



UNIVERSITI PUTRA MALAYSIA

**BIOCHEMICAL AND MOLECULAR EVALUATION
OF JATROPHA MEAL AS BIOFEED**

EHSAN OSKOUFIAN

FBSB 2012 6

**BIOCHEMICAL AND MOLECULAR EVALUATION OF JATROPHA
MEAL AS BIOFEED**

By

EHSAN OSKOUEIAN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirement for the Degree of Doctor of Philosophy**

July 2012

DEDICATION

This thesis is dedicated to my beloved wife, Dr. Forough Barani, my father Ebrahim, my mother Soghra and my brothers Armin, Arshin and Aidin who have supported me all the way since the beginning of my studies. Also, this thesis is dedicated to my supervisor, Prof. Dr. Norhani Abdullah who has been a great source of motivation and inspiration. Finally, this thesis is dedicated to all those who believe in the richness of learning.



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment
of the requirement for the degree of Doctor of Philosophy

**BIOCHEMICAL AND MOLECULAR EVALUATION OF JATROPHA
MEAL AS BIOFEED**

By

EHSAN OSKOUEIAN

July 2012

Chairman: Professor Norhani Abdullah, PhD

Faculty: Biotechnology and Biomolecular Sciences

Jatropha curcas L. (*J. curcas*) plant is well known as a source of seed oil for biofuel production. The plant thrives well in tropical conditions and its planting acreage has increased considerably in Malaysia. In the process of oil extraction of the seed kernel, a residue called Jatropha meal is produced. The meal could be a potential biofeed due to its chemical and bioactive compounds present. However, the bioactive compounds present in the seeds differ among different genotypes of *J. curcas* plant. Thus, before considering the local Jatropha meal as a biofeed, it is imperative to determine the bioactive compounds and biological activities of the meal which would indicate its possible applications and limitations. It is also important to ascertain the *J. curcas* variety that is grown in Malaysia, is either toxic or non-toxic according to the profiles of the bioactive compounds present. Therefore, the hypothesis of this research was, the Jatropha meal is safe and has functional biofeed properties. In order to test the hypothesis a comprehensive study on the bioactive compounds and biological activities of Jatropha meal, physicochemical treatments, and how the meal and isolated phorbol esters from the meal affect rumen microbial activity were first

conducted to evaluate the meal as a biofeed. This was followed by the cytotoxicity evaluation and mode of action elucidation of isolated phorbol esters from the meal on bovine kidney cell line. The results showed that chemical analysis of *Jatropha* meal obtained from local *J. curcas* plant contained 61.8% crude protein, 9.7% neutral detergent fibre and 4.8% acid detergent fibre. The meal also contained high levels of total phenolics (3.9 mg/g DM), total flavonoids (0.4 mg/g DM), total saponins (19 mg/g DM), phytic acids (9.1 %), trypsin inhibitors (34.2 mg/g DM), lectins (102.7 U) and phorbol esters (3.0 mg/g DM). The high performance liquid chromatography (HPLC) analyses of *Jatropha* meal showed the presence of gallic acid, pyrogallol, rutin, myricetin and daidzein with the values of 581.3 ± 0.36 , 631.1 ± 0.47 , 47.6 ± 0.53 , 198.5 ± 0.29 and 297.5 ± 0.27 $\mu\text{g/g DM}$, respectively. The gas chromatography-mass spectrometry analyses (GC-MS) indicated the presence of other metabolites, including 2-(hydroxymethyl)-2-nitro-1,3-propanediol, β -sitosterol, 2-furancarboxaldehyde,5-(hydroxymethyl), furfural (2-furan carboxaldehyde) and acetic acid. The *Jatropha* meal methanolic extract, vitamin C, butylated hydroxytoluene (BHT) and β -carotene showed free radical (2,2-diphenyl-1-picrylhydrazyl) scavenging activity with the IC_{50} values of 1.6, 0.3, 0.3 and 1.5 mg/ml, respectively, while values for the ferric reducing power activity were 3.0, 0.3, 0.3 and 2.6 mg/ml, respectively. *Jatropha* meal extract showed antibacterial activity against several pathogenic bacteria including *Enterobacter aerogenes*, *Klebsiella pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Bacillus subtilis*, *Bacillus cereus*, and *Staphylococcus aureus* with the inhibition range of 0.21-1.63 cm at the concentrations of 1 and 1.5 mg/disc. The *Jatropha* meal methanolic extract, at the concentration of 100 $\mu\text{g/ml}$, inhibited the inducible nitric oxide synthase in macrophages RAW 264.7, comparable to N_0 -L-nitro-arginine

