UNIVERSITI PUTRA MALAYSIA

BIOCHEMICAL AND MOLECULAR EVALUATION OF JATROPHA MEAL AS BIOFEED

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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

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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 µg/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 µg/ml, inhibited the inducible nitric oxide synthase in macrophages RAW 264.7, comparable to N_{ω}-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 ($IVGP_{24}$) 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 µg/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-1β 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-1β 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 OSKOUEIAN

Julai 2012

Pengerusi: Profesor Norhani Abdullah, PhD

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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 penanaman nya semakin meningkat di Malaysia. Semasa proses pengekstrakkan 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. Walaubagaimana pun, terdapat perbezaan dari segi sebatian bioaktif di antara genotip pokok *J. curcas*. Oleh itu, sebelum ia boleh di gunakan 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 sitotoksisiti dan elusidasi 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 μg/g DM, masing-masing. Analisis kromatografi gas spektrometri jisim (GC-MS) menunjukan 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 μg/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 fisikal tidak mempengaruhi parameter fermentasi dengan ketara. Kesan empat tahap forbol ester dari mil Jatrofa iaitu 3, 6, 9 dan 12 mg/30 ml pemampan-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 ($IVGP_{24}$) 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 selulotik 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 sitotoksisiti *in vitro* menunjukan sel mati dengan nilai CC_{50} dengan nilai julat

52.4 to 109.6 µg/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 menunjukan 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-1β 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-1β 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 sitotoksisiti 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 Jatropafa perlu dilakukan sepenuhnya.

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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.

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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:

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