International Food Research Journal 22(1): 421-425 (2015)
Journal homepage: http://www.ifrj.upm.edu.my

Short communication

Screening of tropical medicinal plants for sporicidal activity

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Article history

Received: 18 May 2014
Received in revised form: 2 July 2014
Accepted: 2 July 2014

Keywords

Sporicidal
Bacillus cereus
Eugenia polyantha
Indonesian bay leaf
Spore

Abstract

Bacterial spores have special significance in foods because they are much more resistant to physical and chemical antimicrobial treatment. Nowadays, there is interest in using natural products such as plant extract for food preservation. In this study, 26 of tropical medicinal plants and spices were screened for their sporicidal activity against the spores of *Bacillus cereus*. The spores of *B. cereus* was harvested after incubation at 30°C for 1 week and treated with various plant extracts using the method of Standard Operating Procedure for the AOAC (Association of Official Analytical Chemists) Sporicidal Activity. Glutaraldehyde was used as a positive control. Among them, Indonesian bay leaf (*Eugenia polyantha* Wight) inactivated more than 3 log of spores/ml of *B. cereus* (99.99%) at the concentration of 1% and completely killed *B. cereus* spores at concentration of 2.5%. These results suggest that Indonesian bay leaf extract has strong sporicidal activity against spores of *B. cereus*.

Introduction

Spore-forming bacteria, such as *Bacillus* spp., are difficult to kill due to its ability to generate heat-resistant spores under adverse environmental conditions (Fernandez-No *et al*., 2011). Germination of spores into vegetative cells under favourable conditions is frequently associated with food spoilage and foodborne diseases (Barker *et al*., 2005). *Bacillus cereus* and *B. subtilis* are spore-forming foodborne pathogen, which is ubiquitous in nature, and hence occur, frequently in a wide range of food raw materials (Van Opstal *et al.*, 2004). Ingestion of food associated with the spore can cause gastrointestinal disorder and leading to bacterial food poisoning. Thus, the control of bacterial endospores is desired especially in food products.

Bacterial contamination is a concern in a range of industries, including food and pharmaceutical production, and medical environments. Although sterilization can be achieved by heat, chemical, and UV irradiation treatments, these can be impractical when dealing with food products, compromising quality, taste, nutrition, and other properties important to the consumer (Russell, 1990). In some cases, bacterial contamination is accompanied by the production of endospores. The spores can spread from one food to another through cross-contamination (Stenfors anersen *et al*., 2008). Foods which are associated with *B. cereus* and *B. subtilis* contamination include starchy food, milk, vegetables and fruits. Cooked rice or starchy foods turned slimy after being kept for a short period of time due to the spore formation. Thus, in rice industry, the synthetic compound, glutaraldehyde, was used effectively against bacterial endospores, however, the high concentrations required to eradicate the spores gained concern to apply in food (Russell, 1990).

Many plant-derived antimicrobial compounds have a wide spectrum of activity against foodborne bacteria and this has led to suggestions that they could be used as natural preservatives in foods (Smith-Palmers *et al*., 1998; Cho *et al*., 2008). Besides, consumers are more conscious about the potential health risks associated with the consumption of synthetic components, despite their efficiency (Kechichian *et al.*, 2010). Therefore, recently there is interest in development of natural preservatives derived from plant sources, which can be used in food industries.

