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Comparison of the antimicrobial activities of roselle calyx extracts and chemical sanitizers directly onto contaminated cucumbers

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RESEARCH ARTICLE

Abstract

Cucumbers are associated with foodborne disease caused by *Salmonella*. Fruits and vegetables in restaurants and homes in Mexico are disinfected using sodium hypochlorite and colloidal silver solution. However, a number of studies show that chemical agents used to eliminate pathogenic bacteria have a limited antimicrobial effect. Previous studies show that roselle calyx extracts have antimicrobial effects. Therefore, the present study examined the attachment of 14 foodborne bacteria to whole cucumber in the presence of *Hibiscus sabdariffa* calyx extracts, sodium hypochlorite, acetic acid, and colloidal silver. Cucumbers were inoculated with 14 bacteria: *Listeria monocytogenes*, *Shigella flexneri*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Salmonella* Typhi, *Salmonella* Montevideo, *Salmonella* Choleraesuis, *Escherichia coli* O157:H7, non-O157:H7-Shiga toxin-producing *E. coli*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*, and *Vibrio cholerae* O1. The antibacterial effect of four roselle calyx extracts (in water, methanol, acetone, and ethyl acetate) and some chemical sanitisers including sodium hypochlorite, colloidal silver, and acetic acid were then examined. All foodborne bacteria successfully attached to cucumber. The acetonic and methanolic roselle extracts caused a greater reduction (up to 4 log cfu in some cases) in the concentration of all foodborne bacteria than sodium hypochlorite, colloidal silver, and acetic acid. Thus, roselle calyx extracts may be a potentially useful disinfectant for cucumbers in the field, in processing plants, and in restaurants and homes.

Keywords: disinfection, roselle, *Hibiscus sabdariffa*, antibacterial, cucumber, *Cucumis sativus*

1. Introduction

The cucumber (*Cucumis sativus*) is a major commercial crop in Mexico, accounting for over 817,799 tons of production in 2015 (http://tinyurl.com/ybdgagdo). The product is consumed in a raw state (e.g. in slices) in Mexico and in other countries. However, *Salmonella* outbreaks caused by *Salmonella* serotypes such as Saintpaul (CDC, 2013), Newport (Angelo *et al.*, 2015), and Poona (CDC, 2015) have been traced to the consumption of contaminated cucumbers. In these cases, contamination probably occurred in the production environment (Jung, 2015).

As with other vegetables, pathogenic bacteria are most likely to contaminate cucumbers during growing, harvesting, and packing. A wide variety of pathogens has been identified as a cause of foodborne disease associated with fruits and vegetables. In developing countries such as Mexico, the main pathogens are *Salmonella*, *Shigella*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Vibrio cholerae*, with the diarrheagenic *Escherichia coli* pathotypes (DEPs) becoming prominent more recently (Aguilar *et al.*, 1982; Estrada-Garcia *et al.*, 2009; Parrilla *et al.*, 1993; Secretaría de Salud, 2016). In Mexico, *Salmonella* and DEPs have been isolated from alfalfa sprouts (Castro-Rosas and Escartin,

1999; Rangel-Vargas *et al.*, 2015), parsley, cauliflower, lettuce, spinach (Quiroz-Santiago *et al.*, 2009), jalapeño and serrano peppers (Castro-Rosas *et al.*, 2011; Cerna-Cortes *et al.*, 2012), raw nopalitos (*Opuntia ficus-indica* L.), nopalitos salads (Gómez-Aldapa *et al.*, 2016a), and coriander (Gómez-Aldapa *et al.*, 2016b). *V. cholerae* O1 is a halophilic marine bacterium and commonly *V. cholerae* O1 is isolated from ocean water and marine food. However, in Mexico *V. cholerae* O1 has been isolated from sewage water and the environment (Alam *et al.*, 2014; Choi *et al.*, 2016; Flisser *et al.*, 2002; Sepulveda *et al.*, 2006). In addition, *V. cholerae* O1 also has been isolated from different vegetables in other countries (Mrityunjoy *et al.*, 2013). It is highlighting that *V. cholerae* O1 could contaminate raw vegetables such as cucumbers from contaminated sewage water or environment.

