

Evaluating the Efficacy of Olive, Apple and Grape Seed Extracts in Reducing *Escherichia coli* O157:H7 Contamination on Organic Leafy Greens during the Wash Process

Research Article

Buddhini P. K¹, Jones J², Ravishankar S³, Jaroni D^{1*}

¹Robert M. Kerr Food and Agricultural Products Center and Department of Animal Science, Oklahoma State University, Stillwater, Oklahoma 74078, USA.

²Southern University Agricultural Research and Extension Center, Baton Rouge, Louisiana 70813, USA.

³School of Animal and Comparative Biomedical Sciences, University of Arizona, Tucson, Arizona 85721, USA.

Abstract

Powdered extracts of apple, olive, and grape seed were evaluated for antimicrobial activity against *Escherichia coli* O157:H7 during processing and subsequent short-term storage of organic leafy greens. The leafy greens tested were, baby spinach, mature bunched spinach, romaine lettuce, and iceberg lettuce. The organic leafy greens were thoroughly washed and inoculated with a three-strain cocktail of *E. coli* O157:H7 (6 log CFU ml⁻¹). Antimicrobial treatments of the extracts, prepared in phosphate buffered saline, at 1, 3, and 5% concentrations were used to wash contaminated leafy greens which were then stored for 3 days at 4°C. Surviving *E. coli* O157:H7 populations were enumerated on days 0, 1, and 3 of storage. Significant reductions (P<0.05) in pathogen populations were observed in all the leafy greens following treatment with the plant extracts. Between 2.9-5.3 log CFUg⁻¹ reductions in *E. coli* O157:H7 population were observed with all three concentrations (1%, 3% and 5%) of olive extract by day 3, for all the leafy greens. The 5% olive extract treatment reduced the pathogen to undetectable levels by day 0 for romaine lettuce and by day 1 for iceberg lettuce and baby spinach. Apple extract at 5% concentration showed reductions of 3.4 and 3.9 log CFUg⁻¹ in iceberg and romaine lettuce, respectively. The 5% grape seed extract reduced *E. coli* O157:H7 populations by 4.4 logsCFUg⁻¹ in romaine lettuce. This study demonstrates the potential of olive, apple and grape seed extracts as antimicrobial wash treatments against *E. coli* O157:H7 during the processing of organic leafy greens.

Keywords: *Escherichia coli* O157:H7, Inhibition, Organic Leafy Greens, Plant Extracts, Antimicrobials

*Corresponding Author:

Divya Jaroni,
112 FAPC,
Department of Animal Science,
Oklahoma State University,
Stillwater, Oklahoma 74078, USA.
Tel: +01-405-744-9263
Fax: +01-405-744-4716
E-mail: divya.jaroni@okstate.edu

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Introduction

The past two decades have seen an increase in the amount of fresh produce being consumed in the United States (US). An increase in income, technological improvements, domestic production, healthier lifestyles, and the availability of a wider variety of

produce are some of the factors that have attributed to this trend [31]. Likewise, there has been an increase in the consumption of organic fresh produce [9]. However, foodborne outbreaks originating from minimally processed fresh produce have also seen a parallel increase in the US, with lettuce and sprouts listed as a common source [42]. Fresh produce has also proved to be an important commodity in foodborne outbreaks outside the US, such as the *Escherichia coli* O104:H4 outbreak in Germany in 2011 [4], traced back to contaminated sprouts. In recent years, foodborne outbreaks associated with organic fresh produce commodities have also been reported [5-7]. Shiga-toxin producing *E. coli* has also been implicated in such outbreaks, including the multistate outbreak of *E. coli* O157:H7 infections linked to organic spinach and spring-mix blend in 2012 [7].

It has been well established that fresh produce can contain significant levels of pathogen contamination after harvest, ranging anywhere from 1 to 3 log CFUg⁻¹ [1,2,8-10,15,17,27]. These levels may be influenced by seasonal and environmental conditions, temperature, as well as the type of produce [1,17-18]. Organic produce has also been speculated to have high levels of pathogen contamination due to limited number of effective control strategies that are in place against microbial contamination, both prior to and at processing [26]. Organic growers must follow guidelines set by the United States Department of Agriculture-National Organic Program (USDA-NOP) in order to maintain an organic certification [41]. These include: composted manure or vegetable waste as fertilizers instead of synthetic fertilizers; naturally derived

pesticides; and hydrogen peroxide, peroxyacetic acid or treated water instead of chlorine to wash produce during post-processing [30,41]. However, the use of animal manure-based fertilizers for organic fruit, vegetable and leafy greens has resulted in concerns about an increased risk of enteric pathogens entering the food chain [22,39-40]. Islam *et al.* [16] found that while composting is effective at eliminating pathogen contamination, if the manure compost contains *E. coli* O157:H7, the pathogen can still remain viable in the amended soil for greater than 5 months, allowing for easy contamination of the leafy greens. This highlights the need for effective antimicrobials as industrial washes to remove pathogen contamination in organically-grown salad greens, prior to packaging and before the final product reaches the consumer.

