Antibacterial and antioxidant properties of grape stem extract applied as disinfectant in fresh leafy vegetables

F. J. Vázquez-Armenta¹ · B. A. Silva-Espinoza¹ · M. R. Cruz-Valenzuela¹ · G. A. González-Aguilar¹ · F. Nazzaro² · F. Fratianni² · J. F. Ayala-Zavala¹

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Abstract In the present study total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity and antimicrobial properties of grape (*Vitis vinifera* var. Red Globe) stem extract is reported. Also, the identification of main phenolic compounds was carried out by UPLC-PAD analysis. TPC and TFC of extract were 37.25 g GAE kg⁻¹ and 98.07 g QE kg⁻¹, respectively. Extract showed an antioxidant capacity of 132.60 and 317 g TE kg⁻¹ for DPPH and ABTS radical scavenging capacity, respectively. The main phenolic compounds identified were rutin, gallic acid, chlorogenic acid, caffeic acid, catechin and ferulic acid. Extract inhibited the growth of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, and *Escherichia coli* O157: H7 at MIC range 16–18 g L⁻¹. Extract affected the different phases of bacterial growth. In addition, application of Extract (25 g L⁻¹) as a sanitizer was effective to reduce the populations of all bacteria inoculated in lettuce (0.859–1.884 log reduction) and spinach (0.843–2.605 log reduction). This study emphasizes the potential of grape processing byproducts as an emergent and attractive source of bioactive compounds with antioxidant properties and antimicrobial activity against important foodborne

pathogens. The study demonstrated that stem extract could be used to control the presence of human pathogenic bacteria in fresh leafy vegetables.

Keywords Grape stem extract · Agroindustrial byproducts · Antioxidants · Natural antimicrobials · Leafy vegetables

Introduction

Prevention of food contamination caused by pathogenic microorganisms during their manufacture, processing, and packaging has considerable importance to public health and consequently is a major issue for food industry (Liu et al. 2013). Only in the United States, 9.4 million episodes of foodborne illness occurred in 2011, resulting in 55,961 hospitalizations and 1351 deaths caused by pathogenic bacteria (Scallan et al. 2011). One of the causes of foodborne disease is the consumption of contaminated fresh produce with pathogenic bacteria (Liu et al. 2013). Leafy vegetables, that are consumed raw, are identified as the fresh produce commodity group of highest concern from a microbiological safety perspective (FAO/WHO 2008); because they are often grown in the open field and vulnerable to contamination from soil, sewage, water used for irrigation, and contact with (feces of) wildlife (FAO/WHO 2008). Pathogenic bacteria such as *Escherichia coli* O157: H7, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes*, that were traditionally associated with foods of animal origin, have been isolated from fresh produce including leafy vegetables (Berger et al. 2010).

To reduce the microbial load of fresh produce, prior to delivery to retail markets or during processing for

✉ J. F. Ayala-Zavala
jayala@ciad.mx

¹ Centro de Investigación en Alimentación y Desarrollo A.C. (CIAD, AC), Carretera a la Victoria Km 0.6, La Victoria., 83000 Hermosillo, Sonora, Mexico

² Institute of Food Science ISA-CNR, Via Roma 64, 83100 Avellino, Italy

packaging, are often washed with aqueous sanitizers such as chlorine, hydrogen peroxide, and trisodium phosphate. However, there is an increasing awareness of the consumers on chemical substances used as food preservatives. Also, the use of synthetic disinfectants at sublethal concentrations may result in cross-resistance to antibiotics through phenotypic changes and induction of gene expression in pathogenic bacteria (Potenski et al. 2003). These results highlight the importance of research for more efficient antibacterial compounds that do not have adverse effects and do not confer antibiotic resistance.

In this context, the use of natural antimicrobials from plants has been proposed as an alternative of synthetic sanitizers (Ayala-Zavala et al. 2011). Phenolic compounds, the most numerous and ubiquitous group of plant secondary metabolites, possess antibacterial properties against a wide range of pathogenic bacteria, including *Salmonella* spp., *E. coli* O157: H7, *S. aureus*, *L. monocytogenes* and *Bacillus subtilis*, among others (Ravichandran et al. 2011; Sánchez-Maldonado et al. 2011). It has been proposed that the antibacterial activity could be due to several modes of action, such as damage in the cytoplasmic membrane, inhibition of synthesis of nucleic acids, cell wall components, as well as, cell membrane (Bernard et al. 1997; Borges et al. 2013; Wu et al. 2013). Also, phenolic compounds may exhibit a wide range of biological effects including, antiinflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory action, that has been associated with their antioxidant activities (Del Rio et al. 2013). These properties have gained the attention of food industry due to its potential use as natural antibacterial additives.

