UNIVERSITI PUTRA MALAYSIA

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FSTM 2015 6

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By

LAU KAH YAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

May 2015

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman: Yaya Rukayadi, PhD
Faculty: Food Science and Technology

Spore-forming bacteria, *Bacillus* sp., have often been associated with the contamination of rice and other starchy products. Spores are more resistant to antimicrobial treatments than its vegetative cells. In this study, 26 methanolic plant extracts were screened for sporicidal activity against the spores of *Bacillus cereus* ATCC 33019. The extract of Indonesian bay leaf (*Eugenia polyantha* Wight), a spices used in Indonesian culinary, showed the most potential sporicidal activity against *B. cereus* ATCC 33019. *E. polyantha* extract was selected and further assessed for antibacterial and antispore activity. The Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of *E. polyantha* extract against vegetative cells of *B. cereus* ATCC 33019, 25 of *B. cereus* isolated from various rice samples, *B. subtilis* ATCC 6633, *B. subtilis* KCTC 1028 and *B. subtilis* KCTC 3014 was determined as described by Clinical and Laboratory Standards Institute (CLSI) reference methods. The results showed that *E. polyantha* extract was able to inhibit the growth of vegetative cells of all tested *Bacillus* sp. with MICs ranged from 0.16 to 0.63 mg/mL. The extract can kill all the tested *Bacillus* sp. with MBCs ranged from 0.31 to 2.50 mg/mL. Time-kill curves were established for *B. cereus* ATCC 33019, *B. cereus* BC-NP.8, *B. subtilis* ATCC 6633 and *B. subtilis* KCTC 3014 at concentrations ranging from 0 × MIC to 8 × MIC at 30 ℃ incubation. The bactericidal endpoint for *B. cereus* ATCC 33019 and *B. subtilis* KCTC 3014 were at concentration of 2.50 mg/mL (8× MIC), whereas *B. cereus* BC-NP.8 at 1.25 mg/mL (8 × MIC) and *B. subtilis* ATCC 6633 at 5.00 mg/mL (8× MIC) after 4 h of incubation. The effect of different concentrations, incubation periods, pHs and temperatures on the sporicidal activity of *E. polyantha* extract was determined against spores of *B. cereus* ATCC 33019, *B. cereus* BC-NP.8, *B. subtilis* ATCC 6633 and *B. subtilis* KCTC 3014. Glutaraldehyde, a chemical sporicidal agent, was used as positive control. *E. polyantha* extract inactivated more than 3-$\log_{10}$ (99.99%) of *B. cereus* ATCC 33019, *B. cereus* BC-NP.8, *B. subtilis* ATCC 6633 and *B. subtilis* KCTC 3014 spores at a concentration of 1.0% after 1 h of incubation and the spores was completely killed at 2.5%. The sporicidal activity of *E. polyantha* extract was not affected by different temperatures treatment and alteration of the pHs of extract. These results indicate that the extract is stable against changes in pH 3, 7 and 10 as well as temperature of 50, 80 and 121 ℃. Based on scanning electron microscope

observation, the structure of the *B. cereus* ATCC 33019 and *B. subtilis* ATCC 6633 spores was destroyed after treated with 1% (w/v) *E. polyantha* extract for 1 h. The LC_{50} of *E. polyantha* extract was found to be more than 1 mg/mL meaning that the extract is non-cytotoxic. Hexadecanoic acid, phytol and 9,12-octadecadienoic acid,(Z,Z), found in *E. polyantha* extract using GC-MS analysis as well as citral and eugenol found using LC-MS analysis might be contributing to the antibacterial and sporicidal activity. In summary, *E. polyantha* extract shows potential antibacterial and sporicidal activity against vegetative cells and spores of *Bacillus* sp.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

AKTIVITI ANTIBAKTERIA DAN SPORISIDAL EKSTRAK DAUN SALAM (*Eugenia polyantha* Wight) TERHADAP *Bacillus cereus* DAN *Bacillus subtilis*

