

## Original Research Article

### Antibacterial and sporicidal activity of *Eugenia polyantha* Wight against *Bacillus cereus* and *Bacillus subtilis*

K.Y.Lau<sup>1</sup>, N.S.Zainin<sup>1</sup>, F.Abas<sup>1,2</sup> and Y.Rukayadi<sup>1,2\*</sup>

<sup>1</sup>Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

<sup>2</sup>Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

\*Corresponding author

#### A B S T R A C T

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The spore-forming bacteria, *Bacillus* spp., have often been associated with the contamination of rice and other starchy products. In this study, the extract of Indonesian bay leaf (*Eugenia polyantha*) was assessed for its antibacterial and sporicidal activities against vegetative cells and spores of *Bacillus cereus* ATCC33019 as well as *Bacillus subtilis* ATCC6633. The Minimum Inhibitory Concentration of *E. polyantha* extract against vegetative cells of *B. cereus* and *B. subtilis* were 0.31 and 0.63 mg/mL, respectively, while both their Minimum Bactericidal Concentration were 2.50 mg/mL. Time-kill curves demonstrated that the bactericidal endpoint for *B. cereus* and *B. subtilis* was reached at concentration of 2.50 and 5.00 mg/mL, respectively (8× MIC) after 4 h of incubation. *E. polyantha* extract inactivated more than 3-log (99.99%) of *B. cereus* and *B. subtilis* spores at a concentration of 10.00 mg/mL (1.0%) after 1 h of incubation and the spores was completely killed at 25.00 mg/mL (2.5%). The sporicidal activity of *E. polyantha* extract was not affected by different temperatures treatment and alteration of pH. In conclusion, the potential anti-*Bacillus* activities of *E. polyantha* extract might support its use as a natural food preservative.

## Introduction

Bacterial contamination remains a problem to many including the food industries, as well as medical and pharmaceutical sectors. The application of heat, chemical, and UV irradiation to reduce microbial loads may compromise the organoleptic qualities of food which are important to consumers (Russell, 1990). In addition, some bacteria

can produce spores, which are more difficult to eradicate compared to its vegetative cells, due to their ability to withstand adverse environmental stress, such as heat and chemical treatments (Fernández-No *et al.*, 2011). Food spoilage and foodborne diseases happen when the spores germinate into vegetative cells under favourable

conditions (Barker *et al.*, 2005). Moreover, spores can also spread from one food to another through cross-contamination (Stenfors anersen *et al.*, 2008).

Spore-forming bacteria, such as *Bacillus cereus* and *B. subtilis*, are ubiquitous in nature, and hence occur frequently in a wide range of food raw materials (Van Opstal *et al.*, 2004). Foods that are commonly associated with *B. cereus* and *B. subtilis* contamination include starchy food, milk, vegetables and fruits. Cooked rice will become slimy after being kept for a period of time due to spore formation. Ingestion of food containing spores can cause gastrointestinal disorder and leading to bacterial food poisoning. Therefore, the control of bacterial spores is desired especially in food products.

Glutaraldehyde, a synthetic sporicidal agent, was used commonly used to eradicate bacterial endospores effectively. However, due to its toxic nature, it requires safety precautions and not allowed in food application (Kida *et al.*, 2004). In addition, higher concentrations and exposure times are required to eradicate spores compared to the vegetative cells (Russell, 1990). Thus, the development of natural preservatives derived from plant sources is gaining more attention nowadays. Many plant-derived antimicrobial compounds have been found to have activity against foodborne bacteria and thus, they could be used as natural preservatives in foods (Cho *et al.*, 2008; Smith-Palmers *et al.*, 1998).

*Eugenia polyantha*, a deciduous tropical tree belonging to the Myrtaceae family, is also known as Indonesian bay leaf or *orsalam* (Kato *et al.*, 2013; Sumono and Wulan, 2008). *E. polyantha* leaf extract was proven to possess antibacterial activity against *Staphylococcus aureus* (Grosvenor *et al.*, 1995) and

*Streptococcus mutans* (Sumono and Wulan, 2008) as well as antifungal activities against spoilage fungi *Euroticum* sp., *Aspergillus* sp. and *Penicillium* sp. (Guynot *et al.*, 2005). This prompted us to further investigate the antibacterial and sporicidal activity of *E. polyantha* leaf extract against *B. cereus* and *B. subtilis*. The aim of this study was to determine the antibacterial and sporicidal activity of *E. polyantha* leaf extract against *B. cereus* and *B. subtilis*.