Chlorine (at 50 ppm) is a common and widely used sanitizer in the conventionally-grown fresh produce industry. However, in the organic fresh produce industry, the USDA-NOP prohibits the use of synthetic pesticides, fertilizers, or sanitizers and allows chlorine only at a concentration of 4 ppm [30]. Some of the alternative wash water treatments to wash organic fresh produce include hydrogen peroxide, acetic acid, ozone, and peroxyacetic acid, of which hydrogen peroxide is the most commonly used. The antimicrobial properties of hydrogen peroxide make it a desirable sanitizer for use in the food industry. However, studies have shown that effectiveness of hydrogen peroxide is limited to specific produce and pathogen types [2,29,38]. In a study by Taormina and Beuchat [38], use of hydrogen peroxide spray on alfalfa seeds and sprouts did not control the growth of *E. coli* O157:H7. Less than 1 log CFUg⁻¹ reduction in *Salmonella* population was observed on cantaloupe after treatment with 2% hydrogen peroxide, in a study by Beuchat and Ryu [2]. Treatment with hydrogen peroxide has also shown to induce extensive browning in certain types of produce like lettuce and mushrooms, unless an anti-browning agent is applied in combination [27], and therefore may not be suitable for certain kinds of produce.

In an attempt to discover new antimicrobials for washing fresh produce, the efficacy of plant extracts has previously been studied [13-14,19,25,28]. Combinations of malic acid, lactic acid, and grape seed extracts against *Salmonella* Typhimurium indicated a 2.6 to 3.3 log CFUg⁻¹ reduction on spinach [14]. On the other hand, 2.1 to 4.0 log CFUg⁻¹ reductions on spinach and 1.1 to 2.5 log CFUg⁻¹ reductions on lettuce were seen for *E. coli* O157:H7 [13]. Oregano (leaves) and lime (pulp) extracts showed antimicrobial properties against *Salmonella* spp., *Shigella*, and *E. coli* O157:H7 on cilantro, parsley, and spinach [28]. Clove extract was shown to be highly effective against *S. Typhimurium*, *E. coli* O157:H7, and *L. monocytogenes* on fresh lettuce [19]. However, most of these studies have focused on conventionally-grown fresh produce with very few studies evaluating the effectiveness of plant-derived antimicrobials on organically-grown leafy greens.

The focus of the present study was to test the effectiveness of selected plant extracts for washing organic leafy greens. The antimicrobial efficacy of various concentrations of apple, grape seed, and olive extracts against *E. coli* O157:H7 was evaluated under simulated washing process for organic leafy greens and storage before retail display. A three day refrigeration period was chosen for this study in an attempt to simulate storage conditions in the timeframe between postharvest processing and distribution for retail of organic leafy greens.

Materials and Methods

Bacterial culture preparation and media

A cocktail of three *E. coli* O157:H7 strains was used in this study: ATCC 43888, ATCC 43895, and ATCC 35150. Cryo-preserved cells were activated by obtaining a swab from the frozen stock culture, transferring to tryptic soy broth (TSB; Bacto™, BD, Sparks, MD) and incubating at 37°C for 18-24 hrs. From the resulting culture, 100µl was then subcultured twice into TSB and maintained at 4°C on tryptic soy agar (TSA; Acumedia, Lansing, MI). A single colony was then picked, inoculated in 9 ml TSB, and incubated at 37°C for 18-20 hrs to obtain an overnight culture. This fresh overnight culture was used to prepare a cocktail by mixing equal volumes of overnight cultures for each strain. The cocktail was further diluted in buffered peptone water (BPW; Oxoid Ltd., Basingstoke, Hampshire, England) to obtain a population of 6 logs CFU ml⁻¹ to prepare the dip inoculum for leafy greens.

Antimicrobial treatment preparation

The extracts included in this study were obtained from apple peel (Apple Poly, L.L.C., Morrill, NE), olive (CreAgri Inc., Hayward, CA), and grape seed (local natural foods store). The powdered extracts were weighed appropriately and added to sterile phosphate buffered saline (PBS: sodium chloride, Fischer Scientific, NJ, U.S.A; potassium chloride, sodium phosphate monobasic and sodium phosphate dibasic, Sigma-Aldrich, MO, U.S.A) to make 1%, 3%, and 5% concentrations (wt/v) for each extract. All powdered extracts were mixed thoroughly in PBS, using a stirring plate, until completely dissolved. The pH of the extract concentrations was measured before each experiment (Table 5).

