UNIVERSITI PUTRA MALAYSIA

ANTISTAPHYLOCOCCAL AND ANTIBIOFILM ACTIVITIES OF ETHANOLIC EXTRACT OF Piper cubeba L.

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ANTISTAPHYLOCOCCAL AND ANTIBIOFILM ACTIVITIES OF ETHANOLIC EXTRACT OF *Piper cubeba* L.

By

SELVI VELU

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

April 2018

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DEDICATION

This thesis is dedicated to my beloved family, supervisors and friends

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

ANTISTAPHYLOCOCCAL AND ANTIBIOFILM ACTIVITIES OF ETHANOLIC EXTRACT OF *Piper cubeba* L.

By

SELVI VELU

April 2018

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

Staphylococcus aureus is a very adaptable foodborne pathogen responsible for food outbreaks and a source of cross contamination in fresh and processed foods worldwide. Methicillin-resistant *S. aureus* (MRSA) strains which were initially addressed in humans is being marked as emerging community acquired pathogen in recent years. The resistance of staphylococci towards various novel and existing antimicrobial agents has developed as a problem. Noticeably, the significance of medicinal plants and traditional health practices has gained increasing attentions principally in solving the impact of emergence, spread and resistance of microorganisms of the world. Simultaneously, there is a concern in the field of food safety, quality and preservation to counter the rising of resistant pathogens and the limitation of synthetic chemical additives in the food system. Among the plants investigated to date, one showing enormous potential is the pepper family otherwise known as Piperaceae. *Piper cubeba* L. has infinite medicinal properties and practiced to treat a number of diseases. In this study, the *Piper cubeba* L. extract was examined for its antistaphylococcal and antibiofilm activity against *Staphylococcus aureus* food isolates, *S. aureus* human isolates and a reference strain *S. aureus* ATCC6538P. *P. cubeba* L. berries were extracted using ethanol, methanol and water as solvent. The antimicrobial activity of ethanol, methanol and water extracts of *P. cubeba* L. against *S. aureus* isolates in terms of disc diffusion was performed. Further, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time kill curve were performed using ethanol extract referring to standard method of Clinical and Laboratory Standards Institute (CLSI). The ethanolic *P. cubeba* L. extract was assessed for its stability at various temperature and pH conditions relevant to food production and storage. Scanning electron microscopy (SEM) was used to visualize the effect of ethanolic *P. cubeba* L. extract on representative *S. aureus*. The bioactive compounds present in ethanolic *P. cubeba* L. extract were determined using Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS). Biofilm forming patterns of *S. aureus* isolates and antibiofilm activity of ethanolic *P. cubeba* L. extract were performed in presterilized flat-bottom 96-well microplate and were quantified using

