ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF *Piper cubeba* L. BERRIES EXTRACT AND ITS CYTOTOXICITY EFFECT ON 3T3 HUMAN FIBROBLAST AND HEPG2 CELL LINES

By

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Human-pathogenic bacterial has been increase dramatic in the past decades, then the antibacterial resistance was recognized widely as a grave threat to the global health in the 21st century. The most important oxygen-containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical. This study aimed to determine the antibacterial and antioxidant activities of *Piper cubeb*a L. extract and its toxicity on 3T3 and HepG2 cell lines. Methanol and ethanol were the extraction solvents used for dried *P. cubeba* L. berries using maceration methods. and the selected crude extract was fractionated using liquid-liquid partition with the solvents of hexane, chloroform and ethyl acetate. The antibacterial activities of P. cubeba L. berries crude extract and its fraction were determined in term of disc diffusion assay, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against four bacterial: *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus pumilus* and *Klebsiella pneumoniae*. The total phenolic contents (TPC) were analysed using Folin-Ciocalteau method of *P. cubeba* L. berries extract and its fractions and antioxidant activity of were determined using 2,2-diphenyl-1-picrylhydryzyl (DPPH) radical scavenging assay. The cytotoxicity of *P. cubeba* L. was evaluated using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay on 3T3 human fibroblast and HepG2 cell lines. The active compounds were identified by using Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatograph-Mass Spectrometry (LC-MS). Diameter of inhibition zones of methanol extracts against the selected bacterial were ranged from 8.00 ± 0.10 to 11.00 ± 0.00 nm. MICs of methanol and ethanol crude extracts were ranged from 0.16 mg/mL to 1.25 mg/mL and 0.62 mg/mL to 1.25 mg/mL, respectively. The methanol and ethanol both crude extracts can kill the bacterial with the values of MBC 2.50 mg/mL. The total phenolic of methanol crude extract was 0.59 ± 0.01 mg/GAE/g and ethanol crude extract was 0.66 ± 0.02 mg GAE/g. The DPPH results of methanol and ethanol crude extracts displayed IC_{50} were 546.14 ± 3.28 µg/mL and 472.43 ± 5.21 µg/mL, respectively. The cytotoxic effect on 3T3 fibroblast cell line of methanol and ethanol both crude extracts showed non-cytotoxic, however, the cytotoxic

effect on HepG2 cell line of methanol crude extract with value of IC_{50} 13.14 ± 3.77 μg/mL, while the ethanol crude extract had the IC_{50} value of 28.40 ± 1.44 μg/mL. The inhibition zoon of fractions was ranged from 7.20 ± 0.16 mm to 10.00 ± 0.16 mm. The MICs of fractions were ranged from 0.078 mg/mL to 0.625 mg/mL and the value of MBC of fractions was ranged from 0.156 mg/mL to 1.250 mg/mL. The total phenolic content in the fractions of hexane, chloroform and ethyl acetate was 0.45 ± 0.02 mg GAE/g, 0.69 ± 0.12 mg GAE/g and 0.86 mg GAE/g, respectively. And the DPPH values of fractions of hexane, chloroform and ethyl acetate displayed IC_{50} was 3.614 ± 0.24 mg/mL, 0.197 ± 0.02 mg/mL and 0.098 ± 0.03 mg/mL, respectively. Hexane, chloroform and ethyl acetate fractions illustrated cytotoxic effect on HepG2 cell line with the IC_{50} values of 11.05 ± 0.86 μg/mL, 21.00 ± 0.43 μg/mL, 29.77 ± 0.99 μg/mL, respectively while ethyl acetate displayed non-cytotoxic effect on 3T3 fibroblast cell line. Further purification to isolate the potentially active compound would be taken in the future. The major volatile components were identified using GC-MS, including beta-cubebene, muurolene, alfa-copaene and alfa-cubebene with the antibacterial activity, cubebol, spathulenol and viridflorol with the antioxidant activity and elemene, eugenol and caryophyllene with the anticancer activity. Hemiarensin with antibacterial activity, myricetin with antioxidant and ellagic acid with anticancer activity, were identified using LC-MS.

Keywords: Antibacterial, antioxidant, cytotoxic, liver cancer, HepG2, *P. cubeba* L.

Abstrak tesis yang dipersembahkan kepada Senat Universiti Putra Malaysia untuk memenuhi syarat Ijazah Master Sains

AKTIVITI ANTIBAKTERIA DAN ANTIOKSIDAN EKSTRAK BERI *Piper cubeba* L. DAN KESAN SITOTOKSIK TERHADAP 3T3 FIBROBLAS MANUSIA DAN SEL HEPG2

