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Antimicrobial Activity of *Hibiscus sabdariffa* Aqueous Extracts against *Escherichia coli* O157:H7 and *Staphylococcus aureus* in a Microbiological Medium and Milk of Various Fat Concentrations

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ABSTRACT

Hibiscus sabdariffa L. calyces are widely used in the preparation of beverages. The calyces contain compounds that exhibit antimicrobial activity, yet little research has been conducted on their possible use in food systems as antimicrobials. Aqueous extracts prepared from the brand ''Mi Costenita'' were sterilized by membrane filtration (0.22-μm pore size) or autoclaving (121°C, 30 min) and tested for antimicrobial activity against the foodborne pathogens *Escherichia coli* O157:H7 strains ATCC 43894 and Cider and *Staphylococcus aureus* strains SA113 and ATCC 27708 in a microbiological medium and ultrahigh-temperature-processed milk with various fat percentages. Extracts heated by autoclaving exhibited greater activity than did filtered extracts in a microbiological medium. Against *E. coli,* results of 20 mg/ml filtered extract were not different from those of the control, whereas autoclaved extracts reduced viable cells ca. 3 to 4 log CFU/ml. At 60 mg/ml, both extracts inactivated cells after 24 h. There were reduced populations of both strains of *S. aureus* (ca. 2.7 and 3 log CFU/ml, respectively) after 24 h of incubation in 40 mg/ml filtered extracts. When grown in autoclaved extracts at 40 mg/ml, both strains of *S. aureus* were inactivated after 9 h. Autoclaved extracts had decreased anthocyanin content (2.63 mg/liter) compared with filtered extracts (14.27 mg/liter), whereas the phenolic content (48.7 and 53.8 mg/g) remained similar for both treatments. Autoclaved extracts were then tested for activity in milk at various fat concentrations (skim [<0.5%], 1%, 2%, and whole [>3.25%]) against a 1:1 mixture of the two strains of *E. coli* O157:H7 and a 1:1 mixture of the two strains of *S. aureus*. Extracts at 40 mg/ml inactivated *S. aureus* after 168 h in skim and whole milk, and *E. coli* was inactivated after 96 h in 60 mg/ml extract in all fat levels. These findings show the potential use of *Hibiscus* extracts to prevent the growth of pathogens in foods and beverages.

According to the Centers for Disease Control and Prevention, an estimated 48 million people are sickened by foodborne diseases each year in the United States *(3)*. Shiga toxin–producing *Escherichia coli* O157:H7 causes an estimated 63,000 illnesses annually, resulting in 20 deaths *(28)*. *Staphylococcus aureus* is estimated to cause 240,000 foodborne related illnesses each year, with >1,000 hospitalizations and 6 deaths *(28)*. *S. aureus* is often transmitted from human contamination and produces enterotoxins, which can cause gastrointestinal illness when consumed *(14)*. There are 13 known staphylococcal enterotoxins, and the form(s) of enterotoxin encoded depends on the isolate *(14)*. Foodborne illnesses are also costly, resulting in expenses of approximately $51 billion each year in the United States *(28)*. It is a worthy goal to decrease the number of illnesses from foodborne pathogens.

Novel antimicrobials from natural sources are new tools with the potential to help ensure a safe food supply while decreasing the use of synthetic antimicrobials. *Hibiscus sabdariffa* L. (family Malvaceae) is an annual, tropical or subtropical shrub that is native to an area from Malaysia to India. *Hibiscus* is grown in many countries including Sudan, Mexico, India, and Thailand. While many species of *Hibiscus* are used as ornamentals, the red calyces of *H. sabdariffa* are used in the preparation of a flavorful and tart cold or hot beverage. These calyces have been shown to contain numerous bioactive compounds. The majority of compounds found within the calyces that exhibit antimicrobial activity are polyphenolic compounds, some of which have also been shown to demonstrate antioxidant activity *(12, 30)*. One group of polyphenolic compounds present in *Hibiscus* calyx extract is the flavonoids, which includes the plant pigments called anthocyanins. The two anthocyanins present in the highest amount that have been identified in *Hibiscus*, delphinidin-3-sambubiside and cyanidin-3-sambubioside *(9),* are responsible for the deep red pigment of the calyces and were also found to be the major contributors to antioxidant activity *(30)*. Other compounds found in the calyces include phenolic acids such as gallic and protocatechuic acid *(24)*.

