Antilisterial activity of broccoli stems (*Brassica oleracea*) by flow cytometry


1Food Technology Department, Federal University of Sergipe (UFS), Aracaju, Brazil
2Laboratory for Innovations in Therapy, Education and Bioproducts, Oswaldo Cruz Institute, Rio de Janeiro, Brazil
3Agri-Food industry, Food and Nutrition Department, “Luiz de Queiroz” College of Agriculture, University of São Paulo, Piracicaba, Brazil

Abstract

Studies have demonstrated the presence of antimicrobial compounds in vegetal species. However, natural components can be found not only in raw materials, but also in agro-industrial wastes. Their reuse as source of natural preservatives in food and beverage industry can promote sustainable practices since environmental risks caused by its discard are reduced. The antimicrobial activity of broccoli stems (*Brassica oleracea*) aqueous extracts (1:20 w/v) against *Listeria monocytogenes* was evaluated by detection of Minimal Inhibition Concentration (MIC) and flow cytometry analysis. The chemical composition of major compounds was detected by Gas Chromatography with Mass Spectrometry (CG-MS). Broccoli stems extract showed antimicrobial activity against *L. monocytogenes*, which MIC was 102.4 mg/mL. Analysis by CG-MS allowed the identification of organic acids, as ascorbic and malic acids, and phenolic compounds, as sinapinic, ferulic and caffeic acids. The use of flow cytometry to evaluate the antimicrobial activity of the extracts was very suitable, enabling to infer their action mechanisms. In face of the difficulty to avoid the food contamination by this pathogen, the study of vegetal residues as source of natural preservatives in food and beverage industry is very promising.

Keywords

Broccoli
Wastes
Antimicrobials
*Listeria monocytogenes*
Cytometry

Introduction

Natural products as source of health and welfare is a concept which has become frequent for consumers. In this sense, the demand for foods free of synthetic preservatives has caused changes in the beverage and food industry, which is interested in the potential of natural components for developing various new products (Moreira et al., 2006). The global commerce promotes the production and transportation of food worldwide, so the use of preservatives in foods becomes essential to assure quality foods supply (Davidson and Branen, 2005).

Studies have demonstrated the existence of antimicrobial compounds in vegetal materials, as leaves, flowers, seeds, peels (Baydar et al., 2004). These natural components can be found not only in raw materials, but also in agro-industrial wastes (Martin et al., 2012). Their reuse can promote sustainable practices since environmental risks caused by discard are reduced. Bioactive molecules produced by vegetal species are known as secondary metabolites, which play important roles in protection and defense (Harborne and Williams, 2000). In addition, phenolic compounds have been studied due to their antioxidant and antimicrobial properties (Luciano et al., 2008; Shin et al., 2004).

*Listeria monocytogenes* is a food pathogen that has became the focus of studies in recent years, after appearance of cases and outbreaks in human beings (Lundén et al., 2004; Barbuddhe et al., 2012). This microorganism is largely spread in the nature and can contaminate production, processing and distribution of food, representing risks for food consumers and industry (Chae et al., 2006). Any raw animal food or fresh vegetal can present *L. monocytogenes* contamination, as raw and frozen meat, chicken, seafood, fruits and vegetables (Jay, 2005). In processed food, it occurs in raw and poorly pasteurized milk, besides dairy products, as cheeses and ice-cream. As a psychrotrophic bacteria, its growth is improved in chilled food (Nuttawee, 2009). In this study, broccoli stems extract was evaluated for the presence of antimicrobial compounds with activity against *L. monocytogenes* using flow cytometry and its chemical composition was determined by CG-MS.

Materials and Methods

**Vegetable sample**

Broccoli stems (*Brassica oleracea*) were collected from distribution centers of fruits and
vegetable in the region of Piracicaba, State of São Paulo, Brazil. The samples were freeze-dried for 5 days at 60 - 100 μHg and at -50°C (LiTop® L101) and stored at -20°C until the use.

Extraction procedure

The freeze-dried broccoli stems were ground in mechanical mill (IKA® A11). For preparation of extracts, samples were immersed in distilled water (1:20 w/v), centrifuged at 5,000 rpm for 15 minutes and filtered in qualitative filter paper 12.5 μm (Qualy®). The aqueous extracts were freeze-dried (LiTop® L101) and stored under refrigeration until the time of use. For the antimicrobial analysis, the extracts were dissolved in Tryptic Soy Broth with Yeast Extract (0.6% w/w) (TSB+YE) (Difco®).