methyl ester (L-NAME) indicating appreciable anti-inflammatory activity. Combinations of hydrothermal treatment, alkali and oxidizing agents alleviated the levels of phenolic compounds, saponin and phorbol esters significantly ($p \leq 0.05$), while the level of phytic acid did not decrease. Trypsin inhibitors and lectin activity were fully inactivated. The level of phorbol esters decreased by 76.7% on treatment with heat, 3% (w/w) NaOH and 10% (v/w) NaOCl. *In vitro* fermentation by rumen microbes showed a significant ($p \leq 0.05$) decrease in fermentation parameters when chemically treated meals were used as the substrates, while physically treated meals did not affect the fermentation parameters significantly. Effects of four different levels of isolated phorbol esters from Jatropha meal i.e., 3, 6, 9 and 12 mg/30 ml buffered rumen fluid, on rumen fermentation using ground Guinea grass as the substrate were studied *in vitro*. The results showed that apparent dry matter degradability, metabolisable energy, total volume of gas produced after 24 h of incubation (IVGP₂₄) and total volatile fatty acids decreased significantly ($P \leq 0.05$) in treatments with 9 and 12 mg phorbol esters. Rumen microbial specific enzyme activity (CMCase, FPase, xylanase and β -glucosidase), purine content (index of rumen microbial protein) and rumen microbial protein synthesis showed a significant decrease ($P \leq 0.05$) on treatments with 9 and 12 mg phorbol esters. Similarly, the population of bacteria, fungi, protozoa, methanogens, archaea and major cellulolytic bacteria, including *Fibrobacter succinogenes*, *Ruminococcus albus*, *R. flavefaciens* and *Butyrivibrio fibrisolvens*, significantly ($p \leq 0.05$) decreased when 9 and 12 mg phorbol esters were added. The disappearance of phorbol esters upon rumen microbial fermentation was observed with values ranging from 23.0% to 44.1%. The *in vitro* toxicity evaluation of crude extract obtained from rumen fluid treated with phorbol esters before and after 24 h fermentation on viability of bovine kidney cells

(MDBK) (ATCC: CCL-22) demonstrated a significant ($p \leq 0.05$) decrease in the toxic effect of the phorbol esters where the cell viability improved from 43.6% to 72.3%. The phorbol esters of *Jatropha* meal were isolated into four fractions namely, PE1, PE2, PE3 and PE4. *In vitro* cytotoxicity assay showed cells death with the CC_{50} values ranging from 52.4 to 109.6 $\mu\text{g/ml}$ in bovine kidney cell line when exposed to all fractions of phorbol esters and using phorbol-12-myristate-13-acetate (PMA) as positive control upon 24, 48 and 72 h exposure. The isolated phorbol esters induced cell death in a dose and time dependent manner. The light microscope examination indicated no apparent changes in the morphology of the MDBK cells upon 12 h exposure to all phorbol ester fractions and PMA, while after 24 h exposure, significant morphological changes, detachment, destruction of cells and apoptotic bodies were seen. The expression of the PKC- β II gene from signal transduction pathway, c-Fos, c-Jun and c-Myc from proto-oncogenes, and IL-1 β and Cox2 genes from inflammatory pathway, on 12 h treatment with isolated phorbol esters and PMA at the CC_{50} concentrations, showed significant ($p \leq 0.01$) overexpression in all of the genes, with values ranging from 1.3 to 5.1 fold increase as compared to the untreated cells. Western blot analysis also confirmed the gene expression results, and showed the significant ($p \leq 0.01$) overexpression of PKC- β II, c-Fos, c-Jun, c-Myc, Cox2 and IL-1 β proteins, with values ranging from 1.48 to 3.15 fold increase as compared to the untreated cells. The flow cytometry results confirmed the apoptotic cell death in MDBK cells upon 24 h exposure to isolated phorbol esters and PMA. Consequently, the results of this study showed that the local *J. curcas* plant is of the toxic variety due the presence of phorbol esters, and although the meal could be considered as a potential biofeed, due to the presence of various biological activities and bioactive compounds, however, the cytotoxic effect of phorbol esters could not be

compromised. Although the rumen microbes could detoxify the phorbol esters partially, it is still not sufficient to consider it safe as a biofeed due to their cytotoxic effects even at low concentrations. Therefore, complete removal of phorbol esters from *Jatropha* meal is absolutely necessary.



