ANTIBACTERIAL, ANTISPORE AND PHYTOCHEMICAL ANALYSES OF ETHANOLIC *JAMBU BATU* (*Psidium guajava* L.) LEAF EXTRACT AND APPLICATIONS IN TOFU

By

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

August 2022

FSTM 2022 32

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August 2022

Chair : Assoc. Prof. Yaya Rukayadi, PhD
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Spore-forming bacteria can survive various stages during food processing; thus, eliminating the vegetative bacterial cells and spores become the primary concern to the food industry. *Bacillus* species (spp.) spores are resistant to heat, radiation and disinfectants, which necessitate potent antibacterial agents. The crude extracts of *jambu batu* leaves exhibited profound antimicrobial activities against various foodborne pathogens. Thus, the current research aimed to determine the antibacterial and antispore activities of *jambu batu* leaves extracts on the vegetative cells and spores of *B. cereus* ATCC® 33019™, *B. megaterium* ATCC® 14581™, *B. pumilus* ATCC® 14884™ and *B. subtilis* ATCC® 6633™. The phytochemical constituents of the extracts were also analysed and the effect of the extracts on bacterial population in tofu were evaluated. The *jambu batu* leaves were collected from the Taman Pertanian, Universiti Putra Malaysia. The dried powder of *jambu batu* leaves was extracted with the ultrasonic-assisted maceration method using ethanol as solvent. The extracts were tested for the antibacterial activity against *Bacillus* spp. vegetative cells in terms of well diffusion assay (inhibition zone), minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), as recommended by the Clinical and Laboratory Standard Institute (CLSI). The Time-kill curve analyses were conducted at the concentration of the extract with ½, 1, 2 and 4×MICs at 0, ½, 1, 2 and 4 h of exposure time. The antispore activity of the extract was determined at different concentrations and time exposures. The stability of the extract on the antibacterial activity was conducted by exposing the extracts to various pHs (pH3, 5, 6.7, 7 and 11) and different temperatures (10ºC, 28ºC, 30ºC, 50ºC and 80ºC). The phytochemicals constituents in the extracts were identified using GC-MS and LC-MS/MS analysis. The effect of *jambu batu* leaves extract on bacterial population in tofu samples was evaluated at different extract concentrations (tap water, 0.00%, 0.05%, 0.50% and 5.00%) at different exposure times (1, 2 and 4 h). The *jambu batu* leaves extract generated the minimum and maximum inhibition zones of 13.75 ± 0.95 mm to 16.25 ± 0.95 mm, respectively. It showed that all the tested *Bacillus* spp. were susceptible to the *jambu batu* leaves extract. The growth inhibition pattern was detected in all tested *Bacillus* spp. with MIC ranging from 0.195

mg/mL to 0.781 mg/mL, while killed by an MBC value of 0.781 mg/mL. The bactericidal activities of *jambu batu* leaves extract were determined by the time-kill curve analysis for *B. cereus, B. megaterium, B. pumilus* and *B. subtilis* at 4.0×MIC for 4 h, 4×MIC for 2 h, 1×MIC for 4 h and 4×MIC for 2 h, respectively. The spores of *B. cereus* were inactivated more than 3-$\log_{10}$ (99.9%) at a concentration of 1.0% extract. Subsequently, spores were killed at a concentration of >2.0% upon 4 h incubation. Jambu batu leaves extract caused a stable antibacterial activity at various pHs and temperatures against *Bacillus* spp. The GC-MS analysis identified 30 compounds in the extract; mainly six bioactive compounds were caryophyllene, aromadendrene, alpha-humulene, beta-selinene, alpha-selinene and globulol. However, LCMS/MS analysis detected 26 compounds and the antimicrobial bioactive compounds detected were lasalocid, cannabichromene, asiatic acid, curcumin, 18-β-Glycyrrhetinic acid, acetoxyurs, apigenin and aloesin. Tofu sample resulted in a reduction of 3-$\log_{10}$ of *Bacillus* spp. bacteria when exposed to 0.05% (v/v) *jambu batu* leaves extract for 2 h, and an incubation period of 4 h inhibited all the bacteria. In conclusions, *jambu batu* leaves extract yielded good antibacterial and antispore activities, suggesting that *jambu batu* leaves extract can be utilised or explored as a potential anti-*Bacillus* agent in food.

Keywords: Antibacterial activities, antispore activities, anti-*Bacillus* agent, *Bacillus* spp., *jambu batu* leaves extract.