Leafy greens preparation

The organic leafy greens tested were, romaine and iceberg lettuce, and mature and baby spinach, obtained from local stores in Stillwater, OK. The leafy greens were bought on the day of the experiment, transported on ice-packs in a cooler, and stored under refrigerated conditions (4°C) until use. All the leafy greens were washed thoroughly for 2 minutes under running tap water (room temperature (RT); 23-25°C) to remove dirt and organic particles.

Lettuce (romaine and iceberg) were prepared by discarding the outer leaves and removing the core (for iceberg lettuce only). Using aseptic techniques, the lettuce leaves were cut with sterile scissors into 1.5 x 1.5" pieces. Whole leaves of baby spinach (approximately 1.5 to 2.0" leaves) were used whereas bunched mature spinach samples were prepared by separating individual leaves, trimming off the stalks, and then cutting the leaves into 1.5 x 1.5" pieces with sterile scissors, using aseptic techniques.

Antimicrobial activity of olive, grape seed, and apple extracts against *Escherichia coli* O157:H7 on organic leafy greens

Approximately 300 g of leafy greens were prepared as described above, weighed, placed in a sterile plastic tub (L21.4" x W17.1" x H5.1") and washed three times in sterile distilled water (RT, 23-25°C), using a gentle back-and-forth motion. Each washing step was carried out for 2 minutes. The leafy green samples were then exposed to UV (254 nm) light under a bio-safety cabinet for 30 minutes (15 minutes on each side of the leaf) to reduce background microflora. After UV exposure, a 20 g sample was set aside to be

used as the negative (uninoculated) control. The remaining greens (280 g) were then dip inoculated [21] for 2 minutes in 2800 ml inoculum containing the *E. coli* O157:H7 cocktail, prepared as described above. Inoculated leaves were then removed from the inoculum using sterile forceps and placed for 30 minutes under the bio-safety cabinet to facilitate attachment. A 20 g sample of inoculated leafy greens was set aside to be used as the positive control, while the remaining greens were separated into 20 g samples. Each of these 20 g samples were washed in the appropriate antimicrobial treatment solution (200 ml each) for 2 minutes with gentle agitation using a horizontal, back-and-forth motion. Along with the positive and negative experiment controls, 3% hydrogen peroxide and sterile distilled water were used as industrial controls. Phosphate buffered saline was also used as a control as it was used in the study to dissolve the powdered extracts during preparation of the antimicrobial treatments. Following the wash, leaves were removed from the liquid treatment and any excess liquid shaken off the treated leaves before placing into a sterile Whirl-Pak™ bag (NASCO, Fort Atkinson, WI). Treated leaves were then stored at 4°C for three days. On days 0, 1, and 3, a 5 g sample from each stored sample was evaluated for surviving *E. coli* O157:H7 populations by transferring into sterile Whirl-Pak™ bags and stomaching with 45 ml of sterile buffered peptone water (BPW; BBL™, Difco, BD) at 230 rpm for 1 minute. The resulting sample was then serially diluted in BPW and plated on Sorbitol MacConkey agar (SMAC; Remel Inc., Lenexa, KS) to recover surviving *E. coli* O157:H7 populations and also on tryptic soy agar (TSA, Bacto™, Becton Dickinson, Sparks, MD) to recover injured pathogen populations. Colonies of *E. coli* O157:H7 (CFU g⁻¹) were counted after 18-24 hrs of incubation at 37°C.

Statistical analysis

Each trial, including all the controls and extract concentrations,

was conducted three times. The *E. coli* O157:H7 populations (in CFUg⁻¹) recovered after the antimicrobial treatments at each sampling period were converted to log CFUg⁻¹ and an average of the results per treatment was obtained. The limit of detection was 1 log CFUg⁻¹. The experimental design was a 4 x 13 x 3 factorial in a completely randomized design comparing: 4 leafy greens (baby spinach, mature spinach, romaine and iceberg lettuce); 13 treatments (positive control, hydrogen peroxide, PBS, water, olive extract at 1%, 3%, and 5%, apple extract at 1%, 3%, and 5%, and grape seed extract at 1%, 3%, and 5%) and 3 storage times (0, 1, and 3 days). Results were analyzed using PROC MIXED in SAS v. 9.3 software (SAS Inst., Cary, NC, U.S.A) to determine the analysis of variance (ANOVA) with the significant difference between results estimated at P < 0.05.