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

PENILAIAN BIOKIMIA DAN MOLEKULAR UNTUK MIL JATROFA SEBAGAI BIO-MAKANAN

Oleh

EHSAN OSKOU EI AN

Julai 2012

Pengerusi: Profesor Norhani Abdullah, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Jatrofa curcas L. (*J. curcas*) adalah pokok yang terkenal sebagai sumber biji berminyak untuk penghasilan biodisel. Pokok ini tumbuh dengan baik dalam cuaca tropika dan penanamannya semakin meningkat di Malaysia. Semasa proses pengekstrakan minyak daripada kernel biji, residu yang terhasil dikenali sebagai mil Jatrofa. Mil ini berpotensi sebagai bio-makanan (biofeed) berdasarkan kepada kandungan kimia dan sebatian bioaktif yang terdapat didalamnya. Walaubagaimanapun, terdapat perbezaan dari segi sebatian bioaktif di antara genotip pokok *J. curcas*. Oleh itu, sebelum ia boleh digunakan sebagai satu bio-makanan, adalah perlu menentukan komponen bioaktif dan aktiviti biologi mil tersebut. Adalah juga penting untuk mengetahui samada *J. curcas* tempatan ini bersifat toksik atau tidak berdasarkan profil sebatian bioaktif yang ada. Hipotesis kajian ini ialah mil Jatrofa adalah selamat dan mempunyai ciri bio-makanan berfungsi. Untuk menguji hipotesis ini, kajian yang terperinci ke atas kandungan bahan bioaktif dan aktiviti biologi mil Jatrofa, rawatan fizikokimia dan bagaimana mil serta forbol ester yang diasingkan dari mil mempengaruhi aktiviti rumen mikrob adalah yang pertama dilakukan untuk menilai mil tersebut sebagai satu bio-makanan. Ianya diikuti oleh

penilaian sitotoksitas dan elucidasi mod tindakan forbol ester daripada mil terhadap sel buah pinggang lembu. Keputusan menunjukkan analisis kimia mil Jatrofa yang diperolehi dari pokok *J. curcas* tempatan mengandungi 61.8% protin kasar, 9.7% serabut detergen netral dan 4.8% serabut detergen asid. Mil ini juga mengandungi kandungan fenolik yang tinggi (3.9 mg/g DM), jumlah flavonoid (0.4 mg/g DM), jumlah saponin (19 mg/g DM), asid fitik (9.1%), perencat tripsin (34.2 mg/g DM), lektin (102.7 U) dan forbol ester (3.0 mg/g DM). Analisis kromatografi cecair berprestasi tinggi (HPLC) mil Jatrofa menunjukkan kehadiran asid galik, firogalol, rutin, mirisetin dan daidzein dengan nilai 581.3 ± 0.36 , 631.1 ± 0.47 , 47.6 ± 0.53 , 198.5 ± 0.29 dan 297.5 ± 0.27 $\mu\text{g/g DM}$, masing-masing. Analisis kromatografi gas spektrometri jisim (GC-MS) menunjukkan kehadiran metabolit lain termasuk 2-(hidroksimetil)-2 nitro-1,3-propanediol, β -sitosterol, 2-furankarboksaldehid, 5-(hidroksimetil), furfural (2-furan karboksaldehid) dan asid asetik. Ekstrak metanolik mil Jatrofa, vitamin C, butilated hidroksitoluene (BHT) dan β -karoten menunjukkan aktiviti mengaut radikal bebas (2,2-difenil-1-pikrilhidrazil) pada nilai IC_{50} iaitu 1.6, 0.3, 0.3 dan 1.5 mg/ml masing-masing, manakala nilai untuk kuasa penurunan ferik adalah 3.0, 0.3, 0.3 dan 2.6 mg/ml, masing-masing. Ekstrak mil Jatrofa menunjukkan aktiviti antibakteria terhadap bakteria fatogen seperti *Enterobacter aerogenes*, *Klebsiella pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Bacillus subtilis*, *Bacillus cereus*, dan *Staphylococcus aureus* dengan nilai rencatan berjulat 0.21-1.63 cm pada kepekatan 1 dan 1.5 mg/piring. Ekstrak metanolik mil Jatrofa pada kepekatan 100 $\mu\text{g/ml}$ merencat nitrik oksida sintase teraruh dalam makrofaj RAW 264.7, setara dengan N_{ω} -L-nitro-arginina metil ester (L-NAME), menunjukkan aktiviti ketara anti-radang. Kombinasi rawatan hidroterma, alkali dan agen pengoksidaan mengurangkan kandungan fenolik, saponin

