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Antibacterial and Sporicidal Activities of *Syzygium polyanthum* L. Extract against *Bacillus cereus* Isolated from Rice

(Aktiviti Antibakteria dan Sporisid Ekstrak *Syzygium polyanthum* L. terhadap *Bacillus cereus* yang Dipencil daripada Nasi)

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ABSTRACT

Spore-forming bacteria, Bacillus *sp., frequently been associated with the contamination of rice and other starchy products. Spores are more resistant to antimicrobial treatments than its vegetative cells. The extract of Indonesian bay leaf* (Syzygium polyanthum *L.*) *was assessed for its antibacterial and sporicidal activities against vegetative cells and spores of* B. cereus *isolated from rice (25 strains). The results showed that* S. polyanthum *L. extract was able to inhibit the growth of vegetative cells of all* B. cereus *isolates with MICs ranged from 0.16 to 0.63 mg/mL and can kill with MBCs ranged from 0.31 to 2.50 mg/mL. The bactericidal endpoint for* B. cereus *BC-NP.8 in time kill curve was at 1.25 mg/mL (8× MIC) after 4 h of incubation while for* B. cereus *ATCC 33019 was at 2.50 mg/mL (8× MIC). The sporicidal activity of* S. polyanthum *L. extract was not affected by different temperatures treatment and alteration of the pHs of extract. Therefore, this indicates that the extract was stable after exposed to pH3, 7 and 10 as well as temperature of 50, 80, and 121°C. Observation under on scanning electron microscope the structure of the* B. cereus *ATCC 33019 spores was ruptured after being treated with 1% (w/v)* S. polyanthum *L. extract for 1 h. In conclusion,* S. polyanthum *L. extract had antibacterial and sporicidal activity against vegetative cells and spores of* B. cereus *isolated from rice.*

Keywords: Antibacterial; B. cereus*; rice; sporicidal;* Syzygium polyanthum *L.*

ABSTRAK

Bakteria pembentuk spora seperti Bacillus *sp., sering dikaitkan dengan pencemaran nasi dan produk-produk yang berkanji. Spora mempunyai rintangan yang lebih tinggi terhadap rawatan antimikrob daripada sel-sel vegetatif. Ekstrak daun salam* (S. polyanthum *L.*) *telah diuji untuk aktiviti antibakteria dan antisporisid terhadap sel vegetatif dan spora 25* B. cereus *yang dipencil daripada nasi. Ekstrak* S. polyanthum *L. boleh merencat pertumbuhan semua* Bacillus *sp. yang diuji dengan MIC dalam lingkungan 0.16 hingga 0.63 mg/mL dan boleh membunuh semua* Bacillus *sp. yang diuji dengan MBC adalah dalam lingkungan 0.31 hingga 2.50 mg/mL. Titik akhir bakterisid* B. cereus *BC-NP.8 untuk keluk masa-pembunuhan ialah 1.25 mg/mL (8× MIC) selepas inkubasi selama 4 jam dan untuk* B. cereus *ATCC 33019 ialah pada 2.50 mg/mL (8× MIC). Aktiviti sporisid ekstrak* S. polyanthum *L. tidak terjejas dengan pengubahan pH ekstrak dan rawatan suhu yang berbeza. Keputusan kajian ini menunjukkan bahawa ekstrak tersebut adalah stabil terhadap perubahan kepada pH3, 7 dan 10 serta suhu 50, 80 dan 121°C. Berdasarkan pemerhatian dengan mikroskop elektron imbasan, struktur spora* B. cereus *ATCC 33019 musnah selepas dirawat dengan 1% (w/v) ekstrak* S. polyanthum *L. selama 1 jam. Secara keseluruhannya, ekstrak* S. polyanthum *L. menunjukkan potensi dalam aktiviti antibakteria dan sporisid terhadap sel vegetatif dan spora* Bacillus *sp.*

Kata kunci: Antibakterial; B. cereus*; nasi; sporisid;* Syzygium polyanthum *L.*

INTRODUCTION

Bacillus species, a Gram-positive, facultative anaerobic, motile rod-shaped bacterium, is widely distributed in nature (Kim et al. 2014). The genus Bacillus, includes species such as *B. cereus* and *B. subtilis* can successfully adapt to various changes in the environment. *B. cereus* causes diarrhoea and emetic type of food poisoning. The type of foodborne sources are include meaty foods, vegetables, sauces and milk products (Kim et al. 2014). The emetic type, which causes symptoms such as nausea and vomiting, is often associated with the consumption of rice and other farinaceous foods, such as pasta and noodles (Altayar & Sutherland 2006; Kim et al. 2013). The spores of *B. cereus* may survive and germinate if cooked rice was left at room temperature, resulting in foodborne illness (Choi et al. 2014). *B. cereus* responds to adverse environmental stresses by forming a dormant structure known as endospore (simply termed as spore) through the process of sporulation (Leggett et al. 2012). Spores are able to survive in harsh external conditions, such as nutrient starvation or desiccation and germinate after the favourable growth conditions returned (Tan & Ramamurthi 2013). Bacterial spores' resilient and highly resistant characteristic poses problems to the food industries (Leggett et al.

2012). Germination of spores into vegetative cells under favourable conditions is frequently associated with food spoilage and foodborne diseases (Barker et al. 2005). *Bacillus* spores are highly resistant to various chemical disinfectants. In addition, there are limitations to several chemical sporicidal agents used to eradicate Bacillus spores, such as formaldehyde and glutaraldehyde, which are toxic and require special precaution for use (Kida et al. 2004).