Results and Discussion

The Results of the study, for each leafy green treated with the extracts, along with the controls, are shown in Tables 1-4. These results only show population data for *E. coli* O157:H7 recovered on SMAC agar since no differences were observed when compared to populations recovered on TSA (data not shown). Population data for the negative control is not shown since *E. coli* O157:H7 colonies were not recovered for any of the negative control trials conducted.

The results from this study (Tables 1-4) indicate that in most of the leafy greens tested, olive, grape seed, and apple extracts were able to significantly reduce *E. coli* O157:H7 populations by day 3 in comparison to the hydrogen peroxide, sterile water and PBS controls (P<0.05). The efficacy of all three extracts varied, depending on the produce type. In comparison to the other three leafy greens (mature spinach, romaine and iceberg lettuce), *E. coli*

Table 1. Antimicrobial effects of olive, apple, and grape seed extracts at 1, 3, and 5% concentrations against *Escherichia coli* O157:H7 on baby spinach at 4°C

Treatment	Concentration (%)	Surviving <i>E. coli</i> O157:H7 Population (Log ₁₀ CFU/g)			Log Reductions (Log ₁₀ CFU/g)		
		Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
Positive control		4.6 ± 0.8 ^a	4.8 ± 0.2 ^a	4.7 ± 0.4 ^a	-	-	-
PBS		4.4 ± 0.1 ^a	4.2 ± 0.1 ^{ab}	4.1 ± 0.0 ^{ab}	0.2	0.6	0.6
Water		3.5 ± 0.4 ^{ab}	3.5 ± 0.5 ^{ab}	4.7 ± 0.7 ^a	1.1	1.3	0
HP		2.0 ± 0.9 ^{bc}	3.3 ± 0.9 ^b	3.5 ± 0.4 ^{ab}	2.6	1.5	1.2
Olive extract	1	3.7 ± 0.7 ^{ab}	3.2 ± 0.7 ^b	1.4 ± 0.4 ^c	0.9	1.6	3.3
Olive extract	3	2.8 ± 0.4 ^{bc}	2.1 ± 0.0 ^{bc}	0.8 ± 0.1 ^c	1.8	2.7	3.9
Olive extract	5	1.4 ± 0.6 ^c	ND ^d	ND ^c	3.2	4.8	4.7
Apple extract	1	3.5 ± 0.3 ^{ab}	3.8 ± 0.5 ^{ab}	3.7 ± 1.3 ^{ab}	1.1	1	1
Apple extract	3	3.5 ± 0.7 ^{ab}	3.2 ± 1.0 ^{ab}	3.5 ± 1.2 ^{ab}	1.1	1.6	1.2
Apple extract	5	3.9 ± 0.4 ^{ab}	3.9 ± 0.5 ^{ab}	3.3 ± 1.6 ^{ab}	0.7	0.9	1.4
Grape seed extract	1	3.7 ± 0.7 ^{ab}	3.7 ± 0.2 ^{ab}	3.3 ± 1.1 ^{ab}	0.9	1.1	1.4
Grape seed extract	3	3.9 ± 0.4 ^{ab}	3.2 ± 0.8 ^{ab}	2.4 ± 0.2 ^{bc}	0.7	1.6	2.3
Grape seed extract	5	3.4 ± 0.6 ^{ab}	1.2 ± 0.1 ^{cd}	2.2 ± 0.3 ^{bc}	1.2	3.6	2.5

1 PBS: Phosphate Buffered Saline; HP: Hydrogen Peroxide

2 Values represent average mean of three replications. Standard deviation for surviving *E. coli* O157:H7 population (Log₁₀ CFU/g) is presented following mean value.

3 Mean values with letters a, b, c, etc. provide evidence of significant difference (P<0.05), where different letters represent statistical significance between treatments for the same sampling day.

4 ND = No growth detected

5 ‘-’ Indicates control

Table 2. Antimicrobial effects of olive, apple, and grape seed extracts at 1, 3, and 5% concentrations against *Escherichia coli* O157:H7 on mature spinach at 4°C

