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Full Length Article

Removal of Phorbol Esters Present in *Jatropha curcas* Kernel by Fungal Isolates

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Abstract

Seed kernel from *Jatropha curcas* L. cannot be utilized as animal feed due to the presence of toxic phorbol esters. However, biological treatments may alleviate the concentration of phorbol esters to a safe level. In the present study, two fungal isolates obtained from garden soil and five endophytes from *Achillea fragrantissima* plant in Saudi Arabia were used for treatments of *J. curcas* kernel. These fungi were identified as *Cladosporium cladosporioides* (isolate TUC9), *Fusarium chlamydosporum* (isolates TUF1, TUF10 and TUF11), *Paecilomyces sinensis* (isolate TUP8) and *Trichoderma harzianum* (isolates TUT1 and TUT2), based on their morphological characteristics and internal transcribed spacer regions sequence analysis. Fungal extracts at 250 µg mL^{-1} of all isolates grown in potato dextrose broth (PDB) did not show cytotoxic effect against both human Chang liver and mouse NIH 3T3 fibroblasts cell lines. Treatment of *J. curcas* kernel in submerged fermentation showed the ability of all isolates to grow in 30 ml PDB supplemented with 14 g ground *Jatropha* kernel (5.6 g dry matter) containing 15.57 mg phorbol esters. The levels of phorbol esters decreased from 2.78 mg g^{-1}dry matter of kernel to 0.06 mg $g^{-1}$of spent substrate (97.8%) after 30 d incubation at 28ºC by *T. harzianum* TUT1. Lipase activity was observed in all fungal isolates, but only *P. sinensis* TUP8, *C. cladosporioides* TUC9 and *F. chlamydosporum* TUF10 showed both lipase and esterase activities. Both enzyme activities were significantly higher ($p<0.05$) in the presence of phorbol esters.

Keywords: *Trichoderma* spp.; Endophytes; Submerged fermentation; Cytotoxicity; Lipase; Esterase.

Introduction

Jatropha (*Jatropha curcas* L.) produces seeds rich in oil and protein. The kernel contains about 60% oil and 30% protein. However, the seed kernel could not be used safely as animal feed due to the presence of phorbol esters that have been recognized as the main toxic compounds (Oskoueian *et al*., 2011). The need for an effective treatment either by physical or biological means to eliminate the phorbol esters is a prerequisite to convert this nutritious material to a useful product, in particular as animal feed.

Specific genera of phylum *Ascomycota* such as *Trichoderma*, *Paecilomyces*, *Cladosporium* and *Fusarium* contain numerous species that have attracted a great deal of attention as non-toxic biological agents. *T. harzianum*, *P. sinensis*, *C. cladosporioides* and *F. chlamydosporum* are inhabitants of nearly every types of soil, decaying wood, compost or other organic matter and plant tissues (Persoh *et al*., 2010). Many of these fungal species are non-toxic and non-pathogenic to humans and animals. The soil treated with *T. harzianum* stimulated plant growth (Harman *et al*., 2004), while endophytic fungal species such as *P. sinensis*, *C. cladosporioides* and *F. chlamydosporum* are non-infectious and are important in both agriculture and industrial applications(Moore *et al*., 2002; Meincke *et al*., 2010; Nalini *et al*., 2005; Sunitha *et al*., 2013).

Many of these fungi produce enzymes to degrade recalcitrant substrates as defense mechanism as well as to gather nutrients for their survival in adverse environmental conditions. Certain enzymes, such as lipase and esterase are involved in degrading a variety of plant natural lipids similar in structures to phorbol esters (Joshi *et al*., 2011). Recently, bacterial and fungal species have been evaluated for their ability to degrade phorbol esters under solid or submerged state fermentation. *Bacillus* spp. could detoxify the toxic and anti-nutritional compounds in *J. curcas* seed cake with submerged fermentation better than solid state (Phengnuam and Suntornsuk, 2013), while *Pseudomonas aeruginosa* PseA strain could degrade phorbol esters completely during solid state fermentation (Joshi *et al*., 2011). Similarly, using solid fermentation, fungal species such *Aspergillus niger* and *Neurospora sitophila* showed the ability to degrade phorbol esters from seed cake of *J. curcas* (Kurniati, 2012). The edible mushroom (*Pleurotus ostreatus*), grown on *J. curcas*

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cake with solid fermentation could reduce the phorbol esters by 99% (Rodrigues da Luz *et al*., 2013), while three white-rot fungi (*Bjerkandera adusta*, *Ganoderma resinaceum* and *Phlebia rufa*) could remove phorbol ester compounds from *J. curcas* in the range of 20–97% under submerged fermentation (Barros *et al*., 2011). Submerged fermentation process could provide microorganisms with soluble nutrients and flowing liquid is more efficient for fungal growth compared to solid state fermentation which results in high degradation of different compounds within a short time (Phengnuam and Suntornsuk, 2013).

