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Efficacy of roselle (*Hibiscus sabdariffa*) calyx formulations against *Escherichia coli* O157:H7 during flume-washing of organic leafy greens

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RESEARCH ARTICLE

Abstract

Foodborne outbreaks due to contaminated fresh produce, including organic leafy greens, have increased in the last twenty years. Several of these outbreaks have been associated with *Escherichia coli* O157:H7 infections. The limited availability of approved antimicrobial washes for organic leafy greens warrants the need to seek alternative approaches by the organic produce industry. The present study evaluated the use of roselle (*Hibiscus sabdariffa*) calyx formulations as flume-tank wash treatments for four types of organic leafy greens (iceberg and romaine lettuce, and mature and baby spinach). These formulations were prepared as either 10, 20, or 30% concentrations (v/v) of roselle aqueous extract or as 100% concentration (v/v) of roselle tea. Leafy greens were inoculated with a cocktail of 3 *E. coli* O157:H7 strains and washed with appropriate antimicrobial treatments. Treated leafy greens were tested for surviving *E. coli* O157:H7 populations on 0, 1, and 3 days. Results from the study indicated a 1.4-2.5 log cfu (colony forming units)/g reduction overall for the 20% concentration of roselle extract by day 3, on all leafy greens. Baby spinach treated with 30% roselle extract showed a 3.7 log cfu/g reduction in pathogen population by day 3. Treatment with roselle tea resulted in 2.0-3.4 log cfu/g reductions of *E. coli* O157:H7 for all leafy greens by the third day. This study demonstrates the potential of roselle calyx formulations for reduction of *E. coli* O157:H7 populations during flume-washing of organic leafy greens.

Keywords: inhibition, fresh produce, wash-water, foodborne pathogens, plant extracts, antimicrobials

1. Introduction

In recent estimates by the Centers for Disease Control and Prevention (CDC), approximately 48 million Americans fall victim to foodborne illnesses each year, out of which 128,000 are hospitalised, and 3,000 are expected to die. Of the total number of foodborne disease cases that result in hospitalisation, 62% are caused by bacteria, with *Escherichia coli* O157:H7 listed among the top five (Scallan *et al.*, 2011). Although frequently associated with beef and dairy products, *E. coli* O157:H7 has recently been identified as the causative agent for a number of fresh produce related outbreaks (CDC, 2012, 2015).

Modern lifestyle of today's consumer has resulted in an increased demand for ready-to-eat foods, which include pre-washed and bagged leafy greens. Recent trends have also shown an increased preference for fresh or minimally processed organic fresh produce (Dimitri and Greene, 2002; Pollack, 2001). However, these trends have also been accompanied by an increase in foodborne illnesses associated with fresh produce. A recent example is the multistate outbreak of *E. coli* O157:H7 traced back to contaminated organic spinach and spring-mix blend (CDC, 2012). According to Olsen *et al.* (2000), 61% of foodborne illnesses associated with fresh produce are caused by shigatoxigenic *E. coli* and *Salmonella enterica*. Therefore, there is a need for effective control measures for foodborne pathogens during the production and processing of fresh produce.