Treatment	Concentration (%)	Surviving <i>E. coli</i> O157:H7 Population (Log ₁₀ CFU/g)			Log Reductions (Log ₁₀ CFU/g)		
		Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
Positive control		4.6 ± 0.7 ^a	4.7 ± 1.0 ^a	4.8 ± 0.4 ^a	-	-	-
PBS		3.4 ± 0.7 ^{a,b}	3.6 ± 1.0 ^{a,b}	3.3 ± 0.9 ^{a,b}	1.2	1.1	1.5
Water		3.7 ± 1.0 ^{a,b}	3.3 ± 0.8 ^{a,b}	3.3 ± 0.9 ^{a,b}	0.9	1.4	1.5
HP		2.9 ± 0.3 ^{b,c}	2.3 ± 1.1 ^{b,c}	2.5 ± 0.7 ^b	1.7	2.4	2.3
Olive extract	1	3.0 ± 0.1 ^{a,b}	2.2 ± 1.0 ^{b,c}	1.9 ± 0.5 ^b	1.6	2.5	2.9
Olive extract	3	2.3 ± 0.9 ^{b,c}	1.5 ± 0.4 ^{c,d}	ND ^c	2.3	3.2	4.8
Olive extract	5	1.6 ± 0.3 ^c	0.4 ± 0.1 ^d	ND ^c	3	4.3	4.8
Apple extract	1	3.6 ± 0.9 ^{a,b}	3.5 ± 1.2 ^{a,b}	3.4 ± 1.1 ^{a,b}	1	1.2	1.4
Apple extract	3	3.3 ± 0.9 ^{a,b}	3.0 ± 0.9 ^{a,b,c}	3.2 ± 0.9 ^{a,b}	1.3	1.7	1.6
Apple extract	5	3.0 ± 1.4 ^{a,b}	2.6 ± 1.5 ^{b,c}	2.6 ± 0.2 ^b	1.6	2.1	2.2
Grape seed extract	1	3.8 ± 0.6 ^{a,b}	3.8 ± 0.9 ^{a,b}	2.9 ± 0.3 ^b	0.8	0.9	1.9
Grape seed extract	3	3.3 ± 0.9 ^{a,b}	3.3 ± 1.0 ^{a,b}	2.2 ± 0.1 ^b	1.3	1.4	2.6
Grape seed extract	5	3.7 ± 0.7 ^{a,b}	3.3 ± 0.8 ^{a,b}	2.9 ± 0.1 ^b	0.9	1.4	1.9

1 PBS: Phosphate Buffered Saline; HP: Hydrogen Peroxide

2 Values represent average mean of three replications. Standard deviation for surviving *E. coli* O157:H7 population (Log₁₀ CFU/g) is presented following mean value.

3 Mean values with letters a, b, c, etc. provide evidence of significant difference (P<0.05), where different letters represent statistical significance between treatments for the same sampling day.

4 ND = No growth detected

5 ‘-’ Indicates control

Table 3. Antimicrobial effects of olive, apple, and grape seed extracts at 1, 3, and 5% concentrations against *Escherichia coli* O157:H7 on romaine lettuce at 4°C

Treatment	Concentration (%)	Surviving <i>E. coli</i> O157:H7 Population (Log ₁₀ CFU/g)			Log Reductions (Log ₁₀ CFU/g)		
		Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
Positive control		5.2 ± 1.0 ^a	5.1 ± 1.3 ^a	5.3 ± 1.1 ^a	-	-	-
PBS		3.7 ± 1.1 ^{a,b}	3.9 ± 0.4 ^{a,b}	3.6 ± 1.0 ^{a,b}	1.5	1.2	1.7
Water		3.1 ± 1.0 ^{a,b}	3.6 ± 1.0 ^{a,b}	3.5 ± 0.9 ^{a,b}	2.1	1.5	1.8
HP		1.6 ± 0.6 ^{b,c}	2.0 ± 0.8 ^b	2.3 ± 0.7 ^{b,c}	3.6	3.1	3
Olive extract	1	2.2 ± 0.8 ^b	2.8 ± 1.3 ^b	1.3 ± 0.4 ^{b,c}	3	2.3	4
Olive extract	3	0.5 ± 0.1 ^c	0.8 ± 0.8 ^c	0.8 ± 0.3 ^{b,c}	4.7	4.3	4.5
Olive extract	5	ND ^c	ND ^c	ND ^{c,d}	5.2	5.1	5.3
Apple extract	1	3.4 ± 0.9 ^b	3.7 ± 1.1 ^{a,b}	3.7 ± 0.9 ^{a,b}	1.8	1.4	1.6
Apple extract	3	3.0 ± 0.8 ^b	3.1 ± 1.0 ^{a,b}	2.7 ± 1.3 ^{b,c}	2.2	2	2.6
Apple extract	5	1.3 ± 0.3 ^{b,c}	1.2 ± 1.4 ^{b,c}	1.7 ± 0.9 ^{b,c}	3.9	3.9	3.6
Grape seed extract	1	1.9 ± 0.9 ^{b,c}	3.7 ± 1.4 ^{a,b}	2.5 ± 0.6 ^{b,c}	3.3	1.4	2.8
Grape seed extract	3	1.7 ± 0.9 ^{b,c}	2.8 ± 1.0 ^b	1.0 ± 0.7 ^c	3.5	2.3	4.3
Grape seed extract	5	2.2 ± 0.4 ^b	2.5 ± 0.6 ^b	0.9 ± 0.6 ^c	3	2.6	4.4

1 PBS: Phosphate Buffered Saline; HP: Hydrogen Peroxide

2 Values represent average mean of three replications. Standard deviation for surviving *E. coli* O157:H7 population (Log₁₀ CFU/g) is presented following mean value.