## Materials and Methods

### Bacterial strain and spores preparation

*Bacillus cereus* ATCC33019 and *Bacillus subtilis* ATCC6633 were obtained from the American Type Culture Collection (Rockville, MD, USA). Both *B. cereus* and *B. subtilis* were cultured, grown and maintained statically in nutrient broth (NB; Difco, Sparks, MD, USA) or NB supplemented with 1.5% (w/v) agar (NA). *B. cereus* and *B. subtilis* spores were prepared according to the method described previously by Kida *et al.* (2003) and Rukayadi *et al.* (2009), with modification. *B. cereus* and *B. subtilis* were grown on NA at 30°C for over 1 week.

The spores and vegetative cells were harvested and suspended in sterile 0.85% NaCl solution. Heat shock at 65°C was applied to the suspension for 30 min to kill vegetative cells. Spores were harvested by centrifugation and washed four times with the original volume of sterile 0.85% NaCl solution by centrifugation (13,000 × g for 30 min at 4°C). A 1 mL portion of the spore suspension containing approximately 10<sup>8</sup> spores/mL was stored in a 1.5 mL plastic cryopreservation tube at -18°C until further use.

### **Sample collection and extraction**

*E. polyantha* leaf was purchased from Herbal Market, Pasar Baru, Bandung, Indonesia. The sample was identified and deposited in Laboratory of Natural Products, Institute of Bioscience (IBS), Universiti Putra Malaysia. One hundred gram of dried *E. polyantha* leaf was ground and extracted with 400 mL absolute methanol for seven days at room temperature as stated by Rukayadi *et al.* (2008), with some modification. The mixture was then filtered using Whatman No. 2 filter paper and concentrated by using rotary evaporator at 50°C and speed of 150 rpm. The extract was stored at 4°C prior to use.

### **Extract and glutaraldehyde preparation**

The crude extract was first dissolved in 100% dimethylsulfoxide (DMSO, Gibco) to obtain 100 mg/mL (10%) and the solution was further diluted in 1:10 (v/v) distilled water to obtain 10 mg/mL (1%) stock solution. The final concentration of DMSO was 10% in the stock solution and it was found not to kill *B. cereus* and *B. subtilis* tested in this research, as supported by Rukayadi *et al.* (2008). A standard 25% commercially available glutaraldehyde solution (Merck, Darmstadt, Germany) was diluted 1:25 in distilled water to yield 1% concentration and used as positive control for sporicidal activity experiments.

### **Antimicrobial bioassay**

*E. polyantha* extract was tested for antimicrobial activity using the disc diffusion method as described by Clinical and Laboratory Standards Institute (CLSI, 2003). The *B. cereus* and *B. subtilis* were streaked on Mueller Hinton agar (MHA, Difco, Sparks, MD, USA) 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) *E. polyantha* extract was loaded on the paper discs. 0.1 mg/mL of chlorhexidine (CHX) was used as positive control in the assay. The plates were incubated at 30°C for 24 h. Evidence of clear zone indicates bacterial growth inhibition and the diameter were measured in mm.

### **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 (2003). The MICs and MBCs of *E. polyantha* extract against the vegetative cells of *B. cereus* and *B. subtilis* were performed in a 96-well microtiter plate using two fold standard broth microdilution method with an inoculum of approximately 10<sup>7</sup> CFU/mL. Briefly, a 100 µL of *E. polyantha* extract stock solution (10 mg/mL) was mixed and diluted two-folds with the test organism in 100 µL of Mueller Hinton broth (MHB, Difco, Sparks, MD, USA). Column 12 of the microtiter plate contained the highest concentration of the extract, while column 3 contained the lowest concentration. The first column served as negative growth control (only MHB, no inoculum and antibacterial agent) while the second column is the positive growth 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 for each bacterial species as outlined for MIC by removing the media from each well showing no visible growth and sub-culturing 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).

### **Determination of time-kill curve**

Time-kill assay was done on the vegetative cells of *B. cereus* and *B. subtilis* according to CLSI (2003) reference method, with slight modification. Briefly, the inoculum suspension of *B. cereus* and *B. subtilis* was approximately  $10^6$  CFU/mL. The *E. polyantha* 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 CFU/mL versus time was plotted.

