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Phorbol 12-Myristate 13-Acetate Degradation in *Jatropha Curcas* Seed Meal through Solid State Fermentation

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ABSTRACT

The study aimed at detoxifying JCSM using biological treatments (spontaneously, fungi cocktail and specific). Shimadzu HPLC/ UV was used to quantify phorbol esters in *Jatropha curcas* seed meal under isocratic conditions. After six days of fermentation, cocktail of fungi, and spontaneous fermentation of JCSM was effective in reducing PE concentration. Defattening seed meal using cold maceration in petroleum ether reduced phorbol-12-myristate-13-acetate (PMA) in the JCSM from 40.42 to 10.9 µg/ml prior to solid state fermentation. Spontaneous fermentation of defatted JCSM further reduced PMA from 10.9 to 5.05 µg/ml (53.6%). Fungi isolated during spontaneous fermentation included, *Aspergillus niger* (B), *Aspergillus flavus* (A), *Rhizopus Stolonifer* (C), *Penicillium chrysogenum* (D) and *Fusarium* species. The reduction of PE ranged between 10.71 – 1.46 µg/ml when single or a combination of the fungi isolates were used in solid state fermentation of the defatted seed meal. *P. chrysogenum* reduced phorbol ester levels (PE) in defatted seed 76.5%, *R. stolonifer* 43.3%, *A. flavus* 35.6% and *A. niger* 1.72%. When fungi isolates were paired, *A. niger* + *P. chrysogenum* (BD) reduced PE levels by 13.7%, *A. flavus* + *A. niger* 64.5%, *A. flavus* + *P. chrysogenum* 70.3%, *A. flavus* + *R. stolonifer* 78.8%, *A. niger* + *R. stolonifer* 86.6% and *R. stolonifer* + *P. chrysogenum* 86.6%. The treatment combination of *A. niger* + *R. stolonifer* + *P. chrysogenum* reduced PE levels by 84.0%, *A. flavus* + *A. niger* + *R. stolonifer* to 67.2% and *A. flavus* + *R. stolonifer* + *P. chrysogenum* to 62.3%. *P. chrysogenum* was effective as a single isolate but its activity was suppressed when paired with *A. niger*. Treatments of the deffated seed with, *A. flavus* + *R. stolonifer*; *A. niger* + *R. stolonifer*; *R. stolonifer* + *P. chrysogenum*; and *A. niger* + *R. stolonifer* + *P. chrysogenum* were the only combination that exceeded the phorbol reductive activity of *P. chrysogenum*. It could therefore be concluded that *Jatropha curcas* seed meals can be detoxified by solid state fermentation.

Introduction

Global interest in the exploitation of defatted seed cake of *Jatropha curcas* L., or physic nut, as an ingredient of feed has been growing largely because it is being produced in large quantities as the major by-product in the extraction of physic nut oil for use as biofuel.

The defatted seed cake requires detoxification before it can be considered as an ingredient in feed formulation (Najjar *et al*., 2014). For the sustainable production of biofuel from the physic nut, the safe management of toxic by-products is imperative for the assurance of environmental security.

In Ghana, the gradual collapse of the poultry industry which has been attributed to the high cost of imported feed ingredients has motivated researchers to explore locally available alternate sources of affordable feed ingredients (Akyere, 2011). Fortunately, the introduction of Jatropha in Ghana as a rich source of biofuel and the ability of the crop to grow virtually everywhere in the country has further encouraged researchers to focus on the detoxification of the physic nut seed cake which has the potential to make available for human consumption, food crops currently used as ingredients for feed. Unlike other major biofuel crops such as maize, soya and rapeseed, *Jatropha curcas* is not used for food (Brittaine and Lutaladio, 2012).

In the extraction of oil from *J. curcas* seeds by screw press or mechanical method, about 77% to 79% of *J. curcas* seed meal is produced from the raw material (Chikpah and Demuyakor, 2012). The *J. curcas* seed meal (JCSM) is a rich source of crude protein. The crude protein content, on dry matter basis, ranges from 57 to 64 percent in the Jatropha kernel meal (Makkar and Becker, 1997b) and 24.3% in the seed meal (Tjakradidjaja *et al*., 2007). With the exception of lysine, the amino acid in *J. curcas* meal is higher than FAO reference protein required for animal wellbeing and growth (Makkar and Becker, 1997). The seed cake is rich in lignocellulosic compounds and minerals such as phosphorus, potassium, calcium and magnesium (Chikpah and Demuyakor, 2012). Undoubtedly, Jatropha meal has the potential as an ingredient of feed in animal husbandry (Kasuya *et al*., 2013) and aquaculture (Kumar *et al*., 2011a).