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Hibiscus extracts have been shown to have a wide range of antimicrobial activity against bacteria. Methanol extractions of the calyces have shown to have antimicrobial activity against *S. aureus, Bacillus stearothermophilus, Micrococcus luteus, Serratia marcescens, Clostridium sporogenes, E. coli, Klebsiella pneumoniae, Bacillus cereus,* and *Pseudomonas* sp. at concentrations of 0.30 ± 0.2 to 1.30 ± 0.2 mg/ml *(23)*. Navarro García et al. *(21)* found that the MICs for aqueous calyx (*Hibiscus sabdariffa*) extracts were 0.5 and 1.0 mg/ml for *S. aureus* ATCC 6358 and *E. coli* ATCC 8937, respectively. However, there is limited research regarding the extracts' possible use as antimicrobials in foods and beverages. Ethanol and aqueous extracts of *H. sabdariffa* (5 or 10 mg added to 100 g of ground beef or 100 ml of apple juice) showed dose-dependent inhibitory effects against *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes, B. cereus,* and *S. aureus* after 3 days of storage conditions, with ethanol extracts demonstrating greater antimicrobial activity *(4)*. Recently, a study that examined the use of aqueous extracts of *H. sabdariffa* (100%, vol/vol) as a wash on lettuce against *E. coli* O157:H7 and sprouts against *S. enterica* was performed. Bacterial populations of approximately 4 log CFU of *E. coli* O157:H7 and *S. enterica* per g were eliminated after 24 h *(13)*.

The objective of this study was to determine the antimicrobial effectiveness of aqueous *Hibiscus* extracts against *E. coli* O157:H7 and *S. aureus* in milk as a model food system and to explore the chemical composition of both filtered and autoclaved extracts.

MATERIALS AND METHODS

Preparation of extract. Aqueous extracts from the dried calyces of *H. sabdariffa* of the brand ''Mi Costenita,'' purchased from a local international market (Knoxville, TN), were prepared according to published methods *(1)* with modifications. Dried calyces were finely ground (to <1-mm particles) with a blender (Oster, Boca Raton, FL). Extracts were obtained by adding sterile water at a ratio of 3.6 ml to 1 g of ground tissue and allowed to stand for 2 h at 4°C in the dark with occasional mixing to maximize extraction. The mixture was then centrifuged at 5,000 × *g* for 30 min to remove large particles. Aqueous extracts were then dialyzed (3,500 molecular weight cutoff; SnakeSkin Pleated Dialysis Tubing, Pierce Biotechnology, Rockford, IL) for 36 h against deionized water with three water changes at 4°C in the dark to remove low-molecular-weight compounds. Resulting extracts were centrifuged at 5,000 × *g* for 30 min to remove sediment and then frozen at −80°C. Frozen extracts were lyophilized with the LabConco FreeZone 12 Liter Freeze Dry system (Kansas City, MO) and stored in a sealed container at room temperature.

Preparation of cultures. *E. coli* O157:H7 strains ATCC 43894 and Cider were stock cultures obtained from the Department of Food Science and Technology at the University of Tennessee, Knoxville, and *S. aureus* strains SA113 and ATCC 27708 were obtained from the Center for Environmental Biotechnology at the University of Tennessee, Knoxville (courtesy of Dr. Steven Ripp). Cultures were grown in tryptic soy broth (TSB; BD, Sparks, MD) and stored in glycerol at −20°C. Working cultures were obtained by inoculating 50 ml of TSB with 200 μl of stock cultures and then incubating them for 24 h at 35 to 37°C. *S. aureus* strain SA113 was subcultured once due to slower growth. After incubation, cultures were diluted to ca. 5.0 to 6.0 log CFU/ml and tested for antimicrobial activity.

