Antibacterial Effects of Roselle Calyx Extracts and Protocatechuic Acid in Ground Beef and Apple Juice

Che-Yi Chao¹ and Mei-Chin Yin²

Abstract

The antibacterial effects of roselle calyx aqueous and ethanol extracts and protocatechuic acid against food spoilage bacteria Salmonella typhimurium DT104, Escherichia coli O157:H7, Listeria monocytogenes, Staphylococcus aureus, and Bacillus cereus were examined. Minimal inhibitory concentrations of roselle calyx aqueous and ethanol extracts and protocatechuic acid against these bacteria were in the range of 112–144, 72–96, and 24–44 μg/mL, respectively. Protocatechuic acid content in roselle calyx aqueous and ethanol extracts was 2.8 ± 0.7 and 11.9 ± 1.2 mg/g, respectively. Antibacterial activity of roselle calyx ethanol extract and protocatechuic acid was not affected by heat treatments from 25°C to 75°C and 25°C to 100°C, respectively. After 3 days storage at 25°C, the addition of roselle calyx extracts and protocatechuic acid exhibited dose-dependent inhibitory effects against test bacteria in ground beef and apple juice, in which the roselle calyx ethanol extract showed greater antibacterial effects than the aqueous extract. These data suggest that roselle calyx ethanol extract and protocatechuic acid might be potent agents as food additives to prevent contamination from these bacteria.

Introduction

Salmonella typhimurium DT104, Escherichia coli O157:H7, Listeria monocytogenes, Staphylococcus aureus, and Bacillus cereus are five common food spoilage bacteria (Pan et al., 1997; Meldrum et al., 2006; Vindigni et al., 2007; Little et al., 2008). The contamination from these bacteria in many foods including meat and juice has been reported (Zhao et al., 2002; Al-Holy et al., 2006; Cohen et al., 2006; Moon et al., 2006). It is well known that the contamination from these bacteria can reduce shelf-life of foods, cause foodborne illness, and lead to economic loss for food producers, and foodborne disease outbreaks due to these bacteria in Taiwan have been reported (Pan et al., 1997; Su et al., 2005). Therefore, the development and application of proper agent(s) with antibacterial activity may be necessary in order to ensure food safety.

Roselle (Hibiscus sabdariffa L.) is a functional food in Asia countries such as China, Japan, Korea, and Taiwan. Roselle calyx could be treated as a sugaring preserved fruit or used as an ingredient of tea-like beverage (locally called flower tea) by mixing with rose and chrysanthemum. Roselle calyx juice is a popular drink in Taiwan. Our previous study reported that the aqueous extract of this plant could inhibit several nosocomial infectious bacteria such as methicillin-resistant Staphylococcus aureus and Klebsiella pneumoniae (Liu et al., 2005); however, it is uncertain whether roselle calyx aqueous extract could inhibit the growth of S. typhimurium DT104, E. coli O157:H7, L. monocytogenes, S. aureus, and B. cereus. On the other hand, the

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antibacterial activity of ethanol extract from this plant is unknown, and it is unclear whether heating treatment affects the antibacterial effect of these extracts. If these extracts are able to exhibit antibacterial protection at higher temperatures, they could be useful in a wide variety of food systems. Protocatechuic acid, a polyphenol containing a 3,4-dihydroxy substructure, is a compound that naturally occurs in roselle calyx. Several in vitro studies have indicated that this compound can inhibit the growth of *E. coli* and fungi (Fernandez et al., 1996; Aziz et al., 1998). The information regarding the inhibitory capability of this agent against *S. typhimurium* DT104, *L. monocytogenes*, *S. aureus*, and *B. cereus* is limited.

The major purpose of this study was to investigate the inhibitory effects of roselle calyx aqueous and ethanol extracts and protocatechuic acid against five bacteria associated with food contamination in ground beef and apple juice. The influence of temperature upon the antibacterial activity of these agents was also examined.

