Short communication

Aqueous extracts of Hibiscus sabdariffa calyces as an antimicrobial rinse on hot dogs against Listeria monocytogenes and methicillin-resistant Staphylococcus aureus

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1. Introduction

Listeria monocytogenes, a Gram-positive bacterium, is ubiquitous in nature and has a zero tolerance in foods in the United States according to the ‘Listeria rule’ (9 CFR 430). Listeria can survive and grow at refrigeration temperatures and is difficult to eliminate once it is present in a processing facility. L. monocytogenes has been associated with foodborne illness outbreaks from a variety of foods, mostly of animal origin including dairy, meat products, and produce (CDC, 1999).

Staphylococcus aureus is a Gram-positive bacterium commonly found in the nose and on the skin of animals and humans (Jay, Loessner, & Golden, 2005, chap. 23). While methicillin-resistant S. aureus (MRSA) has been primarily considered a healthcare associated pathogen, it has been associated with and detected in food products, especially meats. In a recent study in the United States, Waters et al. (2011) examined the contamination of meats and poultry by S. aureus and found 37% contamination (14/38) in beef products. For all of the meat samples tested, 52% of the S. aureus isolates detected were multi-drug resistant (Waters et al., 2011). Further, the rates of animal to human transmission of MRSA are increasing (Lewis et al., 2008).

As the trend for ‘natural products’ consumption grows, alternatives to chemical antimicrobials and sanitizers added to ready-to-eat products need to be discovered in efforts to reduce and eliminate pathogens from contaminating the food supply. A variety
of plant extracts have been shown to provide antimicrobial activities (Cowan, 1999). *Hibiscus sabdariffa* is a subtropical plant species grown in countries such as India, Mexico, and Thailand. Calyces from this plant are often used to prepare a tart beverage (*Hibiscus tea*) that is deeply red in color, often consumed for its presumed medicinal benefits. The calyces of *Hibiscus* have known antimicrobial activity against *S. aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Propanibacterium acnes*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Salmonella*, and *L. monocytogenes* (Chao & Yin, 2009; Chomnawang, Surassmo, Wongrinyu, & Bunyaphrathsara, 2009; Liu, Tsao, & Yin, 2005; Olaleye, 2007). The minimum inhibitory concentration (MIC) for an aqueous *H. sabdariffa* extract against *L. monocytogenes* was found to be 136 ± 24 µg/mL and 84 ± 8 µg/mL for an ethanol extract (Chao & Yin, 2009). Against MRSA, an ethanol extraction of *H. sabdariffa* was found to have an MIC of 5000 µg/mL (Chomnawang et al., 2009).

Often, ready-to-eat products are formulated with antimicrobial agents to inhibit the growth of pathogens. Lactates and diacetates are frequently added to the product formulation (USDA, 2012); however, their use alone has been shown to be ineffective over time after heating. Davidson, Stewart, Zivanovic, & Harte, (2012) with modifications. The calyx extract after the absorbance was read at 765 nm.

2.3. Culture preparation

Stock cultures of *L. monocytogenes* Scott A and 101 were obtained from the Department of Food Science and Technology and MRSA strains ATCC 33591 and ATCC 33593 were purchased from the American Type Culture Collection (Manassas, VA). Cultures were grown in tryptic soy broth (TSB; Becton, Dickinson and Co., Sparks, MD) and stored in glycerol at −20 °C. Working cultures were obtained by inoculating 50 mL TSB with 200 µL stock cultures and incubating for 24 h at 32 °C for *L. monocytogenes* and at 35–37 °C for MRSA and each strain was subcultured once. After incubation cultures had a population of ca. 9 log CFU/mL.

