveined, 71-90 count/lb., lot # R502803) were purchased from a local grocery store. Fifty milliliter of each stock solution (50 mg/ml) of CG and PP extract was diluted to 5 mg/ml using sterile de-ironized water.

The shrimp were thawed at 4 °C for 12 h, and divided into four groups. Each group of shrimp was then aseptically transferred into diluted CG extract (5 mg/ml), diluted PP extract (5 mg/ml), 7% methanol, or sterile water. The shrimp were soaked and shaken for 5 min. After the soaking treatment, the shrimp (5.5-7.5 g) were drained and aseptically transferred into a stomacher bag. Bacterial cultures of *L. monocytogenes* and *V. parahaemolyticus* were diluted to approximately 10^5 and 10^7 CFU/ml, respectively, then 100 µl of *L. monocytogenes* or *V. parahaemolyticus* were inoculated into each bag. The shrimp inoculated with *V. parahaemolyticus* were incubated at 12°C for 10 days. On day 1, 4, 7 and 10, two bags were removed. Sterile peptone salt water (0.1% peptone, 2% NaCl in water) was added to make 20 g mixtures, which were then homogenized using a stomacher (AESTM). One hundred microliters of the mixture was removed and plated on TCBS agar. The plates were incubated at 37 °C for 24 h and colony-forming units were counted. Shrimp inoculated with *L. monocytogenes* were divided into two groups, and incubated at 4° or 12 °C, respectively, for 10 days. On day 1, 4, 7 and 10, two samples were removed. Sterile peptone water (0.1%) was added to make a 20 g mixture that was homogenized in a stomacher. Fifty microliters of each homogenized sample were plated on MOX agar using a spiral plater (Spiral Biotech Autoplate[®] 4000). The plates were incubated at 37 °C for 24 h and colony forming units were counted.

2.4. Antimicrobial test on raw seafood model – frozen tuna

Frozen raw tuna steaks were purchased from a local grocery store in Hampton, VA (wild caught ahi tuna steaks raw, lot code 24690-PO20091). The tuna steaks were thawed at 4°C for 12 h and then cut into small pieces (approx. 10 × 10 × 5 mm or smaller) and approximately 20 g were aseptically weighed in a stomach bag. Methanol (7%), sterile water, CG and PP extracts (5 mg/ml in 7% methanol) were prepared, respectively, and 10 ml of either extract were added to one bag. The stomacher bags containing tuna pieces and crude extracts were homogenized in a stomacher, and divided into two groups for inoculation with *V. parahaemolyticus* or *L. monocytogenes*. Cultures of *V. parahaemolyticus* were diluted to approximately 10⁷ CFU/ml, and 100 µl of diluted culture were inoculated in each bag. The bags were manually massaged for 5 min and then incubated at 12 °C for 10 days. On day 1, 4, 7 and 10, the bags of tuna slurry were removed from incubator and massaged for 5 min, then 100 µl was plated on TCBS agar. Plates were incubated at 37 °C for 24 h and counted for colony forming units.

Cultures of *L. monocytogenes* were diluted to approximately 10⁵ CFU/ml and 100 µl was inoculated in each bag. The bags were manually massaged for 5 min and subdivided into two groups that were incubated at 4° and 12°C, respectively, for 10 days. On day 1, 4, 7 and 10, the bags of inoculated tuna were removed and massaged for 5 min and 50 µl was plated on MOX agar using spiral plater. Plates were later incubated at 37 °C for 24 h and colony forming units were counted.

2.5. Statistical Analysis

Colony count results were analyzed in GraphPad Prism Version 6.01. The results were compared using two-way ANOVA. Whenever the overall F value was significant, multiple comparisons were performed using Tukey's Studentized T-test to find differences among treatments (Zar, 2010).

3. Results

3.1. Antimicrobial test on shrimp against *V. parahaemolyticus*

The planned incubation temperature was 12 °C, however, the actual recorded incubation temperature was 14±1 °C. The plate count results indicate that inhibition of *V. parahaemolyticus* growth by CG extract began on day 1, and continued to day 4 (Fig. 1). On day 4, the population of *V. parahaemolyticus* in the CG extract treated samples was below detectable levels. Overall, the CG extract (5 mg/ml) treatment decreased the growth of *V. parahaemolyticus* on cooked shrimp by 4 logs during the 10-day period. The inhibition of *V. parahaemolyticus* growth by the PP extracts was greatest on day 4. Afterwards, a slight increase in population was observed. In contrast, *V. parahaemolyticus* in the control group increased from 10⁴ to over 10⁸ CFU/g. The PP extracts did not completely inhibit *V. parahaemolyticus*, but growth was reduced by 3 logs over the 10-day period compared to a control (water).