2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino) carbonyl]-2H tetrazolium-hydroxide (XTT) reduction assay. The effect of ethanolic *P. cubeba* L. extract was assessed on cut-up parts of chicken (breast, wing and drumstick) as natural sanitizer. Visual attribute sensory acceptability was evaluated on raw and steamed chicken meat treated with ethanolic *P. cubeba* L. extract. Toxicity effect of the extract was evaluated using the brine shrimp lethality assay. The results showed that ethanol and methanol *P. cubeba* L. extract exhibited significant diameter of inhibition zone in the range of 7.23 – 8.50 mm and 7.13 – 8.57 mm respectively, against *S. aureus* isolates. MIC and MBC of ethanolic extract were in the range of 0.625 – 2.5 mg/ml and 1.25 – 5 mg/ml against all tested isolates, respectively. The time-kill curve plots revealed *S. aureus* food isolates CM10 and CM14 were killed within 1 h of incubation at a concentration of 4× MIC. As for *S. aureus* human isolate and ATCC6538P the time-kill curve plot revealed bacteriostatic effect of the *P. cubeba* L. extract. Trends of increasing, decreasing and constant MIC and MBC values were observed upon heat treated ethanolic *P. cubeba* L. extract as compared to non-heat treated extract. Generally, the pH altered extracts varied the MIC and MBC values of the *S. aureus* isolates. Under SEM observation, the treated cells underwent a significant transition from initially smooth surfaces cells to completely distort and shrank cells. The major volatile bioactive compounds determined using GC-MS were β-cubebene, cubebol, α-copaene, α-cubebene, caryophyllene and germacrene-D. Non- volatile compounds identified by LC-MS were 5,7-dihydroxy-3',4'-dimethoxy-6,8-dimethylflavone, brosimacutin B, phellodensin D, 9E,12Z,15Z-octadecatrienoic acid, 2,4-dimethyl-tetradecanoic acid, eriodictyol 7,3'-dimethyl ether 4'-prenyl ether, erioflorin methacrylate, 12-oxo-5E,8E,10Z-dodecatrienoic acid and (S)-β-himachalene. *S. aureus* isolates exhibited strong adherent ability at 37°C and 28°C while weak and non-adherent capability mostly observed at 7°C. The biofilm formation of *S. aurues* can be inhibited by sessile minimal inhibitory concentration (SMIC) of 6.25 – 25 mg/ml and the formed *S. aurues* biofilm can be eradicated by minimal biofilm eradication concentration (MBEC) values of 25 – 50 mg/ml. In general, reduction 3 Log_{10} of *S. aureus,* total plate count, *E. coli* and coliform was started to observe at ethanolic *P. cubeba* L. extract of 0.50% on the cut-up parts of chicken. *P. cubeba* L. sanitized raw and steamed chicken meat at 0.05%, 0.50% and 5.00% was accepted by the panelists. Brine shrimp lethality test exhibited no significant toxicity (LC_{50} = 6.98 mg/ml) against brine shrimp naupili. In conclusion, ethanolic *P. cubeba* L. extract with potential antimicrobial and antibiofilm activities could be prominently marked as a valuable natural antimicrobial, antibiofilm, and sanitizing agent in the food safety field.

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

AKTIVITI ANTISTAPHYLOCOCCAL DAN ANTIBIOFILM EKSTRAK ETANOL *Piper cubeba* L.

Oleh

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April 2018

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Staphylococcus aureus adalah patogen yang dapat disesuaikan dengan makanan dan menyebabkan wabak makanan. Ia menjadi sumber pencemaran dalam makanan segar dan diproses di seluruh dunia. Strain methicillin resistant *S. aureus* (MRSA) yang pada mulanya ditangani pada manusia ditandai sebagai pathogen dikaitkan dengan komuniti pada tahun-tahun kebelakangan ini. Rintangan staphylococci terhadap pelbagai novel dan agen antimikrob sedia ada telah berkembang sebagai masalah. Ketahuilah, pentingnya tumbuhan ubatan dan amalan kesihatan tradisional telah mendapat perhatian yang lebih banyak terutamanya dalam menyelesaikan kesan kemunculan, penyebaran dan penentangan mikroorganisma dunia. Pada masa yang sama, terdapat keprihatinan dalam bidang keselamatan makanan, kualiti dan pemeliharaan untuk mengatasi peningkatan patogen yang tahan dan batasan bahan kimia sintetik dalam sistem makanan. Antara tanaman yang disiasat setakat ini, yang menunjukkan potensi yang besar adalah keluarga lada yang dikenali sebagai Piperaceae. Piper cubeba L. mempunyai sifat perubatan yang tidak terhingga dan diamalkan untuk merawat sejumlah penyakit. Dalam kajian ini, ekstrak *P. cubeba* L. telah diperiksa untuk aktiviti antimikrob dan antibiofilmnya terhadap *Staphylococcus aureus* isolate makanan, *S. aureus* isolate manusia dan *S. aureus* ATCC6538P. Biji *P. cubeba* L. telah diekstrak menggunakan etanol, metanol dan air. Aktiviti antimikrob *P. cubeba* L. terhadap *S. aureus* dari segi cakera penyebaran telah dibuat menggunakan ekstrak etanol, metanol dan air. Kepekatan perencatan minimum (MIC), kepekatan pembunuhan bakteria (MBC) dan lengkungan masa-pembunuhan ekstrak telah dilakukan menggunakan ekstrak etanol merujuk kepada kaedah yang dinyatakan dalam Clinical and Laboratory Standards Institute (CLSI). Kestabilan ekstrak etanol *P. cubeba* L. telah dinilai pada pelbagai suhu dan keadaan pH. Mikroskop electron pengimbas (SEM) telah digunakan untuk menggambarkan kesan ekstrak etanol *P. cubeba* L. pada *S. aureus* yang terpilih. Sebatian bioaktif yang terdapat di dalam ekstrak etanol *P. cubeba* L. telah ditentukan menggunakan Gas