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

ANALISIS ANTIBAKTERIA, ANTISPORA DAN FITOKIMIA EKSTRAK ETANOL DAUN JAMBU BATU (*Psidium guajava* L.) DAN APLIKASINYA DALAM TAUHU

Oleh

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Bakteria pembentuk spora menjadi perhatian utama industri makanan kerana kemampuannya bertahan dalam pelbagai pemprosesan dan langkah yang dirancang untuk membunuh sel dan spora vegetatif mereka. Spora *Bacillus* spp. terbukti bertahan terhadap pemanasan, proses penyinaran dan nyahjangkitan. Kajian sebelum ini menunjukkan bahawa ekstrak daun jambu batu mempunyai potensi yang baik sebagai antimikroorganisma terhadap beberapa patogen bawaan makanan. Kajian ini bertujuan untuk menganalisis aktiviti antibakteria dan antispora ekstrak daun jambu batu pada sel vegetatif dan spora *B. cereus ATCC® 33019™, B. megaterium ATCC® 14581™, B. pumilus ATCC® 14884™ and B. subtilis ATCC® 6633™* sekaligus menganalisis kandungan fitokimia dan menilai keberkesanan ekstrak terhadap populasi mikroorganisma di dalam tofu. Daun jambu batu untuk kajian ini dipetik dan dikumpulkan dari Taman Pertanian, Universiti Putra Malaysia. Serbuk kering daun jambu batu diekstrak menggunakan kaedah maserasi dengan etanol sebagai pelarut dibantu dengan teknik ultrasonik. Ekstrak diuji untuk aktiviti antibakteria terhadap sel vegetatif *Bacillus* spp. dengan ujian kerentanan yang dijalankan untuk mengetahui saiz diameter zon perencatan, kepekatan perencatan minima (MIC) dan kepekatan bakterisida minima (MBC) dilakukan seperti yang disarankan oleh Institut Standard Klinikal dan Makmal (CLSI). Analisis keluk masa-membunuh dilakukan pada kepekatan 0, ½, 1, 2 dan 4 × MIC pada masa pendedahan yang berbeza, iaitu 0, ½, 1, 2 dan 4 jam. Aktiviti antispora bagi ekstrak ini ditentukan pada kepekatan dan pendedahan masa yang berbeza. Kestabilan aktiviti antibakteria ekstrak ini dilakukan dengan mendedahkan ekstrak pada nilai pH 3, 5, 6.7, 7 dan 11 serta suhu 10ºC, 28 ºC, 30ºC, 50ºC dan 80ºC. Pengenalpastian kandungan fitokimia dalam ekstrak dilakukan menggunakan analisis GC-MS dan LC-MS/MS. Keberkesanan pendedahan ekstrak daun jambu batu pada populasi mikroorganisma dalam sampel tauhu diuji pada kepekatan ekstrak yang berbeza (air paip, 0.00%, 0.05%, 0.50% dan 5.00%) dan pada masa pendedahan yang berbeza (1, 2 dan 4 jam). Hasil kajian menunjukkan bahawa semua *Bacillus* spp. yang diuji pendedahan ekstrak daun jambu batu menunjukkan julat zon inhibisi antara 13.75 ± 0.95 mm hingga 16.25 ± 0.95 mm. Ekstrak daun jambu batu mampu menghalang

pertumbuhan *Bacillus* spp. dengan nilai MIC 0.195 mg/mL hingga 0.781 mg/mL. *Bacillus* spp. dapat dibunuh sepenuhnya pada nilai MBC 0.781 mg/mL. Ujian keluk masa-membunuh menunjukkan bahawa *B. cereus*, *B. megaterium*, *B. pumilus* dan *B. subtilis* dapat dibunuh oleh ekstrak daun jambu batu pada 4 × MIC selama 4 jam, 4 × MIC selama 2 jam, 1 × MIC untuk 4 jam dan 4 × MIC selama 2 jam masing-masing. Ekstrak daun jambu batu pada kepekatan 1.0% dapat menyahaktifkan semua *B. cereus* spora yang diuji kepada lebih daripada 3-$\log_{10}$ (99.9%) dan membunuh semua *B. cereus* spora yang diuji setelah tempoh pengeraman selama empat jam pada kepekatan> 2.0%. Aktiviti antibakteria ekstrak daun jambu batu terhadap empat *Bacillus* spp. pada pH yang berbeza (pH 3, 5, 6.7, 7 dan 11) dan suhu yang berbeza (10˚C, 28˚C, 30˚C, 50˚C dan 80˚C) tidak terjejas dengan ketara di mana ini bermaksud aktiviti antibakteria ekstrak daun jambu batu adalah stabil. Analisis GC-MS dapat mengenal pasti kehadiran 30 bahan kimia tumbuhan dalam ekstrak ini terutamanya enam bahan bio aktif seperti *caryophyllene, aromadendrene, alpha-humulene, beta-selinene, alpha-selinene* dan *globulol*. Sebanyak 26 bahan kimia tumbuhan dikesan menggunakan analisis LCMS/MS dimana yang mempunyai fungsi antimikroorganisma adalah *lasalocid, cannabichromene, asiatic acid, curcumin, 18-β-Glycyrrhetinic acid, acetoxyurs, apigenin and aloesin.* Secara umumnya, pengurangan 3-$\log_{10}$ *Bacillus* spp. mula dapat diperhatikan dan direkodkan dengan kepekatan ekstrak daun jambu batu 0.05% (v/v) pada waktu pengeraman 2 jam dan membunuh semua bakteria *Bacillus* sp. yang diuji setelah tempoh pengeraman selama empat jam pada kepekatan 0.05% ke atas sampel tauhu. Kesimpulannya, ekstrak daun jambu batu menunjukkan aktiviti antibakteria dan antispora terhadap *Bacillus* spp. yang diuji. Oleh itu ekstrak daun jambu batu berpotensi melakukan aktiviti anti-*Bacillus* yang boleh diperluaskan lagi untuk digunakan sebagai agen anti-*Bacillus* dalam makanan.

