**UNIVERSITI PUTRA MALAYSIA**

# ANTIBACTERIAL AND ANTISPORE ACTIVITIES OF CRUDE EXTRACT AND BIOACTIVE COMPOUNDS OF *Piper cubeba* L. BERRIES AGAINST *Bacillus* sp. AND APPLICATION OF THE EXTRACT FOR FOOD PRESERVATON

**FATIMAH KHALLEEFAH ALQADEERI**

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**FATIMAH KHALLEEFAH ALQADEERI**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy**

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**ANTIBACTERIAL AND ANTISPORE ACTIVITIES OF CRUDE EXTRACT AND BIOACTIVE COMPOUNDS OF *Piper cubeba* L. BERRIES AGAINST *Bacillus* sp. AND APPLICATION OF THE EXTRACT FOR FOOD PRESERVATON**

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**July 2019**

**Chairman : Associate Professor Yaya Rukayadi, PhD**
**Institute : Bioscience**

In the food industry, the *Bacillus* species, in particular, is known as organisms that cause foodborne diseases and food spoilage. *Bacillus* species are produce of spores and many of the spores of the *Bacillus* species have been shown to be resistant to heat, radiation, and disinfectants. A previous study has shown that crude extracts of tailed pepper (*Piper cubeba* L.) have potential antimicrobial activities against some of *Bacillus* species. The present study aims to analyse the antibacterial and antispore activities of *Piper cubeba* L. berries extracts on the vegetative cells and spores of *Bacillus cereus* ATCC33019, *B. subtilis* ATCC6633, *B. pumilus* ATCC14884, and *B. megaterium* ATCC14581. Results showed that exposing of *Bacillus* sp. to *P. cubeba* L. extract and its compounds resulted in an inhibition zone with a large diameter which ranged between 9.50 to 11.40 mm for the extract and 7.21 to 9.61 mm for the compounds. The MIC of the extract ranged between 0.156 – 0.313 mg/mL and the MBC at 2.5 mg/mL. Moreover, for the compounds the MIC range was between 63.0 to 125.0 µg/mL and MBC at 250.0 to 500.0 µg/mL against *Bacillus* sp. The time-kill curve plots showed that exposing *Bacillus* sp. to a concentration of 8× MIC for a period of four hours resulted in the death of all cells. The values of MIC and MBC showed a fluctuating trend when the bacteria were exposed to *P. cubeba* L. extract treated with different temperature in comparison to untreated extract. Generally, the pH altered extracts caused a variation in the MIC and MBC values of the *Bacillus* sp. The effect of using varying concentrations of extracts and compounds against the *Bacillus* sp. spores for varying periods of incubation were determined. Glutaraldehyde, which is a chemical sporicidal agent, was used as a positive control. *P. cubeba* L. extract at a concentration of 1.0% inactivated more than 3-$Log_{10}$ (90.99%) of the *Bacillus* sp. spores after an incubation period of four hours, and all the spores were killed at a concentration of 2.5%. The image of scanning electron microscope showed that the structure of spores were destroyed after treatment with 1% *P. cubeba* L. extract for one hour. The major volatile compounds, as determined using GC-MS, are $\beta$-cubebene, cubebol, $\alpha$-copaene, $\alpha$-cubebene, caryophyllene, 9,12-octadecadienoic acid, $\beta$-asarone, and germacrene-D. The non- volatile compounds identified through LC-MS are gallic acid, quinic acid, asaronaldhyde, epicatechin, clusin, cubebininolide, hemiarensin, $\beta$-asarone, hinokinin, ellagic acid, myricetin, and $\beta$-cubebene. The identified phytochemical compounds are similar with those in the literature and MS/MS databases. $\beta$-Asarone, asaronaldehyde, cubebin mixture and linoleic acid were successfully isolated and identified from the methanol extract of *P. cubeba* L. In general, a decrease of 3 $Log_{10}$ of *Bacillus* sp., total plate count, *E. coli* and coliform bacteria on the tofu sample was observed when these bacteria were exposed to 0.50% (v/v) *P.cubeba* L. extract. In conclusion, *P.cubeba* L. extracts and its compounds show a promising potential of antibacterial and sporicidal activities against the *Bacillus* sp. and thus can be developed as an anti-*Bacillus* agent.

**Keyword:** antibacterial, antispore, *Bacillus* sp, *P. cubeba* L. berries extract, $\beta$-asarone, asaronaldehyde, and linoleic acid.