Chromatography-Mass Spectrometry (GC-MS) dan Liquid Chromatography-Mass Spectrometry (LC-MS). Corak pembentukan biofilm *S. aureus* dan aktiviti antibiofilm ekstrak etanol *P. cubeba* L. telah dilakukan dalam pra-disterilkan 96-microplate dan diukur menggunakan 2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino) karbonil]-2H-tetrazolium-hidroksida (XTT) assay. Kesan ekstrak etanol *P. cubeba* L. dinilai pada bahagian potongan ayam (dada, sayap dan drumstick) sebagai agent sanitizer semulajadi. Kesesuaian deria dinilai pada daging ayam mentah dan kukus yang telah dirawat dengan ekstrak etanol *P. cubeba* L. Ketoksikan ekstrak etanol *P. cubeba* L. telah dinilai menggunakan ujian kematian udang naupili air garam. Keputusan menunjukkan bahawa ekstrak etanol dan metanol *P. cubeba* L. telah menunjukkan diameter zon perencatan (DIZ) ketara dalam lingkungan 7.23 – 8.50 mm dan 7.13 – 8.57 mm masing- masing terhadap isolate *S. aureus*. Nilai MIC dan MBC ekstrak telah didapati dalam lingkungan 0.625 - 2.5 mg/ml dan 1.25 - 5 mg/ml, masing-masing. Plot masa-pembunuhan telah menunjukkan bahawa *S. aureus* isolate makanan CM10 dan CM14 dapat dibunuh pada masa 1 jam pada kepekatan 4× MIC. Ekstrak *P. cubeba* L. telah menujukkan aktiviti bacteriostatik pada plot masa-pembunuhan *S. aureus* isolate manusia dan ATCC6538P. Tren nilai MIC dan MBC yang meningkat, berkurang dan berkekalan diperhatikan pada ekstrak etanol *P. cubeba* L. yang telah dirawat haba berbanding dengan ekstrak yang tidak dirawat haba. Secara amnya, nilai MIC dan MBC yang berbeza didapati pada ekstrak yang telah berubah nilai pH. Daripada pemerhatian menggunakan SEM, sel-sel yang telah dirawat menjalani peralihan yang ketara dengan keadaan permukaan sel-sel yang terganggu dan merosot. β-cubebene, cubebol, α-copaene, α-cubebene, caryophyllene and germacrene-D adalah sebatian bioaktif yang telah ditentukan menggunakan GC-MS. 5,7-dihydroxy-3',4'-dimethoxy-6,8-dimethylflavone, brosimacutin B, phellodensin D, 9E,12Z,15Z-octadecatrienoic acid, 2,4-dimethyl-tetradecanoic acid, eriodictyol 7,3'-dimethyl ether 4'-prenyl ether, erioflorin methacrylate dan 12-oxo-5E,8E,10Z-dodecatrienoic acid adalah sebatian bioaktif yang telah ditentukan menggunakan LC-MS. Isolate *S. aurues* menunjukkan keupayaan berpengaruh yang tinggi pada suhu 37°C dan 28°C manakala keupayaan lemah dan tidak berpengaruh kebanyakannya diperhatikan pada suhu 7°C. Pembentukan biofilm dapat direncat pada nilai sessile kepekatan perencat minimal (SMIC) pada lingkungan 6.25 – 25 mg/ml dan kepekatan pembasmian biofilm minimal (MBEC) yang telah didapati adalah pada lingkungan 25 – 50 mg/ml. Secara amnya, pengurangan 3 Log_{10} *S. aureus*, jumlah bacteria, *E. coli* dan koliform mula diperhatikan pada ekstrak etanol *P. cubeba* L. 0.50% pada bahagian potongan ayam. Daging ayam mentah dan kukus yang telah disanitize dengan ekstrak etanol *P. cubeba* L. pada kepekatan 0.05%, 0.50% dan 5.00% telah diterima oleh panelis. Ekstrak etanol *P. cubeba* L. didapati tidak menunjukkan ketoksikan yang ketara (LC_{50} = 6.98 mg/ml) terhadap udang naupili air garam. Sebagai kesimpulan, ekstrak etanol *P. cubeba* L. dengan potensi antimikrob dan aktiviti antibiofilm boleh dianggap sebagai agen antimikrob, antibiofilm, dan agen sanitizer semulajadi dalam bidang keselamatan makanan.