### **Sporicidal activity of *E. polyantha* extract**

Sporicidal activity was determined basically as described previously (Kida *et al.*, 2003, 2004; Palhano *et al.*, 2004; Rukayadi *et al.*, 2009) with modification. Briefly, prepared spores suspension ( $10^8$  spores/mL) was thawed and diluted 1:100 in 0.85% NaCl solution (pH 6.6), yielding an adjusted spores suspension of  $10^6$  spores/mL. The stock extract (10%) were diluted 1:10 in adjusted spores suspension of  $10^6$  spores/mL, resulting final concentrations of extract (0.00, 0.05, 0.25, 0.50, 1.00, 2.50 and 5.00 %) and an initial *B. cereus* and *B.*

*subtilis* spore suspension of  $9 \times 10^5$  spores/mL and  $2 \times 10^6$  spores/mL, respectively. One mL of each concentration was then exposed to different incubation times (0, 1, 2, 3 and 4 h) in a water bath (30°C). A 100 µL aliquot was removed and transferred to microcentrifuge tubes, centrifuged ( $12,000 \times g$  at 4°C for 5 min) and rinsed twice with 0.9 mL of 0.85% NaCl solution (pH 6.6) to obtain bacterial-free spores and to avoid effect of vegetative cells residue. Pellets were suspended in 100 µL of 0.85% NaCl solution (pH 6.6) and serially diluted. An appropriate volume (100 µL, 40 µL, 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 duplicate plates were counted and the mean of colony-forming unit (CFU/mL) was calculated.

### **Effect of temperature and pH on the sporicidal activity of *E. polyantha* extract**

The sporicidal activity of *E. polyantha* extract was determined as mentioned previously. The methanolic extract was dissolved in 10% aqueous DMSO to obtain 20 mg/mL. Different temperature treatments of 4, 30, 50, 80 and 121°C for 15 minutes were applied to the extract. The original pH of *E. polyantha* extract was 5 and the pH was altered to pH 3, 7 and 10 using hydrochloric acid (Merck Millipore, Darmstadt, Germany) and sodium hydroxide (Sigma Aldrich, Missouri, United States) diluted to 0.1 M. The treated extracts were then tested for their sporicidal activity against the spores of *B. cereus* and *B. subtilis*. The extract (2%) was diluted in adjusted spores suspension, resulting in final concentrations of extract of 1%. The test solutions were then exposed to incubation at 30°C for 1 h.

## Results and Discussion

### Antibacterial Activity of *E. polyantha* Extract against Vegetative Cells of *B. cereus* and *B. subtilis*

The antibacterial activity of *E. polyantha* extract was screened against vegetative cells of *B. cereus* and *B. subtilis* using the disc diffusion method. Visible clear zone indicates inhibition of bacterial growth. The diameter of inhibition zone against *B. cereus* and *B. subtilis* were 8.0 and 7.5 mm, respectively. Screening served as a preliminary check for antibacterial activity (Burt, 2004); however, the antibacterial activity of plant extract may be more accurately evaluated using MIC values (Jun *et al.*, 2013). The MIC of *E. polyantha* extract against *B. cereus* and *B. subtilis* was 0.31 and 0.63 mg/mL, respectively, while both their MBC was 2.50 mg/mL (Table 1). The MIC was defined as the lowest concentration of antimicrobial agent that resulted in the complete inhibition of visible growth, while MBC is the corresponding concentrations required to kill the microorganisms completely (Rukayadi *et al.*, 2009).

Time-kill curves were determined to assess the correlation between MIC and bactericidal activity of *E. polyantha* extract at concentrations ranging from 0 MIC to 8× MIC. The bactericidal endpoint for *B. cereus* and *B. subtilis* (Figure 1) were reached after 4 h of incubation at 2.50 and 5.00 mg/mL (8 × MIC), respectively. These data demonstrated that the bactericidal ability of *E. polyantha* extract is dependent on the concentration and bacterial species. The antibacterial activity is most likely due to the adsorption of phytochemical constituents causing membrane disruption, subsequent leakage of cellular contents and cell death (Negi, 2012). The antibacterial activity of *E.*

*polyantha* extract against *B. cereus* and *B. subtilis* is an additional benefit, besides the ability of *E. polyantha* solution in reducing the numbers of *Streptococcus* spp., the oral pathogens in dentistry, as reported by Sumono and Wulan (2008). In addition, Setiawan (2002) also reported that salam leaves are able to inhibit the growth of *Salmonella* spp., *B. cereus*, *B. subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas fluorescens*. This effect was reported to be due to the tannin, flavonoid and essential oil content in the plant (Sumono and Wulan, 2008).

