UNIVERSITI PUTRA MALAYSIA

MECHANISM OF HEPATOPROTECTIVE ACTIVITY OF METHANOLIC EXTRACT OF Melastoma malabathricum L. LEAVES

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By

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

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Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Chairman : Associate Professor Zainul Amiruddin Zakaria, PhD
Faculty : Medicine and Health Sciences

The objective of this study was to determine the hepatoprotective activity of methanolic extracts of *Melastoma malabathricum* leaves (MEMM) and its partitions using rats' model, by evaluating the prophylactic effect of the plants extracts administered prior to the induction of liver toxicity using a hepatotoxic agent. The study were design as hepatoprotective potential of MEMM has never been reported. In an attempt to establish the pharmacological properties, the hepatoprotective potential of MEMM was investigated using carbon tetrachloride (CCl_4)- and paracetamol (PCM)- induced hepatotoxicity in rats. Throughout this study, the animals were divided into 22 groups containing 6 rats each group. In the first stage of *in vivo* study, rats were divided into groups and administered orally once daily with 10 % dimethyl sulfoxide (DMSO) as negative control, 200 mg/kg silymarin as positive control, or MEMM (50, 250, 500 mg/kg) for 7 days, followed by hepatotoxicity induction using CCl_4 or PCM. In the second stage, MEMM was partitioned into 3 fractions: petroleum ether extract (PEMM), ethyl acetate extract (EAMM), aqueous extract (AQMM). PEMM, EAMM and AQMM were then tested on PCM-induced hepatotoxicity in rats. Blood sample underwent biochemical analysis to evaluate alanine transferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels; the livers were subjected to microscopic analysis. All extracts (MEMM, PEMM, EAMM AQMM) underwent antioxidant study using oxygen radical absorbance capacity (ORAC) test, Total phenolic compound (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and superoxide scavenging assay, and anti-inflammatory evaluation via lipoxygenase (LOX) and xanthine oxidase (XO) assays. Total phenolic content (TPC), phytochemical screening and high-performance liquid chromatography (HPLC) evaluation were also performed. From the histological observation, lymphocyte infiltration and

marked necrosis were observed in the DMSO-treated groups (negative control). MEMM showed encouraging activity for reducing the toxic effect of CCl_4 or PCM on the liver by reducing the weight of the liver in a dose independent manner; histological observation demonstrated normalization of the histopathological changes, preserving hepatocyte structure, causing a significant decline in AST and ALT levels ($p<0.05$). PEMM, EAMM and AQMM which contains non-polar compounds, intermediate compounds and polar compounds, respectively, attenuated the liver enzyme levels in a dose-independent manner. Overall, EAMM had the best activity for attenuating the liver enzymes. MEMM had the highest TPC value, followed by AQMM, EAMM, and PEMM. All the extracts (MEMM, PEMM, EAMM, and AQMM) demonstrated potential free radical scavenging activity in SOD and DPPH assays. However, the different trend was showed by ORAC assay from that DPPH and SOD. EAMM and AQMM had high ORAC value, which determine the capacity of an extract to act as an antioxidant. In the anti-inflammatory assays, MEMM and EAMM showed the moderate inhibition in LOX activity, but weak anti-inflammatory activity in XO. Phytochemical screening showed that the extracts showed that MEMM, PEMM and EAMM contained flavonoids, triterpenes, tannins, saponins, polyphenolic compounds and steroids, but not alkaloids. In contrast, the AQMM extract contained fewer compounds. HPLC analysis demonstrated that four to eight peaks detected at different wavelengths of the chromatogram of MEMM, PEMM, EAMM and AQMM, which were suggested to be flavonoid-based compounds. In conclusions, MEMM exerted potential hepatoprotective activity that can be partly attributed to its antioxidant activity, and EAMM was considered to have the best activity among the fractions, which warrants further investigation.

