UNIVERSITI PUTRA MALAYSIA

EFFECTS OF ETHANOLIC AZADIRACHTA EXCELSA (JACK) JACOBS. LEAF EXTRACT ON BLOOD PRESSURE AND OXIDATIVE STRESS IN THE SPONTANEOUSLY HYPERTENSIVE RAT MODEL

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By

NUR FARHANA AHMAD SOPIAN

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

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DEDICATIONS

This thesis is especially dedicated to my beloved mother, Salmah Ismail and to my late father Allahyarham Ahmad Sopian Omar. A lot of thanks to my siblings, family and friends for all the tremendous support, prayers and encouragement during my entire postgraduate life.

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

EFFECTS OF ETHANOLIC *Azadirachta excelsa* (Jack) Jacobs. LEAF EXTRACT ON BLOOD PRESSURE AND OXIDATIVE STRESS IN THE SPONTANEOUSLY HYPERTENSIVE RAT MODEL

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August 2017

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Oxidative stress is implicated in the development and maintenance of hypertension. To date the available therapies and oral antihypertensive drugs could only help in treating hypertension regardless of oxidative stress status and organ protective effect in the hypertensive patients. Hence, researchers are searching for antihypertensive plants that could complement oral drugs in hypertension. *Azadirachta excelsa* or locally known as "sentang" tree is one of the plants that have been used in Malay traditional medicine to treat diabetes. However, there were only a few studies regarding its antihypertensive and antioxidant activity on mammals. Therefore, this research aimed to evaluate the antihypertensive effects of *A. excelsa* by measuring the blood pressure of rats, serum analysis on aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine phosphokinase (CPK) and lactate dehydrogenase (LDH). Meanwhile, its antioxidant effect on oxidative stress status was determined through organ analysis on malondialdehyde (MDA) level, antioxidant enzyme activity [Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] and histopathological changes of tissue structures in the liver, kidney, and heart of SHR. In this study, twenty-four male SHR of 12-14 weeks old were assigned randomly into four different groups (n=6) as followed; Group I, SHR control received distilled water; Group II, SHR received 250 mg/kg of *A.excelsa*; Group III, SHR received 10 mg/kg of quercetin; and Group IV, SHR received 40 mg/kg of captopril. Meanwhile, male Wistar-Kyoto (WKY; n=6) rats were used as normotensive control also received distilled water. The treatments were given in 28 days where the blood pressure was measured weekly. Initially, systolic blood pressure (SBP) and diastolic blood pressure (DBP) of SHR were high significantly ($P<0.01$) compared to WKY. At the end of experiment, the SBP and DBP in SHR were alleviated by all treatment groups ($P<0.01$). Meanwhile, only quercetin-treated SHR showed significantly reduced serum enzymes of AST, ALT, LDH, and CPK. MDA level in the liver, kidney, and heart

of SHR were significantly higher ($P<0.05$) compared to WKY indicating impaired organ in these rats. Interestingly, treatment with *A.excelsa* and quercetin lower the MDA level in all organs ($P<0.05$). Furthermore, SOD activity in the heart from all treated SHR was decreased while only GPx activity in the liver and heart of *A.excelsa* treatment was increased. *A.excelsa* and quercetin treatments managed to improve hepatocyte structure. Also, all treatments managed to ameliorate glomerular and renal tubular lesions of the kidney, while only captopril treatment retained normal architecture of myocardial tissue of the heart. In conclusion, current findings suggested that *A. excelsa* ethanolic leaf extract could attenuate hypertension via amelioration of oxidative stress and improved tissue structure in SHR.

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

KESAN EKSTRAK ETANOL DAUN *Azadirachta excelsa* (Jack) Jacobs. TERHADAP TEKANAN DARAH DAN TEGASAN OKSIDATIF KE ATAS MODEL TIKUS HIPERTENSI

