Jatropha curcas L. is known as a valuable source of seed oil for biodiesel production. The meal contained about 60% protein with a good balance of essential amino acids, containing various bioactive phytochemicals, including phenolics, flavonoids, saponins, phytic acids, trypsin inhibitors, and lectins which render it as a potential biofeed for ruminant production. The Jatropha meal exhibited antioxidant, antibacterial and anti-inflammatory activities which corresponded to the various bioactive compounds present in the meal. Among bioactive compounds presence in the meal, the phorbol esters limits its application as ruminant functional feed. The rumen microbes were capable of degrading the phorbol esters to some extent. Moreover, the phorbol esters even in low concentrations induced cytotoxicity in the bovine kidney cells, followed by changes in cell morphology, proliferation, differentiation and finally cell death. Therefore, complete removal of phorbol esters is absolutely necessary before its application as a functional feed for ruminants.

Ehsan Oskoueian holds a PhD in Molecular Biotechnology. He has over 15 years of research experience, and has published around 100 research papers. He has developed over 10 innovative products available on the market. He is currently a part of the managing team of the Industrial and Mineral Research Center, Arka Industrial Cluster, Mashhad, Iran.

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Jatropha Meal as a Novel Biofeed

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Ehsan Oskoueian

Jatropha Meal, a Functional Feed for Ruminants: Benefits & Limitations

A novel bioactive protein source for the future of feed industry

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Jatropha Meal, a Functional Feed for Ruminants: Benefits & Limitations

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By

Dr. EHSAN OSKOUEIAN

Abstract

Jatropha curcas L. (*J. curcas*) plant is well known as a source of seed oil for biofuel production. The plant thrives well in tropical conditions and its planting acreage has increased considerably in Malaysia. In the process of oil extraction of the seed kernel, a residue called Jatropha meal is produced. The meal could be a potential biofeed due to its chemical and bioactive compounds present. However, the bioactive compounds present in the seeds differ among different genotypes of *J. curcas* plant. Thus, before considering the local Jatropha meal as a biofeed, it is imperative to determine the bioactive compounds and biological activities of the meal which would indicate its possible applications and limitations. It is also important to ascertain the *J. curcas* variety that is grown in Malaysia, is either toxic or non-toxic according to the profiles of the bioactive compounds present. Therefore, the hypothesis of this research was, the Jatropha meal is safe and has functional biofeed properties. In order to test the hypothesis a comprehensive study on the bioactive compounds and biological activities of Jatropha meal, physicochemical treatments, and how the meal and isolated phorbol esters from the meal affect rumen microbial activity were first conducted to evaluate the meal as a biofeed. This was followed by the cytotoxicity evaluation and mode of action elucidation of isolated phorbol esters from the meal on bovine kidney cell line. The results showed that chemical analysis of Jatropha meal obtained from local *J. curcas* plant contained 61.8% crude protein, 9.7% neutral detergent fibre and 4.8% acid detergent fibre. The meal also contained high levels of total phenolics (3.9 mg/g DM), total flavonoids (0.4 mg/g DM), total saponins (19 mg/g DM), phytic acids (9.1 %), trypsin inhibitors (34.2 mg/g DM), lectins (102.7 U) and phorbol esters (3.0 mg/g DM). The high performance liquid chromatography (HPLC) analyses of Jatropha meal showed the presence of gallic acid, pyrogallol,

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rutin, myricetin and daidzein with the values of 581.3±0.36, 631.1±0.47, 47.6±0.53, 198.5±0.29 and 297.5±0.27 µg/g DM, respectively. The gas chromatography-mass spectrometry analyses (GC-MS) indicated the presence of other metabolites, including 2-(hydroxymethyl)-2-nitro-1,3-propanediol, β-sitosterol, 2-furancarboxaldehyde,5-(hydroxymethyl), furfural (2-furan carboxaldehyde) and acetic acid. The Jatropha meal methanolic extract, vitamin C, butylated hydroxytoluene (BHT) and β-carotene showed free radical (2,2-diphenyl-1-picrylhydrazyl) scavenging activity with the IC_{50} values of 1.6, 0.3, 0.3 and 1.5 mg/ml, respectively, while values for the ferric reducing power activity were 3.0, 0.3, 0.3 and 2.6 mg/ml, respectively. Jatropha meal extract showed antibacterial activity against several pathogenic bacteria including *Enterobacter aerogenes, Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Micrococcus luteus, Bacillus subtilis, Bacillus cereus,* and *Staphylococcus aureus* with the inhibition range of 0.21-1.63 cm at the concentrations of 1 and 1.5 mg/disc. The Jatropha meal methanolic extract, at the concentration of 100 µg/ml, inhibited the inducible nitric oxide synthase in macrophages RAW 264.7, comparable to N_{ω}-L-nitro-arginine methyl ester (L-NAME) indicating appreciable anti-inflammatory activity. Combinations of hydrothermal treatment, alkali and oxidizing agents alleviated the levels of phenolic compounds, saponin and phorbol esters significantly ($p \leq 0.05$), while the level of phytic acid did not decrease. Trypsin inhibitors and lectin activity were fully inactivated. The level of phorbol esters decreased by 76.7% on treatment with heat, 3% (w/w) NaOH and 10% (v/w) NaOCl. *In vitro* fermentation by rumen microbes showed a significant ($p \leq 0.05$) decrease in fermentation parameters when chemically treated meals were used as the substrates, while physically treated meals did not affect the fermentation parameters significantly. Effects of four different

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levels of isolated phorbol esters from Jatropha meal i.e., 3, 6, 9 and 12 mg/30 ml buffered rumen fluid, on rumen fermentation using ground Guinea grass as the substrate were studied *in vitro*. The results showed that apparent dry matter degradability, metabolisable energy, total volume of gas produced after 24 h of incubation ($IVGP_{24}$) and total volatile fatty acids decreased significantly ($P \leq 0.05$) in treatments with 9 and 12 mg phorbol esters. Rumen microbial specific enzyme activity (CMCase, FPase, xylanase and β-glucosidase), purine content (index of rumen microbial protein) and rumen microbial protein synthesis showed a significant decrease ($P \leq 0.05$) on treatments with 9 and 12 mg phorbol esters. Similarly, the population of bacteria, fungi, protozoa, methanogens, archaea and major cellulolytic bacteria, including *Fibrobacter succinogenes, Ruminococcus albus, R. flavefaciens* and *Butyrivibrio fibrisolvens,* significantly ($p \leq 0.05$) decreased when 9 and 12 mg phorbol esters were added. The disappearance of phorbol esters upon rumen microbial fermentation was observed with values ranging from 23.0% to 44.1%. The *in vitro* toxicity evaluation of crude extract obtained from rumen fluid treated with phorbol esters before and after 24 h fermentation on viability of bovine kidney cells (MDBK) (ATCC: CCL-22) demonstrated a significant ($p \leq 0.05$) decrease in the toxic effect of the phorbol esters where the cell viability improved from 43.6% to 72.3%. The phorbol esters of Jatropha meal were isolated into four fractions namely, PE1, PE2, PE3 and PE4. *In vitro* cytotoxicity assay showed cells death with the CC_{50} values ranging from 52.4 to 109.6 µg/ml in bovine kidney cell line when exposed to all fractions of phorbol esters and using phorbol-12-myristate-13-acetate (PMA) as positive control upon 24, 48 and 72 h exposure. The isolated phorbol esters induced cell death in a dose and time dependent manner. The light microscope examination indicated no apparent changes in the morphology of the MDBK cells upon 12 h

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exposure to all phorbol ester fractions and PMA, while after 24 h exposure, significant morphological changes, detachment, destruction of cells and apoptotic bodies were seen. The expression of the PKC-β II gene from signal transduction pathway, c-Fos, c-Jun and c-Myc from proto-oncogenes, and IL-1β and Cox2 genes from inflammatory pathway, on 12 h treatment with isolated phorbol esters and PMA at the CC_{50} concentrations, showed significant ($p \leq 0.01$) overexpression in all of the genes, with values ranging from 1.3 to 5.1 fold increase as compared to the untreated cells. Western blot analysis also confirmed the gene expression results, and showed the significant ($p \leq 0.01$) overexpression of PKC-β II, c-Fos, c-Jun, c-Myc, Cox2 and IL-1β proteins, with values ranging from 1.48 to 3.15 fold increase as compared to the untreated cells. The flow cytometry results confirmed the apoptotic cell death in MDBK cells upon 24 h exposure to isolated phorbol esters and PMA. Consequently, the results of this study showed that the local *J. curcas* plant is of the toxic variety due the presence of phorbol esters, and although the meal could be considered as a potential biofeed, due to the presence of various biological activities and bioactive compounds, however, the cytotoxic effect of phorbol esters could not be compromised. Although the rumen microbes could detoxify the phorbol esters partially, it is still not sufficient to consider it safe as a biofeed due to their cytotoxic effects even at low concentrations. Therefore, complete removal of phorbol esters from Jatropha meal is absolutely necessary.

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LIST OF ABBREVIATIONS

ADF	Acid detergent fibre
ADM	Apparent dry matter
AST	Aspartate aminotransferase
BAPNA	Benzyl-dl-arginine-para-nitroanilide
BHT	Butylated hydroxytoluene
CC_{50}	Cytotoxic concentration to kill 50% of cells
CMCase	Carboxymethylcellulase
COX	Cyclooxygenase
CP	Crude protein
DAG	Diacyl glycerol
DM	Dry matter
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNSA	Dinitrosalicyclic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
FBS	Fetal bovine serum
FID	Flame ionization detector
FPase	Filter paperase
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GC-MS	Gas chromatography-mass spectrophotometry
GOT	Glutamate oxaloacetate transaminase
GP	Gas production
HeLa	Human cervix carcinoma cell lines
HIV	Human immunodeficiency virus
IC_{50}	Inhibitory concentration
IFN-γ	Interferon-γ
IL-1β	Interleukin-1 beta

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iNOS	Inducible nitric oxide synthase
IVDMD	*In vitro* DM degradability
IVGP	*In vitro* gas production
LB medium	Luria-Bertani medium
L-NAME	N_{ω}-nitro-L-arginine methyl ester
LPS	Lipopolysacharide
ME	Metabolizable energy
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Nutrient agar
NB	Nutrient broth
NDF	Neutral detergent fibre
NO	Nitric oxide
NOS	Nitric oxide synthase
OM	Organic matter
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEs	Phorbol esters
PI	Propidium iodide
PKC	Protein kinase C
PMA	Phorbol-12-myristate 13-acetate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP-HPLC	Reverse phase high performance liquid chromatography
rRNA	Ribosomal ribonucleic acid
SSF	Solid-state fermentation
TCA	Trichloroacetic acid
TIA	Trypsin inhibitor activity
VFAs	Volatile fatty acids

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LIST OF ANNOTATIONS

α	Alpha
β	Beta
γ	Gamma
δ	Delta
ε	Epsilon
η	Eta
θ	Theta
ζ	Zeta
ι	Iota
λ	Lambda
%	Percentage
(v/v)	Volume per volume
(w/v)	Weight per volume
≤	Less or equal
±	Plus and/or minus
µ	Micro
µl	Microliter
µM	Micromolar
g	Gram
h	Hour
Hz	Hertz
kDa	Kilo Dalton
Kg	Kilogram
L	Liter
mg/ml	Milligram per millilitre

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µg/ml	Microgram per millilitre
min	Minute
mmol	Millimole
U	Units
V	Volt
mA	Milliampere
^{o}C	Degree Celsius
S	Second

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CHAPTER 1

INTRODUCTION

Jatropha curcas L. (*J. curcas*) belongs to the *Euphorbiaceae* family. It is a drought disease resistant plant and grows in tropical and sub-tropical countries, such as South America, Africa and South-east Asia. It is a multipurpose tree with significant economic importance due to its several industrial and medicinal uses (Kulkarni *et al.*, 2005). All parts of *J. curcas* have been used in traditional human medicine and for veterinary purposes for a long time (Monteiro *et al.*, 2011). *Jatropha curcas* kernel contains about 40-60%, oil which makes it a potential source for biodiesel production. During the process of kernel oil extraction, the residue called Jatropha meal is produced. It was reported that the meal contained 60% of crude protein and 10% fiber (Makkar *et al.*, 2009).

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Jatropha meal was shown to possess antioxidant (Kim *et al.*, 2010), anti-tick (Akinleye *et al.*, 2012; Prasad *et al.*, 2012), anthelmintic (Monteiro *et al.*, 2011), antibacterial (Namuli *et al.*, 2011; Xiao *et al.*, 2011a), antifungal (Wei *et al.*, 2005) and antitumor activities (Juan *et al.*, 2003). The major bioactive compounds in Jatropha meal are phenolics, saponins, trypsin inhibitors, phytic acid, lectin and phorbol esters (Martinez-Herrera *et al.*, 2006). The chemical composition, bioactive compounds constituents and biological activity observed in Jatropha meal render the meal suitable as a potential biofeed, although the profile and content of bioactive compounds vary among genotypes. According to Makkar *et al*. (2009), the *J. curcas* seed containing phorbol esters is categorized under the toxic variety of *J. curcas* plant. Rakshit *et al*. (2008) reported the reduced appetite and low feed intake accompanied by diarrhea in rats fed with Jatropha meal containing phorbol esters.

They believed that the major toxic compounds present in the meal are lectin and phorbol esters. Aregheore *et al*., (1998) reported that the lectins are heat labile and could be easily inactivated by moist heat treatment. Thus, the phorbol esters would be the main toxicant in the meal (Aregheore *et al.*, 2003) as heating up to 160ºC for 30 min did not destroy the phorbol esters (Makkar *et al.*, 2009b; Li *et al.*, 2010). In order to remove the phorbol esters the Jatropha meal has been subjected to various treatments including water leaching, autoclaving, acid and alkali treatments. Combination of heat and alkali treatments could reduce the phorbol esters up to 89% (Rakshit *et al.*, 2008) and 93% (Aregheore *et al.*, 2003).

Li *et al*. (2010) assessed the toxic effects of the isolated phorbol esters from Jatropha meal in mice and their results showed a loss of body mass and pathological symptoms in lung and kidney tissues. Generally, ruminants are less sensitive to the plant toxins compared to monogastric animals, due to the presence of microbes in the rumen. Rumen microbes are also capable of degrading, biotransforming and detoxifying the plant bioactive compounds to a substantial extent (Dziba *et al.*, 2006; Broudiscou *et al.*, 2007).

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Besides rumen microbiota, liver and sera of ruminants contain enzymes, such as acylcarnitine hydrolase and diacylglycerol lipase, which degrade the phorbol esters to their non-toxic derivatives (Mentlein, 1986; Devappa *et al.*, 2010b). The phorbol ester biological activities are strictly structure dependent and according to their functional groups, different phorbol esters activate different protein kinase C (PKC) isozymes in the cells which could trigger different pathways. Thus, phorbol esters have been reported to show tumor promoter activity (Goel *et al.*, 2007) while in

another experiment induced apoptotic cell death (Day *et al.*, 1994; Gobbi *et al.*, 2009).

Thus, the hypothesis of this research was, the Jatropha meal is safe and has functional biofeed properties. Hence, this study was conducted to characterize and to evaluate Jatropha meal as a biofeed using biochemical and molecular techniques.
The specific objectives of the study were:

1. To determine the chemical constituents, bioactive compounds and biological activities of Jatropha meal.

2. To determine the effects of physicochemical treatments on the chemical constituents, bioactive compounds concentration and *in vitro* rumen fermentation of Jatropha meal.

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3. To investigate the effects of isolated phorbol esters from Jatropha meal on rumen microbial fermentation, microbial protein synthesis, enzyme activity and rumen microbial populations.

4. To evaluate the potential of rumen microbes in the degradation and detoxification of phorbol esters isolated from Jatropha meal.

5. To investigate the biological and molecular mechanisms of action of isolated phorbol esters from Jatropha meal using animal cell models.

CHAPTER 2

LITERATURE REVIEW

2.1 *Jatropha curcas* Plant

Jatropha curcas is a tall shrub that can grow between 2 to 6 metres in height. This plant begins to bear fruit after 1 year, and the full productive stage is at the age of five years. The fruits of the plant are usually 1.8 cm in length and 1.2 cm in width in average, round-shaped, and on ripening contain three black seeds. After growing for five years, its seed yield ranges from 7.5 to 12 tonnes per hectare per year. The productive life of the plant is 40-50 years (Carels, 2009; Monteiro *et al.*, 2011).

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The plant itself is very sturdy and can be an excellent candidate for re-greening of eroded zones, and for those lands that are not suitable for growing more sensitive and demanding crops (Martinez-Herrera *et al.*, 2006). *Jatropha curcas* seeds contain about 50-60% oil, which makes it a good source of oil for biodisel production. In Malaysia, there is a growing interest to grow this plant in various provinces, such as Sabah, Selangor and Terengganu. The plantation area in Malaysia, at the end of 2008 was estimated at 750,000 acres, and was expected to increase to 1.5 million acres in 2009 and 2.5 million acres in 2010 (Tan, 2008).

There are varieties of *J. curcas* known by their origins, for instance from Nicaragua (Cape Verde and Nicaragua), Nigeria (Ife) and Mexico (Papantla) (Makkar *et al.*, 2009a). However, the different varieties contain different levels of bioactive compounds, as affected by both genotypes and geophysical conditions. Makkar *et al.*

(2009a) categorized the *J. curcas* plant as toxic and non-toxic genotypes, where the seeds of the toxic variety contain phorbol esters, while the non-toxic variety do not.

2.2 Jatropha Meal as Novel Biofeed

For the past decades, several feed additives, such as ionophores, antibiotics, growth promoters, synthetic antioxidants and other chemicals have been used to enhance feed conversion efficiency, increase livestock and aquaculture productivity and decrease pollution and environmental damage arising from animal agriculture industry. However, recently in some countries, the use of most of these feed additives has been banned due to the antibiotic resistance in humans caused by derivatives or residues left in animal products, which has raised concerns about product safety for consumers (Cheftel, 2011). Hence, it is important to introduce natural feed additives containing bioactive compounds to livestock and aquaculture industries to enhance their productivity. In this sense, any type of material, which possesses biological activities towards the improvement of animal health and production are called biofeed.

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Agriculture and food industries annually produce large amounts of waste materials, including bagasse, peels, meals, pulps and husks. Most of these residues have nutritional values and are classified as agro-industrial by-products which are gaining importance as biofeeds. Rapeseed, cottonseed and soybean meals are common by-products of the seed oil industry. These by-products are used as feed for livestock production, in particular the poultry industry, due to their high protein and low fibre content (Koivunen *et al.*, 2006). Chemical analyses of these oilseed meals showed the presence of lignin, cellulose, amino acids, proteins and polyphenolic compounds

together with other bioactive compounds (Vollmann *et al.*, 2009). Some of these compounds contain many functional groups, including carboxyl, hydroxyl and methyl groups, as well as high amounts of fixed anionic and cationic moieties which may offer beneficial biological and therapeutic effects, such as antimicrobial, anti-inflammatory and antioxidant activities.

Gallegos-Tintore *et al.* (2011) reported the presence of bioactive peptides in Jatropha meal possessing free radical scavenging, reducing power and antioxidant activities. Besides, Lihua *et al.* (2004) showed that the leaf extract of *J. curcas* plant possesses an *in vitro* antibiotic effect on chicken pathogens, such as *Escherichia coli* and *Staphlococcus aureus.* Moreover, a recent study has shown the anti-tick activity of Jatropha meal in rabbits (Abdel-Shafy *et al.*, 2010; Nasr *et al.*, 2011).

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2.3 Bioactive Compounds in Jatropha Meal and Their Potential

2.3.1 Phenolics

Phenolic compounds are plant metabolites that are widely spread throughout the plant kingdom. They are a class of chemical compounds comprising one or more hydroxyl groups (-OH) bonded directly to an aromatic hydrocarbon group. The hydroxyl groups are usually the functional moieties that determine the biological activity of each phenolic compound. Phenolics are usually found as esters or glycosides rather than as free compounds (Makkar *et al.*, 2010). Previous research reported the presence of various phenolic compounds in *J. curcas* seed, leaf, root and stem (Khafagy *et al.*, 1977; El Diwani *et al.*, 2009; Manpong *et al.*, 2009). Makkar *et al.* (1998a) reported that, Jatropha meal had a total phenolic content ranging from 0.2

to 0.4% tannic acid equivalent and tannins at 0.02 to 0.04%, tannic acid equivalent whereas condensed tannins were not detected.

Some phenolics are considered to be powerful antioxidants, being more potent than, vitamins E, C and carotenoids (Joshi *et al.*, 2011). Besides, phenolic compounds are also known to have antimicrobial activity against pathogens and they act directly against microorganisms by inhibiting virulence factors. Recently, Monteiro *et al.* (2011) evaluated the anthelmintic activity of the hexane, ethyl acetate and ethanol extracts obtained from the seeds of *J. curcas* against *Haemonchus contortus* larvae. This study demonstrated that the ethanol extract was most promising to inhibit larval exsheathment (99.8%). This activity was attributed to the presence of the total phenolic compounds and tannins with values of 108 mg/g (gallic acid equivalent) and 3.2 mg/g (tannic acid equivalent), respectively.

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The response of rumen microbes to polyphenolic compounds varies. Polyphenolics have been reported to decrease the methanogens activity in the rumen (Animut *et al.*, 2008). Various response inhibitory activity of Sainfoin (*Onobrychis viciifolia*) tannins on the growth of *Butyrivibrio fibrisolvens, Ruminobacter amylophilus* and *Streptococcus bovis* confirmed the different response in rumen bacteria depending on species of microorganism and the type of tannin (Sivakumaran *et al.*, 2004). Zuhainis *et al.* (2008) also observed a reduction in the enzymes and fermentation activities of the rumen fungus *Neocallimastix frontalis* B9 in the presence of various phenolic compounds. Makkar *et al.* (1995) reported that quebracho condensed taninn (0.1–0.4 mg/ml medium) significantly reduced the number of total protozoa, entodiniomorphs and holotrichs. However, many ruminants, such as cattle, goats and

sheep have a high ability to digest phenolic compounds, in particular tannins, to alleviate their negative impacts on animal health. Most of the identified microorganisms contributing to the bio-degradation of tannins in the gut belong to the genus *Streptococcus* (e.g., *S. bovis*, *S. caprinus* and *S. gallolyticus*) (Hiura *et al.*, 2010).

2.3.2 Flavonoids

Flavonoids are widely distributed in the leaves, seeds; bark and flowers of plants. Over 4,000 flavonoids have been identified to date (Xiao *et al.*, 2011c). Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyran rings and classified according to carbon skeleton and substitution. They are a large group of structurally related compounds with a chromane-type skeleton, with a phenyl substituent in the C_2 or C_3 position (Nijveldt *et al.*, 2001). The main flavonoids subclasses are depicted as flavone, flavonol, isoflavone, flavanone, chalcone, anthocyanidin, catechin and flavanonol. The subclasses of flavonoids differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups, and in the conjugation between the A- and B-rings (De Rijke *et al.*, 2006). Some of these compounds, are responsible for the flower colours in certain plants offered protection against ultraviolet radiation, pathogens and herbivores (Harborne *et al.*, 2000). The presence of various flavonoid compounds in *J. curcas* leaf, root and stem has been reported by various researchers (Khafagy *et al.*, 1977; El Diwani *et al.*, 2009; Manpong *et al.*, 2009). Flavonoids are attributed to their antioxidant and chelating abilities (Kay, 2010), antimicrobial activities (Cushnie *et al.*, 2005), anti-inflammatory and anti-cancer properties (Benavente-Garcia *et al.*, 2008).

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Flavonoids in the rumen might be metabolized to more active compounds and their interaction with rumen microbes could be positive or negative (Cushnie *et al.*, 2005). Previously, Broudiscou and Lassalas (2000) reported that isoquercitrin, a flavonoid present in *Equisetum arvense,* increased the amount of acetate and propionate during rumen microbial fermentation in batch culture. Recently Serra *et al.*, (2010) reported that a mixture comprising naringin and bitter orange extract could inhibit acidosis caused by *Streptococcus bovis* through inhibition of its growth rate in the intensive fattening of ruminants. Moreover in another study, daidzein supplementation at 10 mg/kg of DM increased the growth of some rumen bacterial species in goats (Griner *et al.*, 2007). Flavonoids are believed to have direct effects against methanogens (Park *et al.*, 2001), and Tavendale *et al.* (2005) observed the suppression of methane production in a broth culture of *Methanobrevibacter ruminantium* strain DSM 1093 when flavonol was added.

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2.3.3 Saponins

Saponins are phytochemical compounds found in many plant genera. Their role in nature is likely to be in defense against pathogens, pests and predators. Saponins are high molecular weight glycosides in which a triterpene or steroidal aglycone moiety is linked to one or more sugar chains. The presence of both polar (sugar) and nonpolar (steroid or triterpene) groups provides saponins with strong surface-active properties that then are responsible for many of its adverse and beneficial effects (Makkar *et al.*, 2007a). The presence of saponins in Jatropha meal obtained from toxic and nontoxic varieties ranged from 2.6 to 3.4 % diosgenin equivalents (Makkar *et al.*, 2009a). The observation of a very similar content of saponins in the toxic and

non-toxic varieties of *J. curcas* suggested their nutritional safety (Makkar *et al.*, 1998a).

Saponins are known for their antimicrobial, antioxidant and cytotoxic activities (Osbourn *et al.*, 2011), and are also characterized by their hemolytic activity and foaming properties. Bitter taste and astringency of plant materials could be as a result of high concentrations of saponin (Makkar *et al.*, 2007a). The beneficial biological activities of saponins include hypocholesterolemic effects, improvement of growth and production in various animal species, anthelmintic activity, antimicrobial activity, improved reproductive efficiency, and have been comprehensively discussed by Rochfort *et al.* (2008). The inclusion of tea saponin (10, 20, 30 and 40 g/kg substrate) to rumen fluid in *in vitro* gas production tests and incubation for 24 h decreased the protozoa counts significantly by 19, 25, 45 and 79%, and a real-time PCR-based technique also confirmed the antiprotozoal effect of the total saponin fraction (Hu *et al.*, 2005; Guo *et al.*, 2008). However, rumen microbes may adapt to the presence of saponins, as was observed in Ethiopian sheep, which have developed a microbial population in the rumen capable of degrading plant saponins (Wina *et al.*, 2005a).

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2.3.4 Phytic Acid

Phytic acid is the hexaphosphoric ester of the hexahydric cyclic alcohol meso-inositol. Phytic acid or phytate (when in salt form) is the principal storage form of phosphorus in many plant tissues. Phytic acid, because it has a negative charge at a wide range of pH values, shows a strong affinity to bind metal ions, such as calcium, zinc, and iron, which leads to malabsorption of minerals. Moreover, phytate forms a strong complex with some proteins, starchs and lipids, and resists their digestibility

(Makkar *et al.*, 2007a; Kumar *et al.*, 2010a). In addition, phytates have been implicated in decreasing protein digestibility by forming complexes, and by interacting with enzymes such as trypsin and pepsin (Reddy *et al.*, 2002). Recently, phytic acid has been suggested to have a protective role in carcinogenesis, antioxidant properties, hypolipidaemic activity, antiplatelet activity, and to be active against diabetes mellitus, human immunodeficiency virus (HIV), dental caries and renal lithiasis (Kumar *et al.*, 2010a).

Phytic acid in monogastric nutrition is more important than in polygastric animals, since polygastric animals benefit from microbial phytase, which allows digestion of phytate (Tsukatani *et al.*, 2008). *Mitsuokella jalaludinii* and *Selenomonas ruminantium JY35* are representatives of a unique class of phytases, which produce bacteria isolated from the rumen (Chu *et al.*, 2004; Lan *et al.*, 2010). The phytate content of *J. curcas* reported by Makkar *et al.* (2009a) and Martinez-Herrera *et al.* (2006) was in the range of 6 to 10%.

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2.3.5 Lectins

Lectins are proteins of non-immune origin which recognize and bind to specific carbohydrate structural epitopes without modifying them (Nilsson, 2007). Many lectins contain covalently bound sugar moieties and are glycoproteins in nature. They are also called phytohemagglutins because they agglutinate red blood cells (Makkar *et al.*, 2007a). The primary effect of lectins is mediated through binding to the mucosa of the intestinal wall, which results, in a decreased absorption of nutrients. This effect may cause a decreased nutrient digestibility, decreased nitrogen retention, reduced weight gain and a less efficient feed conversion (Huisman *et al.*, 2001).

Lectins are widely distributed in the plant kingdom, and have the unique property of binding to carbohydrate-containing molecules, with a high degree of specificity

toward the sugar component. One obvious manifestation of this property is their ability to agglutinate the red blood cells from various species of animals, as a result of the interaction of multiple binding sites on the lectin molecule with specific glycoconjugate receptors on the surface of the cell membrane (Makkar *et al.*, 2007a). Other physiological effects are the lowering of insulin levels in the blood, inhibition of the disaccharidases and proteases in the intestine, causing degenerative changes in the liver and kidneys. The other effects include increasing the endogenous loss of nitrogen and protein catabolism, and the breaking down of stored fat and glycogen, disturbing the mineral metabolism, interfering with the absorption of non-heme iron and lipid from diets, and also impairing immune systems.

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Jatropha meal contains 51 to 102 U (U: 1/mg of meal that produced hemagglutination per ml of assay medium) for nontoxic and toxic genotyopes, respectively (Devappa *et al.*, 2010a) and this range of lectin content was reported to be similar to that of soybean meal (Makkar *et al.*, 2007b). Plant lectins have been widely studied for their biological and therapeutics properties. They bind selectively to cell membrane receptors and induce cytotoxicity, inhibit tumor cell growth, inhibit protein synthesis by binding to ribosomes, down-regulate telomerase activity, and reduce angiogenesis. Curcain is the lectin fraction isolated from the latex of *J. curcas* and showed wound-healing properties, whereas curcin, isolated from the seed of *J. curcas* appeared to have cytotoxic activity against gastric cancer, mouse myeloma and human hepatoma cell lines with IC_{50} values of 0.15-0.32 mg/L, 0.35-0.97 mg/L, and 2.74-3.58 mg/L, respectively (Devappa *et al.*, 2010a). Baintner *et al.* (1993) studied the binding and degradation rate of 15 plant lectins by rumen microbes and observed that the preferential binding of rumen microbes to lectin contributed to their

various degradation rates. Lectins are also heat labile and easily inactivated by heat treatment (Makkar *et al.*, 2009a).

2.3.6 Trypsin Inhibitors

Trypsin inhibitors are widely distributed in plant seeds. Trypsin inhibitors induce pancreatic hypertrophy/hyperplasia. Concomitant with this increase in the size of the pancreas is an increase in the secretion of digestive enzymes, including trypsin, chymotrypsin and elastase. This led to the hypothesis that the growth depression caused by the trypsin inhibitors is the consequence of an endogenous loss of amino acids in the form of enzymes being secreted by a hyperactive pancreas (Makkar *et al.*, 2007a). The major effects of trypsin inhibitors in monogastric farm animals are the reduction of activity of (chymo) trypsin, pancreas hypertrophy, and the increased secretion of pancreatic enzymes (Machado *et al.*, 2008).

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Makkar *et al.* (1997) reported the similarity of trypsin inhibitor activity of Jatropha meal in both toxic and nontoxic genotypes ranging from 18.4 to 27.3 mg/g DM. Martinez-Herrera *et al.* (2006) reported that the amount of trypsin inhibitor in Jatropha meal ranged from 33.1 to 36.4 mg/g, higher than trypsin inhibitor reported by Makkar *et al.* (1997). Generally, trypsin inhibitors are also prone to heat, and could be inactivated by heat treatment at 121°C for 30 min. Despite the negative nutritional effects of trypsin inhibitors, they have been reported to have antifungal activity, antimalarial activity, antiproliferative activity toward leukemia, lymphoma and ovarian cancer cells, and HIV-1 reverse transcriptase inhibitory activity (Zhu *et al.*, 2011). Carp fish (*Cyprinus carpio*) fed Jatropha meal containing 24.8 mg trypsin inhibited/g did not show any remarkable difference in their growth performance, which indicated their tolerance to the presence of high levels of trypsin (Makkar *et al.*, 1999b). Ruminants are also considered to be less sensitive to plant bioactive

compounds as compared to monogastrics since the rumen microbes are able to degrade trypsin inhibitors, lectins and phytate to a substantial extent (Devappa *et al.*, 2010a).

2.3.7 Phorbol Esters

Phorbol is a natural, plant-derived organic compound. It is a member of the tigliane family of diterpenes. It was first isolated in 1934 as the hydrolysis product of croton oil, which is derived from the seeds of *Croton tiglium*. Phorbol esters are polycyclic compounds in which two hydroxyl groups on neighboring carbon atoms are esterified by fatty acids (Abdel-Hafez *et al.*, 2002) (Figure 2.1). They are very soluble in most polar organic solvents. Phorbol esters are widely distributed in plant genera in the families *Euphorbiaceae* and *Thymelaeaceae*.

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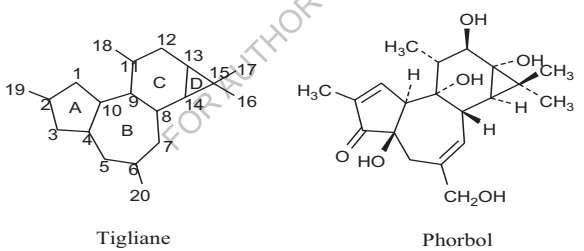

Tigliane Phorbol

Figure 2. 1 Structure of the tigliane nucleus and phorbol

The phorbol esters mimic the action of diacyl glycerol (DAG), activator of protein kinase C (PKC), which regulates different signal transduction pathways and other cellular metabolic activities (Goel *et al.*, 2007). Binding of phorbol esters to PKC is the first step in the activation of PKC, and this binding is saturable and occurs through specific interactions within the C1 domain in the regulatory region of the PKC molecule (Kazanietz, 2005). There are nine PKC genes, which code for

isozymes classified into three groups: classical PKCs (cPKCs: PKC-α, PKC-β I, PKC-β II and PKC-γ), novel PKCs (nPKCs: PKC-δ, PKC-ε, PKC-η and PKC-θ) and atypical PKCs (aPKCs: PKC-ζ and PKC-ι) (Griner *et al.*, 2007). Different phorbol esters may activate different PKC isozymes exerting various biological activities such as growth inhibitory responses, appoptosis, tumorogenesis, or cell differentiation (Figure 2.2).

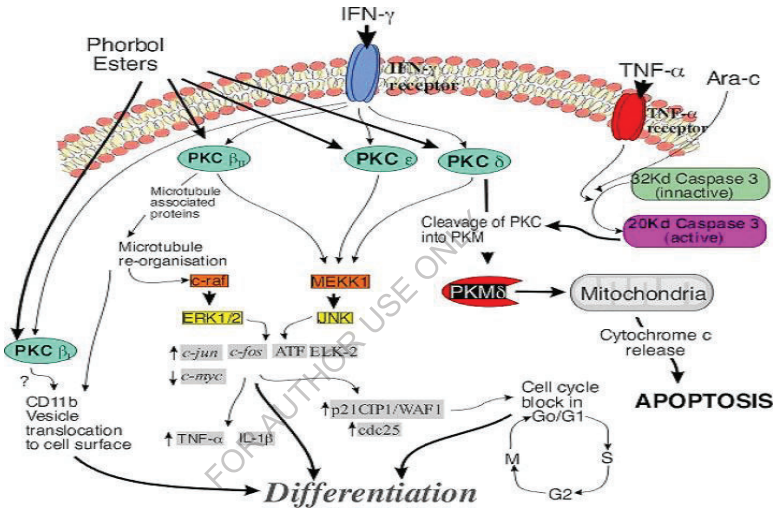

Figure 2. 2 The effects of phorbol esters in the activation of different PKC isoforms involved in differentiation and apoptosis (Hatton *et al.*, 2002)

Previous research indicated that the cellular response to phorbol esters is critically dependant on the experimental conditions as to whether pro-apoptotic or anti-apoptotic effect is observed (Hatton *et al.*, 2002; Gomperts *et al.*, 2009). As shown in Figure 2.2, depending on the experimental conditions, the different type of phorbol ester and its functional groups may activate different PKCs. The activated PKC-β I induces the translocation of CD11b to the cell surface and affects cells differentiation (Figure 2.2). The PKC-β II, PKC-ε and PKC-δ act through activation of proto-

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oncogenes and JNK cascades, followed by TNF-α and IL-1β, and finally affecting cell differentiation (Figure 2.2). Generally, after prolonged exposure phorbol esters the PKC-δ is cleaved to PKM-δ which affects cytochrome c release by the mitochondria leading to apoptosis (Figure 2.2). Amemiya *et al.* (2005) suggested the mechanism of action of phorbol esters through activation of PKC-β, followed by triggering the expression of proto-oncogenes, including c-Fos and c-Jun. Fos family dimerise with c-Jun to form the AP-1 transcription factor, which upregulates the transcription of a diverse range of genes involved in many diverse effects from proliferation and differentiation, to defense against invasion and cell damage. In addition, the overexpression of c-Jun and c-Fos genes may lead to elevation in the expression of COX2 and induction of inflammation (Lee *et al.*, 2007). Gomperts *et al.* (2009) also reported the anti-proliferative and pro-apoptotic signal as a result of prolonged treatment with phorbol-12-myristate 13-acetate (PMA).

The other reported biological activities of phorbol esters include induction of structural changes in the parasite *Leishmania amazonensis* at a PMA concentration of 20 ng/ml of PMA (Chan-Bacab *et al.*, 2001), and antimycobacterial activity of the phorbols isolated from *Sapium indicum* against mycobaterium with the minimum inhibitory concentration between 3.12 and 200 μg/ml (2003).

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2.3.7.1 Jatropha Meal Phorbol Esters

Haas and Mittelbach (2002) confirmed the presence of five phorbol esters in *J. curcas* seed (Figure 2.3). The five phorbol esters were identified and characterised, and appear as four peaks in the high-performance liquid chromatography chromatogram (Li *et al.*, 2010; Devappa *et al.*, 2010a). The phorbol esters are considered to be the most biologically active compounds present in the Jatropha meal (Devappa *et al.*, 2010a).

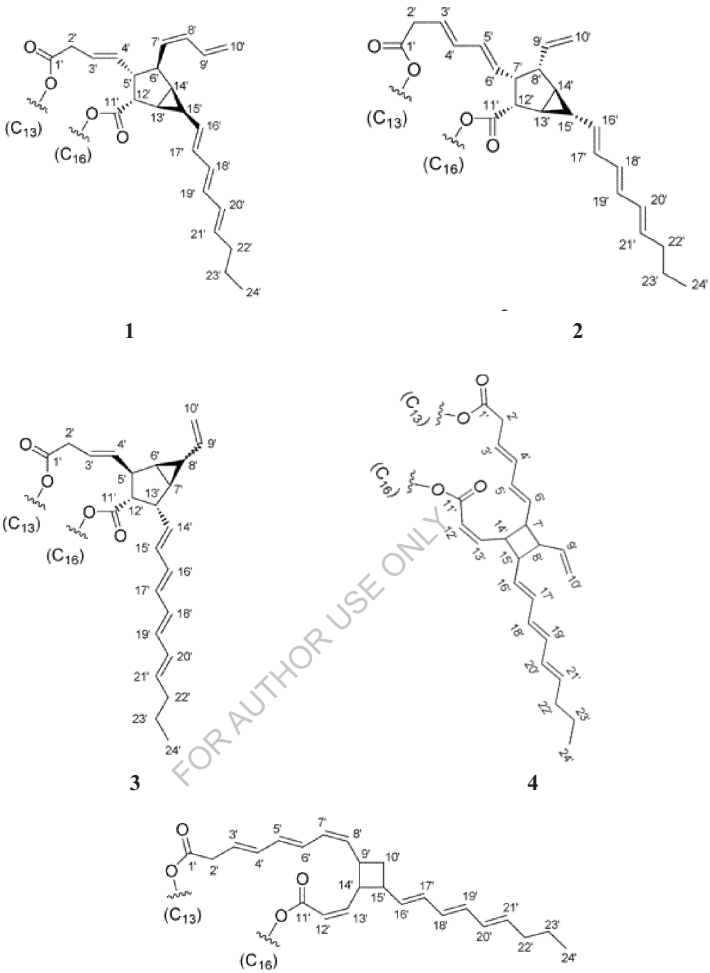

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Figure 2. 3 Phorbol esters of *J. curcas* seed (Haas *et al.*, 2002)

Recently, Saetae and Suntornsuk (2010) reported the antifungal activity of Jatropha meal ethanolic extract against *Fusarium oxysporum, Pythium aphanidermatum, Lasiodiplodia theobromae, Curvularia lunata, Fusarium semitectum, Colletotrichum capsici and Colletotrichum gloeosporiodes*. The phorbol esters were attributed to be the constituents mainly responsible for the antifungal activities observed in this study.

The biodegradation of the phorbol esters from Jatropha meal by soil microorganisms was reported by Devappa *et al.* (2010c). The Jatropha meal containing 0.37 mg/g phorbol esters completely lost the phorbol ester content upon 15 days incubation in soil with 230 g/kg moisture at a temperature of 32 ºC. Isolated phorbol esters at a concentration of 2.6 mg/g soil were completely degraded after 9 days in the soil containing 230 g/kg moisture at 42 ºC. Increasing the temperature and the moisture content increased the rate of phorbol ester degradation.

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In an early study conducted by Horiuchi and Sugimura (1987) the phorbol ester factor I isolated from *J. curcas* seed was shown to possess skin tumor promoting activity, however, Devappa *et al.* (2010a) believed that, the phorbol esters themselves do not induce tumors. In addition, not all phorbol esters are toxic, and their activity is strictly structure dependent with the α form of phorbol being inactive and the β form being active (Goel *et al.*, 2007). Detailed information regarding the biological activity of the phorbol esters present in Jatropha meal has not been elucidated so far.

2.4 Alleviation of Bioactive Compounds in Jatropha meal

2.4.1 Physical Processing

It has been reported that various types of physical processing, including roasting, moist heat treatment (autoclaving) and irradiation have been evaluated for removal of the bioactive compounds present in Jatropha meal (Makkar *et al.*, 1998b; Martinez-Herrera *et al.*, 2006). Overall, heat treatment is necessary to inactivate the trypsin inhibitors and lectins. Adjustment of moisture of the Jatropha meal to 66% and heating at 121ºC for 30 min inactivated the total trypsin inhibitors and lectin content (Makkar *et al.*, 1998a; Makkar *et al.*, 1999b; Aregheore *et al.*, 2003). Moist heat treatment appeared to be the most effective method, compared with irradiation and roasting, in the inactivation of lectin and trypsin inhibitors. However, the content of the saponins, phorbol esters, phytic acid and the total phenolic compounds was not affected.

