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Antimicrobial activities of grape (*Vitis vinifera* L.) pomace polyphenols as a source of naturally occurring bioactive components

Faisal Kabir[1*], Mosammad Shahin Sultana[2] and Heri Kurnianta[3]

[1]Department of General Animal Science and Animal Nutrition, Patuakhali Science and Technology University, Babugonj, Barisal-8210, Bangladesh.
[2]Department of Agricultural Economics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.
[3]Department of Bioscience and Food Production Science, Shinshu University, 8304 Minamiminowamura, Ina, Nagano 399-4598, Japan.

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Grape pomace is a potential source of winery by-products having useful bioactive components. Antimicrobial activities of enzyme-assisted grape pomace polyphenols (GPP) were assessed against *Escherichia coli* IFO 3301 and *Staphylococcus aureus* IFO 12732 using plate count and spectrophotometry assays. GPP have shown credential growth inhibition against *E. coli* and *S. aureus*, respectively. The higher growth inhibition was mediated by the higher GPP concentrations against both *E. coli* and *S. aureus*, which implies dose dependency. GPP also exhibited bactericidal effects against both the Gram-positive and Gram-negative bacteria, whereas, Gram-positive bacteria have shown more susceptibility than Gram-negative bacteria. It is revealed that GPP is a potential source of natural antimicrobial agents.

Key words: Grape pomace, polyphenols, antimicrobial activity.

INTRODUCTION

Grape pomace contains naturally occurring bioactive components. Phenolic compounds, as secondary plant metabolites, play a critical role in human health. Bioactive components of fruits and vegetables have been shown to be beneficial for human health (Liu, 2003; Georgiev et al., 2014), including flavonoids, a major class of phyto-chemicals commonly found in fruits and vegetables (Vinson et al., 2001; Altameme et al., 2015). Considerable attention has been paid to polyphenols because of their diverse biological functions (Gulluce et al., 2003; Almeida et al., 2006). Grape pomace is generally underutilized and thrown away by the wine factory as waste products. Grape skins are the rich sources of anthocyanins, hydroxycinnamic acids,

*Corresponding author. E-mail: fkabir@pstu.ac.bd. Tel: +8801718080017.

Abbreviations: LB, Luria broth; **GPP,** grape pomace polyphenols; **DMSO,** dimethyl sulfoxide; **PBS,** phosphate buffer saline.

flavanols and flavonol glycosides; flavanols were mainly present in the seeds (Kammerer et al., 2004). Anthocyanins, catechins, flavonol glycosides, phenolic acids and alcohols, and stilbenes are the principal phenolic compounds of grape pomace (Schieber et al., 2001). Epidemiological studies indicate that fruits, vegetables and plant-based phenolic metabolites are beneficial to human health because of their potent antioxidant activity and wide range of pharmacologic properties such as antioxidant, anticancer, and platelet aggregation inhibition activities (Waterhouse and Walzem, 1998; Teixeira et al., 2014). Antibacterial activities shown by phenolic compounds may be because of iron deprivation or hydrogen bounding with vital proteins, such as microbial enzymes (Field and Lettinga, 1992). There is no systematic way to use grape pomace.

Finding an effective way of using grape pomace is needed, as it is a good source of natural bioactive components, which could be used as a functional food component. In this study, we made an attempt for the utilization of underutilized grape pomace and to assess the antimicrobial activities of grape pomace polyphenols as a source of naturally occurring bioactive components.

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO) was purchased from WAKO Pure Chemical Industries Ltd. (Tokyo, Japan). Luria broth (LB) medium was purchased from SIGMA-ALDRICH Inc. (Tokyo, Japan). MacConkey agar was purchased from Nippon Seiyaku Co. Ltd. (Tokyo, Japan), and Mannitol salt agar was purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). *Escherichia coli* IFO 3301 and *Staphylococcus aureus* IFO 12732 were collected from the Institute for Fermentation (Osaka, Japan). Other chemicals used were of biochemistry grade.