On the other hand, grapes (*Vitis vinifera* L.) belong to the world's largest fruit crops with a global production of around 77.18×10^6 tons in 2013 (FAOSTAT 2015). Table grapes are common commercialized in clusters; however, the increased interest in ready to eat products has introduced table grapes in the minimally processed market due to easy to consume and the globalization of food trade (Almela et al. 2014). The byproducts generated in minimally processed grapes consist in grape stems, which comprise the woody part of grape clusters. There are few reports on the composition and quantification of phenolic compounds in table grape stems; however, this approach has been applied in grape stems from vinification byproducts. Llobera and Cañellas (2007) reported that total phenolic content (TPC) of Manto Negro grape stem is four times higher than pomace (a mixture of peels and seeds) (116 vs. 26.3 g GAE kg⁻¹ dw, respectively). Similarly,

TPC and total proanthocyanidins content of 10 different cultivars of *V. vinifera* (Cabernet Sauvignon, Callet, Chardonnay, Macabeu, Manto Negro, Merlot, Parellada, Premsal blanc, Syrah, and Tempranillo) stem byproducts ranged from 47 to 115.2 g GAE g⁻¹ dw and from 0.79 to 2.02 g of tannins kg⁻¹ dw, respectively (Gonzalez-Centeno et al. 2012). This highlights the potential of grape byproducts as source of phenolic compounds that could be used as antimicrobial agents.

For these reasons, the present study was aimed to investigate (1) the phenolic composition and antioxidant activity of table grape (*V. vinifera* var. Red Globe) stems extract, and (2) their in vitro antimicrobial activity against pathogenic bacteria, as well as (3) the efficacy as disinfectant of lettuce and spinach leaves as model of fresh leafy vegetables.

Materials and methods

Plant material

Grapes (*V. vinifera* L.) var. Red Globe were obtained from a local market in Hermosillo, Sonora, Mexico, and were manually de-stemmed in our laboratory. Grape stems were chopped into small pieces and stored at -20 °C prior to extraction.

Preparation of phenolic extract from grape stems

Freeze-dried grape stems (3 g) were homogenized in 10 mL solution of ethanol:water (7:3) (IKA® Works, Model T25, Willmington, NC, USA) at room temperature. The homogenate was sonicated for 30 min (Bransonic Ultrasonic Co., Model 2210, Danbury, CT, USA) and then centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatants were collected and the pellet was resuspended again with 10 mL of 70% ethanol, under the conditions previously described twice. The three supernatants were mixed, filtered (Whatman No. 1, Springfield Mill, Maidstone Kent, UK) and solvent was removed using a rotary evaporator (Quirós-Sauceda et al. 2014). Then was hydrolyzed (50 mL of NaOH 4 M) for 4 h in absence of light, subsequently, an acid hydrolysis was performed with HCl (4 M) taking every sample to pH 2. The hydrolyzed extract was freeze-dried to obtain a powder extract. The extraction was performed by triplicate and extract yield, total phenolic, flavonoid contents, antioxidant capacity and antimicrobial activity of the extract were determined (Vega-Vega et al. 2013).

Characterization of phenolic content in grape stem extract

Total phenolic content

Total phenolic content was measured by the method described by Singleton and Rossi (1965), with some modifications. For the colorimetric assay, 75 μL of Folin–Ciocalteu reagent (1:10) and 60 μL of 7.5% Na_2CO_3 were added to 15 μL of the sample. After incubation in the dark for 30 min, optical density (OD) was measured at 765 nm using a microplate reader FLUOstar Omega (BMG Labtech, Chicago, IL, USA). Gallic acid was used as standard and results were expressed as grams of gallic acid equivalents per kilogram of dry weight extract (g GAE kg^{-1} dw). All the samples were determined by triplicate.

