UNIVERSITI PUTRA MALAYSIA

HEPATOPROTECTIVE EFFECT OF BAUHINIA PURPUREA L. METHANOLIC LEAVES EXTRACT

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment 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

HEPATOPROTECTIVE EFFECT OF *BAUHINIA PURPUREA* L. METHANOLIC LEAVES EXTRACT

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December, 2014

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

The objective of this study was to determine the hepatoprotective activity of methanolic extract of *Bauhinia purpurea* (Fabaceae) leaves (MEBP) and its partitions using rat models, i.e., by evaluating the prophylactic effect of the plant extracts administered prior to the induction of liver toxicity using a hepatotoxic agent. The study was designed as a preventive method, as the hepatoprotective potential of MEBP has never been reported. In an attempt to establish the pharmacological properties of *B. purpurea*, the hepatoprotective potential of MEBP was investigated using paracetamol (PCM)- and carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats. Throughout this study, the animals were divided into 22 groups containing 6 rats per group. For the first stage of the *in vivo* study, rats were divided into groups and administered orally once daily with 10% dimethyl sulfoxide (DMSO) (negative control), 200 mg/kg silymarin (positive control), or MEBP (50, 250, 500 mg/kg) for 7 days, followed by hepatotoxicity induction using PCM or CCl_4. In the second stage of the *in vivo* study, MEBP was partitioned into 3 fractions: petroleum ether extract (PEBP), ethyl acetate extract (EABP), and aqueous extract (AQBP). PEBP, EABP, and AQBP activities were tested on PCM-induced hepatotoxicity in rats. Blood samples underwent biochemical analysis to evaluate alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and total protein (TP) levels; the livers were subjected to microscopic analysis. All extracts (MEBP, PEBP, EABP, AQBP) underwent antioxidant study using the 2, 2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), superoxide dismutase scavenging assay (SOD), and oxygen radical absorbance capacity assay (ORAC), and anti-inflammatory study using lipoxygenase (LOX) and xanthine oxidase (XO) assays. Total phenolic content (TPC), phytochemical screening, and high-performance liquid chromatography (HPLC) analysis were also performed. From the histological observation, lymphocyte infiltration and marked necrosis were observed in the DMSO-treated

groups (negative control). MEBP showed encouraging activity for reducing the toxic effect of CCl_4 and PCM on the liver by reducing the weight of the liver in a dose-dependent manner; histological observation demonstrated normalization of the histopathological changes, preserving hepatocyte structure, causing a significant decline in ALT and AST levels ($p < 0.05$) and escalation of TP level. PEBP, which contains non-polar compounds, reduced the liver enzyme levels in a dose-dependent manner and increased the production of TP. EABP and AQBP, which contain intermediate compounds and polar compounds, respectively, attenuated the liver enzyme and LDH levels (concentration-independent). Among the extracts, EABP had the best activity for attenuating the liver enzymes. MEBP had the highest TPC value, followed by EABP, AQBP, and PEBP. EABP and MEBP demonstrated potential free radical scavenging activity in the SOD assay. The trend for the ORAC assay was slightly different from that of the DPPH and SOD assays. AQBP and EABP had high ORAC value, which determines the capacity of an extract to act as an antioxidant. All extracts in the present study had weak anti-inflammatory activity in the inhibition of LOX and XO. Phytochemical screening of the extracts showed that MEBP, PEBP, and EABP contained flavonoids, tannins, polyphenolic compounds, and steroids. However, the phytochemical screening showed that AQBP contained fewer compounds. HPLC analysis demonstrated several peaks detected at different wavelengths of the chromatogram of MEBP, EABP and AQBP, which were suggested to be flavonoid-based compounds. In conclusion, MEBP exerted potential hepatoprotective activity that can be partly attributed to its antioxidant activity, and EABP was considered to have the best activity among the fractions, which warrants further investigation.

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

KESAN HEPATOPROTEKTIF OLEH EKSTRAK METANOL DARI DAUN *BAUHINIA PURPUREA* L.