Preparation of sample

Red grape pomace was collected from Nagano prefecture, Japan. 1:9 samples with PBS (phosphate buffer saline, pH 7.3) were homogenized using blending machine. Cellulase enzyme (0.25 mg/mL) was added to the homogenized samples and then incubated at 55°C for 24 h. After enzymatic digestion the samples were centrifuged at 7500 × *g* for 20 min and supernatant samples were collected. The extracted supernatant was filtered through filter paper (Whatman no. 4) to remove unutilized residues. Supernatant samples were vacuum drying to remove organic solvent and then lyophilized for collection of grape pomace polyphenols (GPP). Dried lyophilized GPP was dissolved in 1% dimethyl sulfoxide (DMSO) using distilled water to make stock solution which was then sterilized by micro-filtration through 0.45 µM Millipore filter and kept at 4°C until use.

Microorganisms and culture conditions

Gram-positive bacterium *S. aureus* IFO 12732, and Gram-negative bacterium *E. coli* IFO 3301 were used in this study. Each bacterial strain was incubated in LB medium at 37°C for overnight. After incubation the bacterial solution was centrifuged two times and washed by using PBS (phosphate buffer saline, pH 7.0), and the test bacterial solution was prepared with PBS to give a concentration of 10^7 CFUs/mL by using a haematometer (Neubauer, LO-Laboroptik GmbH, Friedrichsdorf, Germany).

Determination of microbial growth inhibition

A 500 µL of mid-logarithmic phase bacterial cultures (10^7 CFUs/mL) was inoculated in 4.5 mL LB medium to make the final concentration of 10^6 CFUs/mL. A 2.0 mg/mL of GPP stock solution was prepared and then serial two-fold diluted in a 1.0 and 0.5 mg/mL using LB medium and also control (without samples) were taken to measure the growth inhibition of *E. coli* and *S. aureus*. The GPP was diluted using 1.0% DMSO. The cultures were incubated in a rotary shaker at 37°C and growth inhibition was determined by measuring the absorbance at OD 600 nm using a UV-VIS spectrophotometer SHIMADZU-1700 (Tokyo, Japan). Absorbance readings were taken for 360 min, followed by 60, 120, 180, 240, 300 and 360 min intervals. A growth curve was plotted with the obtained absorbance readings (Farouk et al., 2007). All the measurements were done in triplicate.

Determination of antimicrobial activities as killing effect

The GPP was added to 5.0 ml of PBS (phosphate buffer saline, pH 7.0) containing 500 µL of mid-logarithmic phase bacterial culture (10^7 CFUs/mL) to prepare the final concentration 10.0 mg/mL and 10^6 CFUs/mL. The tested bacterial cultures with samples were incubated at 37°C for 60, 120, 180, 240, 300 and 360 min intervals to determine the log survival ratio. The visible colony forming units (CFUs/mL) was measured with the above time intervals using MacConkey and Mannitol salt agar plate, incubated at 37°C for 24 h to measure the log reduction of *E. coli* and *S. aureus*. In addition, dose-dependent killing effect was also assessed using mid-logarithmic phase bacterial cultures (10^7 CFUs/mL) that were inoculated in a 500 mL PBS (phosphate buffer saline, pH 7.0) to make the final concentration of 10^6 CFUs/mL. In the 500 ml of PBS the final concentration of GPP was 3.0, 6.0, 9.0 and 12.0 mg/mL to measure the killing effect of *E. coli* and *S. aureus* incubated at 37°C for 6 h. After incubation to measure the killing effect of bacteria a serial 10-fold dilution were prepared using PBS (pH 7.0) and plated onto MacConkey and Mannitol salt agar plates and incubated at 37°C for 24 h. And then log survivals were enumerated using visible colonies on agar plates, while the count detection limit was maintained between 5 and 50 CFUs. The bacterial killing effect was calculated using the following formula: killing effect (Log CFUs/mL) = $\log10^{nc} - \log10^{np}$, where nc and np were CFUs/mL of mock and treated cells (Hoq et al., 2008). All the data represents the mean of triplicate tests.

