



UNIVERSITI PUTRA MALAYSIA

***ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF FRACTIONS
FROM *Jatropha curcas* Linn. ROOT EXTRACT AND ATTENUATION OF
PRO-INFLAMMATORY MEDIATORS IN RAW 264.7 CELLS***

SHAHIRAH ATIQA OSMAN

IB 2018 3



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By

SHAHIRAH ATIQA OSMAN

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

February 2017

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

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February 2017

Chair: Professor Dr. Norhani Abdullah, PhD
Faculty: Institute of Bioscience

Jatropha curcas Linn. plant belongs to the Euphorbiaceae family. This plant has been widely used in traditional medicine to treat injuries, fever, mouth infections, jaundice, guinea worm sores, and joint rheumatism. Among the different parts of the plant, the root has been widely used in ethno-medicine. However, extracts prepared from the roots could be variable in terms of efficacy and safety. The method of extraction and solvents used play a crucial role in determining the biological activities. It was hypothesized that liquid-liquid fractionation process could extract metabolites from *J. curcas* root which possessed antioxidant and anti-inflammatory activities without cytotoxic effect in the cell lines used. Therefore, the main objective of the present study was to investigate the biological activity and cytotoxicity of different solvents extract from *J. curcas* root. The metabolites were partitioned by using different solvents with different polarities. The phenolic and flavonoid contents of each solvent fraction were analyzed colorimetrically, and each fraction was evaluated for antioxidant and anti-inflammatory activities in macrophage RAW 264.7 cell lines. The nature of compounds present in each fraction was analysed by Liquid Chromatography Mass Spectrometry (LCMS). The active fraction with anti-inflammatory activity and low cytotoxicity was used to study the expression of pro-inflammatory genes by qPCR. The peripheral roots were collected from three *J. curcas* plants approximately 4 to 5 years old and subjected to 80% methanolic extraction for the preparation of dried root extracts. Then, the methanolic extract was used in the liquid-liquid fractionation technique with five different solvents (hexane, chloroform, ethyl acetate, n-butanol, and

aqueous). This was followed by determining the antioxidant activity of different fractions in vitro using 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) free radical scavenging, ferric-reducing antioxidant power (FRAP) assay and scavenging of ABTS (2,2'-azinobis[3-ethylbenzothiazoline-6-sulphonate])-nitrogen-centred radical cation (ABTS^{•+}) assay. The results showed that ethyl acetate and n-butanol fractions of the three root samples possessed high antioxidant activity with the highest total phenolic (36.74 ± 0.046 GAE $\mu\text{g/g}$ DW and 33.12 ± 0.006 GAE $\mu\text{g/g}$ DW) and total flavonoid content (11.76 ± 0.015 GAE $\mu\text{g/g}$ DW and 10.11 ± 0.009 GAE $\mu\text{g/g}$ DW). The anti-inflammatory activity of all five fractions was evaluated using murine macrophage RAW 264.7 cell line by determining the percentage of nitric oxide (NO) production by Griess reaction. The cytotoxicity activity of each fraction was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method using murine macrophage RAW 264.7 cell line. The results showed that NO inhibition by n-butanol fraction at the concentration of 100 $\mu\text{g/mL}$ in macrophage RAW 264.7 cells was 86% and cell viability was 105%, comparable to curcumin treated cells. The LCMS analysis showed that ethyl acetate contained epigenin and kaempferol glucoside isomer, which were categorised under flavone and phenolics that possessed antioxidant and anti-inflammatory properties. While n-butanol fraction contained high amount of coumaric acid and p-coumaroylquinic acid that could be involved in anti-inflammatory activity. The n-butanol fraction was evaluated using qPCR technique to study the expression of genes (iNOS, TNF- α , IL-6, and IL-1 β genes) involved in inflammatory activity of induced lipopolysaccharide (LPS) and interferon-gamma (IFN- γ) RAW 264.7 cells, where cells treated with curcumin was used as positive control and normalized by expression of housekeeping gene transcript (G3DPH). The results showed that n-butanol fraction at 100 $\mu\text{g/mL}$, significantly ($P < 0.05$) reduced the expression of iNOS, TNF- α , IL-6, and IL-1 β genes. Hence, the anti-inflammatory effect was due to the down-regulation of the pro-inflammatory mediators. The present study showed that liquid-liquid fractionation was successful in partitioning the metabolites present in the *J. curcas* root methanolic extract, where n-butanol fraction was observed to be the most active fraction among other fractions. This fraction contained metabolites which possessed strong antioxidant and anti-inflammatory properties with low cytotoxicity activity and the ability to down-regulated the expression of all four pro-inflammatory genes evaluation.