In addition to the high protein, lipids and mineral content of *J. curcas*, the presence of antinutritional factors and phorbol esters in the seeds and their toxicity to a number of animal species, including rats, mice and ruminants, have been reported (Makkar *et al*., 2008; Becker and Makkar, 1998; Makkar *et al*., 1997). Phorbol esters extracted from *J. curcas* seeds have been identified as the main toxic agent responsible for *Jatropha* toxicity (Makkar *et al*., 2008). Several incidents of *J. curcas* nut

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poisoning in humans after accidental consumption of the seeds have been reported (Adolf *et al*., 1984; Makkar *et al*., 1997). This discouraged the efficient use of JCSM in animal husbandry. The applications of physical, chemical and biological treatments to detoxify Jatropha seed cake have been reported (Becker and Makkar, 1998; 1997; Aderibigbe *et al*., 1997; Aregheore *et al*., 2003). In some instances, heat treatment was unable to destroy phorbol esters (Becker and Makker 1998) which are the principal toxic components of *J. curcas;* phorbol esters are stable at high temperature of 160 °C for 30 min (Makkar and Becker, 1997). Over the years, chemical treatment of *J. curcas* meal has been promising but it is expensive to produce *J. curcas* seed/kernel meal free from phorbol esters (Aregheore *et al*., 2003). Considering the variations in effectiveness and economic viability, research is focusing on solid state fermentation as an effective and less expensive tool for the detoxification of Jatropha seed cake (Najjar *et al*., 2014; Chikpah and Bawa, 2012; De Barros *et al*., 2011; Belewu and Sam, 2010).

The thrust of this study is to evaluate the efficacy of solid state fermentation using isolated fungi from spontaneously fermented *J. curcas* seed meal in degrading phorbol esters.

Materials and methods

Materials

Phorbol 12-myristate 13-acetate, petroleum ether, dimethyl sulfoxide (DSMO) HPLC grade of acetonitrile, water and methanol, were purchased from Sigma-Aldrich Co., St. Loius, MO 63103 USA. All other chemicals and solvents used were of analytical grade and obtained from standard suppliers.

Study area/ Collection of *J. curcas* seeds

The study was conducted at the Spanish grant laboratory of the Department of Biotechnology, University for Development Studies (UDS), Nyankpala campus and Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana. Matured *J. curcas* seeds were collected from Nyankpala (A Guinea savanna zone, Localization Latitude 09° 25‘N, Longitude 00° 58‘ W; Average temperature 28.3 °C; Annual rainfall 1043 mm; Average humidity of 58 %) in the Northern Region of Ghana, during the period of November and December 2013. The seeds were harvested from their fruit pulp. The seeds were cleaned and a portion was shelled, milled, packaged into sterile plastic bags and stored in a refrigerator at 4 ºC until use.

Defattening of *J. curcas* seed for solid state fermentation

Dried *J. curcas* seeds collected were ground into powdery state using an Elbee blender (model: LB-1323). Defattening of *J. curcas* seed was done using cold extraction method with petroleum ether (bp 40 - 60 ^{o}C). The ratio of ground *J. curcas* and petroleum ether by weight was 1:3. The meal was collected and air-dried. The defatted sample (DF) was labeled and kept in sterile polyester plastic containers and refrigerated at 4 ºC for later use.

Sourcing of fungi from spontaneous fermentation of *J. curcas*

The seed meal obtained from Jatropha seeds were divided into four portions. The first portion (A), the control was autoclaved, and while the second (B), third (C), and fourth (D) portions were not autoclave. Each treatment contained 10 grams of defatted *J. curcas* seed and were placed in sterile Petri dish. Approximately 5 ml of sterile distilled water was added to each treatment to make it moist but not wet. Each treatment was replicated thrice. Samples were allowed to ferment spontaneously for 6 days. Each day, the mycology of the fermented samples were determined using potato agar plate method and microscopy for fungi identification. Samples were taken on day 6 of fermentation and heated at 80 ºC for 24 hours before storage. The samples were stored in a refrigerator at 4 ºC until the laboratory analysis was complete.