dan forbol ester dengan perbezaan ketara ($p \leq 0.05$), tetapi tahap asid fitik tidak berkurang. Perencat tripsin dan aktiviti lektin dinyahkan sepenuhnya. Tahap forbol ester berkurangan sebanyak 76.7% dengan rawatan haba, 3% (b/b) NaOH dan 10% (i/b) NaOCl. Fermentasi *in-vitro* oleh mikrob rumen menunjukkan penurunan ketara ($p \leq 0.05$) dalam parameter fermentasi apabila mil yang dirawat secara kimia digunakan sebagai substrat, manakala mil yang dirawat secara fizikal tidak mempengaruhi parameter fermentasi dengan ketara. Kesan empat tahap forbol ester dari mil Jatrofa iaitu 3, 6, 9 dan 12 mg/30 ml pemampatan-cecair rumen, ke atas fermentasi rumen menggunakan rumput Guinea sebagai substrat dikaji secara *in-vitro*. Keputusan menunjukkan pencernaan jelas berat kering, tenaga metabolik, jumlah penghasilan gas selepas 24 jam (IVGP₂₄) eraman dan jumlah asid lemak menurun secara berbeza ($P \leq 0.05$) dengan 9 dan 12 mg forbol ester. Aktiviti spesifik enzim mikrob rumen (CMCase, FPase, zailanase and β -glukosidase), kandungan purina (indeks protin rumen mikrob) dan sintesis protein mikrob rumen menurun dengan perbezaan ketara ($P \leq 0.05$) oleh rawatan 9 dan 12 mg forbol ester. Seperti juga populasi bakteria, fungi, protozoa, metanogen, arkea dan jumlah bakteria selulolitik termasuklah *Fibrobacter succinogenes*, *Ruminococcus albus*, *R. flavefaciens* dan *Butyrivibrio fibrisolvens* menurun secara signifikan ($p \leq 0.05$) bila ditambah 9 dan 12 mg forbol ester. Kehilangan forbol ester semasa fermentasi mikrob rumen dilihat dengan nilai dari 23.0% hingga 44.1%. Penilaian ketoksikan *in-vitro* ekstrak mentah dari cecair rumen yang dirawat dengan forbol ester sebelum dan selepas 24 jam fermentasi keatas viabiliti sel buah pinggang lembu (MDBK) (ATCC: CCL-22) menurunkan kesan toksik ($p \leq 0.05$) dari 43.6% hingga 72.3%. Forbol ester dari mil Jatrofa diasingkan kepada empat fraksi PE1, PE2, PE3 dan PE4. Asai sitotoksiti *in vitro* menunjukkan sel mati dengan nilai CC_{50} dengan nilai julat

52.4 to 109.6 $\mu\text{g/ml}$ dalam sel buah pinggang lembu apabila didedahkan kepada semua fraksi forbol ester dan forbol 12-miristat 13-asitat (PMA) sebagai kawalan positif semasa pendedahan 24, 48 dan 72 jam. Forbol ester mengaruh kematian sel secara bergantung dos dan masa. Pencerapan dengan mikroskop cahaya menunjukkan tiada perbezaan yang ketara pada morfologi sel MDBK semasa pendedahan selama 12 jam kepada semua fraksi forbol ester dan PMA, manakala pendedahan selama 24 jam menunjukkan perbezaan morfologi yang ketara, penanggalan, penghancuran sel dan jasad apoptotik badan dapat dilihat. Ekspresi gen PKC- β II dari aliran trunsduksi isyarat, c-Fos, c-Jun dan c-Myc dari proto-onkogens dan gen IL-1 β and Cox2 dari aliran keradangan semasa 12 jam rawatan dengan forbol ester dan PMA pada kepekatan CC_{50} menunjukkan ekspresi-lebih untuk kesemua gen dengan nilai dari 1.3 sehingga 5.1 kali ganda meningkat bila dibandingkan dengan sel yang tidak dirawat. Analisis Western blot juga mengesahkan keputusan ekspresi gen dan menunjukkan secara ketara ($p \leq 0.01$) ekspresi-lebih PKC- β II, c-Fos, c-Jun, c-Myc, Cox2 dan IL-1 β proteins dengan nilai di antara 1.48 sehingga 3.15 kali ganda bila dibandingkan dengan sel yang tidak dirawat. Keputusan sitometri aliran juga mengesahkan bahawa sel mati apoptotik dalam sel MDBK pada pendedahan 24 jam berlaku kerana forbol ester dan PMA. Dengan sebab itu, keputusan kajian ini menunjukkan yang pokok *J. curcas* tempatan adalah jenis toksik kerana kehadiran forbol ester dan walaupun mil ini boleh dianggap sebagai sumber bio-makanan berpotensi, berdasarkan kepada kehadiran pelbagai aktiviti biologi dan kandungan sebatian bioaktif, tetapi kesan sitotoksiti forbol ester tidak dapat dikompromi. Walaupun mikrob rumen dapat menyahtoksikkan forbol ester secara separa, ia masih tidak mencukupi untuk mil dianggap selamat digunakan sebagai bio-makanan kerana kesan toksik berlaku