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

**AKTIVITI ANTIOKSIDAN DAN ANTI-KERADANGAN FRAKSI DARI
EKSTRAK *Jatropha curcas* Linn. DAN PENGECILAN MEDIATOR PRO-
RADANG DALAM SEL RAW 264.7**

Oleh

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Februari 2017

Pengerusi: Professor Dr. Norhani Abdullah, PhD
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Jatropha curcas Linn. berasal daripada famili Euphorbiaceae. Pokok ini telah digunakan secara meluas di dalam perubatan tradisional untuk merawat kecederaan, demam, jangkitan mulut, jaundis, ubat cacing, dan juga sakit sendi. Di antara kesemua bahagian pokok ini, akarnya sering digunakan di dalam perubatan etho-perubatan. Namun, ekstrak yang diperolehi daripada akar pokok ini berpotensi untuk berubah-ubah dari segi kecekapan dan keselamatan. Cara pengekstrakan serta pelarut yang digunakan adalah sangat penting dalam menentukan aktiviti biologinya. Hipotesis telah menjangkakan bahawa proses fraksi cecair-cecair boleh mengekstrak metabolit daripada akar *J. curcas* di mana mempunyai aktiviti antioksidan dan anti-keradangan tanpa memberi kesan kepada sitotoksik barisan sel yang digunakan. Oleh yang demikian, objektif utama bagi kajian ini adalah untuk mengenalpasti aktiviti biologi dan sitotoksik bagi fraksi ekstrak yang berbeza daripada akar pokok *J. curcas*. Metabolit kemudian diasingkan dengan menggunakan pelarut yang berbeza polariti. Kandungan fenolik dan flavonoid bagi setiap fraksi di analisa menggunakan analisa yang melibatkan bantuan reagen warna, dan setiap aktiviti antiosida dan anti-radang di uji bagi setiap fraksi dengan menggunakan makrofaj sel 264.7. Sifat bagi setiap kompaun yang ada di dalam setiap fraksi di analisa dengan menggunakan teknik Analisis Cecair Kromatografi Spektrometri Jisim (LCMS). Fraksi yang aktif dengan aktiviti anti-radang dan rendah sitotoksik di pilih dan digunakan untuk kajian ekspresi gen pro-radang dengan menggunakan teknik rantai polymerase masa-nyata (qPCR). Akar periferik dikumpulkan daripada tiga pokok *J. curcas* yang berlainan di mana usia pokok adalah di dalam lingkungan 4 ke 5 tahun dan ekstrak akar 80% metanol disediakan untuk menghasilkan ekstrak kering akar. Selepas itu, ekstrak metanol akar digunakan di dalam proses teknik cecair-cecair pemeringkatan dengan menggunakan lima jenis pelarut yang berbeza (hexana, klorofom, etil asetat, n-butanol, dan aqua). Kemudian, kesemua fraksi berbeza yang terhasil di uji