Medicinal plants are used widely in the food industry as spices for flavours and fragrances and some of them contain phytochemical compounds that exhibit antimicrobial activities against a wide spectrum of foodborne bacteria. This led to suggestions that they could be used as natural food preservatives (Cho et al. 2008). The leaves of *S. polyanthum* L., which is also known as '*daun salam*' in Indonesia, are commonly used as spice in culinary due to its aroma besides the sour taste and also as ingredient in the Indonesian traditional medicine '*Jamu*' (Kato et al. 2013). From the study by Lau et al. (2014), it is note that *S. polyanthum* L. extract confers significant antibacterial and sporicidal activity against spore-forming bacteria, *B. cereus*. Therefore, in this study, the antibacterial and sporicidal activities of *S. polyanthum* L. against *B. cereus* isolated from rice samples was determined.

Materials and Methods

Bacillus strains

Bacillus cereus ATCC 33019 was obtained from American Type Culture Collection (Rockville, Maryland, United States) whereas twenty five of *B. cereus* strains which isolated from various rice samples (Sandra et al. 2012) were obtained from Centre of Excellence for Food Safety Research, Faculty of Food Science and Technology, Universiti Putra Malaysia. *B. cereus* were cultured and maintained statically in nutrient broth (NB; Difco, Sparks, Maryland, United States) or NB supplemented with 1.5% (w/v) agar (NA).

Preparation of *Bacillus cereus* spore suspension

The spores of *Bacillus cereus*, were prepared by referring to the methods described previously by Kida et al. (2004) and Rukayadi et al. (2009), with modification. Briefly, *B. cereus* were grown on NA at 30°C for over 1 week. The cells were harvested by dredging using a cotton swab. After harvesting, spores and vegetative cells were suspended in sterile 0.85% NaCl solution and then heat shocked was done at 65°C for 30 min in order to kill vegetative cells. Spores were harvested by centrifugation (12,000 × g for 30 min at 4°C) and washed four times with the original volume of sterile 0.85% NaCl solution. The presence and purity of spores were confirmed through microscopic examination by using Phase Contrast Microscope (Nikon, Japan) after spore staining and the number of viable spores was determined using a viable plate count. Spores suspension were then aliquot into 1 mL portion containing approximately 10^8 spores/mL and stored in a 1.5 mL plastic cryopreservation tube at -18°C until further use (Rukayadi et al. 2009).

Plant extraction and extract preparation

Dried *Syzygium polyanthum* L. was purchased from Herbal Market, Pasar Baru, Bandung, Indonesia. One hundred grams of dried *S. polyanthum* L. were grounded and extracted with 400 mL of absolute methanol (99.8%) (Systerm, ChemAR, Kielce, Poland) for seven days at room temperature (Rukayadi et al. 2008), with some modifications. After seven days, the plant material was filtered using Whatman No. 1 filter paper (Whatman International Ltd., Middlesex, England) and concentrated by using rotary vacuum evaporator (Heidolph VV2011, Schwabach, Germany) at 50°C and speed of 150 rpm for about 3 to 4 h. At the end of extraction, the temperature of the rotary evaporator was increased to 85°C for 30 s to obtain methanol-free extract. The crude extract was then stored at 4°C prior to use. The crude extract was dissolved in 100% dimethylsulfoxide (DMSO) (Fisher Scientific, Leicestershire, United Kingdom) to obtain 100 mg/mL and the solution was further diluted in 1:10 (v/v) distilled water to obtain 10 mg/mL (10,000 µg/mL) stock solutions.

In-vitro susceptibility test

Disc diffusion test *Syzygium polyanthum* L. methanolic extract was tested for antimicrobial activity against vegetative cells of *B. cereus* isolated from rice using the disc diffusion method as described by Clinical and Laboratory Standards Institute (CLSI 2012). The *B. cereus* was streaked on Mueller Hinton agar (MHA, Difco, Sparks, Maryland, United States) plates with a sterile cotton swab. Sterile filter paper discs with 6 mm diameter were placed on top of the agar and 10 µL of 10 mg/mL (w/v) *S. polyanthum* L. extract was loaded on the paper discs. A 0.1 mg/mL of chlorhexidine (CHX) was used as positive control in the assay. The plates were then incubated at 30°C for 24 h. The size of clear zone was measured in mm. This assay was done three times with three replicates ($n = 3 \times 3$).

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The determination of MIC and MBC were done according to methods as recommended by CLSI (2012). The MICs and MBCs determination were performed in a 96-well microtiter plate using two fold standard broth microdilution method with an inoculum of approximately 10^7 CFU/mL. Briefly, a 100 µL of *S. polyanthum* L. extract (10 mg/mL = 10,000 µg/mL) was mixed and diluted two-folds with the tested organism in 100 µL of Mueller Hinton broth (MHB, Difco, Sparks, Maryland, United States). Column 12 of the microtiter plate contained the highest concentration of the extract (5 mg/mL = 5,000 µg/mL), while column 3

contained the lowest concentration (0.0195 mg/mL = 19.50 µg/mL). The first column served as negative control (only MHB), while the second column is the positive control for all samples (only MHB and inoculum). The microtiter plate was then incubated aerobically at 30°C for 24 h. The MIC was defined as the lowest concentration of antibacterial agent that resulted in the complete inhibition of visible growth (Rukayadi et al. 2008). The MBC was determined sub-culturing the media in each well onto MHA plates. The plates were incubated at 30°C for 24 h until growth was seen in the growth control plates. MBC was defined as the corresponding concentration required to completely killing the microorganisms (Rukayadi et al. 2008). MIC and MBC test were repeated thrice with three replicates each ($n = 3 \times 3$).

