UNIVERSITI PUTRA MALAYSIA

BIODEGRADATION OF PHORBOL ESTERS IN Jatropha curcas (Linn.) KERNEL BY FUNGI FOR PRODUCTION OF POULTRY FEED

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By

AZHAR NAJJAR

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

March 2014

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

BIODEGRADATION OF PHORBOL ESTERS IN *Jatropha curcas* (Linn.) KERNEL BY FUNGI FOR PRODUCTION OF POULTRY FEED

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March 2014

Chairman: Prof. Norhani Abdullah, PhD
Faculty: Biotechnology and Biomolecular Science

Poultry industry in Malaysia is highly dependent on imported feeds like corn and soybean meal. The cost of poultry production then depends on the price of feeds according to the global market. With the increase in feed ingredients, the cost of production would be accordingly affected. Hence, it is pertinent to find local alternative feed that can partially replace corn or soybean meal, to reduce the dependency on imported feeds. *Jatropha curcas* has gained importance as a source of seed oil for the production of biodiesel. The seed kernel also shows potential as a feed ingredient for poultry due to its high protein and low fiber content. However, the presence of phorbol esters as the main toxic compound makes the kernel unsafe as animal feed. Hence, the main objective of this study was to treat *Jatropha* seed kernel by fungal fermentation to produce a safe feed ingredient for poultry. The specific objectives of this study were to determine the levels of phorbol esters in the local *Jatropha* seed kernel, to treat the dried ground kernel by submerged fermentation, to conduct enzymes and cells bioassays and to evaluate the fermented kernel as a feed ingredient for poultry. The hypothesis to be tested was that the selected non toxic and non pathogenic fungal strains could degrade the phorbol ester present in *Jatropha* kernel to a safe level for the production of poultry feed.

Two fungal isolates obtained from garden soil and five endophytes from *Achillea fragrantissima* plant in Saudi Arabia were used for degrading the phorbol esters. These fungi were identified as *T. harzianum* (isolates TUT1 and TUT2), *P. sinensis* (isolate TUP8), *C. cladosporioides* (isolate TUC9) and *F. chlamydosporum* (isolates TUF1, TUF10 and TUF11) based on their morphological characteristics and internal transcribed spacer regions (ITS) sequence analysis. All 7 fungal strains were non-toxic

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to both normal Chang liver cells and mouse cell lines. The optimum fungal growth was in potato dextrose broth (PDB) medium at temperature 28°C and pH 5.5.

Phorbol esters in the phorbol esters-rich fraction prepared from the seed kernel were analyzed by LC-DAD-ESIMS. Four phorbol ester derivatives were detected, where Peak 1 was identified to be 12-deoxy-16-hydroxyphorbol. Peaks 2, 3 and 4 were phorbol esters that possess the same diterpene moiety, namely, 12-deoxy-16-hydroxyphorbol. Quantitative analysis of phorbol esters by high performance liquid chromatography showed a value of 2.78 mg phorbol-12-myristate 13-acetate (PMA) equivalent per g dry weight of *Jatropha* kernel. The phorbol ester-rich fraction prepared contained 66.72 mg PMA equivalent per g dry weight. The addition of different levels of phorbol ester-rich fraction (1-3 g) to 30 ml PDB did not inhibit the growth of the 7 fungal strains after 14 days incubation. All fungal strains were able to utilize phorbol esters-rich fraction as a carbon source in PDB as well as in mineral salt broth (MSB) media. The fungal dry weight increased significantly ($p<0.05$) in the presence of 2 g of phorbol ester-rich fraction after 30 days incubation. The values obtained for *T. harzianum* JQ350879.1 and control (without phorbol ester-rich fraction) were 3227.3 and 440.0 mg, respectively. The phorbol esters present in phorbol esters-rich fraction or in methanolic extract or kernel were degraded in the range of 67.7 to 99.7% after 30 days of incubation by the fungal strains. The maximum removal of phorbol esters was by *T. harzianum* JQ350879.1 for all the three different substrates. The level of phorbol esters was reduced by 99.7% by *T. harzianum* JQ350879.1.