Intervention strategies are currently in place for minimally processed leafy greens, with the flume-washing of greens as the primary decontamination step. However, the organic fresh produce industry faces further challenges in that the use of chemical sanitisers or pesticides is prohibited according to the United States Department of Agriculture-National Organic Program standards (http://www.ams.usda.gov/AMSv1.0/nop). If chlorine is to be used in the wash water, only 4 mg/kg of free chlorine is permitted according to the US Environmental Protection Agency standards (Plotto and Narciso, 2006). However, there are concerns over the use of chlorine as a sanitiser. Studies have shown that chlorine may not be sufficiently effective at reducing pathogen populations on fresh produce (Beuchat, 1999; Brackett, 1992; Cherry, 1999; Taormina and Beuchat, 1999). Environmentally, chlorine wash water demonstrates limited reusability and may result in an environmental burden (Olmez and Kretschmar, 2009). Furthermore, chlorine can react with organic matter to produce carcinogenic compounds such as chloramines and trihalomethanes (Gallard and Gunten, 2002; Komulainen, 2004; Olmez and Kretschmar, 2009). Hydrogen peroxide, acetic acid, ozone, and peroxyacetic acid have also been used as alternative wash-treatments for organic fresh produce. Of these treatments, use of hydrogen peroxide is the most common and desirable due to its potent antimicrobial activity. However, it has been indicated by several studies that hydrogen peroxide is not effective on all types of produce or bacterial pathogens (Beuchat and Ryu, 1997; Buddhini *et al.*, 2014; Denton *et al.*, 2015; Park and Beuchat, 1999; Taormina and Beuchat, 1999). For instance, 2% hydrogen peroxide treatment of cantaloupe cubes resulted in less than 1 log reduction of *Salmonella* populations (Beuchat and Ryu, 1997). In a study by Denton *et al.* (2015), hydrogen peroxide reduced *E. coli* O157:H7 populations by less than 2 logs on baby spinach. Furthermore, hydrogen peroxide treatment has shown to cause severe browning in shredded lettuce and mushrooms (Lin *et al*, 2002; Olmez and Kretschmar, 2009) and therefore may not be suitable for all produce types. Due to limited wash-treatment options for organic produce, it is necessary to seek alternative intervention strategies.

Previous research has revealed that natural antimicrobials such as plant-derived extracts and essential oils have the potential to replace traditional sanitising agents (Buddhini *et al.*, 2014; Denton *et al.*, 2015; Friedman *et al.*, 2011, 2013; Ganesh *et al.*, 2010, 2012; Gupta and Ravishankar, 2005; Kim *et al.*, 2010; Orue *et al.*, 2013; Ravishankar *et al.*, 2008, 2009; Uhart *et al.*, 2006). Plant-derived antimicrobials are also generally regarded as safe, and are therefore becoming widely popular in the food industry.

Roselle (*Hibiscus sabdariffa*) is an edible shrub, the leaves, flowers, roots, and seeds of which are used in various food applications. The fleshy edible calyces of this flowering plant have been used for many applications including the production of wine, juices, jams, puddings, syrups, ice cream, or tea. Roselle is also known to have antiseptic, antimutagenic, diuretic and antioxidant properties (Chewonarin *et al.*, 1999; Onyenekwe *et al.*, 1999; Salleh *et al.*, 2002). The flowers contain flavonoids such as anthocyanins (gossipetine and hibiscin) and the flavanol hibiscritin (Duke, 1983). Roselle calyces have also been found to be rich in ascorbic acid, niacin, riboflavin, carotene, calcium, and iron (Duke, 1983).

Previous research involving food matrices such as lettuce, spinach, ground beef and fruit juices has suggested that roselle calyx formulations can be effective in reducing foodborne pathogens. In the present study, effects of aqueous extracts of roselle against *E. coli* O157:H7 were validated for single-wash, flume-tank washing of four types of organic leafy greens.

2. Materials and methods

Bacterial culture preparation and media

On the day of the experiment, a cocktail (1:1; v/v) of three *E. coli* O157:H7 strains (ATCC: 43895, 43888 and 35150) was prepared from the overnight cultures of each strain. The cocktail was further diluted in buffered peptone water (BPW; Oxoid Ltd., Basingstoke, UK) to obtain the dip inoculum (10^6 cfu/ml) for leafy greens. Populations of overnight cultures and dip inoculum were confirmed by plating appropriate dilutions on tryptic soy agar (TSA; Acumedia, Lansing, MI, USA) and Sorbitol MacConkey agar (SMAC; Remel Inc., Lenexa, KS, USA).

Preparation of organic leafy greens samples

Organic baby spinach, mature bunched spinach, romaine lettuce, and iceberg lettuce were obtained from local grocery stores in Oklahoma. Leafy greens (checked for freshness and not very close to the sell-by date) were bought on the day of the experiment, transported on ice, and stored under refrigerated conditions (4 °C) until use. All leafy greens were washed thoroughly for two minutes under running tap water (23-25 °C) to remove dirt and organic particles. Damaged outer leaves of iceberg and romaine lettuce and the core of iceberg lettuce were discarded. The stems of mature spinach were removed and individual leaves separated. Using aseptic techniques, the leaves of romaine and iceberg lettuce and mature spinach were cut into 3.8×3.8 cm pieces with sterile scissors. Baby spinach leaves (approximately 3.8-5.0 cm leaves) were used as such from the bag.

