UNIVERSITI PUTRA MALAYSIA

SOLVENT EXTRACTION AND IDENTIFICATION OF ACTIVE ANTICARIOGENIC METABOLITES IN Piper cubeba L. THROUGH 1H NMR-BASED METABOLOMICS

RAJA NUR ASILA BINTI RAJA MAZLAN

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RAJA NUR ASILA RAJA BINTI MAZLAN

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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 : Associate Professor Yaya Rukayadi, PhD
Institute : Bioscience

Dental caries is a noticeable infection in human. Even though rarely a life threatening, dental caries is still a major hassle for health provider companies. *Piper cubeba* L. with potential in elimination of dental caries has been studied. The aim of this study was to determine the effects of using different solvents for extraction, liquid-liquid partition, concentrations of extracts and fractions on the anticariogenic activity against *Streptococcus mutans* KCCM3309, *S. sobrinus* ATCC33478 and *Actinomyces viscosus* ATCC15987. The potentially active anticariogenic metabolites were identified by mean of proton nuclear magnetic resonance (^{1}H NMR) via multivariate data analysis (MVDA). The disc diffusion assay results of extracts and fractions showed the range of inhibition zone between 7.00 to 12.67 mm. Minimum inhibitory concentrations (MIC) data showed *P. cubeba* L. extracts and fractions at concentrations ranged from 0.10 mg/mL to 3.15 mg/mL have inhibited 99% bacterial growth. The ability of the extracts or fractions to kill at least 99% of bacteria was shown by minimum bactericidal concentrations (MBC) data, wherein the range for extracts and fractions spread from 0.10 mg/mL to 25.0 mg/mL. Bactericidal endpoint was determined to be in the range of 0.20 mg/mL to 3.80 mg/mL through time-kill curve assay of *P. cubeba* L. extracts at concentration of 0× MIC, 1/2× MIC, 1× MIC, 2× MIC and 4× MIC. The results revealed that all tested bacteria were susceptible to *P. cubeba* L. extracts and fractions. Varying solvents used for extraction, liquid-liquid partition and concentrations of extracts and fractions have influenced the antibacterial activity. For MTT cell proliferation assay, the percentage of RAW 264.7 cell viability approximately 80% and above are considered as not toxic. From the assay, methanol extract, ethanol extract, hexane fraction and ethyl acetate fraction were not toxic at the concentration of ≤ 62.5 µg/mL, while hexane extract and aqueous methanol fraction were at ≤ 125 µg/mL. Anti-inflammatory properties of the extracts and fractions were evaluated by nitric oxide (NO) production in stimulated RAW 264.7 cells by

lipopolysaccharide (LPS). The lowest NO production observed in methanol extract was at concentration 62.5 µg/mL with 22.98 µg/mL NO production, suggesting that methanol extract might be a suitable candidate for the anti-inflammatory agent. Twelve metabolites have been identified based on the ^{1}H NMR, which were cubebin **(1)**, yatein **(2)**, hinokinin **(3)**, dihydrocubebin **(4)**, dihydroclusin **(5)**, cubebinin **(6)**, magnosalin **(7)**, *p*-cymene **(8)**, piperidine **(9)**, cubebol **(10)**, D-germacrene **(11)** and ledol **(12)**. The metabolites that contributed to separation in principal component analysis (PCA) between hexane and other extracts were detected as cubebol, ledol, D-germacrene and piperidine. Meanwhile cubebol, ledol, D-germacrene and *p*-cymene caused separation of hexane fraction from ethyl acetate and aqueous methanol fractions. The partial least squares (PLS) model showed that higher biological activity was related more towards the polar solvents. Despite, the active metabolites also present in the non-polar solvents. The metabolites that related to the MBC were identified as cubebol, D-germacrene and ledol. *p*-cymene, cubebol and ledol were observed to contribute to the activity for anti-inflammatory in stimulated RAW 264.7 cells by LPS. In summary, *P. cubeba* L. extracts and fractions exhibited antibacterial and anti-inflammatory activity and have potential to be developed as anticariogenic agent.