Antimicrobial activity

Antimicrobial activity was evaluated in *Listeria monocytogenes* ATCC 7644, from the collection of strains of the Laboratory of Hygiene and Dairy – “Luiz de Queiroz” School of Agriculture (ESALQ/USP). For Minimum Inhibitory Concentration (MIC/MBC), the microbroth dilution method in 96-well microplate was used (Clinical and Laboratory Standards Institute, 2007). The concentrations of extracts were obtained by 2-fold serial dilution in the microplate, resulting in initial concentrations of 102.4 mg/mL, after the addition of inoculated TSB (1-2 x 10^8 CFU/mL). The final volume for each well was 200 L. The controls were composed as follows: positive control (200 μL of TSB + YE added of 0.12% chlorhexidine v/v) and negative control (200 μL of inoculated TSB+YE). Two hundred microliters of sterile TSB were used for broth sterility control. After incubation at 35°C for 24 hours, all wells received 30 μL of resazurin (0.01% w/v) in order to verify, through visual reading, in which wells bacterial growth was detected. Any evidence of color change was considered as indicative of bacterial growth (Cabrál et al., 2009). The experiments were carried out in triplicate for each extract.

Flow cytometry

Flow cytometry methodology (Paparella et al., 2008) was used to analyze the conditions of bacterial cells exposed to broccoli stems extract. *L. monocytogenes* was reactivated in TSB+YE from stock culture at 35°C for 24 hours. The bacterial culture was washed with Phosphate Buffered Saline (PBS), centrifuged at 8,000 rpm at 4°C for 10 minutes, re-suspended in PBS and adjusted to 1-2 x 10^8 CFU/mL. The concentration analyzed was the CIM (102.4 mg/mL), previously detected. For this analysis, broccoli stems were prepared using PBS. The controls were composed as follows: negative control (PBS) and positive control (heating at 70°C for 30 minutes). The extracts were filtered in membrane 0.22 μm (Millipore®) to remove impurities. Three different parameters were used to evaluate the cellular viability during the extract action: 1, 3 and 5 hours of incubation. The cells were marked with esterase substrate Carboxyfluorescein Diacetate (cFDA) (Molecular Probes®, Eugene, Oregon) and collected in the FL1 channel (525 nm ± 20 nm), and marked with Propidium Iodide (PI) (Sigma-Aldrich®, Italy) and collected in the FL2 channel (620 nm ± 15 nm). The tests were carried out by FACScalibur™ (Becton Dickinson, San Jose, CA). For data collection, Cellquest™ Software (collection rate of 10,000 events/sample) was used, as well as Summit Software version 4.3 for data analysis. Forward Scatter (FSC) and Side Scatter (SSC) were measured on a logarithmic scale. The fluorochromes detection was evidenced in different intensities and four regions were assigned to each area of labeled cells (cF-PI+, cF+PI+, cF-PI-ecF+PI-), according Ananta et al., 2004 (Figure 1).

Chemical composition

The broccoli stems extracts were submitted to Gas Chromatography with Mass Spectrometry (GC-MS) in order to determine their chemical composition (Proestos et al., 2006; Markham et al., 1996). Chromatographic analysis: the extracts were analyzed by Shimadzu® gas chromatograph (Model GC-2010) coupled to a Shimadzu® mass spectrometer (QP 2010 Plus). The separation occurred in capillary column RTX5MS (30 m x 0.25 mm x 0.25 μm). The injector temperature was 280°C and the injection volume was 0.5 μl in “splitless” mode. The interface was maintained at 280°C and the detector operated in the “scanning” mode (m/z 40 - 800). Chromatographic conditions were: initial temperature of 80°C (1 min) heating to 250°C, at a rate of 20°C/min (1 min), heating to 300°C (5 minutes) at a rate of 6°C/min.
heating to 310°C (10 minutes) at a rate of 15°C/min, and heating to 320°C (10 minutes) at a rate of 20°C/min, totaling 40 minutes of analysis. The integration was done using the LabSolutions-CGMS software. Flavonoids, phenolic acids and derivatives were identified by comparison with data obtained from GC-MS, such as retention time and ionic fragmentation of authentic standards, silanized and eluted under the same conditions, and with the Wiley 8 Library.

**Results and Discussion**

The antimicrobial analysis of extracts showed a high MIC (102.4 mg/mL). Several studies have demonstrated the biological properties of related species from Brassicaceae family, as antioxidant and antimicrobial activities (Llorach et al., 2003; Ayaz et al., 2008). Despite the high MIC value, we reinforce the potential for extraction of antimicrobial compounds in this material; the extraction optimization could increase the levels of this bioactive components.

