Antimicrobial Activity of Mulberry Leaf Extract on Postharvest Soft Rot Caused by *Erwinia carotovora*

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Abstract

The antimicrobial activity of mulberry (*Morus alba* L.) leaf extracts on bacterial soft rot cause by *Erwinia carotovora* subsp. *carotovora* (ATCC 15713) were studied. Fresh mulberry leaves were dried at 60°C for 12 h and later were processed to powder using 70 mesh size sieve. Extracts were obtained by extracting powder using distilled water into three extract concentrations i.e. 5%, (MP5), 7.5% (MP7.5), and 10% (w/v) (MP10). Antimicrobial activities of all extract concentrations were tested against *Erwinia carotovora* using the paper disc diffusion method on the Luria-Bertani agar, in comparison to those of a range of ampicillin concentrations including 3.125, 6.25 and 12.5 µg/ml, as well as only distilled water (designated as control). The study also investigated total phenolic content and quercetin content of the extracts. Experimental findings showed that the mulberry leaf extract (10g %, w/v) had reasonable antimicrobial activity against the *Erwinia carotovora* upto 5 days. The antimicrobial activities of the extracts are concentration dependent. These are attributed to contents of phenolic compounds mainly as quercetin in the mulberry leaf. Contents of total phenolic and quercetin in the mulberry powder extracts increased with higher concentrations of the extract.

Keywords : Antimicrobial activity, *Erwinia carotovora* subsp. carotovora (ATCC 15713), *Morus alba* L., Mulberry leaf extract

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Introduction

Fresh fruit and vegetable are living tissues which continue their metabolic activities thereafter harvested. Microbial spoilage and proliferation on harvested fresh produce are considered to key undesirable effects which significant reduce the produce's quality and marketability (Obetta et al., 2011). Whilst chemical antimicrobial agents such as chlorine and sodium hypochlorites have extensively been used to control postharvest microorganisms, prohibitions of these chemicals are increasingly effective in several countries for example European countries because of their potential carnogenic properties, leading to cancer and other health related problems (Issa-Zacharia et al., 2010). Natural antimicrobial agents currently have been recognised as alternatives to the chemicals because of their antimicrobial activities, and, importantly, having lower health effects to human (Martín-Diana et al., 2008). Extracts of mulberry leaf have been reported their antimicrobial activities against for example *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (Keyanont et al., 2004; Kumar and Chauhan, 2008; Manjula, 2011; Nirmal and Benjakul, 2011; Sohn et al., 2014). The activities are principally attributed to soluble protein and phenolic compounds including mulberrin (Kuwanon C), albanol A (Mulberrofuran G), albanol B (Zafar et al, 2013), and quercetin (Cushnie and Lamb, 2005). Postharvest bacterial soft rot causes short shelf life to fresh horticultural products including lettuce, celery, broccoli head, tomatoes and potatoes that facilitate entry of pathogens such as *Salmonella spp.* (Mendonca, 2005). *Erwinia carotovora* is one of important bacteria and plant pathogen causing the bacterial soft rot (Ismail et al, 2012; Trias et al., 2008). It can produce extracellular enzymes including pectinase and cellulase which chemically degrade pectin and cellulose, respectively (Murata et al., 1994).

The purpose of this study is to investigate the antimicrobial activity of mulberry leaf extract against *Erwinia carotovora* in comparison to that of ampicillin, a typical antimicrobial agent used in food and horticultural product sanitisations. Ampicillin is extensively known its antibiotic property and has been utilized to control bacterial disease and infections (Sharma et al., 2015). Ampicillin interferes and disrupts cell wall synthesis by binding to penicillin-binding proteins at the bacterial inner membrane of its cell wall. Inactivation of this binding interferes the cross-linkage of peptidoglycan chains which are essential for bacterial cell wall strength and rigidity, resulting in weakness of bacterial cell wall and cell lysis (National center for biotechnology information, 2016). The research was undertaken as *in vitro* assay and *Erwinia carotovora* subsp. *carotovora* (ATCC 15713) was utilised as a model microorganism. At present the information on the antimicrobial activity of interest is considered limited. The present work also investigated total phenolic content as well as the quercetin contents of the mulberry extract in relations to the antimicrobial activities.