Preparation of roselle calyx formulations

The roselle calyx aqueous extracts and tea were prepared as previously described by Jaroni and Ravishankar (2012). On the day of the experiment, the aqueous extract was mixed with appropriate volume of sterile phosphate buffered saline (PBS: sodium chloride, Fischer Scientific, NJ, USA; potassium chloride, sodium phosphate monobasic and sodium phosphate dibasic, Sigma-Aldrich, MO, USA) to obtain concentrations (v/v) of 10%, 20% and 30%.

Efficacy of roselle formulations against E. coli O157:H7 on organic leafy greens

Approximately 200 g samples of each leafy green, prepared above, were washed three times in sterile distilled water (23-25 °C), using gentle back-and-forth motion for two minutes. Leafy greens were then transferred to a bio-hood and exposed to UV (254 nm) light for 30 minutes (15 minutes on each side of the leaf) to reduce potential background microflora. A 20 g sample was set aside to be used as the negative control, while the rest of the greens were dip inoculated (Lang *et al.*, 2004) for two minutes (180 g greens in 1,800 ml inoculum) with the *E. coli* O157:H7 cocktail. Inoculated greens were then placed under the bio-hood for 30 minutes to facilitate attachment. Approximately 20 g of each inoculated leafy greens samples was set aside as the positive control, while the remaining greens were separated into 20 g samples. Using gentle agitation, each of these leafy green samples were washed for two minutes in the appropriate antimicrobial treatments (200 ml each), including sterile distilled water and 3% hydrogen peroxide as industry controls. Since the various concentrations of the roselle aqueous extract were prepared in PBS, a control of PBS was also included to wash the leafy greens. Immediately after washing, the leaves were removed from the liquid and any excess liquid shaken off before placing into a sterile Whirl-Pak™ bag (NASCO, Fort Atkinson, WI, USA). Treated greens were stored at 4 °C for three days. Surviving *E. coli* O157:H7 populations were determined on days 0, 1, and 3 by transferring 5 g of each treated leafy green sample into a sterile Whirl-Pak™ bag containing 45 ml BPW and stomaching (Stomacher® 400 Circulator; Seward Ltd, London, UK) at 230 rpm for 1 minute. Samples were serially diluted in BPW and plated on SMAC or TSA to recover any injured pathogen populations. Plates were incubated at 37 °C for 18-24 hours and colonies of *E. coli* O157:H7 (cfu/g) enumerated.

Statistical analysis

Data was analysed using ANOVA to determine differences among means for leafy greens, antimicrobial treatments, and storage time (in days) using PROC MIXED of Statistical Analysis (SAS version 9.3; SAS Institute, Cary, NC, USA). The experimental design was a 4×8×3 factorial in a completely randomised design comparing four leafy greens (baby spinach, mature bunched spinach, romaine and iceberg lettuce) and eight treatments (positive control, hydrogen peroxide, PBS, water, roselle tea and 10, 20, and 30% roselle aqueous extracts) with three storage times (0, 1, and 3 days). The populations of *E. coli* O157:H7 were converted to log cfu/g. All experiments were conducted in triplicate. The α-level was set as 0.05.