keberkesannya melalui aktiviti antioksidan secara in vitro iaitu 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) memerangkap bebas radikal, aktiviti penurunan kuasa ferik, dan aktiviti penurunan perapasan radikal 2'-Azinobis (3-ethylene benzothiazoline) 6-Sulphonicacid (ABTS). Keputusan menunjukkan fraksi etil asetat dan n-butanol untuk ketiga sampel akar mempunyai sifat antioksidan yang tinggi dengan kandungan fenol tertinggi pada (36.7 ± 0.046 GAE $\mu\text{g/g DW}$ and 33.1 ± 0.006 GAE $\mu\text{g/g DW}$) dan flavonoid (11.76 ± 0.015 GAE $\mu\text{g/g DW}$ and 10.11 ± 0.009 GAE $\mu\text{g/g DW}$). Keputusan analisa LCMS mendapati fraksi etil asetat mengandungi epigenin dan kaempferol glucosid isomer, di mana ianya dikategorikan di bawah kumpulan flavon dan fenolik yang mempunyai ciri-ciri antioksidan dan anti-radang. Kemudian, n-butanol mengandungi asid komerik dan asid p-komerilquinik yang tinggi di mana ianya menyumbang kepada aktiviti anti-radang yang bagus. Aktiviti anti-radang bagi kesemua lima fraksi dikaji dengan menggunakan murin makrofaj barisan sel RAW 264.7 dengan mengenalpasti peratusan nitrik oksida (NO) yang terhasil melalui tindakbalas Griess. Aktiviti sitotoksik bagi setiap fraksi ditentukan dengan cara penurunan 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) yang digunakan pada murin makrofaj barisan sel RAW 264.7. Keputusan ujikaji menunjukkan 86% perencatan nitrik oksida (NO) oleh fraksi n-butanol pada kepekatan 100 $\mu\text{g/mL}$ pada sel makrofaj RAW 264.7 dan daya maju sel sebanyak 105% berbanding curcumin. Analisa LCMS menunjukkan kehadiran epigenin dan isomer kamferol glucosida, di mana ianya diklasifikasikan di bawah kumpulan flavon dan fenolik yang mempunyai ciri-ciri antioksidan dan anti-keradangan. N-Butanol pula mengandungi asid komarik dan asid p-komarolquinik yang berpotensi untuk terlibat dalam aktiviti anti-keradangan. Fraksi n-butanol kemudiannya di nilai menggunakan teknik rantai polimerase masa-nyata (qPCR) untuk mengkaji ekspresi gen-gen (iNOS, TNF- α , IL-6, dan IL-1 β) yang berkaitan dengan aktiviti radang oleh RAW 264.7 yang di rawat dengan lipopolisakarida (LPS) dan interferon-gamma (IFN- γ) di mana sel RAW 264.7 yang di rawat dengan curcumin telah digunakan sebagai pengawal positif dan diselaraskan oleh ekspresi gene pengawal (G3DPH). . Keputusan kajian menunjukkan bahawa fraksi n-butanol pada kepekatan 100 $\mu\text{g/mL}$ dengan ketara ($P < 0.05$) menurunkan ekspresi gen iNOS, TNF- α , IL-6, dan IL-1 β . Oleh itu, kesan anti-keradangan adalah disebabkan penurunan regulasi pengantara pro-keradangan. Kajian ini menunjukkan proses fraksi cecair-cecair telah Berjaya membahagikan metabolit yang hadir di dalam ekstrak metanol bagi akar pokok *J. curcas*, di mana fraksi n-butanol dilihat mempunyai metabolit-metabolit yang memiliki ciri-ciri aktiviti antioksidan dan anti-radang yang tinggi dan sitotoksik yang rendah serta kebolehan mengurangkan kesemua empat ekspresi gen pro-radang yang di uji.

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

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TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	iii
ACKNOWLEDGEMENTS	v
APPROVAL	vi
DECLARATION	viii
LIST OF TABLES	xiv
LIST OF FIGURES	xv
LIST OF APPENDICES	xx
LIST OF ABBREVIATIONS	xxi
LIST OF ANNOTATIONS	xxii
 CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	3
2.1 Plant Secondary Metabolite	3
2.2 Practices in Traditional Medicine	3
2.3 Medicinal Plants	3
2.4 Herbal Medicinal Products	4
2.5 Medicinal plants in Malaysia	4
2.6 Side Effect and Procedure of Producing Traditional Medicine	4
2.7 <i>Jatropha curcas</i> Linn.	5
2.8 Medicinal Uses of <i>Jatropha curcas</i> Linn.	6
2.9 Plant Secondary Metabolites Extraction	7
2.10 Liquid-liquid Fractionation and Solvents Polarity	7
2.11 Bioactive Compound in <i>Jatropha curcas</i> Roots and Its Potential	
2.11.1 Phenolics	8
2.11.2 Flavonoids	8
2.11.3 Antioxidants	9
2.12 Inflammation	9
2.12.1 Contribution of Reactive Oxygen Species and free radicals to Inflammation	10
2.13 Nitric oxide (NO) and Nitric Oxide Synthase (NOS)	11
2.14 Types of Biological NO Generating Enzyme, NOS	12
2.15 The Immune System	12
2.16 Cytokines	13
2.17 Interferon Gamma	13
2.18 Lipopolysaccharide (LPS)	14
2.19 Genes	14