Oleh

LAU KAH YAN

Mei 2015

Pengerusi: Yaya Rukayadi, PhD
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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. Dalam kajian ini, 26 ekstrak methanolik tumbuhan telah diuji untuk aktiviti sporisidal terhadap spora *Bacillus cereus* ATCC 33019. Ekstrak daun salam (*Eugenia polyantha* Wight), satu rempah yang digunakan dalam masakan Indonesia, menunjukkan aktiviti sporisidal yang paling berpotensi terhadap *B. cereus* ATCC 33019. Ekstrak *E. polyantha* telah dipilih dan seterusnya dikaji untuk aktiviti antibakteria dan sporisidal. Kepekatan Perencat Minimal (MIC) dan Kepekatan Bakterisidal Minimal (MBC) ekstrak *E. polyantha* terhadap sel vegetatif *B. cereus* ATCC 33019, 25 *B. cereus* yang diisolasi dari pelbagai sampel nasi, *B. subtilis* ATCC 6633, *B. subtilis* KCTC 1028 dan *B. subtilis* KCTC 3014 ditentukan seperti yang dinyatakan dalam kaedah rujukan *Clinical and Laboratory Standard Institute* (CLSI). Ekstrak *E. polyantha* boleh merencat pertumbuhan semua *Bacillus* sp. yang diuji dengan MIC dalam lingkungan 0.16 hingga 0.63 mg/mL. Ekstrak *E. polyantha* boleh membunuh semua *Bacillus* sp. yang diuji dengan MBC adalah dalam lingkungan 0.31 hingga 2.50 mg/mL. Keluk masa-pembunuhan telah ditentukan untuk *B. cereus* ATCC 33019, *B. cereus* BC-NP.8, *B. subtilis* ATCC 6633 dan *B. subtilis* KCTC 3014 dengan kepekatan antara 0 × MIC hingga 8 × MIC dan diinkubasi dalam 30 ℃. Titik akhir bakterisidal untuk *B. cereus* ATCC 33019 dan *B. subtilis* KCTC 3014 pada kepekatan 2.50 mg/mL (8 × MIC), manakala *B. cereus* BC-NP.8 pada 1.25 mg/mL (8 × MIC) dan *B. subtilis* ATCC 6633 pada 5.00 mg/mL (8 × MIC) selepas inkubasi selama 4 jam. Kesan kepekatan, tempoh inkubasi, pH dan suhu yang berbeza ke atas aktiviti sporisidal ekstrak *E. polyantha* terhadap *B. cereus* ATCC 33019, *B. cereus* BC-NP.8, *B. subtilis* ATCC 6633 dan *B. subtilis* KCTC 3014 juga ditentukan. Glutaraldehida merupakan satu ejen sporisidal kimia, telah digunakan sebagai kawalan positif. Ekstrak *E. polyantha* merencat lebih daripada 3-$\log_{10}$ (99.99%) spora *B. cereus* ATCC 33019, *B. cereus* BC-NP.8, *B. subtilis* ATCC 6633 dan *B. subtilis* KCTC 3014 pada kepekatan 1.0% selepas inkubasi selama 1 jam dan spora dibunuh sepenuhnya pada 2.5%. Aktiviti sporisidal ekstrak *E. polyantha* tidak terjejas dengan pengubahan pH ekstrak dan rawatan suhu yang berbeza. Hasil kajian ini menunjukkan bahawa ekstrak tersebut adalah stabil terhadap perubahan kepada pH 3, 7 dan 10 serta suhu 50, 80 dan

121 ℃. Berdasarkan pemerhatian dengan mikroskop elektron imbasan, struktur spora *B. cereus* ATCC 33019 dan *B. subtilis* ATCC 6633 musnah selepas dirawat dengan 1% (w/v) ekstrak *E. polyantha* selama 1 jam. LC_{50} ekstrak *E. polyantha* didapati melebihi 1 mg/mL dan ini menunjukkan bahawa ekstrak tersebut adalah tidak toksik. Asid heksadekanoik, fitol dan asid 9,12-oktadekadienoik (Z,Z), yang didapati dalam ekstrak *E. polyantha* melalui analisa GC-MS, serta sitral dan eugenol yang didapati melalui analisa LC-MS mungkin merupakan penyebab aktiviti antibakteria dan sporisidal. Secara keseluruhannya, ekstrak *E. polyantha* menunjukkan potensi dalam aktiviti antibakteria dan sporisidal terhadap sel vegetatif dan spora *Bacillus* sp.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude and appreciation to my supervisor, Associate Professor Dr. Yaya Rukayadi for his guidance, advice, encouragement and above all, his confidence in me to complete this project.

Heartfelt appreciation is also extended to my co-supervisor, Associate Professor Dr. Faridah Abas, for her guidance and support throughout the studies. Deep thanks are also extended to Prof. Dr Son Radu for providing and allowing the use of several *Bacillus cereus* strains in this study.

Credit also goes to lab assistants from the Microbiology and Biochemical Laboratory of Faculty of Food Science and Technology including Encik Zulkefli Nordin, Cik Nur Asikin bt Mohd. Yusof, Cik Nur Fatihah, Encik Azman Asmat, Puan Norhafiza and Puan Rosmawati for their help in my research endeavour. Not forgetting also the staff from the Laboratory of Natural Products, Institute of Bioscience, Puan Mazina Mohd Yusoff.