Organic acids and sodium hypochlorite are often used to reduce microbial contamination of fruits and vegetables in the field, processing plants, restaurants, and homes (Al-Zenki *et al.*, 2012). However, a number of studies shows that organic acids and sodium hypochlorite have a limited or no antimicrobial effect (Castro-Rosas and Escartin, 1999; Gutiérrez-Alcántara *et al.*, 2016a,b). In the 21st century, the use of chemical disinfectants is increasingly regarded as environmentally unsound as it is associated with occupational and operational hazards. Indeed, research suggests that excessive use of chlorine can be harmful due to the formation of carcinogenic by-products such as trihalomethanes, chloramines, haloketones, chloropicrins, and haloacetic acids caused by a reaction between residual chlorine and organic matter (Gil *et al.*, 2009). Thus, the use of these compounds is forbidden in European countries such as the Netherlands, Sweden, Germany, and Belgium, and in American countries such as the USA (Ölmez and Kretzschmar, 2009). In response, there is a trend in applying other alternatives, such as antimicrobials from plants. Many studies are now in progress searching for alternative antimicrobials from plants, for example, antimicrobial from roselle calyx.

Roselle (*Hibiscus sabdariffa*) calyces have antimicrobial effects against pathogenic bacteria (Gutiérrez-Alcántara *et al.*, 2016a). Previous studies used only aqueous roselle calyx extracts to disinfect romaine lettuce and alfalfa sprouts contaminated with *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* (Jaroni and Ravishankar, 2012); organic romaine lettuce, iceberg lettuce and baby spinach contaminated with *Salmonella* (Moore *et al.*, 2011). Recently, we reported the antimicrobial effects of four roselle calyx extracts (in water, methanol, acetone, and ethyl acetate) against multidrug-resistant *Salmonella* strains on carrots (Gutiérrez-Alcántara *et al.*, 2016a) and tomatoes (Gutiérrez-Alcántara *et al.*, 2016b). However, no data are available regarding the activity of these roselle calyx extracts against *Salmonella* Typhimurium, *Salmonella* Typhi, *Salmonella* Montevideo, *L. monocytogenes*, *Shigella flexneri*, *S. aureus*, *V. cholerae* O1, *E. coli* O157:H7, Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAEC) present on cucumbers. Roselle extracts may be a potential agent for controlling foodborne bacteria on whole cucumbers. The aim of the present study was to examine the potential of 14 foodborne bacteria to attach to cucumber and to compare the antibacterial effects of different roselle calyx extracts with those of sodium hypochlorite, acetic acid, and colloidal silver.

2. Materials and methods

Bacterial strains

The foodborne bacteria strains used in the study are reported in Table 1. Mutant strains resistant to rifampicin (100 mg/l; R+) were obtained for all experiment strains (Castro-Rosas *et al.*, 2010). These R+ strains were streaked onto tryptic soy agar (TSA; Bioxon, Mexico City, Mexico) slants and maintained at 3 to 5 °C, with weekly transfers onto TSA. All strains maintained rifampicin resistance throughout the study.

Cucumber preparation

Cucumbers (approximately 15 cm long) were obtained from a local grocery store and disinfected with sodium hypochlorite solution (pH=5.0; 200 mg/l free chlorine) by immersion for 5 min. They were then washed for 1 min in sterile tap water to remove any remaining sodium hypochlorite solution before being dried in a strainer and placed on a stainless steel tray in a laminar flow biosafety hood at room temperature for 1 h to remove surface moisture.

Preparation of inocula and inoculation of cucumbers

Cucumbers were inoculated as previously described (Gutiérrez-Alcántara *et al.*, 2016a). Briefly, tryptic soy broth tubes (TSB; 3 ml; Bioxon) were inoculated with rifampicin-resistant (R+) *S.* Typhimurium, *S.* Montevideo, *S.* Typhi, *Salmonella* Choleraesuis, *L. monocytogenes*, *S. aureus*, *S. flexneri*, *E. coli* O157:H7, STEC, EIEC, EPEC, ETEC, EAEC, and *V. cholerae* O1 strains and incubated at 35 °C for 18 h. The cultures were then washed twice in sterile isotonic saline solution (ISS; NaCl, 0.85%) by centrifugation at 1,500×*g* for 20 min, and the pellets were resuspended in sterile peptone water to yield a final bacterial concentration of about 9 log cfu/ml. For each of the four EPEC, ETEC, EIEC, and STEC strains, an inocula cocktail was prepared from the same pathotype by mixing together 1 ml of each washed suspension. Individual suspensions of *S.* Typhimurium, *S.* Montevideo, *S.* Typhi, *S.* Choleraesuis, *L. monocytogenes*, *S.*

Table 1. Foodborne bacteria strains used in the trials.