Oleh

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Tegasan oksidatif dikaitkan dengan perkembangan dan penyelenggaraan tekanan darah tinggi. Setakat ini, terapi dan ubatan antihipertensi oral yang sedia ada hanya mampu membantu merawat hipertensi tanpa mengira status tegasan oksidatif dan melindungi organ pesakit hipertensi. Oleh itu, para penyelidik mencari pokok antihipertensi yang mampu bertindak sebagai pelengkap kepada ubatan oral di dalam hipertensi. *Azadirachta excelsa* atau pokok yang dikenali sebagai "sentang" adalah salah satu pokok yang digunakan dalam perubatan tradisional Melayu untuk merawat kencing manis. Walau bagaimanapun, terdapat hanya beberapa kajian sahaja mengenai aktiviti antihipertensi dan antioksidan pada mamalia. Oleh itu, kajian ini bertujuan untuk menilai kesan antihipertensi *A. excelsa* dengan mengukur tekanan darah tikus, analisis serum terhadap paras aspartate aminotransferase (AST), alanina aminotransferase (ALT), kreatina fosfokinase (CPK) dan laktat dehidrogenase. Sementara itu kesan antioksidan ke atas tegasan oksidatif diukur melalui analisis organ terhadap paras malondialdehide (MDA) dan aktiviti enzim antioksidan [Superoksida dismutase (SOD), katalase (CAT) dan glutathione peroksidase (GPx)] dan perubahan histopatologi struktur tisu di dalam hati, ginjal, dan jantung daripada SHR. Di dalam kajian ini, dua puluh empat tikus SHR jantan berumur 12-14 minggu dibahagikan kepada empat kumpulan yang berbeza secara rawak (n=6) seperti berikut; Kumpulan I, SHR kawalan menerima air suling; Kumpulan II, SHR menerima 250 mg/kg *A.excelsa*; Kumpulan III, SHR menerima 10 mg/kg quercetin; dan Kumpulan IV, SHR menerima 40 mg/kg captopril. Sementara itu, tikus Wistar-Kyoto (WKY; n=6) dijadikan sebagai kawalan normotensif juga menerima air suling. Rawatan diberikan dalam masa 28 hari di mana tekanan darah diambil setiap minggu. Pada mulanya, tekanan sistolik (SBP) dan diastolik (DBP) dari tikus SHR adalah sangat tinggi ($P<0.01$) berbanding tikus WKY. Pada akhir rawatan, semua rawatan telah berjaya mengurangkan tekanan SBP dan DBP ($P<0.01$). Sementara itu, hanya tikus SHR yang dirawat dengan quercetin menunjukkan pengurangan signifikan terhadap paras serum AST, ALT, LDH dan CPK. Paras MDA diukur dalam hati, ginjal dan jantung kawalan SHR menunjukkan peningkatan signifikan ($P<0.05$) berbanding dengan kawalan WKY,

menandakan organ yang terjejas dalam tikus ini. Menariknya, rawatan dengan *A.excelsa* dan quercetin mengurangkan paras MDA dalam semua organ ($P<0.05$). Tambahan lagi, aktiviti SOD dalam jantung daripada semua SHR yang dirawat telah menurun, sementara itu hanya aktiviti GPx di dalam hati dan jantung daripada rawatan *A.excelsa* telah meningkat. Rawatan *A.excelsa* dan quercetin berjaya memperbaiki struktur hepatosit. Dan juga, semua rawatan berjaya mengurangkan luka-luka tiub glomerular pada ginjal, manakala hanya rawatan captopril membaiki struktur normal tisu miokardium jantung. Kesimpulannya, penemuan terkini menunjukkan bahawa ekstrak *A.excelsa* boleh merawat tekanan darah tinggi dengan menangani tegasan oksidatif dan menambah baik struktur tisu dalam tikus SHR.

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I certify that a Thesis Examination Committee has met on 8th August 2017 to conduct the final examination of Nur Farhana Binti Ahmad Sopian on her thesis entitled "Effects of Ethanolic Azadirachta excelsa (Jack) Leaf Extract on Blood Pressure and Oxidative Stress in the Spontaneously Hypertensive Rat Model" 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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This thesis was 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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LIST OF ABBREVIATIONS