Total flavonoid content

Flavonoid content was determined based on the method described by Zhishen et al. (1999), with some modifications. The extract (100 μL) was mixed with 430 μL of mixture A (1.8 mL of NaNO_2 5% with 24 mL of distillate water) and incubated for 5 min. Then, 30 μL of anhydrous AlCl_3 at 10% were added and incubated for 1 min. Later, 440 μL of mix B (12 mL of NaOH 1 M with 14.4 mL of distillate water) were added and 150 μL of this reaction was taken and OD was read at 496 nm in a microplate reader FLUOstar Omega (BMG Labtech, Chicago, IL, USA). Results were expressed as grams quercetin equivalents per kilogram of dry weight of the extract (g EQ kg^{-1} dw). All the samples were determined by triplicate.

Chromatographic analysis of phenolic compounds (UPLC–PDA)

Identification and quantification of phenolic compounds were carried out by using ultra-performance liquid chromatography (UPLC) using an ACQUITY Ultra Performance LCTM system (Waters) linked to a PDA 2996 photodiode array detector (Waters). The ultraviolet-detection wavelength was set at 280 nm. Empower software (Waters) was used for controlling the instrument as well as for data acquisition and processing. The analysis was performed at 30 °C by using a reversed phase column (BEH C18 1.7 μm , 2.1×100 mm; Waters). The mobile phase consisted of solvent A (7.5 mM acetic acid) and solvent B (acetonitrile) at a flow rate of 250 $\mu\text{L min}^{-1}$. Gradient elution was used starting at 5% solvent B for 0.8 min, 5–20% solvent B for 5.2 min, isocratic 20% solvent B for 0.5 min, 20–30% solvent B for 1 min, isocratic 30% solvent B for 0.2 min, 30–50% solvent B for 2.3 min, 50–100% solvent B for 1 min, isocratic 100% solvent B for 1 min, and finally

100–5% solvent B for 0.5 min. At the end of this sequence, the column was equilibrated under the initial conditions for 2.5 min. The pressure ranged from 6000 to 8000 psi during the chromatographic run. The effluent was introduced to a liquid chromatography detector (scanning range, 210–400 nm; resolution, 1.2 nm). Injection volume was 5–10 μL (Fratianni et al. 2011). The identification was made by comparison of UV spectra, using a database previously made with reference substances. Quantification was performed using standard curves of the corresponding compounds and reported as $\mu\text{g g}^{-1}$ dw.

Free radical scavenging capacity

DPPH radical scavenging activity

The total antioxidant activity was determined using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method which measures the ability of antioxidants to quench a DPPH stable radical. The DPPH was adjusted with pure methanol to OD of 0.7. Then 140 μL of the radical, followed by 10 μL of extract were added by triplicate to a microplate. After incubation for 30 min OD was read at 515 nm in a microplate FLUOstar Omega (BMGLabtech, Chicago, IL, USA). The results were expressed as grams of trolox equivalents per kilogram of extract dry weight (g TE kg^{-1} dw). All the samples were determined by triplicate (Vega-Vega et al. 2013).

Trolox equivalent antioxidant capacity (TEAC)

The test consisted of the antioxidant capacity of the extracts to inactivate the radical $\text{ABTS}^{\cdot+}$. The radical cation $\text{ABTS}^{\cdot+}$ was generated by mixing 5 mL of a solution of 7 mM ABTS and 88 μL of a 0.139 mM solution of $\text{K}_2\text{S}_2\text{O}_8$ and allowed to react for 16 h in darkness. Subsequently, the radical was adjusted to an OD of 0.7 at 754 nm. For the test, 5 μL of extract and 245 μL of the $\text{ABTS}^{\cdot+}$ solution were added to a microplate and the OD was read after 5 min. The results were expressed as grams of Trolox equivalents per kilogram of dry weight extract (g TE kg^{-1} dw). All the samples were analyzed by triplicate (Vega-Vega et al. 2013).

