UNIVERSITI PUTRA MALAYSIA

ISOLATION AND IDENTIFICATION OF ANTIBACTERIAL AND ANTISPORE COMPOUNDS FROM Syzygium Polyanthum L. LEAVES

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By

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ISOLATION AND IDENTIFICATION OF ANTIBACTERIAL AND ANTISPORE COMPOUNDS FROM *Syzygium Polyanthum* L. LEAVES

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October 2018

Chairman : Associate Professor Yaya Rukayadi, PhD
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In this study, the antibacterial and antispore activities in *Syzygium polyanthum* L. leaves extract were evaluated against vegetative cells and spores of *Bacillus cereus* ATCC33019, *B. megaterium* ATCC14581, *B. pumilus* ATCC14884 and *B. subtilis* ATCC6633. The susceptibility test showed that all tested vegetative cells of *Bacillus* were inhibited by *S. polyanthum* extract, with the range of inhibition zone between 10.00 to 16.00 mm. The extract could inhibit the growth of *Bacillus* with MIC ranged 0.02 to 0.08 mg/mL. These bacteria can be completely killed at different MBC valueup to 0.63 mg/mL. Time-kill curve study showed that. *Bacillus* sp. can be killed by *S. polyanthum* L. extract at 8× for 1 h. Moreover, *S. polyanthum* L extract deactivated more than 3-Log of *Bacillus* sp. spores at a concentration of 1.0% after 1 hour of incubation. The antibacterial and sporicidal activity of *S. polyanthum* L. extract was stable at different temperatures and pHs treatment. Scanning electron microscope showed that structure of the spores was destroyed after treated with 1.0% *S. polyanthum* L. extract for 1 hour. ^{1}H NMR analyses showed that the young leaves extracts were found to be richer in sugars, and glycosides, whereas the old leaves contained higher levels of fatty acid components. LCMS analysis identified the presence of active compounds in *S. polyanthum* L. extract which included gallic acid, quinic acid, and oleanolic acid. The β-sitosterol and oleanolic acid were successfully isolated from the methanol extract of *S. polyanthum* L. β-sitosterol was able to inhibit the growth of all tested *Bacillus* sp. with the range of inhibition zone between 11.7 and 12.3 mm. The growth of all tested *Bacillus* sp. can be inhibited by MIC value of 7.81 µg/ml and can be killed with MBC value of 15.62 µg/ml. Meanwhile, oleanolic acid was able to inhibit the growth og all tested *Bacillus* sp. with the range of inhibition zone between 12.2 and 13.5 mm. MIC and MBC values of oleanolic acid on all tested *Bacillus* sp. was 31.25 and 500 µg/ml, respectively. Finally, β-sitosterol and oleanolic acid at concentration of 1.00 µg/mL (0.1%) can killed 100% of all tested *Bacillus* sp. spores. In conclusion, *S. polyanthum* extract and its

compounds exhibited antimicrobial and sporicidal activity against *Bacillus* species, thus it can be developed as anti-*Bacillus* agent.

Keyword : Antimicrobial activity, antispore activity, β-sitosterol, oleanolic acid, *S. polyanthum* leaves extract.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGASINGAN DAN PENGECAMAN KOMPOUN ANTIBAKETRIA DAN ANTISPORA DARI DAUN *Syzygium Polyanthum* L.

