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Antibacterial and Sporicidal Activities of Methanolic *Sygygium polyanthum* L. Leaf Extract against Vegetative Cells and Spores of *Bacillus pumilus* and *Bacillus megaterium*

A.M. Abdelgani[1], Y. Rukayadi [1,2]*, K. Shaari[1,3] and I. Safinar[1,3]

[1]Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[2]Faculty of food science and Technology, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.
[3]Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

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The *Sygygium polyantum* L. leaf has been used as spice as well as traditional herbal medicine. The methanolic extracts of *S. polyanthum* L. was tested for antibacterial and sporicidal activities against vegetative cells and spores of *Bacillus pumilus* ATCC14884 and *B. megaterium* ATCC14581. The disc diffusion assay (DDA), minimum inhibition concentration (MIC), minimal bacterial concentration (MBC) and sporicidal activities of the extract on *Bacillus* sp. were analyzed using Clinical and Laboratory Standard Institutes (CLSI) methods. The effect of the extract on spores was visualized using scanning electron microscope (SEM). The results show that the inhibition zone of the extract on *B. pumilus* and *B. megaterium* was 12.16 ± 0.57 mm. The extract has been able to inhibit the growth of *B. pumilus* and *B. megaterium* vegetative cells with MICs of 0.08- and 0.04 mg/ml, respectively. Moreover, the *B. pumilus* and *B. megaterium* can be killed by MBCs of 1.25- and 0.64 mg/ml, respectively. One presence of extract can kill 100% of all *Bacillus* sp. spores during 1 h exposure time. SEM observation showed that the spores were destroyed by the extract. These results suggested that *S. polyanthum* L. extract can be developed as antibacterial and antispore against *Bacillus* sp.

Keywords: Antibacterial activity, *Bacillus pumilus, B. megaterium*, Sporicidal activity, *Sygygium polyanthum* L. extract.

Gram-positive bacteria, such as *Bacillus* and *Clostridium* sp. respond 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 the harsh external conditions, such as nutrient starvation or desiccation, and germinate after the favourable growth conditions returned (Tan and 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). On the other hand, thermal processing is a relatively inexpensive and effective method of producing food safe from undesirable microorganisms and enzymatic reactions. However, the setbacks of thermal processing include reduction in the nutrient content

* To whom all correspondence should be addressed.
Tel: +603-3-8946-8519; Fax: +603-8942-3552;
E-mail: yaya_rukayadi@upm.edu.my

and the organoleptic qualities are affected (Cho *et al.*, 2008). Therefore, the development of effective, safe and stable sporicidal agents is gaining more attentions (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 activity 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 need to develop natural preservatives with potential sporicidal ability or natural sporicidal agent which able to reduce the populations of *Bacillus* spores in rice or starchy foods has prompted the study in determining the sporicidal activity of tropical medicinal plants.

The leaves of *S. polyanthum* L. which is also known as "*daun salam*" in Indonesia are commonly used in dishes as spice in culinary such as "*nasi liwet"* due to its aroma besides the sour taste and also as ingredient in the Indonesian traditional medicine ''*Jamu*'' (Kato *et al.*, 2013). *S. polyanthum* L. is effective against ulcers, hypertension, diabetes, hyperuricemia, diarrheal, gastritis, skin diseases and inflammation (Ismail *et al.*, 2013). In addition to its ability to neutralise residual alcohol, this plant also has diuretic and analgesic effects (Sumono and Wulan, 2008). Research conducted by Sumono and Wulan (2008) reported that the young shoots of *S. polyanthum* L. are consumed as a fresh salad (*ulam*), whereas the mature leaves were regularly added as a flavor enhancer in Malaysian cuisine. Fresh and dried aromatic leaves of *S. polyanthum* L. are useful in cooking for their scent, color and flavor. It is also often used as flavoring spice for meat, fish, and vegetable dishes, or in rice (De Guzman and Siemonsma, 1999). The aim of this study was to evaluate the antibacterial and sporicidal activities of *S. polyanthum* L. against *B. pumilus* and *B. megaterium* vegetative cells and spores.

MATERIALS AND METHODS

Plant materials

Dried leaves of *S. polyanthum* L. was purchased from Herbal Markets (Pasar Baru, Bandung) in Indonesia. The samples were deposited in the Laboratory of Natural Products at Institute of Bioscience (IBS) in the Universiti Putra Malaysia (UPM). Dried leaves were ground using a heavy duty blender (Waring, USA) until fine powder was formed. The powder of *S. polyanthum* L. was kept in a sealed polyethylene plastic bag and stored in a refrigerator (4°C) until required.

