Research Note

Combined treatments of chestnut shell extract, fumaric acid, and mild heat to inactivate foodborne pathogens inoculated on beetroot (*Beta vulgaris* L.) leaves

Shin-Min Park, Ji-Hoon Kang, Hyeon-Jeong Son, Deog-Hwan Oh¹, Sea Cheol Min², and Kyung Bin Song*

Department of Food Science and Technology, Chungnam National University, Daejeon 34134, Korea

¹Department of Food Science and Biotechnology, Kangwon National University, Chuncheon, Gangwon 24341, Korea

²Department of Food Science and Technology, Seoul Women's University, Seoul 01797, Korea

Received March 23, 2016
Revised June 2, 2016
Accepted June 15, 2016
Published online August 31, 2016

*Corresponding Author
Tel: +82-42-821-6723
Fax: +82 42 825 2664
E-mail: kbsong@cnu.ac.kr

pISSN 1226-7708
eISSN 2092-6456

© KoSFoST and Springer 2016

Abstract To evaluate the combined treatments of chestnut shell extract (CSE), fumaric acid (FA), and mild heat (MH) on the inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes* inoculated on beetroot leaves, samples were treated with different concentrations of CSE or FA, as well as combinations of 0.5% CSE/0.5% FA and 0.5% CSE/MH at 50°C/0.5% FA. Among the treatments, the combined treatment of CSE/MH/FA was most effective, reducing the populations of *E. coli* O157:H7 and *L. monocytogenes* on beetroot leaves by 3.18 and 3.76 log CFU/g, respectively. In addition, the initial populations of pre-existing bacteria on beetroot leaves were reduced by 2.58 log CFU/g after the combined treatment. The inactivation effect was retained during storage at 4°C for 8 days. These results indicate that the combined treatment of CSE, FA, and MH can be effective in decontamination from foodborne pathogens and improving in the microbial safety of beetroot leaves during storage.

Keywords: chestnut shells extract, fumaric acid, combined treatment, inactivation, microbial safety

Introduction

Beetroot leaves are known to be a good source of vitamin A, folate, and dietary fiber (1). In recent years, consumers have become more interested in ready-to-eat (RTE) and minimally processed (MP) vegetables because of their convenience. However, outbreaks of foodborne diseases have been increasing since fresh vegetables can become contaminated by foodborne pathogens during processing and storage. Therefore, a suitable method is required for decontamination of fresh vegetables from foodborne pathogens to ensure microbial safety.

To control the microorganisms in RTE and MP vegetables, various types of sanitizers have been used (2,3). While chlorinated water is one of the most commonly used sanitizers for ensuring the safety of fresh produce, health-issues associated with trihalomethanes raise concerns about its use (4). Therefore, an environmentally friendly alternative method is needed.

There have been many studies aiming to replace chemical sanitizers with natural antimicrobial agents, such as essential oils and organic acids (5). Organic acids, such as fumaric acid (FA), have well-known strong antimicrobial properties (6). Recently, new approaches have been developed using food by-product extracts as novel antimicrobial agents (7).

Castanea crenata (chestnut) shells are a by-product from chestnut processing, and comprise approximately 10% of the total chestnut weight (8). Chestnut shells contain considerable amounts of phenolic compounds such as tannins (9), and chestnut inner shell extract has antibacterial properties against several foodborne pathogens (10). Nonetheless, the effect of chestnut whole shell extract on the inactivation of foodborne pathogens and its use as a sanitizer have not yet been studied.

Therefore, the objective of this study was to examine the antibacterial properties of chestnut whole shell extract. In addition, the combined effects of chestnut shell extract (CSE), mild heat (MH), and FA treatments were examined on the inactivation of pathogenic bacteria inoculated on beetroot leaves with the goal of developing a suitable non-thermal treatment for improving microbial safety.

Materials and Methods

Sample preparation and storage condition Beetroot (*Beta vulgaris* L.) leaves were purchased from a local market (Daejeon, Korea). The fresh beetroot leaves were packed in a low-density polyethylene bag (30 cm × 35 cm) and then stored at 4°C for 8 days after the treatment with CSE and FA or the combined treatment with CSE/FA/MH as

described below.

