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Antibacterial stability of *Spondias pinnata* (L. f.) Kurz leaves extract and its mechanism

Asnani[1,2], Winiati Pudji Rahayu[1,3], Betty Sri Laksmi Jenie[1,3], Nancy Dewi Yuliana[1,3]

[1]Department of Food Science and Technology, Bogor Agricultural University, Kampus IPB Darmaga 16680, Bogor, Indonesia.
[2]Department of Aquatic Products Technology, Halu Oleo University Kendari, Kampus Hijau Bumi Tridharma, Anduonohu 93132, Kendari, Indonesia.
[3]Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Centre, Bogor Agricultural University, LPPM - Bogor Agricultural University, Kampus IPB Darmaga 16680, Bogor, Indonesia.
Email wini_a@hotmail.com

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ABSTRACT

Aims: This study was conducted to observe the stability of *Spondias pinnata* leaf (SPL) extract antibacterial at different pH, salt concentration and temperature, to examine its antibacterial effectivity on minced fish, and to determine its fraction's antibacterial mechanism.
Methodology and results: The tested SPL ethanolic extract, n-hexane, chloroform, ethyl acetate and water fractions' antibacterial activity against *Bacillus cereus* and *Vibrio parahaemolitycus*. Its stability against pH, salt, and thermal variation was studied, as well as the mechanism and application in fish. Ethyl acetate fraction and water fraction showed the highest activity against *B. cereus* (MIC 0.62 mg/mL). Protein profile analysis using gel electrophoresis showed that *B. cereus* cells exposed with SPL ethyl acetate fraction and water fraction showed thinner protein bands as compared to control. Severe damage of the cells treated with 3 MIC was also observed under SEM. Antibacterial activity of SPL ethanolic against *Bacillus cereus* and *Vibrio parahaemolitycus* were stable against heat treatment (80–121 °C for 15 min) and NaCl treatment (0–10% w/v), whereas the inhibition zone respectively at pH 4 (10.31±0.25 and 8.09±0.97 mm) was higher than pH 7 (8.45±0.52 and 6.66±1.84 mm). Application of SPL ethanolic extract in fish broth showed higher antibacterial activity than in fish flesh, which gave bactericidal effect at 3 MIC.
Conclusion, significance and impact study: Ethanol extract can be developed as a natural preservative in fish processing.

Keywords: Antibacterial activity, antibacterial mechanism, antibacterial stability, *Spodias pinnata*

INTRODUCTION

Fish is a good medium for bacterial growth such as *Bacillus cereus* and *Vibrio parahaemolyticus*, both are known as food poisoning microbes. In United States during 1998-2004, there were 2 cases of food poisoning associated with seafood consumption caused by *B. cereus* and 25 cases caused by *V. parahaemolyticus* out of a total of 644 microbes related seafood poisoning cases (NACMCF, 2008). Several studies reported some cases of *B. cereus* and *V. parahaemolyticus* contamination in fish. Elhadi *et al.* (2016) reported that 9.4% of fish imported from Asian countries that were marketed in Saudi Arabia was contaminated by *B.cereus*. Noorlis *et al.* (2011) reported that the prevalence of *Vibrio spp* and *V. parahaemolyticus* contamination in catfish and red nile tilapia sold in hypermarkets in Malaysia reached as much as 98.67% and 24% respectively.

The shelf life of fishery products can be extended by cooling and freezing processes, and both methods are good at preserving texture and taste for a long time. To further extend the shelf life, combining cooling techniques and natural antibacterial applications can be done. Abdollahzadeh *et al*. (2014) evaluated the antilisterial activity of thyme EO (essential oil) in minced fish during refrigerated storage. The addition of thyme EO at 0.8 or 1.2% to minced fish meat exhibited a strong antilisterial activity during storage. The addition of EO at 1.2% showed a higher effect against *L. monocytogenes* than the addition at 0.8% during refrigerated storage. The strong antilisterial activity of thyme oil is often attributed to a high percentage of phenolic compounds.

***Corresponding author**

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Spondias pinnata (Anacardiaceae), also known as *wild mango* or *hog-plum*, scattered in primary and secondary forest in Indonesia, Africans normally use SPL as an antipyretic, antihelmintic, anti-inflammatory and anti-diarrhea (Muhammad, 2013). In Indonesia, SPL is usually consumed as fresh vegetables, while in Southeast Sulawesi SPL is a popular herb to improve the taste of meat or fish.

