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ELUCIDATION OF THE ANTI-INFLAMMATORY COMPOUND PRESENT IN Jatropha curcas LINN. ROOT AND ITS MODE OF ACTION

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By

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Thesis Submitted to the School of Graduate Studies,Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the Degree of Doctor of Philosophy

ELUCIDATION OF THE ANTI-INFLAMMATORY COMPOUND PRESENT IN *Jatropha curcas* LINN. ROOT AND ITS MODE OF ACTION

By

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June 2016

Chairman : Professor Norhani Abdullah, PhD
Institute : Bioscience

Jatropha curcas Linn. (family *Euphorbiaceae*) is a drought resistant shrub which is widely grown in Central and South America, South-east Asia, India and Africa. The plant has been considered a traditional herb in many parts of the world. In inflammatory treatment, it has been widely accepted that non-steroidal anti-inflammatory drugs (NSAIDs) can effectively prevent inflammation. However, several studies have also revealed side effects resulting from prolonged use of NSAIDs, which include the possibility of several chronic diseases such as gastrointestinal ulcers, adverse cardiovascular side-effects, and Alzheimer's disease. Hence, alternative medicine based on natural herbs should be considered in inflammation treatment. Furthermore, the use of herbal remedies is gaining acceptance in various pharmaceutical applications. Although several studies have shown different parts of *J. curcas* possessed anti-inflammatory activity, but the nature of the compounds involved and the mode of action are not well understood. Hence, before the herbal products can be made available, detail information regarding the nature of bioactive compounds and the mode of action have to be understood. Thus, the main objective of this study was to elucidate the anti-inflammatory compounds from *J. curcas* plant and to determine the mode of action. The experiments conducted include screening of different parts of plant extracts for the anti-inflammatory activity by using Murine monocytic macrophage RAW 264.7 cells line, purifying and elucidating the structure of the anti-inflammatory compounds, determining the mode of action of the purified compounds on the inflammation pathway and the inflammatory enzymes affected. Anti-inflammatory activity was assayed by determining the inhibition of nitric oxide production in the RAW 264.7 cells, while cytotoxicity activity was determined by the (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay. In the initial study, it was observed that Jatropha root methanolic extract showed anti-inflammatory property higher than other plant parts (leaves, fruits and stem bark), but with high cytotoxicity towards Murine monocytic macrophage RAW 264.7 cells line. Subsequently, the root extract was fractionated into fractions with different solvents (hexane, chloroform and ethyl acetate). The hexane fraction possessed high anti-inflammatory property, but with high cytotoxicity towards cell growth. Analysis of the compounds present in the hexane fraction by gas chromatography mass spectrometry (GC-MS) showed the presence of many compounds belonging to the

terpene group which probably caused the cytotoxicity. Further purification was conducted by using an open column system to isolate and purify the compounds with anti-inflammatory activity without cytotoxicity. Five spots (labeled H-1, H-2,3, H-4 and H-5) from the hexane fraction were obtained. The anti-inflammatory assay showed the compounds present in spot H-4 and H-5 possessed high anti-inflammatory without cytotoxicity activity. Analysis of compounds present in these two spots by GC-MS showed the presence of hexadecanoic and octadecanoic acid groups in both spots. The high performance liquid chromatography (HPLC) analysis of spot H-4 showed two peaks (A and B), with eluent A (peak A) displaying better anti-inflammatory activity than eluent B (peak B), without being toxic to the cell growth. Identification of the compound present in eluent A by liquid chromatography-tandem mass spectrometry (LC-MS/MS) indicated that the compound belonged to the octadecanoic acid group. Further analysis conducted by using NuclearMagnetic Resonance (NMR) showed that this active compound was a long chain of hydrocarbons with a carboxylic group attached at the end, thus confirming that the active compound belonged to octadecanoic acid group. To determine the mode of action in the anti-inflammatory activity, the fluorescence staining assay was conducted to observe the translocation of the NF-κB subunit (involved in inflammatory signaling pathway) from the cytoplasm into the nuclei of the RAW 264.7 cells. The results showed that octadecanoic acid did not inhibit the translocation of p65 subunit, thus could not inhibit expression of inflammatory genes. Similarly, in the gene expression study by qualitative Reverse Transcriptase PCR, genes for phospholipase A_2 (PLA_2), cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX) and inducible nitric oxide synthase (iNOS) involved in inflammatory signaling pathway were not affected by octadecanoic acid added to the cells at various concentrations (0.125 mg/mL – 1.0 mg/mL). In the inflammatory enzyme assays, only PLA_2 activity, but not COX-1, COX-2, and 5-LOX were inhibited. An IC_{50} analysis showed that at concentrations of 0.24 mg/mL, octadecanoic acid inhibited 50% of PLA_2 activity. As a conclusion, the present study showed that *J.curcas* plant possessed anti-inflammatory activity, especially the roots and several compounds belonging to the terpene group present in the root, might contributed to the cytotoxicity of the root extract. The anti-inflammatory compound was identified to be octadecanoic acid and was found to inhibit PLA_2 enzyme activity (as the possible mode of action) by competing with the enzyme substrate as indicated by the IC_{50} analysis, where PLA_2 activity was only inhibited at high concentrations of octadecanoic acid.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENJELASAN TENTANG SEBATIAN ANTI-RADANG DI DALAM AKAR *Jatropha curcas* LINN. DAN MOD TINDAKANNYA