There is also a need to develop a potential sporicidal agent with compounds derived from plant which can be used as natural preservatives for the reduction of spores populations in rice or

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Table 1. Plant part of medicinal plants and spices used in this study

Sample Code	Domestic Name	Scientific Name	Plant Part
H003	Puyang	*Zingiber aromaticum* Vahl.	Rhizome
H006	Temu Ireng (hitam)	*Curcuma aeruginosa* Roxb.	Rhizome
H007	Klabet	*Trigonella foenum-graecum* L.	Seed
H010	Biji Pala (Nutmeg)	*Myristica fragrans* Houtt.	Nutmeg
H012	Biji Peka	*Illicium verum* Hook.	Seed
H014	Kembang Pala (Arir/Mace)	*Myristica fragrans* Houtt.	Fruit
H015	Temu Lawak	*Curcuma xanthorrhiza* Roxb.	Rhizome
H016	Cengkih	*Syzygium aromaticum* Merr.	Flower
H021	Jinten Masak	*Coleus amboinicus Lour.*	Seed
H023	Jinten Hitam	*Nigella sativa L.*	Seed
H026	Panili	*Vanilla planifolia* B.D. Jackson	Bean
H027	Merica	*Piper nigrum*	Seed
H075	Merica Hitam	*Piper nigrum*	Seed
H082	Kunci	*Kaempferia pandurata* Ridl.	Rhizome
H090	Laos = Lengkuas	*Alpinia galanga* Sw.	Rhizome
H100	Lada Putih	*Piper nigrum Linn.*	Seed
H109	Kemukus	*Piper cubeba* L.	Fruit
H110	Gadung Cina	*Smilax china* Linn.	Stem bark
H112	Kunci Pepet	*Kaempferia rotunda* L.	Rhizome
H113	Ginseng	*Panax ginseng*	Root
H123	Kemrunggi	*Caesalpinia crista* Linn.	Fruit
H125	Sambiloto = Sambilata	*Andrographis paniculata* Nees.	Whole Plant
H127	Biji Selasih	*Ocimum basillicum* Linn.	Seed
H138	Salam (Indonesian bay leaf)	*Eugenia polyantha* Wight.	Leaf
H143	Duwet	*Syzygium cumini Skeels.*	Leaf
H146	Daun Sendokan	*Plantago major* Linn.	Whole Plant
H156	Paku Simpari	*Cibotium barometz* (L.) J. Sm.	Whole Plant

starchy foods. Thus, by looking at the need for the development of natural sporicidal agent, this prompted us to perform the study in determining the sporicidal activity of tropical medicinal plants or spices. The aim of this study was to screen and evaluate the sporicidal activity of medicinal plants and spices extracts against *B. cereus* spores.

Materials and Methods

Samples and extract preparation

Twenty six of dried medicinal plants and spices (Table 1) were purchased from Herbal Market, Pasar Baru, Bandung, Indonesia. The samples were then identified and deposited in the Laboratory of Natural Products, Institute of Bioscience (IBS), Universiti Putra Malaysia. Each dried plant material was ground and extracted with 400 ml of 100% (v/v) methanol for seven days at room temperature as stated by Rukayadi *et al*. (2008), with some modification. After seven days, the plant material was filtered using Whatman No. 2 filter paper and concentrated by using rotary evaporator (50°C, 150 rpm) at Biochemical Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia. The extracts were then stored at 4°C prior to use.

Bacterial strain and spores preparation

Bacillus cereus ATCC 33019 was obtained from the American Type Culture Collection (Rockville, MD, USA). *B. cereus* was cultured, grown and maintained statically in nutrient broth (NB; Difco, Spark, MD, USA) or NB supplemented with 1.5% (w/v) agar (NA). *B. cereus* spores were prepared using the method described previously by Kida *et al.* (2003) and Rukayadi *et al.* (2009), with modification. Briefly, *B. cereus* was grown on NA at 30°C for over 4 weeks. After harvesting, spores and vegetative cells were suspended in sterile 0.85% NaCl solution, and heat shocked at 65°C 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 approximately10^8 spores/ml was stored in a 1.5 ml plastic cryopreservation tube at -85°C until further use.