Antimicrobial assays

Minimum inhibitory and bactericidal concentration

The antimicrobial properties of extract were evaluated against *L. monocytogenes* ATCC 7644, *S. aureus* ATCC 6538, *Escherichia coli* O157: H7 ATCC 43890, and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028. The minimum inhibitory concentration

(MIC) and minimum bactericidal concentration (MBC) of extract were determined using a broth microdilution technique. Serial dilutions of grape stem extract ($0\text{--}25\text{ g L}^{-1}$) were made up in sterile Muller Hilton Broth (MHB) in sterile 96-well microplates (Costar 96). To each well was added $5\text{ }\mu\text{L}$ of inoculum and $295\text{ }\mu\text{L}$ of each dilution of extract. The inoculum was prepared using a 16 h culture adjusted by reference to an $\text{OD} = 0.1$ at 600 nm using a microplate reader (FLUOstar Omega, BMG Labtech, Chicago, IL, USA) and further diluted with MHB to achieve approximately $1.25 \times 10^8\text{ CFU mL}^{-1}$, which produced a total bacterial load of approximately 10^6 CFU per well. A positive control (containing inoculums but no extract) and negative control (containing extract but no inoculums) were included on each microplate. The contents of the wells were mixed and the microplates were incubated at $37\text{ }^\circ\text{C}$ for 24 h. The MIC was the lowest concentration of extract at which inhibition of visible growth of the tested bacteria could be observed (Vega-Vega et al. 2013).

Effect of grape stem extract on kinetic parameters of pathogenic bacteria growth

Growth curves of the bacteria exposed to extract were obtained by triplicate based on the MIC assay. The culture conditions were as previously indicated, using a negative control containing $300\text{ }\mu\text{L}$ of culture medium and extract without bacteria. The plates were incubated at $37\text{ }^\circ\text{C}$ during 16 h with intermittent shaking and the OD at 600 nm was read every 30 min. The experimental growth data for each bacterial strain were fitted to the Baranyi function (Baranyi et al. 1993) using a complementary tool for Microsoft Excel (D-model, J. Baranyi, Institute of Food Research, Norwich, UK). Kinetic parameters, including lag time (h), growth rate (OD h^{-1}), and Y_{max} (OD) for each growth curve, were calculated using the Baranyi function (Vega-Vega et al. 2013).

Effect of grape stem extract on disinfection of leafy vegetables

Inoculation was performed according to the following procedure. Spinach and lettuce were immersed in sodium hypochlorite at 250 ppm to reduce the native microbiota. Samples (10 g) were inoculated with different solutions of bacteria (*L. monocytogenes*, *S. aureus*, *E. coli* O157: H7 or *S. enterica* ser. Typhimurium at $1 \times 10^6\text{ CFU mL}^{-1}$, respectively) by immersion during 2 min, and dried for 30 min in a biosafety cabinet (Esco II Airstream). The initial load of each inoculated bacteria was recorded after drying, subsequently, each inoculated vegetable was immersed during 2 min into 200 mL of grape stem extract

solution (25 g L^{-1}); after the treatments the samples were allowed to dry for 30 min. The bacterial load before and after the treatments were determined on aerobic Plate Count Agar (Ortega-Ramirez et al. 2017).

Statistical analysis

A completely randomized design was applied to all experiments. In first objective experiments were independently conducted three times and data were expressed as mean $\pm$ SEM (Standard Error of the Mean). In antimicrobial assays, the effect of extract (0 , $0.5 \times \text{MIC}$ and MIC) on *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *S. enterica* ser. Typhimurium kinetic parameters of bacterial growth (lag phase, growth rate and Y_{max}) was evaluated. The effect extract was evaluated on the log reduction of inoculated pathogenic bacteria in leafy vegetables (lettuce and spinach). All the data were subjected to analysis of variance (ANOVA) and a multiple range test (Tukey's test) ($p \leq 0.05$) performed using NCSS 2007.

Results and discussion

Identification and quantification of the major phenolic compounds and antioxidant activity of grape stem extract