Preparation of CSE Chestnut whole shells were obtained from a local farm (Buyeo, Korea). The chestnut shells were dried at 70°C for 24 h. The dried chestnut shells were ground in a blender and were extracted using the method described by Hao *et al.* (11). The ground samples (50 g) were suspended in 70% ethanol (1:20, w/v) and the mixture was then homogenized with a homogenizer at 12,000 rpm for 5 min, and extracted at 50°C for 3 h. Ethanol was removed using a rotary vacuum-evaporator at 60°C.

Bacterial strains and culture preparation Strains of *Escherichia coli* O157:H7 (NCTC 12079 and ATCC 43889) and *Listeria monocytogenes* (ATCC 19115 and KCTC 13064) were streaked onto Tryptic Soy agar (Difco, Sparks, MD, USA) and Brain Heart Infusion agar (Difco), respectively, and then incubated at 37°C for 24 h. After incubation, a single colony from two strains of *E. coli* O157:H7 and *L. monocytogenes* were selected. The colony of each pathogen was added to 50 mL corning tubes containing 25 mL of selective medium broth and incubated at 37°C for 24 h with shaking (150 rpm). Incubated cultures were centrifuged at 10,000×*g* for 15 min, after which the cell pellets were washed twice using 20 mL of peptone water.

Inoculation of pathogenic bacteria To completely inactivate the pre-existing microorganisms on beetroot leaves, the samples were exposed to UV-irradiation for 10 min on each side on a clean bench. The inoculation solutions of *E. coli* O157:H7 and *L. monocytogenes* were prepared by dilution in sterile peptone water to a final concentration of approximately 7–8 log CFU/mL. One millilitre of the solution was then spot-inoculated onto the surface of beetroot leaves. After inoculation, the beetroot leaves were air-dried on a clean bench.

Single treatment of CSE, MH, and FA Inoculated beetroot leaves were dipped in different concentrations of CSE (0.1, 0.25, 0.5, and 1.0%) or FA (0.1, 0.25, and 0.5%) solution for 3 min, and then air-dried in a clean bench. In addition, for the optimization of MH treatment time, the samples were dipped in the distilled water at 50°C for 1, 3, and 5 min.

Combined treatment For the combined treatment, the inoculated samples were first treated with 0.5% CSE, and then with 0.5% FA or with MH at 50°C for 3 min. In addition, for the combined treatment of CSE, MH, and FA, the beetroot leaves were treated sequentially with the solution of 0.5% CSE with MH at 50°C, and then with 0.5% FA.

Microbiological analysis After treatment, beetroot leaves (10 g) were put in a stomacher bag containing 90 mL of sterile peptone water (0.1%). Samples were homogenized for 3 min with a stomacher, and then serially diluted with peptone water. Pre-existing bacteria

counts were identified by plating diluted samples onto Plate Count Agar (Difco) and incubating at 37°C for 48 h. *E. coli* O157:H7 and *L. monocytogenes* counts were identified by plating diluted samples onto Sorbitol MacConkey agar (Difco) and Oxford Medium Base (Difco), respectively, and incubating each plate at 37°C for 24 h. Microbial counts were conducted in triplicate and expressed as log CFU/g.

Statistical analysis Analysis of variance and Duncan's multiple range tests were conducted to examine the data using SAS program version 9.4 (SAS Institute Inc., Cary, NC, USA). Statistical significance was considered at $p < 0.05$ and all the results are represented as the mean ± SD.

Results and Discussion

The initial populations of *E. coli* O157:H7 and *L. monocytogenes* inoculated on beetroot leaves were 6.31 and 6.74 log CFU/g, respectively. After treatment with 0.1, 0.25, 0.5, and 1.0% CSE, the populations of *E. coli* O157:H7 on beetroot leaves decreased to 4.33, 4.10, 3.68, and 3.64 log CFU/g, respectively, resulting in reductions of 1.98, 2.21, 2.63, and 2.67 log CFU/g, compared with those of untreated samples (Table 1). In a previous study, CSE was reported as having robust antibacterial activity due to the innate presence of tannins and quercetin in its composition (12). In addition, changes in the populations of *L. monocytogenes* by CSE treatment showed a similar pattern as those seen for *E. coli* O157:H7. However, there was no significant difference between 0.5 and 1.0% CSE treatment. Based on these results, 0.5% CSE was chosen as the optimal concentration for the decontamination of foodborne pathogens on beetroot leaves when combined with MH or FA treatments.