There are only a few reports *on S. pinnata* leaves antibacterial activities. Jain *et al.* (2014) tested ethanolic extract of the plant against 8 Gram-positive and Gram-negative bacterial strains. The result indicated that they could inhibit all tested bacteria with the inhibition zone ranged between 8.33±1.53-28.67±0.58 mm with the lowest MIC value was againsts *S. aureus* (2.0 mg/mL). The exact mechanism of this antibacterial action has not been widely documented. In general, the mechanism of microbial inhibition by antimicrobial compounds could be divided into: (1) disruption againsts the cell wall components, (2) reactions of cell membranes resulting on changes in permeability and cell components loss, (3) inhibition of protein synthesis, and (4) disruption to the function of genetic material (Davidson, 2001).

In this research, we extracted SPL leaves using ethanol then fractionated it using n-hexane, chloroform, ethyl acetate and water. Antibacterial activity of all extract and fractions were tested against *B. cereus* and *V. parahaemolyticus*. The most active one was applied as antibacterial in fish broth and minced fish. Their antibacterial activity stability against thermal, salts, and pH variations was studied as well. To study the antibacterial mechanism, bacteria cells exposed to the most active fraction or extract were observed for morphological abnormalities using SEM and their protein profiles were observed using gel electrophoresis. The results of this research provided initial important information on the possibility to develop natural antibacterial compound from *S. pinnata* to be used as alternative preservative especially for fish and fish products.

MATERIALS AND METHODS

Plant materials

The leaves of *Spondias pinnata* (L.f. Kurz) was acquired from local gardens in Kambu, Kendari Regency and was identified at the “Herbarium Bogoriense”, Research Center for Biology-Indonesian Institute of Sciences Bogor. *Bacillus cereus* ATCC 11778 and *Vibrio parahaemolyticus* ATCC 17802 as tested bacteria were obtained from National Agency of Drug and Food Control–Indonesia. Nile tilapia fish flesh (*Oreochromis* sp) was purchased from traditional market.

Extraction and fractionation

Spondias pinnata leaves was dried by oven at 40-45 °C for 3-4 days until the leaves were light brown and brittle. The dried leaves were then powdered (size ±40 mesh) and stored at −20 °C until used. Extraction process was conducted by dispersing SPL powder into ethanol (Merck) with 1:20 (w/v) ratio, following Parhusip *et al*. (2005) procedure with slight modification. The SPL powder (50 g) was added with ethanol (500 mL). Maseration aided by *shaker* was then performed at room temperature (28-30 °C) for 1 h, then sonicated for 45 min and macerated again with shake at room temperature for 1 h. To obtain the filtrate, the solution was filtered using Whatman No.1 paper. The process was repeated by using 300 mL and 200 mL ethanol respectively and the filtrates were combined. The solvent were then evaporated using *rotary evaporator* at the temperature of 50 °C followed by flushing under nitrogen gas.

The ethanolic extract was fractionated using manual liquid-liquid sequential solvent extraction based on the method by Maser *et al*. (2015). Ethanol extract were dissolved in distilled water (1:30 w/v) and partitioned gradually in separator funnel using n-hexane, chloroform, and ethyl acetate, respectively (Merck, 1:1 v/v ratio). The process was repeated three times and the solvent was then evaporated using *rotary evaporator* at the temperature of 50-55 °C. All extract and fractions were kept refrigerated prior use.

Antibacterial activity determination

Antibacterial activity of the extract is determined by well diffusion method as previously performed by Chinnaiyan *et al*. (2013). Two bacteria (*Bacillus cereus* and *Vibrio parahaemolyticus*) were used. 100 mg of SPL extract/fraction was dissolved into 1 mL 100% DMSO (Dimethyl sulfoxide**;** Merck). Tested bacteria were refreshed in MHB medium (*Mueller Hinton Broth*; Oxoid) until their population reached 10^6-10^7 CFU/mL. In 25 mL of MHA (Mueller Hinton order; Oxoid) 25 µL of the fresh bacteria culture was inoculated, poured into a petri dish and allowed to harden. Six mm diameter wells were made on the agar. A 60 µL of the extract/fraction was added into the wells. Chloramphenicol (1 mg/mL, Sigma) and 100% DMSO were used as a positive and negative control respectively. The Petri dish was incubated at 37 °C for 18-20 h. Formed transparent zone was measured using a digital caliper (Nankai, Japan and after subtracted by the well diameter (6 mm), it was expressed as diameter of the inhibition zone (DIZ, mm). The experiment was carried out using Completely Randomized Design (CRD) where each treatment was repeated 3 times.