Oleh

ABDELGANI MOHAMED ABOBAKER

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Dalam kajian ini, penilaian dibuat ke atas aktiviti antibakteria dan antispora dalam ekstrak daun *Syzygium polyanthum* L. terhadap sel vegetatif dan spora *Bacillus cereus* ATCC33019, *B. megaterium* ATCC14581, *B. pumilus* ATCC14884 dan *B. subtilis* ATCC6633. Ujian kerentanan menunjukkan sel *Bacillus* sp vegetatif disekat oleh ekstrak *S. polyanthum* dengan julat zon perencatan antara 10.00 ke 16.00 mm. Ekstraks tersebut boleh menyekat pertumbuhan Bacillus sp dengan julat MIC antara 0.02 ke 0.08 mg/mL. Bakteria ini boleh dibunuh terus pada nilai MBC berbeza iaitu sehingga 0.63 mg/mL. Kajian keluk masa-pembunuhan mendapati. *Bacillus* boleh dibunuh oleh ekstrak *polyanthum* L. pada 8× selama 1 h. Tambahan lagi, ekstrak *S. polyanthum L* ternyahaktif lebih 3-Log spora *Bacillus* sp pada konsentrasi 1.0% selepas diinkubasi selama I jam. Aktiviti antibakteria dan pembunuhan spora oleh ekstrak *S. polyanthum* L adalah stabil pada suhu dan rawatan pH yang berlainan. Pemerhatian imbasan mikroskop elektron mendapati struktur spora telah dimusnahkan selepas dirawat dengan ekstrak 1.0% *S. polyanthum* L selama 1 jam. Analisis ke atas ^{1}H NMR mendapati ekstrak daun muda didapati lebih kaya dengan gula dan glikosida manakala daun tua mengandungi tahap komponen asid lemak yang lebih tinggi. Analisis LCMS telah mengecam kehadiran kompoun aktif dalam ekstrak *S. polyanthum* termasuk asid galik, asid kuinik dan asid oleanolik. β-sitosterol dan asid oleanolik telah berjaya diasingkan dari ekstrak methanol *S. polyanthum* L. β-sitosterol mampu menyekat pertumbuhan semua *Bacillus* sp yang dikaji dengan julat zon perencatan antara 11.7 dan 12.3mm. Pertumbuhan semua *Bacillus* sp yang dikaji boleh disekat dengan nilai MIC 7.81 µg/ml dan boleh membunuh pada nilai MBC 15.62 µg/ml. Sementara itu, asid oleanolik mampu menyekat pertumbuhan semua *Bacillus* dengan julat zon rencatan antara 12.2 dan 13.5 mm. Nilai asid oleanolik MIC dan MBC ke atas semua *Bacillus* sp yang diuji masing-masing adalah 31.25 ke 500 µg/ml. Akhir sekali, β-sitosterol dan asid oleanolik pada konsentrasi 1.00 µg/mL (0.1%) mampu membunuh 100% spora

Bacillus sp yang diuji. Kesimpulannya, ekstrak *S. polyanthum* dan kompounnya mempamerkan aktiviti antimikrob dan pembunuhan spora terhadap spesis *Bacillus.* Oleh itu ianya boleh dikembangkan sebagai ejen anti Bacillus.

Kata kunci : Aktiviti antimikrob, aktiviti antispora, ekstrak daun *S. polyanthum* L.

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

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CC	Column Chromatography
CFU	Colony forming unit
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
D	Doublet
Dd	Doublet of doublet
DMSO	Dimethylsulfoxide
GC-MS	Gas Chromatography – Mass Spectrometry
H	Hour
Hz	Hertz
IBS	Institute of Bioscience
INT	Iodonitrotetrazolium violet
L	Litre
LC-MS	Liquid Chromatography–Mass Spectrometry
m/z	Mass to charge
MBC	Minimum Bactericidal Concentration
Me OH	Methanol
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MHz	Megahertz
MIC	Minimum Inhibitory Concentration
Min	Minute
mL	Milliliter

MS	Mass Spectrometry
NA	Nutrient agar
NMR	Nuclear Magnetic Resonance
PBS	Phosphate buffered saline
PCA	Principal Component Analysis
PLS	Partial Least Squares
Ppm	Part Per Million
Rpm	Revolutions per minute
SEM	Scanning Electron Microscopy
Spp	Species
TLC	Thin layer chromatography
VIP	variable Importance in the Projection
WHO	world health organization
µg	Microgram

CHAPTER 1

INTRODUCTION

1.1 Background

The control of microbial pathogens in foods is a significant concern, and a broad spectrum of methods have been employed to prevent the growth of pathogenic bacteria in food including the use of synthetic and natural antimicrobial agents (Tippayatum & Chonhenchob, 2007). Spore forming bacteria, such as species of *Bacillus* and *Clostridium* genera, respond to opposing stresses from their environments by the formation of a dormant structure referred to as an endospore (bacterial spore or seed) via sporulation (Leggett *et al.*, 2012). Bacterial spores are capable of withstanding harsh external conditions such as scarcity of nutrients or dehydration, and germinate and grow when the conditions become favourable again (Tan & Ramamurthi, 2013). The high resistance of bacterial spores and their resilience present difficulties for food industries (Leggett *et al.*, 2012). Spores that germinate to yield vegetative cells when conditions are favourable are usually associated with foodborne diseases and food spoilage (Barker *et al.*, 2005).