3 Mean values with letters a, b, c, etc. provide evidence of significant difference (P<0.05), where different letters represent statistical significance between treatments for the same sampling day.

4 ND = No growth detected

5 ‘-’ Indicates control

Table 4. Antimicrobial effects of olive, apple, and grape seed extracts at 1, 3, and 5% concentrations against *Escherichia coli* O157:H7 on iceberg lettuce at 4°C

Treatment	Concentration (%)	Surviving <i>E. coli</i> O157:H7 Population (Log ₁₀ CFU/g)			Log Reductions (Log ₁₀ CFU/g)		
		Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
Positive control		5.3 ± 1.7 ^a	5.1 ± 1.2 ^a	5.0 ± 1.0 ^a	-	-	-
PBS		3.8 ± 0.4 ^b	3.6 ± 1.0 ^{bc}	3.8 ± 0.4 ^{ab}	1.5	1.5	1.2
Water		3.4 ± 0.3 ^b	3.5 ± 1.0 ^{bc}	4.0 ± 1.0 ^{ab}	1.9	1.6	1
HP		1.4 ± 0.3 ^{cd}	2.3 ± 0.4 ^c	1.8 ± 0.4 ^{cd}	3.9	2.8	3.2
Olive extract	1	2.7 ± 0.7 ^{bc}	2.8 ± 1.3 ^{bc}	0.3 ± 0.5 ^c	2.6	2.3	4.7
Olive extract	3	1.5 ± 0.4 ^{cd}	ND ^d	ND ^c	3.8	5.1	5
Olive extract	5	1.2 ± 0.1 ^d	ND ^d	ND ^c	4.1	5.1	5
Apple extract	1	3.7 ± 0.4 ^b	3.8 ± 1.0 ^{ab}	2.9 ± 0.5 ^{bc}	1.6	1.3	2.1
Apple extract	3	3.3 ± 0.8 ^b	3.2 ± 0.3 ^{bc}	2.5 ± 0.4 ^{bc,d}	2	1.9	2.5
Apple extract	5	3.3 ± 1.0 ^b	2.9 ± 0.9 ^{bc}	1.6 ± 0.4 ^{dc}	2	2.2	3.4
Grape seed extract	1	3.2 ± 1.0 ^b	3.7 ± 0.7 ^b	3.1 ± 0.4 ^c	2.1	1.4	1.9
Grape seed extract	3	3.0 ± 1.1 ^b	3.4 ± 0.9 ^{bc}	3.2 ± 0.6 ^c	2.3	1.7	1.8
Grape seed extract	5	3.3 ± 0.7 ^b	2.9 ± 1.0 ^{bc}	3.3 ± 0.6 ^c	2	2.2	1.7

1 PBS: Phosphate Buffered Saline; HP: Hydrogen Peroxide

2 Values represent average mean of three replications. Standard deviation for surviving *E. coli* O157:H7 population (Log₁₀ CFU/g) is presented following mean value.

3 Mean values with letters a, b, c, etc. provide evidence of significant difference (P<0.05), where different letters represent statistical significance between treatments for the same sampling day.

4 ND = No growth detected

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O157:H7 survivors on baby spinach (Table 1) were the least susceptible to all treatments. The effectiveness of the antimicrobial treatments was also dependent on both, concentration as well as storage time. The pH of the extracts was found to range from 2.89 to 5.43 (Table 5). However, acidity did not seem to influence the antimicrobial effects of the extract formulations since results show that the pH of the extracts did not correlate with their antimicrobial effects. Apple extract (most acidic) was the least effective in reducing pathogen populations compared to olive or grape seed extracts (Tables 1-4).