Phorbol esters are polycyclic diterpeneswith two hydroxyl groups esterified to fatty acid (Abdel-Hafez *et al*., 2002). Hydrolysis of the ester bond would release the fatty acids from the non-toxic parent compound. Hence, the present study was conducted to evaluate other fungal species, in particular the *Trichoderma* spp. and the endophytes for their ability to remove phorbol esters by producing enzymes involved in phorbol esters hydrolysis.

Materials and Methods

Ripe *J. curcas* L. seeds were supplied by the Malaysian Agricultural Research and Development Institute (MARDI), Serdang. The seeds were dehisced and ground using a laboratory food grinder (Guangzhou Xulang Machinery, China) before used. The ground kernel was air dried at room temperature 25°C for 3 days. The weight of dried ground kernel was recorded.

Fungal Isolates

Fungal isolates were obtained from the Fungi Center, Taif University, Saudi Arabia. Isolates were tentatively identified as two *Trichoderma* spp. from garden soil and five endophytes from *Achillea fragrantissima* plant(common name Lavender cotton). All the isolates were collected in 2010 and stored in potato dextrose agar (PDA) (Bokhari *et al*., 2009).

Morphological Characteristics

Fungal isolates were identified based on morphological characteristics up to the genus level. All isolates were cultivated on PDA to measure the growth and sporulation. The size and pigmentation of colonies were recorded and fungal tissues were studied under the light microscope with lactophenol cotton blue (LPCB) staining for micro morphological characteristics (Najjar, 2007). The size and shape of conidia and phailides were measured. The macro and micro morphological features were compared to the identification key (Pitt and Hocking, 2009).

PCR Amplification of ITS Region

Mandles Andreoti medium 20 mL was prepared in 100-mL Erlenmeyer flasks to cultivate fungal strain for 7 d using anorbital shaker incubator (28°C, 150 rpm). The mycelia were ground with mortar and pestle in liquid nitrogen. The frozen powdered mycelia were then transferred into a 1.5 mL eppendorf microcentrifuge. DNA was extracted using Genomic BYF DNA Extraction Mini Kit (iNtRON Biotechnology, Korea). Polymerase chain reaction (PCR) was conducted to amplify the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) using a thermal Cycler (Peltir, MJ Research PTC-200, INC, USA). The reaction composition (50 µL) contained 3 µL of extracted DNA, 2 µL each of the primers, 25 µL of green PCR mix (Fermentas, Dream Taq^{Tm} Green DNA Polymerase, U.S.) and 18 µL of PCR grade water. The cycling conditions were as follows: an initial denaturation of 2 min at 94°C, followed by 39 cycles at 94°C for 20 s, 40–60°C for 10 s, 72°C for 20 s, and a final extension cycle at 72°C for 5 min. The negative control was prepared with the reaction mixtures in the absence of DNA extract (Luangsa-Ard *et al*., 2005).

DNA Visualization, Quantification and Purification

PCR products were loaded onto 1.5% agarose gel and run for 1 h at 100 V. Electrophoretic gel (OSP-105 electrophoresis, Owl Scientific, Germany) was stained in ethidium bromide and visualized under UV light (versa doc imaging system, Bio-Rad). A standard 100 bp molecular weight DNA marker (Gene Ruler ^{TM} Low range DNA 1 Ladder, Fermentas, U.S.) was used to identify and quantify the PCR product results. Megaquick-Spin^{TM} PCR, Agarose Gel DNA Extraction System (manufacturer protocol iNtRON Biotechnology, Korea) was used to purify the PCR products to obtain clear and sharp bands (Zhang and Yang, 2007).

DNA Sequencing and Analysis

The sequence results from both strands for each PCR product of fungus were assembled using thermo Sequencing-kit by Applied Biosystems 3730 DNA Analyzer. Sequence identities were determined by using BLAST Genbank general databases from Centraalbureau voor Schimmelcultures (CBS) and National center for biotechnology information (NCBI) database (Thompson *et al*., 1997). DNA sequences were aligned first with Clustal X 1.81 TREECON for Windows (version 1.3b, 1998) was used to construct neighbor-joining tree using Jukes-Cantor model (Gherbawy *et al*., 2010).