Special appreciation is also dedicated to my fellow colleagues especially Nurul Syazwani Mohd Zainin, Lew Kok Fang, Nor Asma Husna, Slyvester, Nurul Husna Mazahar, Muhammad Safwan bin Ahamad Bustamam, and Maya Zakaria for their guidance, help and mutual support along the way of completing this project. Not forgetting also Ahmed, Khaleeda and Abdel Gani for their help in this study. Our mutual understanding is better as we shared the same path towards academics and research excellence.

In addition, thanks are also due to all my friends and other post graduate students for their mutual encouragement and exchange of opinions or suggestion in the progress of completing this research. They are always there to share my joy, laughter and even tears of frustration. With their company, this long journey was made easier and reminds me that I am not all alone.

Last but not least, I am very grateful to have a warm and comfortable family who has always been there for me throughout my study. Their presence will always be cherished.

I certify that a Thesis Examination Committee has met on 6 May 2015 to conduct the final examination of Lau Kah Yan on her thesis entitled "Antibacterial and Sporicidal Activity of Indonesian Bay Leaf (*Eugenia polyantha* Wight) Extract against *Bacillus cereus* and *Bacillus subtilis*" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATTC	American Type Culture Collection
B. cereus	*Bacillus cereus*
B. subtilis	*Bacillus subtilis*
B. licheniformis	*Bacillus licheniformis*
Ca^{2+}	Calcium cation
Ca^{2+}-DPA	Calcium cation and dipicolinic acid chelate
CFU	Colony forming unit
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
C. butyricum	*Clostridium butyricum*
C. perfringens	*Clostridium perfringens*
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid
E. polyantha	*Eugenia polyantha*
GC-MS	Gas Chromatography – Mass Spectrometry
GRAS	Generally Recognised as Safe
h	Hour
HPLC	High Performance Liquid Chromatography
IBS	Institute of Bioscience
KCTC	Korean Collection for Types Culture
kV	Kilovolts
L. rhamnosus	*Lactobacillus rhamnosus*
LC_{50}	Median Lethality Concentration
LC-MS	Liquid Chromatography – Mass Spectrometry
M	Molarity
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
min	Minute
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MPa	Mega Pascal
m/z	Mass/charge ratio
NA	Nutrient agar
NB	Nutrient broth
NaCl	Sodium chloride
NIST	National Institute of Standards and Technology
PBS	Phosphate buffered saline
Psi	Pounds per square inch
rpm	Revolutions per minute
SEM	Scanning Electron Microscopy
sp.	Species
S. polyanthum	*Syzygium polyanthum*
UV	Ultraviolet
UPM	Universiti Putra Malaysia
× g	Unit gravity
wk	Week

CHAPTER 1

INTRODUCTION

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 sp., 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 *Bacillus cereus*, *B. subtilis* and others, can successfully adapt to various changes in the environment. *B. cereus* causes diarrhoea and emetic type of food poisoning. The diarrhoeal type is associated with 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 (Kim *et al.*, 2013; Altayar and Sutherland, 2006). The spores of *B. cereus* may survive cooking and germinate if cooked rice was left at room temperature, resulting in foodborne illness (Choi *et al.*, 2014). In addition, *B. subtilis* is not typically considered as a human pathogen but may occasionally contaminate food and cause food poisoning (Fernández-No *et al.*, 2013). Contamination of food products including rice with *B. subtilis* has been shown to underlie foodborne diseases (Kim *et al.*, 2013) and in 2005, an outbreak caused by contaminated milk powder has been reported (Fernández-No *et al.*, 2013).

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 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 agents which are 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 *Eugenia polyantha* Wight, 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). Hence, it would be interesting to know the antimicrobial and sporicidal benefits of this plant. In general, this study aimed to determine the antibacterial and sporicidal activity of *E. polyantha* leaves extract against the vegetative cells and spores of *B. cereus* ATCC 33019, 25 of *B. cereus* isolated from various rice samples as well as *B. subtilis* ATCC 6633, KCTC 1028 and KCTC 3014.

Objectives:

1. To determine the antibacterial activity of *E. polyantha* extract in terms of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill curve against the vegetative cells of *B. cereus* and *B. subtilis*.
2. To evaluate the sporicidal activity of *E. polyantha* extract against spores of *B. cereus* and *B. subtilis*.
3. To analyse the active compounds present in *E. polyantha* extract.
4. To determine the toxicity of *E. polyantha* extract.

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