### Materials and Methods

#### Materials

Fresh roselle calyx was obtained from the botanic garden in Nantou County (Taiwan). A 50-g edible portion of roselle calyx was chopped and mixed with 100 mL of sterile distilled water or 75% ethanol at 25°C for 12 hours, and than homogenized in a Waring blender (Sunwei, Taichung City, Taiwan). After filtration through a Whatman No. 1 filter paper, the filtrate was sterilized by passing through a 22-µm pore size filter (Millipore, France) and further freeze-dried to fine powder. Protocatechuic acid (99.5%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Protocatechuic acid, based on its hydrophobic characteristic, was first dissolved in ethanol (20 mg/mL), and then used for other preparations. The impact of ethanol upon the growth of test bacteria was not significant (data not shown).

#### Protocatechuic acid content analysis

The content of protocatechuic acid in freeze-dried aqueous and ethanol extracts of roselle calyx was analyzed according to an high-performance liquid chromatography method described in Ma et al. (2007).

#### Test organisms

Five food spoilage bacteria, *Salmonella typhimurium* DT104, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus*, were recovered from contaminated beef, chicken, milk, or seafood from March 2007 to June 2007 by using a surface swab technique. The methods described in Meldrum et al. (2006) were used to identify and enumerate isolates. The isolate numbers of *S. typhimurium* DT104, *E. coli* O157:H7, *L. monocytogenes*, *S. aureus*, and *B. cereus* used in this study were 17, 24, 20, 18, and 15, respectively. These microorganisms were routinely maintained on appropriate agar medium at 25°C.

#### Minimal inhibitory concentration (MIC) determination

Microdilution MICs of roselle calyx aqueous extract, ethanol extract, and protocatechuic acid were determined against test bacteria according to Clinical and Laboratory Standards Institute guideline (CLSI, 2003). Each bacterial strain culture at 0.1 mL, containing 10^6 colony-forming units (CFU)/mL, was inoculated into 9.9 mL of nutrient broth supplemented with the test agent at concentrations ranging from 4 to 256 µg/mL in tubes. All incubations were carried out at 35°C for 24 hours. MIC was recorded as the lowest concentration of the test compound to inhibit growth of test bacteria.

#### Heat treatment

A 50-mL beaker with solution of roselle calyx aqueous extract or ethanol extract or protocatechuic acid (20 mg/mL) was sealed with parafilm and placed in a water bath. The temperature of these beakers was maintained at 25°C, 50°C, 75°C, or 100°C for 60 minutes in a water bath. After cooling down to room temperature, the inhibitory zone of these solutions against test bacteria was determined.

#### Inhibitory zone measurement

The inhibitory zone of aqueous extract, ethanol extract, or protocatechuic acid was
determined and compared by disk diffusion method. A blank disk (6-mm diameter) was soaked in solution of roselle calyx extracts or protocatechuic acid for 30 minutes, and then placed on the surface of nutrient agar plate previously seeded with $10^4$ CFU/mL test bacteria. The inhibitory zone was measured after 24-hour incubation at 35°C.

**Ground beef and apple juice preparation**

Beef semimembranous muscle (top round) purchased locally was trimmed of all visible extramuscular fat; the beef muscle was then ground via a 4.5-m/m head on a model TS-285 grinder (Ta-sin Ltd., Taichung City, Taiwan), and divided into several portions for the following experiments. Apple juice (pH 3.4–3.6) was purchased from local supermarkets in Taichung City, Taiwan. Apple juice was filtered and divided into several portions for the following experiments. Apple juice (pH 3.4–3.6) was purchased from local supermarkets in Taichung City, Taiwan. Apple juice was filtered through a 0.45-mm sterile filter (Nalge Nunc International Corp., NY) to remove any bacteria or fungi.

**Antibacterial assay in ground beef and apple juice**

Roselle calyx extracts or protocatechuic acid at 5 or 10 mg was mixed with 100 g of ground beef or 100 mL of apple juice. The control group contained neither roselle calyx extracts nor protocatechuic acid. One milliliter of each test bacterial culture at $10^4$ CFU/mL was added into 100 g of ground beef or 100 mL of apple juice previously treated with or without roselle calyx extracts or protocatechuic acid. The inoculated ground beef was then mixed at low speed in a meat mixer (Model TS-383, Ta-sin Ltd.) to assure uniform distribution of inoculum. The inoculated apple juice was mixed with a food mixer (Model TS-119, Ta-sin Ltd.). Unincubated beef and apple juice samples were also used as negative controls. After 3 days storage at 25°C, 20 g of ground beef was homogenized with 100 mL of deionized water. Then, 1 mL of beef homogenate or juice sample was serially diluted with 9 mL of 0.5% peptone water, and 0.1 mL portion of each dilution was spread on brilliant green agar plate, chromocult coliform agar plate, Baird Parker agar plate, Listeria selective agar plate, or B. cereus selective agar plate for *S. typhimurium* DT104, *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, or *B. cereus* enumeration, respectively. All these agar plates were purchased from Oxoid Ltd. (Basingstoke, UK). Plates were incubated at 35°C for 24 hours, and reported as log CFU/g ground beef or log CFU/mL apple juice.