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2.4.2 Chemical Processing

Aregehore *et al.* (2003) reported the varied efficiency of several treatments on the concentration of phorbol esters from 50% (treatment with sodium hydroxide) to 95% removal (treatment with methanol). The heating treatment at 121ºC, 30 min (with 66% moisture), followed by four times washing with methanol removed the phorbol esters, however, it was not an economically viable option for commercial processing (Makkar *et al.*, 1998b). Similarly, Martinez-Herrera *et al.* (2006) also reported the high efficiency of on phorbol ester removal (98%) through a solvent extraction procedure.

Xiao *et al.* (2011b) reported that, a combination of hydrolytic enzymes (cellulase plus pectinase) and washing with ethanol (65%) could completely remove the phorbol esters. The phytates, phenolic compounds, saponins, trypsin inhibitors and lectin were decreased to acceptable levels, which are lower than those in soybean meal. Kumar *et al.* (2010b), Wang *et al.* (2011) and (Harter *et al.*, 2011) reported the full deactivation of lectin and phorbol esters by extracting with 90% methanol-sodium hydroxide, followed by steam treatment (103.4 kPa and 121°C for 15 min). Recently, Katole *et al.* (2011) soaked the Jatropha meal with sodium chloride, sodium hydroxide and calcium hydroxide (over night), followed by roasting (100°C for 30 min). The lectins were fully inactivated, but the phorbol ester content was reduced to 85.0, 75.0 and 83.2% using sodium chloride, sodium hydroxide and calcium hydroxide, respectively.

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2.4.3 Biological Processing

Solid-state fermentation (SSF) has been developed for the biodegradation and biodetoxification of some bioactive compounds in agro-industrial products and wastes (Pandey *et al.*, 2008). Degradation of the Jatropha meal phorbol esters by *Pseudomonas aeruginosa* PseA strain during solid state fermentation (SSF) has been reported by Joshi *et al.* (2011), although the safety of the new compounds produced as a result of phorbol esters biodegradation have not been investigated by these researchers. In a similar study, Abdel-Hafez *et al.* (2002) reported the potential of several isolates from human intestine, such as *Bifidobacterium adolescentis, Clostridium butyricum, C. innocuum* ES 24-06, *Peptostreptococcus anaerobius* 0240, *P. intermedius* EBF 77/25 and *Proteus mirabillis* S2 in the degradation of more than 90% of the phorbol esters purified from croton oil.

De Barros *et al.* (2011) reported the fermentation of Jatropha meal with *Ganoderma resinaceum*, *Bjerkandera adusta* and *Phlebia rufa* and observed a reduction of phorbol ester content by 20%, 91% and 97%, respectively. Efficiency in the degradation of the phorbol esters by white-rot-fungi was attributed to the enzymatic reactions which occurred during the colonization stage.

2.5 *In Vitro* Digestibility of Jatropha Meal

The *in vitro* rumen digestibilities of Jatropha meal reported for two varieties (Cape Verde and Nicaragua) and for the non-toxic provenance from 24 h of gas production were found to be 78.0%, 78.0%, and 78.4% for the organic matter, and 10.9, 10.7, and 10.8 MJ/kg for the metabolisable energy, respectively (Makkar *et al.*, 1997). Heat treatment caused Maillard reactions, and was useful to suppress the *in vitro* rumen protein degradability of Jatropha meal. Aderibigbe *et al.* (1997) observed the suppression of the *in vitro* digestibility of nitrogen in Jatropha meal upon heating at 160°C for 120 min from 73.3% to 38.6%.

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Jatropha meal is rich in protein, but low in *in vitro* rumen degradable protein as compared to soybean. The presence of more than 50% glutelin in the Jatropha meal leads to the low solubility of Jatropha meal protein in the rumen. Hence, a high amount of rumen undegradable protein available post-ruminally could be utilized by gastric digestion which would enhance polygastric production (Devappa *et al.*, 2010b).

The phorbol esters are considered to be the most biologically active compounds present in Jatropha meal (Devappa *et al.*, 2010a). In spite of the biodegradability

(Abdel-Hafez *et al.*, 2002; Oliveira *et al.*, 2008) and antimicrobial activity (Chumkaew *et al.*, 2003; Saetae *et al.*, 2010) of the phorbol esters, Makkar and Becker (2010) reported that rumen microbes do not degrade phorbol esters present in Jatropha meal, and phorbol esters do not adversely affect rumen fermentation.

The effects of heat treatment in the inactivation of lectin and trypsin inhibitors are well documented. However, the saponins, phorbol esters and phenolic compounds alleviation varied depending on the treatment methods used. The effects of Jatropha meal bioactive compounds and their alleviation on improvement of rumen fermentation have not been extensively studied. Probably, the response of the rumen fermentation to the broad effects of treatments on alteration of chemical constituent was much greater to hide the effects of bioactive compounds alleviation on rumen fermentation.

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2.6 Toxicity and Biological Activity

2.6.1 Animal Trials

The effects of feeding Jatropha seeds containing phorbol esters have been studied extensively in different animal species, including goats, sheep, mice, rats, and fish (King *et al.*, 2009). Emerging literature shows that the direct feeding of Jatropha meal or processed meal shows different physiological effects in different organisms as a result of the different levels of bioactive compounds. The direct feeding of Jatropha meal produced mild to severe symptoms in various organs of different animals.

Young Nubian goats (5−8 months old; 8−22 kg body weight) when fed with 5 and 10 g/kg/d Jatropha seed showed symptoms of toxicity and mortality. The goats showed lack of appetite, reduced water consumption, diarrhea, dehydration, and sunken eyes, as well as decreased glucose levels and increased serum arginase and glutamate oxaloacetate transaminase (GOT) activities as their clinical signs. An investigation of their visceral organs revealed hemorrhage in the rumen, reticulum, kidneys, spleen, and heart, catarrhal or hemorrhagic abomasitis and enteritis, congestion and edema of the lung, and excessive fluid in serous cavities (Adam *et al.*, 1975).

Further, Ahmed and Adam (1979) reported an increase in the serum aspartate aminotransferase (AST) activity, increase in urea concentration, and decrease in total protein and albumin levels, decrease in hemoglobin, percent cell volume, and red blood cells when the sheep and Nubian goats were fed with *Jatropha* seeds at 0.05, 0.5, and 1 g/kg/d.

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Katole *et al.* (2011) conducted a feeding trail with sheep using soybean meal (control) and Jatropha meal treated with sodium chloride and calcium hydroxide as protein sources. Their results showed the dry matter, organic matter, crude protein intake and blood metabolites including packed cell volume, serum albumin, glucose, serum urea, triiodothyronine, thyroxine, testosterone and alkaline phosphatase activity contents decreased in sheep fed treated Jatropha meal when compared to control. The lactate dehydrogenase and aspartate amino transferase activities, also increased in these groups compared to the control. Although, the phorbol ester content was markedly decreased to 0.3 mg/g DM by processing, but this was not sufficient to reach a safe level for feeding sheep.

2.6.2 Animal Cell Models

Lin *et al.* (2003) reported that *J. curcas* kernel possessed antiproliferative activity against different cancer and normal cell lines. The *J. curcas* phorbol esters were also reported to exert cytotoxic properties in several cancer cell model studies (Ali *et al.*, 2009; Devappa *et al.*, 2010a). Aiyelaagbe *et al.* (2011) reported the strong growth inhibition potential of *J. curcas* root extract against L5178y mouse lymphoma and HeLa human cervix carcinoma cell lines, while they cause none or only very low activity against neuronal cells (PC12).

Different varieties of Jatropha contain different levels and types of bioactive compounds responsible for the various biological activities, which could determine the beneficial effects to the animal that consume them. Hence, identification of these compounds present in the local Jatropha meal and their effects have to be evaluated before its application as a biofeed is elucidated.

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CHAPTER 3

BIOACTIVE COMPOUNDS AND BIOLOGICAL ACTIVITIES OF JATROPHA MEAL

3.1 Introduction

Agriculture and food industries annually produce large amount of wastes as agro-industrial by-products possessing beneficial biological activities for both humans and animals. Jatropha meal is a common by-product of the biodiesel production industry. This by-product is used mainly as a fertilizer and feed ingredients for livestock production due to its high protein content (Makkar *et al.*, 2009a). Chemical analyses of this meal showed the presence of lignin, cellulose, amino acids and proteins (Martinez-Herrera *et al.*, 2006). The major bioactive compounds in Jatropha meal depending on the genotypes, are phenolics, saponins, trypsin inhibitors, phytic acid, lectins and phorbol esters (Martinez-Herrera *et al.*, 2006), with known biological activities (Rathee *et al.*, 2009). However, the information on the bioactive compounds and biological activities of Jatropha meal obtained from local *Jatropha curcas* plant remains scarce. Therefore, this research was performed to characterize the bioactive compounds and to determine the biological activities of the local Jatropha meal which would determine its possible applications as biofeed.

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3.2 Materials and Methods

3.2.1 Plant Materials

The *J. curcas* L. plant material was collected from the farm of Faculty of Agriculture, Universiti Putra Malaysia with the GPS location of 3°0'26.91"N latitude and 101°42'13.24"E longitude for identification by Mr. Shamsul Khamis. A voucher

specimen (SK1764/2010) was deposited in the Phytomedicinal Herbarium, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. Upon confirmation of the plant, the mature *J. curcas* seeds were collected. The *J. curcas* plants originated from four states in Malaysia, Sabah, Selangor, Terengganu and Johor Bahru, which were planted at the farm of Faculty of Agriculture, Universiti Putra Malaysia. The age of the plants was about four years. The seeds were collected from at least 20 plants, on three occasions, and each time at least two kilograms were sampled. The seeds, air dried, dehulled, packed, labelled based on their place of origin (Sabah, Selangor, Terengganu and Johor Bahru) and dates of collection and kept at -20ºC for further analysis. The isolated kernels were ground, using a mechanical grinder, and the oil was extracted by Soxhlet apparatus, using petroleum ether (boiling point of 40–60ºC) for 16 h to prepare the Jatropha meal (Martinez-Herrera *et al.*, 2006).

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3.2.2 Reagents

Folin-Ciocalteu reagent, gallic acid, rutin, ascorbic acid, alpha-tocopherol, butylated hydroxytoluene (BHT), 1.1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Fisher Scientific, USA. Sodium phytate, Amberlite AG1–X8 (200–400 mesh), diosgenin, benzyl-DL-arginine-*para*-nitroanilide (BAPNA), trypsin, acid washed sand, phorbol-12-myristate 13-acetate (PMA), Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, sulfanilamide, naphthyl ethylene diaminedihydrochloride, N-ω-nitro-L-arginine methyl ester (L-AME), and lipopolysaccharide (LPS) were obtained from Sigma (Germany) and interferon gamma (IFN-γ) was purchased from eBioscience (USA). The reverse-phase C18 (LiChrospher 100, end-capped 5 μm) 250 x 4 mm column was from Agilent (Agilent

Germany). The other chemicals used in this study were obtained from Merck (Darmstadt, Germany).

3.2.3 Chemical Analyses

Analyses of dry matter (DM), organic matter (OM), ash and crude protein (CP) in Jatropha meal were performed according to AOAC (1990) standard procedures. The neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined as described by Van Soest *et al.* (1991). The gross energy value of Jatropha meal was determined using a bomb calorimeter (Parr 1281, Parr Instruments Company, Moline, Illinois). All analyses were performed in triplicate.

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3.2.4 Total Phenolic Compounds

The methanolic extract for Jatropha meal was obtained by a hydrolytic extraction technique according to Crozier *et al.* (1997). Briefly, 0.5 g of Jatropha meal powder was weighed and placed in a 100 ml conical flask. Forty milliliter of absolute methanol was added, followed by 10 ml 6 M HCl to hydrolize the glycosidic bonds and release the phenolic compounds. The mixture was stirred using a magnetic stirrer. The mixture was transferred to a sample flask (250 ml) and refluxed for 2 hours at 90ºC. The mixture was filtered using Whatman No.1 filter paper (Whatman, England) and the filtrate evaporated a using rotary evaporator (Buchii, Switzerland) at 40ºC. The crude extract was then re-dissolved in 5 ml of methanol and used for analyses. The amount of total phenolics in the extract was determined using Folin-Ciocalteu's reagent (Halici *et al.*, 2005). Different concentrations of gallic acid in methanol as standard were prepared (0, 100, 150, 200, 250 and 300 µg/ml). Five hundred microliters of Jatropha meal crude extract and gallic acid in different

concentrations were transferred into different tubes, followed by the addition of 2.5 ml of Folin-Ciocalteu reagent (diluted 1: 10) and 2.0 ml of 7.5% of sodium carbonate (w/v). The mixture was vortexed and incubated at 30ºC for 90 minutes in the dark. The absorbance of the mixture was read at 765_{nm} using a spectrophotometer (Molecular Devices, USA). The amount of total phenolic compounds in the sample was calculated using the calibration curve of gallic acid standard (Appendix A) and the results expressed as mg gallic acid equivalents (GAE)/g DM of Jatropha meal.

3.2.5 Total Flavonoid Compounds

The amount of total flavonoids in Jatropha meal was determined based on the aluminum chloride colorimetric assay (Ismail *et al*., 2010). The crude extract was prepared based on the method described in section 3.2.4. Different concentrations of rutin in methanol as standard were prepared (0, 100, 150, 200, 250 and 300 µg/ml). An aliquot (0.1 ml) of extract or standard was added to 0.3 ml 5% $NaNO_2$. After 5 min, 0.3 ml 10% $AlCl_3$ was added. At 6 min, 2 ml 1 M NaOH was added and the total volume was made up to 5 ml with distilled water. The solution was mixed well and the absorbance was measured at 510_{nm}. The amount of total flavonoids in the Jatropha meal was calculated using the calibration curve of rutin (Appendix A) as standard, and the results expressed as mg rutin equivalents/g DM of the Jatropha meal.

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3.2.6 Total Saponins

Total saponins were determined according to Makkar *et al.* (1997a). Briefly, 0.5 g of sample was stirred overnight in 10 ml of 80% aqueous methanol using a magnetic stirrer. The contents were centrifuged at 3000×g for 10 min and the supernatant

collected in a 25 ml volumetric flask. The residue was washed three times with 5 ml of 80% aqueous methanol, followed by centrifugation. The supernatants were collected in the same volumetric flask and made up to volume with 80% aqueous methanol. An aliquot (0.5 ml) was taken for the total saponin determination. Different concentrations of diosgenin (0, 25, 50, 75, 100, and 125 µg) as standard were prepared (Appendix A). Extracted saponins or standard solutions were mixed with 0.25 ml of vanillin reagent (8%), and 2.5 ml of 72% (v/v) sulfuric acid. The tubes were mixed and transferred to a water bath at 60°C. After 10 min, the tubes were cooled in ice-cold water for 3 to 4 min, and the absorbance was measured at 544_{nm} against the reagent blank (without diosgenin standard solution). The results were expressed as diosgenin equivalents/g DM of Jatropha meal.

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3.2.7 Phytic Acid

The phytic acid was determined according to the method of Makkar *et al.* (2007a). Five grams of ground plant material was mixed with 100 ml of 3.5% HCl for 1 h at room temperature using a magnetic stirrer. The content was centrifuged at 3000×*g* for 10 min at room temperature and the supernatant was collected. The supernatant was diluted 25 times with distilled water. Ten milliliters of the diluted sample extract were passed through an anion exchange column (0.5 g of Amberlite AG1–X8, 200-400 mesh in 0.75 cm × 25 cm column). Inorganic phosphorus and other interfering compounds were eluted with 15 ml of 0.1 M NaCl, and subsequently the phytate was eluted with 15 ml of 0.7 M NaCl (Latta *et al.*, 1980). Three milliliters of the eluted sample was taken in a separate test tube and 1 ml of Wade reagent (30 mg of $FeCl_3.6H_2O$, 300 mg of sulfosalicylic acid dissolved in 100 ml distilled water) was added. The mixture was vortexed and then centrifuged at 3000×*g* for 10 min. The

absorbance value was measured at 500_{nm} against a reagent blank. Sodium phytate was used as a standard since it was soluble in water and does not require conversion to free phytic acid. A series of standard solutions containing 5 to 40 µg/ml phytic acid in distilled water was prepared. Three milliliters of the standard solution were pipetted into 15 ml centrifuge tubes. The blank tube contained three milliliters of distilled water. One milliliter of the Wade reagent was added into each tube. The solution was vortexed for 5 sec. The mixture was centrifuged at 3000×*g* for 10 min and the absorbance of the supernatant was read at 500_{nm} by using blank to zero the spectrophotometer (Vaintraub *et al.*, 1988).

3.2.8 Lectins

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The haemagglutination assay was used to determine the lectin content according to Makkar *et al.* (2011) using round-bottom well microtitre plates with 1% (v/v) trypsinised cattle erythrocyte suspension in phosphate buffer saline (PBS). The Jatropha meal (0.5 g) was weighed and added to 10 ml of the PBS, and stirred using a magnetic stirrer for 16 h at 4°C. The content was centrifuged at 4000×g for 20 min and the supernatant collected was passed through Whatman No. 540 filter and the filtrate used for the assay (Aregheore *et al.*, 1998). Bovine blood was collected in order to prepare the standard trypsinized erythrocyte suspension. An equal volume of Alsever's solution (dissolve 2.05 g of glucose, 0.8 g of sodium citrate, and 0.42 g of NaCl in 100 ml of distilled water, pH 6.1) containing 1/30 volume of the anticoagulant (dissolve 8 g of sodium citrate in 54 ml of 37% formaldehyde and 100 ml saline) was added and the mixture was centrifuged at room temperature using a clinical centrifuge (1500×g, 5 min). The supernatant was discarded and the erythrocytes were washed three times with saline solution (approximately 5 ml of

saline for each milliliter of packed erythrocytes). The PBS was added to the packed erythrocytes to give a suspension with an absorbance of 2 at 620_{nm}. One milliliter of 1% trypsin (10 mg of trypsin in 1 ml PBS) was added to 10 ml of erythrocytes suspension solution, and the mixture was incubated at 37ºC for 1 h. The trypsinized erythrocytes were washed four times with saline to remove the last traces of trypsin, and then suspended in sufficient saline to give a standard erythrocyte suspension with an absorbance of 1 at 620_{nm} (Liener, 1955).

The Jatropha meal extract was serially diluted with PBS and mixed with an equal volume of 1% erythrocytes (final volume 0.1 ml) in microtitre plates. Sedimentation patterns of erythrocyte suspension were read after 2.5 hour at room temperature. A positive pattern, indicating agglutination, was uniform effacement of the bottom of the well by erythrocytes, and a negative pattern, indicating no agglutination, was a circular clump of erythrocytes surrounded by a concentric, clear zone of the size equal to the blank. Blank consisted of PBS and erythrocyte. The hemagglutination activity was reported as unit (U) where U is 1 mg of Jatropha meal that produced hemagglutination per milliliter of assay medium.

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3.2.9 Trypsin Inhibitors

In this method, the trypsin inhibitors activity was measured indirectly by inhibiting the activity of trypsin. A synthetic substrate, benzyl-DL-arginine-*para*-nitroanilide (BAPNA) was subjected to hydrolysis by trypsin to produce the yellow-colored *p*-nitroanilide. The degree of trypsin inhibitory activity of Jatropha meal extract was measured at 410_{nm} using a spectrophotometer. Trypsin inhibitor activity was expressed as milligrams of pure trypsin inhibited/g DM sample (Makkar *et al.*,

2007a). The Jatropha meal was ground to pass through a sieve of 40 mesh size. One gram of the sample was mixed with 50 ml of 0.01 M NaOH and stirred using a magnetic stirrer at low setting for 3 h at room temperature. The pH of the suspension was adjusted to pH 9.5. This suspension was diluted to the point where 1 ml of the diluted suspension produced trypsin inhibition of 40% to 60% in order to reduce the relative standard deviation (Kakade *et al.*, 1969). The following solutions were pipetted into a series of 10 ml tubes, (a) reagent blank: 2 ml of distilled water; (b) standard: 2 ml trypsin solution (20 mg of trypsin dissolved in 1 L of 0.001 M HCl) and 2 ml distilled water; (c) sample blank: 1 ml diluted sample extract and 1 ml distilled water; (d) sample: 1 ml diluted sample extract, 1 ml distilled water and 2 ml trypsin solution. After mixing and preheating at 37°C for 10 min, 5 ml of prewarmed (37°C) BAPNA solution (40 mg of BAPNA hydrochloride dissolved in 1 ml DMSO and diluted to 100 ml with tris-buffer [tris-buffer: 6.05 g tris-hydroxymethylamino methane and 2.94 g $CaCl_2.2H_2O$ dissolved in 1 L distilled water and pH adjusted to pH 8.2]) was pipetted into each tube and mixed. After exactly 10 min incubation at 37°C, 1 ml of acetic acid (30%) was added to each tube to stop the reaction. Then the trypsin solution (2 ml) was added to the reagent blank and sample blank tubes. After centrifugation (3000×*g* for 10 min at room temperature), the absorbance of the clear solution was read at 410_{nm} using a spectrophotometer (Molecular Devices, USA) (Smith *et al.*, 1980).

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The change in absorbance (A_I) due to trypsin inhibitor / milliliter of diluted sample extract was calculated according to the formula:

$$A_I = (A_b - A_a) - (A_d - A_c)$$

where the subscripts refer to tubes (a) to (d) as above. The percentage inhibition in each sample tube was given by:

$$A_I / (A_b - A_a).$$

If this value was less than 40% or greater than 60%, the assay was repeated, and a more suitable dilution of the sample suspension prepared. This reduced the relative standard deviation. Since 1 µg pure trypsin would give an absorbance of 0.019, the weight of pure trypsin inhibited/ ml diluted sample extract is $A_I/0.019$ µg. From this value, the trypsin inhibitor activity (TIA) was calculated in terms of milligrams of pure trypsin/g sample as weight (mg/g).

$$TIA = (2.632\ D\ A_I)/S$$

where D is the dilution factor, and S is the sample weight (g).

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3.2.10 Total Phorbol Esters Content

The total phorbol esters content was determined according to Makkar and Becker (2007a). Three grams of Jatropha meal were ground in a pestle and mortar with 200 mg of acid washed sand. Twenty milliliters of absolute methanol were added, and the mixture was ground again for 5 min in the pestle. The material was allowed to settle and then the liquid phase filtered using a filter paper. About 20 ml of methanol was transferred to the pestle and the mixture was ground again for about 5 min using the pestle and mortar. The material was allowed to settle and the liquid phase was filtered. This extraction procedure was repeated three more times. The filtrates were pooled from all five extractions. The residue containing sand plus Jatropha meal was transferred into a beaker. Fifty milliliters of methanol was added and the content subjected to ultrasonic waves (105 W for 3 min). The suspension was filtered and the filtrate was pooled with the pooled filtrates from the previous five extractions. The

solvent for the pooled filtrate was removed under vacuum at a temperature not exceeding 40°C. The dried residue was dissolved in 5 ml of tetrahydrofuran, passed through a 0.2 µm nylon filter, and a volume of 10 µl was injected into the high performance liquid chromatography (HPLC).

The HPLC was equipped with an analytical reverse-phase C18 (LiChrospher 100, end-capped 5 µm) 250 x 4 mm column, protected with a guard column (Agilent). Analysis was started with 40% of solvent A (1.75 ml of ortho-phosphoric acid (85%) dissolved in 1 L of distilled water) and 60% of solvent B (acetonitrile). Solvent A was decreased to 25% and solvent B increased to 75% in the next 20 min. Then solvent A was decreased to 0% and solvent B increased to 100% in the next 8 min, and maintained for the next 2 min. Finally, the column was washed with solvent C (tetrahydrofuran) by increasing it to 100% in the next 3 min and maintaining it for the next 1 min. The column was adjusted to the starting conditions (40% solvent A and 60% solvent B) in the next 2 min, and this condition was maintained for the next 2 min before injecting another sample. Separation was performed at room temperature (25°C) at a flow rate of 1.3 ml/min. Four phorbol esters peaks appeared between 24 and 27 min. The four peaks were integrated at 280_{nm}, and the results were expressed as equivalent to phorbol-12-myristate 13-acetate, which appeared at 31.5 min.

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3.2.11 HPLC Analysis of Phenolic and Flavonoid Compounds

The phenolic and flavonoid compounds of Jatropha meal were quantitatively measured by HPLC based on the method described by Crozier *et al.* (1997) with some modifications. Phenolic standards were gallic acid, syringic acid, vanillic acid,

salicylic acid, caffeic acid and pyrogallol. Flavonoid standards used in this research were quercetin, rutin, myricetin, kaempferol, naringin and apigenin, and the isoflavonoid standards were genistein and daidzein. Each standard was prepared at different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml) using methanol as the solvent. The methanolic extract was prepared according to the method described in section 3.2.4 and passed through 0.2 µm syringe filter. An aliquot of Jatropha meal extract was loaded on the HPLC equipped with an analytical column Intersil ODS-3 (5µm 4.6x150 mm, Gl Science Inc). Solvents comprising deionized water (solvent A) and acetonitrile (solvent B) were used. The pH of water was adjusted to 2.5 using concentrated trifluoroacetic acid. The phenolic and isoflavonoid compounds were detected at 280_{nm} while flavonoid compounds were detected at 350_{nm}. The column was equilibrated with 85% solvent A and 15% solvent B. The ratio of solvent B was then increased to 85% over 50 min followed by reducing solvent B to 15% over 55 min. This ratio was maintained for 60 min for each sample analysis with a flow rate of 0.6 ml/min. The analysis was repeated three times.

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3.2.12 Gas Chromatography - Mass Spectrometry Analysis (GC-MS)

The organic compounds present in Jatropha meal extracts were characterized using GC-MS based on the method described by Hossain and Rahman (2011). The methanolic extract was prepared according to the method described in section 3.2.4 while the hot water extract was prepared according to a modified method of Gulcin *et al.* (2004). Five gram samples were mixed with 100 ml boiling water and boiled for 15 min while stirring on the hot plate. Then, the extract was filtered using filter paper (Whatman No. 1). The filtrate was evaporated using a vacuum rotary evaporator (Buchii, Switzerland). The GC-MS used was a Schimadzu QP2010PLUS

system. Six microliters were analysed on a BPX-5 SGE ultra-low-bleed 5% phenyl polydimethylsiloxane capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness). Splitless injection was performed with a purge time of 1 min. The carrier gas was helium at a flow rate of 1 ml/min. The column temperature was maintained at 50°C for 3 minutes, then programmed at 5°C/min to 80°C, and then at 10°C/min to 340°C. The inlet and detector temperatures were 250°C and 340°C, respectively, and the solvent delay was 4 min. The identification of the peaks was based on computer matching of the mass spectra with the National Institute of Standards and Technology (NlST 08 and NIST 08s) library, and by direct comparison with published data. The analyses were carried out in triplicate.

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3.2.13 Antioxidant Activity

3.2.13.1 DPPH Scavenging Activity

The methanolic and hot water extracts were prepared according to the methods described in section 3.2.4 and 3.2.12, respectively. The scavenging activity of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical was evaluated according to the method described by Gulcin *et al.* (2004). Briefly, a 0.1 mM solution of DPPH in methanol was prepared, and 3 ml of this solution was added to 1 ml of extract at different concentrations (100, 150, 200, 250 and 300 µg/ml). The mixture was shaken vigorously for a few seconds and incubated at room temperature for 30 min. The absorbance was measured at 517_{nm} using a visible spectrophotometer (Novaspec II Visible spectro). A lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Vitamin C, butylated hydroxytoluene (BHT) and β-carotene were used as standard antioxidants. The DPPH radical scavenging activity was calculated using the following equation:

DPPH Free radical scavenging activity (%) = $[(A_0 - A_1)/ A_0] \times 100\%$

Where A_0 was the absorbance of the control (Methanol+DPPH) and A_1 was the absorbance of the sample.

3.2.13.2 Ferric-Reducing Antioxidant Power (FRAP) Assay

The methanolic and hot water extracts were prepared according to the methods described in sections 3.2.4 and 3.2.12, respectively. The ferric reducing properties of the extracts were determined using the assay described by Yen and Chen (1995). One milliliter of extract containing 100, 150, 200, 250, and 300 µg crude extract was mixed with 2.5 ml of potassium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1g/100 ml). The mixture was incubated at 50ºC for 20 min. Trichloroacetic acid (10%) was added to the mixture to stop the reaction. An equal volume of distilled water was added, followed by 0.5 ml ferric chloride (0.1g/100 ml) ($FeCl_3$). The tubes were incubated at room temperature for 30 min and the absorbance was measured at 700_{nm} using a spectrophotometer (Novaspec II Visblespectro). The assay was performed in triplicate. Vitamin C, butylated hydroxytoluene (BHT) and β-carotene were used as standard antioxidants. The percentage of antioxidant activity of the samples in the FRAP assay was calculated according to the following formula:

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Antioxidant Activity (%) = $[(A_1-A_0)/ (A1)] \times 100$

A_0 = Absorbance of the control (potassium phosphate buffer + FRAP reagent)

A_1 = Absorbance of sample

3.2.14 Antibacterial Activity

3.2.14.1 Extraction

The methanolic and hot water extracts were prepared according to the methods described in sections 3.2.4 and 3.2.12, respectively. Both methanolic and hot water crude extracts were re-dissolved in 5 ml of dimethyl sulfoxide (DMSO) to be used for the antibacterial test.

3.2.14.2 Pathogenic Bacteria

The antibacterial activity of Jatropha meal extracts were evaluated by the disc diffusion method as described by Boussaada *et al.* (2008) against selected animal pathogenic bacteria including Gram positive (*Micrococcus luteus*, *Bacillus subtilis* B145, *Bacillus cereus* B43 and *Staphylococcus aureus* S1431) and Gram negative (*Enterobacter aerogenes*, *Klebsiella pneumonia* K36, *Escherichia coli* E256 and *Pseudomonas aeruginosa* PI96) bacteria. All of the bacteria species were purchased from the Institute of Malaysian Research (IMR) and maintained in the Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. The positive control without extracts (solvent) and the reference controls using two standard antibiotics (kanamycin and streptomycin) were included in the assay. The inhibitory effects of the extracts were corrected based on negative control (DMSO) and compared with those of reference control (antibiotics). The experiments were conducted in triplicate.

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3.2.14.3 Disc Diffusion Method

Nutrient broth (Merck) medium was prepared by dissolving 8 g of nutrient broth in 1L of distilled water and the pH was adjusted to 7. Three milliliters of the broth was then dispensed into each bijou bottle and autoclaved at 121^{o}C for 15 minutes. The

nutrient agar medium (Merck) was prepared by dissolving 24 g of nutrient agar in 1L distilled water and the pH was adjusted to 7. The medium was autoclaved for 15 minutes at 121°C. Twenty milliliters of the medium were then poured into each sterile petri dish and allowed to cool and solidify at room temperature before being used. All of the microorganisms mentioned above were incubated at 37°C for 24 h by inoculation into the nutrient broth. The culture suspensions were prepared and adjusted to approximately 10^5 c.f.u. /ml. One hundred microliters of the each culture were spread over plates containing sterile nutrient agar and paper discs (diameter 6 mm) impregnated with 10 µl (1 and 1.5 mg/disc) of each extracts (methanol and hot water) and placed on the surface of the media. The plates were left for 30 min at room temperature to allow the diffusion of the extracts, and then incubated at 37°C for 24 h. The inhibition zone around the disc was measured. Inhibition potential of different extracts against particular bacterial species were interpreted as showing weak, slight, moderate, high or strong activity following the ranges suggested by Vaquero *et al.* (2007) as presented in Table 3.1. The bacterial species that showed an inhibition zone of 0.1 cm (exclusive of paper disc) and above were considered to be susceptible to the extract.

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Table 3. 1. Range of inhibition zones used in the susceptibility tests of crude extracts from Jatropha meal*

Inhibition zone[a]	Inhibition zone[b]	Interpretation
< 0.1 cm	no inhibition (NI)	no activity
0.1 cm	0.8-0.89 cm	weak antimicrobial activity
0.2-0.3 cm	0.9-1.09 cm	slight antimicrobial activity
0.4-0.5 cm	1.1-1.29 cm	moderate antimicrobial activity
0.6-0.9 cm	1.3-1.6 cm	high antimicrobial activity
> 0.9 cm	> 1.6 cm	strong antimicrobial activity

[a] -disc diameter excluded
[b]- disc diameter included.
* Vaquero *et al.* (2007)

3.2.15 Anti-inflammatory Activity

3.2.15.1 Extract Preparation

The methanolic and hot water extracts were prepared according to the methods described in sections 3.2.4 and 3.2.12, respectively. The crude extracts were dissolved in DMSO and passed through a 0.2 µm sterile syringe filter. The stock solutions (100 mg/ml) of Jatropha meal extracts were diluted using DMEM without phenol red to yield a working concentration of 200 µg/ml and 0.2% (v/v) DMSO.

3.2.15.2 Cell Culture

The murine monocytic macrophage cell line RAW 264.7 (European Cell Culture Collection, CAMR, UK) was routinely maintained, subcultured and passaged in Dulbecco's Modified Eagle's Media (DMEM) containing 10% fetal bovine serum (FBS) and kept at 37ºC in a 5% CO_2 humidified incubator. All of the animal cell culture experiments were performed in Biohazard cabinet type II (Telstar Bio-II-A, Spain) to prevent contamination.

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3.2.15.3 Cell Seeding and Stimulation

At the confluency of 90%, the cells were scraped using a 24 cm scraper (TPP, Europe) and centrifuged at 1000×g for 10 min. Cells were resuspended in 1 ml DMEM and the viable cells were counted using a standard trypan blue cell counting technique. The cell population was adjusted to 1 x 10^6 cells/ml using diluent B (Table 3.2). Fifty microliters of cells were suspended into each of the wells of a 96 well plate, except for the blank. Then the 96-well plate was incubated at 37ºC for 2 hours. After 2 hours, the unattached cells were removed from the wells. Fifty µl of diluent C containing interferon-gamma (IFN-γ) and LPS (bacterial

lipopolysaccharide (Table 3.2) was added into each of the wells, excluding the blank and the negative control. Later, 50 µl of diluted Jatropha meal extract was added into the wells. The blank and the negative control were added with 100 µl of diluent B (Table 3.2). For the positive control, 50 µl of 800 µM of L-NAME was added. Another control with inducer, but without DMSO, was also prepared to account for the effects of DMSO concentration on the NO inhibition and cell viability. The plate was incubated at 37°C for 17 hours in the presence of 5% CO_2 (appendix B).

Table 3. 2. Preparation of diluents A, B, and C

Diluent	Description
A	DMEM without phenol red, 10% FBS, 0.2% DMSO
B	DMEM without phenol red , 10% FBS
C	DMEM without phenol red , 10%, 200U/ml IFN-γ and 10 µg/ml LPS

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3.2.15.4 Nitric Oxide Inhibitory Activity (Griess Assay)

Nitric oxide production in cells was measured by a microplate assay method. Fifty microliters of medium were drawn out into a new non-sterile plate and incubated with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 mins. The absorbance at 550_{nm} was determined using a UV/Vis microplate reader (Spectramax Plus 384; Molecular Devices Inc., USA). The nitrite concentration in each test solution well was determined from the calibration curve of the $NaNO_2$ (Appendix C). Nitric oxide inhibition was calculated as follow:

% Inhibition = [(nitrite in control) – (nitrite in sample)/ (nitrite in control)] x 100

3.2.15.5 Cell Viability

Cell viability was assessed using the mitochondrial respiration-dependent 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method. The RAW 264.7 cells in the culture plate were used to determine the cell viability. One hundred microliters of DMEM with phenol red containing 10% FBS was added into each well, followed by the addition of 20 µl of MTT solution (5 mg/ml MTT in sterile PBS pH 7.2). The cells were incubated at 37 °C with 5% CO_2 for 4 hours. The medium was then carefully discarded and the formed formazan crystals were dissolved in 100 µl of DMSO. Absorbance values were measured at 570_{nm}. The percentage cell viability was calculated using the following formula:

Cell viability (%) = [(O.D. sample)/ (O. D. control)] × 100%

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3.3 Results and Discussion

3.3.1 Chemical Analyses

The Jatropha kernel and Jatropha meal obtained from *Jatropha curcas* plants originating from Sabah, Selangor, Terengganu and Johor Bahru provinces showed similar results in terms of chemical constituents. Therefore, the data are presented as a mean of their value (Table 3.3). The DM contents of the kernel and meal were 94 and 95%, respectively. Fat content of the kernel was 55.7%. Crude protein in the kernel and the meal were 29.5 and 61.8%, respectively. Oil extraction decreased the OM, but increased the ash, NDF and ADF contents.

Table 3. 3. Chemical analysis of Jatropha kernel and Jatropha meal*

Item	Jatropha kernel	Jatropha meal
DM (%)	94.2±0.5	95.1±0.5
OM (%)	96.0±0.0	91.6±0.0
Ash (%)	3.9±0.0	8.3±0.0
CP (%)	29.5±0.3	61.8±0.1
Fat (%)	55.7±0.4	nd
NDF (%)	4.3±0.5	9.7±0.5
ADF (%)	2.9±0.1	4.8±0.2
GE (MJ/kg)	29.1±0.2	17.7 ± 0.1

nd: not determined

*Values are on dry matter basis.

Values represent the mean and standard deviation of triplicate samples

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As shown in Table 3.3, the meal is high in protein (61.8% w/w), but low in neutral detergent fiber (NDF) (9.7% w/w) and acid detergent fiber (ADF) (4.8% w/w). These results are comparable to those of Jatropha meal originating from Mexico, India, Nicaragua and Cape Verde as reported by Makkar and Becker (2009). Chemical constituents of meal obtained from local *J. curcas* seed showed high protein and low fiber content with values suitable as a animal feed ingredient. In fact, the Jatropha meal is regarded for its high protein content, as this value is higher than that of the fish (55-60%), soybean (38-40%), sunflower (30%), cottonseed (35-40%) and canola (40%) meals (Harter *et al.*, 2011). In addition, the amino acid profile of Jatropha meal was similar to soybean meal and contains a good balance of essential amino acids, with the exception of lysine which was higher in Jatropha meal (Makkar *et al.*, 1998a).

3.3.2 Bioactive Compounds of Jatropha meal

3.3.2.1 Major Bioactive Compounds

The Jatropha meal obtained from *Jatropha curcas* plants originating from Sabah, Selangor, Terengganu and Johor Bahru provinces showed similar results in the bioactive compounds analyses. Therefore the mean of the bioactive compound values was calculated and is presented in Table 3.4. The analyses showed that the total phenolics was 3.9±0.2 mg gallic acid equivalents/g DM and the total flavonoid was 0.4±0.1 mg rutin equivalents/g DM. Total phenolic content was comparable to the value of 3.6 mg gallic acid equivalents/g reported by Makkar and Becker (2009), while the total flavonoid content of Jatropha meal has not been reported previously.

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Table 3. 4 Bioactive compounds present in Jatropha meal*

Items	Total phenolics[a] (mg/g)	Total flavonoids[b] (mg/g)	Total saponins[c] (mg/g)	Phytic acid[d] (%)	Trypsin inhibitors[e] (mg/g)	Lectins[f] (U)	Phorbol esters[g] (mg/g)
Content	3.9±0.23	0.4±0.15	19.0±0.48	9.1±0.51	34.2±1.40	102.7	3.0±0.16

[a] Gallic acid equivalents
[b] Rutin equivalents
[c] Diosgenin equivalents
[d] Phytic acid g/100g DM
[e] mg of pure trypsin inhibited/g sample
[f] U:1 mg of meal that produced hemagglutination per milliliter of assay medium
[g] Equivalents to phorbol 12-myristate-13-acetate
*Analyses were performed in triplicate

Phenolic and flavonoid compounds occur ubiquitously in plants, and a variety of biological activities such as antimicrobial, cytotoxic effects, antioxidant, hypolipidemic and anti-inflammatory activities have been attributed to them. Many of these biological functions correspond to their free radical scavenging and antioxidant activities (Muraoka *et al.*, 2004). The total phenolic compounds result

was consistent with the report of Monteiro *et al.* (2012) who observed a value of 3.2 mg/g in Jatropha meal. They also reported the notable anthelmintic activity of the phenolic compounds present in Jatropha meal against *Haemonchus contortus* larvae with the inhibition of the larval exsheathment by about 99.8%.

Total saponin content was 19 mg diosgenin equivalents/g DM. This value was lower when compared to Jatropha meal from Mexico, Cape Verde and India varieties with values of 28.5, 26.0 and 27.3 mg diosgenin equivalents/g DM, respectively (Devappa, 2008). Saponins are widely distributed amongst plants, and it is believed that saponins naturally act to protect the plant against pathogens. Their main biological activities include haemolytic, molluscicidal, anti-inflammatory, antimicrobial, anti-parasitic, cytotoxic and anti-tumor (Sparg *et al.*, 2004). The presence of both polar (sugar) and nonpolar (steroid or triterpene) groups provide saponins with strong surface-active properties that are responsible for many of their biological activities (Makkar *et al.*, 2007a).

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Makkar and Becker (2009a) reported the range of Jatropha meal saponins to be from 26 to 34 mg/g DM diosgenin equivalents among different varieties including toxic and non-toxic plants. They suggested that the level of saponins present in the Jatropha meal was safe. Therefore, due to the safety of the Jatropha meal saponins, beneficial biological activities including hypocholesterolemic effects, defaunation of the rumen and manipulation of the end products of fermentation toward improvement of growth and production in various animal species, anthelmintic activity and antimicrobial activity would be expected when the Jatropha meal saponins were incorporated in the diet (Wina *et al.*, 2005a).

Phytic acid has the ability to chelate minerals, resulting in mineral deficiency which may cause various diseases. The phytic acid in monogastric nutrition is more important than in polygastric, since polygastric animals benefit from microbial phytase which allows the digestion of phytate (Tsukatani *et al.*, 2008). The phytic acid level in Jatropha meal was 9.1% which could be considered safe, as the non-toxic varieties contain about 8.9% phytic acid (Makkar *et al.*, 2009a). Recently, phytic acid has been suggested to have a protective role in carcinogenesis, anti-oxidant properties, hypolipidaemic activity, antiplatelet activity and against diabetes mellitus, HIV, dental caries and renal lithiasis (Kumar *et al.*, 2010a).