Time-kill assays. Processed lyophilized extracts were resuspended in 10 ml of sterile water and filtered through a 0.22-μm-pore-size Express PES Membrane (Millipore, Billerica, MA) or autoclaved at 121°C for 30 min to sterilize them. The antimicrobial activity levels of filtered and autoclaved samples were compared in a microbiological medium against each strain of *E. coli* O157:H7 (final concentrations of 20, 40, and 60 mg of extract per ml) and *S. aureus* (final concentrations of 2.5, 20, and 40 mg of extract per ml). Autoclaved samples at the final concentrations of 60 and 40 mg/ml were then tested in ultrahigh-temperature-processed (UHT) milk against a 1:1 mixture of *E. coli* O157:H7 and a 1:1 mixture of *S. aureus* strains, respectively. Resuspended extracts were mixed with 2.5 ml of bacterial cultures harvested at late logarithmic phase and diluted to ca. 5.0 to 6.0 log CFU/ml. Bacteria and extracts were incubated in 12.5 ml of TSB at 35 to 37°C or UHT milk at various fat concentrations (skim milk [<0.05% fat; Hershey's, Hershey, PA] and 1% fat, 2% fat, and whole [>3.25% fat] milk [Parmalat, Parma, Italy]) at room temperature. At regular intervals (at 0, 3, 6, and 24 h for *E. coli* or 0, 3, 6, 9, and 24 h for *S. aureus* for TSB and at 0, 6, 24, 48, 96, and 168 h for UHT milk), a bacterial suspension sample was collected, serially diluted in 0.1% peptone, plated in duplicate with tryptic soy agar (BD, Sparks, MD), and incubated for 24 h at 35 to 37°C. The next day, CFU were enumerated. Controls were prepared in a similar way with sterile water. The pH of the milk was decreased to 3.7 after the addition of the *Hibiscus* extracts; thus, the milk used for the controls was adjusted with 1 N HCl so that all milk samples had a pH of 3.7.

Anthocyanin and phenolic content. The pH differential method *(15)* was used to determine the anthocyanin content present in the extracts (either filtered through a 0.22-μm-pore-size membrane or autoclaved at 121°C for 30 min) at the concentration of 1 mg/ml. Absorbance was read at 520 and 700 nm at pH 1.0 and 4.5 with a dilution factor of 3 expressed in milligrams of cyanidin-3-glucoside equivalents per liter (molecular weight = 449.2; ε = 26,900). To determine the amount of polymerized color, extracts were bleached with a bisulfite solution, and the absorbance was measured at 420, 520, and 700 nm *(11)*. Percent polymeric color was calculated by the following formula: (polymeric color/color density) × 100. The polymeric color and color density were calculated as follows: $[(A_{420\ nm} - A_{700\ nm}) + (A_{520\ nm} - A_{700\ nm})]$ × dilution factor. The color density is based on water-diluted extract, whereas the polymeric color is based on the extract diluted with the bisulfite solution *(11)*. Phenolic content was determined via Folin-Ciocalteu's phenol reagent *(19)*. Rehydrated extracts (1 mg/ml) were either filtered through 0.22-μm-pore-size filters or autoclaved. Extracts were additionally filtered through Whatman no. 4 filters after cooling. The phenolic content was quantified with gallic acid as the standard and expressed in milligrams of gallic acid equivalents (GAE) per gram of dry calyx extract after the absorbance was read at 725 nm.

Statistical analysis. Results for the anthocyanin and phenolic content were tabulated as the mean values of three replications ± standard error. For the time-kill assays, the experiments were repeated twice and duplicate plating was also used. Statistical analysis was performed in a completely randomized design by analysis of variance to test for significance. The general linear model was used (SAS 9.3, SAS Institute, Cary, NC). Least