3.2. Antimicrobial test on tuna and *V. parahaemolyticus*

Inhibitory activity of CG against *V. parahaemolyticus* was observed during the first 3 days (Fig. 2). The *V. parahaemolyticus* growth in tuna with CG treatments increased by less than 1 log. On day 4, the raw tuna samples inoculated with *V. parahaemolyticus* showed microbial spoilage (Fig. 2). Numerous yellowish green colonies on the TCBS agar plates indicated that the dominating microorganism was not the inoculated *V. parahaemolyticus* (which forms dark green colonies on TCBS agar plate). In contrast, the *V. parahaemolyticus* numbers in the control group increased during the first three days more than 3 logs from 10^4 to more than 10^7 CFU/g. The PP crude extract (1.7 mg/ml) in raw homogenized tuna produced a 1 log reduction of *V. parahaemolyticus*. The methanol control group also showed a slight inhibition of bacterial growth (<1log) compared to the control.

3.3. Antimicrobial test on shrimp against *L. monocytogenes*

The inhibitory effects of CG and PP on *L. monocytogenes* were tested at both $4\pm1^\circ\text{C}$ (Fig. 3) and $12\pm1^\circ\text{C}$ (Fig. 4), which simulated refrigerated storage and mild temperature abuse storage conditions, respectively. The initial *L. monocytogenes* population in the inoculated samples was 10^3 CFU/g. In the control group, during the 10 day incubation, *L. monocytogenes* at 4°C increased by 3 logs. At 4°C , in the CG treated samples, the

inhibition of *L. monocytogenes* was observed on day 7. On day 10, the growth of *L. monocytogenes* was 2 logs less, compared to the control. The PP extract inhibited growth of *L. monocytogenes* less. On day 10, the *L. monocytogenes* numbers were 1 log less compared to the control samples. The methanol control treatment reduced *L. monocytogenes* growth but the difference not significant.

At 12°C, growth of *L. monocytogenes* increased more than at 4°C. Also, at 12°C, significant inhibition of *L. monocytogenes* from the CG extract began on day 4, and continued until day 10. On day 10, the *L. monocytogenes* growth with the CG treatment was 2 logs lower than the control group. At 12°C, the PP extract did not inhibit the growth of *L. monocytogenes*.

3.4. Antimicrobial test on tuna against *L. monocytogenes*

During the 7-day incubation period at 4°C (Fig. 5) and 12°C (Fig. 6), the *L. monocytogenes* population in the tuna samples increased rapidly (3-4 logs), despite the addition of the crude CG and PP extracts. Samples inoculated with CG or PP extracts had lower *L. monocytogenes* populations compared with the controls, but the difference was less than 1 log between them.

4. Discussion

These studies were conducted using bacterial inoculations (10^4 - 10^5 CFU/g), which are higher than those typically found in the contaminated food. These high inoculum levels may have resulted in greater survival of the *V. parahaemolyticus* and *L. monocytogenes*. Also, incubation temperatures used for this study were 4°C and 12°C. The purpose was to simulate typical refrigerated storage temperatures, as well as a mild temperature abuse, (Hudson and Mott, 1993).

Methanol was used as the solvent for the plant extracts. The potential inhibitory effects of the solvent were addressed by including aqueous methanol as a control. For shrimp, 7% methanol was applied as a dipping solution prior to inoculation with *L. monocytogenes* and *V. parahaemolyticus*. Growth of *V. parahaemolyticus* was suppressed by methanol. For example in Figure 1, no *V. parahaemolyticus* growth was detected in the methanol treated shrimp during the first four days. The growth *V. parahaemolyticus* gradually increased starting on day 7, and was similar to the water control group on day 10.

Other studies applied polyphenol extracts after inoculation (Al-Zorely, 2009; Xi et al, 2012). In this study, a pre-inoculation treatment was chosen to simulate a practical application. For the shrimp study, whole shrimps were soaked in the 5 mg/ml crude extract solution. In contrast, in the tuna study, the plant extracts (5 mg in 1 ml 70% methanol) were mixed with the homogenized tuna (30 g), thus the final concentration of plant extract was approximately 1.7 mg/ml. Therefore, *V. parahaemolyticus* and *L.*

monocytogenes were exposed to a higher concentration of crude plant extract in shrimp compared with the tuna study. This may explain why the crude plant extracts demonstrated a stronger bacterial inhibition on shrimp. Raw tuna and plant extracts formed a white precipitate, possibly due to the interaction between the polyphenolic compounds in the extracts and protein in the tuna (Kawamoto et al. 1997). The food reaction (cloudiness) may have significantly reduced the efficacy of the crude plant extracts. The cooked shrimp, on the other hand, reacted less with the plant extract because the protein was already denatured from cooking.

The results of plant extract treatments against *L. monocytogenes* on shrimp indicate that the CG treatment was unable to completely inhibit *L. monocytogenes* growth, but it did postpone the exponential growth phase. Along with the plant extract treatments, the change of sensory properties of the shrimp and tuna may be a concern (Pérez-Mateos et al., 2001). In this study, the tuna meat was whitened by CG and PP extracts. More research needs to be conducted to determine if this change in sensory properties is significant.