Oleh

AHMAD RAZI BIN OTHMAN

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Jatropha curcas Linn. (famili *Euphorbiaceae*) merupakan pokok renek yang mampu bertahan dalam keadaan kontang dan banyak ditanam di kawasan Amerika Tengah, Amerika Selatan, Asia Tenggara, India dan Afrika. Pokok ini digunakan sebagai ubat tradisi bagi kebanyakan masyarakat di dunia. Di dalam bidang rawatan sakit radang, non-steroidal anti-inflammatory drugs (NSAIDs) telah di buktikan keberkesanannya dalam mengawal sakit radang. Walaubagaimanapun, beberapa kajian mendedahkan kesan sampingan daripada penggunaan ubat NSAIDs yang berpanjangan yang menyebabkan kebarangkalian menghidapi penyakit kronik seperti ulser usus, sakit jantung yang kronik dan penyakit Alzheimer's. Oleh itu, perubatan alternatif berasaskan tumbuhan perlu dipertimbangkan dalam merawat sakit radang. Tambahan pula, penggunaan ubatan herba kini semakin diterima dalam pelbagai bidang farmasutikal. Walaupun beberapa kajian telah menunjukan bahagian-bahagian *J. curcas* memiliki keupayaan sebagai anti-radang, tetapi bahan aktif dan mekanisma tindakannya masih belum difahami sepenuhnya. Oleh yang demikian, sebelum sesuatu produk herba boleh digunakan, maklumat terperinci berkaitan dengan bahan bioaktifnya dan mekanisma tindakannya perlulah difahami terlebih dahulu. Dengan objektif utama kajian ini adalah untuk menentukan bahan aktif yang bersifat anti-radang daripada pokok *J.curcas* dan juga mekanisma tindakannya. Kajian yang dijalankan meliputi pemeriksaan terhadap ekstrak bahagian-bahagian pokok Jatropha yang memiliki sifat anti-radang dengan menggunakan sel Murine monocytic macrophage RAW 264.7, penulenan dan penjelasan tentang struktur bahan aktif tersebut, pembuktian tentang mekanisma tindakan oleh bahan aktif dan enzim-enzim yang terlibat didalamnya. Aktiviti anti-radang ditentukan dengan cara perencatan penghasilan nitrik oksida di dalam sel RAW 264.7, manakala aktiviti sitotoksik ditentukan dengan (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay. Di peringkat awal kajian, dapat diperhatikan bahawa ekstrak bahagian akar Jatropha menggunakan methanol memiliki aktiviti sebagai anti-radang yang lebih tinggi berbanding bahagian-bahagian yang lain (daun, buah dan batang), tetapi toksik terhadap sel Murine monocytic macrophage RAW 264.7. Seterusnya, extrak akar pecahkan kepada beberapa pecahan mengikut perbezaan polariti pelarut (hexane, chloroform dan ethyl acetate). Pecahan dari pelarut hexane memiliki aktiviti anti-radang yang tinggi, tetapi masih menunjukan kadar toksik yang tinggi