Cytotoxicity Assay of Fungal Isolates

Two plugs (5-mm) of each isolate grown in PDA were used to inoculate 30 mL PDB and incubated for 7 d (28°C, 150 rpm). After incubation, the fungal cultures were freeze dried under antiseptic condition (-40ºC and vapor pressure of 0.129 mBar). Dimethyl sulfoxide (DMSO) in the ratio 1:100 (v/w) was added to the dried residues to prepare the fungal

extract. A syringe filter (pore size: 22 µm, biofilm, UK) was used to filter the extract. The cytotoxicity activity of fungal extract was evaluated by using Chang liver (human hepatocytes, CCL-13) and NIH 3T3 (Swiss mouse fibroblasts, CRL-1658) cell lines obtained from the American Type Culture Collection (ATCC). The cytotoxicity effect of each extract was compared to the positive control (cells without fungal extract). A 96-well micro-culture plate was used for cell seeding (5×10^3 cells100 μL^{-1}) in Dulbecco's Modified Eagle Media (DMEM) after treatment with 0.25% trypsin. The cells were exposed to the fungal extracts in the range of 7.81 µg mL^{-1} to 250 µg mL^{-1} and incubated in a humidified atmosphere with 5% CO_2 for 24 h at 37°C. The cell viability was determined by thiazolyl blue tetrazolium bromide dye and measured with a microplate reader (spectra max plus plus 384, U.S.) at wave length 570 nm (Shi *et al*., 2010).

Removal of Phorbol Esters in *Jatropha* Kernel by Fungal Isolates

Jatropha kernel was treated by the fungal isolates in submerged fermentation. Thirty mL of potato dextrose broth (PDB) were placed in 250 mL Schott bottle with 14 g of ground *Jatropha* kernel (5.6 g dry matter) containing 15.57 g of phorbol esters. The initial pH of the culture medium was adjusted to 5.5 by using 10 M NaOH and autoclaved. Treatment flasks were inoculated with 2 plugs (5-mm) of each fungal strain grown on PDA for 7 d at 28°C. Uninoculated medium was used as the control. All flasks were placed in an orbital shaker incubator (28°C, 150 rpm) for 30 d. After incubation, the content of each flask was freeze dried, followed by oil extraction with methanol at a ratio of 1:3 (w/v) (Makkar and Becker, 2010). The samples were stirred at room temperature for 15 min and then centrifuged for 8 min at 3200×g to collect the supernatant. The solvent was removed by using a rotary evaporator at 65°C to recover the oil containing the phorbol esters. The oil was weighed and dissolved in 5 mL methanol, followed by filtration with a syringe filter (0.22 µm). The amount of phorbol esters present in the methanolic extract was determined by high-performance liquid chromatography (HPLC) using phorbol-12-myristate 13-acetate (PMA) as the standard. Twenty µL of the samples were loaded on HPLC (Agilent Technologies, Germany), fitted with a reverse-phase C18 column (250 × 4 mm I.D and 5 µm pore size, Agilent Technologies, Germany), and a UV detector. The running conditions were as previously described by Oskoueian *et al*. (2011).

Lipolytic and Esterolytic Activities

Olive oil (C18) and tributyrin (C4) were used as triacylglycerol substrates to detect lipase and esterase activities, respectively (Hasan *et al*., 2009). The seven fungal strains were screened for extracellular hydrolytic enzymes production on PDA plates that contained either commercial olive oil (1% w/v) (Bertolli, Dal) with rhodhamine B (0.001% w/v) to detect lipase, or tributyrin (1% w/v) to detect esterase. Phorbol esters-rich fraction was prepared (Makkar and Becker, 2010) and added (1% w/v) to the PDA medium (treatment). Each fungal strain was first grown in PDA at 28°C for 7 d and one plug (5 mm) was placed at the centre of PDA medium containing the substrates with or without phorbol esters-rich fraction. The lipase and esterase activity of fungal strain was determined by measuring the size of the halo zone after 7 d at 28°C. This study was conducted in triplicates. The halos on tributyrin plates were visualized in normal day light whereas the halos on olive oil plates were observed under UV light, which showed a bright pink fluorescence. An index of relative enzyme activity (REA) was calculated by using the formula (Peterson *et al*., 2009):

REA =Diameter of halo zone /Diameter of colony.

Statistical Analysis

Statistical analysis was conducted by using GraphPad Prism software (GraphPad prism 5 Software, San Diego, USA). SPSS software (2003, version 19.0, USA) was also used toanalyze lipolytic and esterolytic activities data. The mean values for three replicateswere analyzed by General Liner Models in a Complete Randomized Design. Dunnett's Multiple Range test was used for comparingthe mean values of control and treatments at $P<0.05$.