Keywords: anticariogenic, anti-inflammatory, dental caries, multivariate data analysis, toxicity

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

PENGEKSTRAKAN PELARUT DAN PENGENALPASTIAN AKTIF ANTIKARIOGENIK METABOLIK DI DALAM *Piper cubeba* L. BERASASKAN PENDEKATAN ^{1}H NMR METABOLOMIK

Oleh

RAJA NUR ASILA BINTI RAJA MAZLAN

Mei 2018

Pengerusi : Profesor Madya Yaya Rukayadi, PhD
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Karies gigi merupakan satu penyakit yang dikesan terdapat pada manusia. Walaupun penyakit ini bukanlah suatu penyakit yang mengancam nyawa, namun ia masih menjadi suatu masalah yang besar kepada pembekal dalam perkhidmatan kesihatan. Potensi *Piper cubeba* L. dalam usaha untuk mengurangkan karies gigi telah dikaji. Tujuan utama kajian ini adalah untuk menentukan kesan pelarut yang berbeza untuk pengekstrakan, pembahagian cecair ke cecair dan kepekatan ekstrak dan fraksi terhadap aktiviti antikariogenik menentang *Streptococcus mutans* KCCM3309, *S. sobrinus* ATCC33478 dan *Actinomyces viscosus* ATCC15987. Aktif metabolik yang berpotensi telah dikenalpasti menggunakan proton resonans magnetik nuklear (^{1}H NMR) melalui analisis data multivariat (MVDA). Keputusan bagi assai peresapan cakera untuk ekstrak dan fraksi menunjukkan zon julat perencatan di antara 7.00 sehingga 12.67 mm. Data bagi kepekatan perencatan minimum (MIC) menunjukkan ekstrak dan fraksi *P. cubeba* L. pada kepekatan dari julat 0.10 mg/mL kepada 3.15 mg/mL telah merencatkan 99% pertumbuhan bakteria. Keupayaan ekstrak dan fraksi untuk membunuh 99% bakteria telah ditunjukkan melalui data kepekatan pembunuhan bakteria minimum (MBC), dimana julat untuk ekstrak dan fraksi di antara 0.10 mg/mL kepada 25.0 mg/mL. Titik akhir bakterisidal telah ditentukan pada julat 0.20 mg/mL sehingga 3.80 mg/mL melalui assai lengkungan masa-pembunuhan oleh ekstak *P. cubeba* L. pada kepekatan 0× MIC, 1/2× MIC, 1× MIC, 2× MIC and 4× MIC. Keputusan telah mendedahkan ekstrak dan fraksi *P. cubeba* L. mempunyai kerentanan terhadap semua bakteria yang diuji. Pelbagai pelarut untuk pengekstrakan, pembahagian cecair ke cecair dan kepekatan ekstrak dan fraksi yang digunakan telah memberi kesan kepada aktiviti antibakterial. Bagi assai pemproliferatan sel MTT, sel RAW 264.7 yang berupaya hidup pada peratusan 80% dan ke atas dianggap sebagai tidak toksik. Daripada assai tersebut, ekstrak metanol, etanol, fraksi heksana dan etil asetat adalah tidak toksik pada kepekatan ≤ 62.5 µg/mL, manakala ekstrak heksana