terhadap pertumbuhan sel. Analisa terhadap kandungan yang terdapat di dalam pecahan hexane menggunakan teknik gas chromatography mass spectrometry (GC-MS) menunjukan kehadiran kompaun-kompaun dari kumpulan terpene yang berkemungkinan punca kepada sifat toksik kompaun tersebut. Teknik penulenan seterusnya menggunakan sistem kolum terbuka bagi mengasingkan kompaun yang memiliki sifat anti-radang tanpa bersifat toksik terhadap sel. Lima kompaun dapat diasingkan dan dilabel sebagai H-1, H-2,3, H-4 dan H-5 dari pecahan hexane. Assay anti-radang menunjukan kompaun yang terdapat pada H-4 dan H-5 memiliki sifat anti-radang yang sangat tinggi tanpa menunjukan sifat toksik. Analisa terhadap kompaun yang terdapat didalam H-4 dan H-5 menggunakan kaedah GC-MS menunjukan kehadiran kumpulan asid lemak (heksadekanoik dan oktadekanoik) didalam kedua-dua kompaun H-4 dan H-5. Penulenan H-4 menggunakan kaedah "high performance liquid chromatography" (HPLC) menunjukan kehadiran dua kompaun (A dan B) dengan eluen A memaparkan aktiviti anti-radang yang lebih bagus berbanding eluen B tanpa memiliki sifat toksik terhadap pertumbuhan sel. Pengenalpastian kompaun yang terdapat eluen A menggunakan "liquid chromatography-tandem mass spectrometry" (LC-MS/MS) menunjukan kompaun tersebut tergolong di dalam kumpulan asid lemak oktadekanoik. Analisa selanjutnya menggunakan teknik "NuclearMagnetic Resonance" (NMR) membuktikan bahawa kompaun aktif adalah terdiri daripada rantai panjang hidrokarbon dengan hujungnya memiliki kumpulan karboksilik, lalu mengesahkan bahawa kompaun aktif tersebut adalah dari kumpulan asid lemak oktadekanoik. Bagi menentukan mekanisma tindakan di dalam aktiviti anti-radang, assay menggunakan pewarna berpendafluor dijalankan bagi memerhatikan proses translokasi oleh subunit NK-κB (terlibat di dalam proses radang) dari sitoplasma ke dalam nucleus sel RAW 264.7. Hasil kajian menunjukan asid oktadekanoik tidak menghalang proses translokasi subunit p65, seterusnya gagal menghalang proses penyalinan gen yang terlibat dengan proses radang. Bersamaan dengan kajian ekspresi gen menggunakan kaedah PCR Transkriptase berbalik terhadap gen phospholipase A_2 (PLA_2), cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX) dan inducible nitic oxide synthase (iNOS) yang terlibat di dalam proses radang, asid oktadekanoik tidak menunjukkan sebarang kesan pada kepekatan yang berbeza (0.125 mg/mL – 1.0 mg/mL). Di dalam kajian terhadap aktiviti enzim, hanya enzim PLA_2 yang direncat, tetapi tidak COX-1, COX-2 dan 5-LOX. Analisis terhadap IC_{50} menunjukkan pada kepekatan 0.24 mg/mL, asid oktadekanoik merencat 50% aktiviti PLA_2. Sebagai kesimpulan, kajian ini membuktikan bahawa pokok *J. curcas* memiliki kebolehan sebagai anti-radang, terutamanya pada bahagian akar, tetapi beberapa kompaun daripada kumpulan terpene berkemungkinan penyumbang kepada sifat toksik ekstrak akar berkenaan. Kompaun anti-radang yang dikenalpasti sebagai asid oktadekanoik mampu merencat aktiviti enzim PLA_2 dengan cara bersaing terhadap substrat seperti yang ditunjukkan di dalam analisis IC_{50} di mana aktiviti PLA_2 hanya direncat apabila asid oktadekanoik berkepekatan tinggi.