Kata kunci: Aktiviti antibakteria, aktiviti antispora, agen anti-*Bacillus*, *Bacillus* spp, ekstrak daun jambu batu

ACKNOWLEDGEMENTS

"OM NAMASHIVAYA"

All praise goes to the Almighty, Lord Shiva, the provider and protector. This work has been completed and successful only by HIS Grace and Mercy.

During my candidature as a postgraduate student, many kind-hearted souls contributed directly and indirectly toward completing my Master's research work. The first and the most person I respect Associate Professor Dr Yaya Rukayadi deserve the credit for my research work. He has been very helpful in making my research a success. May the Lord bless him with longevity and good health.

I also want to thank my supervisory committee member, Associate Professor Dr Rabiha Binti Sulaiman, for her timely insights and great comments. My sincere gratitude for Laboratory of Food Microbiology staff in Food 3, UPM for sharing their valuable time, knowledge and experiences.

I like to dedicate this research success to my loving family, father : Mr. Murugan, mother Mdm. Thevey, wife : Renuga Damothran, son : Darrshan Kalidass, Nimallan Kalidass and my sweet daughter : Logajanani Kalidass. Beside my family blessings, my dearest friend Dr. Rajesh Ramasamy's full support is very much appreciated in completing my project successfully.

My intensive and warmest gratitude to my colleagues as well for helping me throughout this research period.

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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LIST OF ABBREVIATIONS

CC	Column Chromatography
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
d	Doublet
dd	Doublet of doublet
DMSO	Dimethylsulfoxide
EMB	Eosin Methylene Blue
Ext.	Extraction
GC-MS	Gas Chromatography – Mass Spectrometry
GRAS	Generally Recognized as Safe
HCHO	Formaldehyde
HPLC	High Performance Liquid Chromatography
LC-MS/MS	Liquid Chromatography – Mass Spectrometry
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MHz	Megahertz
m/z	mass/charge ratio
MYP	Mannitol egg Yolk Polymyxin
NA	Nutrient agar
NIST	National Institute of Standards and Technology
PBS	Phosphate buffered saline

CHAPTER 1

INTRODUCTION

1.1 Background

The toxin-producing *Bacillus* spp. are an important human pathogen and they are the cause of common food poisoning (Enosi et al., 2021). Chemical preservations were used generally in the prevention of food spoilage and food poisoning which it generates side effects to human health and microbial resistance to the used of chemicals in the food and feed chains (Mostafa et al., 2018). *Bacillus* spp. are spore-forming bacteria that are resistant to various aggressive chemical and physical environment because their adaptive physiological features enable them to survive in various environmental conditions such as artic soils, desert sands, gastrointestinal tract of finfish and shellfish (Soltani et al., 2019). *Bacillus* spp. in the form of spore is resistant to physical and chemical treatments applied in the food industry and can persist throughout the food chain (Gauvry et al., 2021). The health problems and concerns arising from such usage of chemical preservatives has induced and increased the importance of finding other source of healthy, effective and safer natural alternative preservatives (Mostafa et al., 2018). Plant extracts can be used as an effective antimicrobial agent and natural alternative control measure to prevent food poisoning diseases and to avoid health hazard of chemical preservative usage (Mostafa et al., 2018).