dan fraksi metanol akueus adalah pada kepekatan ≤ 125 µg/mL. Sifat anti keradangan bagi ekstrak dan fraksi telah dinilai melalui pengeluaran nitrik oksida (NO) didalam sel RAW 264.7 yang telah dirangsang oleh lipopolisakarida (LPS). Pengeluaran NO yang paling rendah telah dilihat didalam ekstrak metanol pada kepekatan 62.5 µg/mL, dengan 22.98 µg/mL pengeluaran NO, mencadangkan bahawa ekstrak metanol mungkin sesuai untuk dijadikan agen anti keradangan. Dua belas metabolik telah ditemukan melalui ^{1}H NMR, iaitu cubebina **(1)**, yatein **(2)**, hinokinin **(3)**, dihydrocubebin **(4)**, dihydroclusin **(5)**, cubebinin **(6)**, magnosalin **(7)**, *p*-cymene **(8)**, piperidine **(9)**, cubebol **(10)**, D-germacrene **(11)** dan ledol **(12)**. Metabolik yang menyumbang kepada pemisahan dalam PCA di antara ekstrak heksana dan ekstrak-ekstrak lain dikenalpasti sebagai cubebol, ledol, D-germacrene dan piperidine. Sementara itu cubebol, ledol, D-germacrene dan *p*-cymene menyebabkan pemisahan fraksi heksana dengan fraksi etil asetat dan metanol akueus. Model PLS menunjukkan bahawa aktiviti biologi yang lebih tinggi berkait dengan pelarut berkutub, meskipun metabolik yang aktif juga hadir didalam pelarut tidak berkutub. Metabolik yang berkait dengan MBC telah dikenalpasti sebagai cubebol, D-germacrene dan ledol, manakala *p*-cymene, cubebol dan ledol menyumbang kepada aktiviti anti keradangan dalam sel RAW 264.7 yang dirangsang oleh LPS. Kesimpulannya, ekstrak dan fraksi *P. cubeba* L. menunjukkan aktiviti antibakterial dan anti keradangan dan berpotensi untuk dibangunkan sebagai agen antikariogenik.

Kata kunci: antikariogenik, anti keradangan, karies gigi, analisis data multivariat, ketoksikan

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This thesis was submitted to the Senate of the 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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This is to confirm that:

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LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
ATTC	American Type Culture Collection
brs	broad singlet
CFU/mL	Colony Forming Unit per mililiter
CO_2	Carbon dioxide
CHX	Clorhexidine
DCM	Dichlroromethane
DMSO	Dimethyl sulfoxide
DPPH	Diphenylpicrylhdrazyl
D_2O	Deuterium oxide
d	doublet
dd	doublet of doublet
EOs	Essential Oils
FBS	Fetal Bovine Serum
FT-IR	Fourier Transform- Infrared Spectroscopy
GCMS	Gas Chromatography- Mass Spectrometry
GSH	Glutathione
HMDB	Human Metabolome Database
HPLC-DAD	High- Performance Liquid Chromatography Diode-Array Detector
HPLC-UV	High- Performance Liquid Chromatography-Ultraviolet
H_3PO_4	Phosphoric acid
Hz	Hertz
IFN-γ	Interferon gamma
IPSs	Intracellular Polysaccharides
IC_{50}	Inhibition Concentration at 50 percent

KCCM	Korean Culture Center of Microorganisms
KH_2PO_4	Potassium dihydrogen phosphate
LPS	Lipopolysaccharides
MSG	Mutans Streptococci Group
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
MVDA	multivariate data analysis
m	multiplet
mg/mL	Miligram per mililiter
mg/kg	Miligram per kilogram
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
$NaHCO_3$	Sodium bicarbonate
$NaNO_2$	Sodium nitrite
NaOD	Sodium deuteroxide
NED	N-(1-naphthyl) ethylene-diamine dihydrochloride
NIR	Near-infrared Spectrsocopy
NO	Nitric oxide
PBS	Phosphate Buffer Saline
PCA	Principal Component Analysis
PLS	Partial least squares
ppm	parts per million
RC_{50}	Rescue Concentration at 50 percent
RF	Radio Frequency
rpm	Revolutions per minute
s	singlet
TLC	Thin Layer Chromatography
VIP	Variable Importance on Projection

%	Percent
°C	Degree Celsius
δ	Delta
^{1}H NMR	Proton Nuclear Magnetic Resonance
2D-JRES	2-Dimensional J Resolved
µg/g	Microgram per gram
µg/mL	Microgram per mililiter
µM/g	Micromolar per gram