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

**AKTIVITI ANTIBAKTERIA DAN ANTISPORA EKSTRAK MENTAH DAN SEBATIAN BIOAKTIF *Piper cubeba* L. BERI TERHADAP *Bacillus* sp. DAN PENGGUNAAN EKSTRAK UNTUK PENGAWETAN MAKANAN**

Oleh

**FATIMAH KHALLEEFAH ALQADEERI**

**Julai 2019**

**Pengerusi : Profesor Madya Yaya Rukayadi, PhD**
**Institut : Biosains**

Dalam industri makanan, spesies *Bacillus*, khususnya, dikenali sebagai organisma yang menyebabkan penyakit bawaan makanan dan kerosakan makanan. Spesis *Bacillus* menghasilkan spora dan banyak spora spesies *Bacillus* telah terbukti tahan terhadap haba, radiasi, dan pembasmian kuman. Kajian terdahulu menunjukkan bahawa ekstrak mentah lada berekor (*Piper cubeba* L.) mempunyai potensi aktiviti antimikrob yang kuat terhadap beberapa spesies *Bacillus.* Objektif kajian ini adalah untuk menganalisis aktiviti antibakterial dan antispora ekstrak beri *P. cubeba* L. ke atas sel dan spora vegetatif *Bacillus cereus* ATCC33019, *B. subtilis* ATCC6633, *B. pumilus* ATCC14884 dan *B. megaterium* ATCC14581. Dapatan menunjukkan bahawa ekstrak *P. cubeba* L. dan sebatiannya memperlihatkan diameter zon inhibisi yang signifikan dalam julat 9.50 - 11.40 dan 7.21 - 9.61 mm, terhadap sel vegetatif *Bacillus* sp. MIC 0.156 – 0.313 mg/mL dan MBC 2.500 mg/mL bagi ekstrak. Sebatian adalah masing-masing dalam julat MIC 63.00 – 125.0 µg/mL dan MBC 250.0 – 500.0 µg /mL terhadap semua strain yang diuji. Plot lengkungan masa-bunuh memperlihatkan *Bacillus* sp. telah dibunuh dalam masa inkubasi ke-4 pada konsentrasi 8× MIC. Tren peningkatan, penurunan dan konstan nilai MIC dan MBC telah diperoleh ketika haba terawat pada ekstrak *P. cubeba* L. Jika dibandingkan dengan ekstrak bukan terawat haba. Umumnya, ekstrak terubah pH membezakan nilai MIC dan MBC *Bacillus* sp. Tambahan pula, kesan ekstrak dan sebatian pada konsentrasi yang berbeza, tempoh inkubasi telah ditentukan terhadap spora *Bacillus* sp. Glutaraldehyde, suatu agen kimia sporisidal , telah digunakan sebagai kawalan positif. Ekstrak *P. cubeba* L. tidak diaktifkan lebih daripada 3-log10 (90.99%) bagi spora strain *Bacillus* pada konsentrasi 1.0% selepas inkubasi ke-4 dan spora tersebut telah dibunuh sepenuhnya pada 2.5%. Berdasarkan pemerhatian mikroskopi elektron penimbal, struktur spora telah dimusnahkan selepas dirawat dengan 1% ekstrak *P. cubeba* L. selama I jam. Sebatian volatil utama yang telah ditentukan menggunakan GC-MS ialah *β*-cubebena, cubebol, *α*-copaene, *α*-cubebena, kariofilena, 9, asid 12-

Oktadekadienoik, *β*-asarona, dan germakrena-D. Sebatian bukan volatil yang dikenal pasti oleh LC-MS ialah are gallic acid, quinic acid, asaronaldhyde, epicatechin, clusin, cubebininolide, hemiarensin, *β*-asarone, hinokinin, ellagic acid, myricetin, and *β*-cubebene. Pengenalpastian sebatian fitokimia telah disokong oleh literatur dan pangkalan data MS. *β*-asarona, asaronaldehida, campuran cubebin dan asid linoleik telah berjaya diasingkan, dan dikenal pasti daripada ekstrak/pecahan metanol *P. cubeba* L. Umumnya, pengurangan 3 $Log_{10}$ *Bacillus* sp. jumlah kiraan plat, *E. coli* dan bakteria koliform telah mula diteliti pada ekstrak *P. cubeba* L. pada 0.50% (v/v) ke atas sampel tofu. Kesimpulannya, ekstrak beri *P. cubeba* L. dan sebatiannya menunjukkan aktiviti antibakterial dan sporisidal yang berpotensi terhadap spesis *Bacillus*, oleh sebab itu, ia dapat dibangunkan sebagai agen anti-*Bacillus*.

