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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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ABSTRACT

Contamination of ready-to-eat meat products by foodborne pathogens is a major concern in the food industry. Novel methods to control foodborne pathogens are made necessary by continuing outbreaks as well as the development of antibiotic-resistant pathogens. *Hibiscus sabdariffa* extracts could be useful as a natural source of antimicrobial rinse on ready-to-eat products to control pathogens. In this study, lyophilized *Hibiscus* flower extracts were examined for their antimicrobial activity as a rinse on all-beef hot dogs against *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* (MRSA). Beef hot dogs were dip inoculated in overnight cultures of 1:1 mixtures of *L. monocytogenes* strains Scott A and 101 or MRSA strains ATCC 33591 and ATCC 33593 and were placed at 4 °C overnight to allow for bacterial attachment. Hot dogs were rinsed with extracts (120, 240 mg/mL) or water (control) for 5, 15, 30, or 60 min and then plated immediately (0 h; no storage) or stored at 4 °C overnight and plated at 24 h. Serial dilutions were plated in duplicate on both TSA and selection media, Modified Oxford (*Listeria*) or Baird Parker (MRSA), and the entire experiment was replicated 3 times. Higher extract concentrations, longer rinse times, and longer storage times were the most effective at inhibiting and/or killing *L. monocytogenes* and MRSA on hot dogs. *L. monocytogenes* was reduced to ca. 1.5 log CFU/g while MRSA was reduced to undetectable levels following rinsing of hot dogs with extracts at 240 mg/mL for 60 min and stored for 24 h. Both *L. monocytogenes* and MRSA were reduced ca. 2 log CFU/g following rinsing of hot dogs with extracts at 120 mg/mL for 60 min and stored for 24 h. This research demonstrates the effectiveness of *Hibiscus* extracts against *L. monocytogenes* and MRSA as an antimicrobial rinse on ready-to-eat meat products.

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

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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*, *Propionibacterium acnes*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Salmonella*, and *L. monocytogenes* (Chao & Yin, 2009; Chomnawang, Surassmo, Wongsariya, & Bunyaprophatsara, 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 to inhibit the growth of *Listeria* (Perumalla et al., 2013). Therefore, combination treatments such as lowering the water activity of the product or changing the pH and including additional post-processing treatments such as steam pasteurization can be used to assist in inhibiting pathogens such as *Listeria* (USDA, 2012). Despite all of these methods used, outbreaks continue to occur. Hot dogs and other ready-to-eat products can become contaminated after the heating process but before they are packaged. In order to prevent outbreaks and/or intoxications from pathogens such as *Listeria* and MRSA, an antimicrobial rinse can be applied before or after heating. *H. sabdariffa* calyx extracts would be a natural option for food manufacturers and could be used in place of or in addition to processes and antimicrobial agents such as the diacetates and lactates that are currently used. The purpose of the present study was to determine the effectiveness of heat treated *Hibiscus* calyx extracts as an antimicrobial rinse on hot dogs against *L. monocytogenes* and MRSA, two pathogens of concern in ready-to-eat meats.

2. Materials and methods

2.1. Preparation of extracts

USDA organic *H. sabdariffa* flowers (calyces) were purchased from Starwest Botanicals (SKU: 209355-31, Sacramento, CA). Extracts were prepared according to published methods (Burris, Davidson, Stewart, Zivanovic, & Harte, 2012) with modifications. Calyces were ground using a blender (Oster, Boca Raton, FL) to less than 1 mm particles and extracted using water at a ratio of 1 g of tissue to 3.6 mL water for 2 h at 4 °C to limit microbial growth in the dark with periodic shaking. Extracts were then centrifuged at $5000 \times g$ for 30 min and filtered through miracloth (EMD Millipore, Billerica, MA) to remove large insoluble particles. Extracts were placed at −80 °C and freeze dried using LabConco FreeZone 12 Liter Freeze Dry System (Kansas City, MO) to concentrate and stored in sealed containers until testing.

2.2. Phenolic content determination

Phenolic content of the extract was determined using Folin–Ciocalteu's phenol reagent (Montreau, 1972). Extracts were re-hydrated in water (1 mg/mL) and autoclaved at 121 °C for 30 min. Extracts were then filtered through Whatman No. 4 after cooling. The phenolic content was quantified using gallic acid as the standard and expressed in mg gallic acid equivalents (GAE)/g dry 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 ($p < 0.05$).