Total extract yield, phenolic content, flavonoid content and antioxidant activities of grape stem extract are presented in Table 1. The powder extract of grape stem showed total phenolic content of $37.25 \pm 1.52\text{ g GAE kg}^{-1}\text{ dw}$ and total flavonoid content of $98.07 \pm 2.60\text{ g QE kg}^{-1}\text{ dw}$. In addition, the extract presented radical scavenger activity of 132.60 ± 3.45 and $317.0 \pm 10.76\text{ g TE kg}^{-1}$ against DPPH $^\bullet$ and ABTS $^{+\bullet}$ free stable radicals, respectively. Our results are in agreement with data reported in literature that highlight grape stems extracts as important source of phenolic compounds with antioxidant activity. de Sá et al. (2014) reported the antioxidant activity of Fernão Pires grape stems extracts ($\text{EC}_{50} = 0.052\text{--}0.090\text{ mg mM}^{-1}$ of DPPH $^\bullet$) that was well correlated ($r = 0.89$) to TPC ($17.0\text{--}19.0\text{ g GAE kg}^{-1}\text{ dw}$) and total phenol index ($20.0\text{--}40.0\text{ g (+)-catechin equivalents kg}^{-1}\text{ dw}$). Similarly, the TPC and total proanthocyanidins of the stems of 10 grape varieties (Cabernet Sauvignon, Callet, Chardonnay, Macabeu, Manto Negro, Merlot, Parellada, Premsal blanc, Syrah and Tempranillo) have been reported to range from 47.04 to $115.25\text{ g GAE kg}^{-1}$ and from 79.1 to $202.3\text{ g tannins kg}^{-1}$ with antioxidant capacities of $99.7\text{--}253.2$,

Table 1 Yield of extraction, total phenols and flavonoids content and antioxidant activity of Red Globe grape stem extract

Yield (%)	Phenol content (g GA kg ⁻¹ dw)	Flavonoid content (g QE kg ⁻¹ dw)	DPPH (g TE kg ⁻¹)	ABTS (g TE kg ⁻¹)
34.2	37.25 ± 1.52	98.07 ± 2.60	132.60 ± 3.45	317.0 ± 10.76
Mean ± SEM				

145.4–378.6, 65.4–170.1 and 101.9–282.1 g TE kg⁻¹, measured by ABTS, CUPRAC, FRAP and ORAC assays, respectively (Gonzalez-Centeno et al. 2012). The antioxidant activity has been attributed to the presence of phenolic compounds. Flavonoids and phenolic acids are able to scavenge free radicals directly by hydrogen atom donation. The free radical P-O[•] may react with a second radical, acquiring a stable quinone structure. However, the antioxidant activity depends on the arrangement of functional groups on its core structure. Both the configuration and total number of hydroxyl groups substantially influence the mechanism of the antioxidant activity (Procházková et al. 2011).

Table 2 shows the main phenolic compounds present in grape stem extract. The main phenolic compounds were rutin (212.57 µg g⁻¹ dw), gallic acid (184.10 µg g⁻¹ dw), chlorogenic acid (173.26 µg g⁻¹ dw), caffeic acid (50.59 µg g⁻¹ dw), catechin (34.31 µg g⁻¹ dw) and ferulic acid (8.19 µg g⁻¹ dw). Previously, grape stems have been reported as an important source of flavonoids, including catechin, epicatechin, epicatechin-3-O-gallate, proanthocyanidins (B1–B3, procyanidin dimers), quercetin and their glycoside derivatives, among others (Gonzalez-Centeno et al. 2012). Whereas gallic acid has been considered as the main hydroxybenzoic acid in grape stems, and hydroxycinnamic acids (*p*-coumaric, ferulic, caffeic and their derivatives) are present only in trace amounts (Anastasiadi et al. 2012). The differences in phenolic profile and/or quantitative amounts of the main components of grape stem extract reported in the literature and our results could be due mainly to grape variety studied and the extraction procedure as previously indicated by Gonzalez-Centeno et al. (2012).

In vitro antimicrobial properties of grape stem extract against pathogenic bacteria

Grape stem extracts have been considered an important source of phenolic compounds with antioxidant activity; however, the antimicrobial properties of its byproduct remains scarcely investigated. The antimicrobial activity of grape stem extract was determined by broth microdilution test against two Gram positive (*L. monocytogenes* and *S. aureus*) and two Gram negative bacteria (*E. coli* O157:H7 and *S. enterica* ser. Typhimurium). The minimum inhibitory and bactericidal concentrations (MIC and MBC) for each bacteria are presented in Table 3. *S. aureus* and *S. enterica* ser. Typhimurium were more sensitive (MIC = 16 g L⁻¹) to grape stem extract than *L. monocytogenes* and *E. coli* O157:H7 (MIC = 18 g L⁻¹). On the other hand, bactericidal concentrations were not found (>22 g L⁻¹) in the tested range, indicating that grape stem extract exhibit a bacteriostatic effect.