2.20	Gene Expression	14
2.21	Real-time Polymerase Chain Reaction	15
2.22	iNOS Expression During Inflammation	15
3	PHYTOCHEMICAL COMPOUNDS OF <i>Jatropha curcas</i> ROOT FRACTIONS	
3.1	Introduction	16
3.2	Material and Methods	
3.2.1	Plant Materials	16
3.2.2	Solvents and Reagents	17
3.2.3	Methanolic Extract of <i>Jatropha curcas</i> Root	17
3.2.4	Fractionation of Crude Extract of <i>Jatropha curcas</i> Root	17
3.2.5	Total Phenolic Content	18
3.2.6	Total Flavonoid Content	18
3.2.7	Statistical Analysis	18
3.3	Result and Discussion	
3.3.1	Total Phenolic Content	18
3.3.2	Total Flavonoid Content	21
3.3.3	Comparison value of TPC and TFC of Roots for Each Fraction	25
3.4	Conclusion	26
4	BIOLOGICAL ACTIVITIES OF <i>Jatropha curcas</i> ROOT FRACTIONS	
4.1	Introduction	28
4.2	Material and Methods	
4.2.1	Root Fractions	28
4.3	Antioxidant Activity of Root Fraction	
4.3.1	1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Scavenging Activity	28
4.3.2	Ferric-Reducing Antioxidant Power (FRAP) Assay	29
4.3.3	Scavenging of ABTS (2,2'-azinobis[3ethylbenzothiazoline-6-sulphonate])-nitrogen-centred radical cation (ABTS•+)	29
4.4	Anti-inflammatory Activity of Root Fraction	
4.4.1	Preparation of Sample Stock	30
4.4.2	Cell Culture	30
4.4.3	Seeding and Stimulation of RAW 264.7 Cell	30
4.4.4	Nitrite Determination	31
4.4.5	Cell Viability	32
4.5	Liquid Chromatography Mass Spectrometry (LCMS)	32

4.6	Statistical Analysis	32
4.7	Result and Discussion	
4.7.1	Antioxidant Activities	
4.7.1.1	DPPH Scavenging Activity	33
4.7.1.2	Ferric reducing Antioxidant Power (FRAP) Assay	37
4.7.1.3	Scavenging ABTS Assay	40
4.7.2	Anti-inflammatory Activities	
4.7.2.1	Hexane Fraction	44
4.7.2.2	Chloroform Fraction	45
4.7.2.3	Ethyl Acetate Fraction	46
4.7.2.4	n-Butanol Fraction	47
4.7.2.5	Aqueous Fraction	47
4.7.3	LCMS Analysis	
4.7.3.1	Hexane Fraction	49
4.7.3.2	Chloroform Fraction	51
4.7.3.3	Ethyl Acetate Fraction	52
4.7.3.4	n-Butanol Fraction	55
4.7.3.5	Aqueous Fraction	57
4.8	Conclusion	60
5	EFFECT OF ACTIVE FRACTION ON INFLAMMATORY GENE EXPRESSION IN RAW 264.7 CELL LINE	
5.1	Introduction	61
5.2	Material and Methods	
5.2.1	Preparation of Samples	62
5.2.2	Cell Culture and Treatment	62
5.2.3	Microscopic Examination	62
5.2.4	RNA Isolation and Purification	63
5.2.5	Quantitative Analysis of RNA	64
5.2.6	PCR Primers	64
5.2.7	Optimization of SYBR Green I Real-time PCR Assay	65
5.2.8	RT-PCR and cDNA Synthesis	65
5.2.9	Real-time PCR, qPCR	66
5.3	Result and Discussion	
5.3.1	Microscopic Examination of RAW 264.7 macrophage cell line	68
5.3.2	Gene Expression Analyses Using Real-time PCR	70
5.3.2.1	Down-regulation effect of n-butanol fraction on iNOS gene expression in RAW 264.7 macrophage cell	

5.3.2.2	Down-regulation effect of n-butanol fraction on TNF- α gene expression in RAW 264.7 macrophage cell	72
5.3.2.3	Down-regulation effect of n-butanol fraction on IL-1 β gene expression in RAW 264.7 macrophage cell	74
5.3.2.4	Down-regulation effect of n-butanol fraction on IL-6 gene expression in RAW 264.7 macrophage cell	75
5.4	Conclusion	77
6	GENERAL DISCUSSION, CONCLUSION, AND RECOMMENDATIONS FOR FUTURE RESEARCH	
6.1	General Discussion	78
6.2	Conclusion	79
6.3	Recommendation for Future Research	79
	REFERENCES	80
	APPENDICES	101
	BIODATA OF STUDENT	120
	LIST OF PUBLICATIONS	121