Trypsin inhibitors naturally contribute towards the resistance of seeds to insect and pathogenic microorganisms (Valueva *et al.*, 2004). However, when they are consumed they inhibit the pancreatic proteases resulting in impaired protein digestion, hypertrophy and hyperplasia of the pancreas, followed by depression in growth rate (Makkar *et al.*, 2007a). The trypsin inhibitors in Jatropha meal was 34.2 mg/ g DM (Table 3.4), higher than those of the toxic varieties with the value of 21.31 mg/g DM reported by Makkar and Becker (2009a). Martinez-Herrera *et al.* (2006) reported that the amount of trypsin inhibitor in Jatropha meal obtained from Mexico were in the range of 33.1 to 36.4 mg/g, which is comparable to the result of this study, but higher than trypsin inhibitor level reported by Makkar *et al.*(1997b), indicating the diversity of trypsin inhibitor content, depending on the genotypes. Despite the negative nutritional effects of trypsin inhibitors, they have been reported to have antifungal activity, antimalarial activity, antiproliferative activity toward leukemia, lymphoma and ovarian cancer cells, and HIV-1 reverse transcriptase inhibitory activity (Gohlke *et al.*, 2012).

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Lectins agglutinate red blood cells, impair lipid, carbohydrate and protein metabolism, and cause hypertrophy or atrophy of organs and tissues (Vasconcelos *et al.*, 2004). The Jatropha meal contains 102 to 51 U Lectins (U: 1 mg of meal that produced hemagglutination per milliliter of assay medium) in toxic and nontoxic genotypes, respectively (Devappa *et al.*, 2010a; Makkar *et al.*, 2011). These levels are reported to be similar to the soybean meal lectin content (Makkar *et al.*, 2007b), which indicated the safety of the lectin levels present in Jatropha meal. Plant lectins have been widely studied for their biological and therapeutics properties. They bind selectively to cell membrane receptors and induce cytotoxicity, inhibit tumor cell growth, inhibit protein synthesis by binding to ribosomes, down-regulate telomerase activity, and reduce angiogenesis. Curcin is one of the active lectins from Jatropha meal which possesses notable anti tumor inhibitory activity against gastric cancer cell (SGC-7901), mouse myeloma (Sp2/0), and human hepatoma with IC_{50} values of 0.23, 0.66 and 3.16 mg/L, respectively (Lin *et al.*, 2003).

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Phorbol esters are esters of tetracyclic diterpenes which are widely distributed in plant genera of the families *Euporbiaceae* and *Thymelaeaceae,* and possess biological activities such as anti-HIV, anti-malaria and antimicrobial activities (Goel *et al.*, 2007). The phorbol esters content of Jatropha meal was 3 mg phorbol-12-myristate-13-acetate (PMA) equivalents/g DM (Table 3.4). Phorbol esters are known to mimic the action of diacylglycerol to activate protein kinase C, which regulates different signal transduction pathways and other cellular metabolic activities, including proliferation, malignant transformation, differentiation and cell death (Goel *et al.*, 2007). The multiplicity effects of phorbol esters on biological systems is associated with the presence of phorbol esters receptors (Fujii *et al.*, 2000) and the

type of phorbol esters. Fujii *et al.* (2000) and Park (2001) reported the induction of apoptosis using phorbol esters in prostate cancer cells and gastric cancer cells. The high phorbol esters content of Jatropha meal (Table 3.4), is considered harmful to animals since the toxic varieties contain 2.7 mg/g DM according to the category described by Makkar and Becker (2009a).

3.3.3 HPLC Analysis of Phenolic and Flavonoids Compounds

In this study, high performance liquid chromatography (HPLC) was used to determine the phenolic and flavonoid compounds in Jatropha meal extract. The analyses showed the presence of gallic acid and pyrogallol as phenolics (Figure 3.1), rutin and myricetin as flavonoids (Figure 3.2), and daidzein as an isoflavonoid compound (Figure 3.1), at the concentrations shown in Table 3.5. Compounds that have been reported from *J. curcas* leaves include apigenin and its glycosides, vitexin and isovitexin, stigmasterol, β-sitosterol and gallic acid (Chhabra *et al.*, 1990) while the root and stem contained gallic acid, ellagic acid, quercetin, coumaric acid, benzoic acid and salicylic acid (El Diwani *et al.*, 2009).

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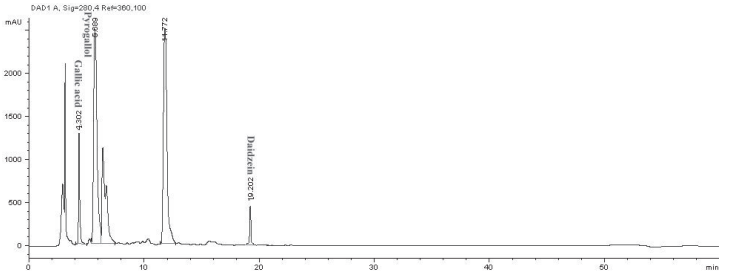

Figure 3.1. HPLC chromatogram of phenolic and isoflavonoid compounds from the methanolic extract of Jatropha meal detected at 280$_{nm}$

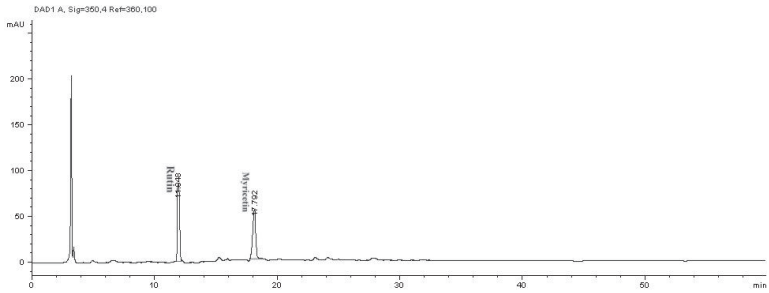

Figure 3.2. HPLC chromatogram of flavonoid compounds from the methanolic extract of Jatropha meal detected at 350_{nm}

Table 3. 5. Concentrations of phenolic, flavonoid and isoflavonoid compounds in Jatropha meal methanolic extract (µg/g)*

Sample	Phenolic (µg/g)		Flavonoid (µg/g)		Isoflavonoid (µg/g)
	Gallic acid	Pyrogallol	Rutin	Myricetin	Daidzein
Jatropha meal	581.3±0.36	631.1±0.47	47.6±0.53	198.5±0.29	297.5±0.27

All values are on DM basis
*Each value represents the mean ± S.E.M of three replicates

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3.3.4 Gas Chromatography - Mass Spectrometry Analysis (GC-MS)

The GC-MS analysis is a rapid and simple method to determine the level and range of compounds present in plant materials. Table 3.6 shows the major organic compounds detected by GC-MS in the methanolic and hot water extracts. More than half of the organic compounds in the methanolic extract consists of 2-(hydroxymethyl)-2-nitro-1,3-propanediol, β-sitosterol, 2-furancarboxaldehyde-5-(hydroxymethyl) and acetic acid and in the hot water extract, the most common compounds present are 2-furancarboxaldehyde-5-(hydroxymethyl), acetic acid and furfural (2-furancarboxaldehyde), adding up to about 60% of the total extracted organic compounds (Appendix D).

Table 3. 6. Major organic compounds detected in the methanolic and hot water extracts of Jatropha meal by GC-MS

Methanolic extract		Hot water extract	
Compounds	**Content (%)**	**Compounds**	**Content (%)**
2-(Hydroxymethyl)-2 nitro-1,3-propanediol	23	2-Furancarboxaldehyde-5-(hydroxymethyl)	30
β-sitosterol	14	Acetic acid	16
2-Furancarboxaldehyde-5-(hydroxymethyl)	9	Furfural (2-furancarboxaldehyde)	15
Acetic acid	11		

Analyses were performed in triplicate

The 2-(hydroxymethyl)-2-nitro-1,3-propanediol has been reported as a bacteriostat in disinfectants (Popendorf *et al.*, 1995). β-sitosterol, a plant sterol possess antimicrobial (Ajaiyeoba *et al.*, 2003), anti-inflammatory and cytotoxic activities (Gupta *et al.*, 1980; Awad *et al.*, 1996). The compound 2-furancarboxaldehyde-5-(hydroxymethyl) also shows antibacterial and antifungal activity applicable in pharmaceuticals, cosmetics and pesticides (Rigal *et al.*, 1983). Furfural is used as a flavor ingredient in foods, in cosmetic products and also in fragrances, pesticides, herbicides, fungicides, insecticides and in germicides. According to Ryssel *et al.* (2009) acetic acid also showed excellent bactericidal effects toward *Proteus vulgaris, Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Therefore, GC-MS results showed the presence of compounds with known activities indicating Jatropha meal as a potential source of functional metabolites.

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3.3.5 Antioxidant Activity

3.3.5.1 DPPH Scavenging Activity

The results shown in Figure 3.3 indicate the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of Jatropha meal extracts at different concentrations. The inhibition of DPPH radical scavenging activity increased in a dose-dependent manner. Table 3.7 shows the IC_{50} (required concentration to scavenge 50% of DPPH radicals) concentration of extracts. Free radical scavenging activity of methanolic extract showed a value of 1.6 mg/ml, but the hot water extract did not show an IC_{50} value, even at the highest concentration used in this study. The methanolic extract of Jatropha meal appeared to be more active as compared to the hot water extract in scavenging the free radicals. Reference antioxidants vitamin C, BHT and β-carotene showed IC_{50} values of 0.3, 0.3 and 1.5 mg/ml, respectively. Both extracts showed lower activities as compared to vitamin C and BHT due to the significant ($p \leq 0.05$) higher IC_{50} values required to inhibit the free radicals (Table 3.7). The methanolic extract activity was similar to β-carotene, as no significant difference was observed between their IC_{50} values (Table 3.7). The strong free radical scavenging activities in the latex, root and stem of *J. curcas* was attributed to the presence of phenolic compounds (El Diwani *et al.*, 2009).

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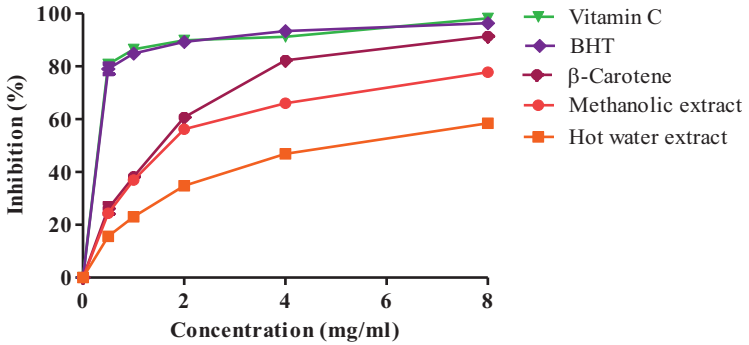

Figure 3. 3. The free radical scavenging activity of Jatropha meal extracts and reference antioxidants

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3.3.5.2 Ferric-Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power (FRAP) of the extracts of Jatropha meal and the reference antioxidants (vitamin C, BHT and β-carotene) are presented in Figure 3.4. Reference antioxidants showed higher reducing power, followed by the methanolic and hot water extracts. The IC_{50} value (the concentration to reduce 50% of Fe^{3+} to Fe^{2+} as an index for antioxidant potential) in FRAP for vitamin C, BHT and β-carotene were 0.3, 0.3 and 2.6 mg/ml, respectively (Table 3.7), while for the methanolic and hot water extracts the IC_{50} values were 3.0 and 8.0 mg/ml, respectively. As observed in the DPPH assay, the FRAP assay also showed similar IC_{50} values between the methanolic extract and β-carotene (Table 3.7).

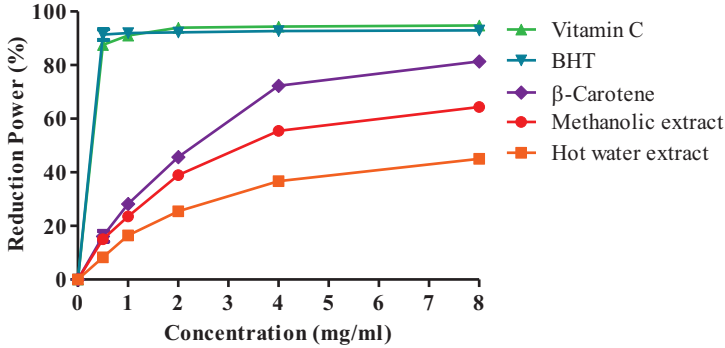

Figure 3. 4. Ferric reducing activity of Jatropha meal extracts and reference antioxidants

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Table 3. 7. The IC_{50} values of Jatropha meal extracts and reference antioxidants

Items	IC_{50} (mg/ml)	
	DPPH	FRAP
Methanolic extract	1.6^{b}	3.0^{b}
Hot water extract	5.0^{a}	$>8^{a}$
Vitamin C	0.3^{c}	0.3^{c}
BHT	0.3^{c}	0.3^{c}
β-Carotene	1.5^{b}	2.6^{b}
*S.E.M	0.09	0.07

Values with different subscriptions in each column are significantly different ($p \leq 0.05$)
Analyses were done in triplicate
* S.E.M: Standard error mean

The antioxidant activity of the Jatropha meal extracts reflects their ability to donate a hydrogen or electron, and to delocalize the unpaired electron within the aromatic

structure. The results indicate that the extracts were more active in scavenging free radicals compared to ferric reducing power activity. In the FRAP assay, the general ability of the extracts to donate electrons is tested, whereas in the DPPH assay reduction by hydrogen atoms are also involved. Hence, the free radical scavenging activity assay is a better indicator of antioxidant activity of Jatropha meal than FRAP based on the reaction mechanisms proposed. In complex systems, such as food, various different mechanisms may contribute to oxidative processes. Therefore, it is important to characterize the extracts by a variety of antioxidant assays (Ismail *et al.*, 2010).

The presence of phenolics (gallic acid, pyrogallol), flavonoids (rutin, myricetin) and isoflavonoid (daidzein) in the Jatropha meal (Table 3.5) may contribute to the antioxidant activity of extracts. These compounds have been identified to be some of the major compounds involved in the antioxidant activity (Crozier *et al.*, 1997). However, saponins in the Jatropha meal (Table 3.4) might be also involved since Sparg *et al.* (2004) reported the antioxidant activity of these compounds. Recently, Gallegos-Tintore *et al.* (2010) reported the presence of bioactive peptides in Jatropha meal possessing noteworthy antioxidant activities, as revealed by free radical scavenging, reducing power and metal chelating activity.

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The study conducted by Hilario *et al.* (2010) suggested that, feeding dairy goat with diet containing natural antioxidants such as total polyphenol, hydroxycinnamic acids and flavonoids improves the profile of bioactive compounds in the milk. Similarly, Descalzo and Sancho (1988) reported that, feeding feedlot cattles with forage containing natural antioxidants, such as phenolics, flavonoids, α-tocopherol, β-

carotene, ascorbic acid and glutathione, led to retarded lipid and protein oxidation in fresh and stored meat, and preserve the color and odor quality of beef, and produced meat with a high antioxidant value. Therefore, the antioxidant activity observed in Jatropha meal could be useful for animal health and production.

3.3.6 Antibacterial Activity

Antibacterial activities of methanolic and hot water extracts of Jatropha meal were tested against four Gram negative and four Gram positive pathogens at two concentrations (1.0 and 1.5 mg/disc). The results shown in Table 3.8 indicate significant differences ($P \leq 0.05$) in the inhibitory activities of methanolic and hot water extracts. The diameter of the inhibition zones of the methanolic extract ranged from 0.35 to 1.25 cm at 1.0 mg/disc, showing moderate to strong antibacterial activity. Whereas the diameter of the inhibition zones of the hot water extract ranged from 0.00 to 0.75 cm at 1.0 mg/disc and indicated no inhibition to moderate antibacterial activity (Appendix E). These activities were categorized according to Vaquero *et al.* (2007) as shown in Table 3.1. The antimicrobial activity of the methanolic extract appeared to be more effective than that of the water extract, since methanol could extract a wide variety of active components compared to hot water. The size of the inhibition zones increased with 1.5 mg/disc dosing. At this concentration, the antibacterial effects of the methanolic extract was similar between both the Gram negative and Gram positive bacteria, except for *P. aeruginosa* which showed a moderate zone of inhibition. This Gram negative bacteria seemed to be less sensitive to other plant extracts (Boussaada *et al.*, 2008).

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Table 3. 8. Inhibition zones of Jatropha meal extracts against pathogenic bacteria at the concentrations of 1.0 and 1.5 mg/disc

Microorganism	Zone of Inhibition (cm)						S.E.M*
	Methanol		Hot Water		Kanamycin	Streptomycin	
	1.0 mg/disc	1.5 mg/disc	1.0 mg/disc	1.5 mg/disc	1.0 µg/disc	1.0 µg/disc	
Gram negative							
Enterobacter aerogenes	1.11^{b}	1.21^{b}	0.21^{e}	0.60^{d}	1.54^{a}	0.96^{c}	0.04
Klebsiella pneumoniae	1.05^{c}	1.20^{b}	0.40^{e}	0.62^{d}	1.44^{a}	1.04^{c}	0.02
Escherichia coli	1.25^{c}	1.63^{b}	0.75^{e}	1.00^{d}	1.70^{a}	1.03^{d}	0.02
Pseudomonas aeruginosa	0.35^{d}	0.58^{b}	0.45^{c}	0.37^{cd}	1.01^{a}	0.41^{cd}	0.02
Gram positive							
Micrococcus luteus	0.51^{c}	1.20^{a}	0.22^{e}	0.30^{d}	1.35^{b}	1.10^{b}	0.02
Bacillus subtilis	1.10^{b}	1.40^{a}	0.65^{d}	0.85^{c}	1.23^{b}	0.86^{c}	0.04
Bacillus cereus	1.00^{d}	1.25^{b}	0.00^{f}	0.77^{e}	1.49^{a}	1.07^{c}	0.02
Staphylococcus aureus	1.15^{b}	1.37^{a}	0.30^{e}	0.75^{d}	1.33^{a}	0.95^{c}	0.04

Means with different superscripts within rows are significantly different (P≤0.05)
Analyses were done in triplicate
* S.E.M: Standard error mean

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E. coli seemed to be the most susceptible bacterial species to the methanolic and hot water extracts at 1.0 and 1.5 mg/disc extract concentration. The antibacterial activities of both kanamycin and streptomycin were as expected, and some of the clearing zones of pathogens were comparable to those of the antibiotics, in particular when 1.5 gm/disc extracts were tested.

Lihua *et al.* (2008) have reported the *in vitro* antibacterial effects of *J. curcas* leaf extract against chicken pathogens, including *E. coli*, and *S. aureus.* Various reports have also shown the antimicrobial activity of extracts from different parts of *J. curcas* plant. However, the ability of the extracts to inhibit microbial growth seemed to vary according to the extraction procedures and assay methods. Igbinosa *et al.* (2009) have reported the *in vitro* antimicrobial activity of crude methanolic and

water extracts of the *J. curcas* stem bark against a wide spectrum of microorganisms with zones of inhibition ranging from 0.8 to 2 and 0 to 0.8 cm for methanol and water extracts at the concentration of 1 mg per well (0.6 mm), respectively. In another study, Atindehou *et al.* (2002) reported the antimicrobial activity of the leaves and roots of *J. curcas* against *E. coli*, *P. aeruginosa, S. aureus* and *E. faecalis*.

Total phenolics generally possess antimicrobial activities which provide chemical barriers for invading microorganism. On the other hand, flavonoids inhibit bacterial growth by the inhibition of DNA gyrase, cytoplasmic membrane function and energy metabolism (Cushnie *et al.*, 2005). Gallic acid and pyrogallol as phenolic compounds, daidzein as an isoflavonoid, and rutin and myricetin as flavonoids (Table 3.5) are substantially effective as antibacterial agents (Cushnie *et al.*, 2005). Additionally, the saponins present in Jatropha meal could also contribute to the antimicrobial activity, as saponins have been shown to possess antibacterial activity against several Gram positive and Gram negative bacteria (Sparg *et al.*, 2004). Furthermore, phorbol esters (Table 3.4) could also act as an antimicrobial agent as this property has been demonstrated by Chumkaew *et al.* (2003).

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Antibacterial agents from plants have been found to suppress methane production through direct action against the rumen methanogens and protozoa (Cheftel, 2011; Chen *et al.*, 2011) and to improve the animal production. In addition, applications of ionophores and antibiotics in animal production have been limited recently, and antimicrobial agents of plant origin are being introduced as an alternative to replace the antibiotic in the feed (Bodas *et al.*, 2012). Thus, according to the results obtained

in this study, Jatropha meal could be a source of natural antibacterial agents with the potential to be used in animal feed.

3.3.7 Anti-inflammatory Activity

The methanolic and water extracts of Jatropha meal were analysed for their inhibitory effects on NO production from macrophages RAW 264.7 cells induced by LPS and IFN-γ. Kim *et al.* (1998) reported that at the concentration of 200 μg/ml, the activity of the plant extract in inhibition of NO production in LPS/IFN-γ stimulated RAW 264.7 cell line could be classified as follows: strongly active (70% and above); moderately active (50-69%); weakly active (30-49%); and very weakly active (29% or less). Figures 3.5 and 3.6 show the NO inhibition and cell viability of the methanolic and water extracts, together with L-NAME as a positive control. According to the above classification, the methanolic extract of Jatropha meal was found to be strongly active (93.6%), while the water extract appeared to be weakly active (44%) in terms of anti-inflammatory properties.

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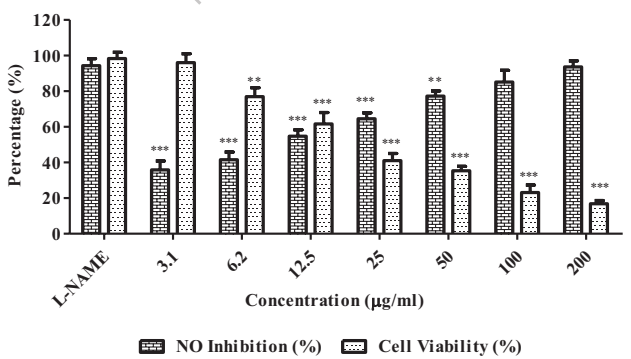

Figure 3. 5. Percentage values of NO inhibition and cell viability of Jatropha meal methanolic extract. All values represent mean ± standard error, ***P ≤ 0.001, P≤0.01 indicates significant difference compared to the control (L-NAME)

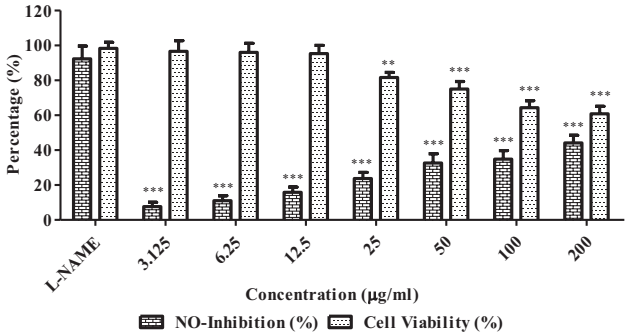

Figure 3. 6. Percentage values of NO inhibition and cell viability of Jatropha meal water extract. All values represent mean ± standard error, ***P ≤ 0.001, **P≤0.01 indicates significant difference compared to the control (L-NAME)

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L-NAME inhibited NO production by 92.3% at the concentration of 250 µM, while the cell viability was 98.3%. The methanolic extract inhibited the NO production in a dose-dependent manner, and starting at 100 µg/ml and above inhibited the NO (85.2%) similar to L-NAME, as no significant difference was observed. Even though this inhibition activity could be considered as strong iNOS inhibitor according to Kim *et al.* (1998), but the reduction in cell viability (23.2 %) of raw 264.7 cell indicated the presence of toxic compounds in the extract. It was noted that, water extract at concentrations between 3.1 to 200 µg/ml (Figure 3.6) did not inhibit the NO production comparable to L-NAME. The water extract was found to be toxic to the RAW 264.7 cells at concentrations of 25 µg/ml and above, as it significantly reduced the cell viability when compared to L-NAME. The cell death observed in this study could be due to the presence of phorbol esters in the extract.

The results obtained suggested that Jatropha meal methanolic extract was a strong iNOS inhibitor contributing to its anti-inflammatory effects. Similar findings on the

anti-inflammatory effect of extracts from different parts of *J. curcas* plants have been reported. Mujumdar and Misar (2004) observed the anti-inflammatory activity of the topical application of *J. curcas* root powder paste on TPA-induced ear inflammation in albino mice. Similarly, Uche and Aprioku (2008) reported the inhibition activity of *J. curcas* leaf extract on the egg albumin-induced inflammation in Wister albino-rats.

The mechanism of the antioxidant activity of phenolic compounds, and their ability to act as free radical and NO scavengers, leading to the formation of phenoxyl radicals have been described by Sumanont *et al.* (2004). Recently, Kazlowska *et al.* (2010) suggested that the inhibition of iNOS in the RAW 264.7 cells is due to the suppressing action of the flavonoids. Furthermore, the isolated saponins from the roots of *Physospermum verticillatum* exerted significant inhibition of NO production in LPS induced RAW 264.7 macrophages (Berger *et al.*, 2012). These findings augur well with the results of the present study, which showed that methanolic extract with high anti-inflammatory activity also contained the highest amount of phenolics, flavonoids and saponins. However, the presence of other phytochemical compounds could also contribute to the anti-inflammatory properties of the methanolic extract.

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3.4 Conclusion

Chemical analyses revealed the similarity of Jatropha meal obtained from local *J. curcas* plant to other genotypes from different regions. The Jatropha meal obtained from local *J. curcas* plants contains a high percentage of crude protein and relatively low fibre, which renders it suitable as an animal feed. Bioactive compound analyses showed the presence of various phenolics and flavonoids, saponins, phytic acid, lectins, trypsin inhibitors and phorbol esters. The HPLC and GC-MS analyses

indicated the presence of various phenolics, flavonoids and isoflavonoids, together with other organic compounds. All of these bioactive compounds are most likely responsible for the antioxidant, antibacterial and anti-inflammatory activities observed. Thus, the results obtained in this study indicated the potential of Jatropha meal as a source of natural bioactive compounds with various beneficial biological activities. However, the presence of phorbol esters in the Jatropha meal confirmed that the *Jatropha curcas* plants originating from Sabah, Selangor, Terengganu and Johor Bahru are categorized under the toxic varieties of *Jatropha curcas*. Therefore, the removal of the phorbol esters before the application of Jatropha meal for any purposes should be considered.

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CHAPTER 4

EFFECT OF JATROPHA MEAL AND ISOLATED PHORBOL ESTERS ON RUMEN MICROBIAL ACTIVITY *IN VITRO*

4.1 Introduction

The results of the previous experiments indicated that Jatropha meal has the potential as a biofeed due to the presence of bioactive compounds. However, the presence of phorbol esters was reported to limit the nutritional application of Jatropha meal (Makkar *et al.*, 2009a). The high level of phorbol esters present in the meal obtained from local Jatropha plants indicated that the *J. curcas* planted in Sabah, Selangor, Terengganu and Johor Bahru is similar to the toxic variety, according to the classification of toxic and non-toxic *J. curcas* plants reported by Makkar *et al.* (2009a). Physicochemical treatment appeared to be promising in the alleviation of harmful bioactive compounds, as previous researchers reported the complete inactivation of lectins and trypsin inhibitors using moist heat treatment (Kumar *et al.*, 2010b; Makkar *et al.*, 2011). In addition, the incorporation of alkaline treatments also appeared to be more effective in reducing the bioactive compounds, in particular the phorbol esters (Rakshit *et al.*, 2008).

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The animal gut microbiota has been reported to possess the ability in biotransformation and biodegradation of harmful plant bioactive compounds to the less toxic or non-toxic derivatives (Laparra *et al.*, 2010). Interestingly, rumen microbes have shown the ability to transform and degrade the terpenoid compounds (Abdel-Hafez *et al.*, 2002; Dziba *et al.*, 2006). The *in vitro* gas production technique has become an important tool to study the activity of rumen microbiota in the presence of plant bioactive compounds (Getachew *et al.*, 1998). The presence of

phorbol esters as the toxic compounds in the Jatropha meal obtained from local *J. curcas* seed may cause determinatal problems to the rumen microbes, if the meal is to be used as a biofeed. Therefore, the present study was conducted to alleviate the bioactive compounds by physical and chemical treatments, and to determine the effects of the treated meal on rumen microbial activity using the *in vitro* gas production technique. This experiment was followed by a study on the effects of isolated phorbol esters on rumen fermentation activity and changes in the rumen microbial population.

4.2 Material and Methods

4.2.1 Reagents

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The 5-bromo-4-chloro-indolyl-galactopyranoside (x-gal) was purchased from Sigma (Sigma, Germany). The QIAamp DNA Stool Mini Kit was from Qiagen GmbH, Hilden, Germany, i-StarTaq DNA polymerase kit, MEGAquick-spin PCR and agarose gel extraction kit and DNA-spin plasmid DNA extraction kit were obtained from iNtRON Biotechnology, Sungnam, Kyungki-Do, Korea. SeaKem® GTG® agarose was purchased from FMC BioProducts, Rockland ME, USA. pCR®2.1-TOPO® TA cloning vector (Invitrogen, Carlsbad, CA, USA), Luria–Bertani (LB) agar, *Eco*R1, HindIII, Loading dye, DNA ladder (100 bp, 1Kb) were from MBI Fermentas, Lithuania, and SYBR green was purchased from Bio-Rad Laboratories, Hercules, CA, and other chemicals used in this study were obtained from Merck (Darmstadt, Germany).

4.2.2 Jatropha Meal Preparation

The Jatropha meal was prepared as described in Section 3.2.1.

4.2.3 Physicochemical Treatments

Hydrothermal, alkali and oxidizing agents were applied to remove the bioactive compounds. One hundred grams of Jatropha meal were weighed in a glass beaker and the moisture content of the sample was adjusted to 66% (Makkar *et al.*, 1998a) with either distilled water or a solution containing alkali (NaOH, $Ca(OH)_2$, KOH) or an oxidizing agent (NaOCl) to give a final concentration of 3% (w/w) or 10% (v/w), respectively. Samples were autoclaved for 30 min at 121ºC, 15 psi (hydrothermal treatment). Samples were washed with distilled water (1:10) and freeze dried. Physical and chemical treatments are summarized in Table 4.1.

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Table 4. 1. Jatropha meal treatments

	Treatments	
Treatments	Chemical	[1]Hydrothermal
a	-	-
b	-	+
c	NaOH	+
d	KOH	+
e	$Ca(OH)_2$	+
f	NaOH + NaOCl	+
g	KOH + NaOCl	+
h	CaOH2 +NaOCl	+
i	NaOCl	+

- Not applied
+ Applied
[1] Autoclaved at 121ºC for 30 min at 15 psi
a= Jatropha meal, b=hydrothermal treatment (HT), c= HT + 3% (w/w) NaOH, d= HT + 3% (w/w) KOH, e= HT + 3% (w/w) $Ca(OH)_2$, f= HT+ 3% (w/w) NaOH + 10% (v/w) NaOCl, g= HT+ 3% (w/w) KOH +10% (v/w) NaOCl, h= HT+ 3% (w/w) $Ca(OH)_2$ +10% (v/w) NaOCl, i= HT+ 10% (v/w) NaOCl

4.2.4 Chemical Analyses

Samples were analysed for DM, OM and CP contents according to the procedure of AOAC (1990). Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined using the method described by Van Soest *et al.* (1991).

4.2.5 Bioactive Compound Analyses

The amount of total phenolics, flavonoids, saponins, phytic acids, lectins, trypsin inhibitors and phorbol esters were determined according to the methods described in Chapter 3.

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4.2.6 Effects of Treated and Untreated Jatropha Meal on Rumen Microbes

The *in vitro* gas production technique (Menke *et al.*, 1988) was used to study the effects of treated and untreated Jatropha meal on rumen microbial activity. Treated and untreated (control) Jatropha meal were milled to pass through a 1 mm sieve and used as substrates. Two hundred mg of substrate were weighed into 100 ml calibrated glass syringes (FORTUNA®Hiberle Labortechnik, Germany). The pistons of the syringes were lubricated with Vaseline to ensure smooth movement of the pistons during fermentation.

Rumen liquor was collected before the morning feed from two rumen fistulated Kedah–Kelantan male cattle (averaged 209 kg BW) fed twice daily with 5.2 kg DM/day (2.5% of BW) consisting of forage (*Panicum maximum*) and commercial cattle concentrate at 60:40 ratio. Mineral lick and water were available *ad libitum*. The rumen fluid was kept in pre-warmed (39^{o}C) thermos flask and transferred immediately to the laboratory. The rumen fluid was homogenised with an ordinary

blender under a stream of CO_2 gas. The homogenised rumen fluid was strained through four layers of muslin gauze into a pre-warmed bottle at 39°C. Throughout the process, the rumen fluid was flushed continuously with CO_2 gas.

4.2.6.1 Preparation of Incubation Medium

The buffer solution was prepared by dissolving 35 g sodium bicarbonate ($NaHCO_3$) and 4 g ammonium carbonate (NH_4HCO_3) in 1 liter solution with distilled water. The macro element solution was made by dissolving 6.2 g potassium dihydrogen phosphate (KH_2PO_4), 5.7 g disodium hydrogen phosphate (Na_2HPO_4), and 0.6 g magnesium sulphate ($MgSO_4.7H_2O$) in 1 liter solution with distilled water. Trace element solution contained 10 g manganese chloride ($MnCl_2.4H_2O$), 13.2 g calcium chloride ($CaCl_2.2H_2O$), 1 g cobalt chloride ($CoCl_2.6H_2O$), 8 g ferric chloride ($FeCl_3.6H_2O$) in 50 ml of distilled water. The resazurine was prepared by dissolving 0.1 g resazurine in 100 ml of distilled water, and the reducing agent was made by dissolving 996 mg sodium sulphide ($Na_2S.9H_2O$) in 94 ml of distilled water followed by addition of 6 ml of 1 M sodium hydroxide solution. All of these solutions were prepared according to Menke and Steingass (1988). The reducing agent was prepared fresh, while the other solutions were stored at 4°C for up to 3 months.

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4.2.6.2 *In Vitro* Gas Production Technique

The solutions were mixed in a 5 liter flask according to the following proportions at 37°C: 474 ml distilled water; 0.12 ml trace element solution; 237 ml buffer solution; 237 ml macro element solution; 1.2 ml resazurin solution and 50 ml reducing agent. CO_2 was bubbled in throughout the preparation until the pink color turned colorless. Five hundred milliliters of rumen fluid was added (ratio 1:2) when the indicator

changed to colourless. Thirty milliliters of buffered rumen fluid were dispensed into each syringe and incubated in a water bath at 39°C for 24 h. *In vitro* gas production (GP) was measured in triplicate at 2, 4, 8, 12 and 24. In each incubation run, three blanks were included to correct the GP values for gas released from endogenous substrates and three standard hays to check the quality of the inoculum.

The incubation was terminated by cooling the syringe in ice and transferring the entire residue into 50 ml centrifuge tube (Nalge Nunc International, Rochester, NY). The pH was measured before samples were centrifuged at 20,000×g for 30 min. The supernatant was carefully removed using a pipette, frozen and stored at -20°C for VFA and ammonia analyses. The syringe was washed twice with 0.4% (w/v) NaCl solution, and the washings were poured into the centrifuge tube containing the pellet. The pellets were washed twice with 0.4% (w/v) NaCl solution. The tubes were again centrifuged at 20,000×g for 30 min. The pellet was oven dried at 105°C overnight and weighed. Apparent dry matter (ADM) and organic matter (OM) degradability of the substrates were determined (Getachew *et al.*, 1998). Metabolizable energy (ME) were estimated according to Menke and Steingass (1988). Total gas values were corrected based on blank gas volume which contained only rumen fluid. Cumulative gas production data were fitted to the model of Ørskov and McDonald (Ørskov *et al.*, 1979) using NEWAY Excel Version 5.0 package (El-Mekkawy *et al.*, 1998). The above procedures were conducted in three individual runs.

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4.2.7 Volatile Fatty Acids Determination

The volatile fatty acids (VFAs), which include acetic, isobutyric, butyric, propionic, valeric, isovaleric and caproic acid (Appendix F) were determined by gas a chromatography (Model G1540N, Agilent Technologies, USA) fitted with a flame

ionization detector (FID) and a packed column 5% Thermon-3000, Shincarbon A60/80. Nitrogen was used as the carrier gas at 40 ml/min and the oven temperature was maintained at 220°C. Injection and FID temperatures were fixed at 260°C.

4.2.8 Ammonia Determination

The phenol hypochlorite color reaction method, as described by Chaney and Marbach (1962), was used to determine the ammonia nitrogen. The reagents consisted of phenol solution (20 g phenol dissolved in 200 ml of 95% v/v ethyl alcohol), sodium nitroprusside (1 g of sodium nitroprusside dissolved in 200 ml of distilled water), alkaline reagent (100 g of sodium citrate and 5 g of NaOH dissolved in 500 ml of distilled water), oxidizing solution (sodium hypochlorite or commercially available chlorox; mixture of 100 ml of alkaline reagent and 25 ml of chlorox). A series of standard solutions using ammonium chloride (NH_4Cl) was prepared, with the concentrations of 0.2, 0.5, 1.0 and 2.0 µg/ml. Five millilitres of diluted samples (or standard) were added to an Erlenmeyer flask followed by adding 0.2 ml phenol solution and mixed. In sequence, 0.2 ml of nitroprusside and 0.5 ml of oxidizing solutions were added, mixed, capped and allowed to stand for one hour. The absorbance was determined at 640_{nm}. The standard curve was plotted using absorbance against concentration, and the amount of ammonia nitrogen was determined in the samples with reference to the standard.

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4.2.9 Isolation of Phorbol Esters

Phorbol esters from Jatropha meal were isolated according to the method of Li *et al.* (2010). Briefly, 4 g of the Jatropha meal was extracted with methanol five times using a pester and mortar. The methanol was evaporated using a rotary evaporator. The crude methanolic extract was dissolved in 5 ml of methanol and an aliquot was

analysed using a high-performance liquid chromatography (HPLC) Waters Alliance 2695 Separations Module with a Waters 996 Photodiode Array Detector (Waters, Milford, MA, USA). It was equipped with a reverse-phase, C18 LiChrospher 100, 250×4 mm I.D and 5µm pore size column (Agilent Technologies, Germany). The separation was performed using a gradient elution with solvents comprising deionized water and acetonitrile. The absorbance was read at 280 nm and peaks were observed at 24.4, 25.5, 26.5 and 26.9 min. Phorbol esters were collected using a fraction collector (Waters, Milford, MA, USA) at the above retention times. The collected fractions were pooled and freeze dried. Isolated phorbol esters were redissolved in absolute ethanol and reanalysed by HPLC to determine the purity and concentration. The concentration of the isolated phorbol esters used in this study was expressed as equivalent to the standard, phorbol-12-myristate-13-acetate (PMA).

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4.2.10 Effects of Isolated Phorbol Esters on Rumen Microorganisms Fermentation Activity

The *in vitro* gas production technique, based on Menke and Steingass (1988) was used to determine the activity of rumen microorganisms in the presence of the phorbol esters isolated from Jatropha meal. The details of the method were described earlier in Sections 4.2.6 to 4.2.8. However, in this experiment 200 mg of ground freeze dried *P. maximum* at 1 mm size was as used the substrate. Four levels of phorbol esters at 0, 3, 6, 9 and 12 mg/30 ml of buffered rumen fluid were tested. Different concentrations of phorbol esters were dispensed into syringe, followed by 30 ml of buffered rumen fluid, and the syringes were incubated in a water bath at 39^{o}C for 24 h. *In vitro* gas production (GP) was measured in triplicate at 2, 4, 8, 12 and 24 h. In each incubation run, three blanks were included to correct the GP values for gas released from endogenous substrates and three standard hays to check the

quality of the inoculum. A total of nine syringes for each treatment were used for total GP production measurement. The content of three syringes were used for *in vitro* DM degradability (IVDMD), pH, fermentation parameters, rumen microbial population quantification, and another three for microbial protein synthesis, and the remaining three syringes were used for the enzyme activity analyses. The above procedures were conducted in three individual runs.

4.2.11 Microbial Enzyme Activity

To extract the enzyme, the whole content of the syringe after 24 h of incubation (section 4.2.10) was transferred into glass beakers and 2.5 ml of lysozyme solution (4 g/L of phosphate buffer, 0.1 mol, pH 6.8) and 2.5 ml of carbon tetrachloride were added. The mixture was incubated at 39°C for 3 h, followed by sonication at 4°C using a sonicator (LabSonic, B. Braun, Germany). The sonicated samples were centrifuged at 20,000 × g for 25 min at 4°C, and the supernatant was collected and used for enzyme assay (Patra *et al.*, 2010). The carboxymethylcellulase (CMCase), filter paperase (FPase), β-glucosidase and xylanase activities were determined by using filterpaper, carboxymethylcellulose, ρ-nitrophenyl-β-d-glucopyranoside and xylan (oat spelt), respectively as substrates. Filterpaperase, CMCase and xylanase activities were determined by measuring the production of reducing sugar using dinitrosalicyclic acid (DNSA), while β-glucosidase activity was determined by measuring the amount of ρ-nitrophenol produced. Glucose and xylose standards in the range of 0–10 µmol, and ρ-nitrophenol standard in the range of 0–100 nmol were prepared. Protein was determined using the Bio-Rad Protein Assay Kit II based on the method described by Kruger (2002). Specific activity of each enzyme (CMCase, FPase, xylanase or β-glucosidase) was expressed as the micromoles (µmol) of product (glucose/xylose) released/min/mg protein under the assay conditions.