Bacteria type[1]	Bacterial strains	Source[2]	Reference
Salmonella Typhimurium	ATCC 14028	n/a	n/a
Salmonella Choleraesuis	ATCC 10708	n/a	n/a
Listeria monocytogenes	ATCC 19115	n/a	n/a
Staphylococcus aureus	ATCC 25923	n/a	n/a
Shigella flexneri	ATCC 12022	n/a	n/a
Salmonella Typhi	SS6	tomatoes	Gutiérrez-Alcántara *et al.*, 2016b
Salmonella Montevideo	Z6	carrots	Gutiérrez-Alcántara *et al.*, 2016a
Escherichia coli O157:H7	E09	donated by E.F. Escartín, Universidad Autónoma de Querétaro, Mexico	n/a
Vibrio cholerae O1 serotype Inaba	87151	donated by E.F. Escartín	n/a
EAEC	EAFP	donated by Dr J.F. Cerna-Cortes, Instituto Politécnico Nacional, Mexico	n/a
EIEC	EISCM13	ready-to-eat salad	Castro-Rosas *et al.*, 2012
	EICJB121	carrot juice	Torres-Vitela *et al.*, 2013
	EIMS79	mung bean sprout	Cerna-Cortes *et al.*, 2013
ETEC	ETSP7	serrano peppers	Cerna-Cortes *et al.*, 2012
	ETJPI	jalapeño peppers	Cerna-Cortes *et al.*, 2012
	ETSAS22	ready-to-eat salad	Castro-Rosas *et al.*, 2012
Non-O157-STEC	STSP41	serrano peppers	Cerna-Cortes *et al.*, 2012
	STJP6	jalapeño peppers	Cerna-Cortes *et al.*, 2012
	STSCM23	ready-to-eat salad	Castro-Rosas *et al.*, 2012
EPEC	EPNW7	nopalitos (*Opuntia ficus-indica*)	Gómez-Aldapa *et al.*, 2016a
	EPNC6	nopalitos (*O. ficus-indica*)	Gómez-Aldapa *et al.*, 2016a
	EPCSA225	ready-to-eat cooked salad	Bautista-De León *et al.*, 2013

[1] EAEC = enteroaggregative *E. coli*; EIEC = enteroinvasive *E. coli*; EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*; STEC = Shiga toxin-producing *E. coli.*
[2] n/a = not applicable.

aureus, *S. flexneri*, *E. coli* O157:H7, EAEC, and *V. cholerae* or a cocktail suspension of each EPEC, ETEC, EIEC, and STEC pathotype were diluted in sterile 0.1% peptone to yield approximately 8 log cfu/ml bacteria. Whole cucumbers were inoculated with approximately 6 log cfu by placing 20 µl of the suspension inside a circle (approx. 0.5 cm in diameter) marked on the cucumber surface. After inoculation, the cucumbers were stored at 22±2 °C for 30 min to allow bacteria to adhere to the surface.

Microbiological counts

We initially observed that the native microorganisms from cucumbers were capable of growing in selective media used for bacterial counts (e.g. violet red bile agar or bismuth sulfite agar; Bioxon) and thus interfered in the plate counts. In response, we used pathogenic bacteria resistant to rifampicin (Rif), a broad-spectrum antibiotic. Rif completely inhibited (<10 cfu) the native bacteria from cucumber on TSA plates containing 100 mg/l Rif. We did not expect the behaviour of these Rif-resistant pathogenic strains on cucumber to differ from that of the native strains, and, indeed, no difference (P>0.05) was observed between the growth patterns of resistant and non-resistant pathogenic strains (data not shown) in TSB monitored by Bioscreen C Automated Growth Curves Analysis System (Growth Curves USA, Piscataway, NJ, USA). These data agree with our results from a previous study showing that attachment of *V. cholerae* O1 Rif-resistant strains to shrimp and crab carapaces did not differ compared with the parent strain (Castro-Rosas and Escartin, 2002).