Lipase activity was significantly higher ($p<0.05$) for all strains grown on olive oil medium containing phorbol esters. However, only three isolates i.e., *P. sinensis* JQ350881.1, *C. cladosporioides* JQ517491.1 and *F. chlamydosporum* JQ517492.1 showed both lipase and esterase activities. The presence of phorbol esters also induced esterase activity significantly ($p<0.05$). In the cytotoxicity bioassay with Chang liver and NIH 3T3 cell lines, cell viabilities were significantly ($p<0.05$) increased (84.3-96.5%) when compared to the control (0.3-0.4%) by fungal treatment of phorbol esters-rich fraction. In the feeding trial experiment, 20% of *Jatropha* kernel treated with *T. harzianum* JQ350879.1 was included in broilers diet (treated group) to replace 50% of soybean meal. Birds in the control group were fed a diet containing 40% soybean meal. The body weight gain and feed consumption of broilers in treatment group were 1996.0 g and 5049.5 g/bird, respectively, and that for control were 2181.8 g and 5596.8 g/bird, respectively. The feed conversion ratio (feed intake over weight gain) was similar between broilers in treated and control groups (2.52 *vs*. 2.56). Blood parameters results were comparable with normal values, showing no signs of toxicity. Mortality rate of birds in the treated group (7/60) was not significantly different from the control group (8/60). There was no histopathological evidence of abnormal change to liver and kidney tissues of broilers in treatment group.

In conclusion, *Jatropha* seed kernel was found to contain four derivatives of phorbol esters at high concentrations, which make the plant a toxic variety. The levels of phorbol

esters were successfully alleviated to a safe level by submerged fermentation with non toxic *Trichoderma* spp. and fungal endophytes. Fungal treated *Jatropha* kernel could be used as a feed ingredient to partially replace soybean in poultry diet without apparent toxicity symptoms.

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

BIODEGRADASI FORBOL ESTER DALAM ISIRONG *Jatropha curcas* (Linn.) OLEH TRICHODERMA SPP. DAN KULAT ENDOFITIK SERTA PENGHASILAN BAHAN MAKANAN UNTUK AYAM

Oleh

AZHAR NAJJAR

Mac 2014

Pengerusi: **Prof. Norhani Abdullah, PhD**
Fakulti: **Bioteknologi dan Sains Biomolekul**

Industri ternakan ayam di Malaysia sangat bergantung kepada bahan makanan yang diimpot seperti jagung dan mil kacang soya. Kos pengeluaran ayam akan bergantung kepada harga bahan mengikut pasaran global. Dengan peningkatan dalam bahan makanan, kos pengeluaran akan terjejas dengan sewajarnya. Oleh itu, adalah penting untuk mencari makanan alternatif tempatan untuk mengurangkan pergantungan kepada bahan yang diimport. *Jatropha curcas* mendapat kepentingan sebagai sumber biji minyak untuk pengeluaran biodiesel. Isirong biji juga menunjukkan potensi sebagai bahan makanan untuk ayam kerana protein yang tinggi dan kandungan serat yang rendah. Walau bagaimanapun, kehadiran forbol ester sebagai sebatian toksik utama, membuat isirong tidak selamat sebagai makanan haiwan. Oleh itu, objektif utama kajian ini adalah untuk merawat isirong biji *Jatropha* melalui penapaian kulat untuk menghasilkan bahan makanan yang selamat untuk ayam. Objektif khusus kajian ini adalah untuk menentukan tahap forbol ester dalam isirong *Jatropha* tempatan, untuk merawat isirong kering melalui penapaian tenggelam, untuk menjalankan enzim dan bioasei sel dan menilai sisirong terperam sebagai bahan makanan untuk ayam. Hipotesis untuk diuji adalah bahawa strain kulat bukan toksik dan bukan patogenik boleh merendahkan forbol ester dalam isirong *Jatropha* ke tahap yang selamat untuk penghasilan makanan ayam.

Dua isolat kulat daripada tanah taman dan lima endofitik dari pokok *Fragrantissima achillea* di Arab Saudi telah digunakan untuk degradasi forbol ester. Kulat telah dikenal pasti sebagai *T. harzianum* (Isolat TUT1 dan TUT2*),* *P. sinensis* (Isolat TUP8), *C. cladosporioides* (Isolat TUC9) dan *F. chlamydosporum* (Isolat TUF1, TUF10 dan TUF11), berdasarkan ciri-ciri morfologi dan analisis *internal transcribed spacer regions (ITS).* Semua 7 strain kulat adalah tidak toksik kepada kedua sel hati Chang

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dan sel tikus normal. Pertumbuhan kulat optimum adalah dalam medium kentang dekstrosa (PDB) pada suhu 28 ° C dan pH 5.5.