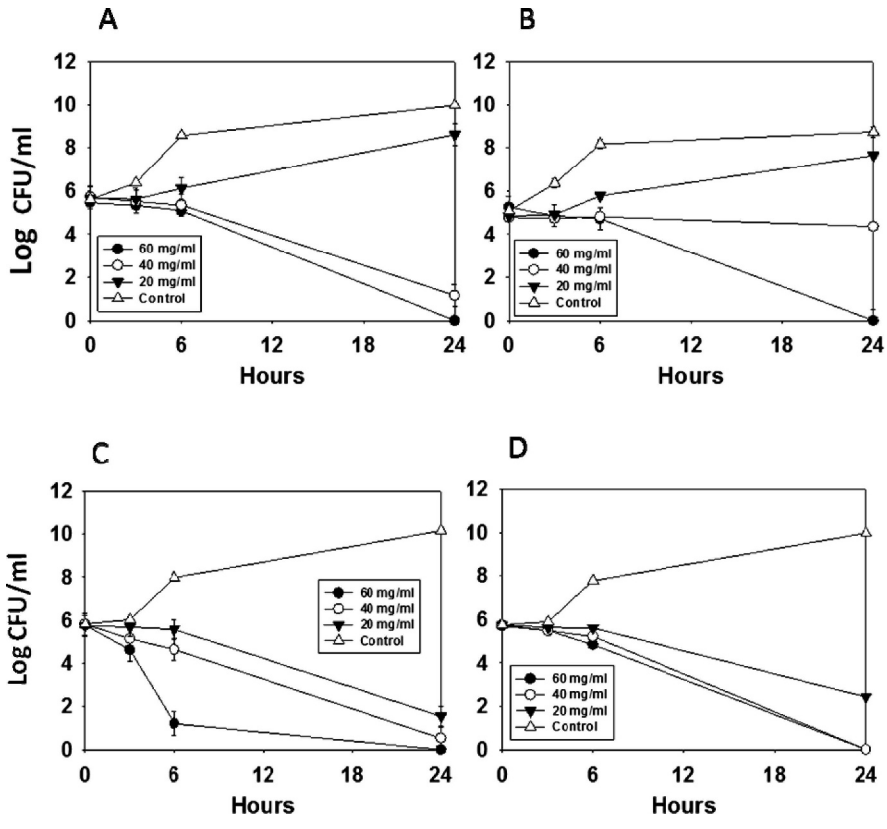

FIGURE 1. *Antimicrobial activity of aqueous extracts filtered through a 0.22-μm-pore-size membrane (A and B) and autoclaved extracts (C and D) from* H. sabdariffa *at concentrations of 0, 20, 40, and 60 mg/ml against* E. coli *O157:H7 strain Cider (A and C) and strain ATCC 43894 (B and D) after 0, 3, 6, and 24 h in TSB at 37°C. Error bars represent 95% confidence intervals for the mean, using LSD (*$P < 0.05$*).*

significant differences (LSD) were used to identify significant differences (at the 0.05 probability level) among treatment mean values. Error bars in figures presented represent 95% confidence intervals with LSD.

RESULTS

Antimicrobial activity of filtered and autoclaved extracts in a microbiological medium. Processed *Hibiscus* extracts demonstrated antimicrobial activity against *E. coli* O157:H7 and *S. aureus* both in a microbiological medium and in UHT milk. In TSB, the extracts lowered the pH to ca. 3.5. Filtered extracts at 20 mg/ml caused an increased lag phase of both strains of *E. coli* O157:H7 of 3 h, but the final levels of growth in extracts at 20 mg/ml reached ca. 7.6 to 8.6 log CFU/ml compared with ca. 8.7 to 9.9 log CFU/ml for the control (0 mg/ml) after 24 h in TSB at 35 to 37°C for strains ATCC 43894 and Cider, respectively (Fig. 1A and 1B). Filtered extracts at 40 mg/ml resulted in growth inhibition of both strains; however, inhibition of ATCC 43894 was essentially bacteriostatic at 24 h, while the same concentration reduced strain Cider by ca. 4.5 log CFU/ml. Both strains of *E. coli* O157:H7 were inactivated to below the level of detection after 24 h in 60 mg/ml filtered extracts. Autoclaved extracts showed enhanced activity at concentrations similar to those of filtered extracts. At 20 mg/ml, autoclaved extracts were more effective than filtered extracts against both strains of *E. coli* O157:H7. The population of strain Cider was reduced by ca. 4 or 5 log CFU/ml at 20 mg/ml and that of ATCC 43894 by ca. 3 log CFU/ml (Fig. 1C and 1D). With 40 mg/ml autoclaved extracts, inhibition of Cider was similar to that observed with the filtered extract, but against ATCC 43894, inhibition was enhanced to at least a 6-log reduction. Autoclaved extracts at 60 mg/ml were similarly effective at inactivating both strains of *E. coli* O157:H7 after 24 h, except that inactivation of strain Cider occurred after 6 h.

Lower apparent concentrations of filtered and autoclaved extracts of *H. sabdariffa* were required to inactivate *S. aureus* than for *E. coli*. (Fig. 2). Filtered extracts at 2.5 mg/ml and 20 mg/ml caused an increase in the lag phase of both strains of *S. aureus,* but only the 20-mg/ml concentration demonstrated bacteriostatic activity after 24 h. Reductions of ca. 2.7 and 3 log CFU/ml of *S. aureus* strains ATCC 27708 and SA113, respectively, were seen for filtered extracts at 40 mg/ml after 24 h (Fig. 2A and 2B). In contrast, populations of SA113 and ATCC 27708 were reduced ca. 5.4 and 1.7 log CFU/ml, respectively, when exposed to autoclaved extracts at 2.5 mg/ml after 24 h. Reductions of ca. 0.7 and 1.9 log CFU/ml of SA113 and ATCC 27708, respectively, were observed for 20 mg/ml extracts. It is not clear why the 2.5 mg/ml extract was more effective than the 20 mg/ml extract against the strains, particularly with SA113. Inactivation to undetectable levels of both strains of *S. aureus* was observed after 9 h at 35 to 37°C when grown in autoclaved extracts at 40 mg/ml.