Antimicrobial efficacy of olive, grape Seed, and apple extracts against *E. Coli* O157:H7 on organic leafy greens

Of all the extracts tested, olive extract (at all concentrations tested) was the most effective, resulting in pathogen reductions between 2.9-5.3 log CFUg⁻¹ for each leafy green by day 3. In romaine lettuce, the 5% olive extract reduced the pathogen populations to undetectable levels by day 0, whereas the 1% and 3% olive extract treatments resulted in 4.0 and 4.5 log CFUg⁻¹ reductions by day 0, respectively (Table 3). For iceberg lettuce, no *E. coli* O157:H7 was recovered on days 1 and 3 after treatment with 3% and 5% olive extract whereas a 4.7 log CFUg⁻¹ reduction was observed by day 3 with the 1% treatment (Table 4). For mature spinach, treatment with 3% and 5% olive extract reduced *E. coli* O157:H7 populations to undetectable levels by day 3 (Table 2). Baby spinach, in comparison to the other leafy greens, showed lower log CFUg⁻¹ reductions in pathogen populations after treatment with 1% and 3% olive extract, resulting in 3.3 and 3.9 log CFUg⁻¹ reductions by day 3, respectively (Table 1). Treatment with 5% olive extract reduced the *E. coli* O157:H7 populations to undetectable levels by day 1 on baby spinach. Overall, the 5% olive extract treatment was the most effective treatment and was statistically significant from

all other treatments (P<0.05).

The trend observed in this study, following treatment of leafy greens with olive extract, is similar to that obtained in previous studies by the authors with *S. Newport* [25], where olive extract showed the highest pathogen reduction in comparison to other plant-derived extracts. In the study by Moore *et al.* [25], the highest log CFUg⁻¹ reduction observed for each olive extract concentration on leafy greens by day 3 was between 2-3 logs CFUg⁻¹. In comparison, the present study resulted in higher log CFUg⁻¹ reductions of *E. coli* O157:H7 (2.9-5.3 log CFUg⁻¹) with olive extract formulations (Tables 1-4). This may suggest that compared to *S. Newport*, *E. coli* O157:H7 is more susceptible to olive extract. Olive extract is known to contain a variety of polyphenols [24,32,33], which have been found to possess antimicrobial activity over a broad range of microorganisms [20,23,37]. Of the many polyphenols found in olive extract, 4-hydroxytyrosol is the most abundant, making 50-70% of the total composition, and has the greatest biological effect [36]. Previous *in vitro* studies have indicated that hydroxytyrosol may have broad range antimicrobial activities against other pathogenic bacteria such as *Staphylococcus aureus*, *Vibrio parahaemolyticus*, and *S. Typhi* [3,12]. The present study also establishes this fact with *E. coli* O157:H7.

Compared to the positive control, grape seed extract also showed significant reductions in *E. coli* O157:H7 populations on all the leafy greens. By day 3, a reduction of 4.3 and 4.4 log CFUg⁻¹ in pathogen populations was seen in romaine lettuce (Table 3) and that of 2.6 and 2.2 log CFUg⁻¹ was seen in mature spinach (Table 2), after treatment with 3% and 5% grape seed extracts, respectively. A 2.5 log CFUg⁻¹ reduction after treatment with 5% grape seed extract was observed in baby spinach (Table 1), while iceberg lettuce showed approximately 2 log CFUg⁻¹ reductions for all

Table 5. pH values of olive, apple, and grape seed extracts at 1, 3, and 5% concentrations in phosphate buffered saline

Extract	Concentration (%)	pH
Grape seed	1	5.43
Grape seed	3	4.96
Grape seed	5	4.76
Olive	1	3.7
Olive	3	3.61
Olive	5	3.51
Apple	1	3.56
Apple	3	3.04
Apple	5	2.89

grape seed extract concentrations by day 3 (Table 4).

A study that investigated the efficacy of grape seed extract as an antimicrobial treatment demonstrated the ability of this plant extract to significantly reduce populations of *Campylobacter* spp. [35], one of the common bacterial pathogens associated with foodborne disease. Furthermore, grape seed extract has been shown to be very effective against *S. aureus* [11] and against *S. Typhimurium* on spinach, in conjunction with malic acid and lactic acid in electrostatic sprays, resulting in 2.3 to 3.3 log CFU g⁻¹ reductions [14]. Similar studies against *E. coli* O157:H7 with spinach and iceberg lettuce, using grape seed extract at 2% and 3% concentration in electrostatic sprays, yielded a 2.1 and 3.8 log reduction, respectively, by day 14 [13]. In the present study, by day 3, treatment with 3% grape seed extract showed reductions of 2.6 log CFUg⁻¹ (mature spinach), 2.3 log CFUg⁻¹ (baby spinach), and 1.8 log CFUg⁻¹ (iceberg lettuce). Additionally, washing the aforementioned leafy greens with higher concentration (5%) of grape seed extract formulation resulted in reductions between 1.7 to 3.6 log CFUg⁻¹ from day 0-3 (Tables 1-2, 4). On the other hand, treatment of romaine lettuce with 3% and 5% grape seed extract yielded over 4 log CFUg⁻¹ reductions by day 3 (Table 4). These results indicate that the type of produce may have an effect on antimicrobial efficacy against *E. coli* O157:H7. The antimicrobial properties of grape seed extract may be attributed to the high concentration of polyphenols found in it [23,34,43]. Results from the present study confirm the effectiveness of grape seed extract as an antimicrobial against *E. coli* O157:H7, and suggest that higher concentrations (3% and 5% treatments) are more effective against the pathogen on organic leafy greens.