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4.2.12 Microbial Protein Synthesis

This method was based on a determination of the purines contents in apparent undegraded residue left after fermentation, as described by Makkar and Becker (1999b). Briefly, the whole content present in the syringe (section 4.2.10) was centrifuged at 20,000 × g for 25 min at 4°C and the supernatant was discarded. The pellet was washed with distilled water followed by centrifugation (20,000 × g for 25 min at 4°C). The washing procedure was repeated two more times. The pellet was lyophilized and 25-100 mg of undigested residue was placed in a 50 ml centrifuge tube and 2.5 ml of perchloric acid (0.6 M) and 0.5 ml of an internal standard (3 mM allopurinol) were added. The mixture was heated in a water bath at 95°C for 1 h. After cooling, 7 ml of 10 mM $NH_4H_2PO_4$ was added and the pH was adjusted to between 6.6 and 6.9 using KOH (8 M) followed by centrifuging at 3000 × g for 15 min to remove the precipitate. Supernatant was filtered through a 0.45 µm filter and injected into the high-performance liquid chromatography (HPLC Agilent-1200) equipped with a UV-Vis photodiode array (DAD) detector, binary pump, vacuum degasser, auto sampler and analytical column Agilent Reverse-phase C18 LiChrospher 100 (250 × 4 mm I.D and 5µm pore size). The solvents comprising solvent A (10 mM $NH_4H_2PO_4$, pH 6 adjusted with 10% NH_4OH) and solvent B (150 ml of acetonitrile added to 600 ml of 12.5 mM $NH_4H_2PO_4$, pH 6 adjusted with 10% NH_4OH). The gradient used was a 30 min linear gradient from 0 to 100% solvent B. After 40 min, solvent A was increased to 100% in the following 5 min, and the column was equilibrated to the starting condition (100% A) in the next 15 min before injecting the next sample. Analysis was conducted at room temperature and the flow rate was 0.8 ml/min. The absorbance was read at 254 nm. Guanine and adenine

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appeared at about 8.3 and 11.1 min respectively. Allopurinol was used as internal standard and appeared at about 16.6 min.

4.2.13 Microbial Population Analysis Using Real Time PCR

4.2.13.1 Sampling

At the end of the incubation (24 h) (Section 4.2.10) the whole content present in the syringe was transferred into a 50 ml centrifuge tube and the contents were mixed. One milliliter of buffered rumen fluid containing digesta was transferred into 2 ml autoclaved centrifuge tube and immediately kept at -80ºC for DNA extraction. The end of the one milliliter tip was cut to make the pipetting easier (Kongmun *et al.*, 2010).

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4.2.13.2 DNA Extraction

The QIAamp DNA Stool Mini Kit (QIAGEN) was used to extract the DNA from rumen fluid (Belanche *et al.*, 2010), and the method used was according to the manufacturer protocol, with some modifications. Frozen samples were thawed on ice and 200 mg glass beads (diameter 425–600 µm; Sigma Chemical, St. Louis, MO, USA) were added to the 2 ml autoclaved tube followed by adding 300 µl of rumen fluid and 1.5 ml of buffer ASL and placed on ice. The tube was vortexed continuously for 5 min. The sample was centrifuged at 20,000 × g for 1 min to pellet the particles. The supernatant (1.4 ml) was pipetted into a new 2 ml microcentrifuge tube and the pellet was discarded. One InhibitEX tablet was added to the tube and vortexed immediately and continuously until the tablet was completely suspended. The suspension was incubated for 1 min at room temperature to allow the inhibitors to adsorb to the InhibitEX matrix. The sample was centrifuged at 20,000 × g for 3

min to pellet the particles and inhibitors bound to the InhibitEX matrix. All of the supernatant was pipetted into a new 1.5 ml microcentrifuge tube and the pellet was discarded. The sample was centrifuged at 20,000 × g for 3 min. Proteinase K (25 µl) was added into a new 2 ml microcentrifuge tube and 600 µl of supernatant from the last step was added to the tube containing proteinase K. Buffer AL (600 µl) was added and vortexed for 15 s. The tube was incubated at 70°C for 30 min, and subsequently at 95°C for 10 min. Absolute ethanol (600 µl) was added to the lysate, and vortexed. The lysate (600 µl) was transferred to the QIAamp spin column and centrifuged at 20,000 × g for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube and the filtrate was discarded. The second aliquot of 600 µl lysate was applied to the QIAamp spin column and centrifuged at 20,000 × g for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube, and the tube containing the filtrate was discarded. The third aliquot of the lysate was also added in the same manner. Buffer AW1 (500 µl) was added to the QIAamp spin column and centrifuged at 20,000 × g for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube, and the collection tube containing the filtrate was discarded. The buffer AW2 (500 µl) was added to the QIAamp spin column and centrifuged at 20,000 × g for 3 min. The collection tube containing the filtrate was discarded. The QIAamp spin column was placed in a new 2 ml collection tube centrifuged at 20,000 × g for 1 min and the collection tube with the filtrate was discarded. The QIAamp spin column was transferred into a new, labeled 1.5 ml microcentrifuge tube and 200 µl of buffer AE was pipetted in the column. After 1 min incubation at room temperature, the QIAamp spin column was centrifuged at 20,000 × g for 1 min to elute the DNA.

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4.2.13.3 DNA Concentration and Quality Assessment

After isolation of DNA, quantification and quality analysis were carried out using gel electrophoresis and spectrophotometric analysis to ascertain the approximate quantity of DNA obtained and the suitability of the DNA samples for further analysis (Sambrook *et al.*, 2001). The OD of 1 µl of DNA was read at 260 and 280_{nm} using a spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The concentration and the ratio of OD260/OD280 were determined. The agarose gel (0.8%) was prepared. One µl of 6X loading dye was added to 100 ng of extracted DNA and the mixture was loaded on the gel and the electrophoresis was conducted at 70V for 1 h. The Hind III DNA marker was also included in the analysis to check the integrity and to estimate the size of genomic DNA. The gel was visualized using a UV transilluminator (Appendix G).

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4.2.13.4 PCR Amplification and Product Purification

The 16S rRNA of bacteria and archaea and 18S rRNA of protozoa and fungi were amplified by PCR using primers, as shown in the Table 4.2. The PCR amplification was performed with the i-StarTaq DNA polymerase kit (iNtRON Biotechnology, Sungnam, Kyungki-Do, Korea) using 1 µl of a template (20 ng μl^{-1}) in 20 µl of reaction solution. A BIORAD MyCycler™ thermal cycler was used with the following program: 1 cycle at 94°C for 5 min, 35 cycles of 94°C for 20 s, 55°C for 30s, 72°C for 1 min, and a final extension at 72°C for 10 min.

The PCR products were detected from a 20 µl volume of the amplification mixture (supplemented with 4-µl loading dye), loaded on to 1.5% SeaKem® GTG® agarose (FMC BioProducts, Rockland ME, USA) containing ethidium bromide, and electrophoresis was carried out at 90 V for 1 h. The PCR products were visualized

under UV illumination and excised from the gel. The PCR product was extracted using MEGAquick-spin PCR & Agarose Gel Extraction kit (iNtRON Biotechnology) (Appendix G).

Table 4. 2. Components of 1X PCR reaction (20 µl)

Ingredients	Volume (µl)
dH_2O	13.8
Buffer (10X)	2
dNTPS (25 mM)	2
Forward primer (10 pmol/ µl)	0.4
Reverse primer (10 pmol/ µl)	0.4
Genomic DNA (20 ng/µl)	1
Taq DNA polymerase (5U/ µl)	0.4

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4.2.13.5 Primers for PCR Amplification

The diversity of various microorganisms in the rumen fluid was determined using several specific real-time PCR primers that encoded the 16S rRNA of bacteria and the 18S rRNA of protozoa and fungi.

Table 4. 3. Characteristics of the PCR primer sets used in this study

Microorganism	Forward	Reverse	Amplicon size (bp)	Reference
General bacteria	cggcaacgagcgcaaccc	ccattgtagcacgtgtgtagcc	130	(Denman *et al.*, 2006)
General fungi	gaggaagtaaaagtcgtaacaaggtttc	caaattcacaaagggtaggatgatt	120	(Denman *et al.*, 2006)
Total protozoa	gctttcgwtggtagtgtatt	cttgccctcyaatcgtwct	223	(Slyvester *et al.*, 2004)
Total methanogens	cgwagggaagctgttaagt	taccgtcgtccactcctt	343	(Yu *et al.*, 2005)
Total archaea	attagatacccsbgtagtcc	gccatgcaccwcctct	273	(Yu *et al.*, 2005)
Ruminococcus albus	ccctaaaagcagtcttagttcg	cctccttgcggttagaaca	175	(Koike *et al.*, 2001)
Ruminococcus flavefaciens	cgaacggagataatttgagtttacttagg	cggtctctgtatgttatgaggtattacc	132	(Denman *et al.*, 2006)
Fibrobacter succinogenes	gttcggaattactgggcgtaaa	cgcctgcccctgaactatc	121	(Denman *et al.*, 2006)
Butyrivibrio fibrisolvens	accgcataagcgcacgga	cgggtccatcttgtaccgataaat	65	(Stevenson *et al.*, 2007)

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4.2.13.6 Cloning and Sequencing

The PCR products were cloned in a pCR®2.1-TOPO® TA cloning vector (Invitrogen, Carlsbad, CA, USA) and transformed into chemically competent *E. coli* TOP10 cells (Invitrogen) according to the kit protocol instructions, with some modifications (Zhou *et al.*, 2011). Briefly, 4 µl of fresh PCR product, 1 µl of salt solution and 1 µl of TOPO® vector were transferred to a 0.2 ml autoclaved centrifuge tube and mixed gently and incubated for 15 minutes at room temperature. From the TOPO® Cloning reaction 4 ul was added to the vial of One Shot® Chemically Competent *E. coli* and mixed gently. The mixture was incubated in ice for 30 min. The competent *E. coli* was given a heat-shock for 30 seconds at 42°C without shaking, and immediately was transferred in ice. The S.O.C. medium (250 µl) was added and the tube was capped tightly and shaken horizontally (200 rpm) at 37°C for 1 hour.

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After incubation, 50 µl of transformed *E. coli* was spread on prewarmed Luria-Bertani (LB) agar (dissolve 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L distilled water adjust the pH at 7) containing 100 µg/ml of ampicillin. The agar surface was previously coated with 32 µl of X-gal solution (50 mg X-gal in 1 ml of dimethylformamide) before spreading the transformed *E. coli*. After 12 h incubation at 37ºC white colonies were picked up using an autoclaved toothpick and were inoculated in LB broth containing 100 µg/ml of ampicillin and incubated at 37ºC overnight (appendix G).

4.2.13.7 Plasmid Extraction

The overnight culture of transformed *E. coli* grown in LB containing ampicillin (100 µg/ml) was used to extract the plasmid. The plasmid extraction was carried out

according to the protocol recommended by the plasmid extraction kit (iNtRON Biotechnology, Sungnam, Kyungki-Do, Korea) with some modifications. Briefly, the bacterial culture (5 ml) was harvested by centrifugation at 8,000×g for 30 sec and the supernatant was discarded. The bacterial pellet was suspended in 250 µl of resuspension buffer and vortexed until no clumps of the cell pellet remained. The lysis buffer (250 µl) was added to the mixture, and mixed gently by inverting the tube several times. Neutralizing buffer (350 µl) was added and mixed gently by inverting the tube several times. The tube was kept on ice for 5 min to improve the yield. The content of the tube was centrifuged at 8,000×g for 10 min at 4ºC. The supernatant was transferred promptly into the column followed by centrifugation at 8,000×g for 60 sec. The collection tube was discarded and then the column was placed in a new collection tube. Washing buffer A (500 µl) was added to the column and centrifuged at 8,000×g for 60 sec. The filtrate was discarded and the spin column placed back in the same collection tube. Washing buffer B (700 µl) was added and the tube centrifuged at 8,000×g for 60 sec. The filtrate was discarded and spin column was placed back in the same collection tube. The washing with buffer B was repeated one more time. The spin column was centrifuged at 8,000×g for 60 sec to dry the filter membrane. The spin column was placed into a clean and sterile centrifuge tube and 50 µl of elution buffer was added. After incubation at room temperature for 1 min the tube was centrifuged at 8,000×g for 60 sec to elute the plasmid (appendix G).

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4.2.13.8 Analyses of Transformants

The plasmids were analysed by the *Eco*R1 restriction enzyme and separated on a 1% (w/v) agarose gel to confirm the presence and correct orientation of the inserts. Plasmids with the right cloned inserts were sequenced in order to assert their identity.

Sequencing of 16S rDNA was performed using capillary electrophoresis on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). BigDye® Terminator v3.1 (Applied Biosystems) was used for sequence reactions. Sequencing files were processed using Appiled Biosystems Sequencing Analysis software v5.2.0 (Appendix G).

4.2.13.9 Sequence Identification and Secondary Structure Analyses

The sequences were analyzed by the Bellerophon (Huber *et al.*, 2004) and Mallard program (Ashelford *et al.*, 2006) to remove chimeric rDNA clones. Further, to determine the sequence similarity value, the sequence was compared to those available in the GenBank (Benson *et al.*, 2008) and Ribosomal Database II (Cole *et al.*, 2007), using the Basic Local Alignment Search Tool (Altschul *et al.*, 1997) and the Sequence Math online analysis tool, respectively. Sequences that were ≥99% similar to the previously published sequence were used as the criterion to indicate species identity.

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4.2.13.10 Real-Time PCR Analysis and Standard Curves

Real-time PCR assays were conducted on a BioRad CFX 96 real-time PCR thermocycler (Bio-Rad, Hercules, United States of America) using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). Sample DNA (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. Primers were identical to those reported previously for conventional PCR (Table 4.2).

Temperature gradient curves were analyzed to determine the optimal annealing temperature for each set of primers. Reaction conditions were as follows: 94°C for 5 min (1X), then 94°C for 20 s, then 55°C for 30 s or 58°C for 30s for total

methanogens and total archaea, and 72 ^{o}C for 20 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 to 95 °C at a rate of 0.2 °C/s with continuous acquisition of fluorescence. Data from the real-time PCR reactions were analyzed using CFX manager software version 2 (Bio-Rad Laboratories). All real-time PCR amplifications were performed in triplicate (appendix H).

4.2.13.11 Standard Curve Construction

Standard curves were constructed using different plasmids carrying the 16S rRNA gene of the targeted microorganisms including general bacteria, general fungi, total protozoa, total methanogens, total archaea, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens* (Zhou *et al.*, 2011). The concentration of each plasmid was determined spectrophotometrically (Nano-Drop Technologies, Wilmington, DE, USA). The copy number of the plasmids was calculated using the following formula:

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Number of copies = [amount (ng)× (6.022×10^{23})] / [length (bp) × (1×10^{9}) × 650]

(Li *et al.*, 2009)

A tenfold dilution series ranging from 10^{3} to 10^{8} copies of the plasmid carrying 16S rRNA gene was performed using nuclease free water. Dilutions of the plasmids were freshly prepared for each experiment. For each standard curve, the 16S rRNA gene copy numbers were plotted against the cyclic threshold (Appendix H).

4.2.14 Ruminal Phorbol Esters Disappearance

The whole content of each syringe was transferred into a glass beker and the content was lyophilized. The ruminal digesta was further extracted using methanol at least

five times with pestle and mortar, and then the methanol evaporated by rotary evaporator. The crude extract was dissolved in dimethyl sulfoxide (DMSO). The phorbol esters were determined according to the methods described in Chapter 3 section 3.2.10.

4.2.15 *In Vitro* Toxicity Test

A bovine kidney cell line (MDBK) (ATCC® Number: CCL-22™) was used to determine the toxicity of rumen fluid containing phorbol esters before and after fermentation. The extracts obtained upon analyses of phorbol ester residue (4.2.14) were used for the toxicity testing. 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 crude extract obtained from the syringe content. After 24h treatment, the supernatant of the 96-well plate containing cells was replaced with fresh media and 20 µl of MTT reagents (0.05 mg/ml) was added into each well. After 4 h, the spent media was 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). All determinations were performed in triplicate. The percentage of cell viability was calculated by using below formula:

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

4.2.16 Statistical Analyses

Data were analysed using the general linear models (GLM) procedure of SAS (2003) in a completely randomized design (CRD), and means were compared with Duncan's Multiple Range test.

4.3 Results and Discussion

4.3.1 Chemical Constituents

The physicochemical treatments were performed to remove the phorbol esters as the major limiting factor in the use of Jatropha meal. Upon treatments, DM and OM decreased with a concomitant increase in crude protein, ash, NDF and ADF as shown in Table 4.4. removal of the soluble fraction during the washing stage resulted in the increase in the percentage values. These findings were in agreement with the results reported by Martinez-Herrera *et al.* (2006) who showed that physical and chemical treatments increased the crude protein, ash, crude fiber and NDF content of Jatropha meal.

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Table 4. 4. Effect of treatments on the chemical constituents of Jatropha meal

Sample	DM lost (%)	Organic Matter (%)	Ash (%)	Crude Protein (%)	NDF (%)	ADF (%)	Gross Energy (MJ/kg)
a	nd	90.3^{a}	9.6^{d}	61.8^{e}	9.7^{c}	4.8^{b}	17.7
b	4.6^{d}	90.0^{a}	9.9^{d}	62.5^{e}	10.4^{c}	5.3^{ab}	17.7
c	25.8^{a}	89.0^{b}	10.9^{c}	67.1^{c}	20.0^{a}	6.0^{a}	17.3
d	24.0^{b}	87.8^{c}	12.1^{b}	70.8^{b}	18.7^{b}	6.5^{a}	17.0
e	19.5^{c}	85.4^{d}	14.5^{a}	64.6^{d}	17.8^{b}	6.2^{a}	17.1
f	26.8^{a}	87.6^{c}	12.0^{b}	67.7^{c}	20.9^{a}	6.6^{a}	17.6
g	25.1^{ab}	87.9^{c}	12.0^{b}	71.8^{b}	19.1^{a}	6.3^{a}	17.6
h	20.6^{c}	86.8^{c}	13.1^{b}	67.8^{c}	18.2^{b}	6.4^{a}	17.3
i	20.8^{c}	90.1^{a}	9.8^{d}	75.1^{a}	18.1^{b}	6.4^{a}	17.5
S.E.M	0.48	0.28	0.28	0.31	0.37	0.34	0.24

a= Jatropha meal, b=hydrothermal treatment (HT), c= HT + 3% (w/w) NaOH, d= HT + 3% (w/w) KOH, e= HT + 3% (w/w) $Ca(OH)_2$, f= HT+ 3% (w/w) NaOH + 10% (v/w) NaOCl, g= HT+ 3% (w/w) KOH +10% (v/w) NaOCl, h= HT+ 3% (w/w) $Ca(OH)_2$ +10% (v/w) NaOCl, i= HT+ 10% (v/w) NaOCl

Means with different superscripts within the same column are significantly different ($P \leq 0.05$)

All of the data are based on DM

nd: not determined

4.3.2 Effect of Treatments on Bioactive Compounds Content

Table 4.5 presents the bioactive compound concentrations as result of various treatments. Reduction of phenolic compounds would be an advantage since they are known for their interaction with proteins and subsequent effects upon feed intake, DM and protein degradability in ruminants. In the present study, the total phenolic compounds were reduced significantly ($P \leq 0.05$) in all treatments with treatments e, g and h showing the lowest content of phenolic compounds. The mechanism of reduction in total phenolic compounds was not clear, however it, could be attributed to the washing, transformation, decomposition of phenolics and formation of phenolic-protein complex under thermal, chemical and pressure conditions.

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Table 4. 5. Effect of physicochemical treatments on bioactive compounds content in Jatropha meal (on dry matter basis)

Treatments*	Total Phenolics[a] (mg/g)	Total Saponin[b] (mg/g)	Phytic acid[c] (g/100g)	Trypsin inhibitor[d] (mg/g)	Lectin activity[e] (U)	Phorbol esters[f] (mg/g)
a	3.9^{a}	19.0^{a}	9.1^{b}	34.2^{a}	102.7^{a}	3.0^{a}
b	3.5^{b}	17.0^{b}	7.8^{c}	0.3^{b}	1.2^{b}	1.3^{d}
c	2.8^{c}	15.0^{c}	11.2^{a}	0.3^{b}	1.2^{b}	1.3^{d}
d	1.8^{e}	9.0^{d}	11.0^{a}	0.2^{b}	1.2^{b}	1.5^{cde}
e	1.4^{ef}	9.0^{d}	11.0^{a}	0.1^{b}	1.2^{b}	1.7^{bc}
f	2.4^{d}	14.0^{c}	11.3^{a}	0.3^{b}	1.2^{b}	0.7^{f}
g	1.3^{f}	8.0^{d}	10.8^{a}	0.3^{b}	1.2^{b}	1.3^{d}
h	1.5^{ef}	7.0^{e}	10.8^{a}	0.2^{b}	1.2^{b}	1.4^{de}
i	1.8^{e}	15.0^{c}	8.8^{b}	0.2^{b}	1.2^{b}	1.6^{cd}
S.E.M	0.11	0.01	0.14	0.60	0.12	0.07

[a] Gallic acid equivalents; [b] Diosgenin equivalents; [c] Phytic acid g/100g DM; [d] mg of pure trypsin inhibited/g sample; [e] U: 1 mg of Jatropha meal that produced hemagglutination per milliliter of assay medium; [f] Equivalent to phorbol 12-myristate-13-acetate

a= Jatropha meal, b=hydrothermal treatment (HT), c= HT + 3% (w/w) NaOH, d= HT + 3% (w/w) KOH, e= HT + 3% (w/w) $Ca(OH)_2$, f= HT+ 3% (w/w) NaOH + 10% (v/w) NaOCl, g= HT+ 3% (w/w) KOH +10% (v/w) NaOCl, h= HT+ 3% (w/w) $Ca(OH)_2$ +10% (v/w) NaOCl, i= HT+ 10% (v/w) NaOCl

Means with different superscripts within a column are significantly different ($P \leq 0.05$)

Saponins are steroid or triterpene glycoside compounds. Previous research reported the beneficial effects of saponins, such as defaunation of the rumen and manipulation of the end products of fermentation, but harmful effects were also observed at higher concentrations (Wina *et al.*, 2005a). The total saponin in Jatropha meal obtained from local *J. curcas* plant was lower (19 mg/g) compared to the meal of toxic and non-toxic varieties with the values of 26 and 34 mg/g, respectively (Makkar *et al.*, 2009a). Total saponin content, as shown in Table 4.5, in the meal decreased significantly ($P \leq 0.05$) as a result of the treatments and washing. Since the total saponin content of Jatropha meal was lower than that of the soybean meal 47 mg/g, (Makkar *et al.*, 1998a), therefore, it might not cause any harmful affect on animal performance.

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Phytic acid has anti-nutritional properties due to its ability to chelate minerals resulting in mineral deficiency which may cause various diseases. The phytic acid in mongastric nutrition is more important than in polygastric animals since polygastric animals benefits from microbial phytase which allows digestion of phytate (Tsukatani *et al.*, 2008). Phytic acid level in Jatropha meal was 9.1 g/100g DM, which could be considered safe as the non-toxic varieties contain about 8.9 g/100g DM phytic acid (Makkar *et al.*, 2009a). As shown in Table 4.5, chemical treatments did not reduce the phytic acid content, and similar outcome was observed by Martinez-Herrera *et al.* (2006). Probably the chemical treatments reduced the solubility of phytic acid, and the increase in the percentage values of phytic acid were due to the loss in DM of the treated samples.

Trypsin inhibitors inhibit pancreatic proteases resulting in impaired protein digestion, hypertrophy and hyperplasia of the pancreas followed by depression in growth rate

(Makkar *et al.*, 2007a). However, they contribute towards resistance of the seeds to insect and pathogenic microorganisms (Valueva *et al.*, 2004). The trypsin inhibitor level in Jatropha meal was 34.2 mg/g DM (Table 4.5), higher than those toxic varieties with the value of 21.31 mg/g DM reported by Makkar and Becker (2009a). The high content of trypsin inhibitor in Jatropha meal might be attributed to the plant genotype. Hydrothermal treatment at 121^{o}C for 30 min at 15 psi fully inactivated the trypsin inhibitors. A similar observation was made by Martinez-Herrera *et al.* (2006). The inactivation of the trypsin inhibitor could be attributed to denaturation of their structural proteins and loss of water solubility due to the heat treatment.

Lectins agglutinate red blood cells, impair lipid, carbohydrate and protein metabolism and cause hypertrophy or atrophy of organs and tissues (Vasconcelos *et al.*, 2004). As shown in Table 4.5, the lectin content of Jatropha meal in the present study was comparable to other varieties including the toxic and non-toxic genotypes containing 102 U according to Makkar and Becker (2009a). Since lectins are protein in nature, therefore heat treatment inactivated them by denaturing their structures.

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Phorbol esters are tetracyclic diterpenoids known for their tumor promoting activity through mimicking the action of diacyl glycerol (activator of protein kinase C) leading to the over expression of protein kinase C and tumor generation (Goel *et al.*, 2007). The phorbol esters content of Jatropha meal was 3 mg/g DM (Table 4.5), considered harmful to animals, since the toxic varieties contain 2.7 mg/g DM according to the category described by Makkar and Becker (Makkar *et al.*, 2009a). All of the treated samples showed significant ($p \leq 0.05$) reduction of phorbol esters to less than 2.7 mg/g DM. In this study, sodium hydroxide was found to be the best

alkali to remove the phorbol esters. The efficiency of phorbol ester removal depends on the extent of saponification in the residual oil, followed by the washing process. Accordingly, a lower level of phorbol esters was observed in treatment c, compared to d and e treatments. Sodium hypochlorite was used as an oxidizing agent, and combined with hydrothermal and alkali treatments, could remove phorbol esters by 76.7% in treatment f.

Removal efficiency varied based on a combination of heat and chemical treatments as reported, e.g., 75% of the total phorbol esters by Martinez-Herrera *et al.* (2006), 87% by Aregheore *et al.* (2003) and 94% by Rakshit *et al.* (2008) where the initial phorbol esters amount in those studies were 3.85, 1.78 and 1.35 mg/g DM, respectively.

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4.3.3 Effects of Treated and Untreated Jatropha Meal on Rumen Microbes

Although hydrothermal treatment could reduce the bioactive compounds, it did not improve the apparent DM, OM degradability, estimated ME and $IVGP_{24}$ significantly (Table 4.6). This result was in agreement with Aderibigbe *et al.* (1997) who observed that hydrothermal treatment (80% moisture at 160^{o}C for 60 min) on Jatropha meal did not affect ruminal *in vitro* apparent OM degradability and estimated ME. On the other hand, a combination of hydrothermal and chemical treatments suppressed the apparent DM, OM degradability, estimated ME and $IVGP_{24}$, which might be due to loss of the soluble fraction, protein denaturation or an alteration of the chemical constituents (Table 4.4) of the Jatropha meal upon processing. Similarly, De Oliveira *et al.* (2008) showed that alkali treatment denaturated the soluble proteins of castor seed meal which possibly reduced protein solubility and rate of ruminal degradation.

Table 4.6 shows that hydrothermal alkali and oxidizing treatments increased the gas production from fraction (a) of Jatropha meal significantly ($p \leq 0.05$). This was possibly due to an increase in the soluble fraction of substrates at initial stage of fermentation. Gas production from fraction (b) of Jatropha meal (Table 4.6) was not affected by hydrothermal treatment, however, alkali and oxidizing agent treatments reduced the gas production from this insoluble fraction which indicated a reduction in degradation. The low rate of fermentation of the insoluble fraction, of the treated Jatropha meal might be beneficial for the synchronization of nitrogen release with available fermentable metabolizable energy to achieve a high yield of rumen microbial biomass.

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As indicated in Table 4.7, pH was not affected significantly by the various treatments. Ammonia nitrogen among treatments were similar except for e and h treatments ($P \leq 0.05$). The combination of alkali and oxidizing treatments in the e and h treatments markedly decreased the apparent dry and organic matter degradability ($P \leq 0.05$), which resulted in low levels of ammonia nitrogen in treatments e and h. This result was supported by Waltz and Loerch (1986) who observed a low level of rumen ammonia nitrogen when soybean meal was treated with 5M sodium hydroxide. Hydrothermal treatment did not affect the total VFAs significantly, and a similar observation was also reported for heat treated cotton seed by Pires *et al.* (1997). Getachew *et al.* (1998) illustrated the positive correlation between the total gas and the VFAs production. Lower gas production was observed in hydrothermal alkali and oxidizing treatments and accordingly lower total VFAs lead to a lower degradation rate of the samples.

Table 4. 6. Effects of treated and untreated Jatropha meal on ruminal fermentation parameters *in vitro*

Items	Treatments									S.E.M
	a	b	c	d	e	f	g	h	i	
DM degradability (%)	67.3^{a}	68.9^{a}	59.3^{b}	61.4^{b}	56.3^{c}	60.2^{b}	60.5^{b}	56.8^{c}	61.0^{b}	0.79
OM degradability (%)	59.3^{a}	61.4^{a}	54.5^{b}	54.1^{b}	51.6^{c}	55.5^{b}	54.8^{b}	51.7^{c}	55.8^{b}	0.74
ME (MJ/Kg DM)	10.6^{a}	10.5^{a}	9.7^{b}	9.8^{b}	9.1^{d}	9.5^{bcd}	9.6^{bc}	9.1^{cd}	9.8^{b}	0.14
Total gas (ml/24h)	33.1^{a}	32.3^{a}	24.5^{b}	24.7^{b}	22.9^{c}	24.3^{b}	24.1^{b}	22.7^{c}	23.7^{bc}	0.39
Gas production parameters*										
a (ml)	0.7^{c}	1.4^{a}	1.2^{ab}	1.3^{a}	1.4^{a}	1.1^{ab}	1.2^{ab}	0.9^{bc}	1.1^{ab}	0.10
b (ml)	40.4^{a}	41.7^{a}	36.8^{b}	38.3^{b}	38.1^{b}	37.7^{b}	36.6^{b}	37.1^{b}	37.9^{b}	0.61
c (% h^{-1})	0.19^{a}	0.10^{b}	0.06^{c}	0.06^{c}	0.04^{c}	0.04^{c}	0.05^{c}	0.05^{c}	0.05^{c}	0.01
a+b (ml)	41.6^{b}	43.6^{a}	38.0^{c}	39.0^{c}	39.1^{c}	38.9^{c}	37.8^{c}	37.9^{c}	39.1^{c}	0.6

a= Jatropha meal, b=hydrothermal treatment (HT), c= HT + 3% (w/w) NaOH, d= HT + 3% (w/w) KOH, e= HT + 3% (w/w) $Ca(OH)_2$, f= HT+ 3% (w/w) NaOH + 10% (v/w) NaOCl, g= HT+ 3% (w/w) KOH +10% (v/w) NaOCl, h= HT+ 3% (w/w) $Ca(OH)_2$ +10% (v/w) NaOCl, i= HT+ 10% (v/w) NaOCl

∗ a, b, c, and a+b were calculated from exponential equation $p=a+b(1-e^{ct})$

a = gas production from the immediately soluble fraction, b = gas production from the insoluble fraction, c = gas production rate constant for the insoluble fraction (b), (a + b) = potential extent of gas production

Means in each row with different superscripts are significantly different ($P \leq 0.05$)

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Table 4. 7. Effects of treated and untreated Jatropha meal on ruminal pH, volatile fatty acids and ammonia

	Treatments									
Items	a	b	c	d	e	f	g	h	i	S.E.M
pH	6.6	6.6	6.7	6.6	6.6	6.6	6.6	6.6	6.6	0.02
Ammonia N (mg/100 ml)	15.2^{ab}	16.3^{a}	15.5^{ab}	15.8^{ab}	15.1^{b}	15.3^{ab}	15.9^{ab}	15.1^{b}	15.9^{ab}	0.31
Total VFA (mM)	57.0^{a}	56.9^{a}	45.2^{b}	46.4^{b}	45.5^{b}	45.2^{b}	45.3^{b}	44.1^{b}	45.4^{b}	1.57

a= Jatropha meal, b= hydrothermal treatment (HT), c= HT + 3% (w/w) NaOH, d= HT + 3% (w/w) KOH, e= HT + 3% (w/w) $Ca(OH)_2$, f= HT+ 3% (w/w) NaOH + 10% (v/w) NaOCl, g= HT+ 3% (w/w) KOH +10% (v/w) NaOCl, h= HT+ 3% (w/w) $Ca(OH)_2$ +10% (v/w) NaOCl, i= HT+ 10% (v/w) NaOCl

Means in each row with different superscripts are significantly different ($P \leq 0.05$)

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The treatments did not influence the individual VFA concentration (Table 4.8) significantly, except for butyric acid which was significantly ($P \leq 0.05$) higher in the untreated Jatropha meal and hydrothermal treatment as compared to others. This might be due to changes in the microbial population involved in the butyric acid fermentation. Molar percentages of VFAs were in accordance with the molar proportion of VFA *in vivo*. i.e., 60-70% acetic acid, 20-25% propionic acid and 10-15% butyric acid (Abdullah, 1986).

Table 4. 8. Molar proportion of volatile fatty acids after 24 h of fermentation with various treated substrates

VFA (Molar %)	Treatments									S.E.M
	a	b	c	d	e	f	g	h	i	
Acetic acid	55.6	56.1	56.2	55.8	56.0	56.1	56.2	56.2	55.9	0.19
Propionic acid	20.7	21.4	21.8	21.7	21.2	21.6	21.3	21.5	21.6	0.37
Isobutyric acid	2.5	2.3	2.7	2.3	2.3	2.7	2.5	2.5	2.1	0.15
Butyric acid	13.4^{a}	11.1^{b}	10.0^{c}	9.7^{c}	9.5^{c}	10.1^{c}	9.8^{c}	9.4^{c}	9.3^{c}	0.25
Isovaleric acid	6.8	6.9	6.9	7.2	6.9	7.1	7.2	7.1	7.2	0.15
Valeric acid	2.2	2.2	2.3	2.4	2.3	2.5	2.5	2.4	2.5	0.06
Caproic acid	0.9	0.6	0.7	0.7	0.6	0.6	0.6	0.7	0.9	0.12
Acetic/ propionic	2.6	2.6	2.5	2.5	2.6	2.6	2.6	2.6	2.5	0.04

a= Jatropha meal, b=hydrothermal treatment (HT), c= HT + 3% (w/w) NaOH, d= HT + 3% (w/w) KOH, e= HT + 3% (w/w) $Ca(OH)_2$, f= HT+ 3% (w/w) NaOH + 10% (v/w) NaOCl, g= HT+ 3% (w/w) KOH +10% (v/w) NaOCl, h= HT+ 3% (w/w) $Ca(OH)_2$ +10% (v/w) NaOCl, i= HT+ 10% (v/w) NaOCl

Means in each row with different superscript are significantly different ($P \leq 0.05$)

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4.3.4 Isolation of Phorbol Esters

The phorbol esters appeared in four peaks named PE1, PE2, PE4 and PE4 (Figure 4.1). All of the fractions corresponding to the peaks were collected, pooled and freeze dried, and the isolated phorbol esters were dissolved in ethanol. The concentrations of the isolated phorbol esters used in this study were 0, 3, 6, 9 and 12 mg/30 ml of buffered rumen fluid.

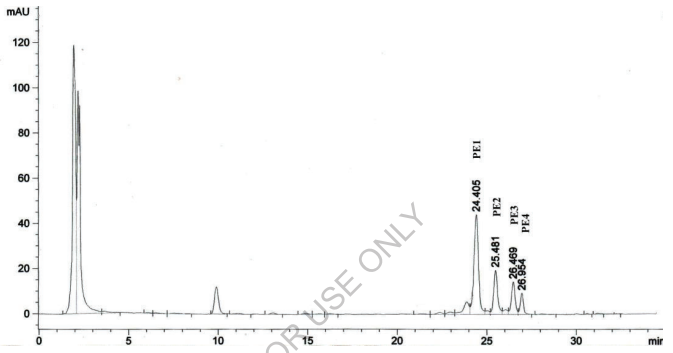

Figure 4. 1. Chromatogram of the phorbol esters present in Jatropha meal

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4.3.5 Effects of Isolated Phorbol Esters on Rumen Microorganisms Fermentation Activity

The *in vitro* gas production technique offers considerable advantages in terms of reliability and reproducibility under defined conditions. Hence, this technique is often used to evaluate the effects of plant bioactive compounds on rumen microbial activity (Makkar, 2005). Phorbol esters are diterpenoids, a major class of plant bioactive compounds derived from isoprene, which are present mostly in plants belonging to the *Euphorbiaceae* and *Thymelaeacae* family. There have been numerous studies concerning the biodegradation and biotransformation of plant originating terpenes in the rumen (Chizzola *et al.*, 2004; Schlichtherle-Cerny *et al.*, 2004).

In vitro gas production parameters are presented in Table 4.9. The phorbol esters carrier (ethanol) did not show any effect on gas production parameters, which indicated that the level of carrier used in the experiment (0.3%) was not detrimental to rumen microbes. The DMD, ME and total volume of gas produced after 24 h of incubation ($IVGP_{24}$) decreased significantly ($P \leq 0.05$) at the 9 and 12 mg phorbol esters levels when compared to the control. The gas production from the immediately soluble fraction (a), insoluble fraction (b), gas production rate constant (c) and potential extent of gas production (a + b) are shown in Table 4.9. Fractions (a) and (c) in the gas production parameters were not affected by the phorbol esters content significantly, while fraction (a+b) and (b) were depressed significantly ($p \leq 0.05$) at the 12 mg phorbol esters level. The significant decrease in gas production, metabolizable energy and fraction (a+b) and (b) could be due to a decrease in dry matter degradability, as observed in the present study.

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Table 4. 9. Effects of different levels of phorbol esters on ruminal fermentation parameters after 24 h fermentation

	Treatments						
Parameters	I	II	III	IV	V	VI	S.E.M
DMD (%)	49.5^{a}	47.7^{ab}	45.4^{b}	46.5^{b}	37.4^{c}	36.6^{c}	0.7
ME MJ/kg DM	6.8^{a}	7.1^{a}	6.9^{a}	6.9^{a}	6.4^{b}	6.1^{c}	0.07
$IVGP_{24}$ (ml/24h)	30.1^{a}	31.6^{a}	31.0^{a}	31.2^{a}	28.1^{b}	24.7^{c}	0.6
Gas production parameters							
a (ml)	1.3	1.3	1.3	1.3	1.1	1.1	0.12
b (ml)	59.1^{a}	58.3^{a}	58.3^{a}	57.3^{a}	58.4^{a}	44.5^{b}	2.79
c (% h^{-1})	0.025	0.026	0.025	0.026	0.027	0.029	0.001
(a+b) (ml)	60.4^{a}	59.6^{a}	59.6^{a}	58.6^{a}	59.4^{a}	45.5^{b}	2.88

200 mg guinea grass were used as substrate
I= grass, II= grass + ethanol (as a carrier), III= grass + 3 mg phorbol esters, IV= grass+6 mg phorbol esters, V= grass + 9 mg phorbol esters VI= grass + 12 mg phorbol esters
DMD: dry matter degradability; ME: metabolizable energy; $IVGP_{24}$: gas produced in 24h
a, b, c, and a+b are calculated from exponential equation $p=a+b(1-e^{ct})$
a = gas production from the immediately soluble fraction, b = gas production from the insoluble fraction, c = gas production rate constant for the insoluble fraction (b), (a + b) = potential extent of gas production.
Means in each row with different superscripts are significantly different ($P \leq 0.05$)

Fermentation parameters are shown in Table 4.10. The pH values and ammonia nitrogen were not significantly different among treatments. However, the total VFA produced by rumen microbes was significantly ($p \leq 0.05$) suppressed at the 9 and 12 mg phorbol esters levels. This finding was in agreement with that of Alexander *et al.* (2007) who reported the suppression of total gas, total volatile fatty acids (VFA) production, acetate to propionate ratio in the presence of *Moringa oleifera* terpenoid compounds.

Table 4. 10. Effects of different levels of phorbol esters on ruminal pH, volatile fatty acids and ammonia

	Treatments						
Parameters	I	II	III	IV	V	VI	S.E.M
pH	7.2	7.2	7.2	7.2	7.2	7.1	0.01
Ammonia N (mg/100 ml)	8.3	8.4	8.3	8.1	7.8	7.9	0.4
Total VFA (mM)	44.9^{a}	44.7^{a}	43.7^{ab}	42.2^{ab}	41.4^{b}	41.7^{b}	0.82

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200 mg guinea grass was used as substrate
I= grass, II= grass + ethanol (as a carrier), III= grass + 3 mg phorbol esters, IV= grass+6 mg phorbol esters, V= grass + 9 mg phorbol esters VI= grass + 12 mg phorbol esters
Means in each row with different superscripts are significantly different ($P \leq 0.05$)

The molar proportion of the individual volatile fatty acids at 24h of *in vitro* incubation with various concentrations of phorbol esters are shown in Table 4.11. Similar to the other gas production results, the 9 and 12 mg of phorbol esters levels decreased the production of the individual fatty acids. The ratio of acetic to propionic acid was also affected significantly ($P \leq 0.05$).

Table 4. 11. Effects of different levels of phorbol esters on molar percentage of volatile fatty acids

VFA (Molar %)	Treatments						
	I	II	III	IV	V	VI	S.E.M
Acetic Acid	61.5^{a}	60.7^{a}	60.2^{a}	59.2^{ab}	55.9^{b}	55.5^{b}	0.94
Propionic Acid	16.0^{a}	14.5^{b}	14.1^{b}	14.1^{b}	14.1^{b}	14.1^{b}	0.12
Isobutyric Acid	1.2^{a}	1.0^{ab}	0.8^{b}	0.8^{b}	0.8^{b}	0.8^{b}	0.06
Butyric Acid	7.4^{a}	7.4^{a}	7.4^{a}	7.4^{a}	7.0^{b}	6.8^{b}	0.12
Isovaleric Acid	2.5^{a}	2.3^{ab}	2.1^{bc}	2.0^{c}	2.0^{c}	2.0^{c}	0.08
Valeric Acid	1.4^{a}	1.2^{ab}	1.0^{ab}	0.8^{b}	0.8^{b}	0.8^{b}	0.14
Caproic Acid	1.4	1.0	0.6	0.4	0.4	0.4	0.25
Acetic/ Propionic Acid	8.6^{a}	8.4^{a}	8.4^{a}	8.4^{a}	7.8^{b}	6.8^{c}	0.12

200 mg guinea grass was used as substrate
I= grass, II= grass + ethanol (as a carrier), III= grass +3 mg phorbol esters, IV= grass+6 mg phorbol esters, V= grass + 9 mg phorbol esters VI= grass + 12 mg phorbol esters
Means in each row with different subscripts are significantly different (P≤0.05)

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This result was supported by Wanapat *et al.* (2008), who reported the reduction in ruminal propionic acid and the ratio of acetic to propionic acids concentrations in the presence of terpenoid compounds originating from garlic powder. Phorbol esters have been shown to possess antifungal activity (Saetae *et al.*, 2010) and antimycobacterial activity (Chumkaew *et al.*, 2003). The biodegradation of phorbol esters by different microorganisms has also been reported in human intestinal bacteria including *Clostridium butyricum*, *Clostridium innocuum* ES 24-06, *Peptostreptococcus anaerobius* 0240, *Peptostreptococcus intermedius* EBF 77/25, *Proteus mirabillis* S2 and *Ruminococcus sp.* P01-3 (Abdel-Hafez *et al.*, 2002), *Pseudomonas aeruginosa* (Joshi *et al.*, 2011) and different white-rot fungi (De Barros *et al.*, 2011). However, Makkar and Becker (2010a) reported that rumen

microbes do not degrade phorbol esters present in Jatropha meal, and phorbol esters do not adversely affect rumen fermentation. This difference in response may result from differences in the rumen microbial population and the inclusion rate of the phorbol esters. High levels of phorbol esters (9 and 12 mg) suppressed the DMD, ME, $IVGP_{24}$, and total VFA. However, the lower levels (3 and 6 mg) of phorbol esters did not affect the rumen fermentation. The impairment of rumen fermentation in the presence of high concentrations of phorbol esters is attributed to the antimicrobial activity of the phorbol esters.