AAPH	2,2'-azobis (2-amidinopropane)
ACE	Angiotensin converting enzyme
ACEI	Angiotensin converting enzyme inhibitors
ADH	anti-diuretic hormone
AGT	Angiotensinogen
AHA	American Heart Association
ALT	Alanine aminotransferase
ANG I	Angiotensin I
ANG II	Angiotensin II
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
BBB	blood-brain barrier
BPM	beats per minute
CAT	Catalase
CI	cardiosomatic index
CVD	Cardiovascular disease
CVO	circumventricular organs
DBP	Diastolic blood pressure
DPPH	2,2-diphenyl-1-picrylhydrazyl
eNOS	endothelial nitric oxide synthase
GR	glutathione reductase
GSSG	oxidized glutathione
GPx	Glutathione peroxidase
H_2O_2	hydroxyl peroxide
HSI	Hepatosomatic index
IC_{50}	half maximal inhibitory concentration
ICV	intracerebroventricular
KSI	kidney somatic index
LDLs	Low density lipoproteins
MDA	Malondialdehyde
MmHg	Millimeter per mercury
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
PKD	polycystic kidney disease
PUFA	Polyunsaturated fatty acid
RAAS	Renin angiotensin aldosterone system
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SEM	Standard error of mean
SHR	Spontaneously hypertensive rat
SOD	Superoxide dismutase
VSMC	vascular smooth muscle cell
WHO	World Health Organization
WKY	Wistar-Kyoto rat

CHAPTER 1

INTRODUCTION

1.1 Background of study

Hypertension is a silent killer disease manifested as the major contributor to the development of many cardiovascular diseases (CVD). It was reported that CVD is the main cause of mortality and morbidity rate in a worldwide population contributed to 16.7 million deaths in worldwide each year (WHO, 2003). Back in 2009, it was estimated about 12.8% of total deaths per year in which 7.5 million from the total deaths is accredited to hypertension (WHO, 2009). However, because of population growth and aging, the number had increased to 16.5% of annual deaths worldwide where hypertension is recognized as leading cause associated with CVD (WHO, 2013). According to WHO (2013), the annual death toll is estimated to reach 23.5 million people by 2030. Commonly, people with high blood pressure are at high risk of CVD such as heart attack, stroke, and atherosclerosis. Other than that, hypertension also lead to renal damage, dementia, or blindness (August, 2004; Freedman and Cohen, 2016). Thus, alleviating hypertension can reduce the risk of mortality associated with CVD and others complications in hypertensive patients (Chobanian et al., 2003).

In current medical practice, there are many types of antihypertensive drugs prescribed for hypertension although sometimes the exact etiology of hypertension remains unknown. Despite this, there is increasing concern about the quality of patient's life receiving treatment for hypertension as these regimes may be associated with various adverse effects. Specifically, elderly hypertensive patients are little more prone to side-effects. Most of the synthetic antihypertensive drugs often cause undesired side effects such as dehydration, vomiting, and cough. As an example, beta-blockers and diuretics drugs only reduce blood pressure but unable to prevent organ damage (Clinical Practice Guidelines for the Management of Hypertension, 2013). In order to achieve an optimal effect of hypotensive action, the efficiency of the drugs are only 34% when two or more antihypertensive drugs from different categories were combined (August, 2004; Yeh et al., 2009; Wang and Xiong, 2012). Major concerns often delay the treatment are represented by their availability and accessibility (Wang and Xiong, 2012), unwanted side effects (Susalit et al., 2011; Wang and Xiong, 2012) and higher costs of antihypertensive drugs (Susalit et al., 2011) when combination of two or more pills are needed. Thus, researchers revealed many potential plant species that possessed antihypertensive properties. For instance, high intake of natural flavonoids from medicinal plants in the diet was reported to have an inverse relationship to risk of CVD (Knekt et al., 2002). They play a protective role against the development of pathological conditions such as hypertension, diabetes, and obesity which associated to oxidative stress. Oxidative stress emerges as a key regulator of blood pressure regulation by

balancing vasoconstrictor and vasodilator mechanisms. Disruption of the mechanism leads to a persistent vasoconstriction and resulted in an elevation of blood pressure. As hypertension is one of the most common metabolic disorders characterized by oxidative stress, there is a growing interest of inhibiting oxidative stress by using natural antioxidants in plants such as phenolic and flavonoid compounds.

Commonly, the hypertensive patients from low-income group, especially rural dwellers in developing countries are seeking alternative approaches such as herbal remedies to control hypertension and its concomitant complications in face of limited socioeconomic resources (Chockalingam et al., 2006). Therefore, plant-based remedies are being widely investigated by researchers to provide alternatives in treating hypertension. Currently, various species of plants have been identified to exert antioxidant associated with antihypertensive properties (Nahida and Feroz, 2011). However, not all of them have received scientific scrutiny and therefore scientific research needs to be done to verify their effectiveness and elucidate safety profile of such herbal remedies.