Oleh

FARHANA BTE YAHYA

December, 2014

Pengerusi: **Profesor Madya Zainul Amiruddin Zakaria, PhD**
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Objektif kajian ini adalah untuk menentukan aktiviti hepatoprotektif ekstrak metanol daripada daun *Bauhinia purpurea* dan pecahannya dengan menggunakan model tikus dengan menilai kesan profilaksis ekstrak tumbuhan yang diambil sebelum induksi ketoksikan hati menggunakan ejen hepatotoksik. Kajian ini berdasarkan kaedah pencegahan kerana potensi hepatoprotektif daripada MEBP tidak pernah didakwa terbukti lagi. Dalam usaha untuk mengenal pasti sifat-sifat farmakologi *Bauhinia purpurea* (Fabaceae), potensi hepatoprotektif dari ekstrak metanol daun *B. purpurea* (MEBP) telah diuji menggunakan rangsangan hepatotoksisiti paracetamol (PCM) - dan karbon tetraklorida (CCl_4) pada tikus. Sepanjang kajian ini, haiwan telah dibahagikan kepada 22 kumpulan dengan 6 tikus setiap kumpulan. Untuk bahagian pertama kajian *in vivo*, tikus (n = 6 bagi setiap kumpulan) dibahagikan kepada beberapa kumpulan dan diberi makan secara oral sekali sehari dengan 10% dimetil sulfoxide (DMSO) (kawalan negatif), 200 mg / kg silymarin (kawalan positif), atau MEBP (50, 250, dan 500 mg / kg) selama 7 hari, diikuti dengan proses rangsangan hepatotoksisiti menggunakan PCM atau CCl_4. Kemudian MEBP di ekstrak kepada 3 pecahan: ekstrak petroleum eter (PEBP), ekstrak etil asetat (EABP), dan ekstrak akueus (AQBP). Dalam bahagian kedua kajian *in vivo*, aktiviti PEBP, EABP dan AQBP telah diuji ke atas rangsangan PCM ke atas hati tikus. Sampel darah yang telah diambil dibuat kajian biokimia untuk menganalisis paras enzim seperti alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), dan total protein (TP). Manakala, sampel hati pula diuji secara mikroskopik. Semua ektrak (MEBP, PEBP, EABP dan AQBP) juga diuji untuk kajian antioksidan menggunakan cerakin 2, 2-difenil-1-picrylhydrazyl radikal (DPPH), pengujian perangkap aktiviti superoxide dismutase (SOD), dan cerakin penyerapan oksigen radikal kapasiti (ORAC), dan kajian anti-radang menggunakan analisis aktiviti lipoxygenase (LOX) dan xanthine oxidase (XO). Kandungan jumlah fenol (TPC), pemeriksaan fitokimia, dan kromatografi cecair berprestasi tinggi (HPLC) analisis juga telah dilaksanakan. Dari segi pemerhatian histologi,

penyusupan limfosit dan nekrosis diperhatikan dalam kumpulan rawatan DMSO (kawalan negatif). MEBP menunjukkan aktiviti yang bagus dalam usaha mengurangkan kesan toksik daripada CCL_4 dan PCM ke atas hati, dengan menyebabkan penurunan berat hati secara kebergantungan pada peningkatan dos, pemantauan histologi menunjukkan pemulihan struktur sel-sel hati, dan menyebabkan penurunan paras ALT dan AST secara signifikan ($P<0.05$), dan peningkatan paras TP. PEBP yang mengandungi sebatian tak berkutub, mengurangkan paras enzim hati secara kebergantungan terhadap dos dan menyebabkan peningkatan TP. EABP dan AQBP yang mengandungi sebatian pertengahan dan sebatian berkutub, masing-masing menyebabkan penurunan paras enzim hati dan LDH (tidak bergantung kepada dos). Antara semua ekstrak, EABP mempunyai aktiviti terbaik dalam penurunan enzim hati. MEBP mempunyai nilai TPC paling tinggi diikuti oleh EABP. AQBP dan PEBP. EABP dan MEBP sekali lagi menunjukkan potensi di dalam aktiviti SOD. Daripada penemuan, trend ORAC sedikit berbeza daripada aktiviti pengujian perangkap DPPH dan superoxide. AQBP dan EABP mempunyai nilai ORAC yang tinggi, ini menunjukkan kebolehan ekstrak di dalam aktiviti antioksidan. Manakala, semua ekstrak mempunyai kadar anti radang yang rendah dalam menghalang aktiviti LOX dan XO. Pemeriksaan fitokimia ekstrak menunjukkan MEBP, PEBP dan EABP mempunyai flavonoid, tannin, sebatian polifenolik, dan steroid. Manakala AQBP yang menunjukkan lebih sedikit sebatian yang di ekstrak. HPLC analisis menunjukkan beberapa puncak yang di kenal pasti pada gelombang yang berbeza di dalam kromatogram MEBP, EABP dan AQBP boleh dikategorikan sebagai jenis-jenis sebatian flavonoid. Kesimpulannya, MEBP mempunyai potensi sebagai agen hepatoprotektif yang juga sebahagiannya mungkin bergantung kepada aktiviti antioksidan. dan EABP dianggap mempunyai aktiviti yang terbaik di antara pecahan ekstrak, yang memerlukan siasatan lanjut.