Results

Fungal Identification

Morphological and molecular techniques were used to identify the isolates.All the seven isolates were identified using the morphological reference keys to the genus level. The colony color and diameter were recorded for all fungal isolates. Pigmentations were produced by *Cladosporium* spp. and *Fusarium* spp. isolates No. TUC9, TUF1, TUF10 and TUF11. The microscopic features in terms of the size and length of conidia and phailides are described in Table 1. These isolates were identified to four genera, i.e., *Trichoderma* (two isolates), *Cladosporium* (one isolate), *Paecilomyces* (one isolate) and *Fusarium* (three isolates) according to published morphological keys (Pitt and Hocking, 2009).

Amplification of Fungal Genomic DNA

PCR amplification of rDNA extracted from all seven isolates was conducted with two universal fungal primer pairs: the first pair was ITS1/2 and the second pair was ITS1/4. In each case, intense bands on agarose gel appeared as PCR products in expected size in the range400-550 bp from all seven isolates. Fig. 1 illustrates the PCR profiles of pair 1 ITS1/4 regions amplified from different isolates. The

Table 1: Macro- and micro-morphological characteristics of strains grown on PDA at 28°C for 7 day incubation

Strain codes	Sources	[a] Colony color and diameter (mm)	Conidia length µm	Conidia feature	Phialide length µm	Phialide feature	Genus
TUT1	Soil	Green, 86.3	3.5-4.1	Clusters	8.1-10	Flask - shaped	*Trichoderma*
TUT2	Soil	Whitish-green,88.3	4.2-4.8	Clusters	8.5-10.2	Flask - shaped	*Trichoderma*
TUP8	Plant	Yellow-brown, 80.6	2.0-4.0	Ellipsoidal	12-20	Cylindrical with wide neck	*Paecilomyces*
TUC9	Plant	[b] Grayish-green, 24.0	4.6-5.5	Lemon- shaped	4.0-5.5	Slender	*Cladosporium*
TUF1	Plant	[b] White-pink, 44.6	4-10	Macro and micro	2.0-3.5	Slender	*Fusarium*
TUF10	Plant	[b] Reddish, 45.6	3.5-12	conidia banana-	1.5-3.0		*Fusarium*
TUF11	Plant	[b] Brownish, 48.3	2.5-12.5	shaped septate	2.0-4.5		*Fusarium*

[a] Results of three replicate measurements of cultures grown on PDA medium. [b] strains were produced pigmentations as observed from the reverse side of the agar plate. Genus identification was based on published morphological keys Pitt and Hocking (2009)

Table 2: Identified fungal species used in this study with relationship to the genus or species and the identity percentage found in the CBS (The Central bureau voor Schimmel cultures) website

No.	Isolate Codes	Accession Numbers	Closely related fungal sequence	Identities (%)
1	TUT1	JQ350879.1	*Trichoderma harzianum* JX082390.1	100
2	TUT2	JQ517493.1	*Trichoderma harzianum* FJ459968.1	99.8
3	TUP8	JQ350881.1	*Paecilomyces sinensis* AY857543.1	100
4	TUC9	JQ517491.1	*Cladosporium cladosporioides* JQ910161.1	99
5	TUF1	JQ350882.1	*Fusarium chlamydosporum* GQ505443.1	100
6	TUF10	JQ517492.1	*Fusarium chlamydosporum* GQ505443.1	99.6
7	TUF11	JQ350880.1	*Fusarium chlamydosporum* GQ505443.1	100

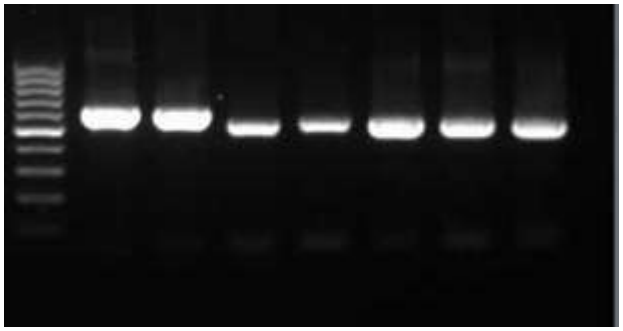

Fig. 1: PCR analyses of seven isolates where TUT1 and TUT2 were identified as *T. harzianum*, TUP8 as *P. sinensis***,** TUC9 as *C. cladosporioides* and TUF1, TUF10 and TUF11 as *F. chlamydosporum***.** All data shown were obtained with ITS1/4 primer. The range of DNA molecular size was from 400 to 550 bp compared to M (100 bp marker)

PCR genetic sequences alignment of isolates were identified as known species based on the 99 – 100 % similarity of their sequences with that of the known species already published NCBI and CBS databases. All seven isolates were identified to species level where TUT1 and TUT2 were *T. harzianum*, TUP8 was *P. sinensis*, TUC9 was *C. cladosporioides*, and TUF1, TUF10 and TUF11 were *F. chlamydosporum*. These isolates were published in GenBank database with the new accession numbers as shown in Table 2.

