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Mode of action of *Jatropha curcas* phorbol esters in bovine kidney cells

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ABSTRACT

Jatropha meal is a potential biofeed for animal production however, the presence of phorbol esters (PEs) in the meal limits its utilization. The PEs caused severe toxic manifestations in animals. The information on the mechanisms of toxicity at the cellular level is rather limited. Therefore, this research was conducted to investigate the mode of action of PEs by evaluating the biochemical, biological and molecular responses of cells exposed to PEs. Phorbol esters from Jatropha meal were isolated and identified as Jatropha factor (JF) JFC1, JFC2, JFC3 and a mixture of JFs (C4, C5 and C6). These PEs exhibited cytotoxic activity towards bovine kidney cells and induced significant morphological changes. The PEs severely altered the redox status of the cells which resulted in the occurrence of oxidative stress. Moreover, the PEs up-regulated the expressions of PKC-βII, proto-oncogenes, pro-inflammatory cytokines and elevated the caspase-3 activity in the cells. All PEs were toxic, but JFC1 and JFC2 appeared to be more toxic to the bovine kidney cells as compared to the JFC3 and the mixture of JFs. The results indicated that the cytotoxic activity of Jatropha meal PEs was elicited through induction of oxidative stress and inflammation of the cells.

1. Introduction

Jatropha curcas Linn. (*J. curcas*) is a multipurpose plant with significant economic importance due to its several industrial and medicinal uses. The seed kernel contains about 40–60% oil which meets the American and European biodiesel standards (Devappa et al., 2012a). The global *J. curcas* planted area increases to 9×10^5 ha by the end of 2008 and Asia, Africa and Latin America, each possessed 85%, 13% and 2% of these plantation areas, respectively. Due to the importance of *J. curcas* kernel as a renewable source of biodiesel, the plantation area is predicted to increase up to 13 million hectares under cultivation worldwide by 2015 (Bayen et al., 2016).

Jatropha meal is produced during the process of kernel oil extraction. Previous studies on the chemical composition and biological activity of Jatropha meal showed the presence of 58–60% crude protein and bioactive phytochemicals with antioxidant, anti-tick, antibacterial, antifungal and antitumor properties (Makkar, 2016). The high protein content and the presence of beneficial phytochemicals such as gallic acid, pyrogallol, rutin, myricetin and daidzein with antioxidant and antimicrobial activities rendered Jatropha meal as a potential biofeed for animal production (Makkar, 2016; Oskoueian et al., 2011). However, the presence of anti-nutritional factors, mainly lectin, trypsin inhibitors and phorbol esters limits its utilization as an animal feed (Makkar, 2016). Several feeding trial experiments conducted in different animal species, including goats, sheep, mice, rats, and fish, indicated the severity of the manifestations, where the animals showed symptoms of toxicity which caused mortality when fed Jatropha meal containing phorbol esters (Harter et al., 2011; Katole et al., 2011; King et al., 2009; Li et al., 2010; Makkar, 2016; Rakshit et al., 2008). The animals suffered lack of appetite, diarrhea and dehydration, as well as changes in blood biochemical parameters. An investigation of their visceral organs revealed hemorrhage in the gastrointestinal tract, kidneys, spleen and heart. Other symptoms include enteritis, congestion and edema of the lung, excessive fluid in serous cavities and the presence of pathological symptoms mainly in kidney tissue (Ahmed and Adam, 1979; Katole et al., 2011; Li et al., 2010; Rakshit et al., 2008).

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It was suggested that phorbol esters present in the Jatropha meal were the main bioactive compounds responsible in eliciting these manifestations (Ahmed and Salimon, 2009; Devappa et al., 2010a). In fact, the biological activities of phorbol esters are dependent on their structures and determined by their functional groups. Thus, different phorbol esters activate different protein kinase C (PKC) isozymes in the cells, which could trigger different pathways and may cause different symptoms in animals (Goel et al., 2007). That is why, some naturally occurring phorbol esters possess the ability to inhibit cells prolifration (Abdel-Hafez et al., 2002; Lee et al., 2009; Muanda et al., 2010), while others increase cell prolifration, induce inflammation leading to the tumor formation (Ahmad et al., 2013; Goel et al., 2007). For instance, phorbol 12-myristate 13-acetate found in coroton oil appeared to be a potent tumor promoter (Goel et al., 2007). Although there have been various reports dealing with the toxicity evaluations of the Jatropha meal using different animal models, but the mode of action of phorbol esters present in Jatropha meal is still not well understood. Thus, this study was performed to elucidate the mode of action of Jatropha phorbol esters through biochemical and molecular evaluations using animal cell model.

2. Material and methods

2.1. Jatropha meal preparation

The ripe *J. curcas* seeds were collected from the farm of the Faculty of Agriculture, Universiti Putra Malaysia (GPS location 3°0′26.91″N latitude and 101°42′13.24″E longitude) and identified by Mr. Shamsul Khamis. A voucher specimen (SK1764/2010) was deposited in the Phytomedicinal Herbarium, Institute of Bioscience, Universiti Putra Malaysia. The seeds were air dried and dehulled. The kernels isolated were ground using a mechanical grinder, and the oil was extracted by a Soxhlet apparatus, using petroleum ether (boiling point of 40–60 °C) for 16 h to obtain the meal.

2.2. Reagents

3,4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (Sigma Germany). Eagle's minimal essential medium (EMEM), trypsin-EDTA, and fetal bovine serum (FBS) were purchased from Calbiochem (San Diego, CA). RNeasy Mini Kit and cDNA Synthesis Kit were from Qiagen GmbH, Hilden, Germany, while the i-StarTaq DNA polymerase kit was obtained from iNtRON Biotechnology, Sungnam, Kyungki-Do, Korea. SeaKem® GTG® agarose was purchased from FMC BioProducts, Rockland ME, USA. Loading dye and DNA ladder (100 bp, 1Kb) were from MBI Fermentas, Lithuania and SYBR Green Supermix was purchased from Bio-Rad Laboratories, Hercules, CA, USA.

2.3. Isolation of phorbol esters

The phorbol esters were extracted from Jatropha meal according to the method described earlier by Makkar and Becker (2007). Briefly, 5 g of Jatropha meal was ground in a pestle and mortar with 200 mg of acid washed sand. The 20 mL of dichloromethane was added in the pestle and the mixture was ground for 5 min with the mortar. The material was allowed to settle and the liquid phase was filtered using a filter paper (No.1). The Jatropha meal residue was extracted in the same manner for three more times. The filtrates were pooled and the dichloromethane was evaporated using a rotary evaporator at the temperature not exceeding 40 °C. The crude extract was dissolved in methanol and used for isolation. Phorbol esters from Jatropha meal were isolated by using high-performance liquid chromatography (HPLC) Waters 2690 separation module equipped with Waters photodiode array detector 2996 and computer with millennium 32 software as described earlier (Oskoueian et al., 2011). The column was chromolith semiprep column, RP-18 endcapped 100–10 mm (Merck, Germany).

A solvent gradient consisting of water and aqueous acetonitrile (85%) was used. Four fractions containing the phorbol esters which were detected at 14.81, 15.46, 16.34 and 16.71 min were collected individually using a fraction collector (Waters, Milford, MA, USA) at the above retention times and named Jatropha factor (JF) JFC1, JFC2, JFC3 and JFs (JFC4 + JFC5 + JFC6 mixture). The collected fractions were freeze dried and dissolved in dimethyl sulfoxide (DMSO) and re-analysed using HPLC to confirm the purity and to determine the concentration. The proportion of individual peak to the total area of all phorbol esters was calculated. The concentration of the isolated phorbol esters used in this study was expressed as equivalent to a standard, phorbol-12-myristate 13–acetate (PMA).

2.4. Cell culture and maintenance

In this study, the kidney cell line was chosen, as earlier reports in animals fed Jatropha meal showed prominent pathological symptoms mainly occurred in the kidney (Katole et al., 2011; Li et al., 2010; Oskoueian et al., 2012b; Rakshit et al., 2008). Bovine kidney cell line (MDBK) was purchased from the American Type Culture Collection (ATCC® Number: CCL-22™). The MDBK cell line was prepared from a kidney of a normal adult steer. The cells were maintained in EMEM containing 2 mM L-glutamine, 1 mM sodium pyruvate, sodium bicarbonate (1500 mg/L) and horse serum to a final concentration of 10%. The cells were sub-cultured at a ratio of 1:2–1:4, twice per week. The cell sheet was rinsed twice with PBS to detach the cells, and the PBS was removed. The flask was kept at 37 °C for 15 min and then the fresh medium was added, aspirated, and dispensed into new flasks. For the cryopreservation, fetal bovine serum containing 5% DMSO was used.