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

MEKANISMA AKTIVITI HEPATOPROTEKTIF OLEH EKTRAK METANOL DARI DAUN *Melastoma malabathricum*

Oleh

SITI SYARIAH BINTI MAMAT

Februari 2017

Pengerusi : Profesor Madya Zainul Amiruddin Zakaria, PhD
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Objektif kajian ini adalah untuk menentukan aktiviti hepatoprotektif ekstrak metanol daripada daun *Melastoma malabathricum* dan pecahannya dengan menggunakan model tikus dengan menilai kesan profilaksi ekstrak tumbuhan yang diambil sebelum induksi ketoksikan hati menggunakan agen hepatotoksik. Kajian ini telah direka kerana potensi hepatoprotektif dari ektrak MEMM tidak pernah dilaporkan. Dalam usaha untuk mewujudkan sifat-sifat farmakologi, potensi hepatoprotektif MEMM telah dikaji dengan menggunakan induksi karbon tetraklorida (CCl4) - dan paracetamol (PCM) pada tikus. Sepanjang kajian ini, tikus telah dibahagikan kepada 22 kumpulan yang mempunyai 6 tikus bagi setiap kumpulan. Dalam peringkat pertama dalam kajian *in vivo*, tikus telah dibahagikan kepada kumpulan dan diberi makan sekali sehari dengan 10% dimetil sulfoxide (DMSO) sebagai kawalan negatif, 200 mg/kg silymarin sebagai kawalan positif, atau MEMM (50, 250, 500 mg / kg) selama 7 hari, diikuti dengan hepatoksisiti induksi menggunakan CCl_4 atau PCM. Pada peringkat kedua, MEMM telah dibahagikan kepada 3 pecahan: ekstrak petroleum eter (PEMM), ekstrak etil asetat (EAMM), ekstrak akueus (AQMM). PEMM, EAMM dan AQMM kemudiannya diuji ke atas ransangan PCM pada tikus. Sampel darah telah diambil untuk menjalani analisis biokimia untuk menilai paras enzim seperti transferase alanine (ALT), aspartat aminotransferase (AST) dan tahap alkali phosphatase (ALP); sampel hati pula dinilai secara mikroskopik. Semua ekstrak (MEMM, PeMM, EAMM AQMM) menjalani kajian antioksidan menggunakan oksigen kapasiti penyerapan radikal (ORAC) ujian, Jumlah sebatian fenolik (TPC), 2,2-difenil-1-picrylhydrazyl (DPPH) aktiviti memerangkap radikal dan ujian perangkap activiti superoxide dismutase (SOD), dan penilaian anti-radang melalui ujian lipoxygenase (LOX) dan xanthine oxidase (XO). Jumlah kandungan fenolik (TPC), pemeriksaan fitokimia dan kromatografi cecair penilaian berprestasi

tinggi (HPLC) juga telah dijalankan. Dari pemerhatian histologi, penyusupan limfosit dan nekrosis diperhatikan dalam kumpulan yang diberikan DMSO (kawalan negatif). MEMM menunjukkan aktiviti yang menggalakkan bagi mengurangkan kesan toksik CCl_4 atau PCM pada hati dengan mengurangkan berat hati selari denga jumlah dos yang diberi; pemerhatian histologi menunjukkan perubahan normal, memelihara struktur sel-sel hati, menyebabkan penurunan yang ketara dalam tahap AST dan ALT ($p < 0.05$). PEMM, EAMM dan AQMM yang mengandungi sebatian bukan kutub, sebatian perantaraan dan sebatian kutub, masing-masing, dapat mengurangkan tahap enzim hati tanpa bergantung pada dos. Secara keseluruhan, EAMM mempunyai aktiviti yang terbaik dalam penurunan enzim hati. MEMM mempunyai nilai TPC tertinggi, diikuti oleh AQMM, EAMM dan PEMM. Semua ekstrak (MEMM, PEMM, EAMM dan AQMM) menunjukkan potensi aktiviti memerangkap radikal bebas dalam ujian SOD dan DPPH. Walau bagaimanapun, ikutan yang berbeza telah ditunjukkan oleh ujian ORAC dari DPPH dan SOD. EAMM dan AQMM mempunyai nilai ORAC yang tinggi, yang menentukan kapasiti untuk bertindak sebagai antioksidan. Dalam ujian anti-radang, MEMM dan EAMM menunjukkan perencatan yang sederhana dalam aktiviti LOX, tetapi aktiviti anti-radang lemah dalam XO. Penyaringan fitokimia menunjukkan bahawa ekstrak menunjukkan bahawa MEMM, PEMM dan EAMM mengandungi flavonoid, triterpenes, tanin, saponin, sebatian polifenolik dan steroid, tetapi tidak alkaloid. Sebaliknya, ekstrak AQMM mengandungi hanya beberapa sebatian sahaja. Analisis HPLC menunjukkan bahawa empat hingga lapan puncak dikesan pada panjang gelombang yang berbeza daripada kromatogram daripada MEMM, PEMM, EAMM dan AQMM, yang telah dikategorikansebagai jenis-jenis sebatian flavonoid. Sebagai kesimpulan, MEMM dikenakan berpotensi aktiviti hepatoprotective yang boleh sebahagiannya disebabkan oleh aktiviti antioksidan, dan EAMM dianggap mempunyai aktiviti yang terbaik dalam kalangan pecahan ekstark, yang memerlukan siasatan lanjut.