### Sporicidal Activity of *E. polyantha* Extract against Spores of *B. cereus* and *B. subtilis*

The sporicidal activity of *E. polyantha* extract was tested at different concentrations of 0.05, 0.25, 0.50, 1.00, 2.50 and 5.00% for 1, 2, 3, and 4 h. Glutaraldehyde was reported to have sporicidal activity against spore-forming bacteria and used as positive control in this study, even though it is not allowed in food application (Russell, 1990).

The reduction in the viability of *B. cereus* and *B. subtilis* spores at different concentrations and incubation time were presented in Figure 2. The initial inoculum of *B. cereus* and *B. subtilis* spores was  $9 \times 10^5$  spores/mL and  $2 \times 10^6$  spores/mL, respectively. *E. polyantha* extract at 1% concentration showed potential sporicidal activity with a sharp reduction in the number of *B. cereus* and *B. subtilis* spores for more than 3 log units (99.99%). However, the complete killing of *B. cereus* and *B. subtilis* spores was achieved after treated with *E. polyantha* extract at 2.50% concentration for 1 h of incubation. The different incubation times of 0, 1, 2, 3 and 4 h were selected to observe the optimal reduction time. Significant reduction was observed for both *B. cereus*

and *B. subtilis* spores after 1 h of incubation. The effects of different temperatures and pH on sporicidal activity of 1% *E. polyantha* extract against *B. cereus* and *B. subtilis* spores at 1 h of incubation were depicted in Figure 3 and 4, respectively. *E. polyantha* extract exposed to temperature of 4, 30, 50, 80 and 121°C for 15 mins was used to treat the *B. cereus* and *B. subtilis* spores. Significant reduction of more than 3-log reduction in the *B. cereus* and *B. subtilis* spores after treated with the 1% *E. polyantha* extract. Thus, *E. polyantha* extract was heat stable and the effect of temperature on the sporicidal activity was not significant. This is important that the extract could be applied in addition to heat treatment in order to enhance the sporicidal effects on food products. The original pH of *E. polyantha* extract was 5. The extract with pH 3, 5, 7 and 10 was used to treat the *B. cereus* and *B. subtilis* spores. Significant reductions of more than 3-log reduction in the *B. cereus* and *B. subtilis* spores were observed after treated with 1% *E. polyantha* extract at all tested pH (Figure 4). Thus, it shows that the *E. polyantha* extract was pH insensitive and there was no significant effect on the sporicidal activity.

To best our knowledge, report of sporicidal agents isolated from plants is still rare. Tassou *et al.* (1991) reported that oleuropein purified from olive extract inhibited both the germination and the subsequent outgrowth of spores of *B. cereus*.

In addition, macelignan isolated from nutmeg exhibit inhibition activity towards the growth of vegetative cells and sporicidal activity against spores of *B. cereus* (Rukayadi *et al.*, 2009). *Torilis japonica* extract showed activity against spores of *B. subtilis* (Cho *et al.*, 2008), while the essential oils of cardamom, tea tree, and juniper leaf was also found to be effective

against spores of *B. subtilis* (Lawrence and Palombo, 2009). On the contrary, lichocalcone A isolated from the roots of licorice (*Glycyrrhiza inflata*) has antibacterial activity against vegetative cells of *B. subtilis*, but did not inhibit the germination *B. subtilis* spores (Tsukiyama *et al.*, 2002). In reality, simple comparisons are difficult because of differences in tested bacteria and the concentrations used. In this study, Indonesian bay leaf extract was found to exhibit inhibition activity to the growth of *B. cereus* and *B. subtilis* spores.

Indonesian bay leaves (*Eugenia polyantha* Wight), synonym to *Syzygium polyanthum*, are commonly used as spice in culinary due to its aromatic smell, as well as sour and astringent taste. In Surinam, the dried brown leaves of *E. polyantha* was applied to meat, while in Indonesia, it was used widely in the cooking of rice (*nasili wet*). *E. polyantha* leaf was found to contain essential oils such as simple phenols, phenolic acids, and lactones sekuisterferenoid, triterpenoids, saponins, flavonoids, and tannins (Davidson and Branen, 1993).