The *Bacillus* sp. comprises multiple species capable of causing emetic or diarrheal foodborne illness (Carroll et al., 2019). Two types of gastrointestinal diseases are caused by *B. cereus* foodborne pathogens which are diarrhoea and emesis. Capability of spore formation, diarrhoea and emetic toxins production and biofilms of *B. cereus* are the major concern for the food industry which causes food safety issues (Huang et al., 2020a). *B. cereus*, *B. megaterium, B. pumilus* and *B. subtilis* were the most distinguishable four *Bacillus* spp. within enormous genetic diversity (Draganić et al., 2017). *Bacillus* spp. food-borned outbreaks were most frequent in starchy and vegetable foods (Glasset et al., 2016). *B. cereus* produced cereulide emetic toxin is heat and acid stable cyclic dodecadepsipeptide that can cause food intoxication with vomiting (Guérin et al., 2017).

Endospores resilience is known by their unique cellular structures which are developed by a complex process often initiated by a response to nutrient deprivation and they are intrinsically resistant to antimicrobial treatment and extreme environmental conditions. These endospores are highly heat-resistant spores (Berendsen et al., 2016). *B. subtilis* is an endospore- forming, Gram-positive bacterium and its soil-derived (Cui et al., 2018). *B. cereus* enterotoxin production and biofilm forming on steel surfaces in dairy plants can lead to serious hygiene problems and economic loss due to spoilage of dairy products (Kumari & Sarkar, 2016). *Bacillus* spp. spore germination can be triggered by activation of germination receptors (GRs) and by opening dipicolinic acid

(DPA) channels present in the inner membrane of the spores (Borch-Pedersen et al., 2017).

Food scientists and fermentation technologists work on effective selective prevention of *Bacillus* spp. sporulation (Sinnelä et al., 2019). *Bacillus* spp. produces extracellular enzymes which contribute to food pathogenicity and food spoilage (Özdemir & Arslan, 2019). Toxin and enzyme producing meat originated *B. cereus* and other *Bacillus* spp. isolates may cause food spoilage and can cause potential health risk for consumers (Özdemir & Arslan, 2019). *B. cereus* isolates from milk often found multiresistant with varied sensitivity to tested vancomycin, tetracycline, clindamycin and macrolides (Bartoszewicz & Czyżewska, 2021). Presence of enterotoxin genes were exhibited by the entire *Bacillus* spp. isolates (Nga Ombede et al., 2020). Increase in the concentrations of resistant forms in final products and food processing equipment were caused by spore-forming bacteria sporulation (Gauvry et al., 2021).

Formaldehyde and glutaraldehyde validated for inactivation of live spores of *Bacillus* spp. isolates (Chua et al., 2019). Formaldehyde, glutaraldehyde, sodium hypochlorite, hydrogen peroxide and guanidium thiocyanate inactivated *Bacillus* spp. spores and viruses by higher $\log_{10}$ level (Sagripanti et al., 2011). Treatment with 20% formaldehyde proved to be superior to several physical and chemical antispore inactivation capacities (Stöckel et al., 2010). Wet heating killed *B. cereus*, *B. megaterium* and *B. subtilis* spores significantly which impacted the killing mechanism or inactivation of *Bacillus* spp. spores by wet heating treatment (Coleman et al., 2010). The combination of thermal treatment with carvacrol and thymol which were natural origin antimicrobial were needed to kill 90% of the spore population (Periago et al., 2006).

Severe outbreak of multidrug resistance food-borne diseases was possible because pathogens are becoming resistance to various antibiotics hence it requires urgent explore to alternative antimicrobial compounds (Bano et al., 2021). Bioactive natural compounds aimed to reduce environmental and health hazards caused by synthetic products and effectively manage the antimicrobial resistance diffusion (Scavo et al., 2019). Natural ingredient terpinen-4-ol effectively inhibited *B. cereus* growth, spore germination and biofilm (Zhao et al., 2021). The ethanolic extract concentrations assessed in vitro for antimicrobial activity against *B. cereus*, *B. megaterium* and *B. subtilis* were most active followed by methanolic extracts (Scavo et al., 2019). *Psidium guajava*'s leaves, flowers, fruits, seeds, root and bark used as food and therapeutic agent in treatment related diseases caused by pathogenic or opportunistic microorganisms and organ malfunction (Morais-Braga et al., 2016).

