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Antimicrobial activity of safflower seed meal extract and its application as an antimicrobial agent for the inactivation of *Listeria monocytogenes* inoculated on fresh lettuce

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ABSTRACT

This study examined the antimicrobial activity of safflower seed meal extract (SSME) against foodborne pathogens, and the microbial reduction effects of combined treatment with SSME and fumaric acid (FA) against *Listeria monocytogenes* inoculated on lettuce. Results with SSME showed antimicrobial activity against the pathogens, with the highest activity against *L. monocytogenes*. With 0.7% SSME treatment for 3 min, the reduction levels of total aerobic bacteria and *L. monocytogenes* in lettuce were 1.55 and 1.58 log CFU/g, respectively, compared with the control. In particular, the combined treatment with SSME and FA reduced the populations further by 1.14 and 1.66 log CFU/g compared to those in SSME treatment alone. The combined treatment did not affect quality indicators such as lettuce color. These results indicate that combined treatment of SSME and FA can be used as a novel antimicrobial agent for inactivating the pathogens in fresh produce.

1. Introduction

Fresh vegetables and fruits are an important source of vitamins and minerals (Nicosia et al., 2016). However, they are susceptible to microbial contamination due to lack of heat treatment before consumption. The survival of these microorganisms in foods is a major problem affecting food safety (Lv, Liang, Yuan, & Li, 2011).

Listeria monocytogenes is a major foodborne pathogen associated with fresh produce because it grows well even at the low temperatures of processing and storage (Odedina, Vongkamjan, & Voravuthikunchai, 2015; Ölmez & Temur, 2010). Outbreaks of *L. monocytogenes* infection have been associated with minimally processed vegetables: according to a CDC report (CDC, 2016), there was an outbreak of *L. monocytogenes* from packaged salads in 2016.

To inactivate the pathogens on fresh produce, various non-thermal treatments such as chlorinated water (Kim, Kim, & Song, 2009; Nagar, Godambe, & Shashidhar, 2015), organic acids (Kang & Kang, 2016; Ramos-Villarroel, Martín-Belloso, & Soliva-Fortuny, 2015), ultraviolet-C (Chun, Kim, & Song, 2010), and ultrasound (Gani et al., 2016) have been used. Chlorinated water is widely used as a sanitizer for fresh vegetables, but customer concern about the safety of these chemical sanitizers is increasing (Joshi, Mahendran, Alagusundaram, Norton, & Tiwari, 2013). Many studies have explored the use of natural substances such as plant extracts as alternative antimicrobial agents (del Carmen Villalobos et al., 2016; Nicosia et al., 2016; Yang et al., 2014). One candidate class of natural antimicrobial agents is bioactive phenolic compounds obtained from food processing by-products. The use of these by-products has some advantages because they are natural and inexpensive (Abdalla, Darwish, Ayad, & El-Hamahmy, 2007; García-Lomillo, González-SanJosé, Del Pino-García, Rivero-Pérez, & Muñiz-Rodríguez, 2014; Zhu, Olsen, Sheng, Xue, & Yue, 2015).

Safflower seed (*Carthamus tinctorius* L.) has been used as a medicine and edible oil in Korea and China (Emongor, 2010). In particular, safflower seed contains various bioactive substances, such as lignan and flavonoid, and has been widely used for osteoporosis and other bone diseases (Hwang et al., 2016). Safflower seed meal is a by-product of production of safflower seed oil, and it contains bioactive polyphenols such as lignan, flavonoids, and serotonin derivatives (Zhang, Nagatsu, Watanabe, & Okuyama, 1997). However, few studies on the antimicrobial activity of safflower seed meal extract (SSME) have been performed, and it has never been studied as a sanitizer on fresh-cut produce.

This study examined the antimicrobial activity of SSME and its application as a natural sanitizer for fresh-cut produce. In addition,

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the combined effect of SSME and fumaric acid (FA) on the inactivation of *L. monocytogenes* on lettuce was investigated.

2. Materials and methods

2.1. Bacterial strains and culture preparation

Salmonella Typhimurium (KCTC 2514, ATCC 14028), *Escherichia coli* O157:H7 (NCTC 12079, ATCC 43889), *Listeria monocytogenes* (ATCC 19115, ATCC 19115, KCTC 13064), and *Staphylococcus aureus* (KCTC 1621) were used in this study. *S.* Typhimurium and *E. coli* O157:H7 were cultured in tryptic soy broth (TSB, Difco, Detroit, MI, USA), and *L. monocytogenes* and *S. aureus* were cultured in brain heart infusion (BHI, Difco) at 37 °C for 24 h. The cultured media were centrifuged at 3000×*g* for 10 min, washed three times with 0.1% peptone water (Difco), and re-suspended in peptone water, resulting in approximately 10^9 CFU/mL. Culture cocktails for subsequent experiments were prepared by combining 10 mL of each bacterial strain.

2.2. Extraction of SSME

Dried safflower seed meal (Yangju, Korea) was ground in a blender at 30,000 rpm, and the samples were extracted with 10 vol (L) of 70% ethanol at 50 °C for 3 h. The extracts were concentrated in a rotary evaporator at 50 °C.