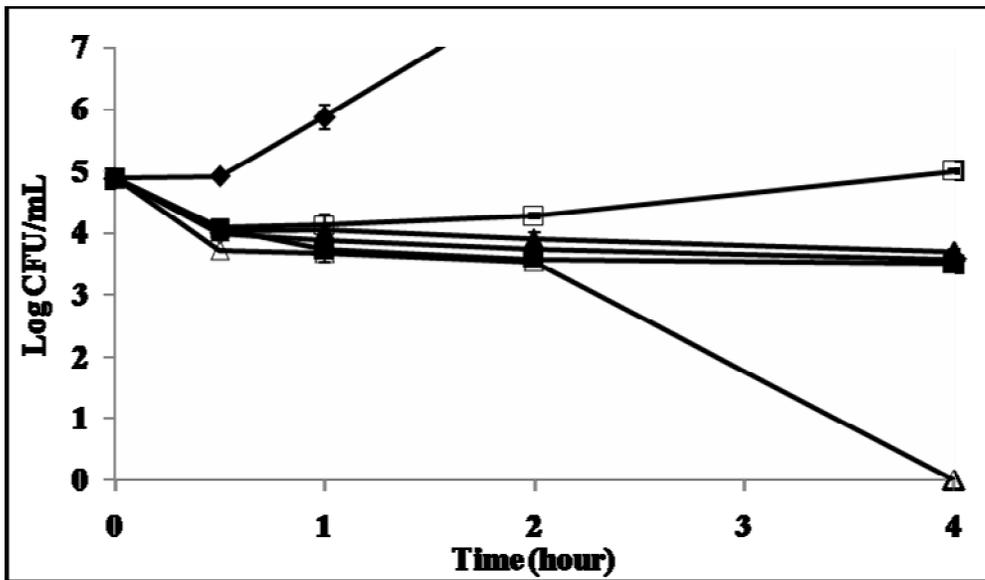
In addition, the *E. polyantha* leaf had been claimed to possess antimicrobial activities against *Salmonella sp.*, *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas fluorescens* due to the presence of tannin (Setiawan, 2002). *E. polyantha* leaf was used in traditional medicine for the treatment of stomach ulcer, diabetes, diarrhea, cataract, hypercholesterolemia, and skin diseases or inflammation (Ismail *et al.*, 2013; Kato *et al.*, 2013). The crude ethanolic extracts of the leaves and fruits of *S. polyanthum* contain terpenoids, phenols, tannins, flavonoids, and alkaloids (Ismail *et al.*, 2013). The antimicrobial properties of medicinal plants are related to the phytochemical components present.

**Table 1.**Diameter of inhibition zone (mm), MIC (mg/mL) and MBC (mg/mL)

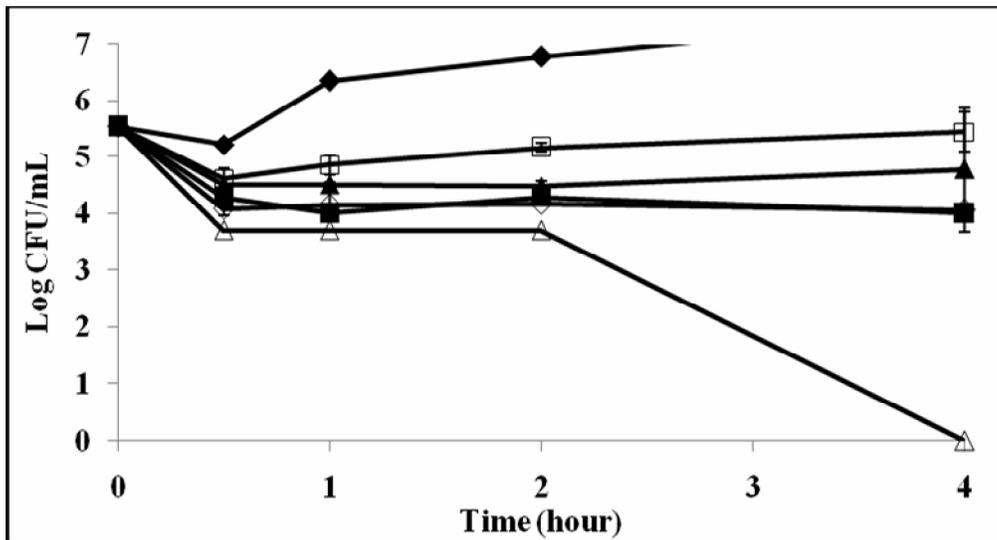
Bacteria	Diameter of inhibition zone (mm)	MIC (mg/mL)	MBC (mg/mL)
<i>Bacillus cereus</i>	8.0	0.31	2.50
<i>Bacillus subtilis</i>	7.5	0.63	2.50

**Figure.1** Time–kill plots for (a) *B. cereus* and (b) *B. subtilis* following exposure to *E. polyantha* extract at 0× MIC (filled diamond), 0.5× MIC (open squares), 1× MIC (filled triangles), 2× MIC (open diamond), 4× MIC (filled squares), and 8× MIC (open triangles) after endpoint (4 h).