MIC (Minimum Inhibition Concentration) determination

MIC value was determined according to a method described by Sadiq *et al*. (2015) with slight modification. 100 mg SPL extract/fraction was dissolved into 1 mL of 10% DMSO (Dimethyl sulfoxide) followed by ultrasonification and centrifugation to obtain the supernatant. Next, a series of two folds dilution was performed using sterile MHB with the volume of 1 mL, to obtain a concentration of 1.56-50 mg/mL for the extract and 0.039-5 mg/mL for fractions, and 0.78-50 µg/mL for

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Chloramphenicol which was used as positive control. The tested bacteria were inoculated onto the sample. The mixtures were incubated at 37 °C temperature for 18-20 h. The lowest concentration of the extract/fraction that indicates no visible growth visually (appeared as a clear solution) was determined as MIC.

Stability of antibacterial activity of *S. pinnata* leaves extract under various pH, NaCl concentration and heating temperature

Stability testing was conducted by a procedure previously performed by Naufalin *et al.* (2006). The stability test of SPL extract on pH was done by dissolving 100 mg of SPL extract in 1 mL of phosphate buffer solution each with pH 4 and 7. The stability test of SPL extract on NaCl was done by dissolving 100 mg of SPL extract in 1 mL of NaCl solution each with 0, 2.5, 5, 7.5 and 10% concentration. The stability test of SPL extract on heating temperature was done by dissolving 100 mg of SPL extract in 1 mL of distilled water, then each heated at the temperature of 80, 100 and 121 °C for 15 min. Measurement of antibacterial activities (DIZ) was performed against *B. cereus* and *V. parahaemolyticus* with a method previously described.

Antibacterial activity determination of *S. pinnata* leaves ethanol extract on fish broth and minced fish

Antibacterial activity in fish broth was conducted based on the method by Rialita (2014) with slight modifications. Fish broth was prepared by dispersing minced nile tilapia (*Oreochromis* sp.) into distilled water (10% w/v) and then sterilized. SPL ethanol extracts was added into the fish broth to obtain the concentration of extract 0 (control), 1, 3 and 5 MIC. As much as 5940 µL broth was pipetted aceptically into sealed sterile vial. Fresh culture (60 µL) of *B. cereus* and *V. parahaemolyticus* were inoculated into the broth (initial population were 10^5-10^6 CFU/mL). All samples were stored in a refrigerator at 5 °C and sampling was done daily for 0-4 days. The number of microbes in the fish broth were counted using *total plate count method* after incubation at the temperature of 37 °C for 24 h. Each test was performed with 3 repetitions using factorial Randomized Design with 2 factors (SPL extract concentration and storage duration).

Antibacterial activity in minced fish was conducted by following Abdollahzadeh *et al.* (2014) method. A total of 2 g of minced fish was put into a sealed via (volume 15 mL), the fish surface was manually flattened, and sterilized. Fresh culture (initial population ±10^6 CFU/mL) was inoculated onto the fish surface and stored at room temperature for 30 min. 1 mL of DSP ethanol extract solution at concentrations of 0, 1, 3 and 5 MIC was then pipetted onto the inoculated surface. Incubation was done in the refrigerator at temperatur of 5 °C, sampling was done daily for 0 to 4 days. The number of bacteria was calculated by total plate count method after incubation at 37 °C for 24 h. The experiment were conducted three times. Application on the minced fish were conducted using factorial experiments in Completely Randomized Design (factor 1: concentration of SPL extract, Factor 2: storage duration).