walaupun pada kepekatan yang rendah. Oleh kerana itu, penyahan keseluruhan forbol ester dari mil Jatropha perlu dilakukan sepenuhnya.



ACKNOWLEDGEMENTS

Thanks God the most compassionate, the most merciful as always upon me during this research project, and indeed, throughout my life. I would like to express my deepest gratitude and appreciation to my main supervisor, Professor Dr. Norhani Abdullah for her patience, advice and continuous support. I am honored to have had the chance to work under her guidance during the completion of my thesis.

I am also grateful to my co-supervisors Dr. Wan Zuhainis Saad, Prof. Dr. Abdul Rahman Omar and Prof. Dr. Ho Yin Wan for their invaluable support and guidance. Special thanks to the staff of the Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences and staff of Natural Product Laboratory, Animal Tissue Culture Laboratory, Laboratory of Vaccines and Immunotherapeutics of the Institute of Bioscience, Universiti Putra Malaysia, in particular Dr. Syahida Ahmad, Dr. Tan Sheau Wei, Mr. Khairul Kamar Bakri, Mrs. Mazina Mohd. Yusoff, Mr. Nagayah Muniandy for their help and contribution to this project.

I gratefully acknowledge Ministry of Science, Technology and Innovation (MOSTI) of Malaysia for the grant provided under the Fundamental Research Grant Scheme (Project No. 01-11-08-660FR) and the financial support under Graduate Research Assistant (GRA) during the course of my PhD program. My sincere thanks to Ehsan Karimi, Rudi Hendra and Mohammad Yahaghi for their friendship, valuable help, contribution and suggestions in this project.

I must express my gratitude to my beloved wife, Forough, for her understanding, kindness and patience during my course of study. I am grateful to my dear parents and brothers for their unconditional love, encouragement, and endless support that are well beyond anything that I could possibly give in return.

I certify that an Examination Committee met on 23rd July 2012 to conduct the final examination of Ehsan Oskoueian on his Doctor of Philosophy thesis entitled “Biochemical and Molecular Techniques for Evaluation of Jatropha Meal as Biofeed” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee are as follows:

Maziah Mahmood, PhD

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Muhajir Hamid, PhD

Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Kamaruzaman Sijam, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Geoffrey A. Cordell, PhD

Professor
Faculty of Pharmacy
University of Illinois at Chicago, United States of America
(External Examiner)

ZULKARNAIN ZAINAL, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Norhani Abdullah, PhD

Professor

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Chairman)

Abdul Rahman Omar, PhD

Professor

Institute of Bioscience

Universiti Putra Malaysia

(Member)

Wan Zuhani Saad, PhD

Senior lecturer

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Member)

Ho Yin Wan, PhD

Professor

Institute of Bioscience

Universiti Putra Malaysia

(Member)

BUJANG BIN KIM HUAT, PhD

Professor and Dean

School of Graduate Studies

Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for other degree at University Putra Malaysia or at any other institution.