Materials and Methods

Microorganisms

Erwinia carotovora subsp. *carotovora* (ATCC 15713) was obtained from the New Zealand Reference Culture Collection Medical Section through the School of Engineering and Advanced Technology, Massey University, Palmerston North, New Zealand.

Fresh mulberry leaf and dried mulberry powder

Mulberry leaves were collected from The Queen Sirikit Department of Sericulture Ubon Ratchathani, Thailand and transported to laboratory (Ubon Ratchathani University Ubon Ratchathani, Thailand) within 2 h. They later were sorted to eliminate damaged leaves and the chosen ones were washed with distilled water for 30 sec. The leaves thereafter were taken from the water and were placed on the clean towel at room temperature (~25°C) allowing the leaves to dry out from excess water (drip) left on the surface by absorbing onto the towel. To process dried mulberry powder, mulberry leaves were dried in according to Katsube et al. (2009) with modifications: tray drying in hot-air oven (Verinox J1900, Italy) at 60°C for 12 h. The approximated moisture content of the dried leaves was 9.26% (dried basis). Dried leaves were allowed to cool down at the room temperature for 30 min and subsequently were ground into fine powder using blender (Moulinex DPA 2, France) and 70 mesh size sieve (SIN 1195330, U.S.A.).

Preparations of dried mulberry powder extract and ampicillin solutions

The extraction method reported by Nirrmal and Benjakul (2011) was employed with modifications to obtain extracted compounds from the dried mulberry powder. The extracting solvent utilized was distilled water. In the present work, there were 3 concentration levels of mulberry powder in water solutions prepared for extractions. The concentrations (g %, w/v) were referred to as MPE5, MPE7.5 and MPE10 of which there were 5, 7.5, and 10 g of mulberry powder in 95, 92.5 and 90 ml distilled water, respectively. Individual solutions were left for 1 h at room temperature. The solutions later were filtered using the Whatman No. 1 filter paper.

To prepare ampicillin solution, ampicillin sodium salt obtained from Sigma-Aldrich (Auckland, New Zealand) was dissolved in distilled water. Ampicillin concentrations including 3.125, 6.25, and 12.5 µg/ml and these were designated as AMP3.125, AMP6.25, and AMP12.5. Individual solutions were filtered through 0.45 µm syringe filter (Sartorius Stedim Biotech, Germany) using 10 ml syringe (Terumo, Philippines) for obtaining sterile solutions which later were kept into 10 ml sterile of glass bottle, at 4 °C for further tests.

Preparations of *Erwinia carotovora* cell suspension

Ten colonies of *Erwinia carotovora* were added into 9 ml of peptone solution (0.1% w/v) then thoroughly shaken using the Vortex (SCILOGEX MX-5, USA). One millitre of this solution was transferred to 9 ml of peptone solution using the pipette and thoroughly shaken for preparing serial dilution until achieving 10^{-4} dilution. One hundred microlilitres of 10^{-4} dilution suspension were inoculated to Luria-Bertani (LB) agar, subsequently spread on the agar surface and left for 20 min. It should be noted that *Erwinia carotovora* numbers were counted using haematocytometer (Cho et al, 2005). Before counting, haematocytometer and cover glass were sanitised by dipping into 70% (v/v) ethanol and cleaned the lens using cleaning paper. Ten microliters of *Erwinia carotovora* suspension were dropped in the space between the haematocytometer and the cover glass. The numbers were counted using microscope (Olympus, Japan) and calculated.The results were expressed as cells per ml. An average *Erwinia carotovora* number was 8.43×10^{6} cells per ml.

Antimicrobial activity determination

Paper disc diffusion method as reported by Bakht et al. (2011) with modifications was

utilized for determining antimicrobial activities of mulberry powder extract and ampicillin against *Erwinia carotovora*. 30uL of individual mulberry powder extractions and ampicillin concentrations prepared were added to sterile paper discussing the pipette (i.e. one concentration per disc). Each paper disc was placed on the LB agar plate which was incubated at 30°C for 24 h. In this study sterile distilled water which was also added to a paper disc was designated as control. To identify the antimicrobial activity, diameters of inhibition zones of individual discs were measured using the digital vernier caliper (Craftright, China).