Plants extraction preparation

Extracts were prepared using the maceration method as described previously by (Rukayadi *et al*., 2008). The dried leaves were ground to a powder using an electric miller, and 100 g of the powder was mixed with 400 ml of 99.8% (v/v) methanol and incubated at room temperature for 48 hours. The plant extracts were filtered with Whatman ûlter paper size No. 1, and concentrated with a rotary vacuum evaporator at 40°C. Firstly, the crude extract was dissolved in 100% dimethylsulfoxide (DMSO) at a concentration of 100 mg/ml (10%), and the solution was then further diluted by 1:10 (v/v) in distilled water to obtain a 10 mg/ml (1%) stock solution. The final concentration of DMSO was 10% in the stock solution. The 10% of DMSO did not kill the bacteria used.

Bacillus strain and spore preparation

Bacillus pumilus ATCC14884 and *Bacillus megaterium* ATCC14581 were obtained from American Type Collection Culture (Rockville, MD, USA). Both *B. pumilus* 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. pumilus* and *B. megaterium* spores were prepared according to the method described previously by Kida *et al.* (2003) and Rukayadi *et al*. (2009), with modification. *B. pumilus* and *B. megaterium* 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 10^8 spores/ml was stored in a 1.5 ml plastic cryopreservation tube at -18°C until further use.

Disc diffusion assay

The methanolic *S. polyanthum* L. leaves

extract was tested for antimicrobial activity using the disc diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI, 2012). The *B. pumilus* and *B. megaterium* were streaked on Mueller Hinton Agara (MHA) (Difco, Spark, USA) plates with a sterile cotton swab. Sterile filter paper discs of 6 mm diameter were placed on top of the agar, and 20 µl of the 10 mg/ml (w/v). *S. polyanthum* L. extract was loaded onto the paper discs. A 0.1 mg/ml chlorhexidine (CHX) was used as a positive control in the assay. Finally, the plates were incubated at 30°C for 24 hours. The presence of a clear zone indicated bacterial growth inhibition, and its diameter was measured in mm.

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

The determination of MIC and MBC were done according to the methods described by CLSI (2012). The MIC and MBC of *S. polyanthum* L. extract against the vegetative cells of *B. pumilus* and *B. megaterium* were determined in a 96 Wells microtiter plate using two-fold standard broth microdilution methods with an inoculum of approximately 10^6 CFU/ml. Briefly, 100 µl of *S. polyanthum* L. extract stock solution (10 mg/ml = 10,000 µg/ml) was mixed and diluted two-fold with the test organism in Mueller Hinton broth (MBH) (Difco, Spark, USA) (100 µl). Column 12 of the microtiter plate contained the highest concentration of the extract (5,000 µg/ml), while column 3 contained the lowest concentration (19.50 µg/ml). Column 2 served as the positive growth control for all the samples (MHB and inoculum), and column 1 as negative growth control (only MHB, no inoculum and antibacterial agent), respectively.

The micro titer plate was then incubated aerobically at 30°C for 24 hours. The MIC was defined as the lowest concentration of antibacterial agent that resulted in the complete inhibition of visible growth. The MBC was determined for each bacterial species as outlined for MIC, by removing the media from each well showing no visible growth, and further sub-cultured onto MHA plates. The plates were then incubated at 30°C for 24 hours until visible growth was seen in the control plates. Similarly, MBC was defined as the equivalent concentration required killing the microorganism completely. Additionally, both MIC and MBC tests were done in duplicate (n = 3 × 2).

Sporicidal assay

The prepared spores suspension was thawed and diluted 1:100 in 0.85% NaCl solution (pH 6.6), yielding an initial *B. pumilus* and *B. megaterium* spores suspension of each approximately 5.00×10^5 spores/ml. The stock extract (10%) was diluted in adjusted spores suspension, resulting in final concentrations of extract (0.10, 0.50 and 1.00%). 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 exposed to 1 h incubation times in a water bath (30°C). A 100 µl aliquot was removed and transferred to microcentrifuge tubes, centrifuged (12, 000 × g at 4°C for 5 minutes) 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), serially diluted and spread onto NA plates and incubated at 30°C for 24 hours or 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 duplicate per each experiment (n = 3 × 2).

Scanning Electron Microscopy (SEM) Analysis

B. pumilus spores were mixed with 1% (w/v) of methanolic S. polyanthum L. extract then incubated for 1 hour at 30°C. Spores were recovered by centrifugation and pellets were fixed with 4% buffered glutaraldehyde for 6 hours at 4°C, washed with 0.1 M sodium cacodylate buffer for 10 minutes and was repeated for 3 times. The spore pellets was then post fixed with 1% osmium tetroxide for 2 hours at 4°C, washed again with 0.1 M sodium cacodylate buffer for 10 minutes and was repeated for 3 times. Then the pellets were dehydrated using 35, 50, 75 and 95% for 15

minutes each. Finally the pellets were dehydrated using 100% acetone (Merck Millipore, Darmstadt, Germany) for 15 minutes and were repeated for 3 times. Cell suspension was transferred into a specimen basket, made from aluminium foil coated with albumin, and put in critical dryer for 0.5 hour. 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).