Solid state fermentation of *J. curcas* seed meal using isolated fungi

Each pure cultured fungi obtained from spontaneously fermented seed meal was inoculated onto 10 grams of sterilised *Jatropha* seed meal with 5ml of sterilized distilled water containing 10^6 spores to keep the substrate moist and to ferment it. The setup was allowed to ferment for 6 days and a combination of fungus (A= *Aspergillus flavus*, B=*Aspergillus niger*, C=*Rhizopus stolonifer*, D =*Penicillium chrysogenum*, AB, AC, AD, ABC, ACD and BCD are combinations of the fungi coded) was carried out. Each treatment was replicated thrice. After fermentation, the samples were heated at 80°C for 24 hours. The fermented samples were packaged in sterile plastic bags for phorbol ester analysis by HPLC.

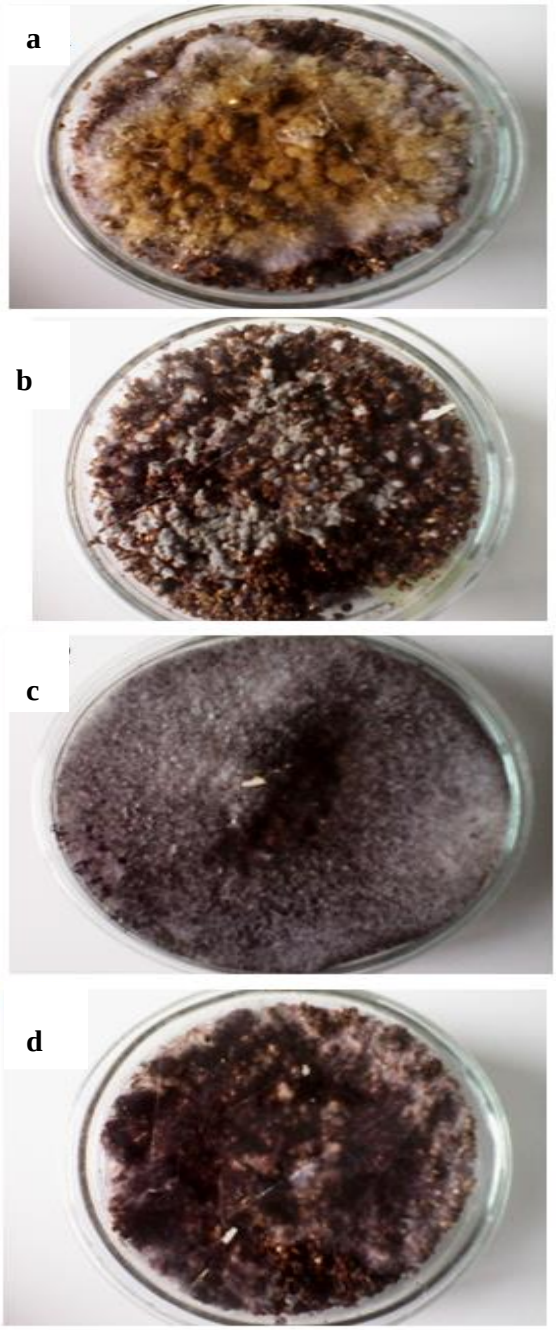

Figure 1. Plate a – d. Solid state fermentation of defatted *J. curcas* seed meal

Quantification of phorbol ester from whole seeds

Extraction of phorbol esters from defatted seed meal

Extraction phorbol esters were carried via vortex method as described by Deppava *et al*. (2013). Fermented *J. curcas* seed meal (0.5g) was weighed out into Eppendorf tube. Absolute methanol (2ml) was added to the sample and mixed via vortex machine for 3 min. The vortexed sample was sonicated for 5 min and centrifuged at 8000 x g to collect methanol layer. The residue obtained after centrifugation was re-extracted three times using methanol by repeating the above procedure. The methanol layer of each extraction was pooled into a glass tube with a known weight. Methanol was evaporated using a hot plate at 60 ºC to obtain an oily residue which was weighed. The residue was dissolved in the mobile phase, vortexed and centrifuged at 8000 x g. The supernatant was transferred into injection vial and analyzed by HPLC.