Medicinal plants extracts and glutaraldehyde preparation

Each extract was dissolved in 100% dimethyl sulfoxide (DMSO) (Gibco) to obtain 1 g/ml (100%) sample solution. Each sample solution was the diluted in 1:10 of sterile distilled water. The final concentration of extract was 100 mg/ml (10%) meanwhile the final concentration of DMSO was 10%. These solutions were called stock solution. DMSO at 10% was found not to kill *B. cereus.* Commercial glutaraldehyde (Merck Darmstadt, Germany) was used as positive control for sporicidal activity experiments. The glutaraldehyde solution was prepared using a standard 25% commercially available solution (Merck Darmstadt, Germany).

Screening of sporicidal activity

Sporicidal activity was determined basically as described by standard operating procedure for the AOAC (Association of Official Analytical Chemists) sporicidal activity (AOAC, 2006) with modifications (Kida *et al.*, 2003, 2004; Palhano *et al.*, 2004; Rukayadi *et al.*, 2009). 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. Individual concentration of stock solution or glutaraldehyde (10%) were diluted 1:10 in adjusted spores suspension, resulting a final concentration of extract or glutaraldehyde of 1%, and an initial spore suspension of 9×10^5 spores/ml. The pH of these test solutions was not changed by addition of extract or glutaraldehyde. A 1 ml of each test solutions were then exposed for overnight 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 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 35-37°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 CFU/ml values of the test solution from those of the control (no antimicrobial). The mean value and standard deviation were calculated using differences from three independent experiments, and the reduction of spore cells in CFU was expressed as sporicidal activity.

Sporicidal activity of Indonesian bay leaf extract

The sporicidal activity of the selected Indonesian bay leaf extract was determined as aforementioned. The stock extract (10%) were diluted 1:10 in adjusted spores suspension of 106 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 spore suspension of 9 × 10^5 spores/ml. One ml of each concentration was then exposed to different incubation times (0, 1, 2, 3 and 4 hours) 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 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 35-37°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.

Results and Discussion

Sporicidal activity of 26 plant extracts at concentration 1% against spores of *B. cereus* is shown in Figure 1. Among them, *lada putih* or white pepper (H100) (*Piper nigrum* Linn.) and *daun salam* or Indonesian bay leaf (H138) (*Eugenia polyantha* Wight) showed potential sporicidal activity; the reduction in the number of *B. cereus* spores by white pepper and Indonesian bay leaf was 94.24% and 99.99%, respectively. Based on these results, Indonesian bay leaf extract was used to treat the spores of *B. cereus* at different concentrations (0.05, 0.25, 0.50, 1.00, 2.50, and 5.00 %) and exposure time (1, 2, 3, and 4 h) (Figure 2). Crude plant extract with 1% concentration is the limit in order to be considered as a good natural food preservative. However, higher concentration was needed to completely kill the spores of *B. cereus*. The different incubation times of 0, 1, 2, 3 and 4 hours were selected to observe the optimal reduction time.

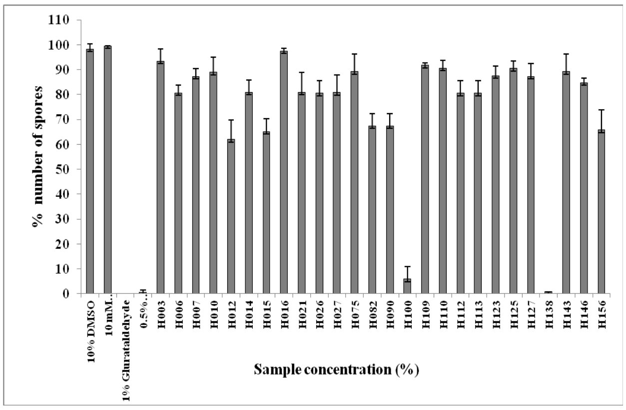

Figure 1. Screening of sporicidal activity of 26 medicinal plants and spices

A sharp reduction of *B. cereus* spores density was reached when the spores were exposed to at a concentration of 1.00%; the reduction in the number of spores/ml was >3 log units (99.99%). The complete killing of *B. cereus* spores was achieved with the treatment by Indonesian bay leaf at concentration 2.50% for 1 h of incubation. Even though glutaraldehyde is not well suited for food preservative, glutaraldehyde was reported to have sporicidal activity against spore-forming bacteria