Statistical Analysis

Mean of data obtained (n = 3 × 2) were calculated using Microsoft Excel 2010 for Windows. Data were then analysed using the Analysis of Variance (One-way ANOVA) procedure of the Minitab ® Version 16. 2. 4 for Windows (Minitab Inc.). When significance was indicated, means were separated using Tukey test (±=0.05).

RESULTS AND DISCUSSIONS

The antimicrobial properties of plants have been recognized for a long time. In many cases, however, mechanisms are not well understood (García-alvarado *et al*., 2008). In this study, the results showed the potency of *S. polyanthum* L. extracts to inhibit and kill the vegetative cells of *B. pumilus* and *B. megaterium* as well as to kill the their spores. Table 1 shows the results of disc diffusion assay of *S. polyanthum* L. on vegetative cells of *B. pumilus* and *B. megaterium*. Visible clear zone indicates inhibition of bacterial growth. The diameter of inhibition zone against *B. pumilus* and *B. megaterium* was 12.66 ± 0.57 mm. Lau *et al*. (2014) reported that methanolic extract of *S. polyanthum* L. leaves can inhibit the growth of *B. cereus* and *B. subtilis* with visible clear zone of 8.0 and 7.5 mm, respectively. The results suggested that *S. polyanthum* L. extract was more susceptible on *B. pumilus* and *B. megaterium*. Moreover, Prabhakaran *et al*. (2011) reported that ethanol and water root and bark extracts of *S. cumini* inhibited bacterial growth of *B. pumilus*, *B. cereus*, *B. megaterium* and *B. subtilis* with inhibition zones ranged between 11mm and 14 mm.

Disc diffusion method is just semi qualitative method as a preliminary check for antibacterial activity (Burt, 2004); however, the antibacterial activity of plant extract may be more accurately evaluated using minimum inhibitory

Table 1. Inhibition zone diameter of *S. polyanthum* L. extracts against *B. pumilus* and *B. megaterium*

Bacterial species	Inhibition zone diameter (mm)
Bacillus pumilus ATCC14884	12.16 ± 0.57
Positive control (0.05% Chlorhexidine)	12.00 ± 0.00
Negative control (10% DMSO)	na
Bacillus megaterium ATCC14581	12.16 ± 0.57
Positive control (0.05% Chlorhexidine)	11.50 ± 0.05
Negative control (10% DMSO)	na

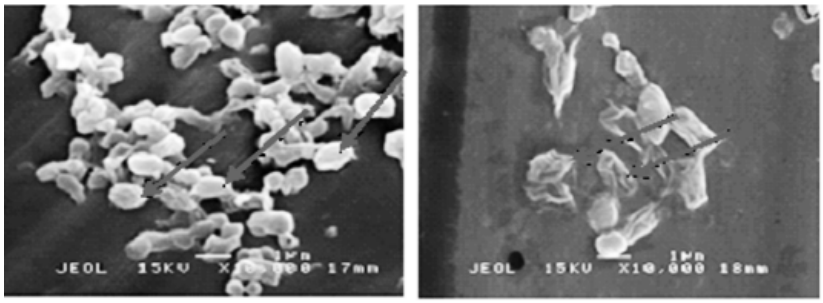
na: No inhibition zone

Table 2. MIC and MBC values of *S. polyanthum* L. extracts against *B. pumilus* and *B. megaterium*

Tested bacteria	MIC (mg/ml)	MBC (mg/ml)
Bacillus pumilus ATCC14884	0.08	1.25
Bacillus megaterium ATCC14581	0.04	0.63

concentration (MIC) and minimum bactericidal concentration (MBC) values (Jun *et al*., 2013). The MICs and MBs values of *S. polyanthum* L. extract against *B. pumilus* and *B. megaterium* is presented in Table 2. 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). Meaning that 0.08- and 0.04 mg/ml of extract can inhibit the growth of *B. pumilus* and *B. megaterium* vegetative cells, respectively. The extract can kill completely the vegetative cells of *B. pumilus* and *B. megaterium* with concentration of extract of 1.25- and 0.63 mg/ml. Lau *et al*. (2014) reported that the methanolic *S. polyanthum* L. extract the growth vegetative cells of *B. cereus* ATCC33019 and *B. subtilis* ACTT6633 with MIC of 0.31- and 0.63 mg/ml, respectively. Moreover, those bacteria can be killed by the extract with concentration of 2.50 mg/ml. These results were also support that the methanolic *S. polyanthum* L. extract was more powerful to inhibit and to kill *B. pumilus* and *B. megaterium*.