Statistical analysis

Statistical analysis was performed using student's *t*-test. Paired tests were done to assess the differences between groups. All data were evaluated as mean ± SD. The differences between means were assessed using analysis of variance (ANOVA) followed by Duncan's new multiple range test. Statistical probability $p<0.05$ were considered significant. All the tests were done in triplicate.

RESULTS AND DISCUSSION

Microbial growth inhibition

Grape pomace polyphenols have shown specific

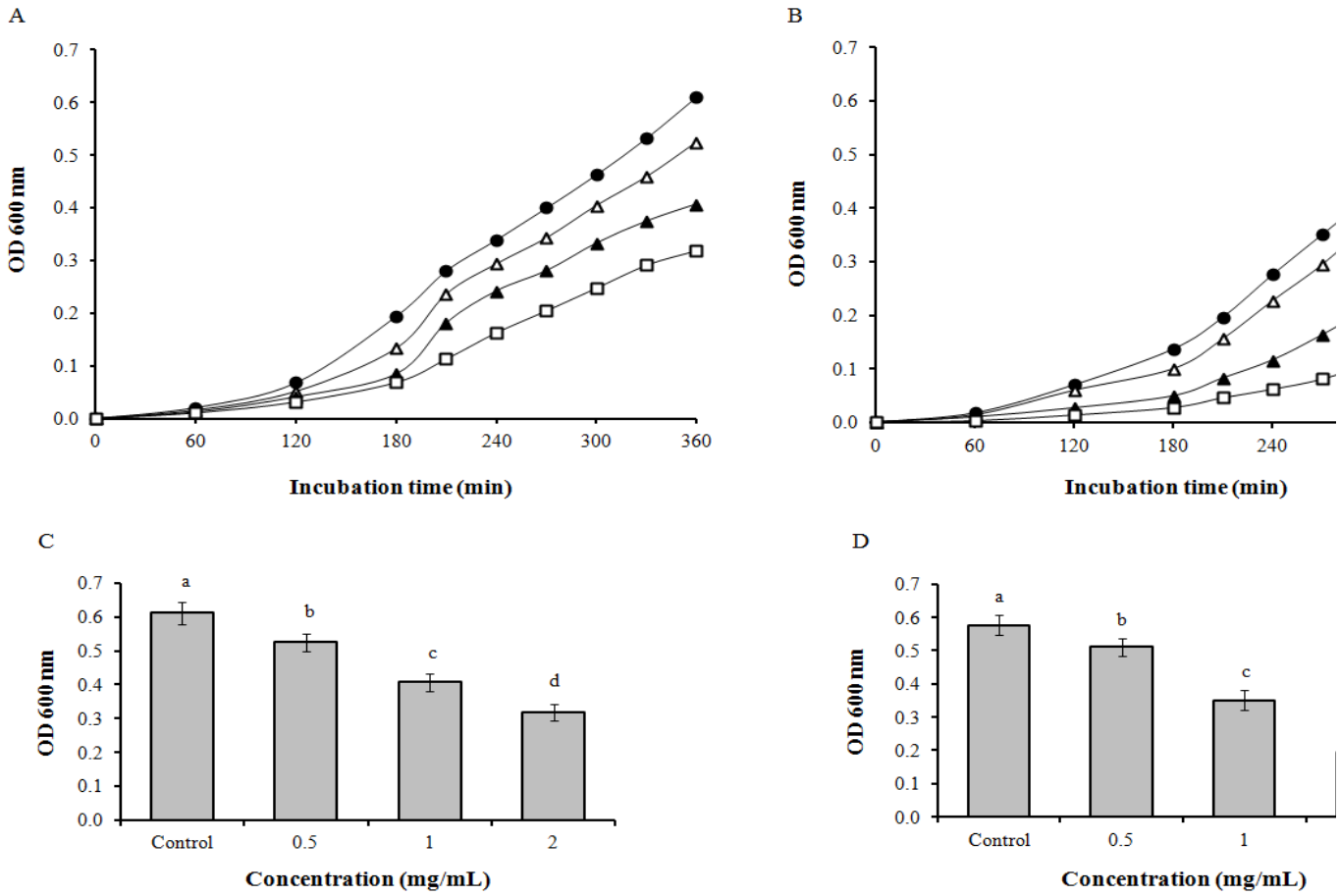

Figure 1. Growth inhibitory activities of GPP against *E. coli* (A) and *S. aureus* (B) under neutral pH condition incubated at 37°C for 360 min. Absorbance readings were measured at OD 600 nm. Data shown in Figure A and B is the representative of three independent experiments. Figure C and D indicate the growth inhibitory effects of GPP after 360 min of incubation against *E. coli* and *S. aureus*, respectively. ● , control; ▲ , 0.5 mg/mL; ▲ , 1.0 mg/mL; ■ , 2.0 mg/mL. All data represents mean ± SD. Different superscripts with uncommon alphabet differ significantly (p<0.05).

antimicrobial activities and corresponding bacteriostatic effects. Figure 1 shows the growth inhibitory activities of GPP against *E. coli* and *S. aureus* at OD 600 nm. As shown in Figure 1A, GPP was indicating gradually increasing growth inhibition against *E. coli* depending on exposure time. In contrast, higher tendency of growth suppression was observed on the higher concentration of GPP, which implies the dose dependency of growth inhibition. Figure 1B shows the growth inhibition of GPP against *S. aureus*. It was observed that growth inhibitory activity was gradually increased