Antimicrobial tests of plant extracts in culture media and on food matrices were conducted at different temperatures. According to the results, *L. monocytogenes* were more resistant to plant extracts at lower temperatures (4°C or 12°C) than at higher temperatures (37°C) during preliminary antibacterial tests on agar. This might be from

the different media used or from the difference in cell membrane permeability at different temperatures (Nikaido, 2003).

5. Conclusion

The use of crude CG extract can significantly reduce the population of *V. parahaemolyticus* in shrimp and tuna during storage under 12°C, and significantly reduce the population of *L. monocytogenes* on shrimp stored at 4°C or 12°C. These plant extracts can be potentially used as natural preservatives, but the storage temperature must be controlled as the prerequisite to ensure food safety.

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Fig. 1. Population (CFU/g) of *V. parahaemolyticus* on cooked shrimp with treatments of Chinese gall (CG) extract, pomegranate peel (PP) extract, 7% methanol, and water, after 0 day, 1 day, 4 days, 7 days and 10 days incubation at 14±1°C

The concentration of plant extracts used as soaking agent was 5 mg/ml in 7% methanol. On Day 1, 4, 7, and 10, Chinese gall (CG) and pomegranate peel (PP) significantly inhibited the growth of *V. parahaemolyticus* on shrimp ($P<0.05$). For each sampling time, columns representing different bacterial populations are labeled with different letters (a, b and c). Error bars represent the standard deviation (SD).

Fig. 2. Population (CFU/g) of *V. parahaemolyticus* on raw tuna with treatments of Chinese gall extract, pomegranate peel extract, 7% methanol, and water, after 0 day, 1 day, and 4 days incubation at 14±2°C

The columns showed bacteria growths (CFU/g) in samples. The concentration of plant extracts in tuna was 1.7 mg/g. The initial populations (Day 0) of bacteria cultures were calculated from the plate count of the inoculum. On Day 2, and 4, Chinese gall (CG) and pomegranate peel (PP) significantly inhibited the growth of *V. parahaemolyticus* ($P < 0.05$) based on two-way analysis of variance. After Day 4, the growth of *V. parahaemolyticus* cannot be quantified due to the boost of natural microflora on tuna. Error bars represent the standard deviation (SD). For each sampling time, columns representing different bacterial populations are labeled with different letters (a, b and c).

Fig. 3. Population (CFU/g) of *L. monocytogenes* on cooked shrimp with treatments of Chinese gall extract, pomegranate peel extract and water, after 0 day, 1 day, 2 days, 4 days, 7 days and 10 days incubation at $4\pm1^{\circ}\text{C}$

The concentration of plant extracts used as soaking agent was 5 mg/ml in 7% methanol. Chinese gall (CG) and pomegranate peel (PP) showed almost no significant inhibition on the growth of *L. monocytogenes* on shrimp ($P>0.05$) until Day 10. Error bars represent the standard deviation (SD). For each sampling time, columns representing different bacterial populations are labeled with different letters (a, b and c).

Fig. 4. Population (CFU/g) of *L. monocytogenes* on cooked shrimp with treatments of Chinese gall extract, pomegranate peel extract and water, after 0 day, 1 day, 2 days, 4 days, 7 days and 10 days incubation at 12±1°C

The concentration of plant extracts used as soaking agent was 5 mg/ml in 7% methanol. Starting from Day 2, Chinese gall (CG) showed significant inhibition on the growth of *L. monocytogenes* on shrimp ($P < 0.05$). Pomegranate peel (PP) did not show significant inhibition on the growth of *L. monocytogenes* on shrimp ($P > 0.05$) except for Day 4, Error bars represent the standard deviation (SD). For each sampling time, columns representing different bacterial populations are labeled with different letters (a, b and c).

Fig. 5. Population (CFU/g) of *L. monocytogenes* on raw tuna with treatments of Chinese gall extract, pomegranate peel extract and water, after 0 day, 1 day, 4 days, and 7 days incubation at $4\pm1^\circ\text{C}$

The concentration of plant extracts in tuna was 1.7 mg/g. On Day 1, 4 and 7, both Chinese gall (CG) and pomegranate peel (PP) inhibited the growth of *L. monocytogenes* on tuna ($P < 0.05$). Error bars represent the standard deviation (SD). For each sampling time, columns representing different bacterial populations are labeled with different letters (a, b and c).

Fig. 6. Population (CFU/g) of *L. monocytogenes* on raw tuna with treatments of Chinese gall extract, pomegranate peel extract and water, after 0 day, 1 day, 4 days, and 7 days incubation at 12±1°C

The concentration of plant extracts in tuna was 1.7 mg/g. On Day 1 and 7, both Chinese gall (CG) and pomegranate peel (PP) inhibited the growth of *L. monocytogenes* on tuna ($P < 0.05$). Error bars represent the standard deviation (SD). For each sampling time, columns representing different bacterial populations are labeled with different letters (a, b and c).