2.5. Cytotoxicity test (MTT assay)

The cytotoxic effect of the isolated phorbol esters was determined using the MTT assay (Sharif et al., 2008). The MDBK cells were seeded at a density of 5×10^3 cells per 100 µL in the 96-well plate and incubated for 24 h. The cells were treated with different concentrations of phorbol ester fractions consisting of JFC1, JFC2, JFC3 and JFs mixture, while the concentration of DMSO was kept at 0.1%. After treatment for 24, 48 and 72 h, the supernatants of the 96-well plate were replaced with fresh media and 20 µL of MTT reagents (0.05 mg/mL) were added into each well and incubated at 37 °C. After 4 h, the spent media were removed and the formazan salts were dissolved by adding 100 µL of DMSO (100%). The absorbance was then measured at 570 $_{nm}$ on a SpectraMax Plus Microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA). The percentage of cell viability was calculated by using the following formula:

$$Cell\ viability\ (\%) = [(OD_{sample})/(OD_{control})] \times 100$$

The concentration required to reduce cell growth or viability by 50% was reported as 50% cytotoxic concentration (CC_{50}).

2.6. Microscopic examination

The cells were seeded at a density of 3×10^5 cells per well in 6-well plates, and allowed to adhere overnight. After 24 h incubation, the medium was removed and fresh media containing different phorbol ester fractions and PMA as a positive control at the CC_{50} concentration were added. The treated cells were then incubated at 37 °C in 5% CO_2 for 24 h. Morphological changes were examined after 24 h incubation, and the cells photographed using an inverted microscope (Nikon; Tokyo; Japan).

2.7. Intracellular antioxidant enzymes activity

The intracellular enzymes activities including catalase (CAT),

superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined calorimetrically using commercial kits (Cayman Chemical, USA) according to the manufacturer's instruction. Briefly, the MDBK cells were seeded at a density of 4×10^4 cells per well in 24-well plates, and allowed to adhere overnight. The medium was removed and fresh media containing different phorbol ester fractions and PMA as a positive control at the CC_{50} concentrations were added. The treated cells were then incubated at 37 °C in 5% CO_2 for 12 h. Then the cells were washed twice with PBS, trypsinized and centrifuged (3000$g \times$ 10 min, 4 °C) to harvest the cells. The cells were lysed by using sonication (5X, 30 s, 4 °C, 50 W, Hielscher-Ultrasonic Gmbh, Germany) in the buffer provided in the kit. The buffer contained 70 mM sucrose, 210 mM mannitol, 1 mM ethylene glycol tetraacetic acid, 20 mM HEPES, pH 7.2. The cell lysate was centrifuged at 10,000 $\times g$ for 20 min at 4 °C, and the clear supernatant was collected for the enzyme activity assay. The enzyme activity was expressed in mili units per mg of protein (mU/mg protein) and results were reported as percentage of the negative control.

2.8. Cellular total antioxidant activity

The cellular antioxidant activity of MDBK cells were determined as described by Dubey et al. (2015). The MDBK cells were seeded and treated as mentioned earlier. At the end of 12 h incubation, the cells were rinsed twice with PBS (pH 7.4), scraped and sonicated in potassium phosphate buffer (0.1% glucose, 0.9% NaCl, 5 mM, pH 7.4). The lysate was centrifuged at 10,000 $\times g$ for 15 min at 4 °C. The cellular antioxidant activity was determined by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) oxidation using a commercial total antioxidant assay kit (Cayman Chemical Company, USA, Cat. No. 709001). The analysis was conducted according to the protocol given by the kit. The cellular total antioxidant activity of each treatment was reported as percentage of the negative control.

2.9. Lipid peroxidation assay

The lipid peroxidation of MDBK cells as an indicator of oxidative stress was determined by measuring malondialdehyde (MDA) using thiobarbituric acid reactive substance (TBARS) assay (Oskoueian et al., 2014). The MDBK cells were seeded and treated as mentioned earlier. At the end of 12 h incubation, the cells were then rinsed with PBS buffer, scraped and transferred into 4 mL ice-cold potassium chloride (1.15%) and homogenized with an Ultra-Turrax homogenizer (Wilmington, USA) at 18,000 RPM for 30 s. An aliquot of 200 µL of homogenized cells, 300 µL distilled water, 35 µL butylated hydroxytoluene, 165 µL sodium dodecyl sulphate (SDS) and 2 mL thiobarbituric acid (TBA) were added into the screw cap glass tubes. The tubes were then heated for 60 min at 90 °C, cooled and immediately added with 3 mL of n-butanol. The tubes were centrifuged at 3000 $\times g$ for 10 min and the absorbance of n-butanol fraction was measured at 532 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA). The extent of cellular lipid peroxidation in each treatment was reported as percentage of the negative control.

2.10. Reactive oxygen/nitrogen species production

The intracellular and extracellular reactive oxygen spices/reactive nitrogen species (ROS/RNS) were measured by using OxiSelect™ *In Vitro* ROS/RNS Assay Kit (cat no. STA-347; Cell Biolabs, CA, USA) according to the manufacturer's instruction. Initially, the MDBK cells were seeded and treated as mentioned earlier. At the end of 12 h incubation, the cells were scraped and 4 mL of culture medium containing scraped cells was transferred to 15 mL tube, kept on ice and homogenized with an Ultra-Turrax homogenizer (Wilmington, USA) at 18,000 RPM for 30 s. The lysate was centrifuged at 10,000 $\times g$ for 15 min at 4 °C and then ROS/RNS was measured in the supernatant using a florescence micro-plate reader (Molecular Devices, Sunnyvale, CA) at 480 nm (excitation) and 530 nm (emission).

2.11. Gene expression analyses

The MDBK cells were seeded at a density of 1$\times 10^6$ cells per 75 cm^2 flask and cultured for 24 h in EMEM. After 24 h incubation, the medium was removed and fresh media containing a different phorbol ester fractions at the CC_{50} concentration were added. The treated cells were then incubated at 37 °C in 5% CO_2 for 12 h. The RNA of the MDBK cells was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the protocol recommended by the manufacturer. The quality and quantity of the extracted RNA was determined (Oskoueian et al., 2012a). For cDNA synthesis, the reverse transcriptase PCR (RT-PCR) was performed using Maxime RT Premix kit (iNtRON Biotechnology, Sungnam, Korea) according to the protocol given by the manufacturer. To design primers, the full sequence of bovine genes were obtained from NCBI GenBank with the accession number of PKC-β II (NM_174587), c-myc (NM_001046074.2), c-Jun (NM_001077827.1), c-Fos (AY322482.1), Cox2 (NM_174445.2), IL-1 beta (NM_174093.1), Beta-actin (NM_173979.3) and GAPDH (NM_001034034.1) and the primers were designed using Primer 3 software (Untergasser et al., 2012). The specificity of the primers was verified using BLAST (Altschul et al., 1997) and further confirmed by conventional PCR using cDNA from bovine kidney cell.

The real-time PCR assays were conducted on a BioRad CFX 96 real-time PCR thermocycler (Bio-Rad, Hercules, USA) using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to analyse the gene expression. cDNA sample (1 µL) was used in a reaction mixture containing 12.5 µL of iQ SYBR Green Supermix, 1 µL forward and 1 µL reverse primers (10 pmol/µL) and 9.5 µL nuclease free water. The primers used are presented in Table 1.

Temperature gradient curves were analysed to determine the optimal annealing temperature for each set of primers. The optimized PCR reaction conditions for PKCβ II and Cox2 were as follows: 94 °C for 5 min (1X), then 94 °C for 20 s, then 60 °C for 30 s and 72 °C for 35 s (40X). The PCR reaction conditions for c-Fos, c-Jun, c-Myc, GAPDH and β-actin genes were as follows: 94 °C for 5 min (1X), then 94 °C for 20 s, then 60 °C for 20 s and 72 °C for 25 s (40X). Fluorescence detection was performed at the end of each denaturation and extension step. A dissociation curve was generated at the end of the assay to analyse the specificity of the amplicon. The dissociation curve was generated by slow heating at temperatures from 70 °C to 95 °C at a rate of 0.2 °C/s

Table 1
Characteristics of the PCR primer sets used in the gene expression.