depending on exposure time, whereas higher concentrations have shown the higher growth suppression activity. Figure 1A and 1B show the growth inhibitory activity of GPP against *E. coli* and *S. aureus* after 6 h of incubation. It was found that significant (p<0.05) growth inhibition was observed against both the Gram-positive and Gram-negative bacteria as compared with control; in addition, the higher concentration indicated significant growth inhibition than against lower concentrations. It was observed that growth inhibitory activities of GPP against both the Gram-positive and Gram-negative bacteria was mediated by exposure time and concentrations, in which GPP have shown higher tendency of growth inhibition against Gram-positive bacteria as compared with Gram-negative bacteria. Antibacterial activities shown by phenolic compounds may be because of iron deprivation or hydrogen bounding with vital proteins (Field and Lettinga, 1992). Polyphenols have shown microbial growth inhibitory activities (Bong-Jeun et al., 2004; Sanhueza et al., 2014) in addition, polyphenols were usually more active against Gram-positive bacteria than Gram-negative bacteria (Lin et al., 1999; Oliveira et al., 2013). It was confirmed that GPP have shown potent microbial growth inhibitory activities.

Antimicrobial activities as killing effect

GPP have shown bactericidal effects under neutral pH at

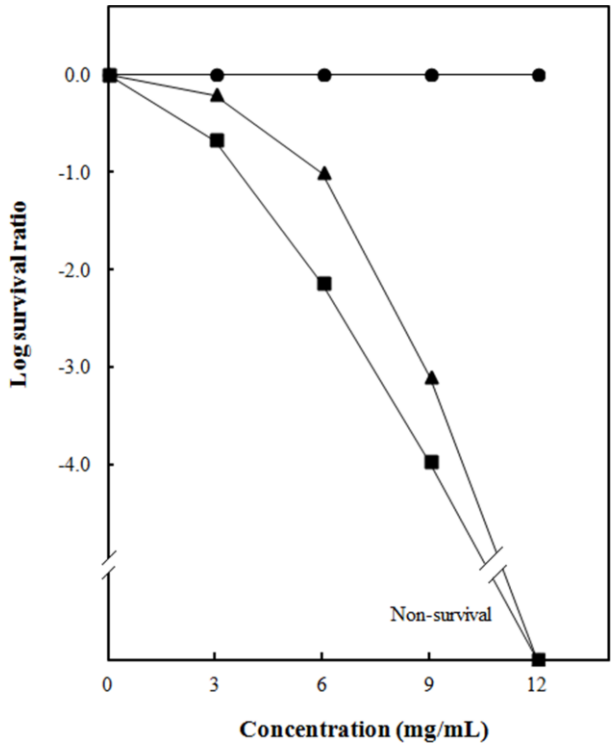

Figure 2. Effect of doses on bactericidal activities of GPP under neutral pH condition at 37°C for 6 h incubation. ●, control; ▲, *E. coli*; ■, *S. aureus*. Log survival ratio was calculated by enumerating viable cells. Showing data are the representative of three independent experiments.