LIST OF TABLES

Table		Page
1.0	Polarity of common solvents	7
2.0	Total phenolic content of root 1, root 2, and root 3 for each fraction from 80% methanol extract of <i>J. curcas</i> Linn. roots.	26
3.0	Total flavonoid content of root 1, root 2, and root 3 for each fraction from 80% methanol extract of <i>J. curcas</i> Linn.	26
4.0	Composition of Diluents A, B, and C	31
5.0	Ic50 Value of FRAP Assay for Each Sample.	39
6.0	Compounds identified by LCMS in <i>J. curcas</i> Root 3 sample for each fraction.	59
7.0	Division of 6 well plates for treatments of n-butanol sample.	61
8.0	Primer sequence used for detection of cytokine gene expression.	63
9.0	Reaction Setup for a Single cDNA Synthesis Reaction.	65
10.0	Reaction Mix Setup	66

LIST OF FIGURES

Figure		Page
1	<i>Jatropha curcas</i> Linn.	5
2	Synthesis of Nitric Oxide	11
3a	Total phenolic content of different solvent fractions prepared from 80% methanolic extract of Root 1 sample. Each histogram represents mean $\pm$ SD (n=3). Vertical bar represents standard deviation.	19
3b	Total phenolic content of different solvent fractions prepared from 80% methanolic extract of Root 2 sample. Each histogram represents mean $\pm$ SD (n=3). Vertical bar represents standard deviation.	20
3c	Total phenolic content of different solvent fractions prepared from 80% methanolic extract of Root 3 sample. Each histogram represents mean $\pm$ SD (n=3). Vertical bar represents standard deviation.	21
4a	Total flavanoid content of different solvent fractions prepared from 80% methanolic extract of Root 1 sample. Each histogram represents mean $\pm$ SD (n=3). Vertical bar represents standard deviation.	22
4b	Total flavanoid content of different solvent fractions prepared from 80% methanolic extract of Root 2 sample. Each histogram represents mean $\pm$ SD (n=3). Vertical bar represents standard deviation.	23
4c	Total flavanoid content of different solvent fractions prepared from 80% methanolic extract of Root 3 sample. Each histogram represents mean $\pm$ SD (n=3). Vertical bar represents standard deviation.	24
4.1a	Scavenging of DPPH radical by different concentration of fractions from 80% methanol extract of <i>J. curcas</i> Linn. Root 1 samples. Each value represents mean $\pm$ SD (n=3). Vertical bar represents standard deviation.	33
4.1b	Scavenging of DPPH radical by different fractions prepared from 80% methanol extract of <i>J. curcas</i> Linn. Root 2 sample. Each value represents mean $\pm$ SD (n=3).	34
4.1c	Scavenging of DPPH radical by different fractions prepared from 80% methanol extract of <i>J. curcas</i> Linn. Root 3 sample. Each value represents mean $\pm$ SD (n=3).	35
4.2a	Ferric reducing activity of different fractions prepared from 80% methanolic extract of <i>J. curcas</i> Linn. Root 1. Each value represents mean $\pm$ SD (n=3).	36

4.2b	Ferric reducing activity of different fractions prepared from 80% methanol extract of <i>J. curcas</i> Linn. Root 2. Each value represents mean $\pm$ SD (n=3).	37
4.2c	Ferric Reducing Activity of Different Fractions from 80% Methanol Extract of <i>J. curcas</i> Linn. Root 3. Each value represents mean+SD (n=3).	38
4.3a	ABTS radical scavenging activity of different fractions from 80% methanol extract of <i>J. curcas</i> Linn. Root 1. Each value represents mean+SD (n=3). Vertical bar represents standard deviation	40
4.3b	ABTS radical scavenging activity of different fractions from 80% methanol extract of <i>J. curcas</i> Linn. Root 2. Each value represents mean+SD (n=3). Vertical bar represents standard deviation.	41
4.3c	ABTS radical scavenging activity of different fractions from 80% methanol extract of <i>J. curcas</i> Linn. Root 3. Each value represents mean+SD (n=3). Vertical bar represents standard deviation.	41
4.5a	Percentage values of NO inhibition and cell viability of hexane fraction from 80% methanol extract of <i>J. curcas</i> Linn. roots. All values represent mean+SD (n=3). ***P $\leq$ 0.001, P $\leq$ 0.01 indicates significant difference compared to the control (curcumin).	43
4.5b	Percentage values of NO inhibition and cell viability of chloroform fractions from 80% methanol extract of <i>J. curcas</i> Linn. roots. All values represent mean+SD (n=3). ***P $\leq$ 0.001, P $\leq$ 0.01 indicates significant difference compared to the control (curcumin).	44
4.5c	Percentage values of NO inhibition and cell viability of ethyl acetate fractions from 80% methanol extract of <i>J. curcas</i> Linn. roots. All values represent mean+SD (n=3). ***P $\leq$ 0.001, P $\leq$ 0.01 indicates significant difference compared to the control (curcumin).	45
4.5d	Percentage values of NO inhibition and cell viability of n-butanol fraction from 80% methanol extract of <i>J. curcas</i> Linn. roots. All values represent mean+SD (n=3). ***P $\leq$ 0.001, P $\leq$ 0.01 indicates significant difference compared to the control (curcumin).	46
4.5e	Percentage values of NO inhibition and cell viability of	47