As described above, after the 30 min of incubation that allowed the bacteria to adhere to cucumber surface, the inoculated area was washed for 1 min applying directly sterile tap water from a wash bottle to remove non-adhered pathogenic cells. The number of pathogenic bacteria was counted by removing the washed inoculated area from each cucumber with a sterile knife (to a depth of approx. 0.5 cm) and placing it in a sterile bag (each sterile bag contained one sample) containing 10 ml of 0.1% sterile peptone water. The sample was then dispersed manually by rubbing the

bag for 2 min. Counts were performed using the plate counting method after spreading appropriate dilutions (1 ml) of each bacterial suspension on TSA plates containing rifampicin (100 mg/l). Plates were then incubated at 35±2 °C for 24-48 h.

Preparation of roselle calyx extracts

A sample (5 kg) of dehydrated roselle (*H. sabdariffa* var. Criolla de Oaxaca) calyces was used. Dried calyces (500 g) were weighed and placed in sterile glass flasks, and 2 l of methanol, acetone, or ethyl acetate (the concentration of each solvent was 99.8%) was added to each flask. The flasks were then sealed and stored at room temperature for 7 days, after which the liquid phase was filtered through filter paper and concentrated in a rotary evaporator (V-800 vacuum controller; Büchi Labortechnik AG, Flawil, Switzerland). Concentrates were placed on glass plates and placing them in a recirculating air incubator (Ambi-Hi-Low Chamber; Labline Instruments, Kochi, Kerala, India) for 24 h at 45±1 °C to eliminate solvents completely from the concentrates.

An aqueous extract was produced by placing dried calyces (500 g) in a sterile glass flask, adding 5 l of distilled water, heating the mixture to boiling for 10 min, and then allowing it to cool to room temperature. Water was eliminated from the concentrate as described above. All dried concentrated extracts were stored in sterile plastic bags at room temperature until used.

A solution was prepared from 10 g of each extract concentrate using 90 ml deionised water (90 ml) for the aqueous and methanol extracts, and a 100 ml solution of tween 80-distilled water (2:10, v/v) for the acetone and ethyl acetate extracts (final extract concentration, 100 mg/ml, and pH=3.5).

Preparation of disinfectant solutions

Disinfectant solutions were prepared as previously described (Gutiérrez-Alcántara *et al.*, 2016a). In brief, 1 l of sodium hypochlorite solution (pH=5.0; 200 mg/l free chlorine) was prepared from a sodium hypochlorite solution (10%; J.T. Baker, Mexico City, Mexico) by dilution in deionised water. Free available chlorine was determined using the method of iodometric titration (Greenberg *et al.*, 1992). The acetic acid solution was prepared by diluting glacial acetic acid (Sigma-Aldrich) in distilled water to yield a 0.5% solution. Colloidal silver (approximately 3.5 mg/l) was prepared by diluting a commercial solution (Microdyn® 0.35% of colloidal silver; Mercancías Saludables S.A de C.V., Mexico City, Mexico) in deionised water in accordance with the instructions on the product label (eight drops of Microdyn per 1 l of water).

Disinfection and microbiological counts

Disinfection and microbiological counts were performed as previously described (Gutiérrez-Alcántara *et al.*, 2016a). Briefly, aliquots (50 ml) of each calyx extract, sodium hypochlorite, acetic acid, colloidal silver, or ISS (control) were placed in individual trays. The inoculated portion of each cucumber was then immersed in a treatment solution or ISS for 10 min. After removal from the tray, the inoculated and treated area (marked by a circle) was washed for 1 min with sterile tap water to remove any treatment solution residue. After washing, pathogenic bacteria were counted as described above.

Experimental design

Each experiment (disinfectant solutions and roselle extracts) performed using individual rifampicin-resistant strains or a cocktail suspension of each *E. coli* pathotype was tested in three independent trials. Each trial was performed in triplicate. The number of bacteria on replicate plates per sample was counted. The results were then converted to log cfu per inoculated area.