The biological properties of phorbol esters are dependent on their structures and the number of their functional groups. The functional groups attach to the surface receptors of the microorganism located on the cell wall, which might trigger different internal pathways. The mechanism of action of plant extracts possessing antimicrobial activity have been shown to be through inhibition of cell wall synthesis, destructions of cell membrane structure or function, inhibition of structure and functions of DNA or RNA and inhibition of protein synthesis (Cowan, 1999). All these responses are triggered by the attachment of a plant bioactive compound functional group to the surface receptors of the microorganism located on their cell wall (Cushnie *et al.*, 2005).

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4.3.6 Rumen Microbial Enzyme Activity

Fibre degradation is the most important aspect of rumen fermentation and ruminant production. The community of anaerobic micro-organisms, including bacteria, protozoa and fungi, in the rumen can rapidly colonize and digest plant cell wall components, through the activity of a vast array of enzymes, to sugars and finally to volatile fatty acids (Wina *et al.*, 2006).

The Gram-negative *Fibrobacter succinogenes*, and two species of Gram-positive bacteria, *Ruminococcus albus* and *Ruminoccocus flavefaciens* are generally considered as the predominant bacterial species with the highest cellulolytic activity in the rumen (Weimer, 1996). Ruminal fungi (*Chytridiomycetes*) are also another important group of cell wall degrading organisms that easily penetrate plant cell walls. *Chytridiomycetes* have been shown to produce several enzymes such as cellulase, xylanase (Lowe *et al.*, 1987), glucosidase (Chen *et al.*, 1994), glycoside hydrolase (Hebraud *et al.*, 1990).

The effect of phorbol esters on rumen microbes enzyme activities are presented in Table 4.12. The significant ($p \leq 0.05$) reductions in CMCase, FPase, xylanase and β-glucosidase enzymes activity in the presence of phorbol esters were observed. The reduction in enzyme activities could be attributed to the antimicrobial activity of phorbol esters.

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Table 4. 12. Effects of phorbol esters on the specific activity of enzymes of rumen microbes *in vitro*

	Treatments						
Enzymes (μmol/min/mg protein)	I	II	III	IV	V	VI	S.E.M
CMCase	0.023^{a}	0.023^{a}	0.023^{a}	0.022^{ab}	0.021^{b}	0.021^{b}	0.0004
FPase	0.022^{a}	0.022^{a}	0.020^{ab}	0.020^{ab}	0.020^{ab}	0.019^{b}	0.0004
Xylanase	0.054^{a}	0.053^{a}	0.048^{b}	0.049^{b}	0.043^{c}	0.025^{d}	0.0006
β-Glucosidase	0.003^{a}	0.003^{a}	0.003^{a}	0.002^{b}	0.002^{b}	0.002^{b}	0.00002

200 mg guinea grass was used as substrate
I= grass, II= grass + ethanol (as a carrier), III= grass + 3 mg phorbol esters, IV= grass+6 mg phorbol esters, V= grass + 9 mg phorbol esters VI= grass + 12 mg phorbol esters
Means within the same row with different superscripts are significantly different ($P \leq 0.05$).

A decrease in cellulytic enzyme activity would affect DMD (Table 4.9), as well as the fermentation end products (Table 4.10). Vourch *et al.* (2001) reported that monoterpenes could inhibit the rumen microbial activity, while Broudiscou *et al.* (2007a) demonstrated that terpenoid undergo degradation in rumen. Recently, Saetae *et al.* (2010) reported the antimicrobial activity of phorbol esters, thus the suppression of enzyme activity observed in this instance could be due to the antimicrobial properties of phorbol esters.

4.3.7 Rumen Microbial Protein Synthesis

The results of microbial protein and the efficiency of rumen microbial protein synthesis in the presence of phorbol esters are shown in Table 4.13. The result clearly demonstrated that the addition of phorbol esters at concentrations of 3, 6, 9 and 12 mg/30 ml of buffered rumen fluid caused a significant ($p \leq 0.05$) decrease in the purine content as an index for rumen microbial protein. Consequently, the efficiency of rumen microbial protein synthesis indicated by the µMolar purine/ml of gas production and µMolar purine/mol of total VFA in the presence of 9 and 12 mg of the phorbol esters were significantly ($p \leq 0.05$) reduced.

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The fermentation of plant carbohydrate and nitrogenous compounds in the rumen can provide volatile fatty acids and microbial biomass that serve ruminants as sources of energy and protein, respectively. Plant bioactive compounds may affect the rumen microbial fermentation leading to changes in rumen microbial protein synthesis. The result obtained in this study was in agreement with Hart *et al.* (2008) who reported a 36% decline in the efficiency of microbial protein synthesis in cattle receiving the plant *Yucca schidigiera*. Moreover, Lu and Jorgensen (1987) found a decrease in the

efficiency of rumen microbial protein synthesis in sheep receiving *Trifolium pratense* containing saponins as the terpenoid constituents.

Table 4. 13. Effects of phorbol esters on purine content (index of microbial protein) and efficiency of rumen microbial protein synthesis

	Treatments						
Analyses	I	II	III	IV	V	VI	S.E.M
Adenine (µmol)	0.85^{a}	0.87^{a}	0.75^{b}	0.76^{b}	0.75^{b}	0.67^{b}	0.03
Guanine (µmol)	0.24^{a}	0.25^{a}	0.21^{b}	0.21^{b}	0.19^{b}	0.20^{b}	0.07
Purines (µmol)	1.10^{a}	1.12^{a}	0.96^{b}	0.98^{b}	0.95^{b}	0.88^{b}	0.03
µmol purine/ml gas produced	0.037^{a}	0.036^{a}	0.033^{ab}	0.034^{ab}	0.031^{b}	0.031^{b}	0.001
µmol purine/mol total VFA	0.82^{a}	0.84^{a}	0.76^{ab}	0.77^{ab}	0.73^{b}	0.69^{b}	0.02

200 mg guinea grass was used as substrate
I= grass, II= grass + ethanol (as a carrier), III= grass + 3 mg phorbol esters, IV= grass+6 mg phorbol esters, V= grass + 9 mg phorbol esters VI= grass + 12 mg phorbol esters
Means in each row with different superscripts are significantly different (P≤0.05)

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The reduction of rumen microbial protein synthesis in the presence of phorbol esters was possibly due to the antimicrobial effects of the phorbol esters, the metabolites produced upon degradation or biotransformation of the phorbol esters, and the lack of adaptation of the rumen microbes to the presence of phorbol esters. Generally, the suppression of fermentation in the rumen fluid that had not been adapted to the bioactive compounds under study could be expected, however, the extent of suppression depends on the nature of the bioactive compounds.

4.3.8 Rumen Microbial Population

In this study, the rumen microbial population was determined by Real time PCR, and the population of rumen microbes was expressed as absolute copy numbers of target DNA in one millilitre of the buffered rumen fluid. The results of quantification of rumen microbial population are presented in Table 4.14. It was observed that all concentrations of phorbol esters were detrimental to rumen microbes, and indicated the significant ($p \leq 0.05$) reduction in population of all rumen microbes. An increase in the concentration of phorbol esters decreased the number of total bacteria, fungi, protozoa, methanogens, archaea, *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens.* The reduction in the population of methanogens, archaea and protozoa may reduce the methane production, which could be an advantage. While, reduction in the population of total fungi, together with major cellulolytic bacteria including *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens,* markedly reduced the feed degradability, in particular the fiber, in the rumen (Table 4.9). Since the feed degradation in the rumen relies on the enzymes produced by various microorganisms, especially the bacteria inhabiting in the rumen, therefore, any reduction in the population of the microorganisms resulted in a reduction of the enzyme levels and would affect feed degradation. The rumen is a complex ecosystem and affected by the nature of dietary feeds, synchronisation of nutrient release and fermentation end-products to be utilized by co-species of rumen microorganisms and the host animal (Waterman *et al.*, 2011). The plant bioactive compounds modulate the rumen microbial ecosystem through changes in the rumen microbial populations. Terpenoid compounds, such as saponins, are known to reduce the rumen protozoa population (Wina *et al.*, 2005a) while tannins, as phenolic compounds, were found to

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suppress the methanogens and archaea (Animut *et al.*, 2008). Different microorganism showed different sensitivity to the plant bioactive compounds. The phenomenon of rumen microbiota adaptation to potentially harmful compounds has been suggested for a number of bioactive compounds, such as oxalic acid (Duncan, 2000), pyrrolizidine alkaloids (Wachenheim *et al.*, 1992), tannins (Goel *et al.*, 2005) and terpenes (Broudiscou *et al.*, 2007b).

The results obtained were in agreement with those researchers who have reported the decrease in rumen protozoa, bacteria and fungi in the presence of the terpenoid compounds of garlic (Wanapat *et al.*, 2008), *Sapindus rarak* (Wina *et al.*, 2005b) and *Euphorbia esula* (Waterman *et al.*, 2011). The decrease in rumen microbial population studied indicated the antimicrobial activity of the phorbol esters. Until now there is no report on the effects of phorbol esters on specific rumen microbial species. Likwise, Jouany and Morgavi (2007) suggested the detrimental effect of triterpenoid or steroid saponins on protozoa through the reaction with the sterols located in the ciliate cell membrane. It is believed that due to the hydrophobic nature of phorbol esters and their affinity for lipids, they can disrupt the cytoplasmic membrane either directly or by damaging the membrane proteins, causing increased membrane permeability and leakage of cytoplasmic constituents (Gomperts *et al.*, 2009). Phorbol esters may also cause cytoplasmic coagulation and eventually lead to cell lysis, similar to other terpenoids (Abdel-Hafez *et al.*, 2002; Chumkaew *et al.*, 2003).

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Table 4. 14. Effect of isolated phorbol esters on rumen microbial population

Items	Treatments						
	I	II	III	IV	V	VI	S.E.M
General bacteria×10^{15} copies/ ml of buffered rumen fluid							
	5.74^{a}	5.15^{a}	4.46^{ab}	4.17^{b}	3.58^{b}	2.93^{c}	0.450
General fungi×10^{7} copies/ ml of buffered rumen fluid							
	5.96^{a}	4.32^{ab}	3.52^{b}	1.95^{c}	1.74^{c}	1.46^{c}	1.047
Total protozoa×10^{6} copies/ ml of buffered rumen fluid							
	5.94^{a}	5.87^{a}	5.11^{b}	3.68^{c}	1.87^{d}	0.58^{e}	0.216
Total methanogens×10^{7} copies/ ml of buffered rumen fluid							
	4.52^{a}	3.41^{a}	2.07^{b}	1.71^{b}	0.83^{c}	0.52^{c}	0.39
Total archaea×10^{8} copies/ ml of buffered rumen fluid							
	5.41^{a}	4.77^{a}	4.25^{ab}	3.78^{b}	3.61^{bc}	3.26^{c}	0.450
Fibrobacter succinogenes×10^{8} copies/ ml of buffered rumen fluid							
	4.68^{a}	4.25^{ab}	3.69^{b}	3.20^{bc}	2.64^{cd}	2.09^{d}	0.550
Ruminococcus albus×10^{6} copies/ ml of buffered rumen fluid							
	7.02^{a}	7.32^{a}	5.50^{b}	3.84^{c}	1.75^{d}	1.27^{d}	0.170
Ruminococcus flavefaciens×10^{6} copies/ ml of buffered rumen fluid							
	5.33^{a}	5.34^{a}	3.02^{b}	0.51^{c}	0.32^{c}	0.15^{d}	0.102
Butyrivibrio fibrisolvens×10^{6} copies/ ml of buffered rumen fluid							
	1.46^{a}	1.37^{a}	1.02^{b}	0.72^{c}	0.18^{d}	0.1^{d}	0.050

200 mg guinea grass was used as substrate

I= grass, II= grass + ethanol (as a carrier), III= grass + 3 mg phorbol esters, IV= grass+6 mg phorbol esters, V= grass + 9 mg phorbol esters VI= grass + 12 mg phorbol esters

Means in each row with different superscripts are significantly different (P≤0.05)

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The susceptibility of rumen microbes to bioactive compounds is alleviated following the adaptation of rumen microbes, and an increase in the number of specific microbes with the ability to degrade that particular bioactive compounds (Poppenga, 2010). In addition, the rumen microbial populations in different ruminants can have different capacities for tolerating or degrading plant bioactive compounds. A gradual increase in the intake of a plant containing toxic bioactive compounds has been indicated to increase the ability of a ruminant to tolerate or degrade that specific bioactive compound.

4.3.9 Ruminal Phorbol Esters Disappearance

The phorbol esters disappearance values upon fermentation by rumen microbes are presented in Table 4.15. The result showed a reduction in the phorbol esters levels upon fermentation by rumen microbes. The incorporation of higher concentrations of phorbol esters led to higher percentages of phorbol esters lost. However, the reasons for the trend in phorbol esters losses are not fully understood. It is possible that, higher levels of phorbol esters may elevate the production of microbial enzymes involved in the degradation or biotransformation of phorbol esters.

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Table 4. 15. Ruminal disappearance of phorbol esters upon fermentation

	Treatments					
	I	II	III	IV	V	VI
Initial phorbol esters (mg)[1]	0	0	3±0.12	6±0.19	9±0.15	12±0.14
Final phorbol esters (mg)[2]	0	0	2.3±0.17	4.1±0.14	5.1±0.49	6.7±0.32
Disappearance (%)	0	0	23.0	32.1	43.7	44.1

200 mg guinea grass was used as substrate
I= grass, II= grass + ethanol (as a carrier), III= grass + 3 mg phorbol esters, IV= grass+6 mg phorbol esters, V= grass + 9 mg phorbol esters VI= grass + 12 mg phorbol esters
[1]Recovered phorbol esters at 0 h fermentation from 30 ml buffered rumen fluid
[2]Recovered phorbol esters at 24 h fermentation from 30 ml buffered rumen fluid

Broudiscou *et al.* (2007a) indicated that the rumen microbial ecosystem has the capacity to degrade large amounts of terpenoids, including α-copaene, myrcene, β-ocimene, α-pinene and sabinene *in vivo*. In addition, Malecky and Broudiscou (2009) reported the higher degradation rate of terpenoids *in vitro* by rumen microflora which were adapted to the high fiber content diet. Thus, rumen microbes showed the ability to degrade the terpenoids, however, it seems that the extent of degradation depends on rumen microbial adaptation and the source inoculum.

4.3.10 *In Vitro* Toxicity Test

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The toxicity results of the crude extract obtained from the rumen fluid containing phorbol esters prior and after fermentation are shown in Table 4.16. It was observed that the extract obtained from treatments I and II did not decrease the MDBK cell viability at 100 µg/ml of crude extract. While the extract obtained from rumen fluid containing phorbol esters at 0 h incubation time has markedly decreased the MDBK cells viability. The crude extracts obtained from 24h fermentation were also assessed for their toxicity and the results revealed the significant ($p \leq 0.05$) improvement in the MDBK cells viability indicating the lower toxicity of the rumen fluid crude extract. This reduction in the toxicity corresponded to the capability of rumen microbes to detoxify the phorbol esters through biodegradation or biotransformation.

Table 4. 16. Toxicity of crude extract obtained from rumen digesta

	MDBK cell viability (100 µg/ml)					
Incubation time	I	II	III	IV	V	VI
0 h	96.6	95.7	79.3[b]	67.3[b]	49.7[b]	43.6[b]
24 h	97.6	96.0	90.3[a]	83.3[a]	75.4[a]	72.3[a]
SEM	4.19	3.58	4.08	4.04	5.07	4.90

I= grass, II= grass + ethanol (as a carrier), III= grass + 3 mg phorbol esters, IV= grass+6 mg phorbol esters, V= grass + 9 mg phorbol esters VI= grass + 12 mg phorbol esters
Means in each column with different superscripts are significantly different ($P \leq 0.05$)

The detoxification of the phorbol esters by rumen microbes could be a promising advantage when combined with physicochemical treatments of Jatropha meal. However, any undegraded phorbol esters even in lower amount might be still detrimental to the animals consuming the meal.

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4.4 Conclusion

Chemical analyses revealed the similarity of the Jatropha meal obtained from the local *J. curcas* plants to other genotypes from different regions. The results showed that the *J. curcas* seed used in the present study could be classified under the toxic variety. Combinations of hydrothermal, alkali and oxidizing treatments alleviated the bioactive compound levels in the Jatropha meal to safe levels, except for the phorbol esters which were only reduced to 0.7 mg/g DM. However, the alleviation of bioactive compounds in Jatropha meal did not improve the *in vitro* rumen fermentation. The rumen microbes could alleviate the phorbol esters content partially and also the cytotoxicity effect on bovine kidney cells. However, the incomplete removal of phorbol esters by both physicochemical treatments and microbial activity could limit the utilization of Jatropha meal as a biofeed.

CHAPTER 5

BIOLOGICAL AND MOLECULAR MECHANISMS OF ACTION OF ISOLATED PHORBOL ESTERS ON BOVINE KIDNEY CELL LINE

5.1 Introduction

Jatropha is a genus of approximately 175 succulent plants, shrubs and trees (Xu *et al.*, 2010) containing about 68 diterpenes. A majority of the diterpenoids, particularly phorbol esters, possess a wide spectrum of activities such as cytotoxic, antitumor, tumor promoter and antimicrobial properties. These compounds possibly could be used in pharmaceutical industry, or as a template to synthesize new compounds with improved biological activities (Devappa *et al.*, 2010a).

The phorbol esters mimic the action of diacyl glycerol (DAG), an activator of protein kinase C, which regulates different signal transduction pathways and other cellular metabolic activities (Goel *et al.*, 2007). Haas and Mittelbach (2002) confirmed the presence of six phorbol esters in *J. curcas* seed. The six phorbol esters have been identified and characterised, but appear as four peaks in the HPLC chromatogram (Li *et al.*, 2010; Devappa *et al.*, 2010a). The phorbol esters are considered to be the most biologically active compounds present in the Jatropha meal (Devappa *et al.*, 2010a).

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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 lead to different responses in animal tissues (Goel *et al.*, 2007). Hence, some naturally occurring phorbol esters are tumor inhibitors and possess the ability to inhibit normal and cancer cell growth (Abdel-Hafez *et al.*, 2002; Lee *et al.*, 2009;

Muanda *et al.*, 2010), while others are tumor promoters (Goel *et al.*, 2007; Ikuta *et al.*, 2008; Shrotriya *et al.*, 2010b).

The results of the previous experiments showed the ability of rumen micobes in detoxification of phorbol esters isolated from Jatropha meal to some extent. The incomplete removal of the phorbol esters raised the question on their effects on animal tissue. Therefore, this experiment was performed to investigate their biological effects and to elucidate the mode of action of the isolated phorbol esters from Jatropha meal in a bovine kidney cell line.

5.2 Material and Methods

5.2.1 Jatropha Meal Preparation

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The mature *J. curcas* seeds were collected from the farm, 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 40–60ºC) for 16 h to obtain the meal according to Martinez-Herrera *et al.* (2006).

5.2.2 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, cDNA Synthesis Kit was from Qiagen GmbH, Hilden, Germany, the i-StarTaq DNA polymerase kit obtained from iNtRON Biotechnology, Sungnam, Kyungki-Do, Korea, SeaKem® GTG® agarose was purchased from FMC BioProducts, Rockland ME, USA, Loading dye, DNA ladder (100 bp, 1Kb) were

from MBI Fermentas, Lithuania and SYBR Green Supermix was purchased from Bio-Rad Laboratories, Hercules, CA.

5.2.3 Isolation of Phorbol Esters

Phorbol esters from Jatropha meal were isolated using high-performance liquid chromatography (HPLC) according to Li *et al.* (2010), and the method was described in detail in Chapter 4 section 4.2.9. However, in this experiment the four peaks representing the phorbol esters which were detected at 24.4, 25.5, 26.5 and 26.9 min were collected individually using a fraction collector (Waters, Milford, MA, USA) at the above retention times and named PE1, PE2, PE3 and PE4. The collected fractions were freeze dried. Different fractions were redissolved in dimethyl sulfoxide (DMSO) and analysed by HPLC to determine the purity and concentration. The concentration of the isolated phorbol esters used in this study was expressed as equivalent to a standard, phorbol-12-myristate 13–acetate (PMA).

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5.2.4 Cell Culture and Maintenance

In this experiment the kidney cell line was chosen, since previous studies observed the prominent pathological symptoms, mainly in the kidney, when animals were fed with Jatropha meal (Rakshit *et al.*, 2008; Li *et al.*, 2010; Katole *et al.*, 2011). Bovine kidney cell line (MDBK) was purchased from the American Type Culture Collection (ATCC® Number: CCL-22™). The MDBK cell line was derived from a kidney of a normal adult steer. The cells were maintained in Eagle's Minimum Essential Medium (EMEM) containing 2 mM L-glutamine, 1 mM sodium pyruvate, sodium bicarbonate (1500 mg/L) and horse serum to a final concentration of 10%. Cells were subcultured at ratio of 1:2 to 1:4, twice per week. To detach the cells, the cell sheet was rinsed

twice with PBS and the PBS was removed. The flask was kept at 37°C for 15 minutes and then the fresh medium was added, aspirated, and dispensed into new flasks. For the cryopreservation, fetal bovine serum containing 5% DMSO was used.

5.2.5 Cytotoxicity Test (MTT Assay)

The cytotoxic effect of the isolated phorbol esters was tested using the MTT reagent. MTT is a water soluble tetrazolium salt which yellow in colour. Metabolically active cells are able to convert the MTT molecule into a water-insoluble dark blue formazan salt by the reductive cleavage of the tetrazolium ion (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 PE1, PE2, PE3 and PE4, while the concentration of DMSO was kept at 0.1%. After treatment, the supernatant of the 96-well plate containing the cells were replaced with fresh media and 20 µl of MTT reagents (0.05 mg/ml) was added into each well. After 4 h, the spent media was 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:

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$$\text{Cell viability (\%)} = [(OD_{sample})/(OD_{control})]\times100$$

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

5.2.6 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 media 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 12 and 24 h. Morphological changes were examined after 12 and 24 h incubation, and the cells photographed using an inverted microscope.

5.2.7 Gene Expression Analyses in the MDBK Cell Line

5.2.7.1 Cell Treatments and RNA Extraction

The MDBK cells were seeded at a density of 1×10^6 cells per 75 cm^2 flask and cultured for 24 h in EMEM. After 24 h incubation, the media was removed and fresh media containing a different phorbol ester fraction at the CC_{50} concentration was 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 RNasey Mini kits (Qiagen, Valencia, CA, USA) according to the protocol recommended by the manufacturer.

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5.2.7.2 Quantification of RNA

The concentration of extracted RNA was quantified using a spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The OD of 1 µl of RNA was read at 260, 280 and 320_{nm} and the concentration of RNA in each sample was determined. The RNA of each sample had a ratio value (A260:A280) of 1.6-2.1. The samples were then separated into 5 µl aliquot each, and stored at -20°C for up to 3 months.

5.2.7.3 RNA Integrity Confirmation

The integrity and size distribution of the total RNA extracted was checked by using denaturing agarose gel electrophoresis and ethidium bromide staining. Briefly, 4 µl of the extracted RNA was mixed with 1 µl of RNA loading dye and incubated at 65°C for 3 to 5 minutes. Then, the samples were loaded into equilibrium formaldehyde agarose gel and run at 90-100 volts. Thereafter, the agarose gel was stained with 1 µg/ml of ethidium bromide for 30 minutes and distained with deinonized water for another 10 minutes. The ribosomal RNA bands were visualised and captured using a UV transilluminator. The respective ribosomal RNAs should appear as 2 sharp bands representing 28S rRNA to 18S RNA; where the apparent ratio should be approximately 2:1 (28S rRNA: 18S rRNA) indicating an intact total RNA was obtained from samples. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation in the RNA extraction process (Appendix I).

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5.2.7.4 cDNA Synthesis and Agarose Gel Electrophoresis

The reverse transcriptase PCR (RT-PCR) was performed using Maxime RT Premix kit (iNtRON Biotechnology, Sungnam, Korea) according to the protocol of the manufacturer. Briefly, 1 µg of total RNA and RNase-free water were added into the Maxime RT PreMix tubes to a total volume of 20 µl. The RT-PCR was performed in Thermal cycle (BioRad, Hercules, CA) with the following program: cDNA synthesis 45^{o}C for 60 min and RTase inactivation step 95^{o}C for 5min. The RT-PCR product was diluted by adding 50 µl RNase-free water into a tube containing the cDNA obtained at RT reactant. Then, the 5 µl of PCR products were subjected to electrophoresis by 1.8 % agarose gel at 108 V for 2 h. The gel was stained with 1

µg/ml of ethidium bromide for 10 min and rinsed with deionized water for 1 min. The image of the gel was visualised and captured using a gel documentation system (Bio-Rad Laboratories, Inc., Hercules, CA) (Appendix J).

5.2.7.5 Primer Design

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 accordingly. Primers for SYBR Green real-time PCR were designed based on a few criteria, i.e. primers should be 18 to 25 nucleotides long, the G/C content should be within 30 to 80%, the last five nucleotides on the 3' end should not contain more than two Gs or Cs, four or more identical nucleotides should be avoided, the amplicon size should be between 50 to 150 bases long and, lastly, the primers should contain no or minimal hairpin structures and dimers. The primers were synthesised by Bioneer Corp (Korea).

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Table 5. 1. Characteristics of the PCR primer sets used in the gene expression analyses

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
Cox2	5'-agcaccattctccctgaaa-3'	5'-aaacctacttctccaccgaaa-3'	136
IL-1 β	5'-ctgatgaccctaaacagatgaaga-3'	5'-cacgatgaccgacaccac-3'	134
β-actin	5'-gccaaccgtgagaagatga-3'	5'-aggcatacagggacagcac-3'	94
GAPDH	5'-gatgctggtgctgagtatgtg-3'	5'-ggcagaaggtgcagagatg-3'	114

5.2.7.6 Validation of the Specificity of Designed Primers

Initially, the specificity of the primers was verified using BLAST (Altschul *et al.*, 1997). The specificity of the primers was further confirmed by conventional PCR using cDNA from bovine kidney cell (Appendix J).

5.2.7.7 PCR Amplifications

The PCR products were analysed by running on a 1% agarose gel. Primer specificity was validated by the lack of a detectable PCR product using non-target template DNA (Appendix J).

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5.2.7.8 Gene Expression Analyses Using Real-Time PCR

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). Sample cDNA (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. Primers are presented in Table 5.1. Temperature gradient curves were analyzed 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 60ºC for 30 s and 72ºC 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 60ºC for 20 s and 72ºC for 25 s (40X). The IL-1β PCR reaction condition was 94°C for 5 min (1X), then 94°C for 20 s, then 62ºC for 20 s and 72ºC 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 to 95°C at a rate of 0.2°C/s 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, IL-1β, Cox2, GAPDH and β-actin) and using the cDNA of the MDBK cell line as template. The concentration of each PCR product was determined by spectrophotometer (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 performed using nuclease free water. Dilutions of the PCR product 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 analyzed using CFX manager software version 2 (Bio-Rad Laboratories). All real-time PCR amplifications were performed in triplicate (Appendix J).

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5.2.8 Protein Expression Analyses Using Western Blot Technique

5.2.8.1 Cells Treatment

The MDBK cells were seeded at a density of 1×10^6 cells per 75 cm^2 flask and cultured for 24 h in EMEM. Once the cells were confluent, the media was removed and fresh media containing different phorbol ester fractions at the CC_{50}

concentration was added. The treated cells were then incubated at 37°C in 5% CO_2 for 12 h and the protein was extracted.

5.2.8.2 Preparation of Whole Cell Lysate

The cells in the control group and the phorbol ester treated groups were scraped and transferred into 10 ml sterile Falcon tubes and centrifuged at 110×g (1000 rpm) for 10 min at 4ºC. The collected cell pellet was resuspended in Tris-sucrose buffer (wash buffer) (Tris-base 1.21 g, sucrose 8.56 g in 1 L deionized water, pH 7) and centrifuged again. The washing step was repeated two more times. The cell pellets were further resuspended with 100 µl of ProteoJET™ Mammalian Cell Lysis Reagent (Fermentas) followed by adding 1 µl of ProteoBlock™ Protease Inhibitor Cocktail (Fermentas). The cells were kept on ice, sonicated at 20 Hz for 30s and incubated on ice for 15 min. The cells were centrifuged at 25000×g at 4ºC for 30 min. Then the supernatant was collected for protein content determination using the Bradford assay. Finally, the protein supernatant was frozen at -80°C in aliquots of 20 µl for further experiments (Appendix K).

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5.2.8.3 Protein Quantification (Bradford Assay)

A series of dilutions of albumin standard was prepared and assayed at the same time with the unknown protein samples. Briefly, bovine serum albumin (BSA) with a concentration ranging from 0 to 20 µg was prepared. Then, 20 µl of each dilution and unknown samples were transferred in triplicates into non-sterile 96-well plate with a U bottom. After that, 200 µl of Bradford reagent (100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml 95% ethanol and 100 ml 85% (w/v) phosphoric acid was added, the solution was diluted to 1 litre and filtered through Whatman no. 1

filter paper) was added into each well of the standard and sample dilutions. Mixtures were mixed thoroughly on a plate shaker for 30 seconds, after which the plate was covered and incubated at 37°C for 10 minutes. Then, the color intensity in the plate was assessed at 575_{nm} with a SpectraMax, Plus 384 microplate reader (Molecular Devices, USA). The intensity of the purple color development was directly proportional to the protein concentration. The average absorbance values were plotted against the concentration of BSA standard curve (0-20 µg). Then, the concentration of each unknown sample was determined by interpolation from the standard curve (Appendix L).

5.2.8.4 Western Blotting Analysis

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In order to determine the expression of the target protein, equal amounts of protein (50- 100 µg) were mixed with loading buffer and incubated at 95°C for 3 min. Then the protein in the loading buffer was separated on a 10% SDS-PAGE discontinuous gel (gel preparation as in Appendix M). The gels were run at 40 mA (20 mA per gel, constant current) for 1 hour. After the separation was completed, the protein bands were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane using a TE 77 SemiPhor Semi-dry Transfer Unit (Amersham Pharmacia Biotech, Amersham, UK). The transfer process was conducted at a constant voltage setting (10 V) for exactly 60 min, and the current was set by multiplying the calculated gel area (cm^2) with 8 mA/cm^2. After transferring, the membrane was blocked for 1 h at room temperature with blocking PBST containing 5% fetal bovine serum (FBS). The targeted proteins with their NCBI accession number were including PKC-β II (NP_777012.1), c-myc (NP_001039539.1), c-Jun (NP_001071295.1), c-Fos (AAP84343.1), Cox2 (NP_776870.1), IL-1 beta

(NP_776518.1), Beta-actin (NP_776404.2) and GAPDH (NP_001029206.1). Different specific primary antibodies were chosen to detect the above proteins individually. The membrane was further incubated with primary antibodies overnight. The primary antibodies consisted of rabbit polyclonal antibody against c-Jun (Biobryt, 1:1000), c-Myc (GenWay 1:1000), PKC-β II (Abnova 1:1000) and Cox2 (Abnova 1:500), Mouse monoclonal antibody against GAPDH (Thermo Scientific 1:1000), IL-1β (AbD Serotec 1:1000) and chicken polyclonal antibody against c-Fos (Thermo Scientific 1:1000).

The membrane was then washed thoroughly with PBST four times and each time for 5 minutes. This was followed by incubation for 1 h with the respective secondary antibody which included IRDye 680 Goat Anti-Mouse IgG (LI-COR Biosciences, 1:10000), IRDye 800 Goat Anti-Rabbit IgG (LI-COR Biosciences, 1:10000) and IRDye 680 Donkey Anti-Chicken (LI-COR Biosciences, 1:10000). After incubation, the membrane was washed four times with PBST and for 5 minutes each wash. The membrane was then visualized using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE), 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 (Appendix M).

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5.2.9 Analysis of Apoptosis by Flow-cytometry

A fluorescent-activated cell sorting (FACS) analysis was performed to detect the apoptosis in MDBK cells treated with different phorbol ester fractions. The MDBK cells were seeded at a density of 1×10^6 cells per 75 cm^2 flask and cultured for 24 h in EMEM. Once the cells were confluent, the media was removed and fresh media

containing different phorbol ester fractions at the CC_{50} concentration was added. The treated cells were then incubated at 37°C in 5% CO_2 for 12 and 24 h. The FITC Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmingen, San Diego, California, USA) was used to stain the cells, following the manufacturer's instructions.

Briefly, the media was transferred to 10 ml centrifuge tube and cells were trypsinized and transferred to the same centrifuge tube and centrifuged. The cell pellet was suspended in cold PBS, centrifuged and the supernatant was discarded. The above washing step was repeated twice to remove the remaining media, and finally the cell pellet was resuspended in 1X Binding Buffer at a concentration of 1×10^6 cells/ml. One hundred microliters of this solution was transferred (1×10^5 cells) to a 5 ml culture tube (BD Biosciences). The FITC Annexin V and PI each 5 µl was added and the cells vortexed gently and incubated for 15 min at RT (25°C) in the dark. Finally, 400 µl of 1X Binding Buffer was added to each tube, the stained cells were monitored by flow cytometry (FACS-Canto II BD Biosciences) and the data were analyzed using Diva software (BD Biosciences).

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5.2.10 Statistical Analyses

Cytotoxicity and flow cytometry 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 (Prism, 2007) was used for all the statistical analyses in gene and protein expression studies. The data were subjected to one-way

analysis of variance (ANOVA) and treatment means were compared to control using Dunnett's Multiple Comparison Test.

5.3 Results and Discussion

5.3.1 Isolation of Phorbol Esters

The phorbol ester extract showed four fractions named PE1, PE2, PE3 and PE4 as shown in Figure 4.1 (Chapter 4). All fractions corresponding to the peaks were collected individually, freeze dried and later dissolved in dimethyl sulfoxide (DMSO) to be used for cytotoxicity, gene expression, protein expression and flow cytometry tests.

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5.3.2 Cytotoxicity Assay

The isolated phorbol ester fractions, PE2, PE3 and PE4 (Table 5.2) exhibited cytotoxic activity toward the MDBK cell line with the CC_{50} ranging from 63.8 to 109.6 µg/ml. The lower CC_{50} in PE1 and PE2 indicated that these two fractions were more active in mediating cytotoxicity as compared to the PE3 and PE4 fractions. The positive control (PMA) 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} represented the higher cell proliferation inhibition activity. The CC_{50} concentrations upon 24, 48 and 72 h incubation (Table 5.2) showed that the Jatropha meal phorbol esters inhibited the MDBK cell proliferation in a dose and time dependent manner.

Table 5. 2. Effects of various isolated phorbol esters from Jatropha meal on the viability of the bovine kidney (MDBK) cell line

Incubation time	Cytotoxic concentration (CC_{50}) [a,b] µg/ml					
	PE1	PE2	PE3	PE4	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
[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
Isolated phorbol esters from Jatropha meal: PE1, PE2, PE3 and PE4

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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 depended on the nature of the phorbol ester, cell type, time of exposure and concentrations used. 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, activators of classical and novel PKC isozymes, led to apoptosis (Abdel-Hafez *et al.*, 2002; Griner *et al.*, 2007; Muanda *et al.*, 2010). It seems that, differences in the experimental conditions led to the observation of different results.

In general, the results observed in this study were in agreement with Avila *et al.* (2005) and Bond *et al.* (2007) who demonstrated the cytotoxic action of PMA on

pancreatic cell line. These authors also suggested that the growth inhibitory of PMA is associated with an increase in apoptosis and contributes to its cytotoxic effects.

5.3.3 Morphological Examination

Figure 5.1 and 5.2 represent the results of morphological changes of MDBK cells upon treatment with the isolated phorbol esters and PMA at CC_{50} concentration after 12 and 24 h incubation observed using a light microscopy. After 12 h incubation no significant changes were observed (Figure 5.1), while at 24 h incubation (Figure 5.2) significant morphological changes, detachment and destruction of cells were observed upon treatment with phorbol esters and PMA. According to these microscopic observations (Figure 5.2), cell damage resembled apoptosis, as the cell walls were not intact and apoptotic bodies were seen. MDBK cells displayed death upon treatment with the phorbol esters and PMA at CC_{50} concentration after 24 h incubation.

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The microscopic examination indicated that the MDBK cells after 24 h exposure to the phorbol esters and PMA started to lose their attachments and began to round up. The cells subsequently detached completely over the following hours of incubation. The characteristics observed in this study were also reported by Bond *et al.* (2007) who observed the detachment of human pancreatic adenocarcinomas cells upon 48 h exposure to PMA. Halaweish *et al.* (2002) reported the changes in morphology, epidermal growth factor binding, arachidonic acid metabolite release and ornithine decarboxylase activity of the cells treated by plant ingenol and phorbol ester diterpenes.

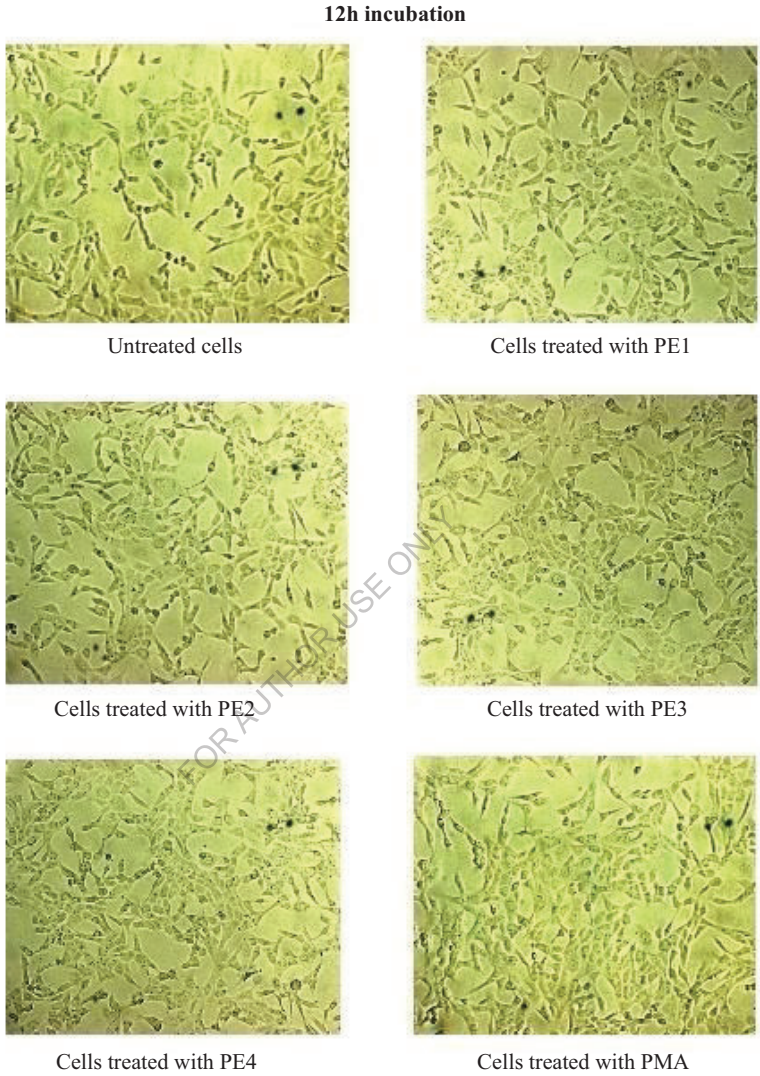

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Figure 5. 1. Morphological characteristics observed in the MDBK cell line upon treatment with isolated phorbol esters and PMA at CC_{50} concentration after 12 h examined by light microscopy at 200X magnification.

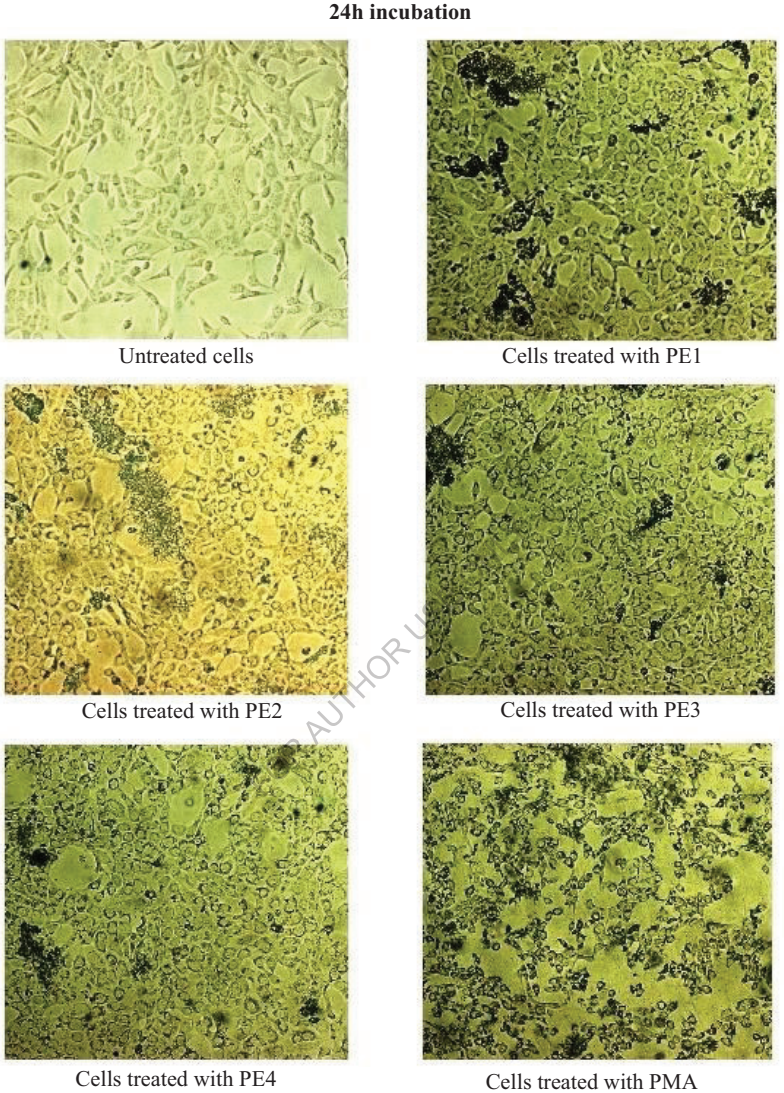

Figure 5. 2. Morphological characteristics observed in the MDBK cell line upon treatment with isolated phorbol esters and PMA at CC_{50} concentration after 24 h examined by light microscopy at 200X magnification.

