Chemistry of Phorbol Ester Toxicity in *Jatropha curcas* Seed – a Review

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Review Article

ABSTRACT

The toxicity of *Jatropha curcas* seeds emanates mainly from phorbol esters. It is known that the seeds are a valuable source of oil, which can be used to produce biodiesel. The cake generated as a by-product, after oil pressing is a protein-rich potential stock for animal feed. Researchers, the world over, have studied these phorbol esters and investigated ways of improving the applications of the seed meal. This review paper outlines the previous research done on the *Jatropha curcas* plant, the toxicity of phorbol esters and the efforts made to detoxify the seed. It also highlights the knowledge gaps concerning the chemistry of phorbol ester toxicity and the probable areas of future research.

1. INTRODUCTION

Jatropha curcas has been touted as a wonder plant. It is drought resistant, making it suitable for low-rainfall areas [1,2]. The seeds contain, on average 33% oil that can be processed to produce good-quality biodiesel and soap [1,3,4,5]. Unfortunately, the remaining high-protein seed cake cannot be used as animal feed because it contains toxic phorbol esters [6].

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Studies have shown that the nutritional value of *J. curcas* seed cake equals that of soya bean, and better than sunflower and cotton seed meal, making it an ideal stock feed, if detoxified [7,8].

Scientists have focused their attention on the detoxification of *J. curcas* seed and a number of methods have been studied. Although physical and microbiological detoxification methods have been forwarded, we believe they are generally not cost-effective and that chemical detoxification techniques can be much faster and cheaper. Studies have also been done by various researchers on the chemical nature and toxicity of phorbol esters. However, the relationship between chemical structure and toxicity of phorbol esters is not well understood. Mechanisms of phorbol-ester degradation in *J. curcas seed* have also not been studied.

2. *Jatropha curcas* PLANT

Family: Euphorbiaceae
Genus: *Jatropha*
Species: *Jatropha curcas Linnaeus*

J. curcas is a genus of approximately 175 succulent plants, shrubs and trees in the Euphorbiaceae family [9]. It has good adaptation capacity to a large variety of soil conditions. Cultivation on dry, stony and very shallow soils is frequent [8]. It can be established on marginal land, paddocks, contour ridges, hilly slopes and gullies [10].

The origin of *J. curcas* is believed to be Mexico and continental Central America. Portuguese people learned about Jatropha's medicinal properties in the 16th Century, and later established commercial plantations for soap and lamp oil production on the Cape Verdean Islands and Guinea Bissau. Later, Jatropha genotypes adopted in West Africa were spread across other Portuguese colonies in Africa (Mozambique, Angola) and into Asia (India, China and Indonesia). *J. curcas* now grows pantropically, from Brazil to the islands of Fiji [1,11]. Two genotypes of *J. curcas* exist: the toxic and the non-toxic varieties [12]. The latter genotype is found in Mexico only [8]. Another non-toxic species, also found in Mexico, is *Jatropha platyphylla Müell* [13].

Seeds of *J. curcas* plant are rich in oil and protein. Significant quantities of macrominerals (Na, K, Mg, Ca, P) and microminerals (Mn, Fe, Zn) have also been found in the seeds [14]. The seeds contain about 300 – 350g/kg oil, which can be used as a fuel directly or in its transesterified form, as a substitute for diesel. The protein quality of the *J. curcas* seed cake is high. Levels of essential amino acids (except lysine) are higher in Jatropha seed cake than in the FAO reference protein for a growing child of 3 – 5 years [7]. *J. curcas* seed cake contains high true protein, high energy and low fibre. The nutritional value of Jatropha meal compares favourably with those from conventional seed meals, such as soya bean [7]. Research done by Chivandi *et al.* [15] indicated higher crude protein, ash, calcium and phosphorus in Jatropha seed meal than in soya bean meal. Makkar and Becker [16] reported that a seven-year old *J. curcas* seed had similar contents of crude protein (25.6%), lipid (57%) and ash (3.4%) in the kernel as those observed for fresh seed. They suggested that the presence of toxins could be a contributing factor to the long shelf-life of the seeds.

J. curcas is famous for being a potential source of raw material for biodiesel. Its popularity stems from the widespread general knowledge that it is a non-edible, oil-yielding tree, well adapted to marginal areas with poor soil and low rainfall, where it grows without competing with annual food crops [5].