Phorbol 12-myristate 13-acetate isolation and quantification using an HPLC

Quantification phorbol 12-myristate 13-acetatae (PMA) in *J. curcas* seed meal was done using a Shimadzu HPLC under isocratic condition. The machine was equipped with a degasser (DGU- $20A_3$) and a photo diode array detector wavelength detector (SPD- M20A). The detector wavelength was set at 254nm. The HPLC pump (LC-20 AB) was set at 7.5 mPa. The separation was performed using a C18 column Slum-pack VP-ODS, with dimension 150 mm × 4.6 mm x 5 µm. The column oven (CTO -10AS VP) was set at 40ºC. The mobile phase was a solution of 83% acetonitrile and the flow rate was 1 ml/min. The injection volume was 20 µl and the run time was 20 min.

Calibration curve of standard

A calibration curve was prepared using phorbol 12-myristate 13-acetate as standard. Stock solution of the standard (5 mg/ml) was prepared in DMSO. Serial dilutions were carried out to obtain concentrations between 0.025 mg/ml and 0.003 mg/ml. The standard solutions were placed in HPLC vials for analysis.

Results and Discussion

Phorbol-12-myristate-13-acetate standard calibration

The retention time of phorbol 12-myristate 13-acetate was 14.4 min. The equation of the calibration curve was, $Y= 5794.6X – 8435.2$ and $R^2 = 0.9977$. Where 'Y' is area of peak and X is concentration of phorbol-12-myristate-13-acetate.

Phorbol-12-myristate-13-acetate degradation

The presence of phorbol ester in all *J. curcas* samples or treatments were expressed as equivalent to the standard phorbol 12-myristate 13-acetate (PMA). The amount of PMA in undefatted whole seed was 40.417 µg/ml before defattening. Defatted seed (DF) using cold extraction (petroleum ether) reduced phorbol ester to 10.9 µg/ml prior to solid state fermentation. Solid state fermentation of fungi via spontaneous fermentation, specific fungus fermentation and cocktail of fungi fermentation reduced further PMA concentration in the defatted seed meal (Table 2).

Petroleum ether based solvent extraction of oil from the *J. curcas* seed significantly reduced phorbol-12-myristate-13-acetate (PMA) concentration in the defatted samples. The reduction of phorbol ester might have resulted from the long period (12 hours) of soaking *J. curcas* seed meal in petroleum ether. Chivandi *et al*. (2004) reported a reduction of phorbol ester by 67.69% (6.5 mg/g to 2.10 mg/g) after oil expulsion from raw shelled kernels. This suggested that phorbol esters were soluble in petroleum ether just as other organic solvents such as methanol, ethanol and hexane. Subjecting defatted *J. curcas* seed meal containing 10.9 µg/ml of phorbol-12-myristate-13-acetate (PMA) to solid state fermentation with *Aspergillus flavus* (A), *Aspergillus niger* (B), *Rhizopus stolonifer* (C), *Penicillium chrysogenum* (D) and cocktail of the isolates revealed that the fungi isolated decreased PMA concentration after six days of fermentation. High performance liquid chromatographic analysis revealed that *Aspergillus niger* was the least effective isolate in degrading PMA, causing only 1.72% degradation of the amount of PMA present in the defatted meal after six days of fermentation. This report is dissimilar to the findings of Belewu and Sam (2010), who reported a reduction of phorbol ester by 77% (from 0.013 % to 0.003 %) after 10 days of fermentation of *J. curcas* kernel cake with *Aspergillus niger*. The difference might be as a result of the longer fermentation time and the approach employed in the quantification of the amount of phorbol ester present in the fermented meal. However, Belewu and Sam (2010) did not indicate the methodology employed in PMA quantification. Defatted *J. curcas* seed meal fermented with *A. niger* for six days and fed to mice resulted in 100% mortality (Tjakradidjaja *et al*., 2009). Similarly, mice that ingested untreated *J. curcas* seed meal died (Tjakradidjaja *et al*., 2009).