The sporicidal activity of *S. polyanthum* L. extract was tested at different concentrations of 0.1, 0.5 and 1.0% for 1 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. pumilus* and *B. megaterium* spores at different concentrations for 1 h of incubation were presented in Table 3. The initial inoculum of *B. pumilus* and *B. megaterium* spores was approximately 5×10^5 spores/ml. The *S. polyanthum* L. extract at 0.5% concentration showed potential sporicidal activity with a sharp reduction in the number of *B. pumilus* and *B. megaterium* spores for more than 3 Log units (99.99%). However, the complete killing of *B. pumilus* and *B. megaterium* spores was achieved after treated with *S. polyanthum* L. extract at 1% concentration for 1 h of incubation.



(a) (b)

Fig. 1. Effect of methanolic *S. polyanthum* L. extract on *Bacillus pumilus* ATCC14884; (a) no extract, (b) with 1% extract for 1 h

Table 3. Sporicidal activity of *S. polyanthum* L. against spores of *B. pumilus* and *B. megaterium*

Concentration of extract or glutaraldehyde (%)	CFU/ml of spores / glutaraldehyde or *S. polyanthum* L. extract / bacterial strain			
	Glutaraldehyde		*S. polyanthum* L. extract	
	B. pumilus ATCC14884	*B. megaterium* ATCC14581	*B. pumilus* ATCC14884	*B. megaterium* ATCC14581
0.0	$^{a}5.53 \pm 0.05$	$^{a}5.48 \pm 0.11$	$^{a}5.31 \pm 0.05$	$^{a}5.31 \pm 0.05$
0.1	$^{c}3.10 \pm 0.07$	$^{b}4.19 \pm 0.07$	$^{b}4.12 \pm 0.07$	$^{b}4.12 \pm 0.07$
0.5	$^{d}0.00 \pm 0.00$	$^{c}3.78 \pm 0.08$	$^{c}3.75 \pm 0.05$	$^{c}3.75 \pm 0.05$
1.0	$^{d}0.00 \pm 0.00$	$^{d}0.00 \pm 0.00$	$^{d}0.00 \pm 0.00$	$^{d}0.00 \pm 0.00$

^{a,b,c}: Significant different (± = 0.05)

The reports that plant extract has sporicidal activity are still limited. *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). Lau *et al*. (2014) reported that methanolic *S. polyanthum* L. extract has sporicidal activity against spore of *B. cereus* and *B. sublitis*; 2.50% of the extract completely killed the spores of those bacteria after 1 h of incubation. In this study, the extract completely killed the spores of *B. pumilus* and *B. megaterium* with concentration of 1.0% after 1 h of incubation. These results suggested that the sporicidal activity of methanolic *S. polyanthum* L. against spores of *B. pumilus* and *B. megaterium* was relatively stronger than that against the spores of *B. cereus* and *B. subtilis*. Moreover, scanning electron microscope observation showed that 1% of the extract for 1 h destroyed the spores of *B. pumilus* (Figure 1).

The sporicidal properties of medicinal plants are related to the phytochemical components present. 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). On the contrary, lichocalcone A isolated from the roots of licorice (*Glycyrrhiza inflate*) 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, the methanolic *S. polyanthum* L. extract was found to exhibit the germination *B. pumilus* and *B. megaterium* spores.

S. polyanthum L. leaf was found to contain essential oils such as simple phenols, phenolic acids, and lactones sekuisterfenoid, triterpenoids, saponins, flavonoids, and tannins (Davidson and Branen, 1993). In addition, Zhang *et al*. (2013) reported that leaves of *S. polyanthum* L. contain polyphenols and flavonoids, and that these bioactive compounds help the plant to resist against microorganisms, thereby providing evidence for anti-infectious properties. The high content of polyphenols and flavonoids indicates antimicrobial properties. These biological activities are related to the molecules structures; through their hydroxyl groups or phenolic rings, phenolic compounds have the capacity to link with proteins and bacterial membranes to form complexes (Cheikna *et al*., 2011). Thus, further research, identification and isolation the active compounds which responsible to sporicidal activity in the extract of *S. polyanthum* L. need to be analysed.

In conclusion, it is remarkable to note that the methanolic *S. polyanthum L.,* extract confers significant antibacterial and sporicidal activity against vegetative cells and spores of spore-forming bacteria, *B. pumilus* and *B. megaterium*. Thus, *S. polyanthum* L. extract might be good to be developed as natural antibacterial and anti-spore.

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