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I certify that a Thesis Examination Committee has met on 16 April 2018 to conduct the final examination of Selvi a/p Velu on her thesis entitled "Antistaphylococcal and Antibiofilm Activities of Ethanolic Extract of *Piper cubeba* L." 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 Doctor of Philosophy.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CHX	Chlorhexidine
Cfu	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
ddH_2O	Deionized distilled water
DIZ	Diameter of inhibition zone
DMSO	Dimethyl sulfoxide
EPS	Extracellular polymeric substances
G	Gram
GC-MS	Gas Chromatography - Mass Spectrometry
GRAS	Generally Regarded as Safe
H	Hour
HPLC	High Performance Liquid Chromatography
IBS	Institute of Bioscience
Lf	Lactoferrin
LC50	Median Lethality Concentration
LC-MS	Liquid Chromatography - Mass Spectroscopy
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MIC	Minimum Inhibitory Concentration
Min	Minute
Ml	Milliliter
µl	Microliter
MRSA	Methicillin resistant *Staphylococcus aureus*

Nacl	Sodium chloride
OD	Optical density
PBS	Phosphate buffered saline
SEM	Scanning Electron Microscopy
SMIC	Sessile Minimum Inhibitory Concentration
spp.	Species
TPC	Total plate count
TSA	Tryptic soy agar
UV	Ultraviolet
UPM	Universiti Putra Malaysia
WHO	World health organization

CHAPTER 1

INTRODUCTION

1.1 Background

Worldwide, there is awareness about the consequences of emerging, spread and resistance of microorganisms. Likewise, there happens to be about 30% rise yearly on people suffering from foodborne diseases significantly in industrialized countries (WHO, 2016). A survey has revealed that about 60% of the foodborne infections are related to microbial transfer from the food processing equipment's to foods that are being processed and vice versa. Disgracefully, people acquired with infections due to food contamination with foodborne pathogens are most likely leads to death (Bridier *et al.,* 2014).The consequences include increased rate of foodborne diseases in several countries, tremendous economic losses due to food spoilage, rising cost of treatment and occurrence of treatment failure.

The significance of medicinal plants and traditional health practices has gained increasing attentions principally in solving the health care problems of the world. Extensively, traditional practices have been linked to history of human interfaces with the environment (Sasidharan *et al.,* 2011). Moreover, in recent years, adversative toxicological reports on many synthetic compounds have led to an enormous request for natural antioxidants/ preservatives. Thus, most of the recent discoveries has been focused towards natural sources, particularly of plant origin (Shah *et al.,* 2014).