DETERMINATION OF TIME-KILL CURVE

Time-kill assay was done on the vegetative cells of *B. cereus* ATCC 33019 and *B. cereus* BC-NP.8 and according to CLSI (2012) reference method, with slight modification. Briefly, the inoculum suspension of *B. cereus* was approximately 10^6 CFU/mL. The *S. polyanthum* L. extract was diluted with the MHB medium containing inoculum to obtain final concentrations of 0× MIC, 0.5× MIC, 1× MIC, 2× MIC, 4× MIC and 8× MIC for each bacterial species. Cultures (1 mL final volume) were incubated at 30°C with 200 rpm agitation. At pre-determined time points (0, 0.5, 1, 2 and 4 h), 100 µL aliquots were removed and transferred to microcentrifuge tubes. The aliquot was serially diluted 1:100 in 1% phosphate buffered saline (PBS) and plated onto MHA. The number of colonies formed on the plates after incubation at 30°C for 24 h was counted and the number of CFU/mL was calculated. Assays were carried out in duplicate. The graph of $\log_{10}$ CFU/mL versus time was plotted. Time-kill curve were done for three times with triplicate data ($n = 3 \times 3$).

DETERMINATION OF SPORICIDAL ACTIVITY IN *SYZYGIUM POLYANTHUM* L. EXTRACT AGAINST SPORES OF *BACILLUS CEREUS* ATCC 33019 AND *BACILLUS CEREUS* BC-NP.8

Effect of different concentrations of Syzygium polyanthum *L. extract on the sporicidal activity at different incubation time* The prepared spores' suspension was thawed and diluted 1:100 in 0.85% NaCl solution (pH 6.6), yielding *B. cereus* ATCC 33019 *B. cereus* BC-NP.8, spores suspension was 1.32×10^6. The stock extract (10%) was added to the adjusted spores suspension, resulting the final concentrations of extract were 0.05, 0.25, 0.50, 1.00, 2.50 and 5.00%. A standard 25% commercially available glutaraldehyde solution (Merck Millipore, Darmstadt, Germany) was used as positive control in the determination of sporicidal activity. The glutaraldehyde was diluted 1:25 in distilled water to yield 1% (w/v) concentration for further testing. The pH of these test solutions was not changed by addition of extract or glutaraldehyde. One mL of each concentration was then incubated for 0, 1, 2, 3 and 4 h in a water bath (30°C). A 100 µL aliquot was removed and transferred to microcentrifuge tubes and then was centrifuged (12, 000 × g at 4°C for 5 min, ALC Microcentrifuge 4214, Milan, Italy). After that, the pellet was rinsed twice with 0.9 mL of 0.85% NaCl solution (pH6.6) to obtain bacterial-free spores and to avoid effect of vegetative cells residue. Pellets were suspended in 100 µL of 0.85% NaCl solution (pH6.6), serially diluted and spread onto NA plates and incubated at 30°C for 24 h or more (until the colonies were seen on the plates). Colonies that formed on the duplicate plates were counted and the mean of colony-forming unit (CFU/mL) was calculated. Differences were obtained by subtracting the $\log_{10}$ CFU/mL values of the test solution from those of the control (no antimicrobial). The reduction of spore cells in CFU was expressed as sporicidal activity. The determination of sporicidal activity was done three times with triplicate per each experiment ($n = 3 \times 3$).

Effect of temperature on the sporicidal activity of Syzygium polyanthum *L. extract* The crude methanolic extract was dissolved in 100% DMSO to obtain 200 mg/mL and the solution was further diluted in 1:10 (v/v) distilled water to obtain 20 mg/mL (2%) stock solutions. Different temperature treatments of 4, 50, 80 and 121°C for 15 min were applied to the extract. The extract was incubated at 30°C is used as control. The treated extracts were then tested for their sporicidal activity against the spores of *B. cereus* ATCC 33019 and *B. cereus* BC-NP.8. The extract (2%) was diluted in adjusted spores suspension, resulting in final concentrations of extract of 1% (w/v). The test solutions were then incubated at 30°C for 1 h. A 100 µL aliquot was removed and transferred to microcentrifuge tubes, centrifuged (12,000 × g at 4°C for 5 min, ALC Microcentrifuge 4214, Milan, Italy) and rinsed twice with 0.9 ml of 0.85% NaCl solution. Pellets were re-suspended in 100 µL of 0.85% NaCl solution and serially diluted. An appropriate volume (1000, 100, 40 or 20 µL) were spread onto NA plates and incubated at 30°C for 24 h or more (until the colonies were seen on the plates). Colonies that formed on the NA plates were counted and the mean of colony-forming unit (CFU/mL) was calculated. The sporicidal activity tests were done for three times with triplicate per each experiment ($n = 3 \times 3$).

Effect of ph on the sporicidal activity of Syzygium polyanthum *L. extract* The diluted *Syzygium polyanthum* L. extract (2%) was found to be at pH5 and it was used as control. Then, the pH was adjusted to pH 3, 7 and 10 using 0.1 M hydrochloric acid (Merck Millipore, Darmstadt, Germany) and 0.1 M sodium hydroxide (Sigma Aldrich, Missouri, United States). The treated extracts were then tested for their sporicidal activity against *B. cereus* ATCC 33019 and *B. cereus* BC-NP.8. The extract (2%) was diluted in adjusted spore suspension, resulting 1% (w/v) as the final concentrations of extract. The test solutions were then incubated at 30°C for 1 h. A 100 µL aliquot was centrifuged (12,000 × g at 4°C for 5 min, ALC Microcentrifuge 4214, Milan, Italy) and rinsed twice with 0.9 mL of 0.85% NaCl

solution. Pellets were re-suspended in 100 µL of 0.85% NaCl solution and then serially diluted. An appropriate volume (1000, 100, 40 or 20 µL) were spread onto NA plates and incubated at 30°C for 24 h or more. Colonies that formed on the plates were counted and the mean of colony-forming unit (CFU/mL) was calculated. The sporicidal activity tests were done three times with triplicate per each experiment ($n = 3 \times 3$).