Targeted gene	Forward	Reverse	Amplicon size (bp)
PKC-β II	5′-cacgaggtgaagaaccacaa-3′	5′-cagacttggcactgaaatcct-3′	116
c-Myc	5′-agcagcaaaagctcaagtca-3′	5′-ggtcaatttaggcgcaaga-3′	102
c-Jun	5′-gaactccgacctcctcacc-3′	5′-ccgttgctggactgtatgatt-3′	90
c-Fos	5′-tacagcccaccctagtctcc-3′	5′-tctgcctcctgtcatggtt-3′	128
β-actin	5′-gccaaccgtgagaagatga-3′	5′-aggcatacagggacagcac-3′	94
GAPDH	5′-gatgctggtgctgagtatgtg-3′	5′-ggcagaaggtgcagagatg-3′	114

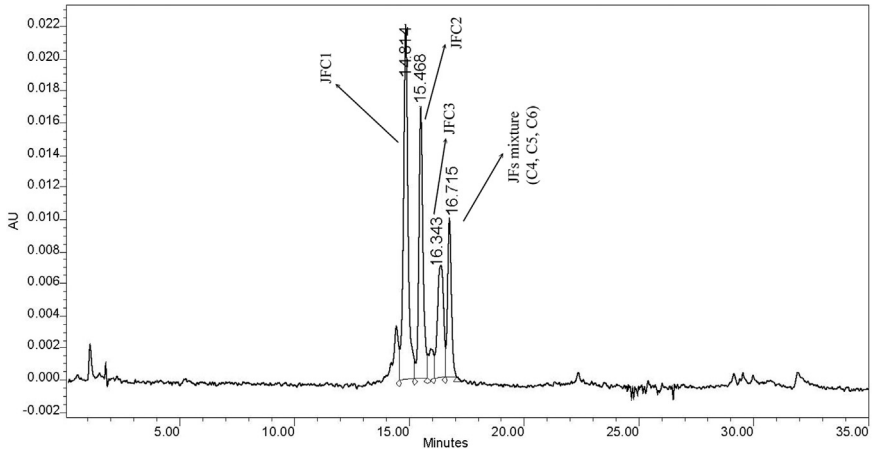

Fig. 1. The HPLC chromatogram of phorbol esters from Jatropha meal. JFC_1: Jatropha factor C1, JFC2: Jatropha factor C2, JFC3: Jatropha factor C3 and JFs (mixture of Jatropha factors C4, C5 and C6).

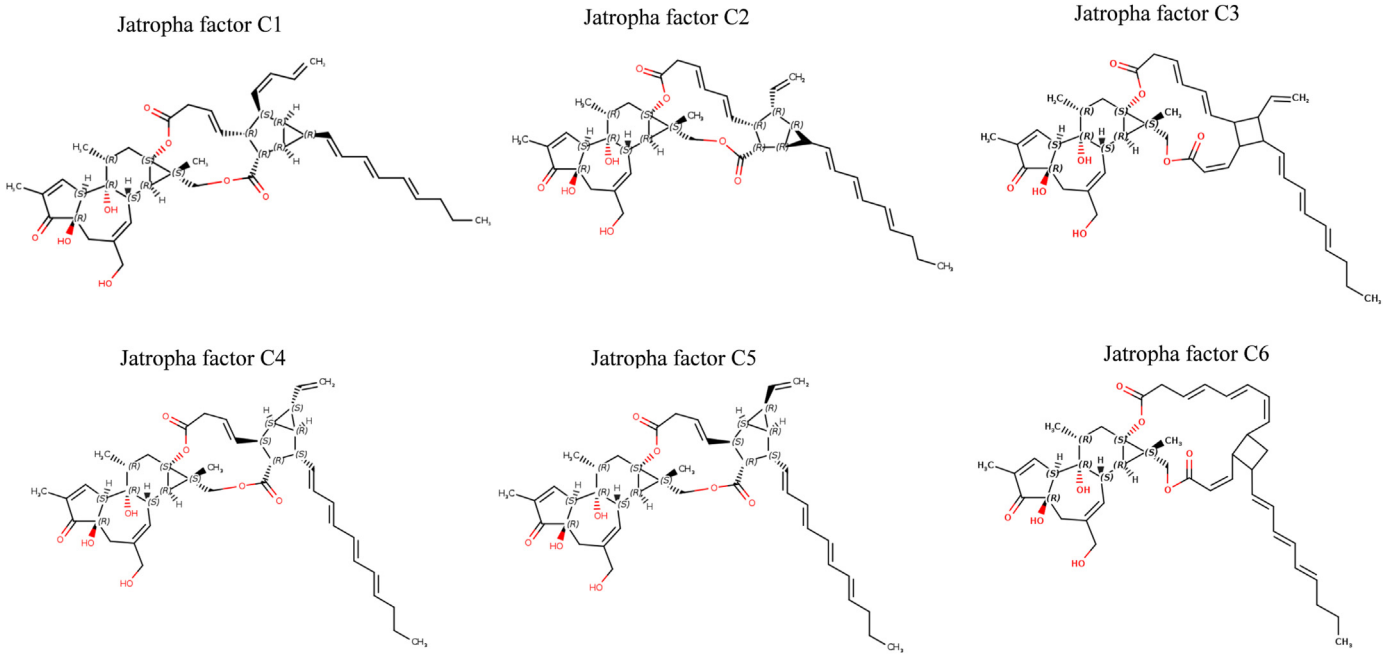

Fig. 2. The phorbol esters present in Jatropha meal (Devappa et al., 2012a; Haas et al., 2002; Hirota et al., 1988).

Table 2
Cytotoxic effects of isolated phorbol esters from Jatropha meal on bovine kidney (MDBK) cell line.

Incubation time	Cytotoxic concentration (CC_{50})[a,b] µg/mL					
	JFC_1	JFC_2	JFC_3	JFs (C_4, C_5,C_6)	PMA[c]	SEM
24 h	93.6[b]	89.0[b]	109.6[a]	108.0[a]	72.8[c]	2.54
48 h	79.8[b]	75.9[b]	92.3[a]	91.4[a]	60.1[c]	2.39
72 h	66.8[b]	63.8[b]	76.6[a]	75.8[a]	52.4[c]	2.68

All values represent the means from three independent experiments.
JFC_1: Jatropha factor C1, JFC2: Jatropha factor C2, JFC3: Jatropha factor C3 and JFs (mixture of Jatropha factor C4, C5 and C6).
Means with different superscripts within a row are significantly ($p < 0.05$) different SEM: Standard error of the mean.
[a] CC_{50}: Concentration of sample at the 50% cell viability.
[b] Equivalents to phorbol 12-myristate-13-acetate.
[c] PMA: phorbol 12-myristate-13-acetate.

with continuous acquisition of fluorescence. Standard curves were constructed using PCR products obtained upon amplification of targeted genes (PKC-β II, c-Myc, c-Fos, c-Jun, GAPDH and β-actin) and using the cDNA of the MDBK cell line as template. The concentration of each PCR product was determined by spectrophotometry (Nano-Drop Technologies, Wilmington, DE, USA). A tenfold dilution series ranging from 10^3 to 10^8 copies of the PCR product carrying the targeted gene was prepared by using nuclease free water. Dilutions of the PCR products were freshly prepared for each experiment. The PCR efficiency result of each standard curve was used for the normalization of targeted genes. The expression of the studied genes were normalized to those of β-actin and GAPDH gene expressions according to Vandesompele et al. (2002). Data from the real-time PCR reactions were analysed using CFX manager software version 2 (Bio-Rad Laboratories). All real-time PCR amplifications were performed in triplicate.