2.4. Hot dog rinse

Beef hot dogs (Kroger Value brand; The Kroger Co., Cincinnati, OH) were inoculated in overnight cultures of 1:1 mixtures of *L. monocytogenes* strains Scott A and 101 or MRSA strains ATCC 33591 and ATCC 33593 (25 mL of each strain for 2 hot dogs) in sterile Whirl-Pak stomacher bags (Nasco, Fort Atkinson, WI). Stomacher bags containing hot dogs were placed at 4 °C overnight to allow for bacterial attachment and were then cut using a sterile blade into 4 equal pieces. Eight pieces per stomacher bag were used and 60 mL of treatments were added (control: water, treatment: *Hibiscus* extract autoclaved for 30 min at 121 °C at the concentrations of 120 and 240 mg/mL). The following rinse times were used: 5, 15, 30, and 60 min, at which time, 2 pieces of hot dog (each piece weighing 10 g) were removed and placed separately into sterile stomacher bags. One piece was used to plate 0 h (no storage) while the other hot dog piece was put at 4 °C overnight, held for 24 h, and plated (24 h storage). For plating, 90 mL of 0.1% peptone was added to the bag and stomached for 30 s at 230 rpm. Serial dilutions were plated in duplicate on TSA and MOX (*Listeria*) or BP (MRSA) and incubated for 48 h at 32 °C for *Listeria* and 24 h at 37 °C for MRSA. The 10 g sample re-suspended into 90 mL 0.1% peptone, a 1:10 dilution, was the lowest dilution plated. A volume of 0.3, 0.3, and 0.4 mL was plated onto 3 different plates, and the counts of all three plates were combined, giving a detection limit of 10 CFU/g. Each experiment was replicated 3 times.

2.5. Statistical analysis

A randomized complete block design was used, blocking on replicate. Split-plot was the treatment design, with extract applied to the whole bag of hot dog pieces and then rinse time (5, 15, 30, and 60 min) and storage time (0 and 24 h) treatment factors applied to the individual pieces. Results were analyzed using ANOVA, using a generalized linear model (SAS 9.3, Cary, NC). Least significant differences were used to determine significant differences among treatment means (p < 0.05).

2. Results

For every 100 g of ground calyces used, approximately 21 g of freeze-dried extract was obtained. The lyophilized extract was also easily re-hydrated. The extract had a pH of approximately 2.5, and the phenolic content was determined to be ca. 16 mg GAE/g freeze-dried extract.

Extracts were more effective at reducing bacterial growth than water for use as a rinse on hot dogs, and extracts at 240 mg/mL were more effective than 120 mg/mL against both *L. monocytogenes* and MRSA (Tables 1 and 2). Also, for both extract concentrations,
increasing rinse times and storage times resulted in significant reductions for both L. monocytogenes and MRSA. Against L. monocytogenes, on TSA, all interactions were significant \((p < 0.05)\) with the exception of the storage and rinse time interaction \((p = 0.34)\) and the extract, storage, and rinse time interaction \((p = 0.06)\). On MOX, all interactions were significant except for the storage and rinse time interaction \((p = 0.45)\). Against MRSA, for both types of media, all interactions were significant \((p < 0.05)\). Overall, higher extract concentrations, longer rinse times, and longer storage times were the most effective at inhibiting and/or killing both L. monocytogenes and MRSA on hot dogs.

### 4. Discussion

In the present study, Hibiscus extracts heated in steam at 121 °C for 30 min were examined for their antimicrobial activity as a rinse on hot dogs to inhibit and/or inactivate the foodborne pathogens, L. monocytogenes and MRSA. The population of L. monocytogenes was reduced when rinsed in the extract but was not eliminated completely. Therefore, a longer rinse time, a higher concentration of extract, or a longer storage time may have reduced the bacterial population further. Higher concentrations of Hibiscus (10 mg compared to 5 mg) when added to ground beef (100 g) were shown to have greater antimicrobial activity against foodborne pathogens (Chao & Yin, 2009).

Autoclaved Hibiscus extracts have been shown to have enhanced antimicrobial activity compared to filtered extracts against S. aureus and E. coli O157:H7 (Higginbotham, Burris, Zivanovic, Davidson, & Stewart, in press). The chemical composition of the bioactive calyces has been partially identified and contains numerous compounds that may be contributing to the observed antimicrobial activity, such as organic acids, phenolic acids, alkaloids, and anthocyanins (Christian & Jackson, 2009; Olaleye, 2007; Tsai, McIntosh, Pearce, Camden, & Jordan, 2002). Gossypetin and protocatechuic acid are two compounds found in Hibiscus that have been shown to have antimicrobial activity (Chao & Yin, 2009; Mounnissamy, Kavimani, & Gunasegaran, 2002). Protocatechuic acid content in an aqueous Hibiscus extract was determined to be 2.8 ± 0.7 mg/g (Chao & Yin, 2009).