aqueous fraction from 80% methanol extract of *J. curcas* Linn. roots. All values represent mean+SD (n=3). ***P ≤ 0.001, P ≤ 0.01 indicates significant difference compared to the control (curcumin).

4.6a	LCMS chromatogram of 15,16-dihydroxy- 9Z,12Z-octadecadienoic acid compound in hexane fraction of <i>J. curcas</i> Root 3 sample.	48
4.6b	LCMS chromatogram of benzenepropanoic acid conjugate compound in hexane fraction of <i>J. curcas</i> Roots 3 sample.	49
4.6c	LCMS chromatogram of coumeric acid based compound in hexane fraction of <i>J. curcas</i> Root 3 sample.	49
4.7a	LCMS chromatogram of 15,16-dihydroxy- 9Z,12Z-octadecadienoic acid compound in chloroform fraction of <i>J. curcas</i> Root 3 sample.	50
4.7b	LCMS chromatogram of coumaric acid conjugate compound in chloroform fraction of <i>J. curcas</i> Root 3 sample.	51
4.8a	LCMS chromatogram of apigenin based 433 in ethyl acetate fraction of <i>J. curcas</i> Root 3 sample.	52
4.8b	LCMS chromatogram of apigenin based compound in ethyl acetate fraction of <i>J. curcas</i> Root 3 sample.	52
4.8c	LCMS chromatogram of kaempferol glucoside Isomer in ethyl acetate fraction of <i>J. curcas</i> Root 3 sample.	53
4.8d	LCMS chromatogram of methyl 2-[cyclohex-2-en-1-yl(hydroxy)methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate compound in ethyl acetate fraction of <i>J. curcas</i> Root 3 sample.	53
4.9a	LCMS chromatogram of coumaric acid compound in n-butanol fraction of <i>J. curcas</i> Root 3 sample.	54
4.9b	LCMS chromatogram of coumaric acid conjugate in n-butanol fraction of <i>J. curcas</i> Root 3 sample.	55
4.9c	LCMS chromatogram of p-coumaroylquinic acid compound in n-butanol fraction of <i>J. curcas</i> Root 3 sample. .	55
4.10a	LCMS chromatogram of quercetin compound in aqueous fraction of <i>J. curcas</i> Root 3 sample.	57
4.10b	LCMS chromatogram of quercetin conjugate in aqueous fraction of <i>J. curcas</i> Root 3 sample.	57
4.10c	LCMS chromatogram of p-coumaroylquinic acid in aqueous	58