Observation of *B. cereus* cell's protein profile

Fresh culture of *B. cereus* was washed twice using PBS, then the pellets were resuspended using PBS to obtain bacteria population density of 10^7 CFU/mL. Ethyl acetate fraction and water fraction of SPL were added into the bacterial suspension (each with the concentration of 0, 1 and 3 MIC). After 2 h of exposure, the sample was centrifuged at 10,000 rpm for 5 min, and then the supernatant was discarded. Cell pellets were rinsed twice and resuspended using PBS. The obtained pellet were prepared and then run on gel electroforesis (SDS-PAGE) as previously performed by Wang *et al*. (2015)

Observation of *B. cereus* cells' morphology transformation

Bacterial pellets was prepared similarly as in the observation of the cell bacteria's protein profile. The morphology of cell pellets were observed using JEOL JSM-6360LA SEM (Scanning Electron Microscopy) as previously conducted by Miksusanti *et al.* (2009).

Data analysis

Data of DIZ and the number of bacteria ($\log_{10}$ CFU) of each treatment analyzed using one and two way ANOVA using the SAS Statistical Program version 9.1. If any significant difference between treatments appeared, Duncan multiple range test (DMRT) were performed with the test level of α=5%.

RESULT AND DISCUSSION

Antibacterial activity of *S. pinnata* leaves

The antibacterial activity of SPL ethanol extract and fractions against *B. cereus* and *V. parahaemolyticus* are shown in Table 1. The ethyl acetate fraction had higher antibacterial activity against *B. cereus* than other fractions (P-value <0.05). Antibacterial activities of ethyl acetate fraction, water fraction and ethanol extract against *V. parahaemolyticus* were higher than other two fractions (P-value <0.05). Chung and Yoon (2008) reported that ethyl acetate fraction of green tea had the highest inhibition zone against *B. subtilis* (17.5 mm) and *V. parahaemolyticus* (25.5 mm) when compared with water, hexane and chloroform fractions. High antibacterial activity in extract/fraction of semi-polar and polar fraction of SPL probably associated with its high phenol and flavonoid content. Ethyl acetate fraction and water fraction of *S. pinnata* fruit exocarp were reported to contain higher total phenol and flavonoids than hexane and dichloromethane fractions (Manik *et al*., 2013). Similarly, ethanol extract and ethylacetate extract of *S. pinnata* leaf were found to have higher total phenol and flavonoid content than those found in hexane extract (Jain *et al*., 2014).

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Antibacterial activity of SPL fraction (n-hexane, chloroform, ethyl acetate and water) againts *B. cereus* was higher than *V. parahaemolyticus*. These results were supported by Bulbul (2016) who reported that antibacterial activity of ethyl acetate fraction of *Momordica charantia* leaves against *B. cereus* was higher (20 mm) than *V. parahaemolyticus* (17 mm). Efdi *et al*. (2015) reported that ethyl acetate fraction of *Enicosanthum membranifolium* and *Enicosanthum cupulare* providing greater inhibition to *B. cereus* (MIC 0.25 and 0.125 mg/mL respectively) than *V. parahaemolyticus* (MIC 2 and >4 mg/mL respectively). Shan *et al.* (2007) found that 46 herbal extracts and spices have greater inhibition impact in Gram positive bacteria rather than in Gram negative ones. Similarly, Santos *et al.* (2013) found that Gram negative bacteria are more resistant to the extract of *Anacardium occidentale* bark and leaves than those of Gram positive bacteria. According to Lambert (2002), the difference in sensitivity between Gram-positive and Gram-negative bacteria to the extract could associate with differences in structure and composition of cell wall membrane between the two groups. The cell wall structure of Gram-positive bacterial consist of peptidoglycan and have a character. The Gram-negative bacteria have an outer membrane (OM) which is a barrier to small hydrophilic molecules penetration. There is a double external defense system, consists of peptidoglycan as well as OM containing various proteins and lipopolysaccharides (LPS). LPS consists of lipid A, the core polysaccharide, and the O side chain, which provides the "quid" that allows Gram-negative bacteria to be more resistant to natural extracts with antimicrobial activity. OM can not be bypassed by hydrophobic or antibacterial antibiotics (Nazzaro *et al*., 2013).

Table 1: Antibacterial activity of ethanol extract and *S. pinnata* leaves' fractions.