Phylogenetic Analysis

The variable ITS region sequences of seven isolates was used for the phylogenetic analysis at taxonomic levels according to CBS and NCBI databases. Neighbor-joining tree based on the used primer pair, classified all seven isolates into four clusters according to the group species (Fig. 2). Since *Trichoderma* and *Fusarium* belong taxonomically to the same order (Hypocreales), the strains belonged to the previously mentioned genera constituted one major cluster with 100% bootstrap percentage. IsolateTUP8 clustered with *Paecilomyces* had 100% bootstrap belonged to the order Eurotiales. Also, TUC9 branched to a new cluster of *Cladosporium* with 100% bootstrap and classified to order Capnodiales.

Cytotoxic Activity of Fungal Isolates

In the present study, the fungal species utilized for treatment of *Jatropha* kernel were evaluated for their cytotoxicity activity. The DMSO extract for all the seven fungal cultures did not show any toxic effect on both human (Chang liver) and mouse (NIH 3T3 fibroblast) cell lines even at high concentrations (250 µg mL^{-1})as the percentage viabilities of cells were in the range of 99.5- 99.9 % (Fig. 3). There was no significant difference ($p>0.05$) of cell viabilities among fungal isolates.

Fungal Detoxification of Phorbol Esters

The HPLC analysis showed the amount of phorbol esters in the *Jatropha* kernel before fungal treatments (control) was 2.78 mg PMA equivalent per g dry weight of *Jatropha* kernel. Fungal isolates used in the present study could reduce the levels of phorbol esters significantly ($P<0.05$) as shown in Fig. 4. The phorbol esters level was reduced to less than 0.2 mg g^{-1} in the treatments with *T. harzianum* TUT1, *T. harzianum* TUT2, *P. sinensis* TUP8 and *C. cladosporioides* TUC9, while the reduction of phorbol esters by *F. chlamydosporum* isolates was lower. The percentage value

Table 3: Relative enzyme activity (REA) of seven fungal strains grown on olive oil or tributyrin substrates in the presence of phorbol esters (PEs) extract at 28°C for 7 days of incubation

Strains	Lipase enzyme		Esterase enzyme	
	Olive oil	Olive oil with PEs	Tributyrin	Tributyrin with PEs
*T. harzianum*TUT1	1.19^{ax}±0.02	1.26^{bx}±0.03	ND	ND
T. harzianum TUT2	1.15^{axy}±0.05	1.21^{bxy}±0.01	ND	ND
*P. sinensis*TUP8	1.10^{axy}±0.02	1.18^{bxy}±0.01	1.11^{cx}±0.05	1.32^{dxy}±0.01
C. cladosporioides TUC9	1.14^{axy}±0.02	1.20^{bxy}±0.02	1.24^{cx}±0.03	1.40^{dx}±0.02
*F.chlamydosporum*TUF1	1.11^{axy}±0.03	1.17^{bxy}±0.02	ND	ND
*F.chlamydosporum*TUF10	1.13^{axy}±0.03	1.19^{bxy}±0.06	1.11^{cx}±0.01	1.25^{dy}±0.03
*F.chlamydosporum*TUF11	1.06^{ay}±0.01	1.13^{by}±0.04	ND	ND

Mean values for three replicates ± standard error. ^{a,b,c,d} Different superscripts indicate significant difference ($p<0.05$) within a row for each isolate. ^{x,y} Different superscripts indicate significant difference ($p< 0.05$) within a column for each enzyme. ND: Not detected

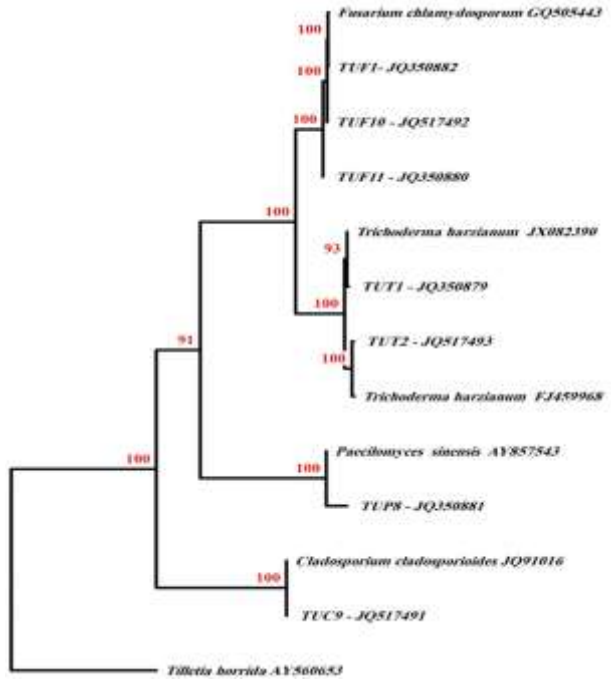

Fig. 2: Neighbor-joining tree based on Internal Transcribed Spacer (ITS) region sequences of the used fungi. Bootstrap percentages from 100 replicates are shown. The tree was rooted with *Tilletia horrid* AY560653 as the out-group

of phorbol esters reduction was highest (97.8%) with *T. harzianum* TUT1 treatment and lowest (86%) with *F. chlamydosporum* TUF11 treatment.