SCANNING ELECTRON MICROSCOPY (SEM) ANALYSIS

Bacillus cereus ATCC 33019 were used in the SEM analysis. Bacteria spores were incubated for 1 h at 30°C in the presence of 1% (w/v) *S. polyanthum* L. extract. Spores were recovered by centrifugation (12,000 × g for 10 min, ALC Microcentrifuge 4214, Milan, Italy) and pellets were fixed with 4% buffered glutaraldehyde for 6 h at 4°C, washed with 0.1 M sodium cacodylate buffer for 10 min and was repeated for 3 times. The spore pellets was then post fixed with 1% osmium tetroxide for 2 h at 4°C, washed again with 0.1 M sodium cacodylate buffer for 10 min for three time. Then the pellets were dehydrated using 35, 50, 75 and 95% acetone for 15 min each. Lastly the pellets were dehydrated using 100% acetone (Merck Millipore, Darmstadt, Germany) for 15 min and were repeated for three times. Cell suspension was transferred into a specimen basket, made from aluminium foil coated with albumin, and was put in critical dryer for 0.5 h. The specimen was mounted on a stub and the sputter was coated with gold. The morphology of the spores was observed and images were obtained using SEM instrument (JSM 6400, JEOL Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

ANTIBACTERIAL ACTIVITY OF *SYZYGIUM POLYANTHUM* L. EXTRACT AGAINST VEGETATIVE CELLS OF *BACILLUS CEREUS*

Bacillus contamination has always been a problem to the food industry because these spore-forming bacteria are hard to eliminate completely in the food processing. Improper food handling and exposure of food to ambient temperature for prolong time may cause food poisoning. Therefore, there is a resurgence of interest in discovering new antimicrobial agent from natural sources, namely from plant sources. Thus, the *S. polyanthum* L. extract at 1% (w/v) concentration was screened for antibacterial activity against vegetative cells of 25 isolated *B. cereus* determined in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The result of susceptibility test is summarized in Table 1. The MICs of S. *polyanthum* L. extract against the *B. cereus* was ranged from 0.16 to 0.63 mg/mL, while the MBCs was ranged between 0.31 and 2.50 mg/mL. The MIC of *S. polyanthum* L. extract against *B. cereus* tested was ranged between 0.16 and 0.63 mg/mL, which is lower compared to a study done by Alzoreky and Nakahara (2003). They found that the MIC of buffered methanolic extract of edible plants from China, Japan, Thailand and Yemen, such as *Artemisia absinthium* (wormwood), *Camellia sinensis* (green tea), *Cissus rotundifolia* (round-leaved vine), *Foeniculum vulgare* (fennel), *Illicium verum* (star anise), *Ocimum basilicum* (basil), *Rhaphanus sativus* (radish), *Rumex nervosus* (sorrel), *Ruta chalepensis* (ruta), *Ruta graveolens* (ruta), *Salvadora perisca* (tooth brush tree), *Thymus serpyllum* (thyme), *Trigonella foenum-graecum* (fenugreek) and *Zingiber officinale* (ginger), against *B. cereus* were ranged between 0.66 and 2.64 mg/mL. However, the MIC value dor for *Azadirachta indica* (neem) and *Cinnamomum cassia* (cassia) were 0.165 and 0.330 mg/mL, respectively. The low MIC value of *S. polyanthum* L. extract showed that the extract has greater antibacterial activity compared to the other plant extracts. In addition, the susceptibility of plant extract towards *B. cereus* due to the absence of lipopolysaccharides in outer membrane which is found in Gram-negative bacteria (Alzoreky & Nakahara 2003). Lipopolysaccharides in

TABLE 1. MIC and MBC of *Syzygium polyanthum* L. extract against vegetative cells of *Bacillus cereus* strains

B. cereus strains	MIC (mg/mL)	MBC (mg/mL)
ATCC 33019	0.31	2.50
BC-NL.1	0.31	0.63
BC-NL.2	0.31	0.63
BC-NL.4	0.63	1.25
BC-NL.7	0.16	0.63
BC-NL.18	0.16	0.31
BC-NB.2	0.31	0.63
BC-NB.3	0.31	0.63
BC-NB.4	0.16	0.31
BC-NB.5	0.63	1.25
BC-NB.9	0.63	2.50
BC-NA.1	0.63	1.25
BC-NA.5	0.31	0.63
BC-NA.11	0.31	0.63
BC-NA.12	0.16	0.31
BC-NA.18	0.63	1.25
BC-NP.4	0.31	1.25
BC-NP-5	0.31	0.63
BC-NP.7	0.63	2.50
BC-NP.8	0.16	0.31
BC-NP.13	0.31	0.63
BC-NP.16	0.31	0.63
BC-KW.1	0.63	1.25
BC-KW.2	0.31	0.63
BC-B.3	0.63	1.25
BC-B.4	0.63	1.25

* BC-NL (*B. cereus* isolated from *nasi lemak*)
BC-NB (*B. cereus* isolated from *nasi biryani*)
BC-NA (*B. cereus* isolated from *nasi ayam*)
BC-NP (*B. cereus* isolated from *nasi putih*)
BC-KW (B. cereus isolated from *keladi wangi*)
BC-B (*B. cereus* isolated from *bario*)

the outer membrane provide an additional barrier against antibacterial agent in the Gram-negative bacteria, whereas the absence of lipopolysaccharides in outer membrane of *B. cereus* vegetative cells will lead them to be inhibited by the *S. polyanthum* L. extract.