Type of extract/fraction	*B. cereus*		*V. parahaemolyticus*	
	DIZ (mm)[1]	MIC[2]	DIZ (mm)[1]	MIC[2]
Ethanol extract	8.40±1,61[c]	3.12	11.47±3,41[a]	3.12
n-Hexane fraction	3.58±1,40[e]	-	2.99±1,42[b]	-
Chloroform fraction	5,3±1,04[d]	-	5.29±1,16[b]	-
Ethyl acetate fraction	13.43±0,39[a]	0.63	12.71±0,73[a]	>5.00
Water fraction	11.47±0.43[b]	0.63	10.48±0,18[a]	1.25
Chloramphenicol	21.13±0.06	0.01	37.70±0.11	0.01

The value in the same column that are not sharing the same superscript letter are significantly different from each other (P-value<0.05). [1]clear zone diameter after substraction of well diameter is 6 mm; [2]MIC=*Minimum Inhibition Concentration* in mg/mL; -, not measured because of relatively small DIZ; DIZ, Diameter of Inhibition Zone.

Stability of ethanol extract of S*. pinnata* leaves to pH, NaCl and temperature

Many factors might influence the ability of antimicrobial compounds to inhibit microbial growth, for examples pH, temperature, and Aw (Naidu, 2000). Figure 1 shows the stability of the SPL ethanol extract to inhibit the growth of *B. cereus* and *V. parahaemolyticus* when it was challenged with pH variation, NaCl addition, and temperature variation. Antibacterial activity of SPL ethanol extract againsts *B. cereus* at pH 4 was higher than that at pH 7 (P-value<0.05), but insignificant againsts *V. parahaemolyticus* at both pH (P-value>0.05). NaCl concentration (up to 10%) in the extract did not affect the extract inhibition activity (P-value>0.05) but heating of the extract up to 121 °C increased the extract's inhibition activity (P-value<0.05).

Study of pH effect on the antibacterial activity of *Mangifera indica* (Anacardiaceae) leaf extract by Doughari and Manzara (2008) was similar to the results of this study. Antibacterial activity of this extract at pH 8 was 4 mm and increased to 9 mm at pH 5 against *B. cereus*. The compond found in *S. pinnata* was not stabil in high pH. In a low pH, especially acid as gallic acid which found in *S. pinnata* (Chaudhuri *et al*., 2015) was undissociated form that means have a potential than in than dissociated form (Naidu 2000).

An increased in antibacterial activities after heating were also found by Doughari and Manzara (2008). Antibacteria activity of mango leaf extract againsts *B. cereus* increased when it was heated at temperature of 60 and 100 °C (from DIZ 11 mm to DIZ 13 mm). In some cases, this might associate with an increase in phenolics content due to heating treatment. Xu *et al.* (2007) found the extract ethanol of orange peels heated at the temperature of 90, 120 and 150 °C for 30 min and 120 °C for 60 and 90 min resulting in an increase of free fraction of phenolic acid, while the ester, glycoside, and ester-bound fractions decreased. Heating treatment to some extent might accelerate the release of phenolics compounds from its matric in plant extract.

During fish processing, SPL were utilized in an intact form, therefore ethanol extract were used to test the stability and effectiveness in the treatment and its application to fishes. Food structure and composition are very influential toward antibacterial activities. Population data of *B. cereus* and *V. parahaemolyticus* bacteria in fish broth and minced fish administered with SPL extract at some level of concentrations and stored at the temperature of 5 °C is presented in Table 2.

The bactericidal effects of SPL ethanol extract against *B. cereus* and *V. parahaemolitycus* in fish broth were observed at 3 and 1 MIC, respectively. The effect was more apparent in fish broth rather than in fish flesh. It was reported that the combination of ginger and galangal

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essential oils inhibited more in the chicken broth than the chicken meat against *B. cereus* and *S.* Typhimurium (Rialita, 2014). Fish meat is a more complex medium with a more dense surface. Application of SPL ethanol extract to the minced fish gave bacteriostatic effect to both tested bacteria (P-value<0.05). The exposure of 5 MIC of SPL extract for 4 days at 5 °C caused the decrease in population of *B. cereus* and *V. parahaemolyticus* by 0.77 and 1.86 CFU logs (compared to 0 day and 0 MIC) respectively.