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The thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

AAPH	2,2'-azobis-2-methyl-propanimide, dihydrochloride
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ANOVA	Analysis of variance
AQMM	Aqueous extract of *Melastoma malabathricum*
AUC	Area under curve
CCl_3	Trichloromethyl free radical
CCl_4	Carbon tetrachloride
Cl_3COO	Trichloromethyl peroxy
COX	Cyclooxygenase
CYP450	Cytochrome 450
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EAMM	Ethyl acetate extract of *Melastoma malabathricum*
H & E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HO	Hydroxyl radical
HPLC	High-performance liquid chromatography
i.p	intraperitoneal
IC50	Median inhibitory concentration
LOX	Lipoxygenase
M. malabathricum	*Melastoma malabathricum*
MEMM	Methanol extract of *Melastoma malabathricum*
NAC	N-acetylcystein
NAPQI	*N*-acetyl *p*-benzoquinonimine
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
o.p	Orally
O_2	Oxygen
ORAC	Oxygen radical absorbance capacity
PCM	Paracetamol
PEMM	Petroleum ether extract of *Melastoma malabathricum*
PPAR-α	peroxisome proliferator-activated receptor-alpha
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SD	Sprague Dawley
SEM	Standard error of mean
SOD	Superoxide Dismutase
TPC	Total phenolic content
UPM	Universiti Putra Malaysia
WHO	World Health Organization
XO	Xanthine oxidase

CHAPTER 1

INTRODUCTION

Nowadays, liver diseases are a major worldwide health problem that occur throughout the world irrespective of sex, age, race and region. According to World Health Organization (WHO), about 46% of global diseases and 59% of the mortality is because of chronic diseases. Almost 35 million people in the world die of chronic diseases and liver disease rates are progressively increase over the years. According to National Statistics in the UK, liver diseases have been ranked as the fifth major cause of death. Deaths from cirrhosis have been estimated to increase and would make it as the 12th leading cause of death in 2020, as reported by Murray and Lopez, 1997.

Although liver damage is stereotypically linked to alcohol or drugs (e.g. paracetamol (PCM) or accidental exposure to certain chemicals (e.g. carbon tetrachloride (CCl_4), the reality is that there are over 100 known forms of liver damage caused by a variety of factors and effecting everyone from infants to older adults such as autoimmune disorders, genetic inheritance, toxins, obesity and cryptogenic. Consequently, these caused chronic damage to the liver, in which scar tissue slowly replaces normal functioning tissue lost, nutrients, hormones, drugs and poisons are not processed effectively by the liver. In addition, protein production and other substances produced by the liver are inhibited.

Even though the modern drug technologies of high-throughput screening and synthetic chemistry of the 20th Century have been expanded upon greatly, nature, particularly plant-based therapies, has remained the most valued resource in the drug development arena (Helmstadter and Staiger, 2014). Drug discovery of current active agents has been discussed from a phytopharmaceutical viewpoint. An analysis from 1981 to 2010 by Newman and Cragg (2012) showed that more than two-thirds of the drug active compounds recently introduced are likely derived from natural sources, and only about 30% are of completely synthetic origin. For decades, therapeutic practices and roles of medicinal herbs for treating disease have been gathered through an array of trials and errors, and have been documented in the history of medicine. Regardless of the abundance of the number of modern drugs in the pharmaceutical market, traditional medicine has been favoured as the primary option for alternative medicines, considering its low cost and effectiveness, and cultural, historical and even religious inclinations (Priya *et al.*, 2010).