Antimicrobial activity of autoclaved extracts in UHT milk. For each of the UHT milk treatments, no growth was observed in any of the uninoculated controls incubated at room temperature after 1 week (data not shown). When grown at pH 3.7 and with the appropriate pH controls, *E. coli* and *S. aureus* increased their population size by ca. 2.0 to 3.0 log CFU/ml by hour 168. Some curdling of the milk was observed after pH was lowered.

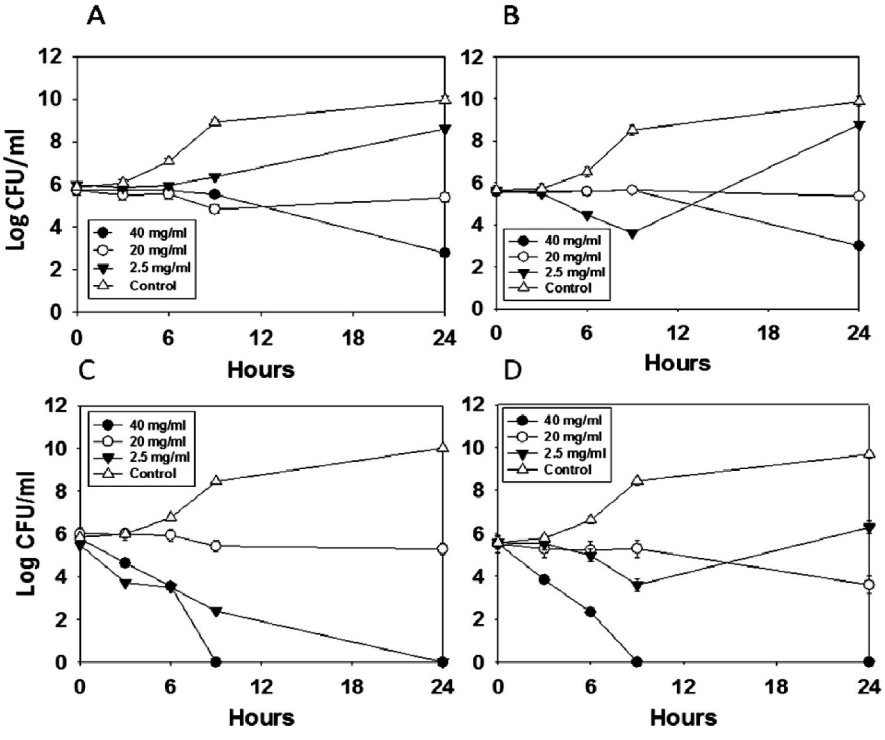

FIGURE 2. *Antimicrobial activity of aqueous extracts filtered through a 0.22-μm-pore-size membrane (A and B) and autoclaved extracts (C and D) from* H. sabdariffa *at concentrations of 0, 2.5, 20, and 40 mg/ml against* S. aureus *strain SA 113 (A and C) and strain ATCC 27708 (B and D) after 0, 3, 6, 9, and 24 h in TSB at 37°C. Error bars represent 95% confidence intervals for the mean, using LSD (*$P < 0.05$*).*

Autoclaved extracts (60 mg/ml) were shown to be effective in milk at all levels of fat against *E. coli* O157:H7 (Fig. 3A). Inactivation to undetectable levels (ca. 5.5-log CFU/ml reduction) of *E. coli* O157:H7 was observed after 48 h in skim milk and after 96 h in 1%, 2%, and whole milk with treatment with 60-mg/ml extracts. For *S. aureus,* 40 mg/ml extracts caused an initial bacteriostasis, but after 168 h the populations were reduced by ca. 5.5 log CFU/ml in skim and whole milk and by ca. 2.6 log CFU/ml in 1 and 2% UHT milk.