**Kata kunci:** Aktiviti antibakterial, aktiviti antispora, *Bacillus* sp, *P. cubeba* L. ekstrak beri, *β*-asarone, asaronaldehyde, and linoleic acid.

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*"In the name of Allah, the most Gracious and the most Merciful"*

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| Signature: | |
| Name of Member of Supervisory Committee: | Associate Professor Dr. Faridah binti Abas |

| | |
|---|---|
| Signature: | |
| Name of Member of Supervisory Committee: | Professor Dr. Khozirah binti Shaari |

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| | |
|---|---|
| $Ca^{2+}$ | Calcium cation |
| $Ca^{2+}$-DPA | Calcium cation and dipicolinic acid chelate |
| CC | Column Chromatography |
| CFU | Colony forming unit |
| CHX | Chlorhexidine |
| CLSI | Clinical and Laboratory Standards Institute |
| *C. butyricum* | *Clostridium butyricum* |
| *C. perfringens* | *Clostridium perfringens* |
| *d* | Doublet |
| *dd* | Doublet of doublet |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| DPA | Dipicolinic acid |
| GC-MS | Gas Chromatography – Mass Spectrometry |
| GRAS | Generally Recognised as Safe |
| h | Hour |
| Hz | Hertz |
| HPLC | High Performance Liquid Chromatography |
| IBS | Institute of Bioscience |
| INT | Iodonitrotetrazolium violet |
| KCTC | Korean Collection for Types Culture |
| L | Litre |
| $LC_{50}$ | Median Lethality Concentration |
| LC-MS | Liquid Chromatography – Mass Spectrometry |
| M | Molarity |
| MHA | Mueller Hinton agar |
| MHB | Mueller Hinton broth |
| min | Minute |
| Me OH | Methanol |
| MIC | Minimum Inhibitory Concentration |
| MBC | Minimum Bactericidal Concentration |
| MHz | Megahertz |
| *m/z* | mass/charge ratio |
| mL | Milliliter |
| MS | Mass Spectrometry |
| NA | Nutrient agar |
| NB | Nutrient broth |
| NIST | National Institute of Standards and Technology |
| NMR | Nuclear Magnetic Resonance |
| PBS | Phosphate buffered saline |

| | |
|---|---|
| Ppm | Part Per Million |
| Rpm | Revolutions per minute |
| SEM | Scanning Electron Microscopy |
| WHO | World health organization |
| µg | Microgram |
| UV | Ultraviolet |
| wk | Week |
| $^{1}$H-NMR | $^{1}$H Nuclear Magnetic Resonance |
| $^{13}$C NMR | $^{13}$C Nuclear Magnetic Resonance |
| CC | Column Chromatography |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

The growth of microbial pathogens in foods has to be controlled, and various methods be utilized to prevent growth of the pathogenic bacteria in food. Several methods for controlling microbial growth involve the utilization of synthetic and natural antimicrobial agents (Tippayatum & Chonhenchob, 2007). Spore forming bacteria, such as *Bacillus* and Clostridium species react to environmental stress by forming a dormant structure known as an endospore (bacterial spore or seed) through sporulation (Leggett et al., 2012). Many of the spores of the *Bacillus* species have been shown to be resistant to heat, radiation and disinfectants. The microbiological risks of these species cannot be contained through refrigeration (Fernández-No et al., 2013). As a result, Bacillus has been frequently shown to cause contamination. In the food industry, the *Bacillus* species in particular is known as organisms that cause foodborne diseases and food spoilage (Kim et al., 2014).The genus Bacillus comprises of *Bacillus cereus*, *B. subtilis*, *B. pumilis*, and *B. megaterium*. These species have been shown to have a good ability to adapt to changes in the environment. Bacillus sp. are rod-shaped, Gram-positive, sporulating, motile, aerobic bacteria and are widespread in the environment (Kim et al., 2014). B. cereus can cause diarrhoea and emetic food poisoning. The type of Bacillus that causes diarrhoea has been associated with foods containing meat, sauces, vegetables, and milk products (Kim et al., 2014). Emetic type *Bacillus* has been found to cause nausea and vomiting and it was associated with the consumption of rice and farinaceous foods such as noodles and pasta (Altayar & Sutherland, 2006; Kim et al., 2013).