2.12. Protein expression analyses

The Western Blot Technique was applied to evaluate the expressions of proteins including cyclooxygenase 2 (Cox2), interleukin 1 beta (IL-1

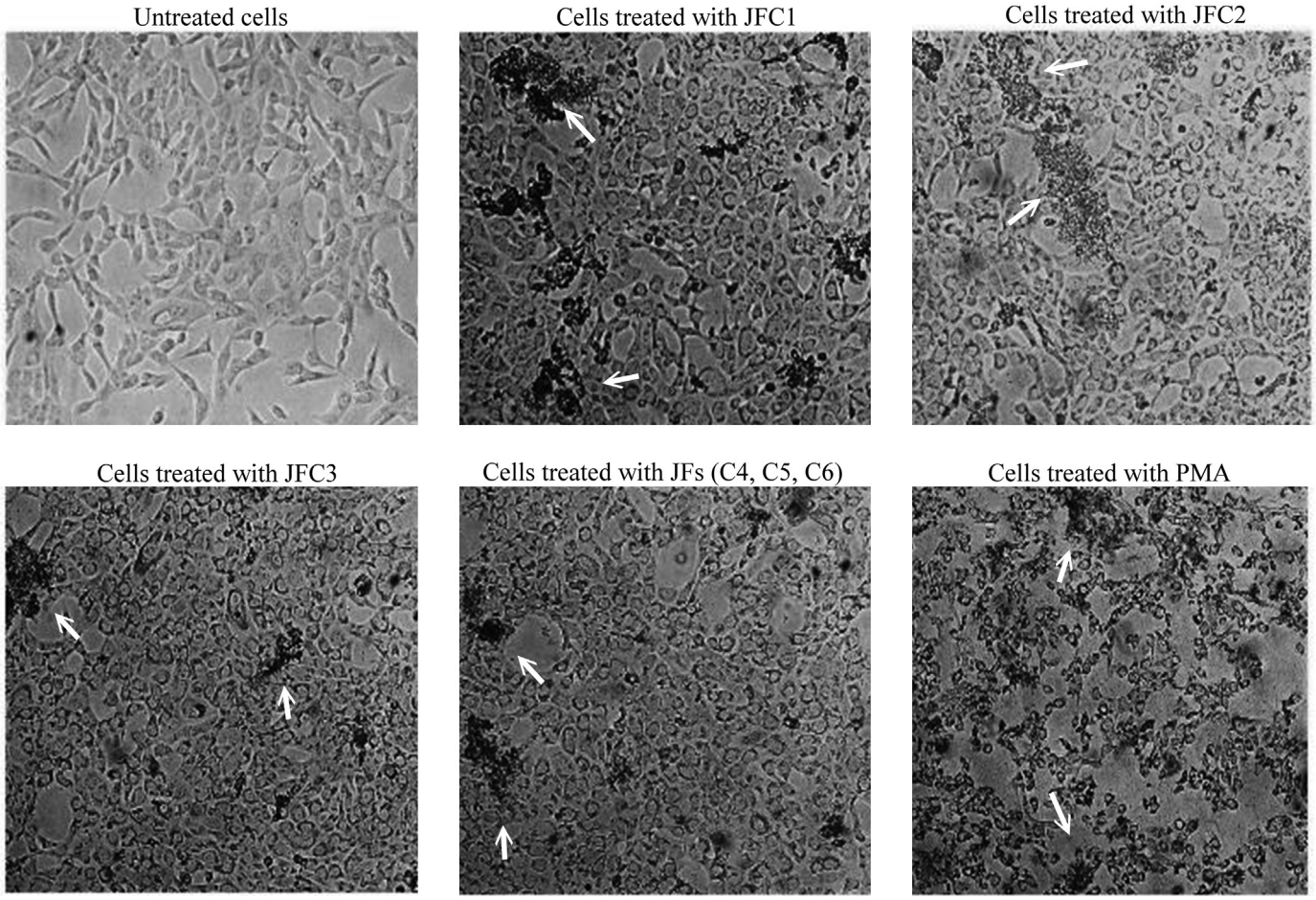

Fig. 3. Morphological characteristics of the MDBK cells upon treatment with phorbol esters [JFC1 (Jatropha factor C1), JFC2 (Jatropha factor C2), JFC3 (Jatropha factor C3) and JFs (mixture of Jatropha factor C4, C5 and C6)] and PMA at CC_{50} concentration after 24 h incubation examined by light microscopy at 200X magnification. Arrows indicate apoptotic bodies (dark regions).

β) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in MDBK cells exposed to Jatropha meal phorbol esters. The MDBK cells were seeded at a density of 1×10^6 cells per 75 cm^2 flask and treated as mentioned earlier. At the end of 12 h incubation, the cells were then rinsed with PBS buffer and protein was extracted (Oskoueian et al., 2014). The concentrations of protein extracted from the treated cells were determined using Protein Assay kit (Bio-Rad, CA, USA). The extracted protein (20 μg) from each treatments were denatured by incubation at 95 °C for 5 min and then analysed using electrophoresis by applying Tris-glycine polyacrylamide gel. The Hoefer Semi-Dry Transfer Unit (Hoefer Instruments, CA, USA) was used to transfer the proteins to the polyvinylidene difluoride (PVDF) membrane and proteins were blocked using Odyssey Blocking Buffer (LI-COR, Lincoln, NE, USA).

After protein transfer, the blocking buffer (Odyssey, LI-COR, Lincoln, NE, USA) was applied to wash the membrane. The membrane was then soaked overnight with the primary antibodies including IL-1β (Santa Cruz sc-12742; 1:500 dilution), COX-2 (Santa Cruz sc-376861; 1:500 dilution) and GAPDH (Thermo Scientific MA1–4711; 1:1000 dilution). The membrane was washed with 0.05% PBST (phorsphate buffer saline and tween 20) three times for 5 min. To view the bands using Odyssey imaging system, a 1:10000 dilution of the IRDye 800 CW Goat Anti-Rabbit Secondary Antibody or IRDye 680 Goat Anti-Mouse Secondary Antibody was used. The membrane was washed the 0.05% PBST for 5 min and repeated three times. The membrane was dried and visualized using Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA) and Odyssey software was used to determine the intensity of the protein bands. The expressions of targeted proteins were normalized to that of GAPDH protein expression (Oskoueian et al., 2012a).

2.13. Caspase-3 activity assay

The caspase-3 activity was determined in MDBK cells using Caspase-3 colorimetric assay kit (Abcam, ab39401, Cambridge, UK) according to the instructions provided by the kit. Briefly, MDBK cells were cultured and treated with isolated phorbol esters at the CC_{50} concentration for 24 h. Then, the cells were trypsinized, washed with PBS and lysed with lysis buffer and sonicated as mentioned in the protein expression analyses section. The cell homogenate was centrifuged for 10 min at $10{,}000 \times g$ and the supernatant was used for colorimetric reaction and the optical density (OD) value was recorded at 405 nm. The OD values were normalized to the protein level of the control.

2.14. Statistical analyses

Cytotoxicity results were subjected to statistical analysis using the GLM procedure (SAS, 2003) employing a complete randomized design following the model: $Yi = \mu + Ti + ei$, where μ is the mean value, Ti is the treatment effect and ei is the experimental error, respectively. Means were compared using Duncan's New Multiple Range test. Differences were considered significant at $P \leq 0.05$. GraphPad Prism 5 software was used for all the statistical analyses in cellular antioxidant enzyme assay, lipid peroxidation, reactive oxygen/nitrogen species assay, caspas-3 activity determination, gene and protein expression