Compounds present in the extracts. Yields of lyophilized extract taken from 100 g of whole dried calyx ranged from 1.78 to 2.14 g. The phenolic content ranged from 48.7 to 53.8 mg GAE/g, while the monomeric anthocyanin content ranged from 2.63 to 14.27 mg of cyanidin-3-glucoside equivalents per liter. For the crude extract and phenolic amounts, the lower value was from the filtered treatment, whereas the lower value for anthocyanins came from the autoclaved treatment (Table 1). The polymerized color of the extracts was 52% in the filtered treatment and 70% for the autoclaved treatment (Table 1). Apparently, autoclaving did not affect total phenolic content; however, it caused a significant degradation of anthocyanins.

DISCUSSION

In this study, we examined the effects of autoclaving *Hibiscus* extracts on their antimicrobial activity and phenolic and anthocyanin content and determined their potential use to prevent the growth of pathogens in the model food system milk. Numerous compounds from *H. sabdariffa* have been identified, many of which are known to have antimicrobial activity, although it is not clear what specific compounds are responsible or if there might be a synergistic effect among all or some of the compounds present.

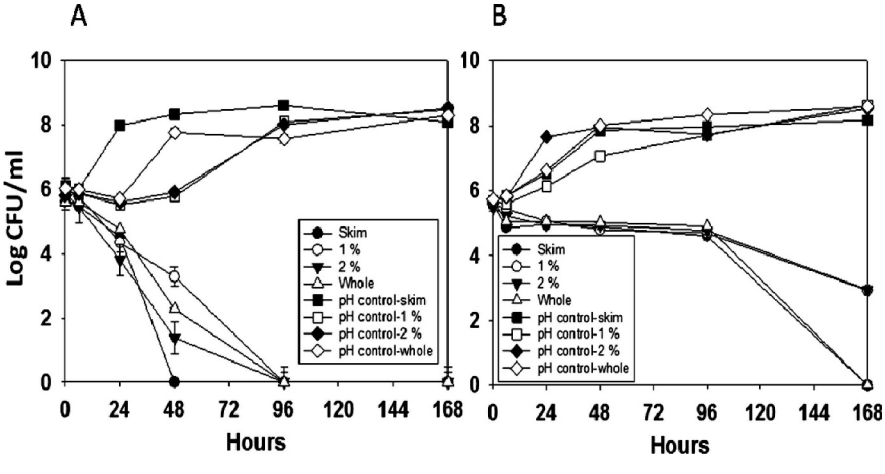

FIGURE 3. *Antimicrobial activity of aqueous extracts from* H. sabdariffa *autoclaved at 60 mg/ml (treatment) against a 1:1 mixture of* E. coli *O157:H7 strains Cider and ATCC 43894 (A) and at 40 mg/ml (treatment) against a 1:1 mixture of* S. aureus *SA113 and ATCC 27708 (B) in milk with various milk fat levels (skim, 1%, 2%, and whole UHT milk) after 0, 6, 24, 48, 96, and 168 h under room temperature storage conditions. The pH controls used were water with no aqueous extracts (0 mg/ml) mixed with milk with the pH adjusted to ca. 3.7. Error bars represent 95% confidence intervals for the mean using LSD (*$P < 0.05$*).*

TABLE 1. *Anthocyanin and phenolic content and polymerized color of commercially available* H. sabdariffa *calyx extracts*

Hibiscus extract treatment	Mean ± SE anthocyanin concn (mg/liter)[a]	Mean ± SE phenolic concn (mg/g)[b]	Polymerized color (%)
Filtered (4°C)	14.27 ± 0.87	48.7 ± 2.9	52
Autoclaved (121°C, 30 min)	2.63 ± 0.04	53.8 ± 0.9	70

[a] Expressed as cyanidin-3-glucoside equivalents.
[b] Expressed as GAE.

Phenolic compounds are known to exhibit antimicrobial activity. Calyx extracts contain many phenolic compounds such as hydroxybenzoic acids and caffeoylquinic acids *(24)*. The protocatechuic acid contents have been examined in both freeze-dried aqueous and ethanol extracts of calyces and were determined to be 2.8 ± 0.7 and 11.9 ± 1.2 mg/g of freeze-dried calyx extract, respectively, and antibacterial activity of protocatechuic acid was also not affected by heat from 25 to 100°C *(4)*. Aqueous calyx extracts were also shown to be effective against methicillin-resistant *S. aureus, K. pneumoniae, Pseudomonas aeruginosa,* and *Acinetobacter baumannii* with MICs of 32 ± 8, 48 ± 2, 56 ± 8, and 48 ± 8 mg/liter, respectively *(16)*. Flavonoids present could be forming complexes with the bacterial cell wall *(8)*. The flavonoid gossypetin, which is present in the calyces, has also been shown to exhibit antibacterial activity against both gram-negative and gram-positive pathogens *(20)*.