Previous studies have reported the antimicrobial activity of stems from vinification grapes varieties. For example, Dias et al. (2015) evaluated the antibacterial capacity of methanolic extracts of grape stems from red and white varieties against Gram positive and Gram negative pathogenic bacteria (*L. monocytogenes*, *S. aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *E. coli* and *Klebsiella pneumoniae*) and found differential sensitivity to distinct stem extracts with MIC values from 66.7 to >134.0 g L⁻¹. Also, a study carried out by Anastasiadi et al. (2009) showed that methanolic extract of red grape (var. Mandilaria) stems was effective against *L. monocytogenes* with a MIC of 3.45 g L⁻¹. The antilisterial effect was attributed to total phenolic content of extract (536.8 g GAE kg⁻¹)

Table 2 Phenolic compounds identified in Red Globe grape stem extract by UPLC-PDA

Compound	Retention time (min)	Peak area (mm ²)	Height (mm)	Concentration (µg g ⁻¹ dw)
Gallic acid	1.035	3,315,229	929,227	184.10
Chlorogenic acid	2.843	826,747	37,931	173.26
Catechin	3.330	1,042,036	67,392	34.31
Caffeic acid	3.840	–	–	50.59
Rutin	5.283	–	–	212.57
Ferulic acid	5.619	116,517	29,441	8.19

Table 3 Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations of Red Globe grape stem extract against pathogenic bacteria

Bacteria	MIC (g L ⁻¹)	MBC (g L ⁻¹)
<i>L. monocytogenes</i>	18	>24
<i>S. aureus</i>	16	>22
<i>E. coli</i> O157: H7	18	>24
<i>S. enterica</i> ser. Typhimurium	16	>22

and its main constituents; (+)-catechin (85.81 µg g⁻¹), procyanidin B3 (31.55 µg g⁻¹), ε-viniferin (31.42 µg g⁻¹) and *trans*-resveratrol (17.56 µg g⁻¹). In comparison with our results, the differences in the MIC values could be due to the extraction method used that influence the phenolic profile in stem extracts as reported by Agustin-Salazar et al. (2014).

In our results, there was no great difference in antimicrobial activity against Gram negative and Gram positive bacteria, which was reported for plant phenolic extracts (Martin et al. 2012), but we found similarities with another grape byproducts. For example, supercritical extracts from Merlot grape pomace obtained at 300 bar/50 °C showed MIC values against *S. aureus* of 625 ± 375 µg mL⁻¹, and 1000 µg mL⁻¹ for *E. coli* and *P. aeruginosa* (Oliveira et al. 2013). Similarly, *S. aureus* was more sensitive to ethanolic and methanolic extracts of Petit Verdot and Pinot Noir grape marcs (MICs = 3.13 to 6.25 g L⁻¹) than *L. monocytogenes* (MICs = 12.5 – > 25 g L⁻¹). However, for *S. enterica* ser. Enteritidis and *E. coli* no antimicrobial activity was observed for any extract (Martin et al. 2012). These results underline the importance of evaluating antimicrobial activity of natural extracts using different pathogenic bacteria.

The presence of grape stem extract affected the different phases of bacterial growth (Table 4). At MICs, the lag phase was extended >16 h for all bacteria tested, for this reason, no growth was observed. On the other hand, at half of the MIC, an extension of lag phase could be observed in all strains exposed to grape stem extract ($p < 0.05$). In *L. monocytogenes* the lag phase was extended ($p < 0.05$) from 1.5 h (control) to 5 h in the presence of grape stem extract at 9 g L⁻¹, and the growth rate decreased ($p < 0.05$) from 0.163 (control) to 0.104 OD/h. Whereas the maximum OD (Ymax) achieved after 16 h of incubation was 0.897 and 0.586 OD for exposed and unexposed bacteria, respectively. In addition, grape stem extract (8 g L⁻¹) extended ($p < 0.05$) the lag phase of *S. aureus* 3.23 h respect to control. However, an increment ($p < 0.05$) in growth rate was observed in exposed bacteria to grape stem extract (0.431 OD/h), despite this, no differences ($p > 0.05$) were found in Ymax parameter.