Lipolytic and Esterolytic Activities of Fungal Extracts

Table 3 shows the relative enzyme activity (REA) of fungal isolates for lipase and esterase activities. Areas of fluorescence were observed around the seven fungal colonies in the absence and presence of phorbol esters-rich fraction on olive oil agar plates. However, significantly ($p<0.05$) higher lipase activity was observed for all isolates grown on olive oil with phorbol esters. On the other hand only three isolates i.e., *P. sinensis* TUP8, *C. cladosporioides* TUC9 and *F. chlamydosporum* TUF10 produced esterase activity. Similar to the lipase activity, the presence of phorbol esters significantly ($p<0.05$) induced esterase activity.

Discussion

The overlapping resemblance of macro and micro morphological characteristics may cause misidentification of fungi to species level (Gherbawy *et al*., 2010). Hence, molecular techniques based on ribosomal DNA fragment amplification and sequencing by ITS universal primes were conducted to complete the identifications to species level. It has been stated that using PCR technique to amplify rDNA-ITS region was a common approach to identify fungi up to species or strain level. The molecular size of the ITS region amplifications of *T. harzianum*, *P. sinensis*, *C. cladosporioides* and *Fusarium* spp. were 650 bp, 537 bp, 350 bp and 389 bp, respectively, in agreement with previous reports (Chen *et al*., 2001; Moore *et al*., 2002; Abd-Elsalam *et al*., 2004; Meincke *et al*., 2010). Phylogenetic analysis indicated the genus of *Paecilomyces* was generally thought to be the anamorphic (asexual) state of the insect parasite *Cordyceps sinensis* (family Clavicipitaceae, order Hypocreales) until the molecular evidence demonstrated that *P. sinensis* was in fact completely unrelated to *C. sinensis*, and was instead in close phylogenetic proximity to *P. variotii* in the family Trichocomaceae (Chen *et al*., 2001). Owing to the phylogenetic species concept, which defines species as the diagnosable taxonomic unit with a clear pattern of parental ancestry, we interpret this conspicuity as species identity. All data shown were obtained with rDNA-ITS sequences of the seven isolates and could be grouped into the phylum *Ascomycota*.

Fungal cytotoxicity is a major concern regarding fungal treatment of feed materials. Many fungal isolates have been reported to be harmless to human and animal cell lines. It has been reported that *Trichoderma* sp. extract did not impair cell viabilities of hepatocellular Chang human cell line at 40 µg mL^{-1} concentration (Shi *et al*., 2010). Similarly, *C. cladosporioides* fungus was non-toxic to mice exposed to high loads of spore indicated by the absence of histological changes of the lung structure (Flemming *et al*., 2004). Furthermore, human epithelia cell line was resistant to *Fusarium* sp. toxin (Calvert *et al*., 2005). Another study conducted (Toledo *et al*., 2012) suggested the safe used of *Paecilomyces* sp. against NIH 3T3 mice fibroblast cell as the fungal species did not produce any toxin in the culture medium. It has also been reported that *P. sinensis* showed positive effects on several pharmacological studies including antitumour, estrogenicity and anti-oxidation activities (Chen *et al*., 2001). It is worth