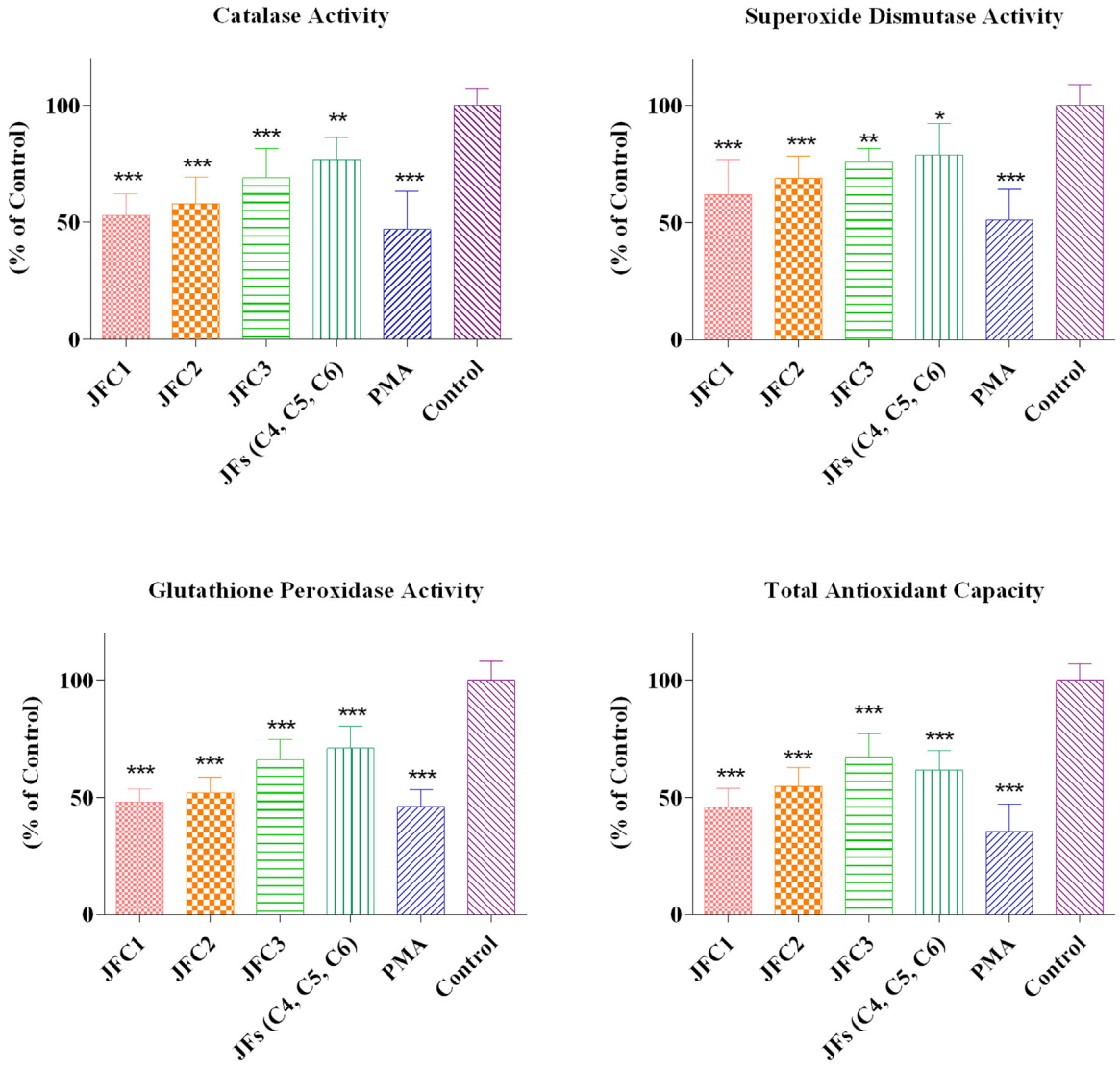

Fig. 4. Antioxidant enzymes and total antioxidant capacity of the MDBK cells treated with different phorbol esters [JFC1 (Jatropha factor C1), JFC2 (Jatropha factor C2), JFC3 (Jatropha factor C3) and JFs (mixture of Jatropha factor C4, C5 and C6)] isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration incubated for 12 h. All values expressed as percentage of control and represent mean ± standard error from three independent experiments, ***P < 0.001, **P < 0.01 and *P < 0.05 indicate significant difference compared to the untreated control.

analyses. The data were subjected to one-way analysis of variance (ANOVA) and treatment means were compared to control using Dunnett's Multiple Comparison Test.

3. Results and discussion

3.1. Jatropha meal phorbol esters

Isolation of phorbol esters was necessary for studying their mode of action on cells in vitro. The high performance liquid chromatography analysis is a well-established technique to detect and quantify the phorbol esters in the Jatropha meal (Devappa et al., 2012b; Oskoueian et al., 2011). In the analysis, phorbol-12-myristate 13-acetate (PMA) was used as the reference compound for the quantitative evaluation of Jatropha meal phorbol esters as other phorbol ester commercial standards for the *J. curcas* are not available. As observed in this study, the Jatropha meal phorbol esters appeared as four peaks in the HPLC analysis (Fig. 1), similar to that reported earlier by Makkar et al. (2007), Li et al. (2010) and Devappa (2012). As shown in Fig. 1, with reference to the results reported by Devappa et al. (2012a), Roach et al. (2012), Devappa (2012) and a recent study conducted by Baldini et al. (2014), the four peaks observed in the present analysis corresponded to Jatropha factor (JF) JFC1, JFC2, JFC3 and JFs (JFC4 + JFC5 + JFC6 mixture), respectively. JFC1 was the most abundant phorbol ester derivative with a concentration of 1.26 ± 0.02 mg PMA equivalents/g DM Jatropha meal (~42.2% of the total phorbol esters). JFC2 was the second most abundant derivative with a concentration of 0.85 ± 0.018 mg PMA equivalents/g DM (~27.2% of the total phorbol esters), while JFC3 showed a concentration of 0.52 ± 0.05 mg PMA equivalents/g DM (16.4% of the total phorbol esters) and JFs mixture which co-eluted as the forth peak showed a concentration of 0.45 ± 0.02 mg PMA equivalents/g DM (~14.0% of the total phorbol esters).

Several authors (Devappa et al., 2012a; Haas et al., 2002; Hirota et al., 1988) had identified six phorbol esters named as Jatropha factors C1, C2, C3, C4, C5 and C6 with the molecular formula of $C_{44}H_{54}O_8$ (Fig. 2). These identified phorbol esters possess similar diterpene moiety identified as 12-deoxy-16-hydroxyphorbol and they differ in their dicarboxylic acid moieties (Nishshanka et al., 2016). The phorbol esters content of Jatropha meal was 3.0 ± 0.18 mg phorbol-12-myristate 13-acetate (PMA) equivalents/g DM. Earlier studies by Makkar et al. (2012) and Devappa et al. (2012a) reported that phorbol esters

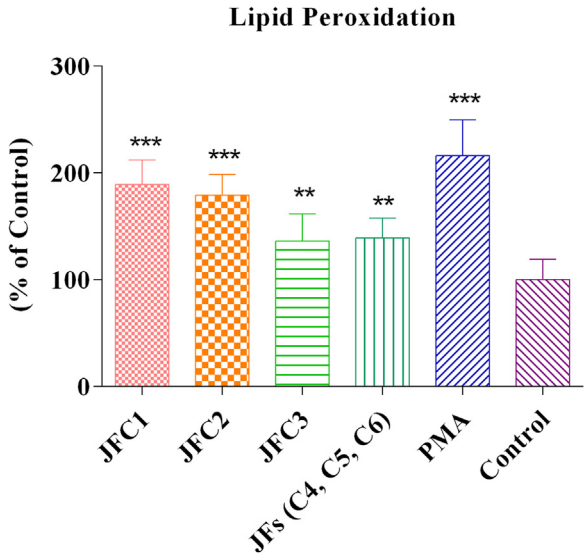

Fig. 5. Lipid peroxidation level in MDBK cells treated with different phorbol esters [JFC1 (Jatropha factor C1), JFC2 (Jatropha factor C2), JFC3 (Jatropha factor C3) and JFs (mixture of Jatropha factor C4, C5 and C6)] isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration incubated for 24 h. All values represent mean ± standard error from three independent experiments, ***P < 0.001, **P < 0.01 indicate significant difference compared to the untreated control.

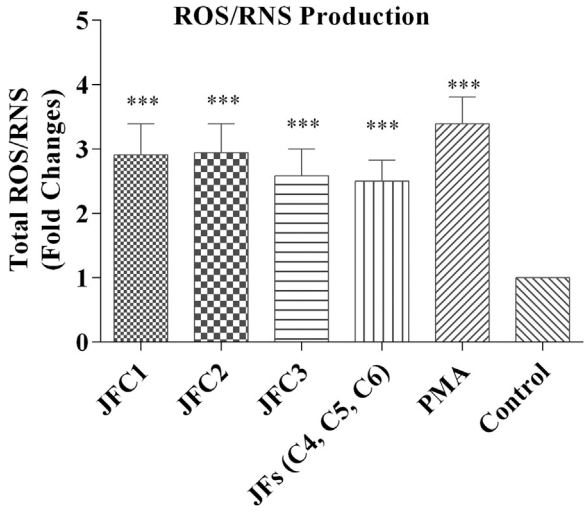

Fig. 6. ROS/RNS production in MDBK cells treated with different phorbol esters [JFC1 (Jatropha factor C1), JFC2 (Jatropha factor C2), JFC3 (Jatropha factor C3) and JFs (mixture of Jatropha factor C4, C5 and C6)] isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration incubated for 12 h. All values represent mean ± standard error from three independent experiments, ***P < 0.001 indicates significant difference compared to the untreated control.

detected in toxic genotypes of Jatropha meal ranged from 1 to 4 mg/g DM.