**Statistical analysis**

All data were expressed as mean ± SD ($n = 15–24$). Differences among means were determined by the Least Significance Difference Test with significance defined at $p < 0.05$.

**Results**

Protocatechuic acid content in freeze-dried roselle calyx aqueous extract and ethanol extract was 2.8 ± 0.7 mg/g and 11.9 ± 1.2 mg/g, respectively. The MICs of two roselle calyx extracts and protocatechuic acid against test bacteria are presented in Table 1. Roselle calyx aqueous and ethanol extracts and protocatechuic acid could inhibit the growth of these bacteria, in which the MICs were in the range of 112–144, 72–96, and 24–44 μg/mL, respectively. Protocatechuic acid exhibited the lowest MICs against five test bacteria and followed by roselle calyx ethanol extract and aqueous extract ($p < 0.05$). The influence of heat upon antibacterial effect of roselle calyx extracts and protocatechuic acid are shown in Tables 2 and 3. After treatment at 75 and 100°C, aqueous extract of roselle calyx had significantly decreased antibacterial activity ($p < 0.05$). However, the heat treatments of 50° and 75°C did not significantly affect the antibacterial activity of roselle calyx ethanol extract ($p > 0.05$). The antibacterial activity of

<table>
<thead>
<tr>
<th>Bacteria (isolates)</th>
<th>RW</th>
<th>RE</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> (17)</td>
<td>120 ± 16c</td>
<td>80 ± 4b</td>
<td>32 ± 4a</td>
</tr>
<tr>
<td><em>E. coli</em> (24)</td>
<td>128 ± 16c</td>
<td>72 ± 4b</td>
<td>24 ± 4a</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> (20)</td>
<td>136 ± 24c</td>
<td>84 ± 8b</td>
<td>36 ± 8a</td>
</tr>
<tr>
<td><em>S. aureus</em> (18)</td>
<td>112 ± 16c</td>
<td>72 ± 8b</td>
<td>24 ± 8a</td>
</tr>
<tr>
<td><em>B. cereus</em> (15)</td>
<td>144 ± 24c</td>
<td>96 ± 8b</td>
<td>44 ± 4a</td>
</tr>
</tbody>
</table>

Means in a row without a common letter differ, $p < 0.05$. 

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**Table 1. Minimum Inhibitory Concentration (μg/mL) of Roselle Calyx Aqueous Extract (RW), Roselle Calyx Ethanol Extract (RE), and Protocatechuic Acid (PA) Against Five Bacteria. Data Are Expressed As Mean ± SD ($n = 15–24$)**
Tables 4 and 5

B. cereus (15) 29
(18) 32
S. aureus
L. monocytogenes (20) 28
(24) 37
E. coli
S. typhimurium (17) 33
8
Bacteria (isolates) 25
levels (log CFU 
8
C. The antibacterial effects of roselle calyx extracts and protocatechuic acid in ground beef and apple juice are presented in Table 4 and 5. After 3 days of storage at 25°C, the addition of roselle calyx extracts and protocatechuic acid dose-dependently inhibited the growth of test bacteria in ground beef and apple juice (p < 0.05), in which ethanol extract showed greater inhibitory effects than aqueous extract in both ground beef and apple juice (p < 0.05), and protocatechuic acid treatments led to bacterial levels (log CFU/g or mL) in ground beef or apple juice lower than 2.