5.3.4 Gene Expression Analyses in the MDBK Cell Line

5.3.4.1 Expression of Signal Transduction Pathway Gene

The expression of PKC-β II in MDBK cells upon treatment with different phorbol ester fractions is shown in Figure 5.3. The results showed that expression of PKC-β II was upregulated significantly ($p<0.001$) upon treatment with Jatropha meal phorbol esters and PMA at 12 h as compared to the untreated cells.

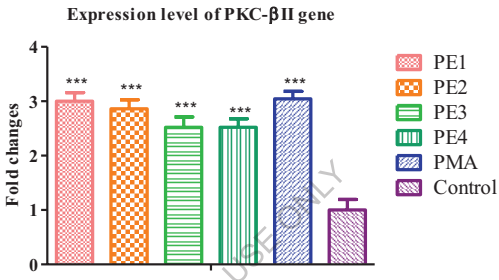

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Figure 5. 3. Fold-changes in the expression levels of the PKC-βII gene determined by SYBR-Green qRT-PCR between cells treated with different phorbol ester fractions isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration incubated for 12 h.

Protein kinase C, also known as PKC, is a family of enzymes which is involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of the serine and threonine amino acid residues on these proteins. The PKC enzymes play an important role in several signal transduction cascades. Binding of phorbol esters to PKC is the first step in the activation of PKC, and this binding is saturable and occurs through specific interactions within the C1 domain in the regulatory region of the PKC molecule (Kazanietz, 2005). Upon activation, PKC enzymes are translocated to the plasma membrane by a receptor for activated C

kinase (RACK) proteins and may phosphorylate potent activators of transcription, leading to increased expression of oncogenes, promoting cancer progression, or interfere with other phenomena (Johnson *et al.*, 2007).

Amemiya *et al.* (2005) found that upon 30 min treatment of rat brain neurons with PMA, PKC-β was activated and triggered the expression of proto-oncogenes, including c-Fos and c-Jun. The c-Fos/c-Jun complexes interact with the AP-1 site on the promoter to regulate the expression of various genes. The upregulation of PKC in this study clearly demonstrated the agonist effect of phorbol esters isolated from Jatropha meal. The PKC agonists, including diacyl glycerol (DAGs) and ingenol-3-angelate, bind to the C1 domains of cPKCs (PKCα, PKCβI, PKCβII and PKCγ) and nPKCs (PKCδ, PKCε, PKCη and PKCθ) leading to their activation (Von Burstin *et al.*, 2010). The role of the various PKC isoforms in tumorigenesis is complex. For example, PKCα might act as a tumor suppressor in one cell type for instance in pancreatic and mammary cell lines (Detjen *et al.*, 2000; Griner *et al.*, 2007), but acts as a supporter of malignant cell growth in another cell type, such as lymphoid and myeloid cells (Jiffar *et al.*, 2004; Kornblau *et al.*, 2006; Kurinna *et al.*, 2006). Therefore, the effect of phorbol esters isolated from Jatropha meal was probably through the activation of PKC-β II downstream genes which determine the response of cells to phorbol esters.

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5.3.4.2 Expressions of proto-oncogenes

The expression analyses of proto-oncogenes, including c-Myc, c-Fos, and c-Jun in MDBK cells upon treatment with Jatropha meal phorbol esters are shown in Figures 5.4, 5.5 and 5.6, respectively. All of the proto-oncogenes showed significant

($p<0.01$) upregulation as compared to the control. Similar significant ($p<0.01$) upregulation was also observed in the cells treated with PMA as the positive control.

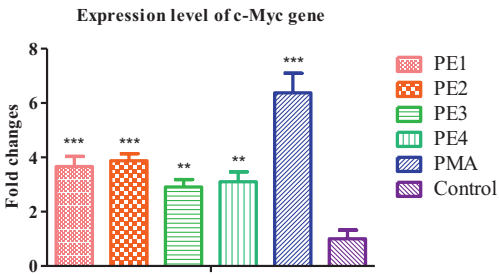

Figure 5. 4. Fold-changes in the expression levels of the c-Myc gene determined by SYBR-Green qRT-PCR between cells treated with different phorbol ester fractions isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration incubated for 12 h.

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The c-Myc gene is responsible for cell growth and proliferation, differentiation and apoptosis. c-Fos is a cellular proto-oncogene belonging to the immediate early gene family of transcription factors AP-1. Transcription of c-Fos is upregulated in response to many extracellular signals, e.g., growth factors, reactive oxygen species (ROS) and peripheral stimuli. In addition, phosphorylation by MAPK, PKA, PKC or cdc2 alters the activity and stability of c-Fos. Members of the Fos family dimerise with c-Jun to form the AP-1 transcription factor, which upregulates transcription of a diverse range of genes involved in everything from proliferation and differentiation to defence against invasion and cell damage.

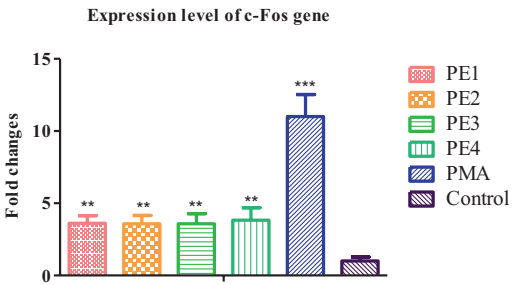

Figure 5. 5. Fold-changes in the expression levels of the c-Fos gene determined by SYBR-Green qRT-PCR between cells treated with different phorbol ester fractions isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration incubated for 12 h.

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PMA was shown to overexpress the c-Jun and c-Fos genes in the mouse skin through activation of mitogen-activated protein kinases (MAPK), and finally leading to elevation in the expression of Cox2 (Chun *et al.*, 2004; Lee *et al.*, 2007). The transcription factor AP-1, whose binding site is a highly conserved enhancer-like element present in many viral and cellular genes, is involved in the phorbol ester response. An early study by Udou *et al.* (2004) confirmed the role of c-Jun in the proliferation and apoptosis of glandular epithelial cells, where overexpression of c-Jun protein prevented the cells from entering into apoptosis during the late secretory phase.

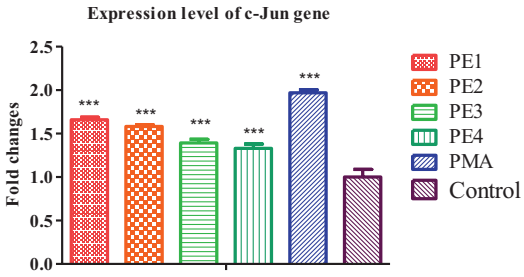

Figure 5. 6. Fold-changes in the expression levels of the c-Jun gene determined by SYBR-Green qRT-PCR between cells treated with different phorbol ester fractions isolated from Jatropha meal and PMA as positive control at the CC_{50} concentration incubated for 12 h.

Although the activation of proto-oncogenes in this study seems to be mediated through PKC, however, it was shown that as a result of oxidative stress, the reactive oxygen species (ROS) are also capable of activating PKC, c-Jun and c-Fos (Frank *et al.*, 2003; Klaunig *et al.*, 2010).

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5.3.4.3 Expressions of Inflammatory Genes

Figure 5.7 shows the changes in expression level of IL-1β upon treatment with the phorbol esters isolated from Jatropha meal and PMA. The phorbol esters significantly ($p<0.01$) upregulated the IL-1β gene expression in MDBK cells as compared to the untreated cells. A similar result was observed when the MDBK cells were treated with PMA.

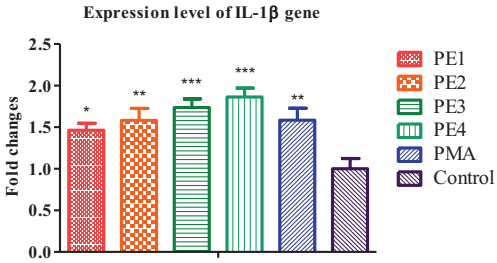

Figure 5. 7. Fold-changes in the expression levels of the IL-1β gene determined by SYBR-Green qRT-PCR between cells treated with different phorbol ester fractions isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration incubated for 12 h.

The IL-1β is a member of the interleukin 1 cytokine family. This cytokine is an important mediator of the inflammatory response, and is 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. Hence, the upregulation of IL-1β revealed the presence of inflammation in MDBK cells upon treatment with phorbol esters of Jatropha meal.

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The expression of the Cox2 gene in the MDBK cells treated with different phorbol esters fraction obtained from Jatropha meal are presented in Figure 5.8. The significant ($p<0.01$) upregulation in the Cox2 gene upon exposure of MDBK cells to isolated phorbol esters was observed. The treatment of MDBK cells with PMA as the positive control has also significantly ($p<0.01$) upregulated the Cox2 in these cells.

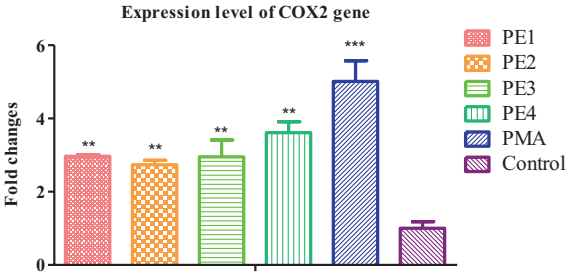

Figure 5. 8. Fold-changes in the expression levels of Cox2 gene determined by SYBR-Green qRT-PCR between cells treated with different phorbol ester fractions isolated from Jatropha meal and PMA as positive control at the CC_{50} concentration incubated for 12 h.

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The molecular links between inflammation and cancer is the proinflammatory enzyme (Cox2), which is a rate-limiting enzyme in the biosynthesis of prostaglandins. The levels of Cox2 are transiently elevated in cells or tissues stimulated with different growth factors, proinflammatory cytokines, endotoxin, and tumor promoters. Cox2 is aberrantly overexpressed in various premalignant and malignant tissues (Shrotriya *et al.*, 2010a). The results obtained were in agreement with Wits (1991) and Klaunig *et al.* (2010) who reported the induction of oxidative stress, cell damage and inflammation in peritoneal phagocytic cells following intraperitoneal administration of 0.1 µg PMA in mice. Furthermore, Lee *et al.* (2005) addressed the overexpression of Cox2 and its mRNA transcript in mouse skin after the topical application of PMA.

Most likely, the underlying mechanisms of Cox2 and IL-1β overexpression upon phorbol esters treatment in MDBK cells could be through activation of c-Fos (Figure 5.5) and c-Jun (Figure 5.6) and upstream kinases, including PKC (Figure 5.3). This activation could be directly due to the phorbol esters acting through the cell surface receptors, or ROS generated during oxidative stress. A similar activation mechanism in inflammatory genes upon PMA exposure was previously suggested by Lee *et al.* (2007).

5.3.5 Protein Expression Analyses in the MDBK Cell Line

5.3.5.1 Expression of Signal Transduction Pathway Protein (PKC-β II)

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The PKC-β II protein expression analysis in MDBK cells treated with Jatropha meal phorbol esters and PMA is presented in Figure 5.9. The results indicated that, expression of the PKC-β II protein was upregulated significantly ($P<0.01$) in MDBK cells upon treatment with all of the phorbol ester fractions obtained from Jatropha meal as compared to the untreated cells. The result was consistent with the result obtained from gene expression analyses (Figure 5.3).

The conventional PKC (cPKC) isoforms (PKC-α, -βI, -βII, and -γ) are activated by phosphatidylserine, calcium and DAG or phorbol esters such as PMA, whereas novel PKCs (nPKCs) (PKC-δ, -ε, -θ, and -η) are activated by phosphatidylserine, DAG or PMA, but not by calcium. The atypical PKCs (aPKCs) (PKC-ζ and -ι/λ) are not activated by calcium, DAG or PMA (Geraldes *et al.*, 2010). The PKC-βII protein was also shown to be upregulated in cancerous colon and prostate tissues (Murray *et al.*, 1999; Koivunen *et al.*, 2006). Thus, the phorbol esters from Jatropha meal were

able to activate the PKC-β II, and affected the growth and differentiation of MDBK cells, as shown in Figure 5.2.

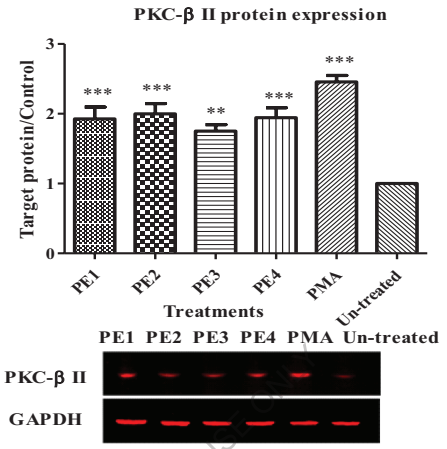

Figure 5. 9. Cells treated with different phorbol ester fractions (PE1, PE2, PE3 and PE4) isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration and incubated for 12 h. Equal amount of total cellular protein was subjected to Western blot analyses for PKC-β II and GAPDH protein expression. All values represent mean ± standard error from three independent experiments, ***P < 0.001, **P<0.01, *P<0.05 indicate significant difference compared to the untreated control.

5.3.5.2 Expression of Proto-Oncoproteins

The expression levels of proto-oncoproteins including c-Myc, c-Fos and c-Jun in MDBK cells upon treatment with the isolated phorbol esters from Jatropha meal and PMA are shown in Figures 5.10, 5.11 and 5.12, respectively. In fact, not all, genes are translated efficiently to their respective proteins, however, the protein expression results in this study were consistent with the results obtained from gene expression analyses, as shown in Figures 5.4, 5.5 and 5.6.

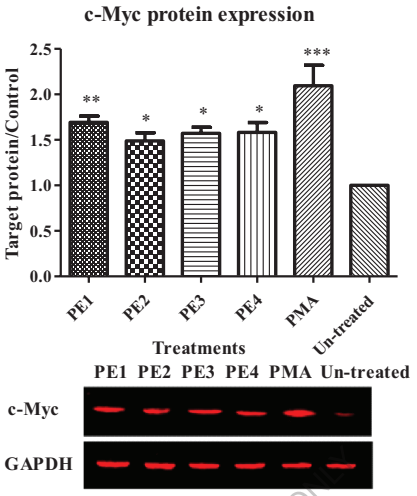

Figure 5. 10. Cells treated with different phorbol ester fractions (PE1, PE2, PE3 and PE4) isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration and incubated for 12 h. Equal amount of total cellular protein was subjected to Western blot analyses for c-Myc and GAPDH protein expression. All values represent mean ± standard error from three independent experiments, ***P < 0.001, **P<0.01, *P<0.05 indicate significant difference compared to the untreated control.

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Proto-oncogenes are normal genes that could become oncogenes due to mutations or overexpression. The resultant proteins are called oncoproteins. Proto-oncoproteins help to regulate cell growth and differentiation. Proto-oncoproteins are often involved in signal transduction and execution of mitogenic signals, usually through their protein products. The proto-oncoproteins regulate the differentiation, proliferation and apoptosis in the presence of a variety of stimuli, including cytokines, growth factors, stress, bacterial and viral infections (Gomperts *et al.*, 2009).

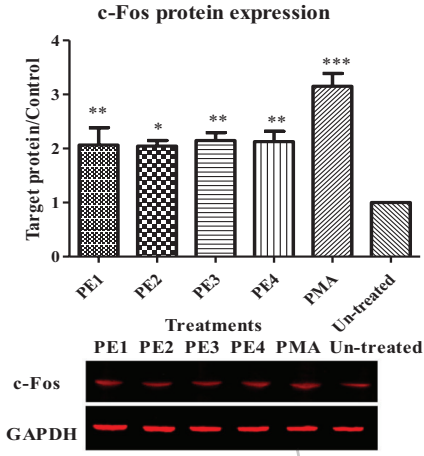

Figure 5. 11. Cells treated with different phorbol ester fractions (PE1, PE2, PE3 and PE4) isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration and incubated for 12 h. Equal amount of total cellular protein was subjected to Western blot analyses for c-Fos and GAPDH protein expression. All values represent mean ± standard error from three independent experiments, ***P < 0.001, **P<0.01, *P<0.05 indicate significant difference compared to the untreated control.

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Moreover, their overexpression have been implicated in transformation and progression of cancer as previously shown by Long *et al.* (2001). Robbins and Zhao (2012) believed that, in the presence of phorbol ester (PMA) during the early stages of tumor promotion, both oncogenes and tumor suppressor genes are activated, resulting in the increase in cell proliferation, followed by an increase in the cell death (Figure 5.13).

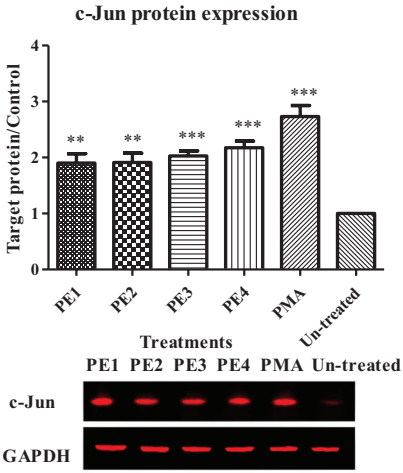

Figure 5. 12. Cells treated with different phorbol ester fractions (PE1, PE2, PE3 and PE4) isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration and incubated for 12 h. Equal amount of total cellular protein was subjected to Western blot analyses for c-Jun and GAPDH protein expression. All values represent mean ± standard error from three independent experiments, ***P < 0.001, **P<0.01, *P<0.05 indicate significant difference compared to the untreated control.

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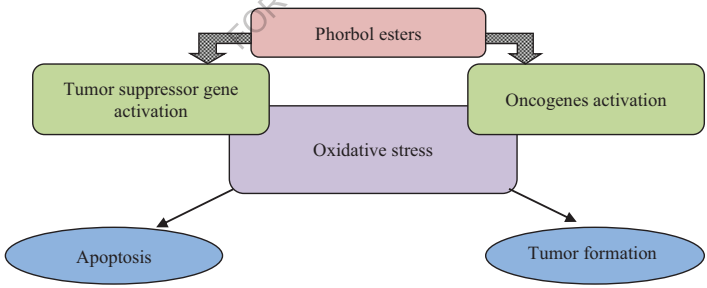

Figure 5. 13. Mechanisms of action of phorbol esters in the early stages of carcinogenesis

The observation of tumor formation or apoptosis as a result of phorbol ester exposure is affected by the time of exposure and the concentration of the phorbol ester in the

way that, the high exposure time (≥24) or concentration can lead to apoptosis, while a short exposure time (≤12) or low concentration can induce the tumor formation.

5.3.5.3 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 Jatropha meal and PMA are shown in Figure 5.14 and 5.15, respectively. Results were in agreement with the results obtained from IL-1β and Cox2 genes expression analyses as shown in Figures 5.7 and 5.8 respectively.

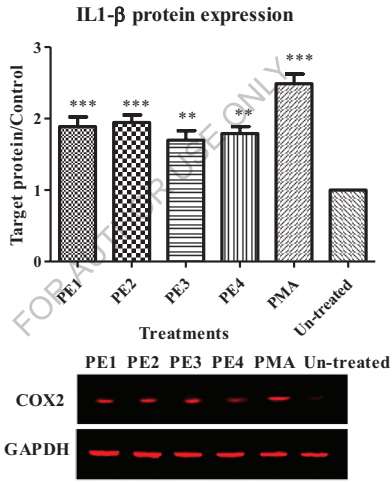

Figure 5. 14. Cells treated with different phorbol ester fractions (PE1, PE2, PE3 and PE4) isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration and incubated for 12 h. Equal amount of total cellular protein was subjected to Western blot analyses for IL-1β and GAPDH protein expression. All values represent mean ± standard error from three independent experiments, ***P < 0.001, **P<0.01, *P<0.05 indicate significant difference compared to the untreated control.

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 infection, injury or peripheral stimuli, such as exposure to phorbol esters. Expression of many of these proteins is under the control of nuclear factor κB (NFκB), AP-1, and interferon regulatory factor (IRF) families (Karin *et al.*, 2005). The activities and/or expression of these factors can be induced by a large number of receptor systems, including pattern recognition receptors for pathogen-associated molecules, and receptors for cell-derived inducers of inflammation, such as IL-1β, TNFα, and interferons (Akira *et al.*, 2004). Upon activation, NFκB, AP-1, and IRF proteins are capable of inducing the expression of proteins in diverse cell types that initiate and amplify inflammatory processes (Ghisletti *et al.*, 2009). Lee *et al.* (2007) has proven the role of c-Jun and c-Fos proteins in the activation of the Cox2 protein in mouse skin treated with PMA.

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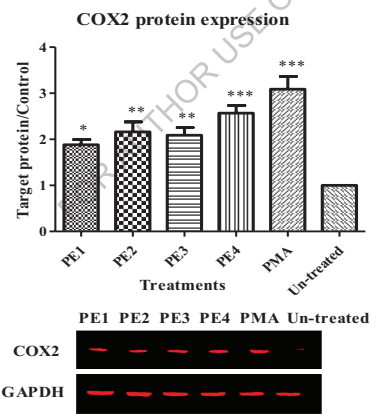

Figure 5. 15. Cells treated with different phorbol ester fractions (PE1, PE2, PE3 and PE4) isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration and incubated for 12 h. Equal amount of total cellular protein was subjected to Western blot analyses for Cox2 and GAPDH protein expression. All values represent mean ± standard error from three independent experiments, ***P < 0.001, **P<0.01, *P<0.05 indicate significant difference compared to the untreated control.

The PMA was found to induce the inflammation through different mechanisms. It induces prompt remodelling of the vasculature, resulting in oedema. It also activates adhesion molecules present on the surface of leukocytes, causing them to bind and then to migrate through the vascular endothelial layer into the tissues underlying the skin. In the tissues, PMA potentiates the antigen-mediated stimulation of T lymphocytes, enhancing the production of the cytokine interleukin-2 (IL-2), essential for the induction of clonal expansion. This is mediated through the activation of PKC θ and the AP-1 complex. The PMA also causes degranulation of neutrophils, releasing proinflammatory cytokines and matrix proteases. It also activates the respiratory burst, vital for first line host defence and also implicated in the tissue destruction associated with chronic inflammatory disease (Figure 5.16) (Goel *et al.*, 2007; Gomperts *et al.*, 2009).

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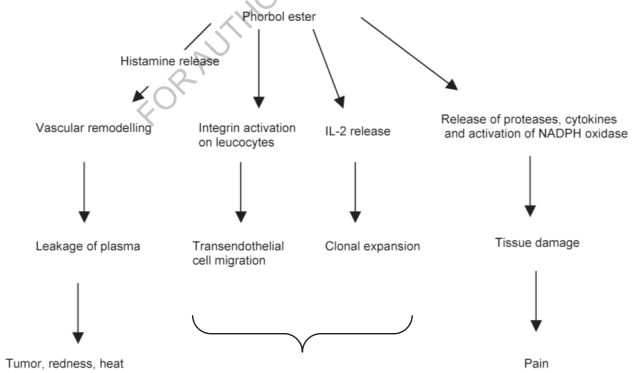

Figure 5. 16. Four, well-recognized characteristics of the inflammatory response upon phorbol ester treatment (Goel *et al.*, 2007).

Studies have suggested that active oxygen species in inflammatory cells are involved in tumor promotion (Klaunig *et al.*, 2010). It is likely that active oxygen species formed during the oxygen burst by stimulated inflammatory cells, as well as active oxygen species formed in the cells, contribute in cellular processes leading to tumor promotion (Wits, 1991). Based on a correlation between promoting activity and the inflammatory activity of phorbol esters, early studies suggested a role for inflammation in tumor promotion. A rapid and transient induction of Cox2 has been observed in cells or tissues exposed to pro-inflammatory and mitogenic stimuli, including cytokines, endotoxins, growth factors, lipopolysaccharides, ultraviolet radiation and phorbol esters (Lee *et al.*, 2007).

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5.3.6 Analysis of Apoptosis by Flow Cytometry

The Figure 5.17 represents a histogram analysis of the flow cytometry data. The M1 marker indicates FITC Annexin V and PI negative (viable cells), while the M2 marker indicates FITC Annexin V and PI positive (dead cells). The histogram shows that cells upon 12 h treatment were still viable with an intact membrane and none of the dyes could penetrate into the cells, thus no shifting of the histogram was observed. However, the M1 part (viable cells) had shifted towards M2 (dead cells) after 24 h treatment with all of the phorbol ester fractions and PMA as the positive control. In general, cells that are considered viable are FITC Annexin V and PI negative; cells that are in early apoptosis are FITC Annexin V positive and PI negative; and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive.

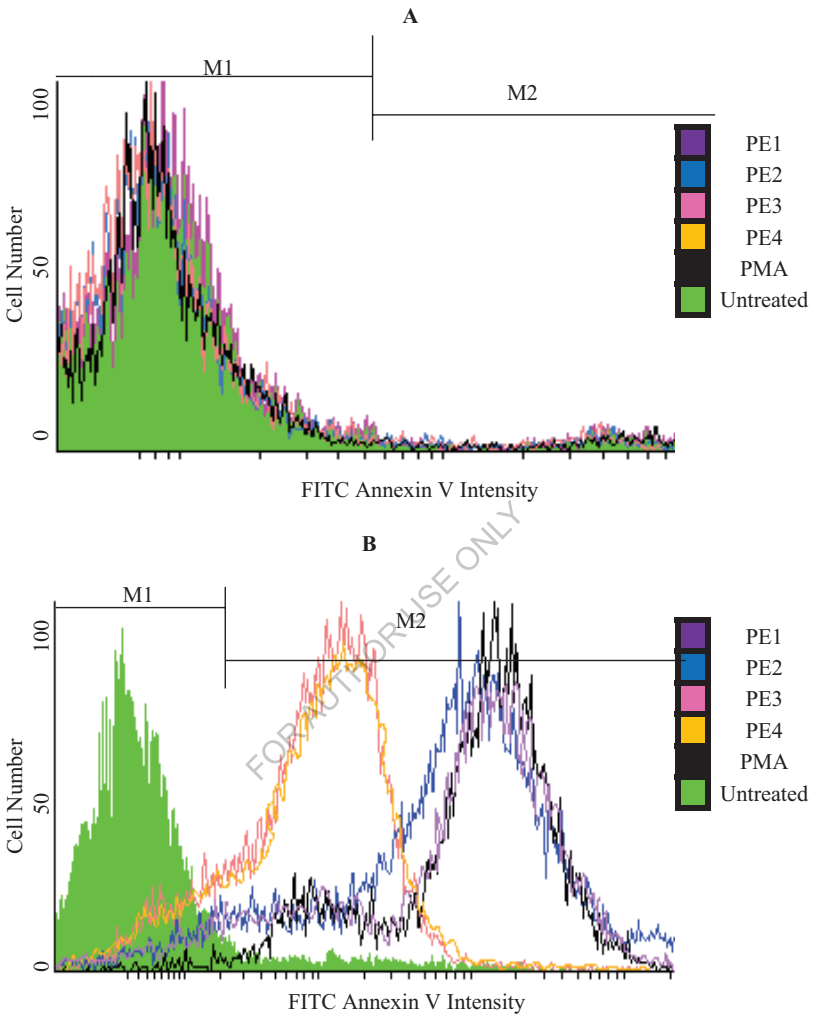

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Figure 5. 17. Cells treated with different phorbol ester fractions (PE1, PE2, PE3 and PE4) isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration and subjected to FITC Annexin V and PI staining followed by flow cytomety analyses. The histogram analysis of flow cytometry data after 12 h (A) and 24 h (B) incubation are shown. M1 marker indicates FITC Annexin V and PI negative. M2 marker indicates FITC Annexin V and PI positive.

Figures 5.18 and 5.19 shows the flow cytometry analyses of MDBK cells treated with various phorbol esters upon 12 h and 24 h incubation, respectively. The flow cytometry results confirmed that all phorbol ester fractions, together with PMA, induced apoptosis cell death upon 24 h exposure, while 12 h exposure did not show apoptosis in MDBK cells.

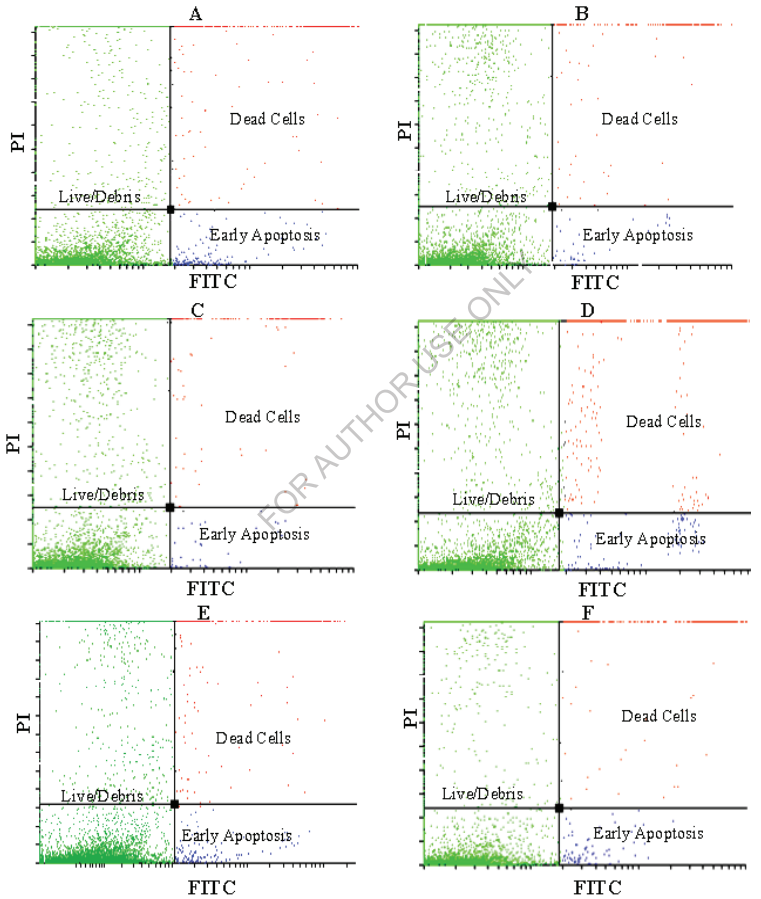

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Figure 5. 18. MDBK cells treated with different phorbol ester fractions (PE1, PE2, PE3 and PE4) isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration and subjected to FITC Annexin V and PI staining followed by flow cytomety analyses after 12 h incubation. A (PE1), B (PE2), C (PE3), D (PE4), E (PMA) and F (untreated).

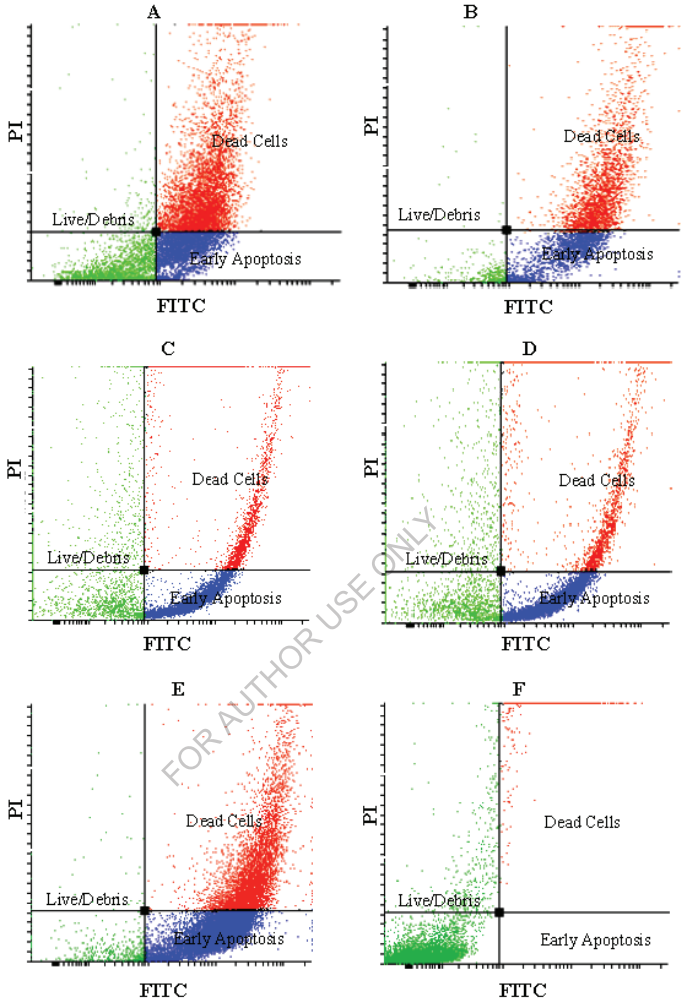

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Figure 5. 19. MDBK cells treated with different phorbol ester fractions (PE1, PE2, PE3 and PE4) isolated from Jatropha meal and PMA as the positive control at the CC_{50} concentration and subjected to FITC Annexin V and PI staining followed by flow cytomety analyses after 24 h incubation. A (PE1), B (PE2), C (PE3), D (PE4), E (PMA) and F (untreated).

The viable cells detected by flow cytometry are indicated as green, early apoptotic cells as blue, and death cells indicated as red. The cells were found to be viable upon 12 h of treatment with different fractions of phorbol ester and PMA, while at 24 h the cells underwent apoptosis, and finally death. Figure 5.20 represents the percentage of dead cells upon 12 and 24 h incubation with each of the phorbol ester fractions and PMA, as computed by flow cytometry. The result of 12 h incubation did not show any significant increase in the percentage of cell death upon treatment with the phorbol ester fractions and PMA as compared to the control. Whereas, the 24 h incubation showed a significant ($p<0.01$) increase in the percentage of cells dead, with the values ranging from 37.4% to 56.3% as compared to the control. Since the cells were treated with phorbol ester fractions and PMA at CC_{50} concentration, therefore no significant differences were observed in terms of cells death percentage among phorbol ester fractions and PMA.

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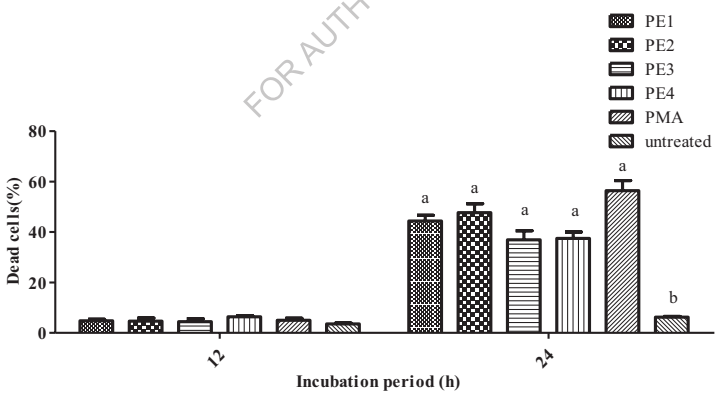

Figure 5. 20. MDBK cells treated with different phorbol ester fractions (PE1, PE2, PE3 and PE4) isolated from Jatropha meal and PMA as positive control at the CC_{50} concentration and subjected to FITC Annexin V and PI staining followed by flow cytomety analyses upon 12 and 24 h incubation. The percentage of dead cells at 12 and 24 h are shown. Each error bar represents standard error of three independent experiments. Columns with different subscripts are significantly different ($p<0.01$)

The role of phorbol esters on the induction of tumorgenesis, apoptosis, inflammation and survival of the cells depends on the type of activated PKC isozymes triggering the different pathways. Indeed, emerging information suggests that phorbol esters serving as PKC activators, may induce apoptosis (Abdel-Hafez *et al.*, 2002; Kazanietz, 2005; Griner *et al.*, 2007; Gobbi *et al.*, 2009; Von Burstin *et al.*, 2010) or tumor formation (Goel *et al.*, 2007; Ikuta *et al.*, 2008; Shrotriya *et al.*, 2010b). The effects and mechanism of action of phorbol esters depends on the types of phorbol ester, the cell type, time of exposure, and the concentrations used. These factors may alter the balance between pro-apoptotic and anti-apoptotic pathways resulting in the observation of tumorigenesis or apoptosis in the cells.

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Another source of variation in the cellular response following activation is that some PKC isozymes may inhibit, while others may promote, apoptosis. Rennecke *et al.* (2000) highlighted the importance of time of exposure to PMA in the cellular response. Thymocytes treated with PMA within the first 2-4 h showed anti-apoptotic effects and Western blot analyses also revealed the overexpression of PKC α, β, δ, η, θ, μ and ζ signals, a very faint PKCϵ and no PKCγ proteins. While, the pro-apoptotic effect of PMA was observed after long-term (>18 h) treatment and the induction of apoptosis by the phorbol esters was reported to be partially due to a downregulation or endogenous inactivation of anti-apoptotic PKC isozymes. With regard to the results obtained in this study, the combination of overexpression of PKC-β II, c-Myc, c-Fos, c-Jun, IL-1β and Cox2 proteins in the MDBK cells could possibly have resulted in the observation of apoptosis, and the time of exposure critically affected the apoptosis induction.

5.4 Conclusion

Phorbol esters from Jatropha meal exerted a variety of effects including changes in cell morphology, proliferation, differentiation and cell death. The effects of the phorbol esters were associated with the time of exposure in the way that short exposure (≤12) increased cell proliferation and induced inflammation, as indicated by the expression of PKC-β II, c-Fos, c-Jun, c-Myc, IL-1β and Cox2 proteins while prolonged exposure (≥24 h) arrested the cell growth and proliferation, which led to apoptosis. The activation of PKC-β II could be directly due to the phorbol esters through the cell surface receptors. The PKC-β II appeared to be responsible for the activation of downstream pathways, including the proto-oncoprtoeins (c-Fos, c-Jun and c-Myc) and the inflammatory proteins, including IL-1β and Cox2. The combination of the expressions of these proteins could possibly result in the apoptosis observed in this study. The present study indicated that all of the fractions of phorbol ester isolated from Jatropha meal are detrimental to the cells, even at low concentrations.

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CHAPTER 6

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

6.1 General Discussion

Jatropha curcas L. thrives well in the tropical climate, and has gained importance as a source of seed oil. In this study, seeds were obtained from *Jatropha curcas* originating from different states in Malaysia, including Sabah, Selangor, Terengganu and Johor Bahru. The seeds contained about 50-60% oil which makes it a good source of oil for biodiesel production. The meal contained about 60% protein, indicating it also to be a good source of protein feed. The amino acid profile of Jatropha meal is similar to soybean meal, and contains a good balance of essential amino acids, with lysine being higher in Jatropha meal (Makkar *et al.*, 1998a).

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The presence of various bioactive compounds, including saponins, phytic acids, trypsin inhibitors, lectins, phenolics and flavonoids, indicated the potential of Jatropha meal as a biofeed. It was observed that a methanolic extract of Jatropha meal demonstrated various biological activities, including antioxidant, antibacterial and anti-inflammatory effects which enhance its property as a biofeed. It was reported that the levels of these bioactive compounds in the seeds are dependent on the genotypes (Makkar *et al.*, 2009a). However, the presence of phorbol esters at the concentration of 3 mg/g DM Jatropha meal confirmed that the seeds obtained locally belong to the toxic variety of *Jatropha curcas,* thus limiting its application as a biofeed. Makkar *et al.* (2009a) also recommended to avoid utilizing the Jatropha

meal containing phorbol esters for any nutritional application unless the phorbol esters are removed.

In order to remove the phorbol esters, the Jatropha meal was subjected to various physicochemical treatments. Combination, of hydrothermal, alkali and oxidizing treatments reduced the bioactive compounds in Jatropha meal to safe levels, except for the phorbol esters. The maximum phorbol esters removal was observed in the treatment consisting of hydrothermal, alkali and oxidizing agents, where the level was reduced to 0.7 mg/g DM, or 76% removal. This indicates the thermostability of the molecule even at high temperature and pressure. Rakshit *et al.* (2008) reported that physical treatments, followed by chemical treatments were able to remove the phorbol esters with an efficiency ranging between 50% to 95%, depending on the initial content of phorbol esters.

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Degradability of the treated Jatropha meal by rumen microbes *in vitro* did not show any improvement with the reduction of phorbol esters. The lower ruminal degradability of treated Jatropha meal was also reported by Makkar *et al.* (1998a), and it is believed to be the result of the Millard reaction during treatments, which reduced the availability of Jatropha meal for rumen microbes, and may not be due to the presence of the phorbol esters. Incomplete removal of the phorbol esters upon physicochemical treatments led to the evaluation of rumen microbial activity as an alternative means to remove the phorbol esters. Hence, the isolated phorbol esters at different concentrations were used to determine the ability of rumen microbes to reduce or degrade the phorbol esters. The results showed that phorbol esters above 6 mg/30 ml of buffered rumen fluid were detrimental to rumen microbial fermentation,

as shown by the reduction in DMD, $IVGP_{24}$, total VFA, enzyme activities, microbial protein synthesis and microbial population. These observations may be attributed to the antimicrobial activity of phorbol esters, as previously shown by Saetae *et al.* (2010). Yet, the disappearance of phorbol esters was observed in this study, which could be due to the biodegradation or biotransformation of phorbol esters by certain rumen microbial species. Concomitant with this result, Broudiscou *et al.* (2007) and Malecky and Broudiscou *et al.* (2009) reported the large capacity of rumen microbes in degradation of terpenoids, and the extent of degradation increased through adaptation. This ability, definitely enhances the removal of the phorbol esters to a greater extent. However, the question regarding the safety of the detoxified products needs to be answered. Therefore, the extract obtained from the fermentation fluid treated with phorbol esters was tested against a bovine kidney cell line (MDBK). The results showed a reduction in the toxicity of the crude extract. The report of Laparra *et al.* (2010) supports the potential of rumen microbiota for the biotransformation and biodegradation of harmful plant bioactive compounds to their safe derivatives.