3.2. Cytotoxic activity of phorbol esters

The isolated phorbol esters, JFC1, JFC2, JFC3 andJFs (C4, C5, C6) (Table 2) exhibited cytotoxic activity towards the MDBK cell line with the CC_{50} ranging from 63.8 to 109.6 µg/mL. The lower CC_{50} in JFC1 and JFC2 indicated that these two fractions were more active in mediating cytotoxicity as compared to the JFC3 and the mixture of JFs (C4, C5, C6). The positive control (PMA), an activator of classical and novel PKC isozymes also induced cytotoxicity with the CC_{50} values ranging from 52.4 to 72.8 µg/mL. All phorbol ester fractions obtained from Jatropha meal showed lower cell proliferation inhibition as compared to the PMA since the lower CC_{50} value of PMA represented higher cell proliferation inhibition activity.

The cytotoxic effects of isolated phorbol esters and PMA were found to be in the ascending order: PMA > JFC2 ≥ JFC1 > JFs (C4, C5, C6) ≥ JFC3. The CC_{50} concentrations upon 24, 48 and 72 h incubation (Table 2), showed that the Jatropha meal phorbol esters inhibited the MDBK cell proliferation in a dose and time dependent manner.

The free hydroxyl groups present in the structure of the phorbol esters as previously shown by Haas et al. (2002) could play an important role in mediating cytotoxicity. The previous reports which demonstrated the different responses of the cells to phorbol esters were dependent on the nature of the phorbol esters, cell types, time of exposure and concentrations. As such, Deng et al. (2010) reported the tumor promoter activity of phorbol ester in mice fibroblast cells. On the other hand, treatment of several cancer cells with PMA led to apoptosis (Abdel-Hafez et al., 2002; Griner and Kazanietz, 2007; Muanda et al., 2010; Oskoueian et al., 2012b). It seems that, differences in the experimental conditions led to dissimilar results. In general, the results observed in this study were in agreement with that reported by Avila et al. (2005) and Bond et al. (2007) who demonstrated the cytotoxic action of PMA on pancreatic cell line. The results of cytotoxicity assay suggested that the growth inhibitory of Jatropha meal phorbol ester and PMA is associated with an increase in apoptosis which contributed to their cytotoxic activities.

3.3. Cells morphology

Fig. 3 represents the morphological changes of MDBK cells upon treatment with the isolated phorbol esters and PMA at CC_{50} concentration after 24 h incubation observed by using a light microscope. Substantial morphological changes, detachment and destruction of cells treated with phorbol esters and PMA were observed. According to these microscopic observations, cell damage resembled apoptosis, as the cell walls were not intact and apoptotic bodies were seen. The MDBK cells displayed death upon treatment with the phorbol esters and PMA at CC_{50} concentration after 24 h incubation. The cells detached completely at further incubation period. The characteristics observed in this study were also reported by Bond et al. (2007) who observed the detachment of the human pancreatic adenocarcinomas cells upon 48 h exposure to PMA.

3.4. Antioxidant enzymes and intracellular antioxidant activity

The results of antioxidant enzymes activity together with cellular total antioxidant capacity of the MDBK cells upon exposure to the isolated phorbol esters and PMA are shown in Fig. 4. These results indicated significant decrease ($p^{<}0.01$) in antioxidant enzymes activity and total antioxidant capacity of the cells exposed to phorbol esters and PMA when compared to the untreated control cells. This significant reduction indicated that all the isolated phorbol esters and PMA induced oxidative stress in the MDBK cells. The cellular intrinsic antioxidant defence mechanism depends on various antioxidant enzymes such as CAT, SOD and GPx. The SOD generally converts the cellular superoxide to hydrogen peroxide, which is subsequently decomposed into water by CAT and GPx enzymes (Zareian et al., 2015). In addition to the antioxidant enzymes, other cellular components such as proteins, lipids, vitamins, glutathione and uric acid contributed in total antioxidant capacity of the cells and protect the cells from free radical damages (Dubey et al., 2015).

3.5. Lipid peroxidation

Fig. 5 shows the results of lipid peroxidation in MDBK cells upon

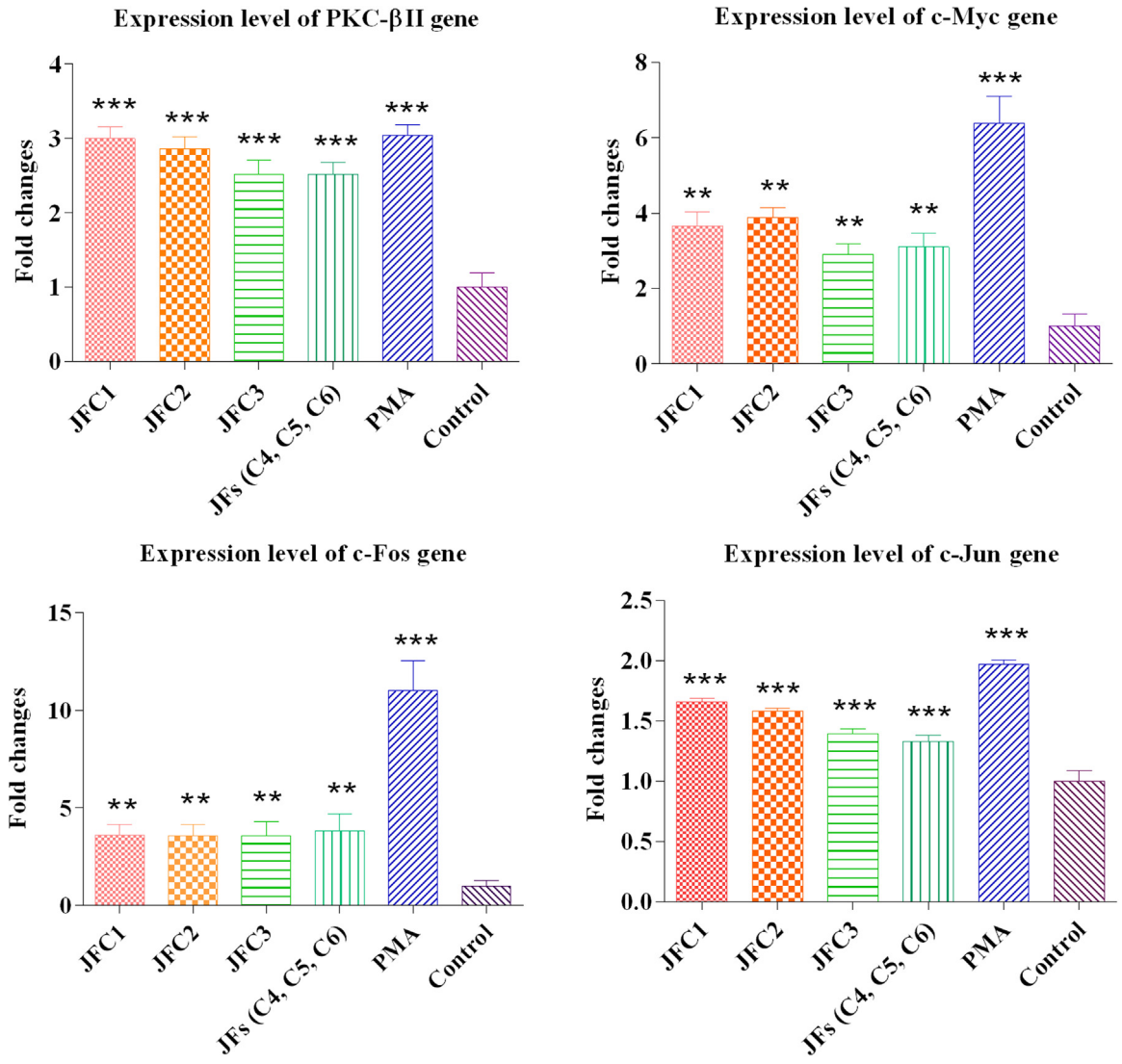

Fig. 7. Fold-changes in the expression levels of the PKC-βII, c-Myc, c-Fos and c-Jun genes determined by SYBR-Green qRT-PCR of cells treated with different phorbol esters [JFC1 (Jatropha factor C1), JFC2 (Jatropha factor C2), JFC3 (Jatropha factor C3) and JFs (mixture of Jatropha factor C4, C5 and C6)] isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration incubated for 12 h. All values represent mean ± standard error from three independent experiments, ***P < 0.001, **P < 0.01 indicate significant difference compared to the untreated control.