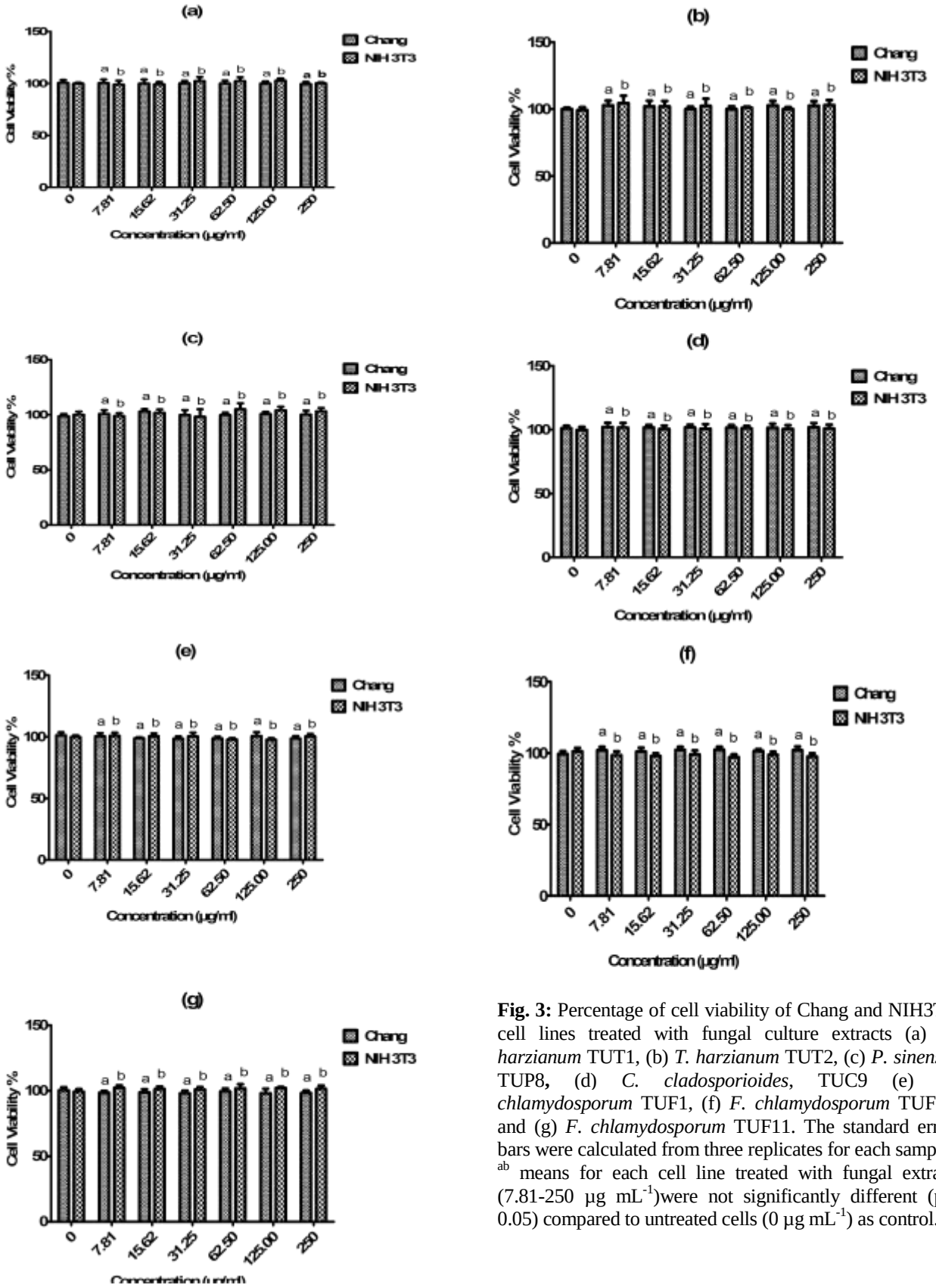

Fig. 3: Percentage of cell viability of Chang and NIH3T3 cell lines treated with fungal culture extracts (a) *T. harzianum* TUT1, (b) *T. harzianum* TUT2, (c) *P. sinensis* TUP8**,** (d) *C. cladosporioides*, TUC9 (e) *F. chlamydosporum* TUF1, (f) *F. chlamydosporum* TUF10 and (g) *F. chlamydosporum* TUF11. The standard error bars were calculated from three replicates for each sample. [ab] means for each cell line treated with fungal extract (7.81-250 µg mL^{-1})were not significantly different ($p > 0.05$) compared to untreated cells (0 µg mL^{-1}) as control.

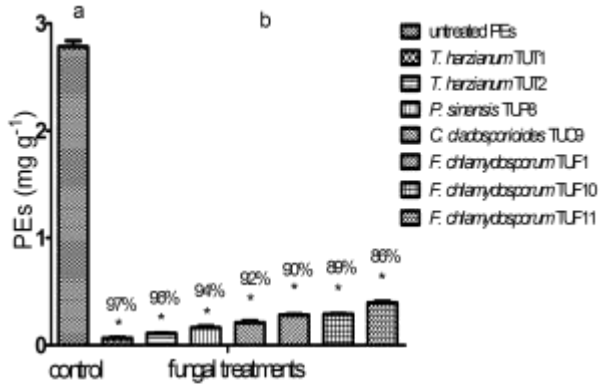

Fig. 4: Phorbol esters (PEs) in mg g^{-1} of spent substrate after 30 d incubation at 28°C with different fungal strains. The standard error bars were calculated from three replicates for each sample. (a) Untreated phorbol esters (control), (b) Fungal treated phorbol esters (treatments). All the treatment means were significantly ($p<0.05$) different compared to the control

noting that Chang liver cell from human show similar sensitivity to NIH 3T3 fibroblast cell from mouse to fungal extracts, indicating the biological similarity between the two cell lines. The subtropical soil and plant revealed promising source of fungi that are rarely considered and poorly understood as biological agents (Klich, 2002).