Discussion

Many studies have indicated that S. typhimurium DT104, E. coli O157:H7, L. monocytogenes, S. aureus, and B. cereus caused contamination in beef and/or apple juice (Cieslak et al., 1996; Troutt and Osburn, 1997; Cody et al., 1999; Brown, 2000). The foodborne disease outbreaks due to these bacteria in Taiwan have been reported (Pan et al., 1997; Su et al., 2005). Thus, exogenous addition of appropriate agent for these foods may be helpful in order to enhance their safety. Our previous study found that roselle calyx aqueous extract could inhibit the growth of several clinical nosocomial pathogens (Liu et al., 2005). The results from our present study not only extended the antibacterial activity of roselle calyx aqueous extract to these five common food spoilage bacteria but also revealed roselle calyx ethanol extract possessed marked antibacterial activities. These data support that roselle calyx extracts could be considered as effective agents for beef, apple juice, or other foods to prevent contamination from these bacteria. Furthermore, our present study revealed that heating treatment diminished antibacterial activity of roselle calyx aqueous extract; thus, this extract could be used for foods only after heating process. However, we also found that the antibacterial activity of roselle calyx ethanol extract was not affected by heating treatments at 50° and 75°C; thus, this ethanol extract could be used for foods that require only mild heating treatment such as high-temperature short-time pasteurization used for milk products. Based on the lower MIC values, greater heat-resistant activity and more efficient antibacterial effects in ground beef and apple juice, roselle calyx ethanol extract was a greater antibacterial agent than aqueous extract.

Protocatechuic acid is a compound naturally occurred in roselle calyx. Several in vitro studies have indicated that this compound is able to inhibit the growth of E. coli or fungi (Fernandez et al., 1996; Aziz et al., 1998). Our previous studies also observed that protocatechuic acid was an effective agent against nosocomial infectious bacteria and H. pylori (Liu et al., 2005, 2008). In our present study, we found this compound exhibited heat-resistant antibacterial activity and effectively inhibited the growth of...
five food spoilage bacteria in media, ground beef, and apple juice. These findings support that this compound is a potent agent for food systems to prevent bacterial contamination. On the other hand, our present study found that ethanol extract of roselle calyx contained more protocatechuic acid than aqueous extract. This finding partially explained the greater antibacterial activity of roselle calyx ethanol extract observed in our present study.

Roselle calyx is an edible plant; thus, both aqueous and ethanol extracts should be safe when they are used for food systems to prevent bacterial contamination. Besides protocatechuic acid, it has been reported that roselle calyx contained other phenolic compounds such as anthocyanidins and hydroxycinnamic acids (Farombi and Fakoya, 2005; Sayago-Ayerdi et al., 2007). It is possible that those phenolic compounds also contributed to the observed antibacterial effect of roselle calyx extracts. Since roselle calyx extracts and protocatechuic acid could dose-dependently inhibit several bacteria as observed in our previous and present studies, these agents might also be considered as bactericides and used for farms or slaughter-houses to enhance environmental sanitation. Besides antimicrobial activity, the anti-oxidative effects of roselle calyx extract and protocatechuic acid have been reported (Liu et al., 2002; Farombi and Fakoya, 2005; Hirunpanich et al., 2005; Ali et al., 2006). Thus, the application of protocatechuic acid and the extracts from this plant might provide both antibacterial and anti-oxidative protection for foods, and benefit food preservation.

In conclusion, roselle calyx aqueous and ethanol extracts and protocatechuic acid effectively and dose-dependently inhibited the growth of S. typhimurium DT104, E. coli O157:H7, L. monocytogenes, S. aureus, and B. cereus in ground beef and apple juice. The antibacterial effects of roselle calyx ethanol extract and protocatechuic acid were dose dependent and heat resistant. These findings support that roselle calyx ethanol extract and protocatechuic acid might be potent agents for foods to prevent contamination from these food spoilage bacteria.