Statistical analysis

Statistically significant differences ($P<0.05$) were examined using analysis of variance and Duncan's test. All statistical analyses were performed using the STATISTICA ver. 8 program (StatSoft, Inc., Tulsa, OK, USA).

3. Results and discussion

Attachment study

Contamination of fresh produce can occur as a result of pre-harvest factors, including the indigenous microbial population in the soil environment and the level of fertiliser in the soil, which ultimately affects the microbial load. Post-harvest factors include handling, container sanitation, and food processing procedures (Beuchat and Ryu, 1997). Cucumbers have a very thick shell-like, waxy, rough skin that primarily functions as a permeable barrier against moisture and gas loss. The epicuticular wax covering the outer surface also repels water. Other functions include scattering of short wave radiation, preventing the attachment of microorganisms, and preventing adherence between the developing organs of the plant (Jeffree, 2008).

Foodborne bacteria attached to the cucumber skin may contaminate the edible parts of the fruit when it is manipulated.

The inoculation method used in this study involves direct inoculation of the cucumber surface. The presence of the pathogens was therefore expected. All tested foodborne

bacteria attached to cucumbers (Table 2). In general, there was no significant difference in the number of pathogenic bacteria attached to the cucumbers (P>0.05) (Table 2).

Attachment is a pre-requisite for colonisation and subsequent spread via the edible parts of fruits and vegetables. Indeed, once attached, it is very difficult to remove the pathogens by washing alone (Beuchat and Scouten, 2002). In addition, once foodborne bacteria have attached to the fruit, they may remain there throughout its shelf life. It is worth mentioning that this is the first report in the literature to show the ability of attachment of *S. aureus*, non-O157:H7-STEC, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli* and *V. cholerae* O1 to cucumbers.

Disinfection study

The ability of the roselle calyx extracts and the disinfectants to reduce the counts of foodborne bacteria present on the surface of cucumbers is shown in Table 3. Overall, the effect of colloidal silver on the number of pathogenic bacteria attached to the cucumber surface was not significantly different from that of control treatment (P>0.05). Colloidal silver solution is widely used to disinfect fruit and vegetables in restaurants and home in Mexico. Even, the Health Secretariat of Mexico promotes their use as disinfectants of fruit and vegetables (COFEPRIS, 2015; Secretaría de Salud, 2015). However, there is little information published in scientific journals about the use of colloidal silver as a disinfectant of fruits and vegetables. In Mexico, Microdyn (colloidal silver in gelatine) is sold in supermarkets to disinfect salad vegetables and drinking water. However, as noted, in this study colloidal silver did not significantly reduce the concentration of pathogens on cucumber. García-Gómez *et al.* (2002), have reported a limited reduction in concentration of *Salmonella* when they disinfected lettuce and coriander with Microdyn. In addition, Soto Beltran *et al.* (2013), have reported limited effect of Microdyn in the reduction in concentration of *S.* Typhimurium from bell peppers. On the other hand treatment with hypochlorite and acetic acid led to a greater reduction in bacterial counts than colloidal silver (P<0.0.5) (Table 3), although there was a significant different between hypochlorite and acetic acid (Table 3): acetic acid treatment resulted in a 0.8-1.3 log cfu reduction in bacterial counts whereas sodium hypochlorite led to a 1.3-1.8 log cfu reduction. The water and ethyl acetate calyx extracts also led to a greater reduction (P<0.05) in the counts of all pathogenic bacteria than colloidal silver or control treatment (Table 3). In most cases, these reductions were not significantly greater than those observed for sodium hypochlorite (P>0.05); however, the EIEC, EAEC, and *V. cholerae* counts were significantly lower than those obtained using sodium hypochlorite (Table 3).

The water and ethyl acetate calyx extracts caused reductions on foodborne bacteria levels of between 1.3 to 2.4 log cfu and 1.3 to 2.5 log cfu, respectively (Table 3). On the other hand, the reductions in bacterial counts after treatment with methanolic (2.1-3.5 log cfu) and acetonic (2.3-4 log cfu) roselle calyx extracts were greater (P<0.05) than those

Table 2. Attachment values (numbers in log cfu ± standard deviation) of fourteen foodborne bacteria per inoculated area of cucumber.