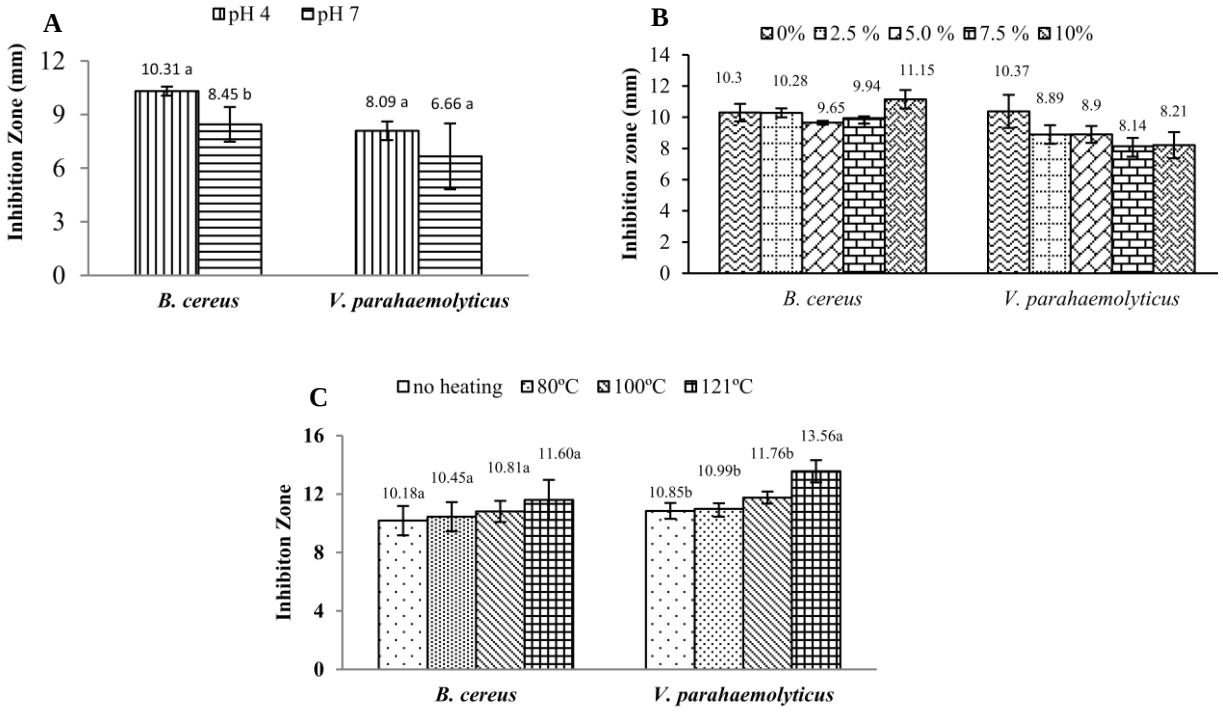

Figure 1: Inhibition zone of ethanol extract at various pH (A) and NaCl levels (B) and temperature of heating (C) againsts *B. cereus* and *V. parahaemolyticus.*

Table 2: Population of *B. cereus* and *V. parahaemolitycus* bacteria in fish broth (log CFU/mL) and minced fish (log CFU/g) exposed to SPL ethanol extract.

Days	Fish broth + extract (MIC)				Minced fish +extract (MIC)			
	0	1	3	5	0	1	3	5
B. cereus								
0	6.50±0.15	6.17±0.09	<1.00	<1.00	6.67±0.06^{bc}	6.56±0.11^{c}	6.22±0.05^{efg}	6.17±0.05^{gh}
1	6.04±0.00	6.06±0.10	1.72±0.48	1.39±0.10	6.95±0.03^{a}	6.72±0.16^{b}	6.27±0.04^{defg}	6.07±0.07^{h}
2	7.04±0.04	7.10±0.40	<1.00	<1.00	6.73±0.10^{b}	6.70±0.07^{bc}	6.41±0.08^{d}	6.21±0.16^{fgh}
3	7.07±0.08	7.20±0.44	<1.00	<1.00	6.88±0.01^{a}	6.71±0.07^{bc}	6.39±0.01^{d}	6.34±0.02^{def}
4	6.98±0.59	6.34±0.55	<1.00	<1.00	6.72±0.05^{b}	6.37±0.01^{de}	6.26±0.16^{defg}	5.90±0.00^{i}
V. parahaemolyticus								
0	5.33±0.19	3.08±0.07	<1.00	<1.00	5.66±0.05^{a}	5.49±0.02^{abc}	5.47±0.05^{abc}	5.30±0.14^{abc}
1	3.11±0.10	<1.00	<1.00	<1.00	5.52±0.02^{ab}	5.31±0.15^{abc}	5.21±0.13^{bcd}	5.11±0.05^{cde}
2	3.11±0.09	<1.00	<1.00	<1.00	5.31±0.19^{abc}	4.64±0.21^{f}	4.84±0.18^{def}	4.63±0.23^{def}
3	3.12±0.08	<1.00	<1.00	<1.00	4.75±0.17^{ef}	4.69±0.29^{ef}	3.83±0.08^{g}	3.69±0.08^{g}
4	3.17±0.31	<1.00	<1.00	<1.00	4.76±0.24^{ef}	4.80±0.11^{def}	3.90±0.05^{g}	3.80±0.06^{g}