Increasing concentrations of FA treatments also significantly decreased the populations of *E. coli* O157:H7 and *L. monocytogenes* on the beetroot leaves (Table 1). The greatest reduction level was

Table 1. Antimicrobial effects of chestnut shell extract, fumaric acid, and mild heat at 50°C against *E. coli* O157:H7 and *L. monocytogenes* inoculated on beetroot leaves (Unit: log CFU/g)

Treatment	<i>E. coli</i> O157:H7	<i>L. monocytogenes</i>
Control	6.31±0.23 ^{a,1)}	6.74±0.07 ^a
Chestnut shell extract (%)	0.10 4.33±0.19 ^d	5.59±0.36 ^b
	0.25 4.10±0.17 ^{de}	5.60±0.24 ^b
	0.50 3.68±0.39 ^f	5.13±0.14 ^c
	1.00 3.64±0.28 ^{fg}	5.14±0.30 ^c
Fumaric acid (%)	0.10 3.86±0.34 ^{ef}	4.43±0.51 ^e
	0.25 3.75±0.22 ^f	3.94±0.11 ^f
	0.50 3.35±0.21 ^g	3.72±0.21 ^f
Water washing at 50°C	1 min 5.35±0.18 ^b	5.27±0.15 ^c
	3 min 4.78±0.21 ^c	4.80±0.15 ^d
	5 min 4.77±0.25 ^c	4.78±0.23 ^d

¹⁾Values are mean ± SD ($n=3$). Any means in the same column followed by different letters (a–g) are significantly ($p < 0.05$) different.

Table 2. Effects of combined treatment on the inactivation of *E. coli* O157:H7 and *L. monocytogenes* inoculated on beetroot leaves (Unit: log CFU/g)

Treatment ¹⁾	<i>E. coli</i> O157:H7	<i>L. monocytogenes</i>
Control	6.25±0.17 ^{a,2)}	6.95±0.09 ^a
CSE/MH	3.52±0.16 ^b	4.96±0.01 ^b
CSE/FA	3.22±0.26 ^{b,c}	3.88±0.38 ^c
CSE/MH/FA	3.07±0.09 ^c	3.19±0.07 ^d

¹⁾CSE, chestnut shell extract (0.5%); MH, mild heat at 50°C for 3 min; FA, fumaric acid (0.5%)

²⁾Values are mean±SD (n=3). Any means in the same column followed by different letters (a-d) are significantly (p<0.05) different.

achieved with 0.5% FA treatment, resulting in *E. coli* O157:H7 and *L. monocytogenes* counts of 3.35 and 3.72 log CFU/g, respectively, comprising reductions of 2.96 and 3.02 log CFU/g, compared with the control. In similar studies, 0.5% FA treatments were more effective than any other single chemical or physical treatment at reducing microorganisms on clover sprouts and strawberries (13,14). This is consistent with our results, indicating that 0.5% FA is the most effective treatment for inactivating foodborne pathogens inoculated on beetroot leaves. The antimicrobial effects of organic acids are mainly due to decreases in pH and penetration of bacterial membranes by undissociated forms of the organic acids (15). Based on our results, 0.5% FA was selected for the combined treatment with 0.5% CSE.

To determine the optimal MH treatment time, inoculated beetroot leaves were treated with MH (50°C) for 1, 3, and 5 min (Table 1). The microbial reduction levels for both *E. coli* O157:H7 and *L. monocytogenes* significantly improved when the treatment time was increased from 1 to 3 min. However, the inactivation effects for 3- and 5-min treatment times were not significantly different. Based on these results, the 3-min MH treatment time was chosen as the optimal time for the combined treatment.