Total phenolic content and quercetin content measurements

Total phenolic content (TPC) in mulberry powder extracts were determined according to the Folin Ciocalteu assayas reported by Bico et al. (2009) and Memon et al. (2010) with modifications. To measure, 1 ml of individual mulberry extracted solutions were mixed with 5 ml of 10% Folin Ciocalteu solution (Sigma-Aldrich, Auckland, New Zealand) and 4 ml of 7.5% (w/v) Na_2CO_3. The mixture was left at 20°C for 1 h. To determine the TPC, absorbances of individual mixtures were measured at 760 nm using the spectrophotometer (T60U, Beijing, China). The results were expressed as microgram of gallic acid equivalent per ml of mulberry powder extracted solution.

Quercetin content of mulberry extracts was determined using the High Performance Liquid Chromatogram (HPLC) (Dionex Ultimate 3000, Thermo Fisher Scientific , Auckland, New Zealand) as described by Zheng et al. (2007) with modifications.20 uL of individual mulberry extracted solutions were analysed.Samples were injected at ambient temperature into a Waters µBondapak C18 (Milford, USA) (3.9 ×300 mm diameter) column. The mobile phase consisted of 2.5% aqueous formic acid (A) and HPLC grade acetonitrile (B). The mobile phase was acidified water containing 2.5% percent formic acid (A) and acetonitrile (B) The mobile phase was set as a linear gradient from 5 to 20% B in the first 15 min, followed by a linear gradient from 20 to 30% B for 5 min, then an isocratic mixture for 5 min, followed by a linear gradient from 30 to 90% B for 5 min, and then isocratic mixture for 2 min before returning to the initial conditions. The flow rate was 1.0 ml/min and the wavelength detection was set at 368 nm. Retention times and peak area were compared to standard curve. Quercetin dehydrated solution at 50, 100 and 200 µg/ml were used as standard. The results were expressed as µg/ml.

Experimental design and statistical analysis

Experiments conducted to measure antimicrobial activities, TPC and quercetin contents of mulberry powder extracts were undertaken in accordance to completely randomized experimental design (CRD) with three replications. All data obtained from individual treatments were subjected to Analysis of Variance (ANOVA) and the differences among treatments subsequently were analyzed using Duncan Multiple range test ($p = 0.05$) using SPSS for Window version 13.0 (2005).

Results and Discussion

All mulberry powder extract concentrations demonstrate antimicrobial activities against *Erwinia carotovora* during storage period (Table 1). It is found that the antimicrobial activities of MPE10 apparently were higher than those of MPE7.5 and MPE5. The MPE10 showed its antimicrobial activities until Day 5 whilst other extract concentrations only maintain their activities up to 3 days. The research findings highlight so-called concentration-dependence of antimicrobial activities of MPE i.e. antimicrobial activity becomes increased when the extract

concentration was increased. MPE inhibited bacterial motility and DNA synthesis causing alterations of cell growth and leading to cell death (Zafar et al., 2013). It is unsurprisingly about the antimicrobial activities of all ampicillin concentrations utilized. The research findings observed in the present work are consistent to those reported in the literature. For example Kumar and Chauhan (2008) reported that ethanolic extracts of mulberry leaves had antimicrobial activities against *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. In contrast to the extracts and ampicillin, the control (using sterile distilled water) did not showed antimicrobial activity.

Table 1 Antimicrobial activities represented by inhibition zones of mulberry powder extracts and ampicillin against *Erwinia carotovora* subsp. *carotovora* (ATCC 15713)