However, in a study done by Jun et al. (2013), the MIC of *S. polyanthum* L. extract against *B. cereus* was slightly higher compared to the extract of *Dryopteris erythrosora* (autumn fern), *Siegesbeckia glabrescens* herb, *Morus alba* (mulberry), *Carex pumila* (dwarf sedge), *Citrus paradisi* (grapefruit), *Siegesbeckia pubescens* herb, *Lastrea japonica* (Harigane-warabi) and *Vitidis vinifera* (grape vine), which ranged from 0.0156 to 0.1250 mg/mL. On the other hand, the MIC of *Carex pumila* (sand sedge), *Agrimonia pilosa* (hairy agrimony), *Rhus chinensis* (nutgall) and *Dryopteris crassirhizoma* (wood fern) extract against *B. cereus* were ranged from 0.2500 to > 2.000 mg/mL (Jun et al. 2013). Although the MIC value of *S. polyanthum* L. extract was slightly higher compared to the plant extracts tested by Jun et al. (2013), it is important to highlight that those extracts do not necessarily possess sporicidal activity. This might be due to the high presence of soluble phenolic and polyphenolic compounds in the extract (Darah et al. 2013).

In this study, *S. polyanthum* L. extract exhibit both antibacterial and sporicidal activity against vegetative cells as well as spores of *B. cereus*. Time-kill curves were established for vegetative cells of *B. cereus* ATCC 33019 and *B. cereus* BC-NP.8 which was isolated from *nasi putih*. This analysis was done to assess the correlation between MIC and bactericidal activity of *S. polyanthum* L. extract at concentrations ranging from 0 MIC to 8× MIC. *B. cereus* BC-NP.8 was selected to further analysis due to lower MIC and MBC values compared other strains. Besides that, this strain was chosen because it was isolated from *nasi putih* which is most popular starchy food in Malaysia compared to others. Therefore, this strains was more interested to be study in order to get detail data regarding antibacterial and antisporicidal activity of *S. polyanthum* L. against *B. cereus* isolated from *nasi putih*. Besides that, the data will be give basic information in application of *S. polyanthum* L. as preservative to control *B. cereus* in rice. The bactericidal endpoint for *B. cereus* ATCC 33019 (Figure 1(a)) and *B. cereus* BC-NP.8 (Figure 1(b)) were reached after 4 h of incubation at 2.50 mg/mL (8× MIC) and at 1.25 mg/mL (8× MIC), respectively.

Sumono and Wulan (2008) also reported that *S. polyanthum* L. solution was able to reduce the numbers of *Streptococcus* sp., the oral pathogens in dentistry. In addition, Setiawan (2002) also reported that *salam* leaves are able to inhibit the growth of *Salmonella* sp., *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas fluorescens*. This ability may be due to present of tannin, flavonoid and essential oil content in the plant (Sumono & Wulan 2008). Therefore, it is suggested that the plant extract had a potential as antimicrobial agent in food industry.

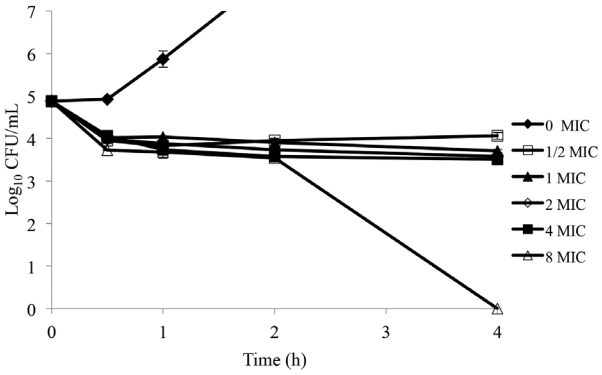


FIGURE 1(a). Time-kill curve for *Bacillus cereus* ATCC 33019 following exposure to *Syzygium polyanthum* L. extract at 0×, 1/2×, 1×, 2×, 4× and 8× MIC (0, 0.16, 0.31, 0.63, 1.25 and 2.50 mg/mL, respectively) at 30°C

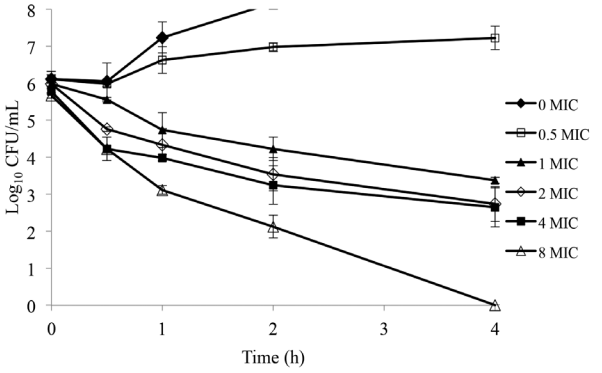


FIGURE 1(b). Time-kill curve for *Bacillus cereus* BC-NP.8 following exposure to *Syzygium polyanthum* L. extract at 0×, 1/2×, 1×, 2×, 4× and 8× MIC (0, 0.08, 0.16, 0.31, 0.63 and 1.25 mg/mL, respectively) at 30°C

SPORICIDAL ACTIVITY OF *SYZYGIUM POLYANTHUM* L. EXTRACT AGAINST SPORES OF BACILLUS CEREUS

Effect of different concentrations of Syzygium polyanthum L. *extract on the sporicidal activity at different incubation time* The *Syzygium polyanthum* L. extract was tested at different concentrations which were 0.05, 0.25, 0.50, 1.00, 2.50 and 5.00% for its ability to reduce viability of *B. cereus* spores after incubated at 30°C for 1, 2, 3, and 4 h. The effect of different concentrations of *S. polyanthum* L. extract and glutaraldehyde (positive control) on the sporicidal activity against *B. cereus* ATCC 33019 and *B. cereus* BC-NP.8 spores at different incubation time was determined and the results were presented in Tables 2(a), 2(b), 3(a) and 3(b), respectively.