Autoclaved (121°C, 30 min) extracts were more effective against both *E. coli* O157:H7 and *S. aureus* than were the filtered extracts in a microbiological medium. Autoclaving may have produced smaller or additional compounds in the extracts that were more effective against these pathogens. Liu et al. *(16)* examined the effect of heating *H. sabdariffa* at 60 and 100°C for 60 min and found that the antimicrobial activity of the *Hibiscus* extract was not affected. However, Chao and Yin *(4)* examined the effect of heat on aqueous extracts of *H. sabdariffa* and determined the antimicrobial activity, after heating the extract to 75 and 100°C, against *S. aureus, E. coli* O157:H7, *L. monocytogenes, B. cereus,* and *Salmonella* Typhimurium to be significantly reduced, but activity of the ethanol extracts was not affected by temperatures of 50 and 75°C. Elsayed and Elshafei *(10)* also examined the effects of boiling and autoclaving calyx extracts and found that the MIC against *Bacillus mycoides, E. coli,* and *C. albicans* was not significantly affected compared with room temperature extracts. In the present study, autoclaved extracts exhibited enhanced activity compared with filtered extracts. The major difference in the present study was that extracts were autoclaved following the entire extraction process while other studies heated the extracts prior to concentrating them.

Differences in phenolic and anthocyanin content were examined in both filtered and autoclaved extracts, given that calyces of *H. sabdariffa* are rich in both anthocyanins and phenolic compounds. Anthocyanins are not heat stable. Ramírez-Rodrigues et al. *(25)* also found that anthocyanins in *Hibiscus* beverages degrade over time during storage, more so in heat-treated than in non–heat-treated beverages. In the present study, the anthocyanin content of the filtered extract (14.27 mg of cyanidin-3-glucoside equivalents per liter) is lower than that shown in other published studies. Ramirez-Rodrigues et al. *(24)* used dried calyces and a cold (25°C) aqueous extraction (ratio, 1:40) and determined the anthocyanin content to be 128.94 mg of delphinidin-3-glucoside equivalents per liter. Cisse et al. *(6)* examined four varieties of calyx extracts made with water at a ratio of 1:10. The varieties used were Guatemala, Koor, Vimto, and Thai, which had anthocyanin contents of 315, 250, 718, and 306 mg of cyanidin-3-glucoside equivalents per liter, respectively. In the present study, the autoclaved extract's polymeric color, an indication of the complexes that the anthocyanins form with other compounds such as tannins *(11),* showed that it had approximately 20% more polymerization than the filtered extract. The phenolic content in the filtered and autoclaved extracts (48.7 and 53.8 mg of GAE/g) is somewhat higher than data found in the literature. In this study, the extract was dialyzed and lyophilized, which could have concentrated the phenolic compounds. The phenolic content of an ethanol extraction of traditional red calyces was found to be 16 mg of GAE/g, which was performed with 1 g of fresh freeze-dried calyces extracted with ethanol that was later evaporated *(5)*. The phenolic content of an extraction done with hot water (1 g of calyx tea in 100 ml of hot water) was found to be 13.3 mg GAE/g of dry tea *(22)*. The differences observed may be the result of the extraction process. Similarly, variation in content has been observed in other plants depending on the extraction method. Oboh and Rocha *(22)* extracted 1 g of green tea in 100 ml of hot water and determined the phenolic content to be 24.5 mg of GAE/g of dry tea. Rusak et al. *(27)* examined various extraction methods and determined the phenolic content in green and white teas. Green and white teas (2 g), either bagged or loose, were extracted in 200 ml of water, water with lemon juice, or ethanol at 10, 40, and 70% for times of 5, 15, and 30 min. The researchers found that aqueous ethanol at 40% for 30 min was the best combination to extract both teas in bagged form, and white tea contained around 2,100 mg of GAE/liter, while green tea contained approximately 2,400 mg of GAE/liter *(27)*. The extraction methods used are important to consider when comparing levels of components found in plant material. The phenolic content also did not change after autoclaving. This could be due to several reasons. Xu et al. *(32)* examined the phenolic content of citrus peel extract after heating (120°C for 30, 60, 90 min), and the levels of free phenolic acids were found to increase, whereas the ester, glycoside, and ester-bound forms were found to decrease. They also found that

antioxidant activity increased after some heating; however, heating for too long (120°C for 90 min) could destroy some phenolic compounds such as flavanone glycosides *(32)*. In our study, extracts were autoclaved at 121°C for 30 min. Longer times may have resulted in destruction of phenolic compounds.