Treatments	Inhibition zones (mm)						
	Day1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
MPE5	$0.35\pm0.00^{d\,W}$	$0.17\pm0.04^{d\,Y}$	$0.25\pm0.04^{d\,X}$	$0^{d\,Z}$	$0^{d\,Z}$	$0^{d\,Z}$	$0^{d\,Z}$
MPE7.5	$0.49\pm0.03^{d\,W}$	0.13 ± 0.05^{dY}	$0.33\pm0.07^{d\,X}$	$0^{d\,Z}$	$0^{d\,Z}$	$0^{d\,Z}$	$0^{d\,Z}$
MPE10	$0.59\pm0.03^{d\,W}$	$0.31\pm0.08^{d\,X}$	$0.40\pm0.07^{d\,X}$	$0.43\pm0.02^{d\,X}$	$0.36\pm0.03^{d\,X}$	$0^{d\,Y}$	$0^{d\,Y}$
AMP3.125	$3.77\pm0.62^{c\,W}$	$3.24\pm0.55^{c\,W}$	$3.45\pm0.66^{c\,W}$	$3.08\pm0.67^{c\,W}$	$2.92\pm0.68^{c\,W}$	$2.79\pm0.70^{c\,W}$	$2.69\pm0.70^{c\,W}$
AMP6.25	$7.74\pm0.58^{b\,W}$	$6.72\pm0.73^{b\,W}$	$7.17\pm0.63^{b\,W}$	$6.74\pm0.72^{b\,W}$	$6.56\pm0.70^{b\,W}$	$6.40\pm0.72^{b\,W}$	$6.13\pm0.79^{b\,W}$
AMP12.5	$16.93\pm0.43^{a\,W}$	$15.98\pm0.92^{a\,W}$	$16.47\pm1.02^{a\,W}$	$15.96\pm1.02^{a\,W}$	$15.81\pm1.00^{a\,W}$	$15.55\pm1.07^{a\,W}$	$15.35\pm1.03^{a\,W}$
Control	0	0	0	0	0	0	0

$^{a\text{-}d}$ Values within a column with different letter are significantly different (p = 0.05)

$^{W\text{-}Z}$ Values within a row with different capital letter are significantly different (p = 0.05)

MPE = Mulberry powder extract (g %), AMP = Ampicillin solution (µg/ml)

Table 2 Total phenolic content (TPC) and quercetin content in mulberry powder extracts

Treatments	TPC(µg /ml)	Quercetin content(µg/ml)
MPE5	668.09 ± 10.78	1.30 ± 0.03
MPE7.5	1,002.14 ± 16.17	1.95 ± 0.05
MPE10	1,336.18 ± 21.57	2.60 ± 0.06

Total phenolic content (TPC) and quercetin contents of all mulberry powder extracts are shown in Table 2. There is clearly seen from this tabular information that the TPC and quercetin contents positively increase with the extract concentrations. The relationship between TPC and quercetin was supported by the correlation analyses (data not shown) of which the Pearson's correlation coefficient equals to 1, suggesting the extremely strong linear correlation between these variables. Whilst there are several phenolic compounds in mulberry leaf, quercetin is a major content of which this knowledge is confirmed by the

research findings on quercetin contents. Cushnie and Lamb (2005) reported that quercetin had antimicrobial against bacteria such as *Escherichia. coli*. The antibacterial mechanism of quercetin has been reportedly involvement with bacterial DNA gyrase inhibition through binding with Gyr B subunit of DNA gyrase, subsequently interfering bacterial DNA synthesis. In addition, quercetin inhibited bacterial motility which disrupted bacterial proton motive force and increased the permeability of inner bacterial membrane that negatively influenced to membrane potential and cellular activities. In addition to quercetin, other phenolic contents including mulberrin (Kuwanon C), albanol A (Mulberrofuran G), and albanol B in mulberry leaf reportedly had antimicrobial activities (Zafar et al, 2013). These were hypothesized to form complex compounds with extracellular and soluble proteins as well as with bacterial cell walls causing changes in protein content of the wall, subsequently damaging bacteria (Gunjal et al., 2015). Sohn et al. (2014) demonstrated that both albanol A and albanol B had antimicrobial activities against *E.coli*, *Salmonella typhimurium*, *Staphylococcus epidermis* and *Staphylococcus aureus*, meanwhile mulberrin inhibited both gram-positive and gram-negative bacteria.

Conclusions

The experimental results clearly show antimicrobial activity and potential applications of the mulberry powder extract for postharvest treatments to minimize microbial loads of *Erwinia carotovora*, in turn, minimizing incidences of bacterial soft rot. The research findings also highlight that extents of antimicrobial activity are dependent on the extract concentrations which are attributed to phenolic compound levels of mulberry leaf.

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