	fraction of <i>J. curcas</i> Root 3 sample.	
4.10d	LCMS chromatogram of leuteolin conjugate in aqueous fraction of <i>J. curcas</i> Root 3 sample.	58
5.0	Morphological characteristics of the RAW 264.7 macrophage cells during seeding, cells for treatment, control I, control II, control III. Cells were treated with 1 µg/mL n-Butanol fraction, 0 µg/mL n-Butanol fraction, and 100 µg/mL n-Butanol fraction after 24 hours of incubation examined by light microscopy at 100x magnification. Scale bar: 50 µm.	68
5.1	Effect of n-butanol fraction on gene expression of inflammatory-related gene, iNOS. RAW 264.7 macrophage cells were treated with 1 µg/mL, 10 µg/mL, and 100 µg/mL of n-butanol fraction for 24 hours in the presence of LPS and IFN-γ. Total RNA was extracted, and mRNA expression was analyzed by RT-qPCR. The fold expression result was normalized by <i>GAPDH</i> . Data are expressed as the mean ± SEM of three separate experiments. A value of *P<0.05, **P<0.01 and ***P<0.001 were considered significantly different to LPS-stimulated control group.	69
5.2	Activation of NFκB and STAT signaling pathway. LPS binding protein (LBP) transfers LPS to CD14 receptor on the cell membrane. Binding LPS activates NFκB or AP-1. IκB- NFκB complex is inactive in the cytosol. After activation of IκB- NFκB complex, free NFκB transfers into the nucleus and induces the expression of iNOS. IFN-γ binds to IFN-γ R and phosphorylates STAT1. Activated Jak-STAT signaling pathway increase iNOS and NO production (Rao, 2000).	71
5.3	Effect of n-butanol fraction on gene expression of inflammatory-related gene, TNF-α. RAW 264.7 macrophage cells were treated with 1 µg/mL, 10 µg/mL, and 100 µg/mL of n-butanol fraction for 24 hours in the presence of LPS and IFN-γ. Total RNA was extracted, and mRNA expression was analyzed by RT-qPCR. The fold expression result was normalized by <i>GAPDH</i> . Data are expressed as the mean ± SEM of three separate experiments. A value of *P<0.05, **P<0.01 and ***P<0.001 were considered significantly different to LPS-stimulated control group.	73
5.4	Effect of n-butanol fraction on gene expression of inflammatory-related gene, IL-1β. RAW 264.7 macrophage cells were treated with 1 µg/mL, 10 µg/mL, and 100 µg/mL of n-butanol fraction for 24 hours in the presence of LPS and IFN-γ. Total RNA was extracted, and mRNA expression was analyzed by RT-qPCR. The fold expression result was	74

normalized by *GAPDH*. Data are expressed as the mean $\pm$ SEM of three separate experiments. A value of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ were considered significantly different to LPS-stimulated control group

- 5.5 Effect of n-butanol fraction on gene expression of inflammatory-related gene, IL-6. RAW 264.7 macrophage cells were treated with 1 μ g/mL, 10 μ g/mL, and 100 μ g/mL of n-butanol fraction for 24 hours in the presence of LPS and IFN- γ . Total RNA was extracted, and mRNA expression was analyzed by RT-qPCR. The fold expression result was normalized by *GAPDH*. Data are expressed as the mean $\pm$ SEM of three separate experiments. A value of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ were considered significantly different to LPS-stimulated control group. 76

LIST OF APPENDICES

Appendix		Page
A	Uses of different plant parts of <i>Jatropha curcas</i> in folk and traditional medicine in selected regions.	100
B	Standard Curves of Total Phenolics and Flavonoids	103
C	Preparation of Buffers, Interferon- γ (IFN- γ) and Lipopolysaccharide (LPS) Stock Solution	104
D	Preparation of Diluents and Solutions for Griess and MTT Assay	105
E	Formaldehyde (FA) Agarose Gel Preparation for RNA Integrity Analysis	107
F	PCR Product DNA Extraction for All Genes	108
G	Real Time PCR Standards, Amplification Curves and Melting Curves Used in Rumen Microbial Quantification	110

LIST OF ABBREVIATIONS

DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
EA	Ethyl acetate
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
IC ₅₀	Inhibitory concentration
IFN- γ	Interferon- γ
IL-1 β	Interleukin-1 beta
iNOS	Inducible nitric oxide synthase
LCMS	Liquid Chromatography Mass Spectrometer
L-NAME	N ^o -nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase Polymerase chain reaction
Real-time PCR	Real-time Polymerase chain reaction
ABTS	Scavenging of ABTS(2,2'-azinobis[3-ethylbenzothiazole-6-sulphonate])-nitrogen-centred radical cation.
TPC	Total phenolic content
TFC	Total flavonoid content
SD	Standard deviation

LIST OF ANNOTATIONS

α	Alpha
β	Beta
γ	Gamma
%	Percentage
(v/v)	Volume per volume
(w/v)	Weight per volume
λ	Lambda
$\pm$	Plus and/or minus
$\leq$	Less or equal
$^{\circ}\text{C}$	Degree celcius
μ	Micro
μL	Microliter
μM	Micromolar
g	Gram
h	Hour
kDA	Kilo Dalton
Kg	Kilogram
L	Liter
mg/mL	Miligram per millilitre
$\mu\text{g/mL}$	Microgram per millilitre
Min	Minute
Mmol	Millimole
U	Units
V	Volt
S	Second

CHAPTER 1

INTRODUCTION

Jatropha curcas Linn. (*J. curcas*) is a multipurpose plant which belongs to the Euphorbiaceae family. This plant species is resistant to drought and disease and mostly grows in low to high rainfall area such as Africa and South-east Asia including Malaysia. Usually, this plant could be found in the farm as a commercial crop or on the boundaries as a hedge for protection from grazing animals and to avoid erosion (Henning, 1996; Gubitz, 1997; Uche, 2008).