In retrospect, there has been tremendous drug discovery from natural products since World War I, but surprisingly, less than 10% of the 250,000 species from worldwide biodiversity has been studied for medicinal purposes (Ramasamy *et al*., 2011), leaving many species awaiting therapeutic exploration. As a land rich with flora and faunal properties, Malaysia believed to be a reservoir of a large collection of potential medicinal plants. An increasing trend in Malaysia was the recent swing in

interest from synthetic allopathic drugs to herbal medicine. In 1999, the herbal and natural product domestic market was reported to be RM4.55 billion, and the current appraisal growth rate is estimated to be worth 15-20% annually (Nordin *et al.*, 2008). Alongside economic factors, the increased interest in the herbal industry in Malaysia has apparently been caused by changes in lifestyle, increased heath consciousness, and the costliness of synthetic medicine (Aziz, 2003). An in depth report by the Ministry of Natural Resources and Environment on Biodiversity in Malaysia (2006) showed that Malaysia enjoys the advantage of genetic natural products, and indigenous knowledge (Biodiversity in Malaysia 2006).

Current drug used for treating liver injury, silymarin and N-acetylcysteine offer protection to the liver from damage or help to regenerate hepatic cells. However, current drugs mainly associated with have limited efficacy, causing adverse drug reactions and yet expensive (Stickle and Schuppan, 2007). Thus, there is an urgent need to find new hepatoprotective agents from natural sources to overcome those problems.

The plant kingdom is undoubtedly valuable as a source of new medicinal agents. Plants have always been a rich source of biochemical compounds. Many of these biochemical compounds are useful drugs in themselves and others have been the basis for synthetic drugs.

To explore these sources for hepatoprotective study, *Melastoma malabathricum* was selected to be investigated on a large scale. *M. malabathricum* is a primitive plant in Malaysia that has been widely tested and documented for its promising pharmacological properties, such as antinociceptive, anti-inflammatory, antipyretic, antiviral, antibacterial, antiparasitic, antioxidant, anticoagulant, antivenom, antiulcer, and antidiarrheal and wound healing activities. Nevertheless, its hepatoprotective properties in particular have not been explored properly. As such, further research on its hepatoprotective activity is significant for nominating another plant to the list of potential medicinal hepatoprotective plant-based products.

1.1 Problem statement

In spite of the advanced development of modern medicine, there are several obstacle faced by the publics, such as the high cost of available drugs, the presence of drug side effects that prevent patients with certain health conditions from consuming a certain drug, and lack of drug availability. Therefore it is highly recommended to search for alternative medicine treating liver ailments as a substitute for currently used drugs that have fewer or no side effects and are cheaper and widely available. Encouraging research on medicinal plants indicates that phytochemicals can be exploited for treating many health problems. Extensive studies have been conducted on plant natural products, and most of these products have shown potential as new promising hepatoprotective agents; thus, this study, which aimed to discover the potential of hepatoprotective activity of *M. malabathricum* leaves, might add another candidate to the list. Scientifically, *M. malabathricum* is not traditionally known to

have hepatoprotective properties. Nevertheless, the factors that might be involved in its cytoprotective effects can be evaluated and further studied for future plant-derived drug development. Previous studies on *M. malabathricum* reported the presence of antioxidant and anti-inflammatory activity that is relevant evidence for hepatoprotective activity. Therefore, this study is expected to discover the capacity of *M. malabathricum* for hepatoprotective activity.

1.2 Hypothesis

1.2.1 Null Hypothesis

Methanol extract of *M. malabathricum* (MEMM*)* leaves possess hepatoprotective activity in carbon tetrachloride (CCl_4) and PCM-induced liver toxicity assays, and one or more of its partition is expected to have hepatoprotective activity in PCM-induced liver toxicity.

1.2.2 Alternative Hypothesis

Methanol extract of *M. malabathricum* (MEMM*)* leaves and its partition do not possess hepatoprotective activity in PCM-induced liver toxicity.

1.3 Objectives of the Study

1.3.1 General Objective

The objectives of the proposal study are as follows:

1. To determine the hepatoprotective activity of *M. malabathricum* methanol leaf extract using various hepatotoxicity induced models in rats.

1.3.2 Specific Objectives

The specific objectives of the proposed study are:

1. To determine hepatoprotective effect of methanolic extract of *M. malabathricum* leaves against carbon tetrachloride and paracetamol-induced liver toxicity models in rats; and then find out the most effective partition of MEMM; petroleum ether, ethyl acetate and aqueous extracts on liver toxicity study,
2. To examine the involvement of antioxidant and anti-inflammatory activities of the extracts as part of the hepatoprotective pathway,
3. To screen for the bioactive constituents present in MEMM and its partition using high performance liquid chromatography (HPLC)

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