Regarding *E. coli* O157: H7, grape stem extract at 9 g L⁻¹ extended the lag phase 2.45 h more than the lag phase of control and no difference ($p > 0.05$) were found in growth rate. However, the Ymax was higher ($p < 0.05$) in exposed bacteria (1.264 OD) than control (0.559 OD). Finally, the presence of grape stem extract at 8 g L⁻¹ extended the lag phase (5.36 h), and decreased growth rate (0.087 OD/h) and Ymax (0.714 OD) of *S. enterica* ser. Typhimurium.

Antibacterial effect of grape stem extract can be associated with the presence of phenolic compounds. It has been reported that the flavonoid rutin (present in grape stem extract) selectively promoted cleavage of topoisomerase IV, an essential enzyme in *E. coli* survival (Bernard et al. 1997). Also, the antibacterial activity of quercetin has been attributed to the inhibition of DNA gyrase enzyme (Wu et al. 2013). Whereas phenolic acids such gallic and ferulic acid (also identified in grape stem extract) could diffuse through the cytoplasmic membrane, increasing its permeability and consequent leakage of essential intracellular constituents (Borges et al. 2013). It has been proposed that hydroxycinnamic acids (such as ferulic and caffeic acid) may interact better with the cell membrane due to its aliphatic group which makes them less polar compared to hydroxybenzoic acids (Nohynek et al. 2006). So, the antimicrobial activity of grape stem extract could be through a similar mechanism of action.

Effectiveness of grape stem extract to remove pathogens from fresh leafy vegetables

The results of the antibacterial effect of grape stem extract (25 g L⁻¹) on lettuce and spinach leaves inoculated with pathogenic bacteria (*L. monocytogenes*, *S. aureus*, *E. coli* O157: H7 or *S. enterica* ser. Typhimurium) are presented in Table 5. Distilled water was used as control treatment. Initial populations of pathogenic bacteria in lettuce leaves ranged from 4.189 to 4.617 log CFU g⁻¹, whereas in spinach were 4.410–4.705 log CFU g⁻¹. Reductions of 0.715–1.012 log CFU g⁻¹ were achieved with water in inoculated lettuce and 0.058–0.596 log CFU g⁻¹ reductions in inoculated spinach. This phenomenon was mainly attributed to the physical removal of pathogen cells from vegetables surfaces (Wang et al. 2013).

On the other hand, treatment with grape stem extract at 25 g L⁻¹ significantly reduced the numbers of *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *S. enterica* ser. Typhimurium in lettuce and spinach leaves. The log reductions of grape stem extract were higher ($p > 0.05$) than water for *S. aureus*, *E. coli* O157: H7 and *S. enterica* ser. Typhimurium in lettuce and for all strains in spinach leaves. In lettuce, the major effectiveness was observed in *E. coli* O157: H7 (1.884 log reduction), followed by *S. enterica* ser. Typhimurium, *S. aureus* and *L. monocytogenes* with 1.604,

Table 4 Effect of different concentrations of Red Globe grape stem extract on growth parameters of pathogenic bacteria

Bacteria	Treatment	Lag (h)	Growth rate (OD/h)	Ymax (OD)
<i>L. monocytogenes</i>	Control	1.50 a	0.163 a	0.586 a
	9 g L ⁻¹	5.08 b	0.104 b	0.897 b
	18 g L ⁻¹	>16	0	0
<i>S. aureus</i>	Control	0.60 a	0.197 a	1.049 a
	8 g L ⁻¹	3.84 b	0.431 b	1.008 a
	16 g L ⁻¹	>16	0	0
<i>E. coli</i> O157: H7	Control	1.52 a	0.189 a	0.559 a
	9 g L ⁻¹	3.97 b	0.154 a	1.264 b
	18 g L ⁻¹	>16	0	0
<i>S. enterica</i> ser. Typhimurium	Control	2.44 a	0.201 a	2.075 a
	8 g L ⁻¹	5.36 b	0.087 b	0.714 b
	16 g L ⁻¹	>16	0	0

Means in the same column not followed by the same letter are significantly ($p < 0.05$) different for each bacteria and kinetic parameter

Table 5 Effect of 25 mg mL⁻¹ of Red Globe grape stem extract in the reduction of pathogenic bacterial load (log CFU g⁻¹) in fresh lettuce and spinach leaves