EHSAN OSKOEIAN

Date: 23 July 2012



COPYRIGHT

TABLE OF CONTENTS

	Page
ABSTRACT	iii
ABSTRAK	viii
ACKNOWLEDGEMENTS	xiii
APPROVAL	xiv
DECLARATION	xvi
LIST OF TABLES	xxii
LIST OF FIGURES	xxiv
LIST OF APPENDIXES	xxvii
LIST OF ABBREVIATIONS	xxviii
LIST OF ANNOTATIONS	xxx
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	4
2.1 <i>Jatropha curcas</i> Plant	4
2.2 Jatropha Meal as Novel Bio-Feed	5
2.3 Bioactive Compounds in Jatropha Meal and Their Potential	6
2.3.1 Phenolics	6
2.3.2 Flavonoids	8
2.3.3 Saponins	9
2.3.4 Phytic Acid	10
2.3.5 Lectins	11
2.3.6 Trypsin Inhibitors	13
2.3.7 Phorbol Esters	14
2.4 Alleviation of Bioactive Compounds In Jatropha Meal	19
2.4.1 Physical Processing	19
2.4.2 Chemical Processing	19
2.4.3 Biological Processing	20
2.5 <i>In Vitro</i> Digestibility of Jatropha Meal	21
2.6 Toxicity and Biological Activity	22
2.6.1 Animal Trials	22
2.6.2 Animal Cell Models	24
3 BIOACTIVE COMPOUNDS AND BIOLOGICAL ACTIVITIES OF JATROPHA MEAL	25
3.1 Introduction	25
3.2 Materials and Methods	25
3.2.1 Plant Materials	25
3.2.2 Reagents	26

3.2.3	Chemical Analyses	27
3.2.4	Total Phenolic Compounds	27
3.2.5	Total Flavonoid Compounds	28
3.2.6	Total Saponins	28
3.2.7	Phytic Acid	29
3.2.8	Lectins	30
3.2.9	Trypsin Inhibitors	31
3.2.10	Total Phorbol Esters	33
3.2.11	HPLC Analysis of Phenolic and Flavonoids Compounds	34
3.2.12	Gas Chromatography - Mass Spectrometry Analysis (GC-MS)	35
3.2.13	Antioxidant Activity	36
3.2.13.1	DPPH Scavenging Activity	36
3.2.13.2	Ferric-Reducing Antioxidant Power (FRAP) Assay	37
3.2.14	Antibacterial Activity	38
3.2.14.1	Extraction	38
3.2.14.2	Pathogenic Bacteria	38
3.2.14.3	Disc Diffusion Method	38
3.2.15	Anti-inflammatory Activity	40
3.2.15.1	Extract Preparation	40
3.2.15.2	Cell Culture	40
3.2.15.3	Cell Seeding and Stimulation	40
3.2.15.4	Nitric Oxide Inhibitory Activity	41
3.2.15.5	Cell Viability	42
3.3	Results and Discussion	42
3.3.1	Chemical Analyses	42
3.3.2	Bioactive Compounds of Jatropha meal	44
3.3.3	HPLC Analysis of Phenolic and Flavonoids Compounds	48
3.3.4	Gas Chromatography - Mass spectrometry Analysis (GC-MS)	49
3.3.5	Antioxidant Activity	51
3.3.5.1	DPPH Scavenging Activity	51
3.3.5.2	Ferric-Reducing Antioxidant Power (FRAP) Assay	52
3.3.6	Antibacterial Activity	55
3.3.7	Anti-inflammatory Activity	58
3.4	Conclusion	60
4	EFFECT OF JATROPHA MEAL AND ISOLATED PHORBOL ESTERS ON RUMEN MICROBIAL ACTIVITY IN VITRO	62
4.1	Introduction	62
4.2	Material and Methods	63