There are many other uses of *J. curcas.* Traditionally, the plant has been used as a hedge and living fence, not edible by livestock. Growing Jatropha reclaims eroded and waste land. The seed oil can be used as a fuel for lighting and is a raw material for making high quality soap. Residue from seed pressing is good organic fertilizer [17]. All parts of *J. curcas* (seeds, leaves and bark) have been used in traditional medicine and for veterinary purposes for a long time [1,18]. This is typical of most members of the Euphorbiaceae family, whose medicinal properties may be due to stress factors such as drought and extreme temperatures that induce synthesis of survival and defence chemicals [19]. A 2% etheral solution of *J. curcas* seed oil was observed to have insecticidal properties against *Callosobruchus maculatus* – a seed beetle in cowpeas [20]. Just 0.05% methanolic solution of the seed oil caused a 70% larval mortality for *Cnaphalocrocis medinalis* – rice leaf folder and *Helicoverpa armigera* – cotton boll worm [21]. Ethanolic root extract of *J. curcas* was found to have antibiotic effect on *Escherichia coli, Neisseria gonorrhoea* and *Staphylococcus aureus*, with a minimum inhibitory quantity of 0.5ml [22]. The toxicity of *J. curcas* to mollusks has been investigated because of its relevance in schistosomiasis control [23]. Methanolic extracts of *J. curcas* seed oil showed toxicity against tested organisms, with LC_{100} values (concentrations killing 100% of the organism) of 25ppm for cercariae and the snail *Biomphalaria glabrata* and 1ppm for the snails *Bulinus truncatus* and *B. natalensis* [24]. Phorbol esters were seen to be the active compounds in activity against snails [25]. At least 1% ethanolic solution of crude extract caused complete inhibition of mycelial growth of *Fusarium oxysporum, Pythium aphanidermatum, Lasiodiplodia theobromae, Curvularia lunata, Fusarium semitectum, Colletotrichum capsici* and *Colletotrichum gloeosporioides* [26].
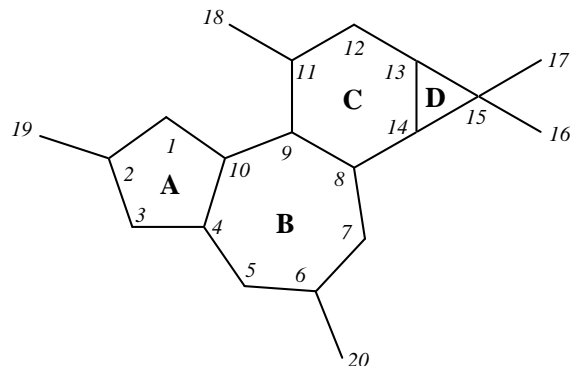
3. PHORBOL ESTERS