Changes in pathogen populations on inoculated beetroot leaves treated with the three different treatment combinations (CSE/MH, CSE/FA, or CSE/MH/FA) were evaluated (Table 2). The initial populations of *E. coli* O157:H7 and *L. monocytogenes* were 6.25 and 6.95 log CFU/g, respectively. Following CSE/MH treatment, *E. coli* O157:H7 and *L. monocytogenes* counts were 3.52 and 4.96 log CFU/g, resulting in reductions of 2.73 and 1.99 log CFU/g, respectively.

The combined treatment of CSE and FA also reduced the populations of *E. coli* O157:H7 and *L. monocytogenes* by 3.03 and 3.07 log CFU/g, respectively, when compared with the control. Relative to the other treatment combinations, the combination of CSE/MH/FA was most effective in reducing *E. coli* O157:H7 and *L. monocytogenes* populations, resulting in reductions of 3.18 and 3.76 log CFU/g, respectively. The additional MH treatment could destabilize the stability of bacterial membranes and have CSE and FA to penetrate easily inside the bacteria, resulting in synergy effect regarding the inactivation of pathogenic bacteria (16).

The effects of the combined treatments of CSE/FA and CSE/MH/FA on the inactivation of endogenous bacterial populations on beetroot leaves were evaluated during 8 days of storage at 4°C (Table 3). Prior to treatment, the pre-existing population of bacteria on the fresh beetroot leaves was 5.68 log CFU/g. Water washing treatment reduced this count to 4.40 log CFU/g, whereas combined treatment of CSE/FA decreased the count to 3.48 log CFU/g. Combined treatment of CSE/MH/FA had the greatest reduction of pre-existing bacteria on the beetroot leaves, resulting in a reduction of 2.58 log CFU/g, compared with that of the control. During storage, populations of pre-existing bacteria on the beetroot leaves in the control and water washing treatment groups significantly increased, whereas the reduced microbial levels were maintained in the combined treatment groups. After 8 d of storage, pre-existing bacterial populations in the control and water washing treatment groups reached 6.01 and 5.30 log CFU/g, respectively. However, bacterial populations in the combined CSE/FA and CSE/MH/FA treatment groups were 3.91 and 3.72 log CFU/g, respectively, which are comparable to the counts observed at an early stage of storage. Thus, the overall bacterial counts remained low. These results suggest that CSE can be used as a natural sanitizer for decontaminating foodborne pathogens, and that combined treatment of CSE, MH, and FA may be an effective bacterial inactivation method for vegetables, such as beetroot leaves.

Acknowledgments This research was supported by a grant from IPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries, Project No. 314059-03), Republic of Korea.

Disclosure The authors declare no conflict of interest.

Table 3. Effects of combined treatment on the inactivation of pre-existing bacteria on beetroot leaves during storage at 4°C (Unit: log CFU/g)

Treatment ¹⁾	Storage time (day)				
	0	2	4	6	8
Control	5.68±0.23 ^{Aa,2)}	5.78±0.39 ^{Aa}	5.88±0.06 ^{Aa}	5.82±0.32 ^{Aa}	6.01±0.32 ^{Aa}
Water washing	4.40±0.26 ^{Cb}	4.61±0.14 ^{Cb}	4.71±0.18 ^{BCb}	5.16±0.17 ^{ABb}	5.30±0.34 ^{Ab}
CSE/FA	3.48±0.32 ^{Bc}	3.97±0.31 ^{Ac}	3.93±0.07 ^{Ac}	3.89±0.14 ^{Ac}	3.91±0.17 ^{Ac}
CSE/MH/FA	3.10±0.17 ^{Bc}	3.52±0.05 ^{Ac}	3.74±0.08 ^{Ad}	3.70±0.19 ^{Ac}	3.72±0.23 ^{Ac}

¹⁾CSE, chestnut shell extract (0.5%); MH, mild heat at 50°C for 3 min; FA, fumaric acid (0.5%)

²⁾Values are mean±SD (n=3). Any means in the same row (A-C) or same column (a-d) followed by different letters are significantly (p<0.05) different.