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To further study the cytotoxicity test, flow cytometry analyses, gene and protein expression studies were conducted using a bovine kidney cell line to determine the biological effects and mode of action of Jatropha meal phorbol esters. The methanolic extract from the meal was subjected to HPLC analysis, where four phorbol ester fractions were collected. The *in vitro* cytotoxicity study with normal bovine kidney cell line showed that all of the four fractions induced cytotoxicity towards the cells similar to phorbol 12-myristate-13-acetate (PMA). All of the fractions inhibited the MDBK cell proliferation in a dose and time dependent manner. The microscopic examination indicated that the MDBK cells after 12 h incubation with all of the fractions of the phorbol esters and PMA did not show any

apparent changes, while after 24 h exposure, the cells began to lose their attachments and to round up. Similar morphological observations were reported by Bond *et al.* (2007) when the human pancreatic adenocarcinomas cell line (Aspc-1) was exposed to phorbol ester (PMA) for 48 h. It was suggested by Haas *et al.* (2002) that the free hydroxyl groups present in the structures of same phorbol esters play an important role in mediating cytotoxicity. Cytotoxic activity of phorbol esters has also been observed in cancer cell lines. Von Burstin *et al.* (2010) demonstrated the cytotoxic activity of phorbol ester (PMA) towards LNCaP prostate cancer cells.

Further studies demonstrated the overexpression of the PKC-β II gene and the encoded protein, indicating activation of the signal transduction pathway in response to phorbol ester exposure for 12 h in MDBK cells. This result is in agreement with Amemiya *et al.* (2005) who reported the activation of PKC-β leading to overexpression of proto-oncogenes, including c-Fos and c-Jun upon 30 min treatment of rat brain neurons with PMA. In this study, the overexpression of proto-oncogenes, including c-Fos, c-Jun and c-Myc genes and their related proteins upon 12 h exposure to each of the phorbol ester fractions confirmed the downstream pathway and the abnormality in the cell differentiation leading to tumorgenesis. These results support the data of Lee *et al.* (2007) who demonstrated the overexpression of c-Jun and c-Fos genes in mouse skin as a response to PMA exposure, which resulted in elevation of COX2 expression. The overexpression of IL-1β and COX2 genes and their derived proteins upon 12 h exposure to each of the phorbol ester fractions showed the presence of inflammation in the MDBK cells upon treatment with different phorbol ester fractions. The results obtained were in agreement with Klaunig *et al.* (2010) who reported the induction of oxidative stress, cell damage and

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inflammation in peritoneal phagocytic cells following intraperitoneal administration of 0.1 µg PMA in mice. Most likely, the underlying mechanisms of IL-1β and COX2 induction upon phorbol ester treatment in MDBK cells is through activation of c-Fos and 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 reactive oxygen species (ROS) generated during oxidative stress.

The flow cytometry results confirmed that, 12 h exposure to all fractions of phorbol esters did not induce apoptosis as the cells were negative to FITC Annexin V and PI staining. While, upon 24h exposure, apoptotic cell death occurred as the cells in early apoptosis were positive for FITC Annexin V and PI. The individual roles of the PKCs in the regulation of apoptosis have been reported in various systems. In most systems, PKCα, ε and ι act as anti-apoptotic kinases, whereas PKC β, µ and δ act as pro-apoptotic kinases (Oh *et al.*, 2005). Laouar *et al.* (2001) reported the induction of apoptosis through activation of the caspase cascade proteins through overexpression of PKC-β in the presence of PMA in the HL-60 cell line. In the present study, the combination in overexpression of PKC-β II, c-Myc, c-Fos, c-Jun, IL-1β and COX2 proteins in MDBK cells could possibly result in the observation of apoptosis, and the time of exposure critically affected apoptosis induction. The effects of phorbol esters were associated with the time of exposure in the way that short time exposure increased the cell proliferation and induced inflammation as indicated by the expression of signal transduction, proto-oncoproteins and inflammatory proteins, while, the prolonged exposure (≥24 h) could arrest cell growth and proliferation, which led to apoptosis.

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6.2 Conclusions

The Jatropha meal used in this study exhibited antioxidant, antibacterial and anti-inflammatory activities which corresponded to the various bioactive compounds present in the meal. Jatropha meal possessed biofeed potential. However, the presence of phorbol esters limits its application. A combination of hydrothermal, alkali and oxidizing treatments reduced the phorbol esters to 0.7 mg/g DM. The rumen microbes were capable of degrading the phorbol esters to some extent, but, phorbol esters above 6 mg/30 ml of buffered rumen fluid inhibited the rumen microbial activity. The phorbol esters even in low concentrations (63 µg/ml) induced cytotoxicity in the MDBK cells, followed by changes in cell morphology, proliferation, differentiation and finally cell death. The present study indicated that each of the phorbol ester fractions isolated from Jatropha meal behaved similarly to the PMA, and they are detrimental to the cells even at low concentrations. Therefore, complete removal of phorbol esters is absolutely necessary before its application as a biofeed.

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6.3 Recommendations for Future Research

The difficulty in removing the phorbol esters, as well as the cytotoxic effects of phorbol esters definitely prevents the meal from being used as a biofeed, even after physicochemical treatments. The best solution is to search for a non-toxic *Jatropha curcas* variety (devoid of phorbol esters). Failing which, exhaustive treatment methods should be considered to remove the phorbol esters to a greater extent, if not completely, and coupled to the ability of rumen microbes in detoxifying the phorbol esters, the meal may still be considered a valuable functional biofeed. To this end, development of microbial consortia or specific enzymes that could degrade the

phorbol esters in Jatropha meal should be explored. Another aspect that is highly recommended is the isolation of the bioactive compounds like phenolics, lectins, saponins, sugars and peptides present in Jatropha meal that offer the prospect of value-added products for pharmaceutical applications.

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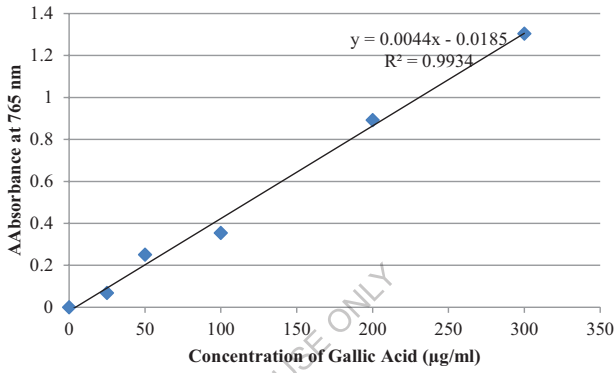
APPENDIXES

APPENDIX A

Standard Curves of Total Phenolics, Flavonoids and Saponins



A standard curve for the total phenolic assay using gallic acid as standard

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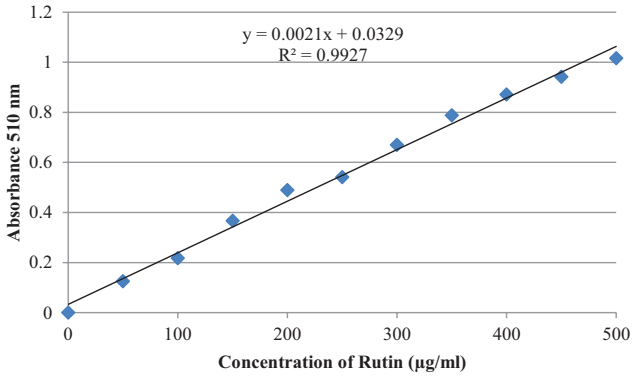
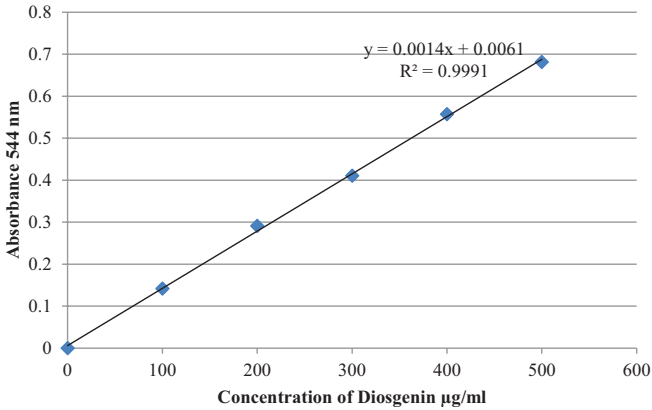


A standard curve for the total flavonoid assay using rutin as a standard



A standard curve for the total saponins assay using diosgenin as a standard

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APPENDIX B

Preparation of Dulbecco's Modified Eagle's Medium with Phenol red, Buffers, Interferon -γ (IFN-γ) and Lipopolysaccharide (LPS) Stock Solution

Incomplete Dulbecco's modified Eagle's medium (DMEM)
(With phenol red, L-glutamine, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate, but without sodium pyruvate)

DMEM powder (Sigma, Germany)	13.38 g
Sodium bicarbonate, $NaHCO_3$ (FW84.01, Sigma)	3.7 g
Deionized water (Millipore)	800 ml

Make up to 1 L. Adjust to pH 6.8 with 1 M HCl. Sterilize immediately by filtering with a membrane with a porosity of 0.22 µ and store at 4°C.

Sterile Phosphate Buffer Saline (PBS), pH 7.2
(1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4 and 154 mM NaCl)

Sodium dihydrogen phosphate, NaH_2PO_4 (FW120, Sigma)	0.23 g
Di-sodium hydrogen phosphate, Na_2HPO_4 (FW141.96, Merck)	1.15 g
Sodium chloride, NaCl (MW58.44, Merck)	9 g
Deionized water (Millipore)	900 ml

Make up to 1 L. Adjust to pH 7.2 with 1 M HCl. Sterilize immediately by filtering with a membrane with a porosity of 0.22 µ and store at 4°C.

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1 x 10^6 U/ml (100 µg/ml) of Recombinant Mouse Interferon-γ stock solution

100 µg/ml of IFN-γ (1× 10^7 U/mg; eBioscience, USA)	100 µl

Sepreate into aliquots of 5 µl each and freeze in -80°C

25 mg/ml of Lipopolysaccharide Stock Solution (*E.coli* strain with Serotype 055:B5)

LPS (Sigma Chemical Co., USA)	10 mg
Incomplete DMEM	400 µl

Freeze at -80°C in aliquots of 20 µl.

31 mM of Trypan blue solution
(Trypan blue in sterile PBS, pH 7.2).

Trypan blue powder, (FW960.8, Sigma)	3 g
Sterile PBS, pH 7.2	100 µl

APPENDIX C

Preparation of Diluents and Solutions for Griess and MTT Assay

Diluents for Griess Assay

A. Diluent A (Complete DMEM with 0.2% DMSO)
(DMEM without phenol red, supplemented with 5 % FBS, 2 mM L-glutamine and 0.2% DMSO)

Diluent B	5 ml
100% DMSO (Merck)	10 µl

B. Diluent B (Complete DMEM)
(DMEM without phenol red supplemented with 5 % FBS, 2 mM L-glutamine)

Incomplete DMEM without phenol red	100 ml
Foetal Bovine Serum (Flowlab, Australia)	5 ml

C. Diluent C (Complete DMEM with IFN-γ/LPS)
(DMEM without phenol red supplemented with 5% FBS, 2 mM L-glutamine and 200 U/ml IFN-γ and 10 µg/ml LPS)

Diluent B	10 ml
$1x10^6$ U/ml of IFN-γ	2 µl
25.0 mg/ml of LPS	4 µl

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500 µM of $N_ω$-nitro-L-arginine methyl ester (L-NAME) hydrochloride
(L-NAME in culture media with 0.2% DMSO)

L-NAME powder (FW 269.7, Sigma)	1.35 g
Diluent A	10 ml

2.5% of Phosphoric acid solution

Phosphoric acid, H_2PO_4 (85%, Sigma)	3 ml
Deionized water (Millipore)	99 ml

Store in the dark at 4°C.

Griess reagent
(1% sulphanilamide and 0.1% naphthyl ethylene diamine dihydrochloride in 2.5% phosphoric acid)

Sulphanilamide, $C_6H_8N_2O_2S$ (FW 172.2, Sigma)	1 g
N-(1-Naphthyl)ethylenediamine dihydrochloride	

$C_{12}H_{14}N_2.2HCl$ (FW259.2, Sigma)	0.1 g
2.5% H_2PO_4	100 ml

Note: Prepare fresh. Make sure the solution is always covered before usage to minimize oxidation.

200 µM of Sodium Nitrate stock solution

Sodium nitrite, $NaNO_2$ powder (FW69, Sigma)	0.2 mg
Deionized water (Millipore)	100 ml

5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)

MTT powder (Fluka)	5 mg
Sterile PBS, pH 7.2	1 ml

Note: Prepare fresh. Keep solution away from light.

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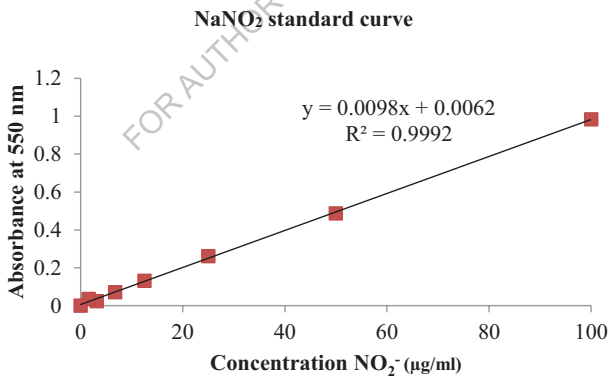

Plate design for Griess and MTT Assay

Controls:
Control 1: DMEM without phenol red with 5% FBS only (100 µl Diluent B)
Control 2: Cells in media with 0.1% DMSO, 200 U/ml IFN-γ and 10 µg/ml LPS (50 µl Diluent B + 50 µl Diluents C)
Control 3: Cells in media with 200U/ml IFN-γ and 10 µg/ml LPS (50 µl Diluent A + 50 µl Diluents C)
Control 4: Cells in media only (100 µl Diluent B)
Control 5: Cells in media with 0.1% DMSO, 200 U/ml IFN-γ plus 10 µg/ml LPS, and 250 µM (final concentration) of L-NAME; a NOS inhibitor. (50 µl Diluent C + 50 µl L-NAME)

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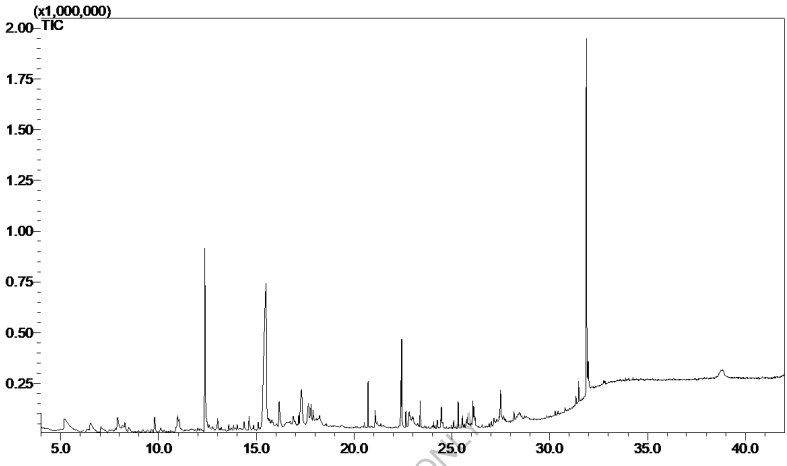
Concentration (µg/ml)	Well	1	2	3	4	5	6	7	8	9	10	11	12
200	A	100 µl Sample A + 100 µl Diluent B			100 µl Sample B + 100 µl Diluent B			100 µl Sample C + 100 µl Diluent B			100 µl Sample D + 100 µl Diluent B		
100	B	100 µl Sample A + 100 µl Diluent A			100 µl Sample B + 100 µl Diluent A			100 µl Sample C + 100 µl Diluent A			100 µl Sample D + 100 µl Diluent A		
50	C	100 µl Sample A + 100 µl Diluent A			100 µl Sample B + 100 µl Diluent A			100 µl Sample C + 100 µl Diluent A			100 µl Sample D + 100 µl Diluent A		
25	D	100 µl Sample A + 100 µl Diluent A			100 µl Sample B + 100 µl Diluent A			100 µl Sample C + 100 µl Diluent A			100 µl Sample D + 100 µl Diluent A		
12.5	E	100 µl Sample A + 100 µl Diluent A			100 µl Sample B + 100 µl Diluent A			100 µl Sample C + 100 µl Diluent A			100 µl Sample D + 100 µl Diluent A		
6.25	F	100 µl Sample A + 100 µl Diluent A			100 µl Sample B + 100 µl Diluent A			100 µl Sample C + 100 µl Diluent A			100 µl Sample D + 100 µl Diluent A		
3.12	G	100 µl Sample A + 100 µl Diluent A			100 µl Sample B + 100 µl Diluent A			100 µl Sample C + 100 µl Diluent A			100 µl Sample D + 100 µl Diluent A		
	H	Control 1		Control 2	Control 3		Control 4	Control 5					

APPENDIX D

GC-MS Chromatogram of Jatropha Meal Methanolic Extract



Gas chromatography/mass spectrometry (GC/MS) chromatogram of Jatropha meal methanolic extract

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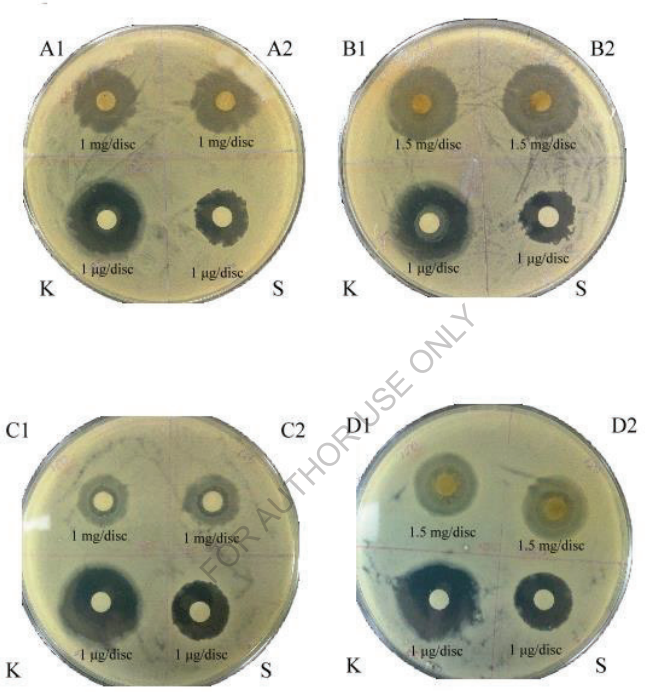
APPENDIX E

Antibacterial Activity of Jatropha Meal Extracts



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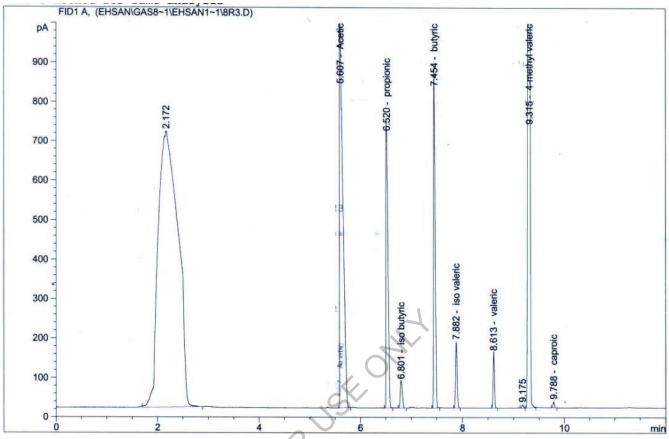
Inhibition zones of Jatropha meal methanolic extract (A1, A2, B1 and B2) and hot water extract (C1, C2, D1 and D2) against *Eschericia coli.* K= Kanamycin; S: Streptomycin

APPENDIX F

The Chromatogram of Rumen Microbial Volatile Fatty Acids analyzed by Gas Chromatography

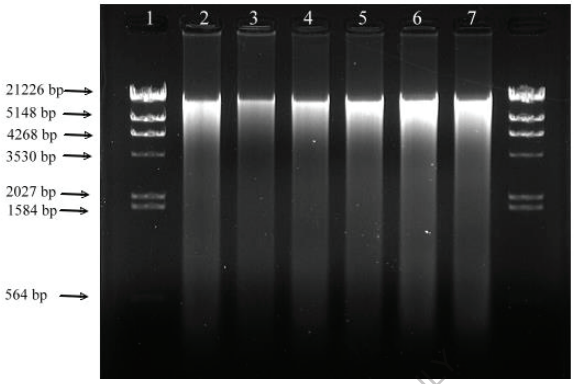


Profile of rumen volatile fatty acids determined using a gas chromatography. The volatile fatty acids consisted of acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid, valeric acid and caproic acid. 4-Methyl valeric acid was used as internal standard

FOR REVIEW USE ONLY

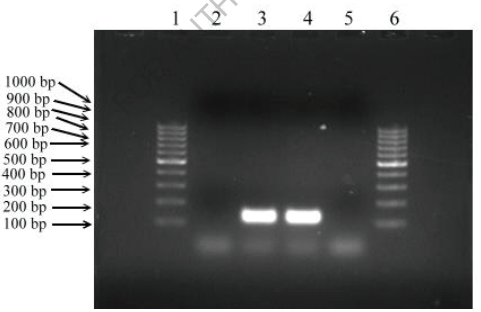
APPENDIX G

DNA Extraction, PCR Amplification, PCR Product Gel Purification and Plasmid Digestion

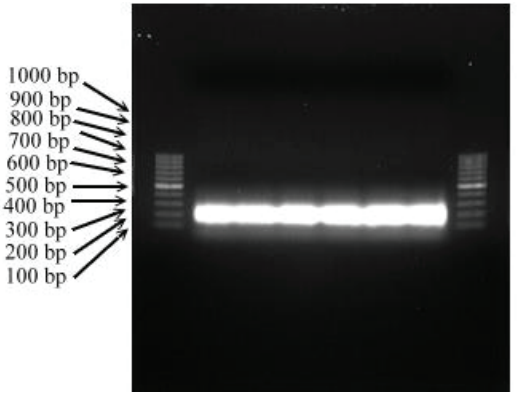


Gel electrophoresis of extracted DNA

AUTHOR USE ONLY



Specificity verification of the primers



PCR product gel purification

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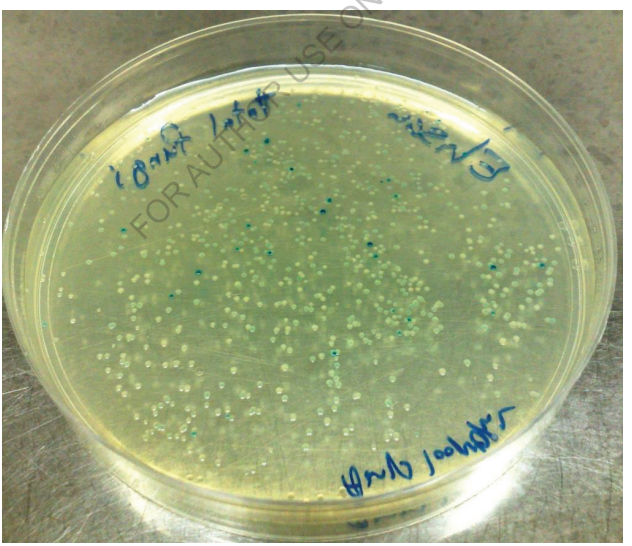
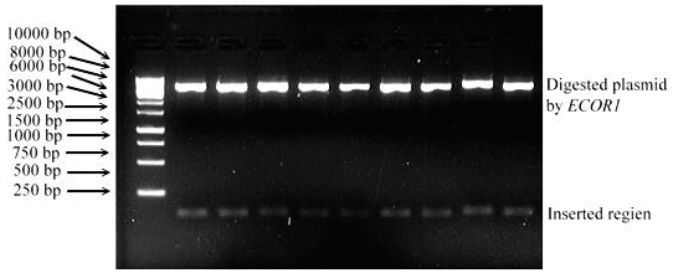


Cloning and blue/white screening of the *E. coli* colonies for insert. The colonies were grown on LB agar covered by X-gal incubated for 12 h



Digestion of plasmid to confirm the presence and correct orientation of the insert

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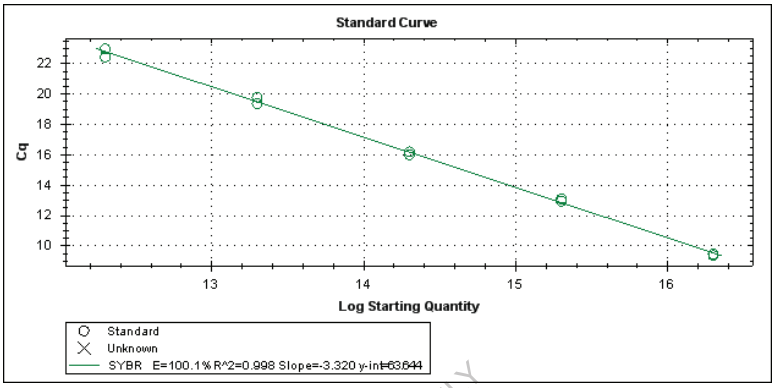
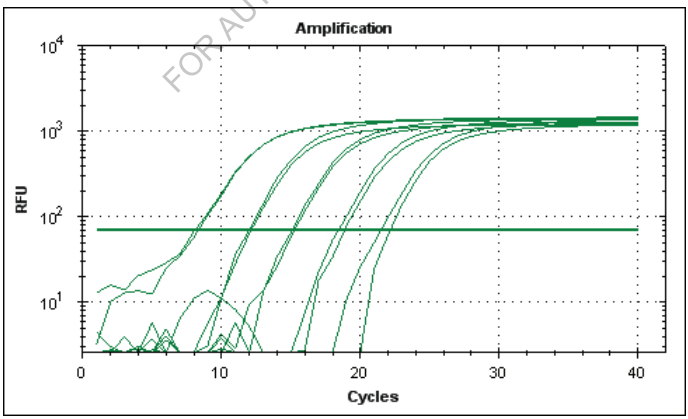
APPENDIX H

Real Time PCR Standards, Amplification Curves and Melting Curves Used in Rumen Microbial Quantification

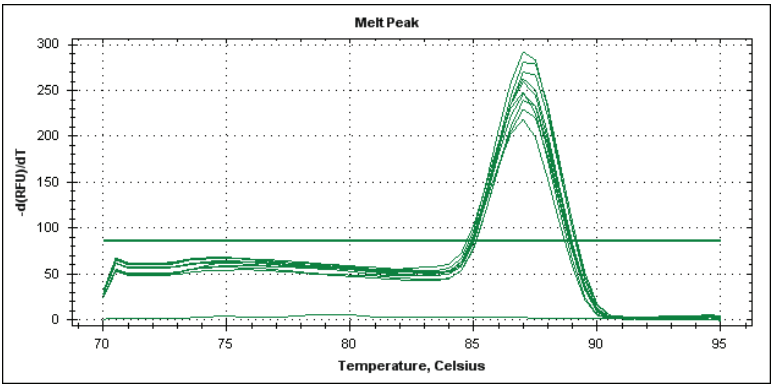


Standard curves of real-time PCR generated from serial dilutions of plasmids containing the targeted 16S rRNA genes of **general bacteria** ranging from 10^{12} to 10^{16} copies. The Ct values were plotted against the copy numbers of the target gene

FOR AUTHOR USE ONLY

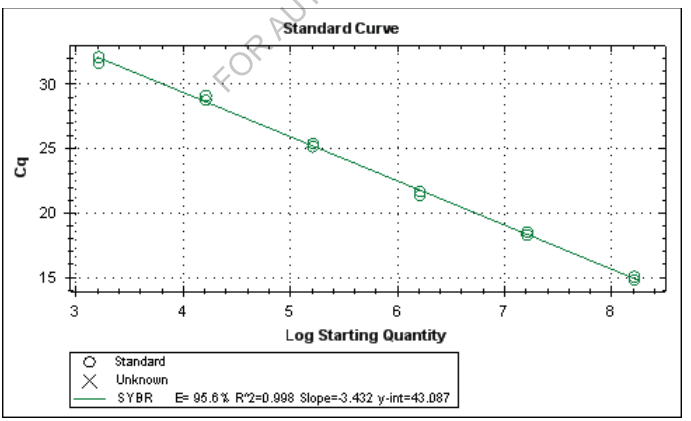


Amplification plots generated from the amplification of 16S rRNA genes of **general bacteria** in recombinant plasmid with various known concentrations using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number

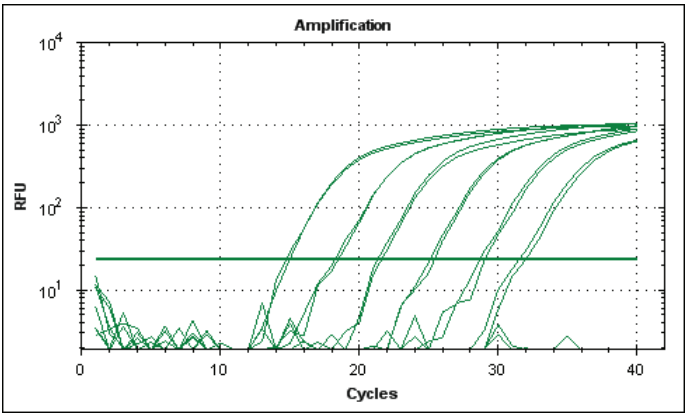


Dissociation curve generated from amplification of **general bacteria**. A melting temperature of 87°C was observed.

FOR AUTHOR USE ONLY

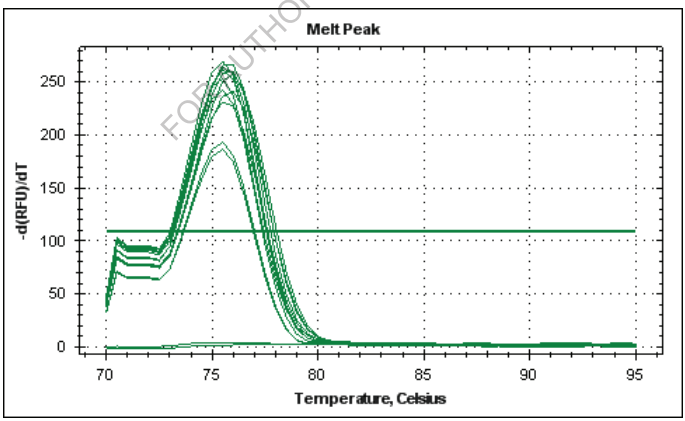


Standard curves of real-time PCR generated from serial dilutions of plasmids containing the targeted 18S rRNA genes of **general fungi** ranging from 10^3 to 10^8 copies. The Ct values were plotted against the copy numbers of the target gene.

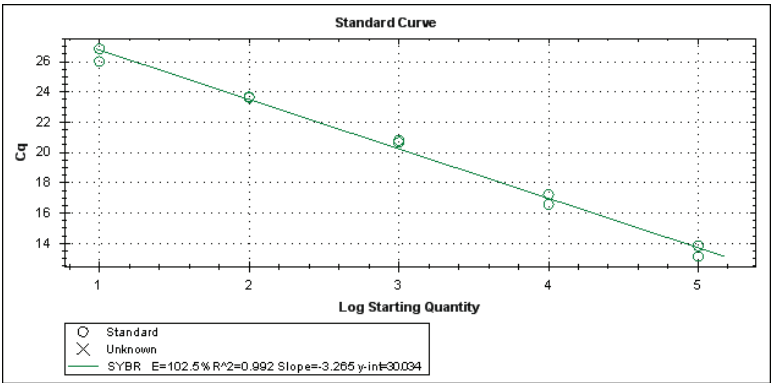


Amplification plots generated from the amplification of 18S rRNA genes of **general fungi** in recombinant plasmid with various known concentrations using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number

FOR AUTHOR USE ONLY

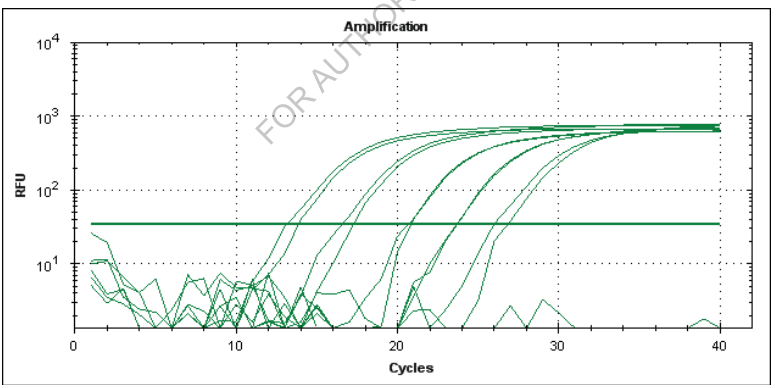


Dissociation curve generated from amplification of **general fungi**. A melting temperature of 76°C was observed.

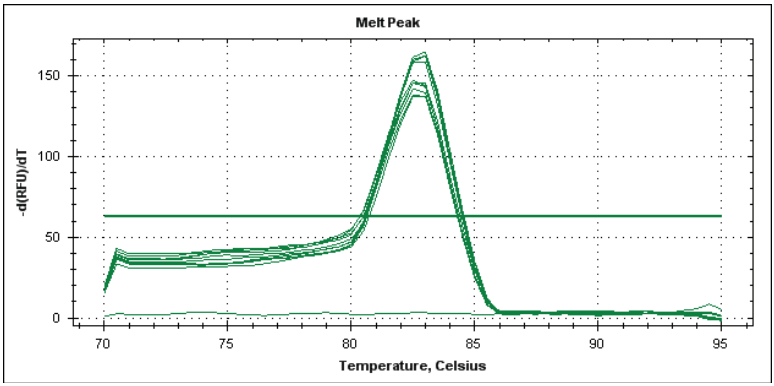


Standard curves of real-time PCR generated from serial dilutions of plasmids containing the targeted 18S rRNA genes of **general protozoa** ranging from 10^1 to 10^5 copies. The Ct values were plotted against the copy numbers of the target gene.

FOR AUTHOR USE ONLY

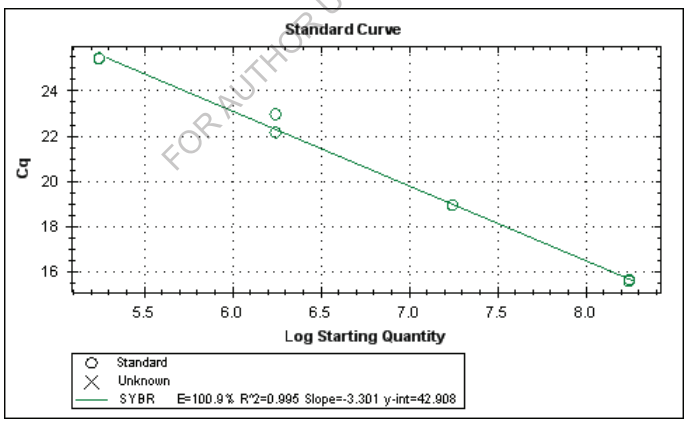


Amplification plots generated from the amplification of 18S rRNA genes of **general protozoa** in recombinant plasmid with various known concentrations using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number

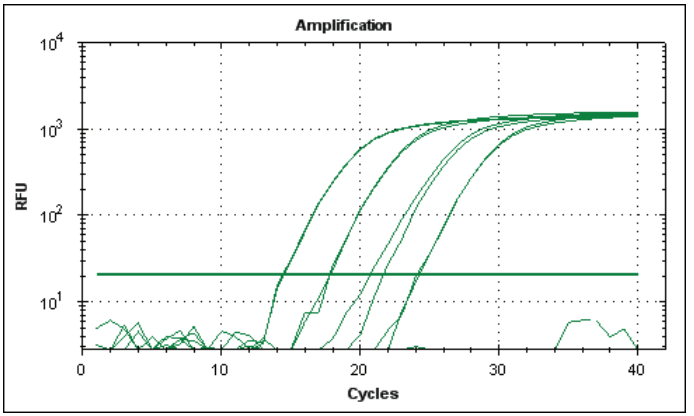


Dissociation curve generated from amplification of **general protozoa**. A melting temperature of 83°C was observed.

FOR AUTHOR USE ONLY

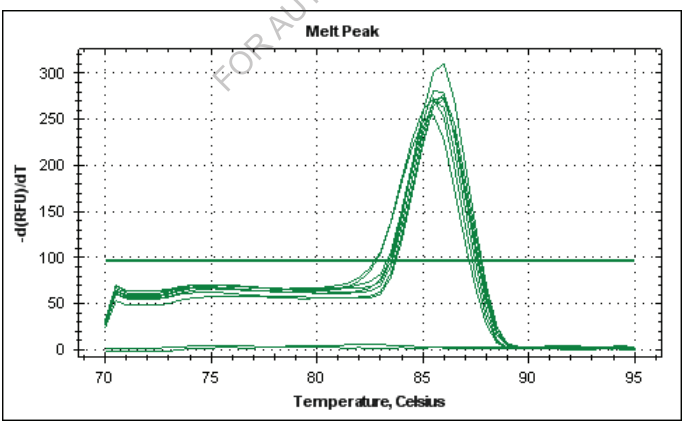


Standard curves of real-time PCR generated from serial dilutions of plasmids containing the targeted 16S rRNA genes of ***Ruminococcus albus*** ranging from 10^5 to 10^8 copies. The Ct values were plotted against the copy numbers of the target gene.

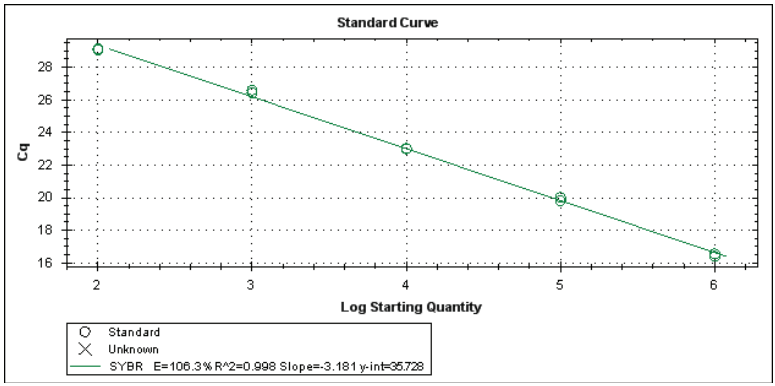


Amplification plots generated from the amplification of 16S rRNA genes of ***Ruminococcus albus*** in recombinant plasmid with various known concentrations using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number

FOR AUTHOR USE ONLY

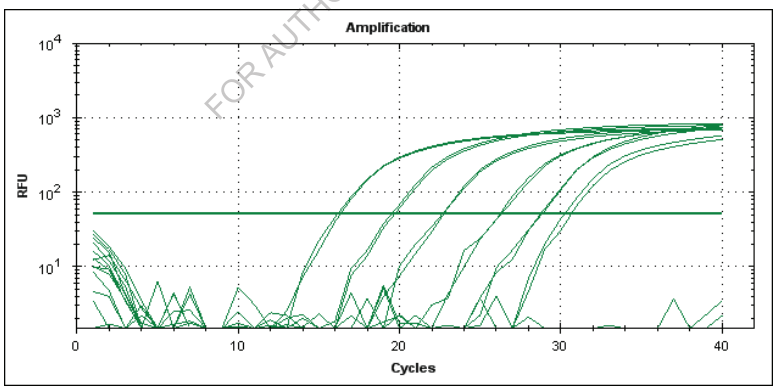


Dissociation curve generated from amplification of ***Ruminococcus albus***. A melting temperature of 86°C was observed.

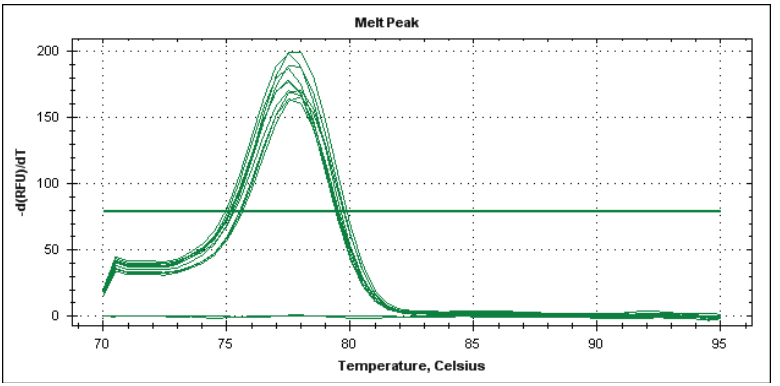


Standard curves of real-time PCR generated from serial dilutions of plasmids containing the targeted 16S rRNA genes of ***Butyrivibrio fibrisolvens*** ranging from 10^2 to 10^6 copies. The Ct values were plotted against the copy numbers of the target gene.

FOR AUTHOR USE ONLY

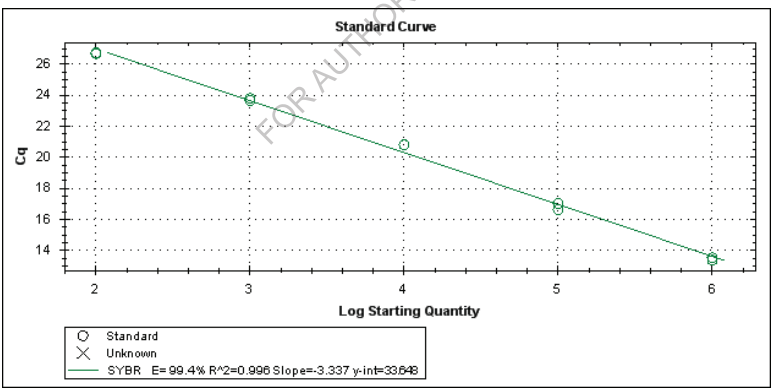


Amplification plots generated from the amplification of 16S rRNA genes of ***Butyrivibrio fibrisolvens*** in recombinant plasmid with various known concentrations using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number

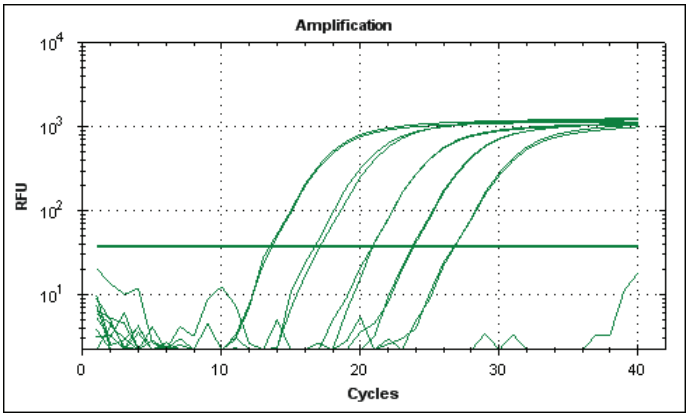


Dissociation curve generated from amplification of ***Butyrivibrio fibrisolvens***. A melting temperature of 77.5°C was observed.