The term 'phorbol esters' is used today to describe a naturally occurring group of compounds mainly distributed in plant species of the Euphorbiaceae family. Phorbol esters are esters of phorbol, a tetracyclic diterpenoid with a tigliane skeletal structure [27]. Terpenoids are classified according to the number of carbon atoms they carry: monoterpenoids have 10 carbons, diterpenoids have 20, and so on.
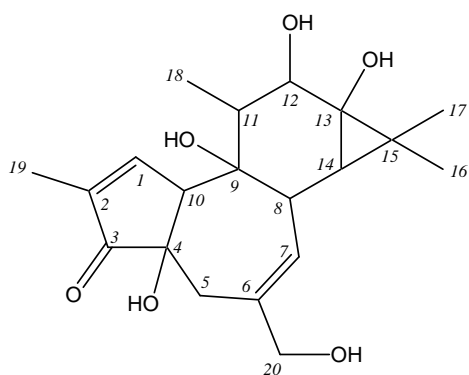
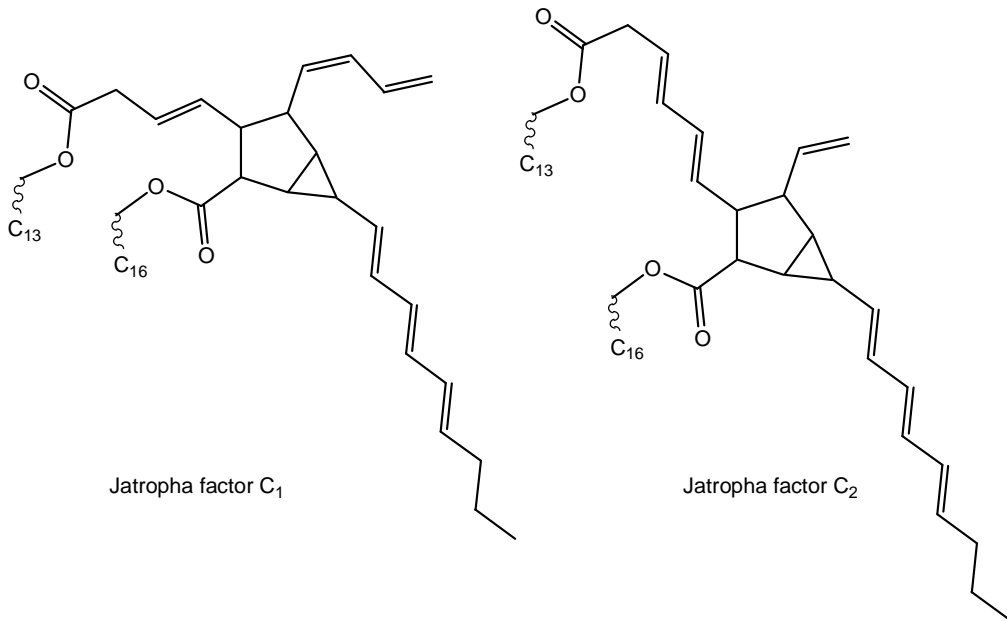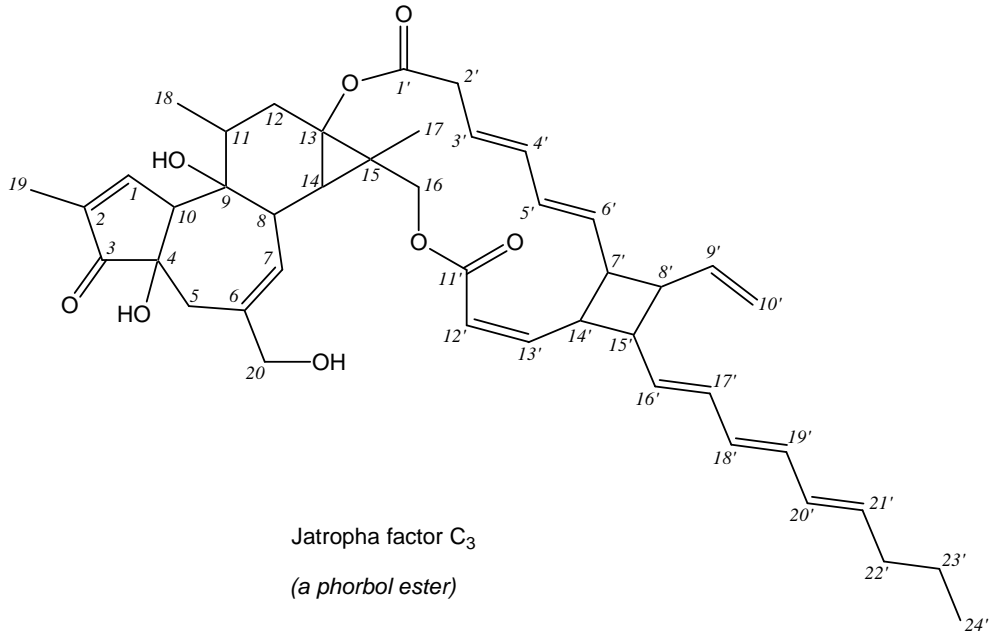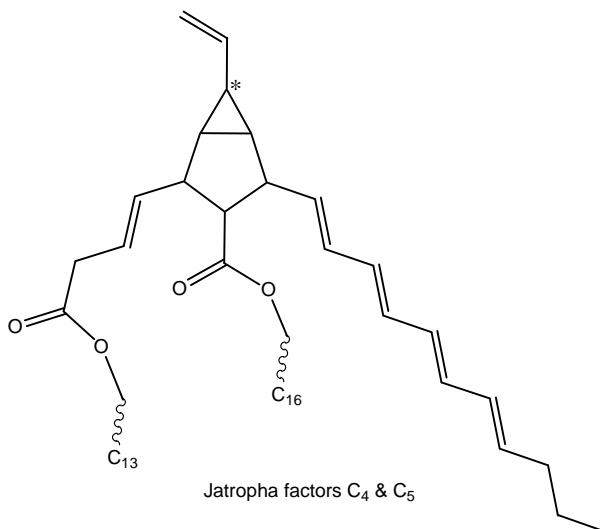phorbol
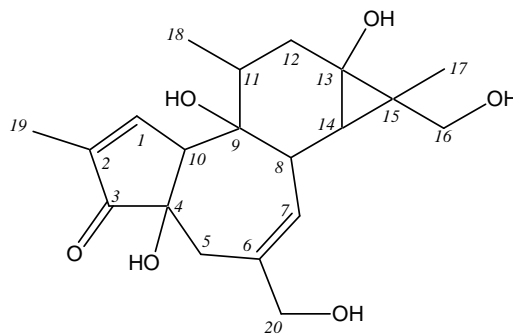
12-deoxy-16-hydroxyphorbol