4.2.1	Reagents	63
4.2.2	Jatropha meal Preparation	63
4.2.3	Physicochemical Treatments	64
4.2.4	Chemical Analyses	64
4.2.5	Bioactive Compound Analyses	65
4.2.6	Effects of Treated and Untreated Jatropha Meal on Rumen Microbes	65
	4.2.6.1 Preparation of Incubation Medium	66
	4.2.6.2 <i>In Vitro</i> Gas Production Technique	66
4.2.7	Volatile Fatty Acids Determination	67
4.2.8	Ammonia Determination	68
4.2.9	Isolation of Phorbol Esters	68
4.2.10	Effects of Isolated Phorbol Esters on Rumen Microorganisms Fermentation Activity	69
4.2.11	Microbial Enzyme Activity	70
4.2.12	Microbial Protein Synthesis	71
4.2.13	Microbial Population Analysis Using Real Time PCR	72
	4.2.13.1 Sampling	72
	4.2.13.2 DNA Extraction	72
	4.2.13.3 DNA Concentration and Quality Assessment	74
	4.2.13.4 PCR Amplification and Product Purification	74
	4.2.13.5 Primers for PCR Amplification	75
	4.2.13.6 Cloning and Sequencing	77
	4.2.13.7 Plasmid Extraction	77
	4.2.13.8 Analyses of Transformants	78
	4.2.13.9 Sequence Identification and Secondary Structure Analyses	79
	4.2.13.10 Real-Time PCR Analysis and Standard Curves	79
	4.2.13.11 Standard Curve Construction	80
4.2.14	Ruminal Phorbol Ester Disappearance	80
4.2.15	<i>In Vitro</i> Toxicity Test	81
4.2.16	Statistical Analyses	81
4.3	Results and Discussion	82
	4.3.1 Chemical Constituents	82
	4.3.2 Effect of Treatments on Bioactive Compounds Content	83
	4.3.3 Effects of Treated and Untreated Jatropha Meal on Rumen Microbes	86
	4.3.4 Isolation of Phorbol Esters	91
	4.3.5 Effects of Isolated Phorbol Esters on Rumen Microorganisms Fermentation Activity	91
	4.3.6 Rumen Microbial Enzyme Activity	95
	4.3.7 Rumen Microbial Protein Synthesis	97
	4.3.8 Rumen Microbial Population	99

4.3.9	Ruminal Phorbol Esters Disappearance	102
4.3.10	<i>In Vitro</i> Toxicity Test	103
4.4	Conclusion	104
5	BIOLOGICAL AND MOLECULAR MECHANISMS OF ACTION OF ISOLATED PHORBOL ESTERS ON BOVINE KIDNEY CELL LINE	105
5.1	Introduction	105
5.2	Material and Methods	106
5.2.1	Jatropha Meal Preparation	106
5.2.2	Reagents	106
5.2.3	Isolation of Phorbol Esters	107
5.2.4	Cell Culture and Maintenance	107
5.2.5	Cytotoxicity Test (MTT Assay)	108
5.2.6	Microscopic Examination	108
5.2.7	Gene Expression Analyses in the MDBK Cell Line	109
5.2.7.1	Cell treatments and RNA Extraction	109
5.2.7.2	Quantification of RNA	109
5.2.7.3	RNA Integrity Confirmation	110
5.2.7.4	cDNA Synthesis and Agarose Gel Electrophoresis	110
5.2.7.5	Primer Design	111
5.2.7.6	Validation of the Specificity of Designed Primers	112
5.2.7.7	PCR Amplifications	112
5.2.7.8	Gene Expression Analyses Using Real-Time PCR	112
5.2.8	Protein Expression Analyses Using Western Blot Technique	113
5.2.8.1	Cell Treatment	113
5.2.8.2	Preparation of Whole Cell Lysate	114
5.2.8.3	Protein Quantification (Bradford Assay)	114
5.2.8.4	Western Blotting Analysis	115
5.2.9	Analysis of Apoptosis by Flow-cytometry	116
5.2.10	Statistical Analyses	117
5.3	Results and Discussion	118
5.3.1	Isolation of Phorbol Esters	118
5.3.2	Cytotoxicity Assay	118
5.3.3	Microscopic Examination	120
5.3.4	Gene Expression Analyses in the MDBK Cell Line	123

5.3.4.1	Expression of Signal Transduction Pathway Gene	123
5.3.4.2	Expressions of Proto-oncogenes	124
5.3.4.3	Expressions of Inflammatory Genes	127
5.3.5	Protein Expression Analyses in the MDBK Cell Line	130
5.3.5.1	Expression of Signal Transduction Pathway Protein (PKC- β II)	130
5.3.5.2	Expression of Proto-Oncoproteins	131
5.3.5.3	Expression of Inflammatory Proteins	135
5.3.6	Analysis of Apoptosis by Flow Cytometry	138
5.4	Conclusion	144
6	GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH	145
6.1	General Discussion	145
6.2	Conclusions	150
6.3	Recommendations for Future Research	150
	REFERENCES	152
	APPENDICES	171
	BIODATA OF STUDENT	216
	JOURNAL PUBLICATIONS	217
	CONFERENCE PRESENTATION	217
	WORKSHOPS/SEMINAR ATTENDED	218
	AWARDS	220