CHAPTER 1

INTRODUCTION

1.1 Background

Dental caries is a noticeable infection in human and even though the mortality case is unlikely occurred, it becomes a major hassle for health provider companies (Forssten *et al.,* 2010). Dental caries occurred when pathogenic bacteria produce an acid through fermentation process from food particles aggregated on the tooth surface and promotes tooth demineralization (Karpinski and Szkaradkiewicz, 2013). Biofilm is a sludge layer comprising of a huge number of bacterial cells, salivary polymers and food particles coated on the tooth surface and can achieve masses number of cells. The biofilm, additionally referred to as plaque, provides tremendous adhesion site for colonization and development for significant number of bacterial species (Forssten *et al.,* 2010).

People are vulnerable to this disease at some stage in their lifetime. A current overview of the accessible epidemiological records from numerous nations demonstrates that there is an increment of dental caries incidence. This disease affects kids, adults, primary and permanent teeth, coronal and also root surfaces (Bagramian *et al.*, 2009). A previous study reported nearly 36% of the people worldwide were affected by dental caries. Meanwhile, it affects nearly 9% of the population in baby teeth (Karpinski and Szkaradkiewicz, 2013). Among Malaysian adults, the occurrence of dental caries is 90% with over ten teeth on average being affected. Moreover, the occurrence of active decay disease experience is greater for people residing in rural compared with urban areas. These data are presumed to be discouraging as The School Dental Service (SDS) in Malaysia has started since the 1950's and in 1985 expand into an extensive dental health care service for students (Esa *et al.*, 2014).

Currently, several solutions for the dental caries have been proposed including elimination of the dental plaque by mechanical removal, dental treatment and usage of chemical as anticariogenic agent. The elimination of the dental plaque by mechanical method is the most productive strategy to forestall dental caries, yet only minority of people involved in this process (Ambrosio *et al.*, 2008). In addition, the cost of dental treatment is high and only accessible to people in developed nations (More *et al.,* 2008). Considerable works has been done to seek chemical anticariogenic compounds that can be integrated into dental formulations. A few antibiotics, for example, ampicillin, chlorhexidine, metronidazole, phenolic-antiseptics, sanguinarine and quaternary ammonium-antiseptics have been utilized. Amongst these antibiotics, chlorhexidine has been acknowledged as a best quality anticariogenic compound. The usage has been endorsed by American Dental Association Council on Dental Therapeutics (Ambrosio *et al.,* 2008). Nonetheless, frequent usage of dental products consisting of these compounds are regularly related with several side effects such as

tooth and restoration staining, modification in the flavour of foods and a burning sensation at the tip of the tongue (Porto *et al.*, 2009b; Greenberg *et al.*, 2008; More *et al.*, 2008). With these issues arised therefore, imply that discovering a new, safe and compelling anticariogenic compounds is still required.

Nature has been great resources for medicinal agents and a significant number of current medications have been isolated, many of which based on their utilization in conventional medicine. Only 1% of the natural resources has been phytochemically examined out of approximately 500,000 plant species existing worldwide (Palombo, 2011). Phytomedicine refers to the utilization of the whole plant parts included seeds, berries, roots, leaves, bark, or flowers for medicinal purposes (Barrett *et al.*, 1999).

Piper cubeba L. is a medicinal plant selected for this current study and *P. cubeba's* potential as antibacterial and anti-inflammatory agents will further investigated. *Piper cubeba* L. is native to Java, Prince of Wales Island, Southern Borneo, Sumatra, and other islands in the Indian Ocean (Lim, 2012). The genus *Piper* belongs to the Piperaceae family, known to have more than 1000 species across the world. In the tropical zone, members of the genus *Piper* are utilized for plenty of purposes for instance, foods and spices, fish poison and oils (Barrett, 1994; Joly 1981).