tigliane

Phorbol itself was isolated as a non-toxic crystalline solid from the plant, *Croton tiglium L.* [28], but its ester, TPA (12-*O*-tetradecanoylphorbol-13-acetate), present in croton oil, is the most toxic and most studied phorbol ester, known to be a co-carcinogen [29,30]. However, phorbol esters from *J. curcas* are derivatives of 12-deoxy-16-hydroxyphorbol [31]. Phorbol esters with the same diterpenoid moiety have also been extracted from the latex of *Euphorbia cooperi* [32,33].

Adolf *et al.* [34] were the first to identify phorbol esters in *J. curcas.* Although they concluded that the irritant principles of the plant represented 12-deoxy-16-hydroxyphorbol-13-acylates with highly unsaturated acid moieties, they could not elucidate their exact chemical structures. Hirota *et al.* [35] identified the macrocyclic, dicarboxylic acid, diester structure of one of the phorbol esters and showed that it is a tumor promoter with weaker activity than TPA. Later, Haas *et al.* [31] isolated and did a thorough structural determination on all phorbol esters found in *J. curcas*.

Six phorbol esters have been characterized from *J. curcas* seed oil and designated as Jatropha factors C_1, C_2, C_3, epimers C_4 & C_5 and C_6, with the molecular formula $C_{44}H_{54}O_8$. All of them are intra-molecular diesters of the same diterpenoid. Jatropha factor C_6 was found to be the least stable. It has been suggested that the diester group biosynthetically arises from two originally separated monoacids [31]. The full structure of jatropha factor C_3 is shown below; the other esters are given without the 12-deoxy-16-hydroxyphorbol portion.

Jatropha factor C$_3$

(a phorbol ester)

Jatropha factor C$_1$

Jatropha factor C$_2$

Jatropha factors C$_4$ & C$_5$

(They are isomeric at C*; C$_4$ has S and C$_5$ has R configuration)

Jatropha factor C$_6$
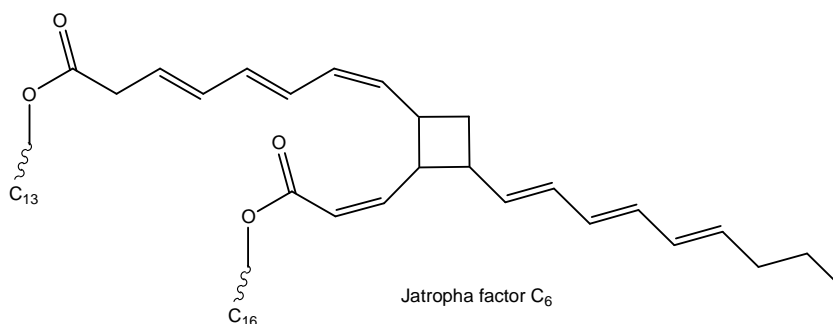
The content of phorbol esters in *J. curcas* can vary according to the region in which the plant grows. Ahmed and Salimon [36] have shown a variation in the phorbol ester content of the *J. curcas* seed oil from Malaysia (0.23%), Indonesia (1.58%) and India (0.58%). Saetae and Suntornsuk [37] demonstrated a variation in phorbol ester content in *J. curcas* seeds from different provinces of Thailand. However, Makkar et al. [7] had previously found similar levels of phorbol esters in seed kernels from Cape Verde (0.27%), Nicaragua (0.22%) and Nigeria (0.23%).

Although the phorbol esters are distributed in different parts of the *J. curcas* plant, they are concentrated in the seed kernel, of which 70% of them report to the oil and the remainder being found in the deoiled seed cake [8]. Hipal *et al*. [38] analyzed *J. curcas* seeds collected from various regions of Gujarat, India; the phorbol ester content in the oil had an average of 4.56%, whereas that of the seed cake was 1.21%. Studies carried out by Devappa *et al*. [39] showed that 86% of kernel phorbol esters are localized in the endosperm.