Microorganism[1]	Initial inoculum on cucumber	Values after washing of cucumber (attached cells)[2]
Listeria monocytogenes	5.92±0.24	4.45±0.18[a]
Shigella flexneri	5.96±0.18	4.04±0.22[a]
Staphylococcus aureus	5.98±0.16	3.96±0.25[a]
Salmonella Typhimurium	5.92±0.20	4.25±0.20[a]
Salmonella Typhi	5.76±0.18	4.14±0.24[a]
Salmonella Choleraesuis	6.00±0.22	4.18±0.16[a]
Salmonella Montevideo	5.76±0.20	4.06±0.18[a]
Escherichia coli O157:H7	5.96±0.16	4.35±0.18[a]
STEC	6.04±0.24	4.28±0.24[a]
EPEC	6.05±0.20	4.26±0.20[a]
ETEC	6.05±0.18	4.35±0.18[a]
EIEC	5.86±0.24	4.16±0.16[a]
EAEC	5.95±0.18	3.94±0.26[a]
Vibrio cholerae	5.98±0.24	3.98±0.18[a]

[1] EAEC = enteroaggregative *E. coli*; EIEC = enteroinvasive *E. coli*; EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*; STEC = Shiga toxin-producing *E. coli.*

[2] Different superscript letter in same column indicate significant differences (P<0.05) according to Duncan's multiple range test.

Table 3. Reductions in concentration (numbers in log cfu recovered after treatments per inoculated area ± standard deviation) of fourteen foodborne bacteria on cucumber in response to roselle calyx extracts, sodium hypochlorite, colloidal silver and acetic acid treatments 1.98.[1,2]

Microorganism	Initial concentration	Control (ISS)	Colloidal silver	Acetic acid	Sodium hypochlorite	Water extract	Ethyl acetate extract	Methanol extract	Acetone extract
Listeria monocytogenes	4.45±0.18^{a}	4.40±0.18^{a}	4.08±0.25^{a}	3.17±0.24^{b}	2.97±0.22^{b}	2.94±0.23^{b}	2.84±0.25^{b}	1.96±0.18^{c}	1.84±0.19^{c}
Shigella flexneri	4.04±0.22^{a}	3.96±0.24^{a}	3.75±0.22^{a}	2.98±0.18^{b}	2.74±0.24^{b}	2.66±0.22^{b}	2.57±0.25^{b}	1.76±0.22^{c}	1.64±0.24^{c}
Staphylococcus aureus	3.96±0.25^{a}	3.94±0.22^{a}	3.74±0.17^{a}	2.94±0.22^{b}	2.55±0.25^{b}	2.51±0.24^{b}	2.64±0.18^{b}	1.86±0.24^{c}	1.66±0.20^{c}
Salmonella Typhimurium	4.25±0.20^{a}	4.14±0.23^{a}	3.85±0.23^{a}	3.09±0.19^{b}	2.57±0.16^{c}	2.64±0.05^{c}	2.46±0.24^{c}	1.96±0.20^{d}	1.62±0.22^{d}
Salmonella Typhi	4.14±0.24^{a}	4.10±0.25^{a}	3.75±0.25^{a}	3.12±0.20^{b}	2.56±0.19^{c}	2.57±0.20^{c}	2.46±0.22^{c}	1.97±0.17^{d}	1.64±0.24^{d}
Salmonella Choleraesuis	4.18±0.16^{a}	4.24±0.20^{a}	3.85±0.23^{a}	3.24±0.24^{b}	2.67±0.20^{c}	2.70±0.20^{c}	2.45±0.25^{c}	1.76±0.23^{d}	1.56±0.18^{d}
Salmonella Montevideo	4.06±0.18^{a}	3.97±0.25^{a}	3.65±0.25^{a}	3.06±0.20^{b}	2.65±0.24^{c}	2.62±0.22^{c}	2.34±0.22^{c}	1.78±0.20^{d}	1.45±0.25^{d}
Escherichia coli O157:H7	4.35±0.18^{a}	4.25±0.25^{a}	3.94±0.24^{a}	3.10±0.20^{b}	2.65±0.25^{c}	2.62±0.24^{c}	2.33±0.21^{c}	1.76±0.22^{d}	1.51±0.23^{d}
STEC	4.28±0.24^{a}	4.14±0.22^{a}	4.00±0.20^{a}	3.23±0.21^{b}	2.48±0.24^{c}	2.56±0.25^{c}	2.35±0.25^{c}	1.67±0.21^{d}	1.43±0.21^{d}
EPEC	4.26±0.20^{a}	4.20±0.25^{a}	4.07±0.25^{a}	3.37±0.23^{b}	2.63±0.25^{c}	2.45±0.20^{c}	2.42±0.23^{c}	1.86±0.20^{d}	1.54±0.25^{d}
ETEC	4.35±0.18^{a}	4.25±0.24^{a}	4.05±0.25^{a}	3.47±0.23^{b}	2.61±0.23^{c}	2.43±0.24^{c}	2.35±0.25^{c}	1.76±0.21^{d}	1.45±0.23^{d}
EIEC	4.16±0.16^{a}	4.11±0.23^{a}	4.09±0.24^{a}	3.25±0.25^{b}	2.49±0.21^{c}	1.96±0.20^{d}	1.82±0.25^{d}	1.05±0.20^{e}	0.46±0.20^{f}
EAEC	3.94±0.26^{a}	3.83±0.25^{a}	3.64±0.24^{a}	2.96±0.22^{b}	2.26±0.20^{c}	2.05±0.23^{cd}	1.58±0.25^{d}	1.00±0.20^{e}	0.0^{f}
Vibrio cholerae	3.98±0.18^{a}	4.00±0.20^{a}	3.65±0.25^{a}	2.82±0.25^{b}	2.20±0.22^{c}	1.66±0.20^{d}	1.56±0.20^{d}	0.52±0.25^{e}	0.0^{f}