3. 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,

Table 1
Log CFU/g,[a] plated on non-selective tryptic soy agar (TSA) and selective Modified Oxford (MOX) media, of *Listeria monocytogenes* (mixture of 1:1 of strains 101 and Scott A) 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.

		Control		*Hibiscus* extract (120 mg/mL)		*Hibiscus* extract (240 mg/mL)	
		Storage time (h)					
	Rinse time (min)	0	24	0	24	0	24
TSA	5	5.67A	5.44AB	5.61A	5.35AB	5.74A	4.81CD
	15	5.47AB	5.64A	5.66A	5.04BC	5.62A	4.10FG
	30	5.53A	5.47AB	4.75CDE	4.55DEF	4.39EF	3.17I
	60	5.53A	5.29AB	3.84GH	3.68GH	3.41HI	1.62J
MOX	5	5.59A	5.32ABCD	5.52ABC	5.34ABCD	5.61AB	4.58FG
	15	5.27ABCD	5.34ABCD	5.33ABCD	5.06DF	5.33ABCD	3.77HI
	30	4.99DEF	5.12CDE	4.68EG	4.05H	4.15H	2.96J
	60	5.19BCD	5.23ABCD	3.82H	3.40IJ	3.23J	1.41K

[a] Log CFU/g values in rows and columns, separated by media type, not followed by a like letter are significantly different ($p < 0.05$).

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,[a] 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.

		Control		*Hibiscus* extract (120 mg/mL)		*Hibiscus* extract (240 mg/mL)	
		Storage time (h)					
	Rinse time (min)	0	24	0	24	0	24
TSA	5	5.64AB	5.38AB	5.71AB	5.67AB	5.61AB	5.27AB
	15	5.48AB	5.61AB	5.79A	5.21B	5.38AB	4.21C
	30	5.58AB	5.35AB	4.56C	4.66C	4.61C	2.49E
	60	5.52AB	5.59AB	4.57C	3.50D	4.63C	NDF
BP	5	5.71A	5.43ABCD	5.81A	5.55ABC	5.68AB	5.28ABCDE
	15	5.33ABCD	5.38ABCD	5.79A	5.44ABCD	5.60ABC	4.31G
	30	5.63ABC	5.36ABCD	5.05BCDEF	4.66EFG	4.89DEF	2.37I
	60	5.56ABC	5.67AB	5.02CDEF	3.42H	4.52FG	NDJ

ND = below detection level of 10 CFU/g.
[a] 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 ± 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 resulted in reductions of 0.67–1.05 and 1.15–1.71 log CFU/g respectively of *L. monocytogenes* on hot dogs where storage time was not a factor (Singh, Singh, Bhunia, & Singh, 2003). However, essential oils are known for their pungent odor and taste and may result in undesirable sensory effects, especially at levels necessary to see comparable reductions to our *Hibiscus* rinse. Singh et al. (2003) also found that increasing the fat content of the hot dog resulted in decreased effectiveness of the essential oils. The fat content of the hot dogs used in this study was 28.5%, which would be considered a full-fat hot dog. Another option to control pathogens on hot dogs, which was recently studied, demonstrated the effects of adding natural extracts of green tea (0.35%) and grape seed (0.22%) along with two antimicrobial preservatives, potassium lactate (1.5%) and sodium diacetate (0.15%) and found that growth of *L. monocytogenes* was inhibited over a 28 d period, but the use of the antimicrobial preservatives alone were not as effective (Perumalla et al., 2013). The hot dogs used in the present study contained potassium lactate, sodium lactate, sodium diacetate, and sodium nitrite, which might have acted additively or synergistically with our *Hibiscus* extracts to further inhibit the growth of *L. monocytogenes* and MRSA.

5. Conclusions

Aqueous extracts of *H. sabdariffa* calyces were effective against both *L. monocytogenes* and MRSA as a rinse on hot dogs. A significant bactericidal effect was observed when high concentrations of the extract were applied to hot dogs followed by 24 h refrigeration storage. *H. sabdariffa* extracts could be used as a natural alternative to traditional antimicrobial preservatives to prevent growth and/or eliminate pathogens in food products, especially since the calyces are extracted in water, and *Hibiscus* is considered generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (21 CFR 172.510). Additional sensory analysis would need to be performed to ensure consumer acceptance since hot dogs were observed to increase in red color with higher concentrations of extract and longer rinse times.

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