Interestingly, nature has been an utmost continuous source of medicinal plants that harbor infinite resources of bioactive compounds. About 80% of world requirements of medicines for health requirements are obtained from botanical preparations in the form of plant extracts or their bioactive components. World Health Organization (WHO, 2005) has outlined medicinal plants as plants/ part of plants that possess compounds/ components that reveal therapeutic properties or those that synthesize metabolites that can be converted into useful drugs and antimicrobial agents. The wide ranges of pure compounds or standardized extracts were being utilized widely for the treatment of infectious as well as chronic diseases. Remarkably, these phytochemicals are being well defined as safe with less adverse effects (Sasidharan *et al.,* 2011). Indeed, the research on alternative antimicrobials is more crucial than ever (Salaheen *et al.,* 2014).

Pepper family otherwise known as Piperaceae is one that exhibits enormous potential among those medicinal plants researched to date. *Piper cubeba* L. belonging to Piperaceae family known as cubeb or tailed pepper. It has been commonly incorporated in food preparations and into food to boost flavor and impart piquancy/ spiciness despite being well known as a spice. It has infinite medicinal properties and

practiced to treat a number of diseases. Therefore, *P. cubeba* L. extract could be identified as an infinite novel natural antimicrobial/ antibiofilm agent in inhibiting human pathogen/ foodborne pathogen, *S. aureus*.

1.2 Problem Statements

Pathogens perseverance in the food chain is majorly due to the bacterial contamination on the food contact surfaces. *Staphylococcus aureus* is a very adaptable foodborne pathogen that has been recurrently related to food processing environments especially on poultry and meat industries and mostly associated with slaughtering process in the food processing facilities. Remarkably, it is concerned as a source of cross contamination and reveals behavior of biofilms production in the food production and processing conditions (Schneid *et al.*, 2016). Moreover, persistence of *S. aureus* has been associated with hospital environments inclusive issues on biofilm formations (Mari *et al.*, 2007). Noticeably, in current years, the resistance of staphylococci towards various novel and existing antimicrobial agents has developed as a problem.

Methicillin-resistant *S. aureus* (MRSA) strains were initially addressed in humans in the year of 1960s. However, in recent years MRSA is being classified as emerging community acquired pathogen. Those strains associated in hospital environments and farms are now commonly found in foods such as poultry, dairy products, milk, meat and fish products which prompted awareness among researchers. Moreover, these MRSA has been reported for its occurences in meat producing animals (Gutierrez *et al.*, 2012; Doulgeraki *et al.*, 2016; Oniciuc *et al.*, 2017).

Simultaneously, there is a concern and consumer awareness in the scope of food safety, quality and conservation to counter the limitation of synthetic chemical additives in the food system. Consumer preference on natural preservatives found to be in a firm rising trend. Substantial source of valuable bioactive constituents are persistently obtained from plants. Consequently, wide range of plant products been assessed for natural antioxidants to preserve and advance the overall quality of food products especially meat and meat products (Shah *et al.*, 2014). *P. cubeba* L. was commonly known as condiment. It was widely accepted in food preparations as a flavour enhancer and to impart piquancy/ spiciness. *P. cubeba* L. extract consisting of prominent antimicrobial activities could lead it to be a natural preservative in the food system. Therefore, in this study, antistaphylococcal and antibiofilm activites of ethanolic *P. cubeba* L. extract was studied in addition to its application in chicken meat.

1.3 Objectives

The general objective of this study is to evaluate the antistaphylococcal and antibiofilm activities of *Piper cubeba* L. extract. The specific objectives of this study are:

1. To determine the antistaphylococcal activity of *P. cubeba* L. extract and to identify the bioactive compounds present in ethanolic *P. cubeba* L. extract.
2. To assess *in vitro* biofilm formation of *S. aureus* isolates and to evaluate the antibiofilm activity of ethanolic *P. cubeba* L. extract.
3. To analyse the effect of ethanolic *P. cubeba* L. extract on microflora in chicken meat, the sensory acceptability of ethanolic *P. cubeba* L. extract sanitized raw and steamed chicken meat and to determine the toxicity effect of ethanolic *P. cubeba* L. extract on eukaryotic cells.

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