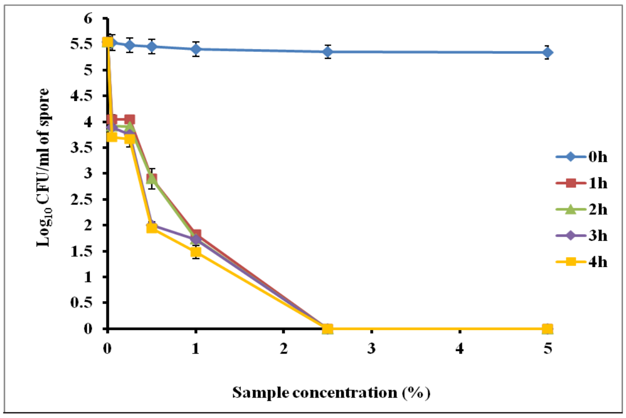

Figure 2. Sporicidal activity of Indonesian bay leaf (*Eugenia polyantha*) extract at different concentrations (0.00, 0.05, 0.25, 0.50, 1.00, 2.50 and 5.00 %) and different exposure times (0 h, blue rhombus; 1 h red quadrangle; 2 h, green triangle; 3h, purple rhombus; and 4 h, yellow quadrangle)

(Russell, 1990). Thus, glutaraldehyde was used as positive control in this study.

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 contrast, lichocalcone A isolated from the roots of licorice *(Glycyrrhiza inflate)*, which has various uses in the food and pharmaceutical industries, has antibacterial activity against vegetative cells of *B. subtilis*, but did not inhibit the germination *B. subtilis* spores (Tsukiyama *et al*., 2002). Moreover, Rukayadi *et al*. (2009) reported that macelignan isolated from nutmeg exhibited inhibition activity to the growth of vegetative cells and sporicidal activity against spores of *B. cereus*. In reality, simple comparisons are difficult because of differences in tested bacteria and the concentrations used. In this report, Indonesian bay leaf extract was found to exhibit inhibition activity to the growth of *B. cereus* spores.

Indonesian bay leaves (*Eugenia polyantha* Wight), synonym to *Syzygium polyanthum*, are commonly used as spice in culinary due to the aromatic smell produced by the volatile components. The dried brown leaves of *E. polyantha* which taste a bit sour and astringent was applied to meat in Surinam, while in Indonesia, it was used widely in the cooking of rice *(nasi liwet)*. Indonesian bay 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, the Indonesian bay leaf was claimed to possess antimicrobial activities and able to inhibit the growth of microorganisms that cause diseases, such as *Salmonella* sp., *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas fluorescens* due to the presence of tannin (Setiawan, 2002). Indonesian bay 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). Indonesian bay leaf was also found to possess high antioxidant activities due to its phenolic and β-carotene contents (Perumal *et al.*, 2012). Thus, there is interest to find out its function. In this study, the extraction of Indonesian bay leaf was done using absolute methanol. The antimicrobial properties of medicinal plants are related to the phytochemical components present, such as alkaloids, acids, essential oils, steroids, saponins, tannins, etc. 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. Moreover, enzyme in plant tissues does not function in methanol (Hirai, 1986).

In summary, it is remarkable to note that Indonesian bay leaf confers significant sporicidal activity against the spore of a spore-forming bacterium, *B. cereus*. It also reported that Indonesian bay leaf extract has antibacterial activity against vegetative cell of *B. cereus* (Setiawan, 2002). Thus, Indonesian bay leaf extract might be good to be developed as a food preservative.

Acknowledgements

This work was supported by Geran Putra IPS to Yaya Rukayadi Project Number GP-IPS/2013/9396700.

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