The three concentrations of apple extract did not differ significantly ($P < 0.05$) from each other in their effectiveness against *E. coli* O157:H7. By day 3, 1%, 3% and 5% apple extracts showed 1.0, 1.2 and 1.4 log CFUg⁻¹ reductions in baby spinach, respectively (Table 1) and 1.4, 1.6, and 2.2 log CFUg⁻¹ reductions in mature spinach, respectively (Table 2). By the third day of storage, treatment with 3% apple extract was able to produce a reduction of 2.6 log CFUg⁻¹ and treatment with 5% apple extract resulted in a reduction of 3.6 log CFUg⁻¹ in romaine lettuce (Table 3). For iceberg lettuce, treatment with 3% and 5% apple extracts produced reductions of 2.5 log CFUg⁻¹ and 3.4 log CFUg⁻¹ respectively, by day 3 (Table 4).

Apple-skin extract has been tested against other foodborne pathogens and has been shown to be an effective antimicrobial against *Salmonella*, *S. aureus* and *L. monocytogenes* [11,25]. The present study was also able to establish this fact with *E. coli* O157:H7. The

highest reduction in *E. coli* O157:H7 population (3.9 log CFUg⁻¹) was observed with the 5% apple extract on romaine lettuce by day 0. Overall, treatment with apple extract at all concentrations yielded lower reductions than treatment with olive or grape seed extract. A similar study by Moore *et al* [25] also showed similar trends, where treatment of leafy greens with apple extract resulted in lower reductions in *S. Newport* populations than olive extract (1-2 log CFUg⁻¹ reductions in comparison to 2-3 log CFUg⁻¹ reductions with olive treatment). The present study was able to achieve higher log CFUg⁻¹ reductions of *E. coli* O157:H7 with apple extract than those observed with *S. enterica* by Moore *et al* [25]. These results indicate that, compared to *S. Newport*, *E. coli* O157:H7 may be more susceptible to apple extract formulations.

Hydrogen peroxide (3%) was used in this study as an organic fresh produce industry control and its effectiveness as an antimicrobial agent, in comparison to apple, olive, and grape seed extracts, was also studied. The highest reduction seen after treatment with 3% hydrogen peroxide was 3.9 log CFUg⁻¹ by day 0, for iceberg lettuce (Table 4). Reductions of 1.7-3.6 log CFUg⁻¹ were observed for the rest of the greens by day 0 (Tables 1-3). Compared to the previous studies with *S. Newport* [25], where the maximum reduction observed after treatment with 3% hydrogen peroxide was about 1 log CFUg⁻¹ in organic leafy greens, higher reductions were observed in the present study. This suggests that hydrogen peroxide may be more effective against *E. coli* O157:H7 than *S. Newport*. However, it is noteworthy in the present study that by the third sampling day, two of the four leafy greens (baby spinach and romaine lettuce) showed an increase in surviving pathogen populations after treatment with hydrogen peroxide (Tables 1 and 3). These results suggest that hydrogen peroxide does possess antimicrobial properties, but may not be able to maintain long term antimicrobial effects against *E. coli* O157:H7 in certain produce types. Based on these results, the use of hydrogen peroxide alone as an antimicrobial wash for organic leafy greens may therefore be inadequate.

All three plant extract formulations demonstrated significant ($P < 0.05$) reductions in *E. coli* O157:H7 populations on all four types of organic leafy greens tested. This study demonstrates the potential of extracts derived from olives, grape seeds, and apple skins to inactivate multiple strains of *E. coli* O157:H7 on organic mature and baby spinach, and romaine and iceberg lettuce. The antimicrobial activity of these extracts was greater than that of hydrogen peroxide and continued to decrease pathogen populations over a three day period. Hence, there is potential for these extracts to be used as natural alternatives to chemical sanitizers for washing organic leafy greens during processing. However,

further investigation needs to be carried out to determine the effectiveness of these antimicrobial treatments against other food-borne pathogens in organic leafy greens as well as other types of fresh produce. Further studies also call for sensory analysis of the tested leafy greens to determine consumer acceptability.

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