3. Results and discussion

Antimicrobial efficacy of roselle calyx aqueous extract against *E. coli* O157:H7 on organic leafy greens

Results of the study are shown in Table 1-4. In comparison to the positive control, all three concentrations (10, 20, and 30%) of roselle calyx aqueous extracts significantly reduced *E. coli* O157:H7 populations ($P<0.05$) on all the leafy greens by day 3. Of the three concentrations tested, 30% concentration of roselle calyx aqueous extract was the most effective, reducing pathogen populations by 1.4-3.7 logs cfu/g on the four types of leafy greens. In baby

Table 1. Antimicrobial effects of roselle tea and 10, 20, and 30% roselle calyx aqueous extract against *Escherichia coli* O157:H7 on baby spinach at 4 °C.[1,2]

	Surviving *E. coli* O157:H7 population (log_{10} cfu/g)			Log reduction		
Treatment	Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
PC	4.6^{a}	4.8^{a}	4.7^{a}	–	–	–
PBS	4.4^{a}	4.2^{a}	4.1^{a}	0.3	0.6	0.6
HP	2.0^{b}	3.3^{b}	3.5^{b}	2.6	1.5	1.2
Water	3.5^{a}	3.5^{b}	4.7^{a}	1.1	1.3	0.0
RC 10%	3.3^{a,b}	3.0^{b}	2.4^{c}	1.4	1.8	2.3
RC 20%	2.0^{b}	2.6^{b}	2.2^{c,d}	2.6	2.2	2.5
RC 30%	2.3^{b}	0.9^{c}	1.0^{d}	2.4	3.9	3.7
RT	3.1^{a}	2.8^{b}	2.4^{c}	1.5	2.1	2.3

[1] PC = positive control; PBS = phosphate buffered saline; HP = hydrogen peroxide; RC = roselle calyx aqueous extract; RT = roselle tea.
[2] Values represent average mean of three replications, with letters a, b, c, etc., within a column, indicating significant difference ($P<0.05$) between treatments.

Table 2. Antimicrobial effects of roselle tea and 10, 20, and 30% roselle calyx aqueous extract against *Escherichia coli* O157:H7 on mature spinach at 4 °C.[1,2]

	Surviving *E. coli* O157:H7 population (log_{10} cfu/g)			Log reduction		
Treatment	**Day 0**	**Day 1**	**Day 3**	**Day 0**	**Day 1**	**Day 3**
Control	4.6[a]	4.7[a]	4.8[a]	–	–	–
PBS	3.4[a]	3.6[a,b]	3.3[b]	1.2	1.2	1.5
HP	2.9[b]	2.3[b]	2.5[b]	1.6	2.4	2.2
Water	3.7[a]	3.3[b]	3.3[b]	0.9	1.4	1.5
RC 10%	3.7[a]	3.8[a,b]	3.4[b]	0.9	0.9	1.3
RC 20%	3.3[a,b]	3.7[a,b]	3.0[b]	1.3	1.1	1.8
RC 30%	3.2[a,b]	3.1[b]	3.1[b]	1.4	1.6	1.7
RT	4.5[a]	4.4[a]	3.7[b]	1.3	2.4	2.0

[1] PC = positive control; PBS = phosphate buffered saline; HP = hydrogen peroxide; RC = roselle calyx aqueous extract; RT = roselle tea.
[2] Values represent average mean of three replications, with letters a, b, c, etc., within a column, indicating significant difference ($P<0.05$) between treatments.

Table 3. Antimicrobial effects of roselle tea and 10%, 20%, and 30% roselle calyx aqueous extract against *Escherichia coli* O157:H7 on romaine lettuce at 4 °C.[1,2]

	Surviving *E. coli* O157:H7 population (log_{10} cfu/g)			Log reduction		
Treatment	**Day 0**	**Day 1**	**Day 3**	**Day 0**	**Day 1**	**Day 3**
Control	5.2[a]	5.1[a]	5.3[a]	–	–	–
PBS	3.7[b]	3.9[a]	3.6[b]	1.5	1.2	1.7
HP	1.6[c]	2.0[c]	2.3[c]	3.6	3.1	3.0
Water	3.1[b]	3.6[b]	3.5[b]	2.0	1.5	1.8
RC 10%	3.6[b]	3.5[b]	4.1[b]	1.6	1.6	1.2
RC 20%	3.2[b]	3.5[b]	3.9[b]	2.0	1.6	1.4
RC 30%	3.5[b]	2.9[b,c]	2.2[c]	1.6	2.2	3.1
RT	3.3[b]	2.3[c]	1.9[c]	1.9	2.8	3.4