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Thank you so much.

I certify that a Thesis Examination Committee has met on 16 December 2014 to conduct the final examination of Farhana Bte Yahya on her thesis entitled "Hepatoprotective Effect of *Bauhinia purpurea* L. Methanolic Leaves Extract" 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 Master of Science.

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LIST OF ABBREVIATIONS

AAPH	2,2'-azobis-2-methyl-propanimidamide, dihydrochloride
ALP	Alkaline phosphatise
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AQBP	Aqueous extract of Bauhinia purpurea
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUC	Area under the curve
$CCl_3\cdot$	Trichloromethyl free radical
CCl_4	Carbon tetrachloride
$Cl_3COO\cdot$	Trichloromethyl peroxy
COX	Cyclooxygenase
CYP450	Cytochrome P450
CYP450	Cytochrome P450
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
GSH	Glutathione
H&E	Haematoxylin & Eosin staining
H_2O_2	Hydrogen peroxide
HCV	Chronic viral hepatitis C
HO·	Hydroxyl radical
HPLC	High performance liquid chromatography
i.p	Intraperitoneally
IC50	Median inhibitory concentration
LDH	Lactate dehydrogenase
LOX	Lipooxygenase
MEBP	Methanol extract of Bauhinia purpurea
MeOH	Methanol
NAC	N-acetyl cystein
NAPQI	N-acetyl-p-benzoquinoneimine
NBT	Nitroblue tetrazolium
o.p	Orally
O_2	Oxygen
$O_2\bullet^-$	Superoxide anion
ORAC	Oxygen radical absorbance capacity
PCM	Paracetamol
PEBP	Petroleum ether extract of Bauhinia purpurea
PPAR-α	Peroxisome proliferator-activated receptor alpha
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SEM	Standard error mean

SOD	Superoxide dismutase
TP	Total protein
TPC	Total phenolic content
WHO	World Health Organization
XO	Xanthine oxidase

CHAPTER 1

INTRODUCTION

Continual damage to the liver by acute liver insult eventually results in the development of hepatic fibrogenesis. Advanced fibrogenesis leads to the development of severe life-threatening complications in patients, which promotes structural changes to the tissue, known as cirrhosis. Cirrhosis is a common and predictable aftermath of irreversible damage to the liver parenchyma triggered by a variety of etiologies. The leading causes of liver cirrhosis in the Western countries are mostly attributed to chronic hepatitis C and alcohol abuse (Qua and Goh, 2011). The increasing incidence of mortality due to liver diseases has been reported to be the tenth leading cause of death in the United States (Liver Center, Saint Louis University). Meanwhile, chronic hepatitis B appears to be the most prevalent cause of liver cirrhosis in the Asian Pacific region. Malaysia is universally acknowledged to have a unique multiracial composition of Asian populations that comprises three main races: Malay, Chinese, and Indian (Qua and Goh, 2011). In the medical field, the diagnosis of liver cirrhosis among these races is no longer a recently discovered, uncommon ocurrence. The only issue is that the etiologies of liver cirrhosis of each ethnic group are apparently different according to their religions, cultural beliefs (Qua and Goh, 2011), lifestyle, and environmental factors.

Chronic liver disease encompasses not only the aforementioned factors, but also a broad spectrum of principles: it also involves drug toxicity, autoimmune disease, fatty infiltration, hereditary linkage, and cryptogenic (unidentifiable) causes. It is understood that hepatic fibrogenesis has a potential reversible component; hindrance of liver trauma has become a reliable therapeutic strategy to minimize the progression of advanced liver disease.