The amount of phorbol esters in the *Jatropha* kernel was close to the value of 3 mg PMA equivalent per g of Malaysian *Jatropha* meal (Oskoueian *et al*., 2011). A slight difference could be due to the samples determined. Oskoueian *et al*. (2011) analysed the defatted sample, whereas, in the present study, the seed kernel was used. Different levels of phorbol esters have been observed in *Jatropha* seed cake (defatted kernel). Brazilian *Jatropha* seed cake showed low level of phorbol esters (0.82 mg g^{-1}) (Barros *et al*., 2011), while Indian *Jatropha* seed cake contained high level (5.45 mg g^{-1}) (Roach *et al*., 2012). This variation could be attributed to a number of factors including different geographical regions, extraction methods, types of solvents and extraction conditions (Devappa *et al*., 2012). In the present study, the amount of phorbol esters in seed kernel after treatment with *T. harzianum* TUT1 was 0.06 mg g^{-1} of dried spent substrate, nearly half the amount of phorbol esters at 0.11 mg g^{-1} of the nontoxic Mexican *J. curcus* (Rodrigues da Luz *et al*., 2013). According to these researchers, humans and chickens in Mexico consume the non-toxic *Jatropha* kernel without any hazardous effects.

The fungal isolates in this study were able to remove the phorbol esters from *Jatropha* kernel to varying degrees. Similarly, other fungal species including *A. niger, Penicillium chrysogenum, Rhizopus oligosporus, R. nigricans* and *T. longibrachitum* could detoxify phorbol esters and other anti-nutritional components present in *J. curcas* kernel cake (Belewu and Sam, 2010). On the other hand, fungal species such as *A. flavus, Botrytis cinerea, F. oxysporum, F. moniliforme, Curvularia lunata* and *Penicillium notatum* were susceptible to phorbol esters rich fraction, indicating the fungicide effect of these compounds. This latter finding demonstrates the possibility of using phorbol esters rich fraction as natural antifungal product in agricultural practices (Devappa *et al*., 2012).

It is known that *Trichoderma* spp. and endophytes in particular exist in mutualistic association with the host plant, producing various enzymes as their defense mechanisms (Nalini *et al*., 2005). It was therefore speculated that these endophytes as well as the *Trichoderma* spp. could produce enzymes for neutralizing toxic compounds like phorbol esters or for exploiting these compounds as nutrients for growth. The use of solid media supplemented with emulsified olive oil (C18, long chain triglycerides) or tributyrin (C4, short chain triglycerides) is specific for the detection of lipase and esterase activities. Lipase catalyzes the hydrolysis of relatively long and short chain triacylglycerols, but esterase only catalyze the reactions involving short chain triacylglycerols (Chul-Hyung *et al*., 2011). These enzyme activities are theoretically regarded as important natural agents to remove phorbol ester compounds from *Jatropha* kernel (Barros *et al*., 2011). In the present study *T. harzianum* only produce lipase but showed the highest percentage of phorbol esters removal. The presence of phorbol esters stimulated the production of lipase enzyme indicating the potential of *T. harzianum* TUT1 and TUT2 in removing phorbol esters from *Jatropha* kernel. This result also indicates that *Jatropha* kernel contains phorbol esters with long chain fatty acids. On the other hand, the other fungal species produce both lipase and esterase but showed lower percentage of phorbol esters removal, probably due to lower lipase activity. The results also indicate that esterase is not a major detoxifying enzyme for the phorbol esters present in the local *Jatropha* kernel. The high lipase activity in *T. harzianum* TUT1 resulted in the highest percentage of phorbol esters removal when compared to other fungal isolates.

In conclusion, Saudi Arabian fungal isolates: *T. harzianum* TUT1, TUT2, *P. sinensis* TUP8, *C. cladosporioides* TUC9, *F. chlamydosporum* TUF1, TUF10 and TUF11 were identified by morphological characteristics and molecular technique. These isolates showed high ability to remove phorbol esters from *J. curcas* kernel in the range of 86% to 97%. The fungal isolates did not have toxic effects on human and animal cell lines and produced enzymes to break down the phorbol esters that made them potential candidates for biological treatments of phorbol esters in *Jatropha* kernel. *Jatropha* kernel or *Jatropha* meal after fungal treatment would nevertheless be a good source of protein and with its low fiber content, may become an alternative feed ingredient for poultry.

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