In some instances, bacterial contamination is followed by the production of endospores. *B. cereus and B. subtilis* are known to be causative agents in food spoilage and food poisoning. Cross contamination occurs when the spores are transferred from contaminated food to uncontaminated foods (Stenfors Arnesen et al., 2008). Among the types of food often contaminated by *B. subtilis* and *B. cereus* are milk, starchy food, vegetables and fruits. Consumption of the contaminated food could cause two types of gastrointestinal disorder, including diarrhoeal and emetic syndromes. Diarrhoea is caused by different toxins that are formed in food as well as in the small intestine of humans, while emetic illness is caused by preformed toxins that are present in food (Rukayadi et al., 2009a). Some foodborne illnesses are caused by the spores of B. cereus that has the ability to survive cooking temperatures, and leaving cooked rice at room temperature could facilitate germination of spores (Choi et al., 2014). B. subtilis is typically considered as pathogen to humans. This Bacillus may contaminate food and could occasionally cause food poisoning (Fernández-No et al., 2013). Foodborne diseases have been shown to have the underlying factors that are associated with food products (including rice) that are contaminated by B. subtilis (Kim et al., 2013). For example in 2005, an epidemic was determined to be caused by spoiled milk powder

(Fernández-No et al., 2013). *Bacillus* spores are highly resistant to several types of chemical disinfectants. In addition, the commercially available chemical sporicidal agents which can destroy *Bacillus* spores is very limited, and these agents, when available, have to be handled with special precautions. This includes the handling of glutaraldehyde and toxic formaldehyde (Kida et al., 2004). On the other hand, thermal food processing is an effective and cheap method for producing safe food that is free from unwanted microbes and enzymatic reactions. It should be noted that thermal processing has its limitations, including deterioration of organoleptic qualities and reduced nutrient content (Cho et al., 2008). Consequently, increasing attention is being given to developing safe, effective, and stable natural antibacterial and antispore agents (Kida et al., 2004). This increases the interest to identify antimicrobial compounds from plant sources.

Natural products contain ingredients that have a promising potential of new types of therapeutic agents (Newman et al., 2003). Globally, there are about 500,000 species of plants; however, considering that phytochemical investigations have been performed on only 1% of these plant species, it is very likely that novel bioactive compounds would be discovered in the future (Palombo, 2011). The scientific name of cubeb or tailed pepper (named on account of the attached stalks) is *Piper cubeba* L. The tailed pepper which a perennial climbing plant is also known in Indonesia as the Javanese pepper (Al-Tememy, 2013). The pepper normally has between four and six leaves, round branches and a climbing stem, and it is usually about half an inch long and between half an inch to two inches wide (Al-Tememy, 2013). There are over 700 species under the genus *Piper* which can be found in both hemispheres of the earth. The family of Piperaceae contains the species *P. cubeba* L., which has been used as a spice in countries such as Indonesia, India, Morocco, and Europe since the middle ages (Silva et al., 2007).

*P. cubeba* L. is used to treat illnesses, for instance dysentery, syphilis, abdominal pain, diarrhoea, enteritis, and asthma (Usia et al., 2005). Many plants from the *Piper* genus are used in traditional herbal medicine. Species from the Piper genus are known to have antifungal, insecticidal, anthelminthic, and antitumor properties. Polyhydroxy cyclohexanes that are isolated from *P. cubeba* L. have been shown to have tumor inhibition, antileukemic and antibiotic properties. Piperine is an alkaloid from the pyridine group and occurs naturally in plants of the Piperaceae family. Piperine is known to be utilised rather extensively in medicinal preparations, including herbal cough syrups; it has been shown to be anti-inflammatory, anti-malarial (Nahak & Sahu, 2011). This study investigates the antibacterial and antispore properties of P. cubeba L. extracts against *B. cereus, B. subtilis, B. pumilus* and *B. megaterium*.

## 1.2 Problem Statements

Biological products derived from plant sources exhibited a wide range of antibacterial properties against microorganisms, including pathogenic microbes. Several recents studies on antimicrobial elements in food products were able to purge the microbes responsible for causing food spoilage, thereby extending the expiry date of food products (Tajkarimi et al., 2010). Natural products are used in food by virtue of their safety and pleasant fragrance, and their use has attracted the interest of consumers. It is imperative to discover natural additives with sporicidal activities, or natural sporicidal agents that have the ability to prevent *Bacillus* spores from contaminating rice or starchy foods. This has inspired the present study to determine the sporicidal activity of plants which have a wide range of medicinal properties.