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 (Helmstädter and Staiger, 2014; Balunas and Kinghorn, 2005; Fabricant and Farnsworth, 2001). Drug discovery of current active agents has to be 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 (Newman and Cragg, 2012). 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 favored as the primary option for alternative medicines, considering its low cost and effectiveness, and cultural, historical, and even religious inclinations (Priya et al., 2010). The World Health Organization (WHO)

estimated that about 80% of developing societies consider natural products their most preferred healthcare option (WHO, 2002).

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; McChesny et al., 2007), leaving many species awaiting therapeutic exploration. Malaysia has been acknowledged as a land of floral and faunal prosperity, and is 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 Malaysian ringgit (RM)4.55 billion, and the current appraisal growth rate is estimated to be worth 15–20% annually (Nordin et al., 2008; Aziz, 2003). Alongside economic factors, the increased interest in the herbal industry in Malaysia has apparently been caused by changes in lifestyle, increased health consciousness, and the costliness of synthetic medicines (Aziz, 2003). From the perspective of the herbal-based market, particularly herbal medicines, the natural herb heritage in Malaysia merits a favorable position in the industry. 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 resource diversity, lush tropical climate, growing demand for specialty natural products, and indigenous knowledge (Biodiversity in Malaysia, 2006).

To exploit these sources for prospective research, particularly hepatoprotective studies, *Bauhinia purpurea* was selected to be investigated on a large scale. *B. purpurea*, from the family Fabaceae and locally known as *tapak kuda*, is a native plant in Malaysia that has been widely tested and documented for its promising pharmacological properties, such as antioxidant (Joshi *et al.*,2009; Zakaria *et al.*,2011a; Annegowda *et al.*,2012), antiulcer (Zakaria *et al.*,2011b; Zakaria *et al.*,2012), anti-inflammatory (Boonphong *et al.*,2007), antinociceptive, antipyretic (Zakaria *et al.*,2007; Zakaria *et al.*,2009), antiproliferative (Zakaria *et al.*,2011a), antimicrobial (Murugan and Mohan, 2011), and wound healing (Ananth *et al.*,2010). 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.

Problem statement

Liver diseases have been acknowledged as one of the major threats to community health. Contributory factors of these problems are mainly attributable to chemicals such as paracetamol (PCM; overdoses), excessive alcohol consumption, autoimmune disorders, and infections. PCM, a mild analgesic and antipyretic drug developed in the past few decades, causes severe liver injuries (necrosis) in humans and experimental

animals following overdose of the drug. Alcoholic liver disease is the second most common reason for liver transplantation (Adewusi and Afolayan, 2010). Marzilawati *et al.* (2012) reported that acute liver failure caused by PCM toxicity is a major problem leading to death worldwide, whereas acute liver failure among Asians is commonly caused by viral hepatitis, and infrequent cases of PCM toxicity are reported. Nevertheless, it has been highlighted that data analysis of N-acetylcysteine (NAC) therapy, currently one of the most dependable drugs for countering PCM toxicity, cannot be taken for granted, which stated that it is not cost-effective in managing Asian patients with accidental PCM overdose. Moreover, toxicology research on NAC documented by the United States National Library of Medicine states that it has several side effects, usually involving anaphylactoid responses.

Justification for studying the hepatoprotective potential of *B. purpurea*

In spite of the advanced development of modern medicine, there are several obstacles faced by the public, 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 for 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 hepatoprotective activity of *B. purpurea* leaves, might add another candidate to the list. Scientifically, *B. purpurea* 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 *B. purpurea* reported the presence of antioxidant and anti-inflammatory activity that is relevant to hepatoprotective activity. Considering these reports, the antioxidant and anti-inflammatory activity indicate different pathways assisting the hepatoprotective effect. In general, free radicals or reactive oxygen species (ROS) generated from drug or chemical metabolism appear to be the fundamental mechanisms underlying most human ailments. The antioxidant and anti-inflammatory properties of plants facilitate the free radical scavenging process and regulate the inflammatory response, respectively, which are believed to initiate their detrimental effects on the liver. Therefore, this study is expected to discover the capacity of *B. purpurea* for hepatoprotective activity.

Hypothesis

Methanolic extract of *B. purpurea* leaves (MEBP) exerts hepatoprotective activity in PCM- and carbon tetrachloride (CCl_4)- induced liver toxicity assays, and one or more of its partitions is expected to have good hepatoprotective activity in PCM-induced liver toxicity.

General objectives:

- To determine hepatoprotective activity of methanol extract of *Bauhinia purpurea* leaves and its partitions using rat models

Specific objectives:

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

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