The FSC and SSC graphic of *L. monocytogenes* demonstrated the status of this population under different conditions (Figure 2). The incubated samples with broccoli stems extract indicated an altered standard to cell morphology in comparison with the negative control (PBS). This changes in the cells morphology provided information about possible mechanisms of action for the vegetal extract. R1 region represents viable population, without any membrane damage (negative control) (Figure 2A). R2 and R3 regions represent conditions of granulosity increase and size and granulosity increase, respectively. In the positive control, we observed a short appearance of the R3 region (Figure 2B). For samples submitted to broccoli stems extracts, the appearance of R2 e R3 regions was considerable (Figure 2C).

This substantial increasing can be explained by two reasons: size and granulosity rising or aggregates formation from cells at different physiological status (viable, dead and damaged cells). Thus, to evaluate the real condition of *L. monocytogenes* population on R3 region, we optimized data from granulosity graphic, excluding cells aggregates and obtaining the percentage of individual cells for the three incubation periods. The percentage of individuals cells of *L. monocytogenes* population under broccoli stems extract action decreased considerably from period of 1 hour of incubation (12.0%) to 3 hours (9.5%) and 5 hours (1.9%). Thus, the extract was capable to form cell aggregates and inhibit the bacterial growth.

The delimited regions from granulosity and size graphics (R1, R2 and R3) were analyzed for fluorescence intensity to the CFDa and PI fluorochromes (Figure 3). As the R1 and R2 regions from this graphic showed the same fluorescence percentage for both markers, populations related to these regions were analyzed together. Therefore, the following results express cell conditions relative to subpopulations present only in these areas, which comprise size and granulosity similar to those of the negative control and increase of size and granulosity. The combined use of the markers exposed 4 different areas of labeled cells, which represent the status of the bacterial population evaluated. In the negative control (PBS), the subpopulation of dead cells consisted of 0.76% of the cell total, viable cells 51.35% and damage cells, 1.27% (Figure 3A). The positive control (heating at 70°C for 30 minutes) showed subpopulations values of 20.61%, 35.97% and 10.92%, respectively (Figure 3B). These results demonstrated considerable functionality for esterase, as opposed to findings in previous study (Paparella et
The chemical composition of broccoli stems extracts analyzed by CG-MS technique is presented in Table 1. Phenolic acids (ferulic, sinapinic and caffeic acid), malic and ascorbic acid were their major components. Sinapinic acid was the most abundant among the phenolic acids (6.33%); in others studies, this compound was found in cauliflower and kale’s bioactive extracts (Llorach et al., 2003; Ayaz et al., 2008), species from the same broccoli family (Brassicaceae). Acid ferulic found in fermentation less was associated to antimicrobial activity against L. monocytogenes and Staphylococcus aureus (Martin et al., 2012). The ascorbic acid, an organic acid widely present in vegetal materials, was found in high concentrations in broccoli stems extract (24.79%). This component is known like a important antioxidant; previous studies have indicated their potential to increase the antimicrobial activity from others compounds (Davidson and Branen, 2005; Golden et al., 1995). Despite the low concentrations (1.16%), caffeic acid present in the extracts was observed in others vegetable materials with antilisterial activity, as grape marcs, peanut peel and guava bagasse (Martin et al., 2012; Anastasiadi et al., 2008).

The mechanism of action of phenolic compounds is not yet completely elucidated, but its effect can be related to cell membrane/wall disruption, action on genetic material, enzymatic inactivation and active transport’s interruption (Sikkema et al., 1995; Burt et al., 2004). Lysis of membrane cell appears to be the primary mechanism of action of phenolic compounds (Beltrame et al., 1988). Probably, the cells of different sizes detected by flow cytometry were due to cell disruption and leakage of internal contents when submitted to the extract action. Consequently, the cells became incapable to control the balance osmotic, resulting in input of external liquid and increasing the size.

**Conclusion**

Broccoli stems extract presented antimicrobial potential against L. monocytogenes. Despite the high levels found for the CIM, this antibacterial activity could be improved by optimization of the extraction procedures. Flow cytometry was very useful to reveal possible mechanisms involved in the inhibitory action of the extract on L. monocytogenes, enabling the identification of viable, dead and damaged cells. In face of the difficulty to avoid the food contamination by this pathogen, the study of vegetal residues as source of natural preservatives in food and beverage industry is very promising.

**Acknowledgement**

The authors wish to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo for financial support and to the CAPES for the fellowships.
References