Synthetic antibiotics usages to inhibit pathogenic microorganisms were currently prohibited in many countries which induced the researchers to look for plant source antimicrobial agents (Velarde et al., 2020). The leaves of *jambu batu* contains full of bioactive compounds and non-toxic to human health application in tofu (Ali et al., 2019). Flavonoids and phenolics compounds found in *jambu batu* leaves extract phytochemicals effectively inhibited growth of *B. pumilus* and *B. subtilis* thus can be formulated to combat the multidrug resistance food-borne diseases (Bano et al., 2021). *Jambu batu* were an important food crop and medicinal plant commonly found in tropical and subtropical countries, belonging to family Myrtaceae and reported widely used as antibacterial, antioxidant, anti-diarrheal, hypoglycemic, lipid lowering and hepatoprotective (Mishra et al., 2017). This study investigated the antibacterial and antispore properties of *jambu batu* leaves extract against *B. cereus*, *B. megaterium*, *B. pumilus* and. *B. subtilis.*

Tofu is a favorite food for many people today. But tofu has very short shelf life which it needs a preservative to extend the shelf life. Soaking tofu in dried *jambu batu* leaves extract showed potential natural preservatives chracteristics (Dewayani et al., 2019). Spores of *B. cereus* diarrheal and emetic were able to form biofilm on stainless steel and found to multiply and grow faster with good survival ability at 11˚C than 9 ˚C in tofu (Kwon et al., 2019). Tofu samples bought local supermarkets in Seoul, Korea were intentionally stored at 30 ˚C for 24h found the *B. cereus* were the most abundant colonies harvested and confirmed (Lee et al., 2017). Predictive growth and survival model developed for *B. cereus* detected 12.9% average contamination level at 1.84 log CFU/g for packaged tofu sample at 6 retail markets in Korea which key factor were temperature and time of packaged tofu (Kwon et al., 2020).

1.2 Problem Statements

Gastroenteritis and child diarrhea commonly can be treated with jambu batu by those who cannot afford or do not have access to antibiotics (Ara et al., 2017). *Jambu batu* leaves methanol, hexane and dichloromethane aqueous extract have effectively inhibited *B. pumilus* and *B. subtilis* (Bano et al., 2021). *Jambu batu* leaves extract through maceration with methanol and shade dryring shown antibacterial activity against *B. subtlis* (Muhammad Abbas et al., 2018). Natural alternatives are much preferred in food for their food safety assurance by consumers. This has inspired the current research to determine the antibacterial activity of *jambu batu* leaves ethanolic extract. Moreover, the specific antispore activity of *jambu batu* leaves ethanolic extract against *B. cereus*, *B. megaterium*, *B. pumilus* and *B. subtilis* have not been assessed yet.

A previous study has shown that quercetin isolated from maceration process of *jambu batu* leaves extract with methanol, ethyl acetate and chloroform has shown antibacterial activity against *B. subtilis* (Ranjan et al., 2017). Ca-Carrophyllene (22.70%), alpha cubebene (11.2%) and alpha-Humulene (5.91%) were the main phytoconstituents found in the extracts of *jambu batu* leaves tested and analyzed with GC-MS and HPLC (Afzal et al., 2019). Jambu batu leaves constituents' phenolic compound traditionally been used in regulating blood-glucose levels (Díaz-de-Cerio et al., 2017). *Jambu batu* leaf extract were reported with the presence of various bioactive compounds such as antimicrobial, antioxidant, antitumor and anticancer properties by GC–MS analysis (Shaheena et al., 2019). However, the antibacterial and antispore activities of ethanolic extract of *jambu batu* leaves extract and its fractions against *B. cereus, B. megaterium, B. pumilus* and *B. subtilis* have not been assessed. The phytochemical compounds in the *jambu batu* leaves ethanolic extract that are responsible for the antibacterial and the antispore activities against *Bacillus* spp. on tofu have never been examined. The active compounds of *jambu batu* leaves that are responsible for antibacterial and antispore activities against *Bacillus* spp. should be isolated. Thus, the general objective of the present study is to assess the antibacterial and antispore activities of *jambu batu* leaves extract and its active compounds against *B. cereus*, *B. megaterium, B. pumilus* and *B. subtilis* and its application in tofu.

1.3 Objectives

The objectives of this study are:

1. To determine the antibacterial activity of *jambu batu* extract on vegetative cells of *B. cereus*, *B. megaterium, B. pumilus* and *B. subtilis* and the stability of the extract on different pH and temperatures.
2. To determine the antispore activity of *jambu batu* extracts against spores of *B. cereus*, *B. megaterium, B. pumilus* and *B. subtilis.*
3. To analyse the pythochemicals present in the extract of *jambu batu* leaves through GC-MS and LC-MS/MS analyses.
4. To evaluate the effect of *jambu batu* leaves extract on bacterial population including *Bacillus* spp. in tofu.

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