(a)

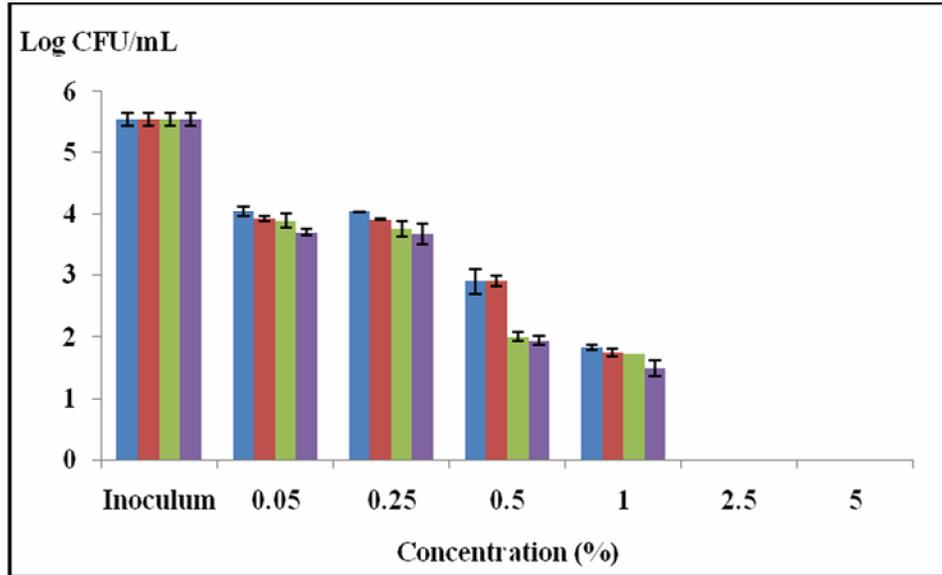


(b)

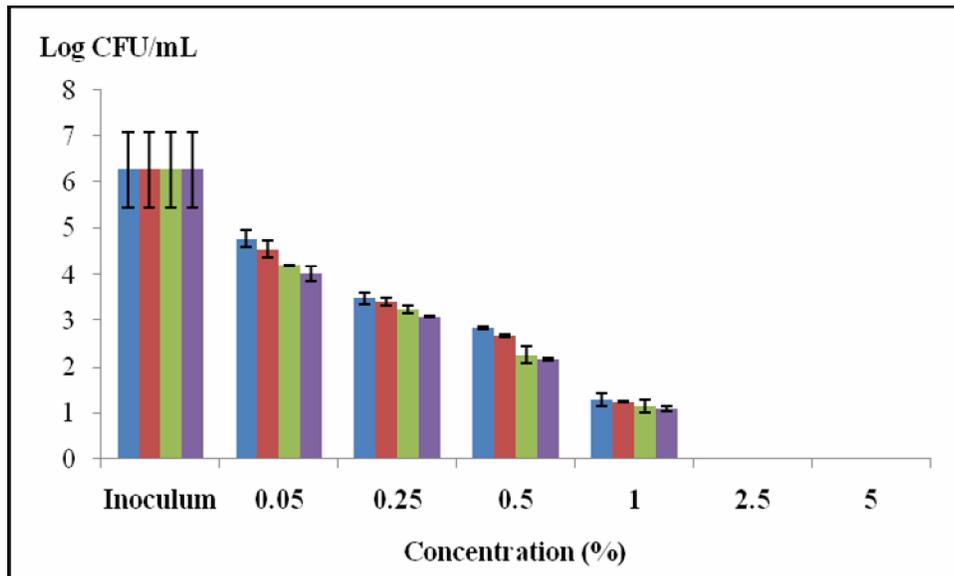


**Figure.2** Sporicidal activity of *E. polyantha* extracta gainst the spores of (a) *B. cereus* and (b) *B. subtilis* following exposure for 1 h (blue), 2 h (red), 3h (green), and 4 h (purple) at concentration of 0.05, 0.25, 0.50, 1.00, 2.50 and 5.00%.