A previous study has shown that crude extracts of tailed pepper (*P. cubeba* L.) have powerful antimicrobial activities against B. cereus and B. subtilis vegetative cells (Vaghasiya et al., 2007; Singh et al., 2008). The antimicrobial activity was achieved through various combinations of phenols, triterpenoids, lignins, alkaloids, saponins, tannins and flavonoids (Visweswari et al., 2013). Unfortunately, the antibacterial and antispore activities of methanolic extract of P. cubeba L. berries and its fractions against other species of *Bacillus,* such as *B. pumilus* and *B. megaterium*, have not been assessed. To the best of the researcher's knowledge, the phytochemicals compounds in the *P. cubeba* L. berries that are responsible for the antibacterial and the antispore activities against *Bacillus* sp. have never been examined. The active compounds of *P. cubeba* L. berries that are responsible for antibacterial and antispore activities against *Bacillus* sp. should be isolated. Thus, the general objective of the present study is to assess the antibacterial and antispore activities of *P. cubeba* L. berries extract and its active compounds against *B. cereus, B. pumilus, B. megaterium* and *B. subtilis.*

## 1.3 Objectives

1) To determine the antibacterial activity of *P. cubeba* L extract and its fractions on vegetative cells of *B. cereus*, *B. subtilis*, *B. pumilus* and *B. megaterium* through disc diffusion test, minimum inhabatory concentration, minimum bacteriocidal concentration and killing time curve.
2) To determine the sporicidal activity of *P. cubeba* L. extracts and its fractions against *B. cereus, B. subtilis, B. pumilus,* and *B. megaterium* spores and examine their effects using scanning electron microscopic (SEM).
3) To analyse the chemical constituents present in the extract and active fractions of *P. cubeba* L. through GC-MS and LC-MS analyses.
4) To isolate, and identify the bioactive compounds from P. cubeba L. berries that are responsible for antibacterial and antispore activities.
5) To evaluate the effect of *P. cubeba* L. extract on microorganism in tofu samples.

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**BIODATA OF STUDENT**

The student, Fatimah Alqadeeri, was born on 11 March 1979 at Benghazi, Libya. She studied the primary and secondary schools in her city during the period from 1985 to 1997. In 1998 she did her bachelor degree at Benghazi University Sciences Faculty. She graduated in 2001 and obtained a Degree in Botany. She worked for nine years in a secondary school as a teacher. In April 2008 she studied in Academy of Libya University Sciences Faculty Botany field. In 29 December 2010 she obtained the degree of Master. She worked for four years in Sciences Faculty at Zawia University as a lecturer. In first of May 2015 she came to Malaysia and studied English in Malvern College, Kuala Lumpur for five months. In October 2015 she studied English in ELS School, Kuala Lumpur for two months and got Ielts certification. She obtained admission in Institute of bioscience Universiti Putra Malaysia to persue her Ph.D program under the supervision of the esteemed Associate Prof. Dr Yaya Rukayadi.

# LIST OF PUBLICATIONS

Fatimah Alqadeeri, Faridah Abas, Khozirah Shaari, and Yaya Rukayadia. (2019). Antibacterial and Antispore Activities of Isolated Compounds from *Piper cubeba* L., *Molecules.* 24, 3095; doi: 10.3390/molecules24173095.

Fatimah Alqadeeri, Faridah Abas, Khozirah Shaari, and Yaya Rukayadia. (2019). Identification of the Chemicals Constituent Present in the Extract/Fractions of *Piper cubeba* L. Berries through GC-MS and LC-MS Analyses. (Manuscript were submitted to Journal of Analytical Method in Chemistry).

Fatimah Alqadeeri, Faridah Abas, Khozirah Shaari, and Yaya Rukayadia*. (2019). Antibacterial and Sporicidal Activities of Tailed Pepper (*Piper cubeba* L.) Berries Extracts and its Fractions against Vegetative Cells and Spores of *Bacillus* sp. (Manuscript will be submitted to Journal of Pharmacy and Pharmacology).

**Proceedings:**

**Fatimah Alqadeeri,** Yaya Rukayadi, Faridah Abas and Khozirah Shaari. Antibacterial and Antispore Activities of Tailed Pepper ( *Piper cubeba* L.) Berries Extracts against *Bacillus* sp . International Conference on Natural Products 2018 (oral presentation). Penang, Malaysia.

**Fatimah Alqadeeri,** Yaya Rukayadi, Faridah Abas and Khozirah Shaari. Phytochemical Constituents and Antibacterial Activity of *Piper cubeba* L. fractions Against Vegetative cell of *Bacillus* sp. Monash Science Symposium 2018, Malaysia (oral presentation).

Yaya Rukayadi, **Fatimah Alqadeeri** and Abdelgani Mohamed . Identification and isolation Active Antispore metabolites from *Sygygium polyanthum* L. leaves and *Piper cubeba* L. Berries Extract through GCMS, LCMS and H-NMR-Based Metabolomics. International Conference on Science, Technology and Humanities 2018. Badung, Bali.

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