The genus *Bacillus* is composed of species such as *B. cereus* and *B. subtilis* which can favourably adapt to a variety of environmental changes. *Bacillus* is motile, rod-shaped, facultative anaerobic, Gram-positive, that is widely found in nature (Kim *et al.*, 2014). Some food poisonings characterised by diarrhoea and emesis are caused by *B. cereus*. The category of diarrhoea is associated with foods with a meat base, sauces, milk products and vegetables (Kim *et al.*, 2014). It is the ingestion of farinaceous foods like noodles, pasta and rice that leads to emetic symptoms such as nausea and vomiting usually associated with *B. cereus* (Kim *et al.*, 2013; Altayar & Sutherland, 2006). In some cases, bacterial contamination is accompanied by the production of endospores. *B. cereus* and *B. subtilis* have been recognised as causative agents in food spoilage and poisoning. Cross-contamination can spread the spores from one food to another (Stenfors anersen *et al*., 2008). Foods associated with *B. cereus* and *B. subtilis* include starchy food, milk, vegetables and fruits. Consumption of contaminated food leads to two types of gastrointestinal disorder including diarrhoeal and emetic syndromes. Diarrhoea is caused by different toxins formed in the food as well as in the human small intestine, whereas emetic disease is caused by preformed toxins in food only (Rukayadi *et al*., 2009a).

The spore of *B. cereus* may tolerate cooking temperatures and so subsequent growth could occur when cooked rice was left at room temperature, resulting in foodborne infection (Choi *et al.*, 2014). Moreover, *B. subtilis* is not conventionally seen as a human pathogen but may occasionally contaminate food, leading to food poisoning (Fernández-No *et al.*, 2013). Other studies also proved that rice contaminated with *B. subtilis* caused foodborne illnesses (Kim *et al.*, 2013). Also in 2005, an epidemic was

reported due to soiled milk powder (Fernández-No *et al.*, 2013). *Bacillus* spores can resist several chemical disinfectants. Furthermore, there are limited chemical sporicidal agents commercially available in destroying *Bacillus* spores, and if available, they require special precautions for handling, examples include toxic formaldehyde and glutaraldehyde (Kida *et al.*, 2004).

Moreover, thermal food processing is a relatively effective and cheap method of producing safe food that is free from enzymatic reactions and undesirable microbes. However, thermal processing is associated with some problems including a reduction in nutrient content, as well as a reduction in organoleptic qualities (Cho *et al*., 2008). Therefore, the development of effective, safe and stable natural antibacterial and sporicidal agents is garnering more attention (Kida *et al*., 2004), along with a rise in interest in searching for antimicrobial compounds of plant origin.

The leaves of *S. polyanthum* L. which is also known as "*daun salam*" are commonly used in dishes as spice in culinary due to its aroma besides the sour taste and also as ingredient in the traditional medicine (Kato *et al.*, 2013). *S. polyanthum* L. was reprted 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 & Siemonsma, 1999).

1.2 Problem Statements

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 foods materials or food products has prompted the study in determining the sporicidal activity of tropical medicinal plants.

Lau *et al*. (2014) reported that methanolic extract of *S. polyanthum* L. leaves has antibacterial and antispore activity against vegetative cells and spores of *B. cereus* and *B. subtilis*. Unfortunatelly, antibacterial and antispore of methanolic extract of *S. polyanthum* L. leaves and its fractions against other species of *Bacillus* such as *B. pumilus* and *B. megaterium* have not been evaluate. To the best our knowledge, the phytochemicals compunds which are respposible for antibacterial and antispore

against Bacillus sp. in *S. polyanthum* L. leaves also have no investigated yet. It is also important to isolate the actvive compounds which are respposible for antibacterial and antispore against *Bacillus* sp. in *S. polyanthum* L. leaves. Thus, the general objective of this study was to evaluate the the antibacterial and antispore activity og *S. polyanthum* L. leaves against vegetative cells and spores of four species of *Bacillus*, then to indentify and isolate the activive compounds which responsible for antibacterial and antispore activity in the leaves of *S. polyanthum* L.

1.3 Objectives

1) To determine the antibacterial activity of *S. polyanthum* L. leaves extract and the fractions against vegetative cells of *B.cereus*, *B. subtilis*, *B. pumilus* and *B. megaterium.*
2) To evaluate the antispore activity of *S. polyanthum* L. leaves extract on the spores of *B.cereus*, *B. subtilis*, *B. pumilus* and *B. megaterium*
3) To analyse the phytochemicals in *S. polyanthum* L. leaves extract using NMR based metabolomics.
4) To identify the phytochemiclas in *S. polyanthum* L. leaves extract that are responsible for antibacterial and antispore activity using LCMS.
5) To isolate and characterize the active compounds from *S. polyanthum* L. leaves that are responsible for antibacterial and antispore activity.

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