It has been reported that all parts of *J. curcas* such as seeds, bark, leaves, and also roots have been used in traditional medicine for humans as well as for animals (Dalziel, 1995; Duke, 1988; Reddy Prasad, 2012). This plant is used for treating scabies, ringworm and gonorrhoea caused by either fungal or bacterial infections (Aiyelaagbe et al., 2007).

There are several parts of *J. curcas* that are traditionally used to treat inflammation, including latex, seeds, leaves and roots. Since 1989, De Feo had discovered *J. curcas* latex to possess anti-inflammatory activity when the latex was rubbed to the traumatic area. The seeds have been used to treat inflammation as well as other ailments like convulsions, burns, and fever (Osoniyi and Onajobi, 2003). Leave extracts from *J. curcas* showed anti-inflammatory activity when tested using *in vivo* technique involving mice and Wister albino rats (Uche et al., 2008).

There are several reports regarding the presence of metabolites such as alkaloid compounds, phenolic, flavonoid, and saponin in different parts of *J. curcas* plant (Thomas et al., 2008). According to Seyum et al. (2006) and Miliauskas et al. (2004), phenolic and flavonoids compound present in plants are well correlated to the free radical scavenging, nitric oxide (NO) scavenging, and also total antioxidant activities. This indicates that antioxidant is correlated with several compounds involved in anti-inflammatory activity.

Inflammation mediators, cytokines are glycoproteins that were synthesized in various types of cells under stress conditions. Generally, cytokines can be classified as pro-inflammatory or anti-inflammatory depend on how they influence inflammation. Pro-inflammatory cytokines such as iNOS, TNF- α , IL-1 β and IL-6 are known as initiators and amplified the inflammatory process, while the anti-inflammatory cytokines like IL-10 inhibit the inflammatory mediator (Abebayehu et al. 2012). These genes were used in gene expression study by using real-time PCR (qPCR) to study the mechanism of action involve.

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 showed side effects resulting from prolonged use of NSAIDs, which include several chronic diseases such as gastrointestinal (GI) ulcers, adverse cardiovascular side effects, and Alzheimer's disease (Sostres et al., 2010, McGeer and McGeer, 2007). Therefore, an alternative way of using natural resources from medicinal plant should be used in pharmaceutical industries to treat inflammation as it is more safe, gentle, and with no or less side effects.

Many studies have been carried out to determine potential plant-derived compounds with significant anti-inflammatory effects. Discovery of plant metabolites for treatment and/or controlling inflammatory diseases is important for developing new drugs. Oskoueian et al. (2011) had reported that the methanolic extract of root from *J. curcas* possessed anti-inflammatory activity, but the extract was toxic to the cells. This observation indicated the presence of bioactive compounds that are anti-inflammatory and cytotoxic to the cells. Therefore, it was necessary to obtain a root extract that contain bioactive compounds that are anti-inflammatory but non-toxic to the cells. Different solvents with different polarities (non-polar to polar) would be used to partition the *J. curcas* root and depending on the biological activity of the compounds, the correct solvent would be determined. It was hypothesized that liquid-liquid fractionation process could extract metabolites from *J. curcas* root which possessed antioxidant and anti-inflammatory activities without cytotoxic effect in the cell lines used.

Hence the main objective of this study was to obtain an active fraction from a methanolic extract of *J. curcas* root with anti-inflammatory activity without cytotoxicity activity and to evaluate its effects on the expression of the pro-inflammatory mediators. The specific objectives were:

1. To partition methanolic extract of *J. curcas* root into different solvents fractions with different polarities and to determine the compounds present in the different fractions.
2. To determine the antioxidant and anti-inflammatory activities of *J. curcas* roots fractions.
3. To evaluate pro-inflammatory mediators including iNOS through gene expression study in RAW 264.7 cell lines treated with an active fraction from *J. curcas* roots.

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