24 h exposure to the isolated phorbol esters and PMA. The lipid peroxidation as indicator of oxidative stress, increased significantly ($p < 0.01$) in the MDBK cells treated with phorbol esters and PMA. Based on the results obtained, all oxidative stress biomarkers were suppressed indicating the ability of phorbol esters to induce oxidative stress in the cells. The JFC1 showed comparable results to that of PMA as positive control.

3.6. Reactive oxygen/nitrogen species production

Besides lipid peroxidation, the production of ROS/RNS has also been used as reliable biomarker to detect oxidative stress. Thus, both intracellular and extracellular ROS and RNS were measured and reported as total ROS/RNS. As shown in Fig. 6, all phorbol esters isolated from Jatropha meal and PMA significantly ($p < 0.001$) elevated the production of ROS/RNS in MDBK cells. The production of ROS/RNS in the treated cells increased almost up to two fold. The ROS/RNS are second messengers which are generally produced in well-regulated manner to maintain cellular homeostasis. Under stressful condition, overproduction of ROS/RNS together with the lack of antioxidant enzymes or non-enzymatic antioxidants led to imbalance in the equilibration of proxidant/antioxidant status. This proxidant/antioxidant status regulates divergent effects on cellular functions, e.g., cell growth and differentiation, growth factor signaling, mitogenic responses, modulation of extracellular matrix, production and breakdown apoptosis, inactivation of nitric oxide (NO), oxygen sensing, and stimulation of pro-inflammatory genes and many kinases (Dhawan, 2014). The results of ROS/RNS together with the results of antioxidant enzyme activity, total cellular antioxidant and lipid peroxidation showed that phorbol esters and PMA severely altered the redox status of MDBK cells and these results confirmed the occurrence of oxidative stress which ultimately caused the cytotoxic effect of Jatropha phorbol esters.

3.7. Gene expression in the MDBK cell line

The expression of PKC-βII, c-Myc, c-Fos and c-Jun genes in MDBK cells upon treatment with different phorbol ester fractions is shown in Fig. 7. The results showed the significant ($p < 0.01$) up-regulation of these genes upon treatment with Jatropha meal phorbol esters and PMA at 12 h as compared to the untreated control cells.

The Jatropha meal phorbol esters apparently acted similarly to the

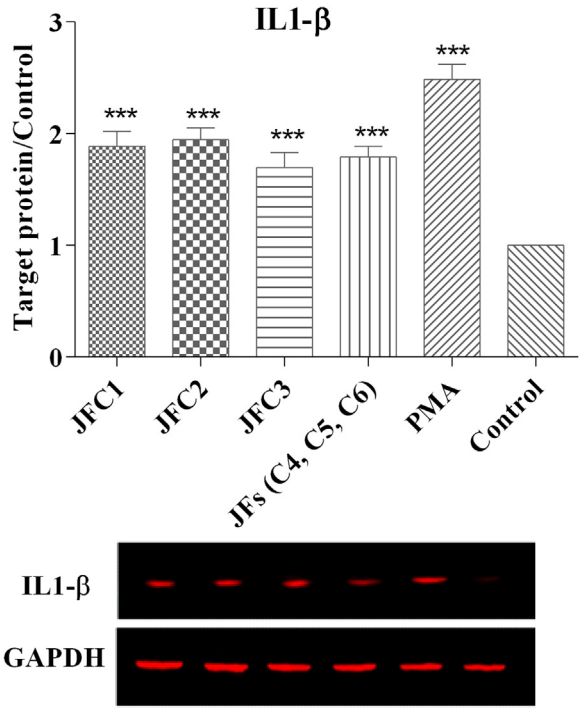

Fig. 8. Cells treated with different phorbol esters [JFC1 (Jatropha factor C1), JFC2 (Jatropha factor C2), JFC3 (Jatropha factor C3) and JFs (mixture of Jatropha factor C4, C5 and C6)] isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration and incubated for 12 h. Equal amount of total cellular protein was subjected to Western blot analyses for IL-1β and GAPDH protein expression. All values represent mean ± standard error from three independent experiments, ***P < 0.001 indicates significant difference compared to the untreated control.

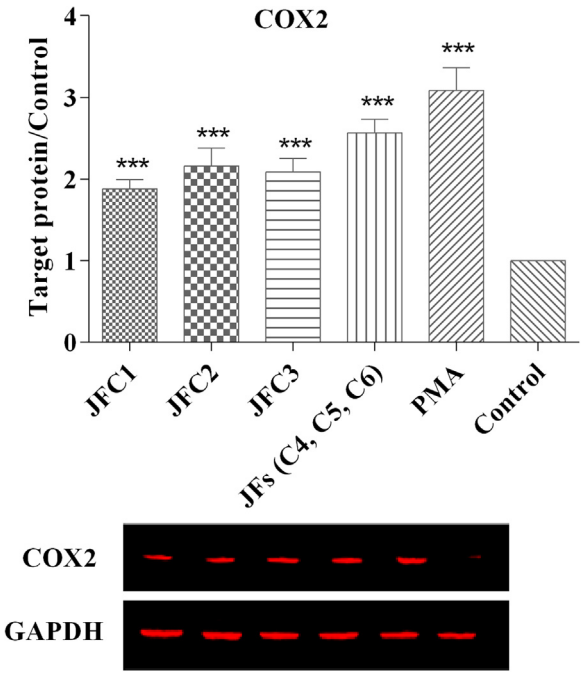

Fig. 9. Cells treated with different phorbol esters [JFC1 (Jatropha factor C1), JFC2 (Jatropha factor C2), JFC3 (Jatropha factor C3) and JFs (mixture of Jatropha factor C4, C5 and C6)] isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration and incubated for 12 h. Equal amount of total cellular protein was subjected to Western blot analyses for Cox2 and GAPDH protein expression. All values represent mean ± standard error from three independent experiments, ***P < 0.001 indicates significant difference compared to the untreated control.

other PKC agonists such as diacylglycerol (DAGs) and ingenol-3-angelate in activating PKCs (Cerveró et al., 2010). Upon activation, PKC enzymes are translocated to the plasma membrane by RACK proteins (receptors for activated C-kinase) and as a result, potent activators of transcription may be triggered, leading to increased expression of oncogenes or promotion of cancer progression (Cerveró et al., 2010). The types of activated PKC and downstream pathway would determine the cells response to the phorbol esters. For example, activation of PKCα triggered tumor suppression in pancreatic and mammary cell lines (Griner and Kazanietz, 2007; Weber et al., 2008), but acted as a growth stimulator in lymphoid and myeloid cells (Leshchinsky and Klasing, 2001; Lu et al., 2009). In this study, Jatropha meal phorbol esters activated the PKC-βII and this activation may activate downstream proto-oncogenes which subsequently affect the response of cells to phorbol esters.

The c-Myc gene plays a major role in cell growth, proliferation, differentiation and apoptosis while c-Fos dimerise with c-Jun to form the AP-1 transcription factor, which up-regulates transcription of a diverse range of genes involved in various activities from proliferation and differentiation to defence against invasion and cell damage (Gomperts et al., 2009). In this study, the up-regulation of PKC-βII and proto-oncogenes including c-Fos, c-Myc and c-Jun in MDBK cells upon exposure to phorbol esters are consistent with the results reported earlier by Mohandas et al. (2013) who observed the PKC-β and proto-oncogenes (c-Fos and c-Jun) over-expressions upon 30 min treatment of rat brain neurons with PMA. Another study by Wang et al. (2013) revealed the overexpression of PKC, c-Jun and c-Fos genes in the mouse skin treated by PMA finally leading to elevation in the expression of Cox2 gene. Although the activation of proto-oncogenes in this study seemed to be mediated through PKC, however, it was also possible that as a result of oxidative stress, the reactive oxygen species (ROS) were also capable of activating PKC, c-Jun and c-Fos.