[1] EAEC = enteroaggregative *E. coli*; EIEC = enteroinvasive *E. coli*; EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*; ISS = isotonic saline solution; STEC = Shiga toxin-producing *E. coli.*

[2] Different letters in the same row indicate significant differences (P<0.05) according to Duncan's multiple range test.

achieved by sodium hypochlorite or by the acetic acid, water, and ethyl acetate calyx extracts (Table 3).

On the other hand, the differences in antimicrobial activity between roselle extracts (aqueous, ethyl acetate, and acetonic and methanolic) could be attributed to the differences in polarity of solvents used. It has been reported that in the roselle calices there are different compounds with antimicrobial activity such as flavonoids (e.g. gossypetin) (Mounnissamy *et al.*, 2002), organic and phenolic acids (e.g. citric acid, hydroxycitric acid, hibiscus acid, protocatechuic acid) (Liu *et al.*, 2005). Many of these antimicrobial compounds have a polarity similar to methanol or acetone (Ali *et al.*, 2005; Borrás-Linares *et al.*, 2015). The antimicrobial activity of roselle calyces has been attributed to compounds such as protocatechuic acid and anthocyanins (Liu *et al.*, 2005), but no published research has yet identified the specific compounds responsible for this activity. Further research will be needed to identify the active compounds and their effective concentrations.

Roselle calyx extracts have been used to disinfect carrots (Gutiérrez-Alcántara *et al.*, 2016a), tomatoes (Gutiérrez-Alcántara *et al.*, 2016b), romaine lettuce and alfalfa sprouts (Jaroni and Ravishankar, 2012), and organic leafy vegetables (romaine lettuce, iceberg lettuce, and baby spinach) (Moore *et al.*, 2011). Gutiérrez-Alcántara *et al.* (2016a), treated carrots contaminated with multidrug-resistant *Salmonella* serotypes (Typhi, Typhimurium, Montevideo and Gaminara) isolated from carrots. Carrots contaminated with multidrug-resistant *Salmonella* serotypes were treated by immersion in roselle calyx extracts (water, methanol, acetone and ethyl acetate) concentrates at 5% by 10 min. Application of the four calyx extracts concentrates resulted in a 2 log reduction in the concentration of the multidrug-resistant *Salmonella* serotypes at rate at least double than sodium hypochlorite and acetic acid. In other study, Gutiérrez-Alcántara *et al.* (2016b), treated tomatoes contaminated with multidrug-resistant *Salmonella* serotypes (Typhi and Typhimurium) isolated from tomatoes. Tomatoes contaminated with multidrug-resistant *Salmonella* serotypes were treated by immersion in roselle calyx extracts (water, methanol, acetone and ethyl acetate) concentrates at 5% by 5 min. Application of the roselle calyx extracts resulted in a 2-2.5 log cfu reduction in the concentration of the two multidrug-resistant *Salmonella* serotypes. Jaroni and Ravishankar (2012), reported an approximately 1 log cfu reduction in the number of *S.* Newport bacteria on alfalfa sprouts and romaine lettuce immediately after exposure to aqueous roselle extracts. Moore *et al.* (2011), treated leafy greens (romaine lettuce, iceberg lettuce, and baby spinach) contaminated with *S.* Newport by immersion in water roselle calices extracts (at 10, 20, and 30%, wt/wt) for 2 min. The *S.* Newport