There were significant reduction in the number of *B. cereus* ATCC 33019 and *B. cereus* BC-NP.8 spores after being treated with 0.05% *S. polyanthum* L. extract and there was more than 3-$\log_{10}$ reduction (99.99%) at 1.00% concentration after 1 h of incubation. *S. polyanthum* L. extract at 2.50 and 5.00% was able to completely kill the both *B. cereus* ATCC 33019 and BC-NP.8 spores after 1 h of incubation. Similar reduction trend was observed

TABLE 2(a). Sporicidal activity of *Syzygium polyanthum* L. extract at different concentration against spores of *Bacillus cereus* ATCC33019

Concentration (% w/v)	Time (h)			
	1	2	3	4
0.00	5.95 ± 0.11^{a}	5.95 ± 0.11^{a}	5.95 ± 0.11^{a}	5.95 ± 0.11^{a}
0.05	4.05 ± 0.09^{b}	3.92 ± 0.04^{b}	3.89 ± 0.12^{b}	3.70 ± 0.05^{b}
0.25	4.04 ± 0.01^{b}	3.91 ± 0.01^{b}	3.76 ± 0.13^{b}	3.67 ± 0.16^{b}
0.50	2.90 ± 0.20^{c}	2.90 ± 0.08^{c}	2.00 ± 0.07^{c}	1.94 ± 0.07^{c}
1.00	1.83 ± 0.04^{d}	1.74 ± 0.06^{d}	1.72 ± 0.00^{c}	1.48 ± 0.13^{d}
2.50	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
5.00	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}

* The data are expressed as $\log_{10}$ CFU/mL
** The results are presented as the mean ± standard deviation. Significant differences in means (n=3×3) within the same column are indicated with different letters (p<0.05)

TABLE 2(b). Sporicidal activity of glutaraldehyde at different concentration against spores of *Bacillus cereus* ATCC 33019

Concentration (% w/v)	Time (h)			
	1	2	3	4
0.00	5.95 ± 0.11^{a}	5.95 ± 0.11^{a}	5.95 ± 0.11^{a}	5.95 ± 0.11^{a}
0.05	4.33 ± 0.15^{b}	3.75 ± 0.21^{b}	3.60 ± 0.02^{b}	3.30 ± 0.05^{b}
0.25	2.16 ± 0.01^{c}	2.15 ± 0.05^{c}	2.00 ± 0.01^{c}	2.00 ± 0.01^{c}
0.50	1.56 ± 0.05^{d}	1.48 ± 0.04^{d}	1.48 ± 0.02^{d}	1.48 ± 0.02^{d}
1.00	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}

* The data are expressed as $\log_{10}$ CFU/mL
** The results are presented as the mean ± standard deviation. Significant differences in means (n=3×3) within the same column are indicated with different letters (p<0.05)

TABLE 2(c). Sporicidal activity of *Syzygium polyanthum* L. extract at different concentration against spores of *Bacillus cereus* BC-NP.8

Concentration (% w/v)	Time (h)			
	1	2	3	4
0.00	6.12 ± 0.21^{a}	6.12 ± 0.21^{a}	6.12 ± 0.21^{a}	6.12 ± 0.21^{a}
0.05	4.76 ± 0.07^{b}	4.23 ± 0.32^{b}	4.12 ± 0.21^{b}	3.98 ± 0.09^{b}
0.25	3.38 ± 0.38^{b}	3.14 ± 0.41^{b}	2.98 ± 0.08^{b}	2.76 ± 0.06^{b}
0.50	2.66 ± 0.06^{c}	2.54 ± 0.45^{c}	2.34 ± 0.43^{c}	2.22 ± 0.22^{c}
1.00	1.65 ± 0.05^{d}	1.52 ± 0.02^{d}	1.52 ± 0.01^{c}	1.51 ± 0.05^{d}
2.50	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
5.00	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}

* The data are expressed as $\log_{10}$ CFU/mL
** The results are presented as the mean ± standard deviation. Significant differences in means (n=3×3) within the same column are indicated with different letters (p<0.05)

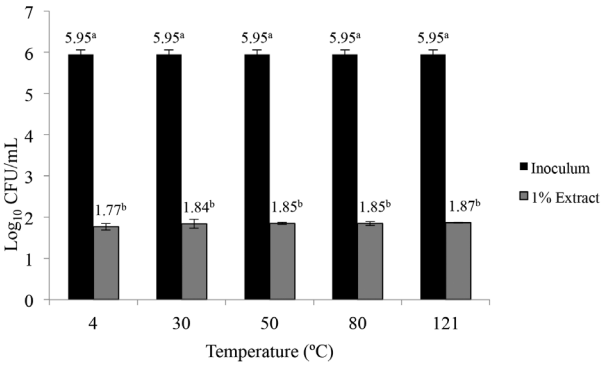
TABLE 2(d). Sporicidal activity of glutaraldehyde at different concentration against spores of *Bacillus cereus* BC-NP.8

Concentration (% w/v)	Time (h)			
	1	2	3	4
0.00	6.12 ± 0.21[a]	6.12 ± 0.21[a]	6.12 ± 0.21[a]	6.12 ± 0.21[a]
0.05	4.45 ± 0.54[b]	4.23 ± 0.23[b]	4.12 ± 0.21[b]	3.98 ± 0.09[b]
0.25	2.46 ± 0.64[c]	2.23 ± 0.32[c]	2.18 ± 0.08[c]	2.16 ± 0.16[c]
0.50	1.66 ± 0.06[d]	1.54 ± 0.20[d]	1.54 ± 0.05[d]	1.50 ± 0.05[d]
1.00	0.00 ± 0.00[e]	0.00 ± 0.00[e]	0.00 ± 0.00[e]	0.00 ± 0.00[e]