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Radikal bebas terlibat dalam banyak penyakit dalam beberapa tahun terakhir. Dalam serangan tubuh membran mempunyai radikal bebas yang berbeza yang menghasilkan pengoksidaan lipid, menurunkan kelancaran biofilm, kehilangan aktiviti enzim yang berbeza, kehilangan aktiviti reseptor, dan inaktivasi sel disebabkan oleh pemusnahan protein dalam membran. Beberapa radikal bebas adalah faktor patogen barah, radikal bebas menyerang DNA sel, yang menyebabkan mutasi dan akhirnya menyebabkan barah. Kerana penyakit ini, saintis dapat menggunakan antioksidan semula jadi untuk menyembuhkan degenerasi sel. Kajian ini bertujuan untuk mengetahui aktiviti antibakteria dan antioksidan ekstrak Piper cubeba L. dan ketoksikannya pada garis sel 3T3 dan HepG2. Metanol dan etanol digunakan untuk pengambilan minyak mentah dan ekstrak kasar terpilih yang digunakan untuk partisi cecair-cecair. Hasil kajian menunjukkan bahawa zon penghambatan ekstrak kasar *P. cubeba* L. terhadap bakteria yang diuji berkisar antara 8.00 ± 0.10 hingga 11.00 ± 0.00 nm. Ekstrak menghalang pertumbuhan bakteria yang diuji dengan nilai kepekatan minimum (MIC) 0.16 mg / mL hingga 1.25 mg / mL, dan dengan kepekatan minimum bakterisida (MBC) pada 2.50 mg / ml membunuh bakteria. Lebih-lebih lagi, 2,2-diphenyl-1-picrylhydryzyl (DPPH) assay scavenging radikal dan jumlah kandungan fenolik (TPC) yang dikira sebagai setara asid gallic (GAE) yang digunakan untuk aktiviti antioksidan, ekstrak *P. cubeba* L. mempunyai IC_{50} 546.14 ± 3.28 µg / mL ekstrak etanol dan IC_{50} 472.43 ± 5.21 µg / mL ekstrak metanol, juga mempunyai perencatan 70.3% dan 79.4% pada kepekatan 1.25 mg / mL ekstrak, masing-masing. Pecahan heksana, kloroform dan etil asetat mempunyai IC_{50} 3.62 ± 0.24 mg / mL, 197.08 ± 0.36 µg / mL, 98.45 ± 0.57 µg / mL, dengan 21.65%, 87.07% dan 93.84% perencatan pada 1.25 mg / mL sampel, masing-masing. Sementara itu, nilai TPC ekstrak metanol *P. cubeba* L., ekstrak etanol, masing-masing 0.66 ± 0.02 mg GAE / g, 0.59 ± 0.01 mg GAE / g. Selanjutnya, sitotoksisiti ekstrak dan pecahan dinilai dalam sel fibroblas manusia 3T3 dan sel HepG2 dengan menggunakan ujian 3-[4,5-dimethylthiazole-2-yl] -2,5-diphenyltetrazolium bromida (MTT). Ekstrak metanol dan ekstrak etanol serta pecahan etil asetat menunjukkan tidak sitotoksik kepada 3T3 fibroblas manusia. Sementara itu, ekstrak metanol menunjukkan kesan sitotoksik yang

lebih kuat dengan nilai IC_{50} 13.14 ± 3.77 µg / mL berbanding ekstrak etanol dengan nilai IC_{50} 28.40 ± 1.44 µg / mL. Pecahan heksana, kloroform dan etil asetat menunjukkan kesan sitotoksik pada garis sel HepG2 dengan nilai IC_{50} 11.05 ± 0.86 µg / mL, 21.00 ± 0.43 µg / mL, 29.77 ± 0.99 µg / mL, masing-masing. Pemurnian lebih lanjut untuk mengasingkan sebatian aktif yang berpotensi akan diambil di masa depan. Komponen volatil utama dikenal pasti menggunakan GC-MS, termasuk cubebol, beta-cubebene, alfa-cubebene, 2-methylene-4,8,8-trimethyl-4-vinyl-bicyclo [5.2.0] nonane, germacrene-D, alfa-copaene, muurolene, humulene, spathulenol, caryophyllene, epi-cubenol, caryophyllene oxide dan beta-selinene. Clusin, asid ellagic, cubebinolide, acid quinic dan beta-cubebene, dikenal pasti menggunakan LC-MS. Kesimpulannya, ekstrak beri *P. cubeba* L. mempunyai aktiviti antibakteria dan antioksidan dan mempunyai kesan sitotoksisitas yang kuat pada sel HepG2. Oleh itu, ia berpotensi untuk dikembangkan sebagai sumber agen antikanker semula jadi yang lain.

Kata kunci: Antibakteria, antioksidan, sitotoksik, barah hati, HepG2, *P. cubeba* L.