2.3. Disc diffusion test

The antimicrobial activity of SSME was screened using an agar disc diffusion test (Pesavento et al., 2015) against four pathogenic bacteria. Bacterial suspensions (0.1 mL) of approximately 10^6 CFU/mL were spread on Mueller Hinton Agar (Difco). A sterile filter paper disc (diameter, 8 mm) was soaked in 50 μL of SSME and placed on the surface of the plates. The agar was incubated at 37 °C for 24 h and the inhibition zone in which no growth of bacteria was determined using the digimatic caliper (Model 500-181-20, Mitutoyo Corp., Kawasaki, Japan). The tests were performed in triplicate.

2.4. Released cell constituents

The cell integrity of *L. monocytogenes* was examined by measuring the amount of released reducing sugar (Zhao, Zhang, Hao, & Li, 2015). The content of reducing sugar was measured using 3,5-dinitrosalicylic acid (DNS) assay. *L. monocytogenes* was cultured in 25 mL of BHI at 37 °C for 24 h. The medium was then collected by centrifugation at 3000×*g* for 10 min, and the sample was washed with 0.1 M phosphate buffer solution (PBS, pH 7.2) and re-suspended in the buffer. Cell suspensions were incubated at 37 °C for 12 h in the presence of different amounts (0.1, 0.4, 0.7, and 1%) of SSME, with a bacterial suspension without SSME as the control. The change in the amount of released reducing sugar was measured every 3 h. Each sample (2 mL) was collected and centrifuged (3000 × *g* at 4 °C for 10 min). After adding 1 mL of DNS reagent to the supernatant (1 mL), it was heated at 90 °C for 10 min. After cooling for 5 min, 10 mL of distilled water was added, and the absorbance at 540 nm was measured using a spectrophotometer. Glucose was used as the standard to quantify the concentration of reducing sugar.

2.5. Scanning electron microscopy (SEM) analysis

To examine the effect of the SSME on the morphological change of bacteria, microscopic images of bacteria were collected using a focused ion beam scanning electron microscope (Tescan, Warrendale, PA, USA) as described by Zhang, Liu, Wang, Jiang, and Quek (2016) with minor modifications. Bacteria were treated with 1% SSME, and the sample without SSME treatment was used as the control. The samples were incubated in a shaking incubator at 37 °C for 12 h. After incubation, cells were collected by centrifugation at 3000×*g* for 10 min and washed twice with 0.1 M PBS (pH 7.2). The samples were then re-suspended in 25 mL of the 0.1 M PBS containing 2.5% glutaraldehyde and kept at 4 °C for 2 h to fix the cells. After centrifugation at 3000 × *g* for 10 min, the cells were repeatedly dehydrated at various ethanol concentrations (30, 50, 70, 90, and 100%). The samples were fixed on the cover glass and coated with platinum for analysis.

2.6. Sample preparation and inoculation of pathogens

Lettuce leaves were obtained from a local market (Daejeon, Korea) and used for the experiment within 24 h. Damaged leaves of lettuce were removed prior to the experiment. For inoculation, samples were placed on sterile aluminum foil and treated with UV-C on a clean bench for 10 min on each side of samples. After UV-C treatment, *L. monocytogenes* was not detected in the lettuce leaves. Prepared inoculum (100 μL), diluted to approximately 10^6 CFU/mL, was applied to 10 g of samples by spot inoculation on the sample surface. The samples were dried at 22 °C for 1 h to allow the microorganisms to attach to the surface.

2.7. SSME treatment and combined treatment with FA

For treatment with a single antimicrobial agent, inoculated samples (20 g) were immersed in SSME (0.1, 0.4, 0.7, and 1.0%) or FA (0.1, 0.3, and 0.5%) solutions for 1, 3, and 5 min. The concentrations of SSME applied in this study were chosen based on a preliminary study, and those of FA were selected by a previous study (Park et al., 2016). The solution for combined treatment was prepared by mixing SSME (0.7%) and FA (0.5%); samples (20 g) were immersed in the combined solution for 3 min. After each treatment, the samples were dried to remove residual solution on the surface.

2.8. Packaging of samples and storage condition

Samples were packaged in low-density polyethylene bags (25 × 30 cm, 60 μm thickness), and packaged samples were kept at 4 °C for 8 days.

2.9. Microbiological analysis

Samples (10 g) were homogenized with a Stomacher blender for 3 min. After homogenization, each sample was serially diluted in peptone water, and diluents were plated onto specific media: plate count agar (Difco) for total aerobic bacteria and Oxford medium base (Difco) for *L. monocytogenes*. All media were incubated at 37 °C for 48 h before counting the colonies.

2.10. Color measurement

The color change of lettuce leaves during storage was determined with a colorimeter (Minolta Camera Co., Osaka, Japan). Color was expressed as Hunter values *L*, *a*, and *b*, which indicate lightness, redness, and yellowness, respectively.

2.11. Statistical analysis

Experimental data were analyzed using a Statistical Analysis System program version 9.4 (SAS Institute Inc, Cary, NC, USA). All experimental data were compared using Duncan's multiple range