* The data are expressed as $\log_{10}$ CFU/mL.
** The results are presented as the mean ± standard deviation. Significant differences in means (n=3×3) within the same column are indicated with different letters (p<0.05)

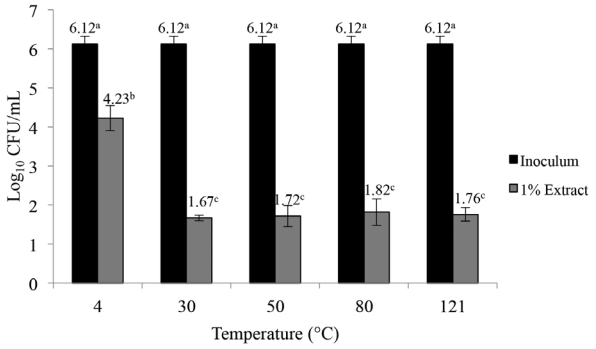
in the 2, 3 and 4 h. Higher reduction in spores was observed after 4 h of incubation compared to the first hour as there is longer contact time for *S. polyanthum* L. extract to act on the spores, and this trend applies to all the different concentration tested. The sporicidal activity *S. polyanthum* L. extract may be due to the distortion of spore coat protein. The binding of the polar group of the spore coats with hydrophilic and hydrophobic groups of *S. polyanthum* L. extract may cause the distortion of spore coat and initiates the spore germination process. The germinated spores are easier to be killed and inactivated by *S. polyanthum* L. extract (Rukayadi & Hwang 2007). Glutaraldehyde was used as positive control in the determination of sporicidal activity. Significant reduction in the number of *B. cereus* spores was observed after being treated with 0.05% glutaraldehyde and there was more than 3-$\log_{10}$ reduction (99.99%) at 0.25% after 1 h of incubation. Glutaraldehyde at 1.00% concentration was able to completely kill the *B. cereus* spores after 1 h of incubation. The sporicidal activity of glutaraldehyde was found to be more efficient against *B. cereus* spores compared to *S. polyanthum* L. extract. From the results obtained, 1.00% glutaraldehyde was able to completely stop the *Bacillus* spores tested from germinating, while S. *polyanthum* L. extract require 2.50%. The lower effectiveness of *S. polyanthum* L. extract compared to glutaraldehyde may be due to some of the phytochemical compounds presence in the glycosidic form and the presence of sugar, can decrease its susceptibility (Negi 2012). Although glutaraldehyde is more effective sporicidal agent compared to S. *polyanthum* L. extract, glutaraldehyde is not suited to be used as food preservation due to its pungent odour and health effects when it comes in contact (Ballantyne & Jordan 2001). Glutaraldehyde is also well known for its toxicity and requires extra precautions for use (Kida et al. 2004).

Effect of temperature on the sporicidal activity of Syzygium polyanthum *L. extract* Heat treatment was applied to *Syzygium polyanthum* L. extract at 50, 80 and 121°C for 15 min as well as kept in the chiller at 4°C and room temperature of 30°C prior treated the *B. cereus* spores. The effect of different temperatures on sporicidal activity of 1% (w/v) *S. polyanthum* L. extract against *B. cereus* ATCC 33019 and *B. cereus* BC-NP.8, *B. subtilis* spores after being treated for 1 h, are shown in Figure 2(a) and 2(b), respectively. There was significant reduction in the *B. cereus* ATCC 33019 and *B. cereus* BC-NP.8 after being treated with the heat treated extract. However, higher survival of spores was observed after being treated with the extract that kept in 4°C. The low temperature may cause the solution to be crystallize and may need longer time to be completely thawed and rendered effective. The *S. polyanthum* L. extract was still able to exhibit sporicidal activity against the tested *B. cereus* spores even after heated to 50, 80 and 121°C. Thus, *S. polyanthum* L. extract was heat stable and the effect of temperature on its sporicidal activity was not significant. These results also proved that the heating at 85°C for 2 × 30 s in the extraction process does not affect the antibacterial and anti-spore activity of the extract. This result shows that the extract could be applied in in high temperature condition. However, it is recommended that the extract was completely thawed to give higher efficacy of the sporicidal effect.



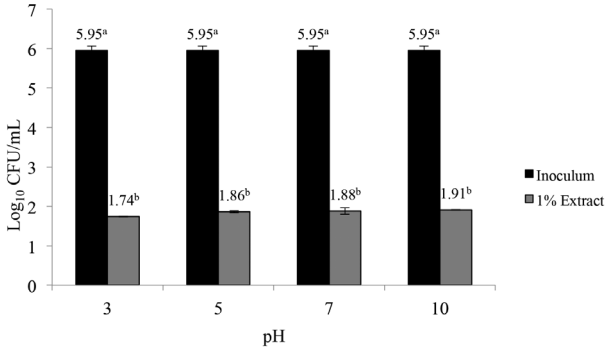
*The results are presented as the mean ± standard deviation. Significant differences in means (n=3×3) are indicated with different letters (p<0.05)

FIGURE 2(a). Effect of temperature on the sporicidal activity of *Syzygium polyanthum* L. extract against spores of *Bacillus cereus* ATCC 33019