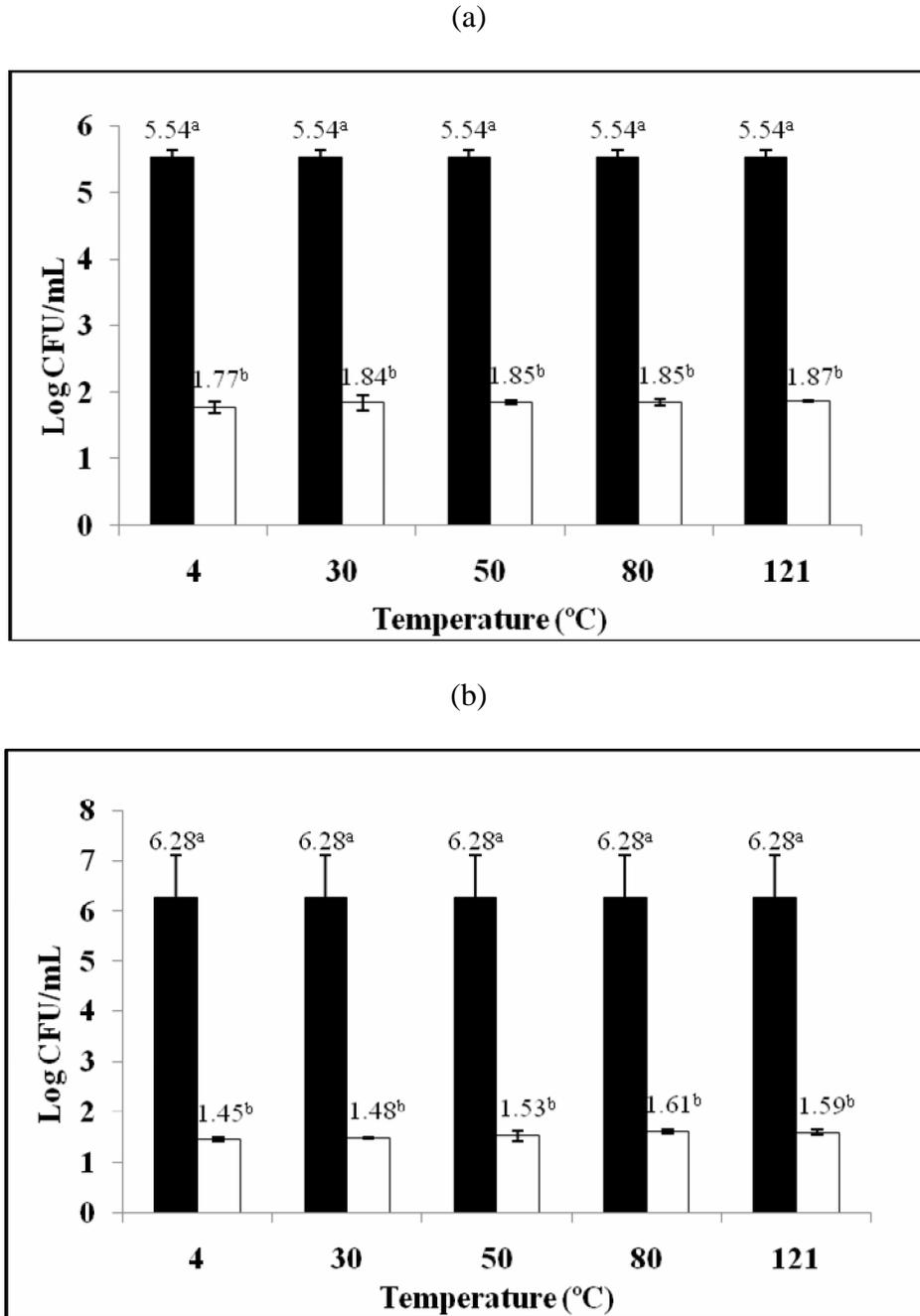
(a)