[1] PC = positive control; PBS = phosphate buffered saline; HP = hydrogen peroxide; RC = roselle calyx aqueous extract; RT = roselle tea.
[2] Values represent average mean of three replications, with letters a, b, c, etc., within a column, indicating significant difference ($P<0.05$) between treatments.

spinach, the antibacterial effects of 20% and 30% roselle extracts were seen immediately following the treatment (day 0), where *E. coli* O157:H7 populations were reduced by 2.6 and 2.4 logs cfu/g, respectively (Table 1). The highest reduction in pathogen populations (3.7 log cfu/g), by day 3, was observed in baby spinach washed with 30% roselle calyx extract. In romaine lettuce (Table 3), the 30% roselle calyx extract reduced *E. coli* O157:H7 by more than 2.0 logs cfu/g on day 1. The 20% roselle calyx extract showed a reduction of 1.3-2.0 logs cfu/g by day 0 on mature spinach and iceberg and romaine lettuce. Treatment with 10% roselle calyx aqueous extract resulted in a reduction of 2.3 logs cfu/g on baby spinach (Table 1) and between 1.2-1.3 logs cfu/g on the rest of the greens by day 3 (Tables 2-4). Visual observations of the leafy greens washed with roselle calyx formulations did not reveal any changes to their sensory properties.

In studies conducted by Jaroni and Ravishankar (2012), treatment with 100% roselle calyx aqueous extract resulted in undetectable levels of *E. coli* O157:H7 populations by day 1 on romaine lettuce and by day 3 *in vitro*. The present study did not yield such high reductions, however, significant reductions ($P<0.05$) with all three concentrations of roselle calyx extract were observed (Tables 1-4). The 30% roselle calyx aqueous extract was able to reduce pathogen populations by 3.7 and 3.1 logs cfu/g on baby spinach and romaine lettuce (Tables 1 and 3), respectively, by day 3.

Table 4. Antimicrobial effects of roselle tea and 10%, 20%, and 30% roselle calyx aqueous extract against *Escherichia coli* O157:H7 on iceberg lettuce at 4 °C.[1,2]

	Surviving *E. coli* O157:H7 population (log_{10} cfu/g)			Log reduction		
Treatment	Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
Control	5.3[a]	5.1[a]	5.0[a]	–	–	–
PBS	3.8[b]	3.6[b]	3.8[b]	1.6	1.5	1.2
HP	1.4[c]	2.3[c]	1.8[c]	3.9	2.8	3.2
Water	3.4[b]	3.5[b]	4.0[b]	1.9	1.6	1.0
RC 10%	3.7[b]	3.4[b]	3.7[b]	1.7	1.7	1.3
RC 20%	3.8[b]	3.2[b]	3.5[b]	1.7	1.9	1.5
RC 30%	3.3[b]	3.2[b]	3.4[b]	2.0	1.9	1.6
RT	3.3[b]	3.0[b]	2.7[b]	2.0	2.1	2.3

[1] PC = positive control; PBS = phosphate buffered saline; HP = hydrogen peroxide; RC = roselle calyx aqueous extract; RT = roselle tea.

[2] Values represent average mean of three replications, with letters a, b, c, etc., within a column, indicating significant difference ($P<0.05$) between treatments.

These findings are also consistent with those of Moore *et al.* (2011), where the 30% roselle calyx extract yielded the highest reduction in *S. enterica* populations on organic greens compared to the 10% and 20% concentrations. This suggests that roselle calyx aqueous extracts could potentially be used at concentrations lower than 100% to significantly reduce pathogen populations on fresh produce. However, the efficacy of these concentrations may vary with the type of leafy greens.