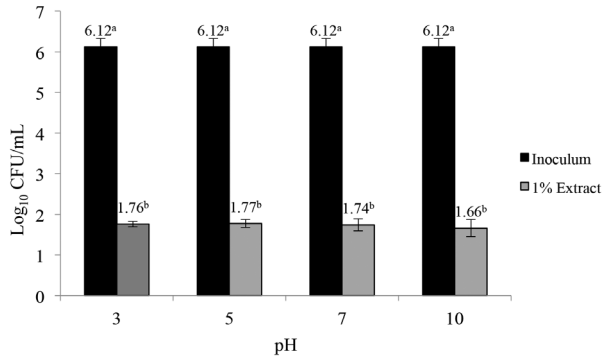
*The results are presented as the mean ± standard deviation. Significant differences in means (n=3×3) are indicated with different letters (p<0.05)

FIGURE 2(b). Effect of temperature on the sporicidal activity of *Syzygium polyanthum* L. extract against spores of *Bacillus cereus* BC-NP.8



*The results are presented as the mean ± standard deviation. Significant differences in means (n=3×3) are indicated with different letters (p<0.05)

FIGURE 3(a). Effect of pH on the sporicidal activity of *Syzygium polyanthum* L. extract against spores of *Bacillus cereus* ATCC 33019



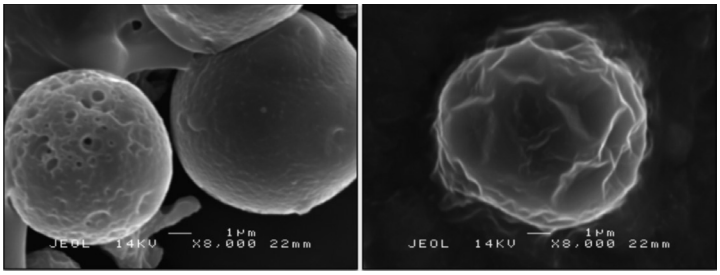
*The results are presented as the mean ± standard deviation. Significant differences in means (n=3×3) are indicated with different letters (p<0.05)

FIGURE 3(b). Effect of pH on the sporicidal activity of *Syzygium polyanthum* L. extract against spores of *Bacillus cereus* BC-NP.8

Effect of ph on the sporicidal activity of Syzygium polyanthum *L. extract* The effect of different pH on sporicidal activity of 1% (w/v) *Syzygium polyanthum* L. extract against *B. cereus* ATCC 33019 and *B. cereus* BC-NP.8 are shown in Figure 3(a) and 3(b). Significant reductions of more than 3-$\log_{10}$ reduction in the *B. cereus* spores tested were observed after being treated with 1% (w/v) *S. polyanthum* L. extract at pH3, 5, 7 and 10. The extract with their pH altered and the control (pH5) were all able reduce the number of *B. cereus* ATCC 33019 and *B. cereus* BC-NP.8 spores significantly. Thus, it shows that the *S. polyanthum* L. extract was pH stable and there was no significant effect on the sporicidal activity. Since *S. polyanthum* L. extract is stable at different pH condition, therefore, further researches should be focused on the use of extract in combination with current treatments. In addition, fast acting killing of an antimicrobial agent against vegetative cells of spore forming bacteria is very important (Rutala & Weber 1999). Generally, food manufacturers rely on preservation by moist heat to produce food products stable under ambient conditions. However, if heat activation of germination of the spores were to occur in this process, subsequent outgrowth might result in food spoilage and food poisoning during preservation (Ciarciaglini et al. 2000). Therefore, it is important to evaluate activity against spore-forming bacteria to determine whether antimicrobial compounds inhibit spore germination or vegetative growth.

SCANNING ELECTRON MICROSCOPY (SEM)

The effect of *Syzygium polyanthum* L. extract on the morphological structure of the *Bacillus* spores was observed using scanning electron microscopy. Figure 4(a) shows the structure of untreated *B. cereus* ATCC 33019 spores, while Figure 4(b) shows the *B. cereus* ATCC 33019 spores being treated with 1% (w/v) *S. polyanthum* L. extract for 1 h, under the magnification of 8000×. The untreated spores showed normal spherical shape and distorted surface. On contratry, surfaces morphology of *B. cereus* spores was effected by *S. polyanthum* L. extract and was clearly observed in Figure 4(b). Although the underlying molecular changes that cause the inactivation of the *B. cereus* spores could not be identified by scanning electron microscope, the partially disrupted surfaces and deformed shapes were believed to be responsible. The distorted structure of the spores is probably due to the reaction between the polar group on the spores coat with the hydrophilic and hydrophobic groups of the various compounds in the extract. The degradation of spore coat and cortex remove the physical restriction to spore core expansion, allowing full core hydration and thus, activating the enzyme activity as well as initiation of metabolism, macromolecular synthesis, and spore outgrowth. Germinated spores are more susceptible to the sporicidal activity of *S. polyanthum* L. extract. Analysis of electron micrograph at the longer exposure time and higher concentrations may result in clearer observation regarding the disruption of cell as shown in Figure 4(a) and 4(b).



(a) (b)

FIGURE 4. Scanning electron micrographs of *Bacillus cereus* ATCC 33019 spores (a) untreated and (b) being treated with 1% (w/v) *Syzygium polyanthum* L. extract for 1 h

CONCLUSION

In conclusion, *Syzygium polyanthum* L. leaves, a commonly used spice in culinary, is a potential candidate for natural food preservative due to its potential anti-Bacillus activity. *S. polyanthum* L. extract was effective to act against vegetative cells and also possessed sporicidal activity against spores of the tested *B. cereus*. This new findings are important in controlling spore-forming bacteria and their contamination in food, such as the *B. cereus*. This novel antibacterial and sporicidal agent from natural source might be safer to be utilized as compared to the chemical agents, besides fulfilling the current demands of the industry and consumer for natural antibacterial agent. For recommendations, it is suggested that *S. polyanthum* L. extract may also be further developed and applied to food to control food spoilage due to the germination and growth of *B. cereus*.

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