Azadirachta excelsa or locally known as "sentang" belongs to Meliaceae family where it is a plant family that recognizable with various benefits associated with medical properties. A number of studies have investigated the biological activities of *A. excelsa* such as antiseptic, anti-inflammatory, antimicrobial, antifeedant and insecticidal agent (Ng et al., 2003; Akhtar et al., 2008; Mustafa and Al-Khazraji, 2008). Meanwhile, our previous finding on animal studies had proven that *A. excelsa* acts as an antidiabetic agent by reducing blood glucose level in type 1 and type 2 of diabetes mellitus (Nurdiana et al., 2013; Nurdiana et al., 2014; Nurul 'Izzati 2016). However, *A. excelsa* has not been studied extensively regarding its antihypertensive activity. Thus, the current study was designed primarily to determine its effect on blood pressure in genetic hypertension animal model. This study may also provide an alternative source of natural herbs potential namely *A. excelsa* as antihypertensive agents with probably fewer or no adverse side effect.

1.2 Significance of study

The study was designed primarily to evaluate the antioxidant activities of *A. excelsa* leaf extract on Spontaneously Hypertensive Rat (SHR) model. This study allows the plant to be scientifically prove as remedies for hypertension as well as its ability in scavenging free radicals. As encouraged by World Health Organization (WHO), the present study can be a turning point in enhancing more research involving natural herbs as drugs of tomorrow to improve our quality life.

1.3 Objectives of study

Main objective;
To determine the antioxidant activities of *A. excelsa* leaf extract on blood pressure and oxidative stress in SHR animal model.

Specific objectives;

1. To quantify the total phenolic, total flavonoids content and the IC_{50} value of *A. excelsa*.
2. To investigate the effects of *A. excelsa* leaf extract on elevated blood pressure in SHR model and serum level of cardiac injury.
3. To evaluate the effects of *A. excelsa* leaf extract on oxidative stress marker and antioxidant enzyme activities in SHR's organs.
4. To determine the effects of *A. excelsa* leaf extract on serum level of liver toxicity and histopathological changes of liver, kidney and heart in SHR model.

1.4 Hypotheses

It was hypothesized that ;

Antioxidant activities of *A. excelsa* exhibited an antihypertensive effect on SHR model by ameliorating serum level of cardiac injury.

A. excelsa mitigates ROS level and lipid peroxidation process in SHR's organs by altering the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) antioxidant enzymes.

Administration of *A. excelsa* ethanolic extract for 28 days reduced serum liver toxicity as well as improve the histopathological structure and the extent of oxidative stress damage.

1.5 Justifications of the study

Recently, several studies were reported on the antidiabetic effect of *A. excelsa* in type I and type II diabetic rats (Nurdiana et al., 2013 and 2014; Nurul 'Izzati, 2016). However, the effect of *A. excelsa* on oxidative stress, specifically in hypertensive rats remains elusive and yet to be investigated. On those studies, the approach used to induce diabetes was by an injection of streptozotocin (STZ). The injection generates free radicals from β-cells of pancreatic islet that are vulnerable and sensitive to oxidative stress (Robertson et al., 2003). As the main underlying cause of diabetes and hypertension is oxidative stress, the current

study was done to investigate effects of *A. excelsa* on oxidative stress in genetic hypertension of SHR model.

The uses of SHR as an animal model to study the pathogenesis of hypertension and screening any potential antihypertensive plants are well documented. While, an elevation and progression of blood pressure in this genetic animal model were attributable by oxidative stress (Hirooka et al., 2011; Fanelli and Zatz, 2011). Therefore, this study was conducted to evaluate the effect of *A. excelsa* on blood pressure in SHR while examining oxidative stress level in organs.

Quercetin was chosen at 10 mg/kg based on the average of human intake in the diet (Duarte et al., 2001b). It was included in the current study to compare between the treatment of quercetin with crude extract from *A. excelsa,* since quercetin was the purified plant phenols found in *Azadirachta* sp (Sithisarn et al., 2007). Meanwhile, captopril was chosen as a positive control in the treatment because it causes little side effects and possessed antioxidant as well as renoprotective effects (Dzau et al., 2001).

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