This suggested that, despite defattening and solid state fermentation with *A. niger*, *J. curcas* seed meal was still toxic. Hence, *A. niger* was not efficient in detoxifying the seed meal as was observed in this study. When *J. curcas* seed meal (JCSM) was fermented with *Aspergillus flavus*, phorbol ester was reduced by 35.6%. *Rhizopus stolonifer* and *Penicillium chrysogenum*, contributed 43.3% and 76.5% reduction of phorbol ester respectively after seven days of fermentation. According to Belewu and Akande (2010), serum biochemical and haematological parameters of West African dwarf goats remained within normal ranges after being fed with *Penicillium* species treated *J. curcas* seed meal. They concluded that, since animals fed on the treated meal did not experience any adverse effect, toxicity from the treated meal was probably negligible. This implies that *Penicillium* species was efficient in detoxifying the *J. curcas* kernel meal. With exception of *Penicillium chrysogenum*, the use of individual fungi to detoxify *J. curcas* seed meal did not sufficiently reduce phorbol esters. Treatment combinations of *A. flavus* + *R. stolonifer* (AC); *A. flavus* + *P. chrysogenum* (AD); *A. flavus* + *A. niger* (AB); *A. niger* + *P. chrysogenum* (BD); *A. niger* + *R. stolonifer* (BC) as well as *R. stolonifer* + *P. chrysogenum* (CD) were able to reduce phorbol esters concentration (Table 2). It was observed from the studies that generally the cocktails of fungi were more efficient than individual fungi fermentation. The use of three fungi in *J. curcas* seed meal, *A. niger* + *R. stolonifer* + *P. chrysogenum* (BCD), *A. flavus* + *A. niger* + *R. stolonifer* (ABC) and *A. flavus* + *R. stolonifer* + *P. chrysogenum* (ACD), also resulted in significant reduction of phorbol ester concentration. Spontaneously fermented (SP) *J. curcas* meal resulted in 53.6% reduction of phorbol ester after fermentation. This implies that preparation of a cocktail of fungi is a more efficient means of degrading phorbol ester in defatted *J. curcas* seed meal. As a result, some researchers have suggested the use of fungi cocktail fermentation to detoxify or reduce phorbol esters in *J. curcas* (Tjakradidjaja *et al*., 2009).

Table 1. Shows the standard concentrations of PMA analyzed and each area under curve at the retention time of 14.4 min

Conc (µg/ml)	Mean of AUC
0	0
3.125	6808.5 ± 659.7
6.25	23555.5 ± 3483.9
12.5	64775.0 ± 1019.6
25	131157.5 ± 942.6
50	284443.0 ± 6307.4

AUC means Area under curve

Table 2. Phorbol ester concentration in fermented *J. curcas* seed meal by various fungi and fungi cocktail

Treatment	Mean (µg/ml)	% Reduction
WS	40.417[a]	
DF	10.9[b]	
A	7.024 [c]	35.6
B	10.712 [b]	1.72
C	6.178 [cd]	43.3
D	2.56 [efg]	76.5
AB	3.861 [defg]	64.5
AC	2.314 [fg]	78.8
AD	3.23 [efg]	70.3
BC	1.456 [g]	86.6
BD	9.407 [b]	13.7
CD	1.456 [g]	86.6
ABC	3.57 [efg]	67.2
ACD	4.105 [def]	62.3
BCD	1.748 [fg]	83.9
SP	5.054 [cde]	53.6

*Means in column with different superscript ([a,b,c,d,e,f,g]) was significantly different at $p<0.05$

A=*Aspergillus flavus*, B=*Aspergillus niger*, C=*Rhizopus stolonifer*, D=*Penicilium chrysogenum*, WS= *Jatropha curcas* whole seeds SP= spontaneously fermented defatted *Jatropha curcas* seed meal, DF= Defatted *Jatropha curcas* seed. AB, AC, AD, ABC, ACD and BCD are combinations of the fungi coded above.

According to Belewu *et al*. (2011), the outcome of cocktail of fungi employed in solid state fermentation of *J. curcas* kernel cake was an appreciable reduction in the anti-nutrient contents of the kernel cake but phorbol esters was not highlighted. This study has shown that a cocktail of two or more fungi is appropriate and effective in detoxification or reduction of phorbol esters in defatted *J. curcas* seeds.

Conclusion

This study has shown the detoxification of JCSM by spontaneous fermentation using mycoflora, within six days. It was observed that *Aspergillus flavus* (A), *Aspergillus niger* (B), *Rhizopus stolonifer* (C), *Penicillium chrysogenum* and *Fusarium* species were associated with the JCSM. Defattening process of *J. curcas* via cold maceration using petroleum ether removed most of the PMA present in the seed meal. However, some amount of PMA present was degraded by the natural fungi associated with the sampled seed meal. *Penicillium chrysogenum* was the most efficient single isolate during detoxification of PMA in JCSM. Cocktails of two or more fungi during JCSM fermentation were efficient in reducing PMA levels. Hence, we recommend that in the detoxification of *J. curcas* seed meal, it is appropriate to use a cocktail of fungi.

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