1.2 Problem statements

In oral environment, the bacteria associated with dental caries are often referred to as cariogenic bacteria. These bacteria are responsible for the occurrence of dental caries (Gouda *et al.*, 2017). If left untreated, dental caries can give rise to pulpitis, a disease related to an inflammation of the dental pulp (Park *et al.*, 2015). Therefore, to prevent dental caries and pulpitis, the study about plants with anticariogenic properties which includes both antibacterial and anti-inflammatory activities are required. In this current study, the antibacterial activity of dried berries of *Piper cubeba* L. with different extraction solvents and fractions are evaluated. Toxicity and anti-inflammatory properties of selected extracts and fractions are also evaluated. The active anticariogenic metabolites will be identified using proton nuclear magnetic resonance (^{1}H NMR) and multivariate data analysis (MVDA).

For many years, *P. cubeba* L. has been used in food preparations as well as for medicinal purposes. Currently, there are increasing studies of *P. cubeba* L. extracts and compounds for its use against oral microorganisms. Silva *et al.* (2007) has evaluated the activities of crude ethanol extract against oral bacteria. From the study, *Streptococcus salivarius* was found to be the most susceptible to the ethanol extract of *P. cubeba* L. with MIC value 80 µg/mL. Another study conducted by Chitnis *et al.* (2007) used different types of solvent for extraction of *P. cubeba* L. which are dichloromethane (DCM), methanol and n-hexane, however the antibacterial activity of those extracts has not been tested against oral bacteria. Despite the previous studies on *P. cubeba* L., there are some limitation in aspect of types of solvent used for

extraction, concentrations of extract and types of bacteria. An approach of using different solvents for extraction and liquid-liquid partition with different extract and fraction's concentrations might be useful in understanding the antibacterial activity at its best. Solvents with different polarities and concentrations will yield extracts with different compounds thus vary in aspect of antimicrobial activity. Hence, the objective of this study is to determine antibacterial activity of *P. cubeba* L. extracts and fractions with different solvents and concentrations against oral bacteria in term of disc diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time-kill curve assay.

Eventhough natural products have been widely accepted, the issues regarding the safety usage of natural products are still rising. Toxicity or remedy properties of natural products are determined by the specific dosage. Based on previous literature, cytotoxicity of *P. cubeba* L. crude extract on normal fibroblast (L929), normal breast (MCF-12A) and three breast cancer cell lines (MCF-7, MDA-MB-468 and MDA-MB 231) have been studied (Graidist *et al.,* 2015). However, the toxicity of the extracts and fractions of *P. cubeba* L. especially in toxicity and anti-inflammatory properties utilizing RAW 264.7 cells are still not widely explored. Therefore, a study need to be conducted to comprehend about the toxicity and anti-inflammatory properties of RAW 264.7 cells in regard of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cells viability assay and nitric oxide (NO) production in lipopolysaccharide (LPS) stimulated RAW 264.7 cells.

Metabolomics permits a scientific approach to investigate an intricate mixture for example, a phytochemical preparation, which can be linked to observations acquired *via* biological testing systems without necessity for isolation works (Yuliana *et al.,* 2011). To the best of our knowledge, there are still no study regarding the identification of active anticariogenic metabolites of *P. cubeba* L. through metabolomics study. Therefore, this study is aimed to identify the active anticariogenic metabolites through proton nuclear magnetic resonance (^{1}H NMR) followed by multivariate data analysis (MVDA).

1.3 Objectives

The objectives of this study are:

1. to determine the antibacterial activity of *P. cubeba* L. extracts and fractions with different solvents, extracts and fractions against *Streptococcus mutans*, *Streptococcus sobrinus* and *Actinomyces viscosus*.
2. to evaluate the toxicity and anti-inflammatory activity of selected extracts and fractions by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cells viability assay and nitric oxide (NO) production in lipopolysaccharide (LPS) stimulated RAW 264.7 cells.

3. to identify anticariogenic metabolites in *P. cubeba* L. extracts and fractions by using proton nuclear magnetic resonance (^{1}H NMR) via multivariate data analysis (MVDA).

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