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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 Master Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µg	Microgram
mg	Milligram
g	Gram
kg	Kilogram
µl	Microlitre
mL	Millilitre
L	Litre
w/v	Weight per volume
mg/mL	Milligram per milliliter
rpm	Revolutions per minute
ppm	part per million
Min	Minute
W	Weigh
V	Volume
oC	Degree celcius
%	Percent
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
DPPH	1,1-diphenyl-2-picryl-hydrazyl
µg/µL	Microgram per microliter
cm	Centimeter
mm	Millimetre
UV	Ultraviolet
CFU/mL	Colony Forming Unit per milliliter
CHX	Clorhexidine
IC50	Inhibition Concentration at 50 percent
MHz	Megahertz
m/z	mass/charge ratio
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
DMSO	Dimethyl sulfoxide
IBS	Institute of Bioscience
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
PBS	Phosphate Buffer Saline
NIST	National Institute of Standards and Technology
GC-MS	Gas Chromatography – Mass Spectrometry
LC-MS	Liquid Chromatography – Mass Spectrometry
CO2	Carbon dioxide

CHAPTER 1

INTRODUCTION

1.1 Background

It’s known that the important sources of drugs are natural products which are from plants (Newman *et al.*, 2012). A lot of plant sources of molecules have shown promising results in treatment (Lokhande *et al.*, 2007). Plants play an important role in human’s life, meeting his daily needs (Szallasi *et al.*, 2005). Recently years, in many diseases free radicals are involved (Ahmad *et al.*, 2011). The most important oxygen-containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical. These are highly reactive species, capable in the nucleus, and in the membranes of cells of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids (Ahmad *et al.*, 2011; Kochhar, 2008). Some free radicals are the pathogenic factors of cancer. First, free radicals attack cell DNA, which causes mutations and eventually induces cancer (Abdullahi *et al.*, 2011; Ahmad *et al.*, 2010). Because of these diseases, scientists can use natural antioxidants to cure cell degeneration (Ahmad *et al.*, 2011; Obinna *et al.*, 2009).

Oxidants are reactive molecules that are produced both inside your body and the environment that can react with protein, DNA and lipids other cellular molecules in human body. Free radicals are waste substances produced by cells as the body processes food andreacts to the environment. If the body cannot process and remove free radicalsefficiently, oxidative stress can result. This can harm cells and body function. Freeradicals are also known as reactive oxygen species (ROS) (Valko et al., 2007; Diplock et al., 1998). Oxidative stress has been linked to heart disease, cancer, arthritis, stroke, respiratory diseases, immune deficiency, emphysema, Parkinson’sdisease, and other inflammatory or ischemic conditions. Free radicals are highlyreactive chemicals that have the potential to harm cells. They are created when anatom or a molecule (a chemical that has two or more atoms) either gains or loses anelectron (a small negatively charged particle found in atoms). Free radicals are waste substances produced by cells as the bodyprocesses food and reacts to the environment. If the body cannot process and remove free radicals efficiently, oxidative stress can result. This can harm cells and body function. Free radicals are also known as reactive oxygen species (ROS) (Valko *et al*., 2007; Diplock *et al*., 1998). Oxidative stress has been linked to heart disease, cancer, arthritis, stroke, respiratory diseases, immune deficiency, emphysema, Parkinson’s disease, and other inflammatory or ischemic conditions. Free radicals are highly reactive chemicals that have the potential to harm cells. They are created when an atom or a molecule (a chemical that has two or more atoms) either gains or loses an electron (a small negatively charged particle found in atoms).

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).

1.2 Problem Statements

The products of natural plant are important sources of new drugs are widely explored and have been favoured by scientists as they not only exhibit a large range of antibacterial properties against microorganisms, including pathogenic microbes, but they have also been sourced as antioxidant and anticancer agents which have piqued the interest of scientists. Among the cancer mortality, liver cancer is the second leading cause. Hep G2 cells are a suitable model to study the intracellular trafficking and dynamics of bile canalicular, sinusoidal membrane proteins, and lipids in human hepatocytes *in vitro* because of their high degree of morphological and functional differentiation. Additionally, HepG2 cells are an ideal in vitro model to study the intracellular dynamics of cell surface domains, such as bile canaliculi and sinusoidal membrane proteins and lipids in human hepatocytes. Due to the sharp rise in the incidence rate of liver cancer, the lack of proper treatment methods and the serious side effect of synthetic drugs had made it necessary to find some new and more effective molecules. Discovering natural products with antimicrobial and antioxidant activities is imperative as natural products have been investigated to have fewer side effects when consumed compared to modern synthetic medicine even though the latter exhibits better properties. This has inspired the present study the herbal medicines or natural products derived from herbal medicines.

1.3 Objectives

The objectives of this study are:

1. To extract the *P. cubeba* L. berries used solvent extraction and to fractionate used liquid-liquid partition

2. To determine antibacterial activity of *P. cubeba* L. extract and its fractions against *B. megaterium*, *B. subtilis*, *B. pumilus* and *K. pneumoniae* in term of disc diffusion assay (DDA), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

3. To determine antioxidant activity of P. cubeba L. extract and its fractions through total phenolic content (TPC) and 2,2-diphenyl-1-picryllhydrazyl (DPPH) radical scavenging assay.

4. To evaluate cytotoxic effect of *P. cubeba* L. extract and its fractions using MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay against 3T3 fibroblast and HepG2 cells.

5. To analyses phytochemicals constituents in *P. cubeba* L. and its active fraction using GC-MS and LC-MS analyses.

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