Higher concentrations of antimicrobials are typically required for protection in food systems than in less complex systems, such as microbiological media, to achieve the same levels of bacterial inactivation. Complex food systems contain components such as fats, proteins, and carbohydrates, which may interfere with antimicrobials. In the present study, the highest effective concentration of the lyophilized extract in a microbiological medium was used in milk. Extracts were less effective against *E. coli* O157:H7 in 1%, 2%, and whole milk than in skim milk, while *S. aureus,* in a lower concentration of extract, survived through 96 h in milk at all fat levels. This lack of activity may be attributed to the fat and protein components present in these media. In a previous study, the antimicrobial activity of essential oils from clove and cinnamon against *L. monocytogenes* was also shown to decrease when fat levels in milk were increased *(2)*. Similarly, lipids from corn oil were found to interfere with the antioxidant butylated hydroxyanisole, reducing its activity against bacteria such as *S. aureus* ATCC 12600 and *Pseudomonas fluorescens* ATCC 15456 *(26)*.

While pH 3.7 is a low pH in which bacterial pathogens often do not grow, in this study, growth of both *E. coli* O157:H7 and *S. aureus* was observed. This phenomenon could be explained by several factors. First, the components present in milk, such as fat and proteins, may have provided a matrix to protect the bacteria. Further, milk is known to have buffering capacity. Heat-processed milk (120°C, 10 min) has been shown to have increased buffering capacity compared to milk that was not heat treated *(29)*. Additionally, the pH may have recovered slightly after acid treatment or increased over time, allowing the bacteria to survive the stress and grow. Further, *E. coli* O157:H7 is more acid tolerant than other *E. coli* strains, and in particular, strains ATCC 43889 and 43895 were found to be able to survive in apple cider for up to 21 days *(18)*. These strains also survived in a pH 2 medium for 24 h with only a minimal drop in CFU per milliliter *(18)*. Uljas and Ingham *(31)* found that when grown in TSB at pH 4.0, *E. coli* O157:H7 strain ATCC 43894 did not decrease at 4 or 21°C. When grown in apple juice at pH 3.5, ATCC 43894 survived 7 days at 4°C but did not survive at 21°C. The strain used in our studies (ATCC 43894) was found to be more acid tolerant than ATCC 43889 *(31),* and strain Cider was isolated from an apple cider beverage, which can help to explain its acid tolerance. When grown in TSB with 0.6% yeast extract acidified with organic acids (acetic, citric, malic, lactic, or tartaric) to lower the pH, at 25°C, *E. coli* O157:H7 increased by 2 to 4 log CFU/ml at pH of ≥4 over a 56-day period *(7)*. Growth of *S. aureus* was also observed in the pH controls. It has been shown that *S. aureus* is acid tolerant to an extent and is able to grow in the wide pH range of 4.0 to 9.8 *(17)*. All of these factors combined may have contributed to the survival and growth of these pathogens in the pH 3.7 milk controls over 7 days.

Extracts of *H. sabdariffa* have the potential to be used as antimicrobials in a food beverage system. Because the calyces are extracted in water and *H. sabdariffa* is accepted as a natural flavoring substance by the U.S. Food and Drug Administration (21 CFR 172.510), its extracts might be good candidates to be applied to existing food and beverage systems as antimicrobials. In addition, activity has shown to be enhanced by autoclaving, enabling *Hibiscus* extracts to be added to a wide variety of foods prior to processing (i.e. pasteurization). Also, although autoclaving degraded anthocyanins, it did not adversely affect the phenolic content, which is important for antimicrobial activity. *Hibiscus* extracts have the potential to contribute additional health benefits due to their antioxidative properties and can be used as a natural alternative to synthetic antimicrobials. Future work is needed to identify the active compounds. In addition, while milk was used as a model food in this study, it is not a practical beverage for *Hibiscus* extract treatment since the resulting milk will have a red color. More-practical beverage applications for protection using *Hibiscus* extracts would be juice beverages. When *Hibiscus* extracts are added to, say, apple juice, a red coloration could translate to a desirable aesthetic for consumers and could also be associated with enhanced safety against foodborne pathogens.

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