Treatments	Lettuce		Spinach	
	Log CFU g ⁻¹	Log reduction	Log CFU g ⁻¹	Log reduction
<i>L. monocytogenes</i>				
Initial	4.189 a	–	4.410 a	–
Water	3.405 b	0.784	3.814 b	0.596
Extract	3.330 b	0.859	3.355 c	1.055
<i>S. aureus</i>				
Initial	4.617 a	–	4.705 a	–
Water	3.902 b	0.715	4.271 b	0.434
Extract	3.304 c	1.313	3.851 c	0.854
<i>E. coli</i> O157:H7				
Initial	4.494 a	–	4.659 a	–
Water	3.761 b	0.733	4.507 a	0.152
Extract	2.610 c	1.884	3.816 b	0.843
<i>S. enterica</i> ser. Typhimurium				
Initial	4.223 a	–	4.605 a	–
Water	3.211 b	1.012	4.547 a	0.058
Extract	2.619 c	1.604	2.0 b	2.605

Means in the same column not followed by the same letter are significantly ($p < 0.05$) different for each bacteria and vegetable

1.313 and 0.859 log reductions, respectively. Whereas in spinach the major reduction was observed for *S. enterica* ser. Typhimurium with 2.605 log reduction, followed by *L. monocytogenes*, *E. coli* O157: H7 and *S. aureus* with reductions of 1.055, 0.854 and 0.843 log CFU g⁻¹, respectively. These results indicate that grape stem extract could be used to effectively reduce the presence of human pathogenic bacteria in fresh leafy vegetables.

Similar reductions could be achieved with chlorine sanitizer, which is widely used to wash produce. For example, the chlorination treatment (150 ppm) reduced the initial level of *E. coli* O157: H7 by 1.23 log CFU g⁻¹

in fresh-cut lettuce (Posada-Izquierdo et al. 2013). Similarly, chlorinated water (20 ppm) were not capable of reducing microbial populations by more than 1 log in lettuce and bell peppers (Alexopoulos et al. 2013). Despite this, the concern in the formation of potential carcinogenic products derived of chlorine-based sanitizer in wash water has created the need to investigate the effectiveness of alternative decontamination techniques (Eyonganyoh et al. 2012). So, grape stem extract could be considered as a natural alternative disinfectant for decontamination of leafy vegetables based on their antimicrobial activity.

As mentioned previously, there are no studies that evaluate the effectiveness of grape stem extract as disinfectant in fresh leafy vegetables; however, some studies have been carried out with this purpose applying natural plant extracts. A study conducted by Bisha et al. (2010), showed that *L. monocytogenes* was reduced by ≈ 2 log units on tomatoes surfaces when exposed to commercial grape seed extract solution (0.125%) (Bisha et al. 2010). Also, washing (10 min) with tannin crude extract (containing gallotannins) or methanol extract (containing methyl gallate and penta-O-galloylglucose) from mango (*Mangifera indica* L.) kernels reduced the counts of *L. monocytogenes* in inoculated lettuce and spinach by ≈ 2.8 to $3.5 \log \text{CFU cm}^{-2}$ when applied at $0.1\text{--}1.0 \text{ g L}^{-1}$ (Engels et al. 2012). However, no differences were found among washing step with water and with tannin solutions in the reduction of *E. coli* O157: H7 and total coliforms in lettuce and spinach (Engels et al. 2012). While washing lettuce samples with oregano aqueous extract for 2 min resulted in $2.1 \log \text{CFU g}^{-1}$ reduction of *E. coli* O157: H7 and when combined with Citrox[®] (containing citric acid and phenolic compounds), $3.7\text{--}4.0 \log \text{CFU g}^{-1}$ reduction was achieved on spinach and lettuce samples (Poimenidou et al. 2016). These results indicated that plant extracts are effective to reduce the pathogenic load in fresh vegetables. In the case of the present study, our results demonstrated that grape stem extract is effective to reduce *L. monocytogenes*, *S. aureus*, *E. coli* O157: H7 and *S. enterica* ser. Typhimurium from lettuce and spinach leaves surfaces.

Conclusion

Results obtained in the present work demonstrated that grape stems extracts are rich in phenolic compounds with antioxidant properties and antimicrobial activity against foodborne pathogens. Also, demonstrated that grape stem extract could be applied as disinfectant to reduce the populations of human pathogenic bacteria in fresh leafy vegetables.

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