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I certify that a Thesis Examination Committee has met on 10 June 2016 to conduct the final examination of Ahmad Razi bin Othman on his thesis entitled "Elucidation of The Anti-Inflammatory Compound Present in *Jatropha curcas* Linn Root and Its Mode of Action" 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 Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follow:

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

δ	Chemical shift in ppm
°C	Degree in Celsius
^{13}C	Carbon-13
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethylsulfoxide
EtOAc	Ethyl acetate
GC-MS	Gas Chromatography-Mass Spectrometry
^{1}H	Proton
gHMBC	Gradient Heteronuclear Multiple Bond Correlation
gHSQC	Gradient Heteronuclear Single-Quantum Coherence
gCOSY	Gradient Correlation Spectroscopy
J	Coupling in Hz
m	Multiplet
m/z	Mass per charge
MeOH	Methanol
MS	Mass Spectrum/ Mass Spectrometry
NMR	Nuclear Magnetic Resonance
s	Singlet
TLC	Thin Layer Chromatography
UV	Ultraviolet
IC	Inhibition concentration
PLA_2	Phospholipase A_2
COX-1	Cyxlooxygenase-1

COX-2	Cyclooxygenase-2
iNOS	Inducible Nitric Oxide Synthase
5-LOX	5-Lipoxygenase
FLAP	Five Lipoxygenase Activated Protein
CRP	C-Reactive Protein
NF-κB	Nuclear Factor Kappa B
I-κB	Inhibitor Kappa B
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
AA	Arachidonic Acid
NO	Nitric Oxide
DMEM	Dulbecco's Modified Eagle Medium
LPS	Lipopolysaccharide
IFN-γ	Interferon gamma
ROS	Reactive Oxygen Species
NSAID	Non-steriodal Anti-Inflammatory Drug
ILs	Interleukins
LTs	Leukotriene
PGs	Prostaglandins
TNF-α	Tumor Necrosis Factor Alpha
TLR	Toll Like Receptor
DNA	Deoxyribonucleic Acid
mRNA	Messenger Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
PIC	Pro-Inflammatory Cytokines
MAPK	Mitogen Activated Protein Kinase

CDCl3	Deuterated chloroform
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
µg	microgram
mg	milligram
mM	millimolar
µl	microliter
ml	milliliter
U/mg	Unit per milligram
U/ml	Unit per milliliter
g	gram
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

CHAPTER 1

INTRODUCTION

Inflammation is defined as the biological process which occurs when the immune system responds to pathogenic infections or damaged cells (Hansel et al., 2010). Inflammation normally causes several symptoms such as swelling, redness, pain, increase in temperature, and numbness. These common symptoms are due to the increased blood flow and capillary permeability (Hansel et al., 2010). Inflammation is also apparent in several joint illnesses such as osteoarthritis (OA), rheumatoid arthritis and gout (Siebuhr et al., 2014, de Lange-Brokaar et al., 2012, Punzi et al., 2012) which occur as a result of prolonged or chronic inflammation.

Inflammation is a condition brought about by mediators commonly known as cytokines. These are glycoproteins that are synthesized in various types of cells under stress conditions. There are numerous types of cytokines, such as interleukin-6 (IL-6), interferon (IFN) and tumor necrosis factor (TNFs) (Hansel et al., 2010). TNF-α is synthesized by macrophage and B-lymphocytes upon stimulation (O'Connor et al., 2009), which also acts as proinflammatory cytokine that triggers the release of other cytokines (IL-4 and IL-6) and C-reactive protein (CRP) in inflammation signaling pathway (Inoue et al., 2009, Ingle and Patel, 2011).