FOR AUTHOR USE ONLY

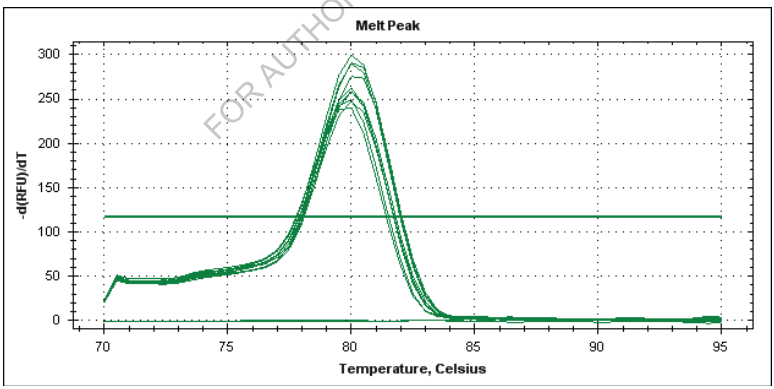


Standard curves of real-time PCR generated from serial dilutions of plasmids containing the targeted 16S rRNA genes of ***Ruminococcus flavefaciens*** ranging from 10^2 to 10^6 copies. The Ct values were plotted against the copy numbers of the target gene.

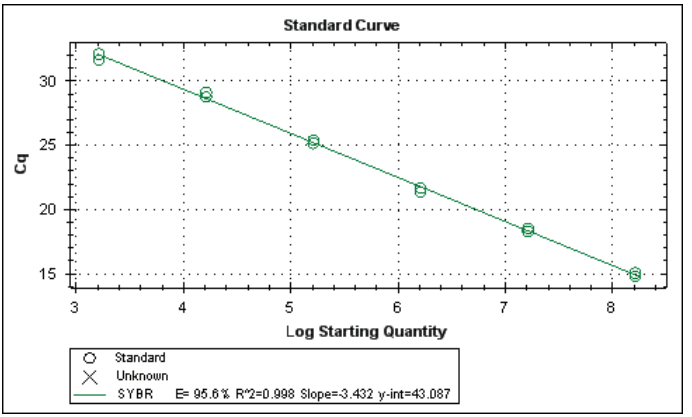


Amplification plots generated from the amplification of 16S rRNA genes of ***Ruminococcus flavefaciens*** in recombinant plasmid with various known concentrations using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number

FOR AUTHOR USE ONLY

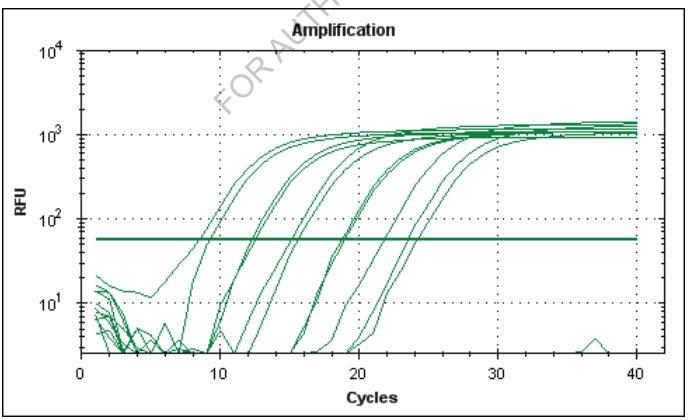


Dissociation curve generated from amplification of ***Ruminococcus flavefaciens***. A melting temperature of 80°C was observed.

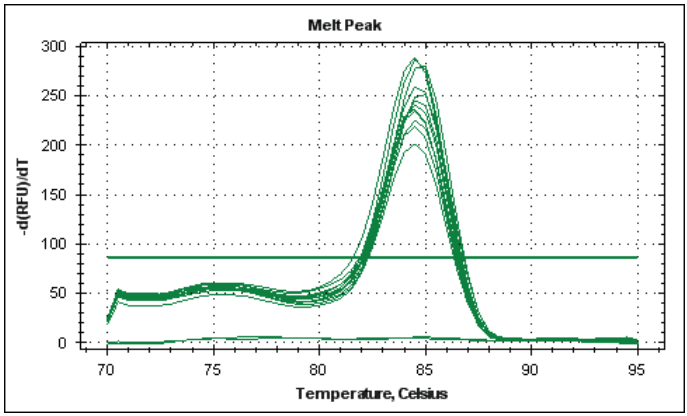


Standard curves of real-time PCR generated from serial dilutions of plasmids containing the targeted 16S rRNA genes of ***Fibrobacter succinogenes*** ranging from 10^3 to 10^8 copies. The Ct values were plotted against the copy numbers of the target gene.

FOR AUTHOR USE ONLY

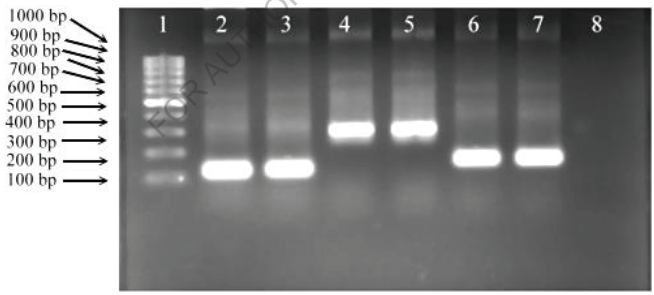


Amplification plots generated from the amplification of 16S rRNA genes of ***Fibrobacter succinogenes*** in recombinant plasmid with various known concentrations using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number



Dissociation curve generated from amplification of ***Fibrobacter succinogenes***. A melting temperature of 84°C was observed.

FOR AUTHOR USE ONLY



Confirmation of primers specificity, real time PCR product quality and lack of unspecific band upon real time PCR amplification

APPENDIX I

Formaldehyde (FA) Agarose Gel Preparation for RNA Integrity Analysis

FA gel buffer stock (10X)

[200 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 50 mM Sodium acetate,

10 mM EDT A, pH 7.0]

MOPS (231.25, Sigma)	23.125 g
Sodium acetate (82.03, Sigma)	2.05 g
EDT A (MW 372.24, Amersham Pharmacia)	1.86 g
Deionized water (Millipore)	400 ml

Adjust pH to 7.0 with HCl Make up the volume to 500 ml with deionized water. Can

be store in room temperature for up to 6 months.

FOR AUTHOR USE ONLY

FA gel running buffer

10X FA gel buffer	100 ml
Formaldehyde (37%, Sigma)	20 ml
RNase free water (Qiagen)	880 ml

5X RNA loading buffer

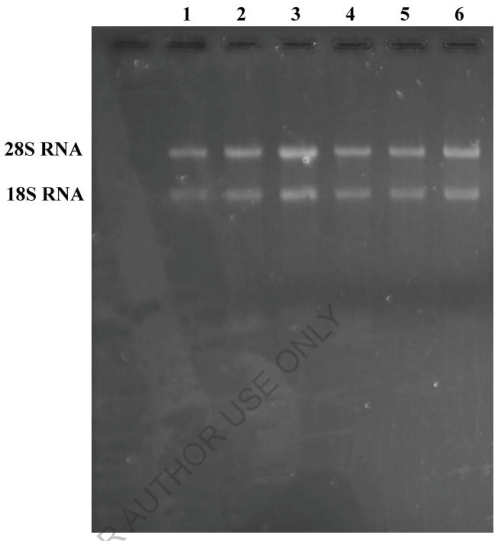
EDTA (500 mM, pH 8.0)	80 µl
Formaldehyde (37%, Sigma)	720 µl
Glycerol	2 ml
Formamide	3.084 ml
F A gel buffer (10X)	4 ml

Small amount of bromophenol blue was added and keep in 4°C up to 3 months.

4. FA gel preparation (1.2%)

Agarose	3.6 g
FA gel buffer (10 X)	3 ml
RNase free water (Qiagen)	30 ml

Heat the mixture until all ingredients completely dissolved. Add 591 µl of formaldehyde and pour the solution into gel tank and wait for it to solidify.

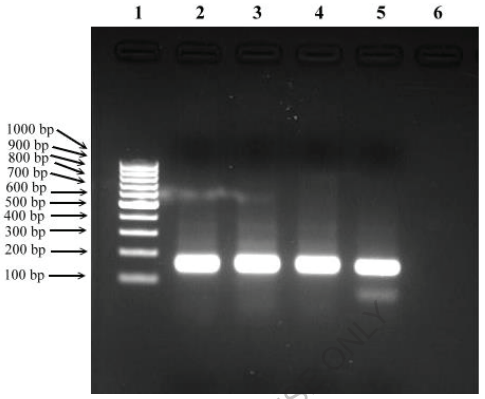


FOR AUTHOR USE ONLY

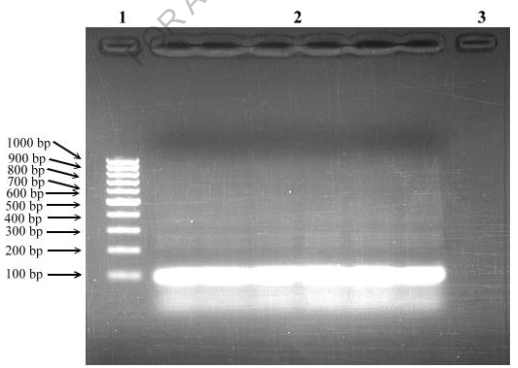
The extracted RNA from MDBK cells and quality analysis using denaturing agarose gel stained with EtBr

APPENDIX J

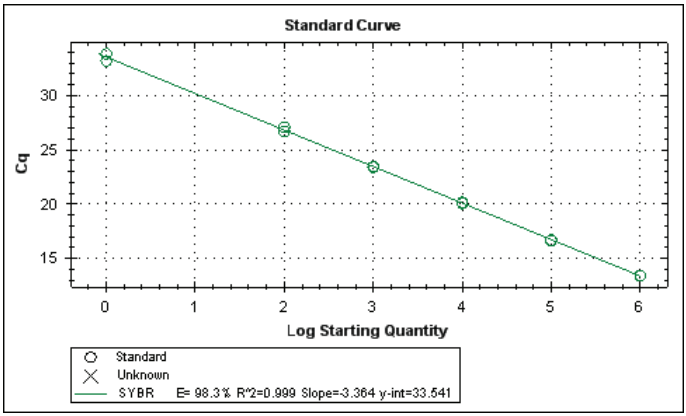
Primer Design and Specificity Verification, Real Time PCR Standards, Amplification Curves and Melting Curves used in Gene Expression Study in Bovine Kidney Cell line



Specificity verification of the designed primers using cDNA as template

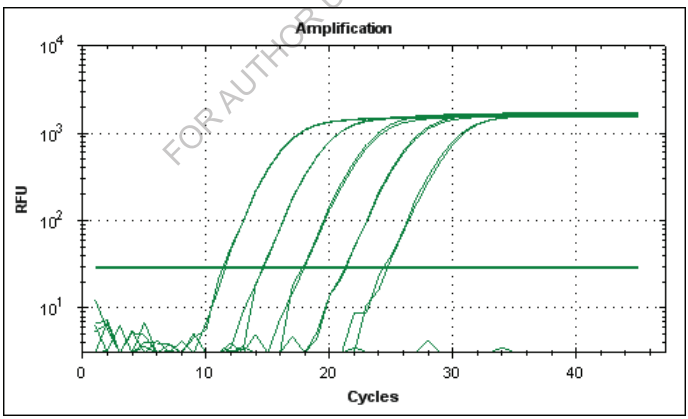


Purification of PCR product to be applied in standard curve generation for real time PCR

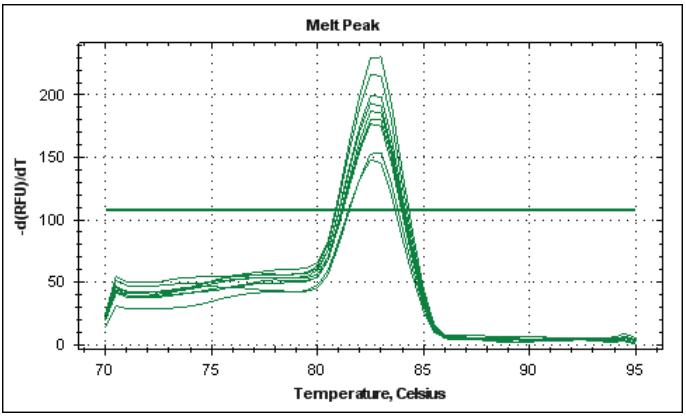


Standard curves of real-time PCR generated from serial dilutions of PCR product and amplification of **Protein Kinase C beta II (PKC-β II)** gene using specific primers. The Ct values were plotted against the copy numbers of the target gene.

FOR AUTHOR USE ONLY

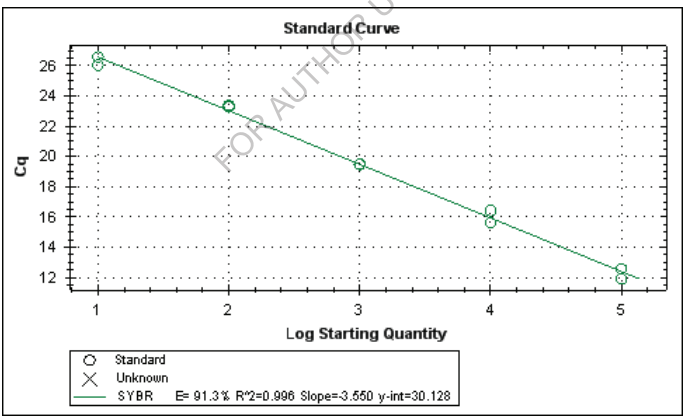


Amplification plots generated from the amplification of **Protein Kinase C beta II (PKC-β II)** gene in various known concentrations of purified PCR product using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number.

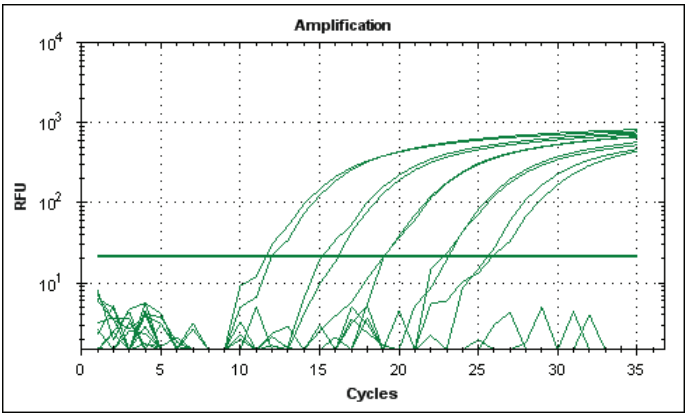


Dissociation curve generated from amplification of **Protein Kinase C beta II (PKC-β II)** gene in the cDNA obtained from MDBK cells. A melting temperature of 82.5°C was observed.

FOR AUTHOR USE ONLY

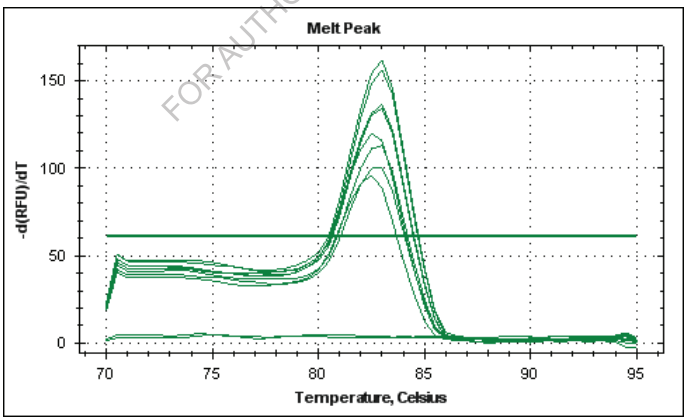


Standard curves of real-time PCR generated from serial dilutions of PCR product and amplification of **c-Fos** gene using specific primers. The Ct values were plotted against the copy numbers of the target gene.

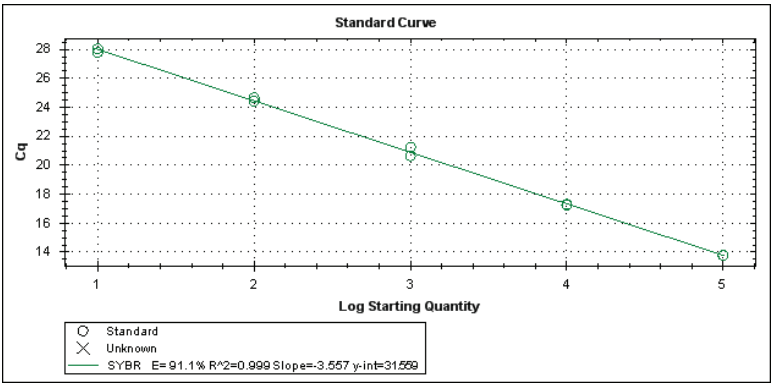


Amplification plots generated from the amplification of **c-Fos** gene in various known concentrations of purified PCR product using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number.

FOR AUTHOR USE ONLY

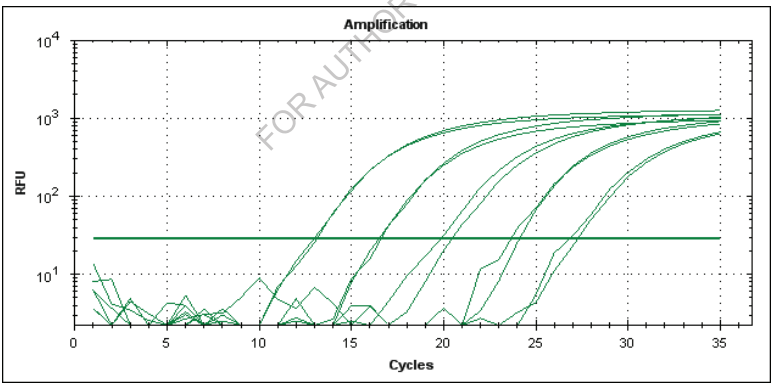


Dissociation curve generated from amplification of **c-Fos** gene in the cDNA obtained from MDBK cells. A melting temperature of 83°C was observed.

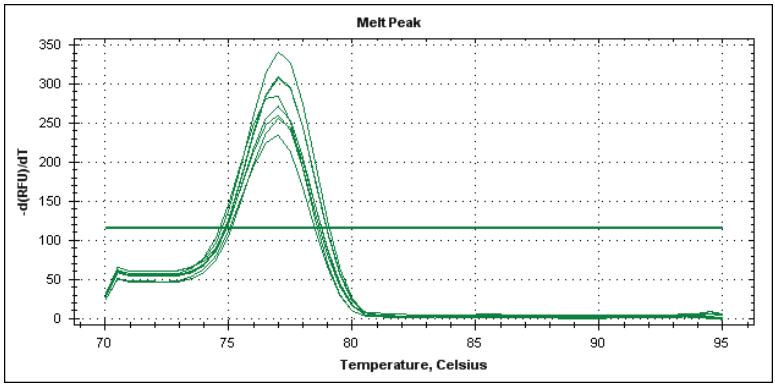


Standard curves of real-time PCR generated from serial dilutions of PCR product and amplification of **c-Myc** gene using specific primers. The Ct values were plotted against the copy numbers of the target gene.

FOR AUTHOR USE ONLY

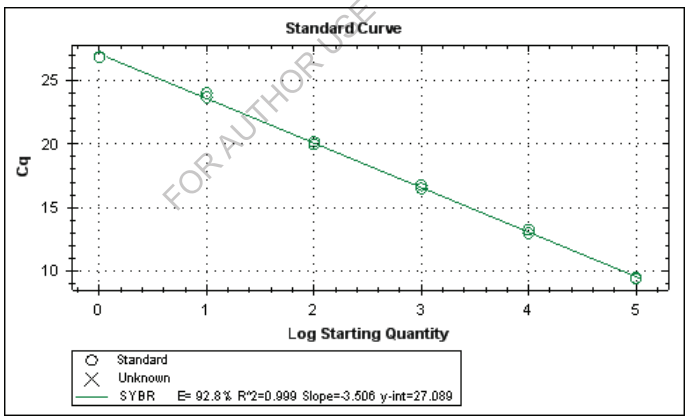


Amplification plots generated from the amplification of **c-Myc** gene in various known concentrations of purified PCR product using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number.

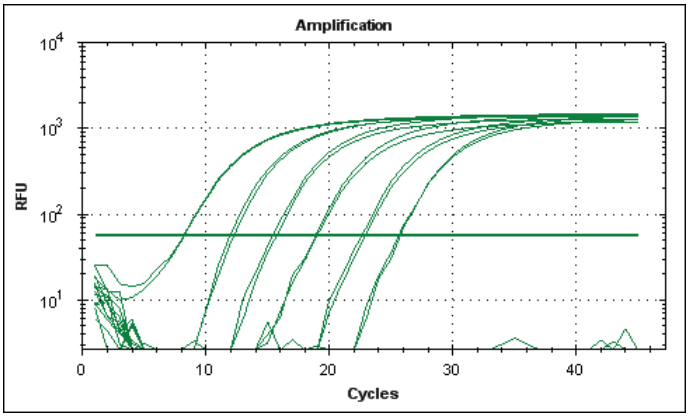


Dissociation curve generated from amplification of **c-Myc** gene in the cDNA obtained from MDBK cells. A melting temperature of 77°C was observed.

FOR AUTHOR USE ONLY

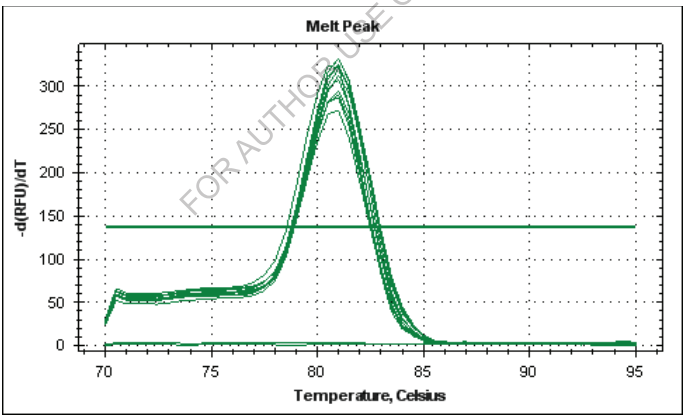


Standard curves of real-time PCR generated from serial dilutions of PCR product and amplification of **interleukin 1 beta (IL1-β)** gene using specific primers. The Ct values were plotted against the copy numbers of the target gene.

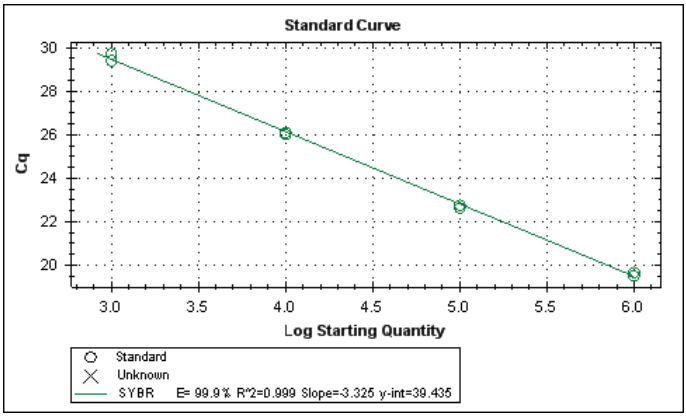


Amplification plots generated from the amplification of **interleukin 1 beta (IL1-β)** gene in various known concentrations of purified PCR product using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number

FOR AUTHOR USE ONLY

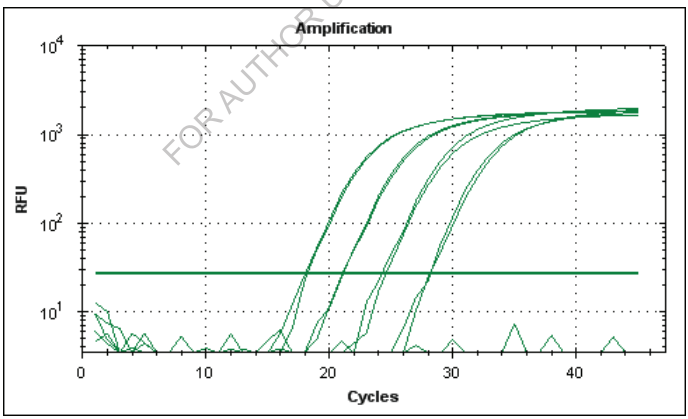


Dissociation curve generated from amplification of **interleukin 1 beta (IL1-β)** gene in the cDNA obtained from MDBK cells. A melting temperature of 81.5°C was observed.

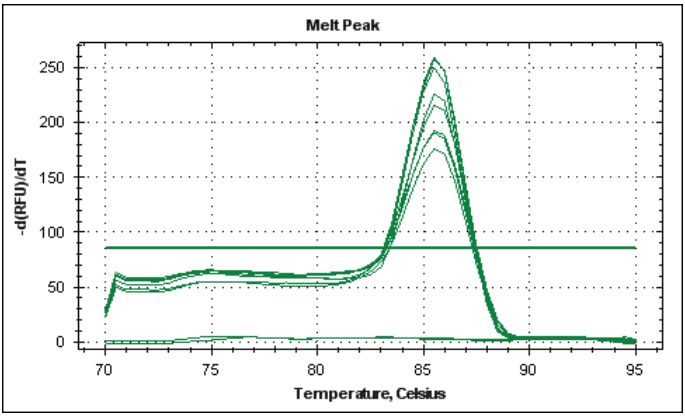


Standard curves of real-time PCR generated from serial dilutions of PCR product and amplification of **Cyclooxygenase-2(COX2)** gene using specific primers. The Ct values were plotted against the copy numbers of the target gene.

FOR AUTHOR USE ONLY

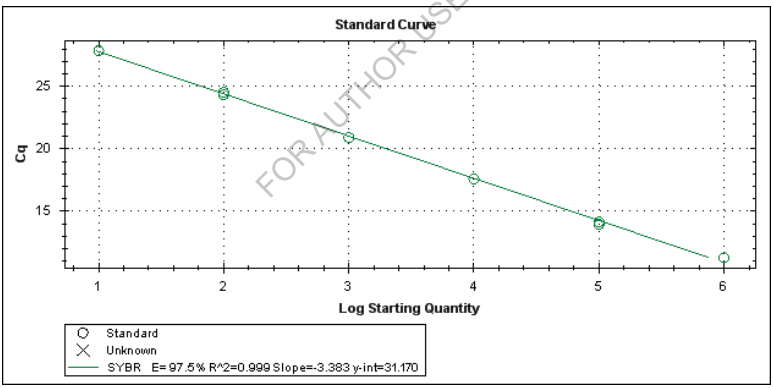


Amplification plots generated from the amplification of **Cyclooxygenase-2(COX2)** gene in various known concentrations of purified PCR product using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number.

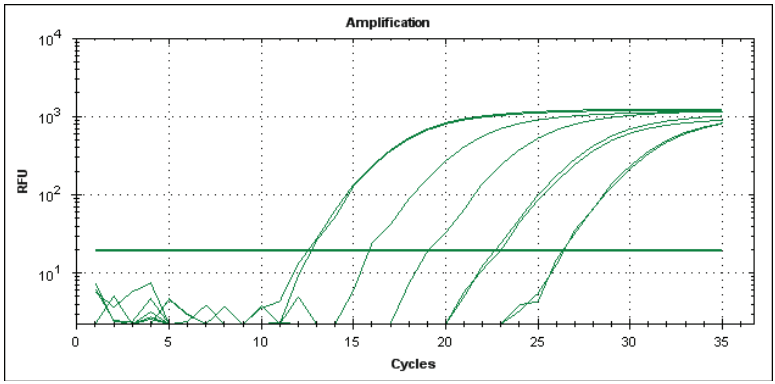


Dissociation curve generated from amplification of **Cyclooxygenase-2(COX2)** gene in the cDNA obtained from MDBK cells. A melting temperature of 86°C was observed.

FOR AUTHOR USE ONLY

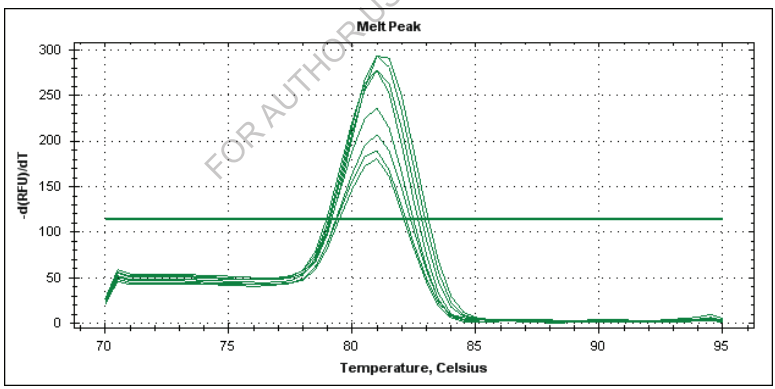


Standard curves of real-time PCR generated from serial dilutions of PCR product and amplification of **GAPDH** gene using specific primers. The Ct values were plotted against the copy numbers of the target gene

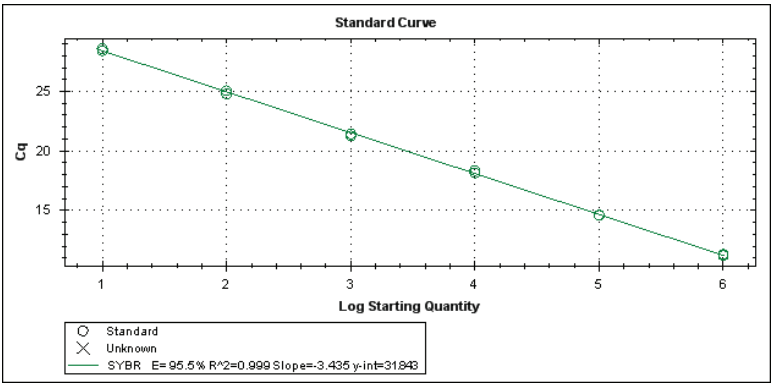


Amplification plots generated from the amplification of **GAPDH** gene in various known concentrations of purified PCR product using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number.

FOR AUTHOR USE ONLY

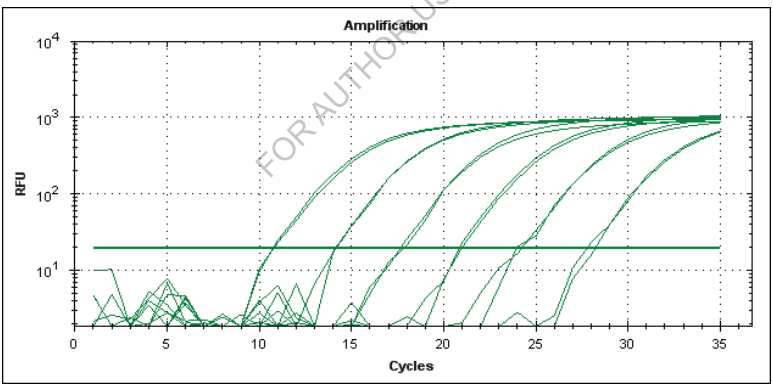


Dissociation curve generated from amplification of **GAPDH** gene in the cDNA obtained from MDBK cells. A melting temperature of 81°C was observed.

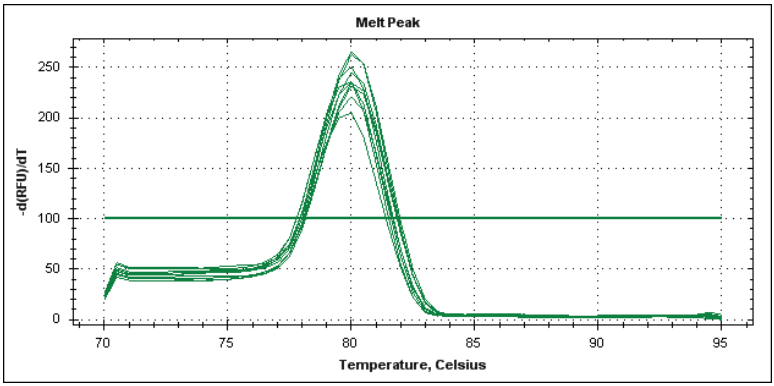


Standard curves of real-time PCR generated from serial dilutions of PCR product and amplification **β-actin** gene using specific primers. The Ct values were plotted against the copy numbers of the target gene.

FOR AUTHOR USE ONLY



Amplification plots generated from the amplification of **β-actin** gene in various known concentrations of purified PCR product using specific primers. The Relative fluorescence intensity was plotted against PCR cycle number.



Dissociation curve generated from amplification of **β-actin** gene in the cDNA obtained from MDBK cells. A melting temperature of 80°C was observed

FOR AUTHOR USE ONLY

APPENDIX K

Preparation of Wash Buffer for Protein Extraction

Tris-sucrose wash buffer

In place of PBS, which contaminates the cells with too much salt, Tris-buffered sucrose was used (containing 10 M Tris, 250 M sucrose, pH 7).

Tris-base (FW121.14, Pharmacia Biotech, UK)	1.21 g
Sucrose (FW 342.30, Pharmacia Biotech, UK)	8.56 g

Make up to 1 L with deionized water. Then titrate to pH 7 with 1 M HCl.

FOR AUTHOR USE ONLY

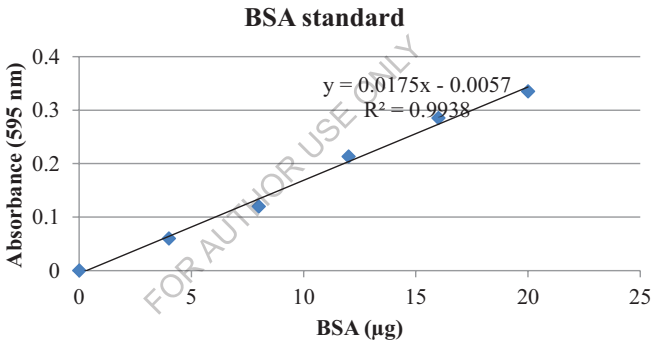
APPENDIX L

Preparation of Bradford Reagent

5X Bradford Reagent
[0.05% (w/v) Coomassie blue, 25% (v/v) ethanol and 42.5% (v/v) phosphoric acid]

Coomassie Brilliant Blue G (FW 854.04, Amresco)	50 mg
Ethanol	25 ml
Phosphoric Acid (85%, Sigma)	50 ml
Deionized water	25 ml

The mixture is filtered through Whatman filter paper No.2 and the reagent stored in a dark bottle and kept at room temperature



FOR AUTHOR USE ONLY

Standard curve of bovine serum albumin (BSA) for Bradford Assay

APPENDIX M

Preparation of Stock Solution for SDS-PAGE, Coomassie Blue Staining and Western Blot analysis

Preparation of stock solution for SDS-PAGE

A. Running Gel Buffer
(1.5 M Tris-HCl, pH 8.8)

Tris (MW 121.14, Pharmacia Biotech, UK)	18.15 g
Deionized water (Millipore)	80 ml

Adjust pH to 8.8 with 1 M NaOH. Make up the volume to 100 ml with deionized water. Can be store in 4°C for up to 6 months.

B. Stacking Gel Buffer
(0.5 M Tris-Cl, pH 6.8)

Tris (FW 121.1)	3.0 g
Deionized water (Millipore)	40 ml

Adjust pH to 6.8 with HCl. Make up the volume to 50 ml with deionized water. Can be store in 4°C for up to 6 months.

FOR AUTHOR USE ONLY

B. Monomer stock solution
[Acrylamide / Bis 30 % (30 % T/ 2.67 % C)]

Acrylamide (FW 71.08 g, Pharmacia Biotech, UK)	60.0 g
N, N'-methylene bisacrylamide (FW: 154.17, Pharmacia Biotech, UK)	1.6 g
Deionized water (Millipore)	200 ml

Mix thoroughly and filter using 0.45 μm filter and store at 4°C for up to 3 months.
Notes: Keep solution mixture away from light.

C. 10 % of Sodium dodecyl sulfate (SDS) stock solution

SDS (MW: 228.38, Pharmacia Biotech, UK)	5.0 g
Deionized water (Millipore)	50 ml

Keep at room temperature.

D. 10 % of Ammonium persulfate (APS) stock solution

APS (FW228.2, Pharmacia Biotech, UK)	0.1 g
Deionized water (Millipore)	1 ml

Note: Always prepare fresh.

E. Non-reducing loading buffer

(0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% SDS, Bromophenol blue)

Deionized water (Millipore)	3.8 ml
0.5M Tris-HCl, pH 6.8	1 ml
100% glycerol	0.8 ml
10% SDS	1.6 ml

Keep at room temperature.

Loading Buffer: Add 5 µl of β–mercaptoethanol plus 95 µl of non-reducing buffer at least before loading the sample.

F. Electrophoresis Buffer 10X (Tank buffer)

Tris-base	30 g
L-Glycine	144 g
SDS	10 g
Deionized water (Millipore)	1 L

The pH need not be adjusted. It should be between 8.2 and 8.4. Keep at room temperature.

FOR AUTHOR USE ONLY

The table below shows the running gel and stacking gel recipes for 0.75 and 1.5 mm thick gels.

Final Gel Concentration (10 ml; 2 x 0.75 mm thick SE 250 gels)

	10%
Monomer solutions	3.3 ml
Running Gel Buffer (1.5 M Tris-Cl, pH 8.8)	2.5 ml
10% SDS	0.1 ml
ddH_2O	4.0 ml
10% APS	50 µl
TEMED*	15 µl

Final Gel Concentration (20 ml; 2 x 1.5 mm thick SE 250 gels)

	10%
Monomer solutions	6.7 ml
Running Gel Buffer (1.5 M Tris-Cl, pH 8.8)	5 ml
10% SDS	0.2 ml
ddH_2O	11.4 ml
10% APS	100 µl
TEMED*	6.7 µl

FOR AUTHOR USE ONLY

Stacking gel solutions (4% acrylamide; for two gels)

Gel thickness:	0.75 mm
Monomer solutions	0.44 ml
Stacking Gel Buffer (0.5 M Tris-Cl, pH 6.8)	0.83 ml
10 % SDS	33 µl
ddH_2O	2.03 ml
10% APS	16.7 µl
TEMED*	10 µl

* Add last

G. Coomassie Blue staining

Staining solution
(0.025% Coomassie Brilliant blue R 250, 40% methanol, 10% acetic acid)

Coomassie Brilliant blue R	0.5 g
Methanol	400 ml
Stir until a homogenous solution is formed.	
Then add:	
Acetic acid	100 ml
Deionized water (Millipore)	1 L

Filtering is not required. Can be store at room temperature for up to 6 months.

Destaining solution
(40% methanol, 10% acetic acid)

Methanol	400 ml
Acetic acid	100 ml
Deionized water (Millipore)	1 L

Can be store at room temperature indefinitely.

FOR AUTHOR USE ONLY

H. Western Blot buffer stock solution (Semi-Dry Transfer Unit)

Towbin Buffer solution
(25 mM Tris, 192 mM glycine, 20% v/v methanol, 0.1% SDS, pH 8.3, 1 liter)

Tris (FW 121.1)	3.0 g
Glycine (FW 75.07)	14.4 g
SDS* (FW 288.4)	1.0 g
Deionized water (Millipore)	1 L

The adjustment of pH is not required. It should be between 8.2 and 8.4. Add 15% of methanol into Towbin Buffer (for PVDF membrane). Adding methanol can improve transfer results. Note: Buffers containing methanol may deteriorate if stored for long periods. Thus, methanol is added just prior to transfer. Optional: chill before use.

Working Transfer buffer
(15% methanol in Towbin Buffer solution)

Add 15 ml of methanol into 86 ml of Towbin buffer solution and mix.

Phosphate buffer saline (PBS)
(0.081 M disodium hydrogen phosphate, 0.015 M sodium dihydrogen phosphate, 0.15 M sodium chloride, pH 7.2)

Na_2HPO_4 (MW 141.96)	11.49 g
NaH_2PO_4 (MW 156.01)	2.28 g
NaCl (MW 58.44)	8.77 g

Add 90 ml of deionized water and adjust pH to 7.2. Make up to 1L with deionized water. Can keep up to 6 months at room temperature.

PBST
(PBS plus 0.05% Tween-20)

Add 0.25 ml of Tween-20 into 500 ml of PBS and mix.
Prepare fresh.

Blocking Solution
(PBST plus 3% skim-milk or 5% serum)

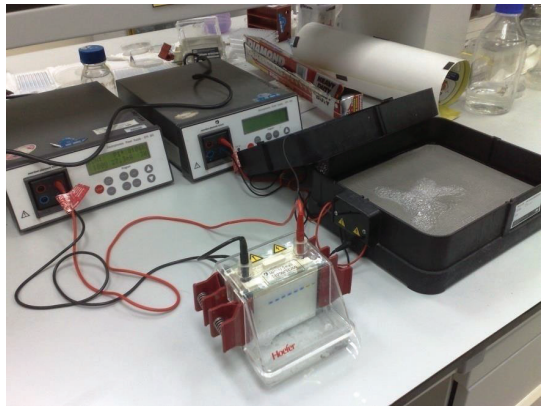
Weigh 3 g of skim-milk or add 5 ml of serum into 100 ml of PBST and mix.
Always prepare fresh.

FOR AUTHOR USE ONLY

Stripping solution
(100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7)

Tris-HCL (MW 157.64)	9.85 g
SDS	20 g
Deionized water (Millipore)	1 L
2-Mercaptoethanol*	1 ml

Add 900 ml of deionized water and adjust pH at 6.7. Adjust deionized water to 1 L. Keep for up to 1 month at room temperature.
* Add prior to use.



SDS-PAGE and SemiPhor Semi-dry Transfer Unit

FOR AUTHOR USE ONLY

Odyssey Infrared Imaging System used for Western Blotting and protein quantification. To detect the specific proteins fluorescent dyes conjugated to respective secondary antibodies were applied