Forbol ester dalam fraksi-kaya forbol ester yang disediakan dari isirong biji telah dianalisis dengan LC-DAD-ESIMS. Empat derivatif forbol ester dikesan, di mana Puncak 1 telah dikenalpasti sebagai 12-deoksi-16-hidroksiforbol. Puncak 2, 3 dan 4 adalah derivatif forbol ester yang memiliki moieti diterpene yang sama, iaitu 12- deoksi-16-hidroksiforbol. Analisis kuantitatif forbol ester dengan kromatografi cecair prestasi tinggi menunjukkan kandungan sebanyak 2.78 mg forbol-12-13-miristati asetat (PMA) ekuivalen per g berat kering isirong *Jatropha*. Fraksi-kaya forbol ester yang disediakan mengandungi 66.72 mg PMA ekuivalen per g berat kering. Penambahan tahap yang berbeza fraksi-kaya forbol ester (1-3 g) ke 30 ml PDB tidak merencat pertumbuhan 7 strain kulat selepas 14 hari pengeraman. Semua strain kulat boleh menggunakan fraksi-kaya forbol ester sebagai sumber karbon dalam PDB serta media garam mineral (MSB). Berat kering kulat meningkat dengan ketara ($p < 0.05$) dengan fraksi-kaya forbol ester selepas 30 hari pengeraman. Nilai yang diperolehi oleh *T. harzianum* JQ350879.1 dan kawalan (tanpa fraksi-kaya forbol ester) adalah 3227.3 dan 440.0 mg, masing-masing. Forbol ester dalam fraksi-kaya forbol ester, atau dalam ekstrak metanol atau isirong didegradasikan dalam lingkungan 67.7-99.7% selepas 30 hari pengeraman oleh strain kulat. Penyingkiran maksimum forbol ester adalah oleh *T. harzianum* JQ350879.1 bagi ketiga-tiga substrat yang berbeza. Tahap ester phorbol telah dikurangkan sebanyak 99.7% oleh *T. harzianum* JQ350879.1.

Aktiviti lipase adalah jauh lebih tinggi ($p < 0.05$) bagi semua strain bila ditumbuhkan dalam media minyak zaitun mengandungi forbol ester. Walau bagaimanapun, hanya tiga strain iaitu, *P. sinensis* JQ350881.1, *C. cladosporioides* JQ517491.1 dan *F. chlamydosporum* JQ517492.1 menunjukkan kedua-dua aktiviti lipase dan esterase. Kehadiran forbol ester juga meningkatkan aktiviti esterase secara ketara ($p < 0.05$). Dalam bioesei sitotoksisiti dengan sel hati Chang dan sel NIH 3T3, viabiliti sel meningkat (84.3-96.5%) dengan ketara ($p < 0.05$) berbanding kawalan (0.3-0.4%) bila fraksi-kaya forbol ester dirawat oleh kulat. Dalam trial pemakanan, 20% isirong terawat dengan *T. harzianum* JQ350879.1 telah dicampur ke dalam diet ayam pedaging (kumpulan rawatan) untuk menggantikan 50% kacang soya. Ayam dalam kumpulan kawalan diberi makan diet mengandungi 40% mil kacang soya. Keuntungan berat badan dan jumlah makanan ayam pedaging dalam kumpulan rawatan adalah 1996.0 g dan 5049.5 g/ayam, masing-masing, dan untuk kawalan (diet tanpa isirong terawat) adalah 2181.8 g dan 5596.8 g/ayam, masing-masing. Nisbah penukaran makanan (pengambilan makanan berbanding berat badan) adalah sama antara ayam daging dalam kumpulan dirawat dan kawalan (2.52 *vs* 2.56). Parameter darah adalah setanding dengan nilai-nilai biasa, menunjukkan tiada tanda-tanda ketoksikan. Kadar kematian ayam dalam kumpulan yang dirawat (7/60) tidak berbeza dengan ketara daripada kumpulan kawalan (8/60). Tiada bukti histopatologikal terhadap perubahan abnormal dalam hati dan buah pinggang tisu ayam pedaging dalam kumpulan rawatan.

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Kesimpulannya, isirong *Jatropha* didapati mengandungi empat derivatif forbol ester pada kepekatan yang tinggi, yang menyebabkan pokok itu toksik. Kandungan forbol ester dapat dikurangkan dengan jayanya ke tahap yang selamat melalui rawatan dengan kulat *Trichoderma* spp. dan endofit yang tidak toksik melalui penapaian tenggelam. Isirong *Jatropha* terawat kulat boleh digunakan sebagai bahan makanan untuk menggantikan kacang soya sebahagiannya dalam diet ayam tanpa gejala keracunan yang jelas.

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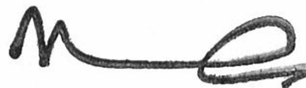

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