References

1. Zieliska-Przyjemka M, Olejnik A, Dobrowolska-Zachwieja A, Grajek W. *In vitro* effects of beetroot juice and chips on oxidative metabolism and apoptosis in neutrophils from obese individuals. *Phytother. Res.* 23: 49-55 (2009)
2. Kim YJ, Lee SH, Park J, Park J, Chung M, Kwon K, Chung K, Won M, Song KB. Inactivation of *Escherichia coli* O157: H7, *Salmonella typhimurium*, and *Listeria monocytogenes* on stored iceberg lettuce by aqueous chlorine dioxide treatment. *J. Food Sci.* 73: 418-422 (2008)
3. Crowe KM, Bushway A, Davis-Dentici K. Impact of postharvest treatments, chlorine and ozone, coupled with low-temperature frozen storage on the antimicrobial quality of lowbush blueberries (*Vaccinium angustifolium*). *LWT-Food Sci. Technol.* 47: 213-215 (2012)
4. Olmez H, Kretzschmar U. Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. *LWT-Food Sci. Technol.* 42: 686-693 (2009)
5. Barbosa IM, Medeiros JAC, Oliveira KAR, Gomes-Neto NJ, Tavares JF, Magnani M, Souza EL. Efficacy of the combined application of oregano and rosemary essential oils for the control of *Escherichia coli*, *Listeria monocytogenes* and *Salmonella Enterica* in leafy vegetables. *Food Control* 59: 468-477 (2016)
6. Ramos B, Miller FA, Brandao TRS, Teixeira P, Silva CLM. Fresh fruits and vegetables-An overview on applied methodologies to improve its quality and safety. *Innov. Food Sci. Emerg.* 20: 1-15 (2013)
7. Salaheen S, Nguyen C, Hewes D, Biswas D. Cheap extraction of antibacterial compounds of berry pomace and their mode of action against the pathogen *Campylobacter jejuni*. *Food Control* 46: 174-181 (2014)
8. Vázquez G, Fontenla E, Santos J, Freire MS, González-Álvarez J, Antorrena G. Antioxidant activity and phenolic content of chestnut (*Castanea sativa*) shell and eucalyptus (*Eucalyptus globulus*) bark extracts. *Ind. Crop. Prod.* 28: 279-285 (2008)
9. Barreira JC, Ferreira IC, Oliveira MBP, Pereira JA. Antioxidant activities of the extracts from chestnut flower, leaf, skins and fruit. *Food Chem.* 107: 1106-1113 (2008)
10. Lee NK, Jung BS, Na DS, Yu HH, Kim JS, Paik HD. The impact of antimicrobial effect of chestnut inner shell extracts against *Campylobacter jejuni* in chicken meat. *LWT-Food Sci. Technol.* 65: 746-750 (2016)
11. Hao JJ, Liu H, Donis-Gonzalez IR, Lu XH, Jones AD, Fulbright DW. Antimicrobial activity of chestnut extracts for potential use in managing soilborne plant pathogens. *Plant Dis.* 96: 354-360 (2012)
12. You TT, Zhou SK, Wen JL, Ma C, Xu F. Chemical composition, properties, and antimicrobial activity of the water-soluble pigments from *Castanea mollissima* shells. *J. Agr. Food Chem.* 62: 1936-1944 (2014)
13. Kim YJ, Kim MH, Song KB. Combined treatment of fumaric acid with aqueous chlorine dioxide or UV-C irradiation to inactivate *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes* inoculated on alfalfa and clover sprouts. *LWT-Food Sci. Technol.* 42: 1654-1658 (2009)
14. Kim JY, Kim HJ, Lim GO, Jang SA, Song KB. The effects of aqueous chlorine dioxide or fumaric acid treatment combined with UV-C on postharvest quality of 'Maehyang' strawberries. *Postharvest Biol. Tec.* 56: 254-256 (2010)
15. Kang JH, Song KB. Inactivation of pre-existing bacteria and foodborne pathogens on perilla leaves using a combined treatment with an organic acid and a surfactant. *Hort. Environ. Biotechnol.* 56: 195-199 (2015)
16. Forghani F, Oh DH. Hurdle enhancement of slightly acidic electrolyzed water antimicrobial efficacy on Chinese cabbage, lettuce, sesame leaf and spinach using ultrasonication and water wash. *Food Microbiol.* 36: 40-45 (2013)