Furthermore, the study by Moore *et al.* (2011) was carried out with *S. enterica* Newport, showing reductions of 0.3, 1.0, 1.0, and 0.1 log cfu/g by day 3 on romaine lettuce, iceberg lettuce, mature spinach, and baby spinach, respectively, when treated with 30% roselle calyx formulation. The present study yielded higher reductions in *E. coli* O157:H7 populations with 30% roselle aqueous extract (Tables 1-4), suggesting that roselle calyx formulations may be more effective against *E. coli* O157:H7 than *S. enterica* Newport. Another study by Chao and Yin (2008) demonstrated a reduction of 1.6-2.5 logs cfu/g when roselle calyx aqueous extracts were used against antibiotic resistant *Campylobacter* species in ground beef. In the present study, the highest reduction in pathogen population was 3.7 logs cfu/g, observed with baby spinach. These differences may be attributed to the type of bacterium, food matrix, and methods used. Investigations into the antimicrobial activity of roselle extracts against *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *E. coli* strains by Khalaphallah and Soliman (2014) also revealed that the effects of roselle extracts varied with the type of bacterium. Similarly, a study by Chao and Yin (2009), investigating the effectiveness of roselle calyx extracts against foodborne pathogens in ground beef and apple juice showed that higher concentrations of roselle aqueous extracts were required for inhibiting *E. coli* O157:H7 (128 ug/ml) than for *Salmonella* (120 ug/ml).

Treatment with hydrogen peroxide resulted in 1.6-3.9 log cfu/g reductions by day 0 in all the leafy greens. These reductions were not significantly different ($P>0.05$) from those seen after treatment of baby and mature spinach with all three concentrations of roselle calyx aqueous extract (Tables 1-2). It was also observed that in baby spinach, the effect of hydrogen peroxide was reduced over the three-day storage, when compared with the three concentrations of roselle aqueous extract that continued to reduce pathogen populations (Table 1).

In the present study, when washed with either of the three concentrations of roselle calyx extract, baby spinach showed the highest reduction in *E. coli* O157:H7 compared to the rest of the leafy greens (Table 1). A study by Higginbotham *et al.* (2013) demonstrated that 2% concentration of roselle aqueous extract was able to reduce *E. coli* O157:H7 by 3-4 logs cfu/ml in skim and whole milk. Likewise, in a study by Yin and Chao (2009), treatment with 5 or 10 mg of roselle calyx extract in 100 g ground beef or 100 ml apple juice resulted in *E. coli* O157:H7 population reductions of 3.8 log cfu/g and 4.9 log cfu/g, respectively. In the present study, higher concentrations of roselle extract were required to bring about similar pathogen population reductions. These results may be attributed to the differences in the food matrices tested in the studies, as well as the methods used. Results from the present study indicate that the interaction between pathogen and fresh produce surface may have an effect on the efficacy of antimicrobials and therefore, may be an important factor to consider when evaluating antimicrobial efficacy of roselle calyx formulations.

Antimicrobial efficacy of roselle tea against *E. coli* O157:H7 on leafy greens

Romaine lettuce treated with roselle tea showed the highest reduction (3.4 log cfu/g) in *E. coli* O157:H7 populations by day 3 when compared to the positive control (Table 3). Microbial reductions observed by day 3 on the rest of the leafy greens were as follows: 2.3 log cfu/g on iceberg lettuce and baby spinach, and 2.0 log cfu/g on mature spinach. For baby spinach, a significantly higher reduction ($P<0.05$), in comparison to treatment with hydrogen peroxide, was observed by the third day of storage (Table 1). In a previous study by Jaroni and Ravishankar (2012), romaine lettuce treated with roselle tea and stored at 4 °C showed a 3.8 log cfu/g reduction in *E. coli* O157:H7 populations by day 3. Similar results were observed in the present study, where a 3.4 log cfu/g reduction in *E. coli* O157:H7 population by day 3 resulted after treatment with roselle tea (Table 3).

4. Conclusions

Roselle calyx aqueous extracts and tea may have the potential to be used as flume-tank wash treatments of organic leafy greens for inhibition of *E. coli* O157:H7. Using them in combination with other natural sanitising agents may enhance their antimicrobial efficacy and calls for further examination. Even though no visual sensory attribute changes were observed for leafy greens washed with roselle formulations, further studies are required to determine consumer acceptability and palatability, and for sensory evaluation of the tested leafy greens.

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Conflict of interest

The authors have no conflict of interest to declare.

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