Table 4. Bacterial Level (Log CFU/g) in Ground Beef Treated by Roselle Calyx Aqueous Extract (RW), Roselle Calyx Ethanol Extract (RE), or Protocatechuic Acid (PA) at 5 or 10 mg After 3 Days Storage at 25°C. Data Are Expressed as Mean ± SD (n=15–24)

<table>
<thead>
<tr>
<th>Bacteria (isolates)</th>
<th>Control</th>
<th>RW, 5 mg</th>
<th>RW, 10 mg</th>
<th>RE, 5 mg</th>
<th>RE, 10 mg</th>
<th>PA, 5 mg</th>
<th>PA, 10 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhimurium (17)</td>
<td>7.6±0.8e</td>
<td>3.9±0.8d</td>
<td>3.0±0.4c</td>
<td>2.5±0.9c</td>
<td>1.6±0.5b</td>
<td>1.9±0.6b</td>
<td>0.6±0.4a</td>
</tr>
<tr>
<td>E. coli (24)</td>
<td>7.4±0.6e</td>
<td>4.2±0.7d</td>
<td>3.6±0.8c</td>
<td>3.1±1.0c</td>
<td>1.7±0.4b</td>
<td>1.3±0.4b</td>
<td>NDa</td>
</tr>
<tr>
<td>L. monocytogenes (20)</td>
<td>8.0±0.7f</td>
<td>4.6±1.0e</td>
<td>3.9±0.6d</td>
<td>3.1±0.7c</td>
<td>2.0±0.5b</td>
<td>1.8±0.5b</td>
<td>0.8±0.3a</td>
</tr>
<tr>
<td>S. aureus (18)</td>
<td>7.7±0.4e</td>
<td>4.0±0.6d</td>
<td>2.9±0.7c</td>
<td>2.4±0.8c</td>
<td>1.6±0.7b</td>
<td>1.2±0.2b</td>
<td>NDa</td>
</tr>
<tr>
<td>B. cereus (15)</td>
<td>8.2±0.7e</td>
<td>4.5±0.8d</td>
<td>3.8±1.0c</td>
<td>3.3±0.5c</td>
<td>1.9±0.3b</td>
<td>1.4±0.4b</td>
<td>0.5±0.2a</td>
</tr>
</tbody>
</table>

Means in a row without a common letter differ, p < 0.05.

CFU, colony-forming units.

Table 5. Bacterial Level (Log CFU/mL) in Apple Juice Treated by Roselle Calyx Aqueous Extract (RW), Roselle Calyx Ethanol Extract (RE), or Protocatechuic Acid (PA) at 5 or 10 mg After 3 Days Storage at 25°C. Data Are Expressed as Mean ± SD (n=15–24)

<table>
<thead>
<tr>
<th>Bacteria (isolates)</th>
<th>Control</th>
<th>RW, 5 mg</th>
<th>RW, 10 mg</th>
<th>RE, 5 mg</th>
<th>RE, 10 mg</th>
<th>PA, 5 mg</th>
<th>PA, 10 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhimurium (17)</td>
<td>6.8±0.9e</td>
<td>4.0±0.9d</td>
<td>2.6±0.3c</td>
<td>3.1±0.6c</td>
<td>1.6±0.4b</td>
<td>1.7±0.5b</td>
<td>0.9±0.3a</td>
</tr>
<tr>
<td>E. coli (24)</td>
<td>7.0±1.0f</td>
<td>4.3±0.5e</td>
<td>2.1±0.5c</td>
<td>3.2±0.8d</td>
<td>1.4±0.8b</td>
<td>1.5±0.4b</td>
<td>0.4±0.2a</td>
</tr>
<tr>
<td>L. monocytogenes (20)</td>
<td>6.6±0.5f</td>
<td>4.5±0.8e</td>
<td>2.5±0.6c</td>
<td>3.6±1.0d</td>
<td>1.6±0.5b</td>
<td>1.3±0.3b</td>
<td>0.5±0.2a</td>
</tr>
<tr>
<td>S. aureus (18)</td>
<td>6.7±0.7e</td>
<td>3.8±0.4d</td>
<td>2.2±0.4c</td>
<td>2.6±0.8c</td>
<td>1.2±0.2b</td>
<td>1.4±0.4b</td>
<td>0.8±0.3a</td>
</tr>
<tr>
<td>B. cereus (15)</td>
<td>7.1±0.8e</td>
<td>4.2±0.4d</td>
<td>2.8±0.9c</td>
<td>3.3±0.5c</td>
<td>1.5±0.4b</td>
<td>1.9±0.6b</td>
<td>0.7±0.4a</td>
</tr>
</tbody>
</table>

Means in a row without a common letter differ, p < 0.05.

CFU, colony-forming units.
References


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