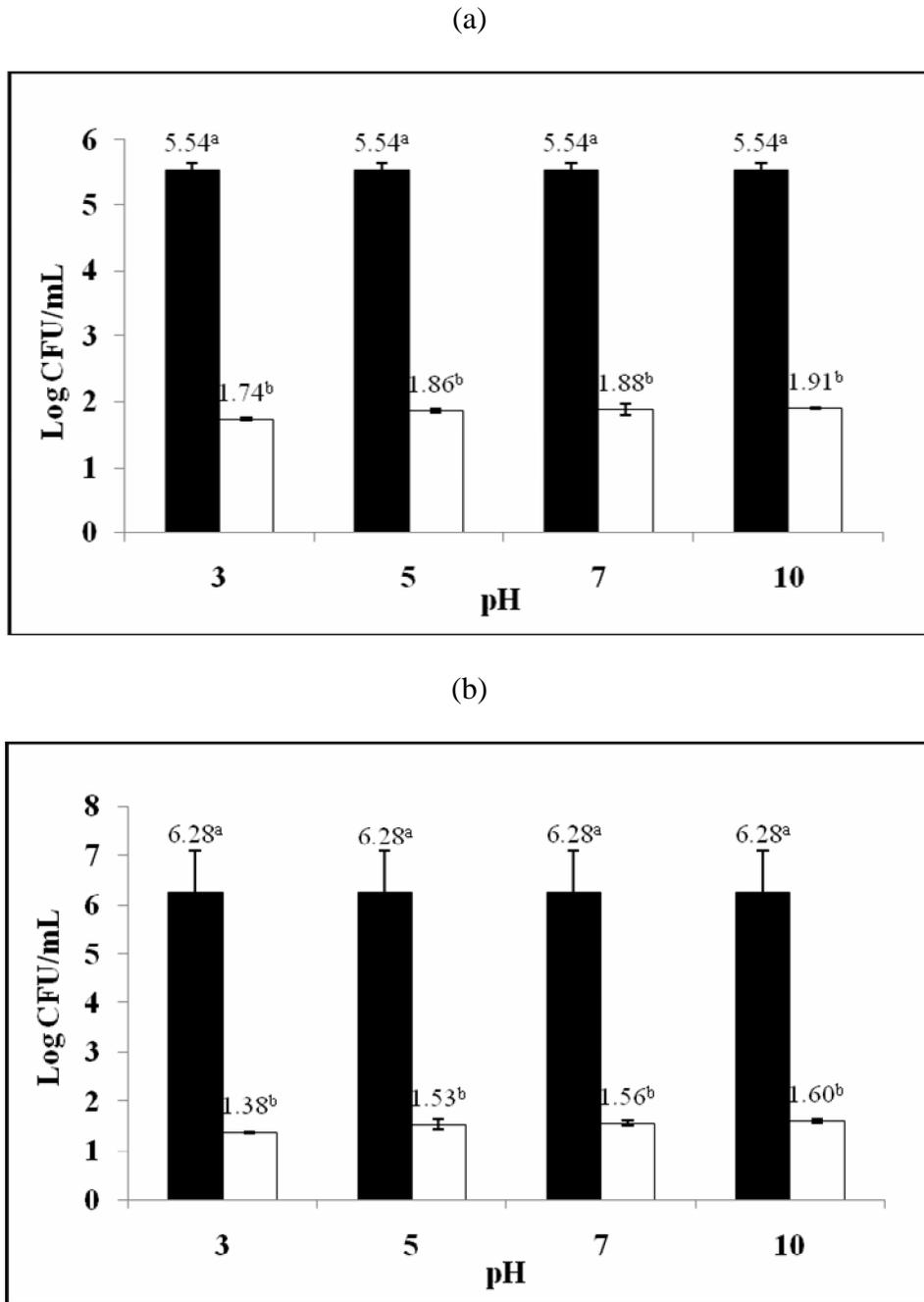
(b)



**Figure.3** Sporicidal activity of 1% *E. polyantha* extract following exposure to temperature of 4, 30, 50, 80 and 121°C for 15 mins against (a) *B. cereus* and (b) *B. subtilis*



**Figure.4** Sporicidal activity of 1% *E. polyantha* extract following exposure to pH of 3, 5, 7 and 10 against (a) *B. cereus* and (b) *B. subtilis*



In this study, the extraction of Indonesian bay leaf was done using absolute methanol. Methanolic extracts show better antimicrobial activities in contrast to

aqueous extract, which may be due to the organic nature of methanol and its ability to dissolve more organic and active antimicrobial compounds (Cowan, 1999).

The high polarity of methanol also attribute to the consistent extraction of different types of sesquiterpenoids (Mohamed *et al.*, 2014). The polar methanol solvent is able to produce higher yield with higher antibacterial and antioxidant activities. Besides that, enzyme in plant tissues does not function in methanol (Hirai, 1986).

In summary, it is remarkable to note that *E. polyantha* extract confers significant antibacterial and sporicidal activity against spore-forming bacteria, *B. cereus* and *B. subtilis*. Thus, *E. polyantha* extract might be good to be developed as a food preservative.

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