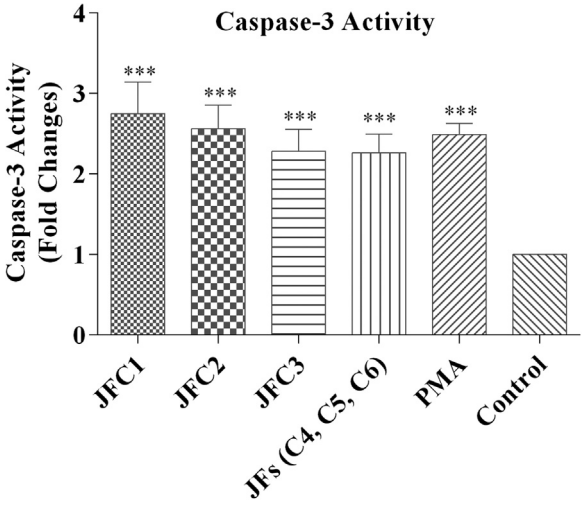

Fig. 10. Caspase-3 activity in MDBK cells treated with different phorbol esters [JFC1 (Jatropha factor C1), JFC2 (Jatropha factor C2), JFC3 (Jatropha factor C3) and JFs (mixture of Jatropha factor C4, C5 and C6)] isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration incubated for 24 h. All values represent mean ± standard error from three independent experiments, ***P < 0.001 indicates significant difference compared to the untreated control.

3.8. Expression of inflammatory proteins

The expression levels of inflammatory proteins including IL-1β and Cox2 in MDBK cells upon treatment with isolated phorbol esters from

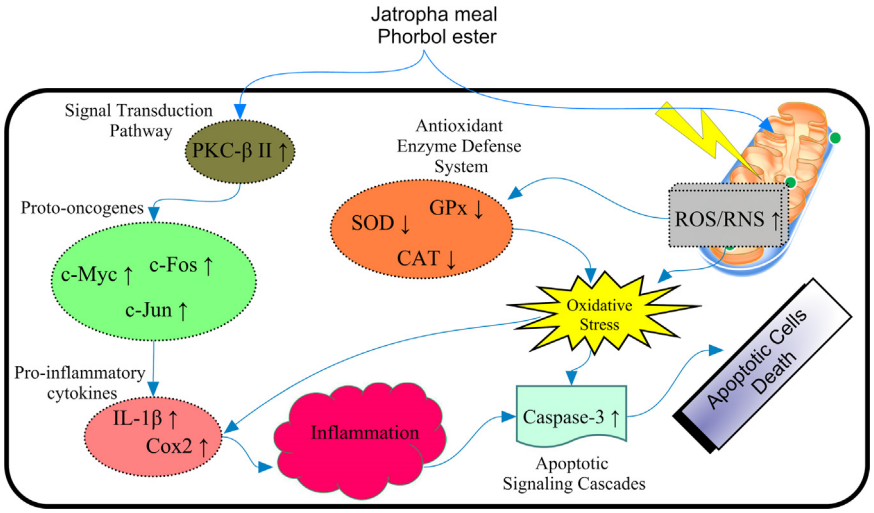

Fig. 11. The proposed mode of action of Jatropha meal phorbol esters in MDBK cells. Arrows indicate increase (↑) or decrease (↓) expression or activity.

Jatropha meal and PMA are shown in Figs. 8 and 9, respectively.

The phorbol esters significantly ($p < 0.01$) up-regulated the IL-1β and Cox2 protein expressions in MDBK cells as compared to the untreated cells. The IL-1β is a member of the interleukin 1 cytokine family and it is an important mediator of the inflammatory response involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The IL-1β plays an important role in the induction of Cox2 and it is unexpressed under normal conditions in most cells, but elevated levels are found during inflammation. The treatment of MDBK cells with PMA as the positive control had also significantly ($p < 0.01$) up-regulated the IL-1β and Cox2 in MDBK cells.

At the cellular level, proteins that mediate inflammatory responses must be kept tightly suppressed under normal conditions, and must also be rapidly and highly induced in the setting of a variety of stimuli such as infection, injury or peripheral incitements, such as exposure to phorbol esters. Most likely, the underlying mechanisms of Cox2 and IL-1β overexpression in the current study could be through activation of c-Fos, c-Jun and upstream kinases, including PKC-β II. This activation could be directly due to the phorbol esters acting through the cell surface receptors, or ROS/RNS generated during oxidative stress.

3.9. Caspase-3 activity

Fig. 10 shows the caspase-3 activity in MDBK cells treated with various phorbol esters upon 24 h incubation. The results showed that all phorbol ester fractions, together with PMA, increased the caspase-3 activity. In this study, the caspase-3 activity was determined based on the hydrolysis of acetyl-Asp-Glu-Val-Asp p-nitroanilide (DEVD-p-NA) by caspase-3 resulting in the cleavage of chromophore p-nitroaniline moiety from labelled substrate DEVD-p-NA. The caspase-3 belongs to the executioner caspases which carries out the mass proteolysis that leads to apoptosis (Oskoueian et al., 2012a). The activation of caspase-3 as a hallmark of apoptosis resulted in a range of substrates cleavage including downstream caspases, nuclear proteins, plasma membrane proteins and mitochondrial proteins, finally leading to apoptotic cells death. Hence, the activation of caspase-3 together with the results of morphological examination confirmed the presence of apoptotic cells death in MDBK cells upon 24 h exposure to Jatropha meal phorbol esters. Although, previous studies had reported that phorbol esters induced apoptosis (Abdel-Hafez et al., 2002; Gobbi et al., 2009; Griner and Kazanietz, 2007; Gülçin et al., 2012; Kazanietz, 2005), but other reports indicated tumor formation (Ahmad et al., 2013; Goel et al., 2007). Nevertheless, based on the results obtained in this study, the cytotoxic effects of Jatropha meal phorbol esters on kidney cells are mediated through a number of biochemical changes including alteration in the redox status of the cells which resulted in oxidative stress, up-regulating pro-inflammatory cytokines (IL-1β and Cox2) and elevating caspase-3 activity, which subsequently led to apoptosis. Although the phorbol esters up-regulated the expressions of PKC-βII and proto-oncogenes (c-Fos, c-Myc and c-Jun), but the effects were not manifested. However, it could be stated that the effects of Jatropha meal phorbol esters may vary depending on types of phorbol esters, concentrations used, cell types and the time of exposure as these factors may alter the balance between pro-apoptotic and anti-apoptotic pathways differently, resulting in either apoptosis or tumorigenesis of the cells.

The mode of action of phorbol esters proposed is as shown in Fig. 11. When cells are exposed to Jatropha meal phorbol esters, PKC-β II via signal transduction pathway is activated and this may result in up-regulation of proto-oncogenes like c-Myce, c-Fos and c-Jun. Most probably, the prolonged up-regulation of proto-oncogenes accompanied with pro-inflammatory cytokines overexpression triggered cellular inflammation process. The hydrophilic nature of phorbol esters facilitates their penetration into the cells. The presence of phorbol ester increases the mitochondrial PKC activity, which disrupts the mitochondrial membrane function, and decreases the mitochondrial complex I and pyruvate dehydrogenase activities resulting in increased mitochondrial ROS/RNS production (Wang et al., 2006). These free radicals are frequently associated with oxidative stress, cytotoxicity and often being described as damaging toxic compounds. The overproduction of ROS/RNS depletes the antioxidant enzymes, impairs cellular antioxidant capacity and promotes oxidative stress. The sustained oxidative stress, redox signaling and inflammation may act individually or in synergy leading to initiation and orchestration of apoptosis cells death through activation of caspase-3 from apoptotic signaling cascades.

4. Conclusion

The present study showed that Jatropha factor C1 C2, C3 and the mixture of Jatropha factor C4, C5 and C6 isolated from Jatropha meal were detrimental to the bovine kidney cells by inducing oxidative stress, activating inflammatory response, arresting cells proliferation and finally triggering apoptotic cells death. Although all Jatropha meal phorbol esters were detrimental to the cells, Jatropha factor C1 and C2 appeared to be more toxic to the bovine kidney cells when compared to the Jatropha factor C3 and the mixture of C4, C5 and C6. Thus, considering the severe cytotoxicity effects of phorbol esters in Jatropha meal, it is imperative that a proper strategy for ensuring the complete removal of phorbol esters is developed before the meal can be used as animal feed.

Competing interests

The authors have declared that no competing interests exist

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