The value in the same column that are not sharing the same superscript letter are significantly different from each other (P-value < 0.05); 0 MIC, control; 1 MIC,3.12 mg/mL; 3 MIC,9.36 mg/mL; 5 MIC,15.60 mg/mL.

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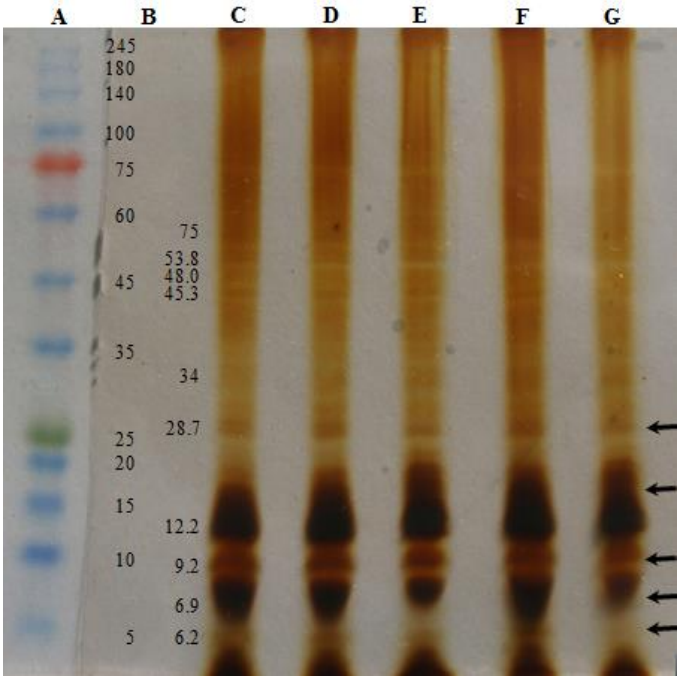

Figure 2: Protein profile of *B. cereus* cells using SDS-PAGE method (A, Marker; B, Protein molecular weight, kDa; C, Unexposed bacteria (0 MIC); Exposed bacteria: D, 1 MIC of ethyl acetate fraction; E, 3 MIC of ethyl acetate fraction; F, 1 MIC of water fraction; G, 3 MIC of water fraction).