Enzymes also play a major role in regulating inflammation pathway. These enzymes are Phospholipase A_2 (PLA_2), Cycloxygenase-1 (COX-1), Cycloxygenase-2 (COX-2) and 5-Lypoxygenase (5-LOX). These enzymes are induced by the activation of Nuclear Factor-κB (NF-κB) cells in cytoplasm. Activation of NF-κB exposes the nuclear localization sequence, thus leads to the translocation of NF-κB from cytosol to nucleus and expresses specific genes for inflammation process (Delfino and Walker, 1999). In general, PLA_2 synthesizes arachidonic acid from *sn*-2 position of phospholipids in membrane (Murakami et al., 2010). Cycloxygenase enzymes (COX-1 and COX-2) convert arachidonic acid to prostaglandins (PGs) and thromboxane A_2 (Marnett, 2000). 5-LOX converts arachidonic acid to leukotriene that is involved in regulation of inflammation in lung tissues (Steinhilber, 1994, Liu and Yokomizo, 2015). There have been numerous studies related to regulation of inflammatory enzymes especially COX enzymes (van Esch et al., 2013, Meskell and Ettarh, 2011). Drugs developed specifically to inhibit COX activity in human cells are named as non-steriodal anti-inflammatory drugs (NSAIDs).

Research has been conducted for decades to find a cure for inflammation and its related symptoms. Now it is widely accepted that non-steroidal anti-inflammatory drugs (NSAIDs) can effectively prevent inflammation (Shen et al., 2011, Raz, 2002). However, several studies have also revealed side effects resulting from prolonged use of NSAIDs, which include the possibility of several chronic diseases such as gastrointestinal (GI) ulcers, adverse cardiovascular side-effects, and Alzheimer's disease (Sostres et al., 2010, Niranjan et al., 2011, McGeer and McGeer, 2007). Because of the

side effects related to NSAID use, many studies have been conducted to find alternative anti-inflammatory drugs based on natural products, as these remedies may have no or less side effects. One of the plants that has been associated with anti-inflammatory properties is *Coptis chinesis*. This plant is widely used by the Chinese in treating inflammation traditionally. Scientific research proves that the presence of alkaloids group (berberine) in the plant *Berberis koreana* was the major contributor for its anti-inflammatory effect (Kim et al., 2010). *Sophora subprostrata* is another example of plant that has been used traditionally by the Chinese in treating inflammation. This plant showed an inhibition towards *in vitro* COX activity and also acted as antioxidant due the presence of quinolizidine alkaloids matrine and oxymatrine (Souto et al., 2011). *Ceanothus thyrisiflorus* was used by the American tribes to treat various types of diseases related to inflammation. The tribes used plant roots as drinks (tea) to treat sore throats and bronchitis. The presence of alkaloids group and saponins were the major compounds that contribute to anti-inflammatory effect in this plant (Darshan and Doreswamy, 2004). *Jatropha curcas* L. is another plant that has been used as a traditional medicine in treating inflammation. *Jatropha curcas* was used by tribes in South America, particularly in Brazil, to treat inflammation (Villegas et al., 1997).

Jatropha curcas is a drought tolerant, versatile perennial plant in the family of Euphorbiaceae. It originated from South America, but now abundant in South and Central America, Africa and Asia (Mandpe et al., 2005). The plant can grow in harsh conditions with low or high rainfall. This plant is considered to have enormous potentials, not only as a source of oil seed but also as a source of bioactive compounds for medicinal purposes. Different parts of the plant have been shown to possess biological activities which have been associated to the presence of phenolics, terpenoids and flavonoids (Oskoueian et al., 2011b).

However, the nature of the active compound has not been completely elucidated, and the mode of action in the anti-inflammatory activity remained unclear. Thus, the general objective of the present study was to elucidate the chemical nature of anti-inflammatory compounds present in *J. curcas* plant and to determine its effects on the inflammatory signaling pathway and enzymes.

The specific objectives were:

1. To screen the anti-inflammatory activity of extracts from different parts of *J. curcas* in Murine monocytic macrophage RAW 264.7 cell line.
2. To purify and elucidate the structure of the anti-inflammatory compounds extracted from *J. curcas* plant.
3. To determine the effects of anti-inflammatory compound on the inflammatory signaling pathway in Murine monocytic macrophage RAW 264.7 cell line.
4. To determine the effect of anti-inflammatory compound on enzymes involved in the inflammation signaling pathway.

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