Extracts were more effective against MRSA than Listeria. These results could be explained by that fact that hot dogs were stored at 4 °C following rinse treatments. Listeria is able to grow in refrigeration, with a minimum growth temperature of 1.7 ± 0.5 °C (Junttila, Niemela, & Hirn, 1988). At temperatures of 5 and 10 °C, L. monocytogenes were shown to have a greater ability to survive higher salt and lower pH conditions than when kept at 30 °C (Cole, Jones, & Holyoak, 1990). In contrast, S. aureus can survive in refrigeration, but typically does not grow (Notermans & Heuvelman, 1983; Schmitt, Schuler-Schmid, & Schmidt-Lorenz, 1990).

Numerous other rinses to inactivate pathogens on ready-to-eat meat products have been examined. Cetylpyridinium chloride (CPC) at 1% reduced L. monocytogenes on frankfurters ca. 1.4–1.7 log CFU/g after spraying (Singh et al., 2005). However, CPC is a quaternary ammonium compound and might not have the appeal of a natural product with equivalent efficacy, such as a rinse with

### Table 2

Log CFU/g* plated on non-selective tryptic soy agar (TSA) and selective Baird–Parker (BP) media, of methicillin-resistant Staphylococcus aureus (mixture of 1:1 of strains ATCC 33591 and ATCC 33593) following a rinse (5, 15, 30, 60 min) with either water (0 mg/mL) or Hibiscus extract (120 or 240 mg/mL) to inoculated hot dogs and stored at 4 °C for 0 or 24 h.

<table>
<thead>
<tr>
<th>Rinse time (min)</th>
<th>Storage time (h)</th>
<th>Control</th>
<th>Hibiscus extract (120 mg/mL)</th>
<th>Hibiscus extract (240 mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>24</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td><strong>TSA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.64AB</td>
<td>5.38AB</td>
<td>5.71AB</td>
<td>5.67AB</td>
</tr>
<tr>
<td>15</td>
<td>5.48AB</td>
<td>5.61AB</td>
<td>5.79A</td>
<td>5.21B</td>
</tr>
<tr>
<td>30</td>
<td>5.52AB</td>
<td>5.38AB</td>
<td>4.56C</td>
<td>4.66C</td>
</tr>
<tr>
<td>60</td>
<td>5.52AB</td>
<td>5.59AB</td>
<td>4.57C</td>
<td>3.50D</td>
</tr>
<tr>
<td><strong>BP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.71A</td>
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<tr>
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<td>5.31ABCD</td>
<td>5.38ABCD</td>
<td>5.79A</td>
<td>5.44ABCD</td>
</tr>
<tr>
<td>30</td>
<td>5.63AB</td>
<td>5.36ABCD</td>
<td>5.05BCDEF</td>
<td>4.66EFG</td>
</tr>
<tr>
<td>60</td>
<td>5.56ABC</td>
<td>5.67AB</td>
<td>5.02BCDEF</td>
<td>3.42H</td>
</tr>
</tbody>
</table>

* Log CFU/g values in rows and columns, separated by media type, not followed by a like letter are significantly different \((p < 0.05)\).
Hibiscus extracts. A similar study found that a spray consisting of a combination of 5% lactic acid and 0.5% sodium lauryl sulfate applied to frankfurters after being inoculated with L. monocytogenes resulted in a reduction of \(2.8 \pm 0.2\) log CFU/cm\(^2\) after being stored for 90 days (Byelashov, Kendall, Belk, Scanga, & Sofos, 2008). Compared to the present study, after only 24 h, a ca. 3.7 log CFU/g and greater than 5 log CFU/g reduction was observed for L. monocytogenes and MRSA, respectively; however, storage times extending beyond 24 h were not examined. Essential oils of thyme and clove at 5 mL/L reduced growth of L. monocytogenes and MRSA, respectively; however, storage times extending beyond 24 h were not examined. Essential oils of thyme and clove at 5 mL/L and sodium diacetate (0.15%) and found that growth of L. monocytogenes was eliminated pathogens in food products, especially since the calyces and temperature on the survival and growth of Listeria monocytogenes. Journal of Applied Microbiology, 69, 63–72.