populations on all three greens showed an immediate 1 log cfu reduction upon exposure to the 30% roselle extract, but no statistically significant reductions (P>0.05) were observed after exposure to the 10 and 20% concentrations compared with the control. In that study, the effect of roselle calix aqueous extract was limited on leafy greens, this differences could be attributed to type of food, type of bacteria, and type of method used to obtain roselle calyx extracts. The antimicrobial activity of water roselle calyx extracts was also evaluated using contaminated ground beef. Chao and Yin (2008), treated ground beef and apple juice contaminated with *S.* Typhimurium, *E. coli* O157:H7, *L. monocytogenes*, *S. aureus*, and *Bacillus cereus*. Liquid (with solvent) roselle calyx water and ethanol extracts at 5 or 10 mg was mixed with 100 g of ground beef or 100 ml of apple juice and stored at 25 °C for 3 days. After storage, the addition of roselle calyx extracts 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 in both ground beef and apple juice (P<0.05). Higginbotham *et al.* (2014a) treated milk at various fat concentrations with aqueous roselle calyx extracts. Aqueous extracts were sterilised by autoclaving. Autoclaved extracts were then tested for antibacterial activity in milk pasteurised with various fat concentrations (<0.5% (skim milk), 1, 2, and >3.25% (whole milk)) against *E. coli* O157:H7 and *S. aureus*. Extracts at 40 mg/ml inactivated *S. aureus* after 168 h in skim and whole milk, and *E. coli* O157:H7 was inactivated after 96 h in 60 mg/ml extract in all fat levels. In other study, Higginbotham *et al.* (2014b), treated hot dogs contaminated with *L. monocytogenes* and methicillin-resistant *S. aureus* with aqueous roselle calyx extracts. Aqueous extracts were autoclaved for 30 min at 121 °C at the concentrations of 120 and 240 mg/ml. Hot dogs were rinsed with extracts for 5, 15, 30, or 60 min and stored for 24 h at 4 °C. Higher extract concentrations, longer rinse times, and longer storage times were the most effective at inhibiting *L. monocytogenes* and methicillin-resistant *S. aureus*; *L. monocytogenes* was reduced to ca. 1.5 log cfu/g while methicillin-resistant *S. aureus* was reduced to undetectable levels. Both *L. monocytogenes* and methicillin-resistant *S. aureus* 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.

It is important to highlight that roselle calyces are considered GRAS (generally recognised as safe) and are approved for use as a colouring agent in the USA by the Food and Drug Administration; roselle calyces are listed as acceptable natural substances and natural adjuvants in the Code of Federal Regulations (21 CFR 172.510; CFR, 2017). In addition, roselle extracts are characterised by a very low degree of toxicity; the LD_{50} of *H. sabdariffa* calyx extracts in rats have been found to be above 5,000 mg/kg (Ali *et al.*, 2005). In view of its reported pharmacological and antimicrobial properties and relative safety, *H. sabdariffa* extracts could be a source of useful products.

Roselle calyx extracts are a promising alternative method of reducing or eliminating foodborne bacteria from the surface of cucumbers. Thus, water, methanolic and acetonic roselle calyx extracts (completely free of methanol and acetone solvents) and antimicrobial compounds from these extracts may be a useful addition to disinfectants used to treat cucumbers in the field, in processing plants, and in restaurants and homes.

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