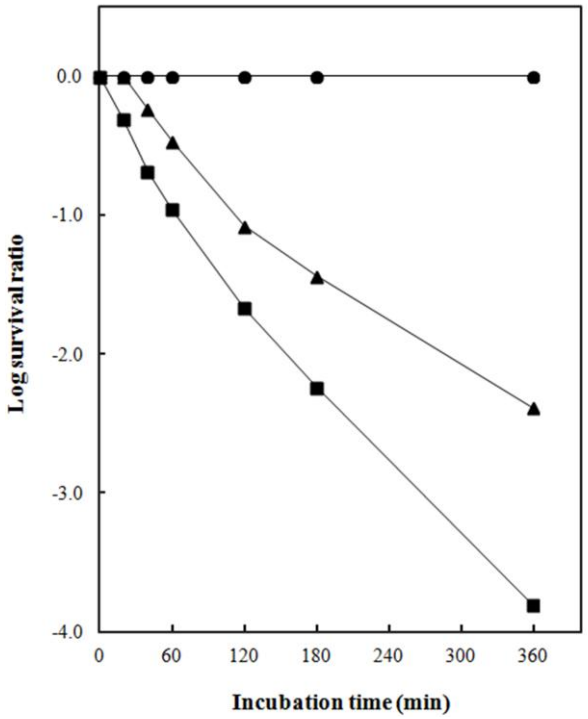

Figure 3. Effect of exposure time on the lethal effects of GPP under neutral pH, incubated at 37°C for 6 h. ●, control; ▲, *E. coli*; ■, *S. aureus*. Log survival ratio was calculated by enumerating viable cells. Data are the representative of at least three independent experiments.

37°C for 6 h of incubation with different sample concentrations. Figure 2 shows the log survival ratio of GPP against *E. coli* and *S. aureus*. It was observed that GPP have shown weak killing effect at lower concentration (3.0 mg/mL) against both the Gram-positive and Gram-negative bacteria, although Gram-positive bacteria have shown higher tendency of log reduction, whereas a moderate and strong killing effect was observed in higher doses of GPP (6.0 and 9.0 mg/mL).

In contrast, no survivability was detected in the concentration of 12.0 mg/mL against both the Gram-positive and Gram-negative bacteria. It was revealed that GPP possess strong bactericidal effects irrespective of Gram-positive and Gram-negative bacteria, which was mediated by doses and implies the dose dependency. It was reported that bacterial killing effect was dose dependent (Kao et al., 2010). Polyphenols exhibited antibacterial activities with protein-related polyamide polymers (Haslam, 1996). In contrast, polyphenols showed higher antibacterial effect on Gram-positive bacteria than Gram-negative bacteria (Viskelis et al., 2009). It was observed that GPP exhibited dose dependent bactericidal effects.

GPP exhibited bacterial killing effect against both *E. coli* and *S. aureus*. As shown in Figure 3, a quick and drastic log reduction was observed against both the Gram-positive and Gram-negative bacteria depending on the exposure time.

A weak killing effect was detected after 1 h of exposure, whereas a rapid and strong killing effect was observed after 2 to 6 h of exposure. In addition, Gram-positive bacteria have shown potent bactericidal effects as compared with Gram-negative bacteria. Polyphenols revealed the higher antimicrobial properties against Gram-positive bacteria than against Gram-negative bacteria (Burdulis et al., 2009). It was also observed that bacterial killing effect was associated with exposure time (Paulo et al., 2010).

Conclusion

It was demonstrated that bactericidal effects of GPP was mediated by the exposure time. Thus, it was revealed that grape pomace polyphenols exhibited bacteriostatic as well as bactericidal activities against both the Gram-positive and Gram-negative bacteria and that these underutilized GPP could be a good source of antimicrobials for further utilization in the food manufacturing industry to control or prevent food-borne pathogens.

Conflict of interests

The authors did not declare any conflict of interest.

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