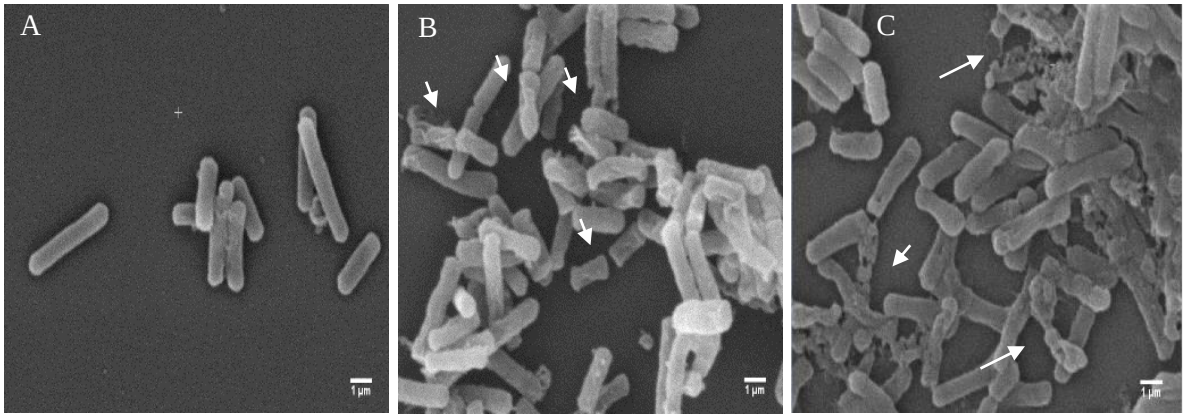

Figure 3: The morphology of *B. cereus* cells using SEM after 1 h of incubation in the PBS containing antibacterial (A, 0 MIC, control; B, 3 MIC ethyl acetate; C, 3 MIC water fraction; arrows indicate the damaged cells).

In Table 2, there was a decrease in the number of *V. parahaemolyticus* by 2.22 CFU logs in fish broth without SPL after 1 day incubation at 5 °C. Whereas with 1 MIC SPL (on the same medium), the number of the bacteria was <1.00 log CFU/mL after one day of incubation. The decline in bacterial population in this study, in addition to the presence of SPL Extract, also due to the use of low temperatures. According to Muntada-Garriga *et al.* (1995), *V. parahaemolyticus* will be inactive when stored at low temperatures. The inactivation time depends on the amount of the initial bacteria and the incubation temperature. Wang *et al.* (2008) found a decrease in the number of *V. parahaemolyticus* during storage at 5 °C in live and slurry Jinjiang oyster and APW (alkaline pepton water) with the greatest decrease in the slurry (>2 logs CFU, incubation time < 24 h).

Protein profile of *B. cereus* cells exposed to ethyl acetate fraction and water fraction of S*. pinnata* leaves

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Protein profile of *B. cereus* cells both unexposed and exposed to ethyl acetate fraction and water fraction is shown in Figure 2. There are differences of the protein bands thickness in the treated cells (D, E, F and G) as compared to the control group. It indicated that protein loss occured in *B. cereus* cells after being exposed to ethyl acetate and water fractions.

At the concentration of 3 MIC, protein bands of *B. cereus* cells are thinner as compared to those exposed under 0 and 1 MIC, especially the bands of 6.9, 9.2 and 28.7 kDa. Even the 6.2 kDa protein bands were not visible at 3 MIC treatment. Wang *et al.* (2015) showed the differences of electrophoresis protein bands profile of *Salmonella*, *E. coli* and *Listeria* cells treated with lactic acid compared to the control group. In control group, the protein bands were greater in number and clearer in appearance, whereas in the lactic acid treatment, the protein bands were more vague and some even disappeared.

The morphology of *B. cereus* cell exposed to ethyl acetate fraction and water fraction of S*. pinnata* leaves

The morphological changes of *B. cereus* cells after being exposed to ethyl acetate and water fractions were observed using SEM. The result is shown in Figure 3. The ethyl acetate fraction-exposed there are many cells with defective cell wall (B, arrow), while destroyed cells are widely found in those exposed to water fraction (C, arrow).Miksusanti *et al.* (2009) demonstrated that after being exposed to *Kaempheria pandurata* essential oil, *B. cereus cells* showed rougher cell surface, multiplication of cell debris, and empty cells appearance under SEM. *Polygonum cuspidatum* root extracts was observed to cause morphological changes and damages to the cell wall of 6 tested bacteria including *B. cereus* (Shan *et al*., 2008). Damages to the cell wall and cytoplasmic membrane indicating the loss of membrane integrity and ability as permeability barrier, leading to cell death (Shan *et al*., 2008).

CONCLUSION

Ethanolic extract of *S. pinnata* leaves demostrated good antibacterial activities against *B. cereus* and *V. parahaemolyticus* with equal MIC value (3.12 mg/mL). After fractionation, ethyl acetate fraction had the highest antibacterial activity (MIC 0.63 mg/mL) against *B. cereus*. The mechanism was proposed due to its ability to damage the cell wall. The ethanol extract of SPL was stable under pH 4 and heating temperature up to 121 °C. At 3 MIC, it was more effective to inhibit *B. cereus* and *V. parahaemolyticus* in fish broth than in minced fish.

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