4. PHORBOL ESTER TOXICITY

Phorbol esters are the most potent tumor promoters known. They exhibit a remarkable ability to amplify the effect of a carcinogen but are themselves not carcinogenic [40]. Their promoter dose can be as low as 5 nanomoles [41]. The seeds from *J.curcas* have been

reported to be orally toxic to humans, rodents and ruminants and phorbol esters have been identified as the main toxic agent [42]. Pure phorbol esters can kill when administered in quantities of micrograms [1]. Symptoms of intoxication in humans are: burning and pain in mouth and throat, vomiting, delirium, muscle shock, decrease in visual capacity and high pulse [6]. In Nigeria and Burkina Faso, Jatropha seeds and seed oil are added to Strophanthus arrow poison, whereas in Gabon the seeds are grated with palm oil to kill rats [6]. The Shambaa of Usambara region of Tanzania used Jatropha seeds as an ordeal poison [43]. According to Adam and Magzoub [44], toxicity to goats was recorded, with doses of seed at 10g/kg per day having fatal consequences in two days. Post-mortem examination of the goats revealed hemorrhage in the rumen, reticulum, kidney, spleen and heart [44]. Ahmed and Adam [45] studied toxicity of *J. curcas* seeds on calves; they found that feeding the animals at doses of 0.25g/kg caused deaths within 19 hours. Some of the symptoms of toxicity were abdominal pain, salivation, diarrhea, sunken eyes and dehydration [45]. Common symptoms of topical application of isolated phorbol esters to rabbits, mice and rats are erythema, oedema, necrosis, diarrhea, scaling and thickening of skins [46]. Ingestion of phorbol esters (LD_{50} for mice being 27mg/kg body mass) can cause lung and kidney damage, resulting in fatality [47].

A summary of the effects of phorbol esters on different animals is given in Table 1 below.

Table 1. Toxicity of phorbol esters to different animals

Animal	Application	Symptoms
Humans	Ingestion	Burning and pain in mouth and throat, vomiting, delirium, muscle shock, high pulse, death [6]
Ruminants	Ingestion	Diarrhea, gastrointestinal inflammation, dehydration, salivation [45]
Rabbits and rodents	Topical	Erythema, oedema, necrosis, diarrhea, scaling and thickening of skin, tumor formation [46]
	Ingestion	Diarrhea, depression, loss of weight, death [48]
Goats	Ingestion	Lack of appetite, reduced water intake, diarrhea, dehydration and hemorrhagic effects in different organs [44]
Fish	Ingestion	Lower metabolic rate, increase in fecal mucus production, rejection of feed [42]

The toxicity of phorbol esters is mainly due to their action on biological cell membranes [23]. The phorbol esters are amphiphilic molecules and have a tendency to bind to phospholipid membrane receptors. The receptors are usually the primary targets of the phorbol esters [23]. The most investigated activity of the phorbol esters is their binding and activation of protein kinase C (PKC), an enzyme that plays a critical role in signal transduction pathways and also regulates cell growth and differentiation [49,50,51]. It has been proposed that the phorbol esters convert PKC into a constitutive active form that is irreversibly inserted into the membrane [49]. During normal signal transduction, the enzyme is activated by diacylglycerol, which is then rapidly hydrolyzed. Upon activation, PKC enzymes are translocated to the plasma membrane to conduct various other signal transduction pathways [52]. The phorbol esters act as analogues for diacylglycerol and are stronger PKC activators that are hardly metabolized by the cell [51]. They hyperactivate PKC and trigger cell proliferation, thus amplifying the efficacy of carcinogens [23].

Because phorbol esters activate PKC, they affect insulin binding by stimulating phosphorylation of insulin receptors on the protein [53].

The relationship between the chemical structure and toxicity of phorbol esters has received considerable attention from scientists, without conclusive results. For instance, the lack of toxicity of phorbol, as contrasted to its esters, both *in vivo* and *in vitro* had strongly indicated that adequate hydrophobicity was one of the requirements for activity [54]. Studies on effects of phorbol myristate acetate (PMA), also known as PTA, in mouse skin showed that its metabolite, phorbolol myristate acetate (PHMA) was nearly as effective a tumor promoter as PMA [55] – here, the carbonyl group in PMA is converted to a hydroxyl group to give PHMA. Other esters found to bind specifically to PKC are phorbol-12,13-didecanoate, phorbol-12,13-dibutyrate, phorbol-12,13-dibenzoate, phorbol-12,13-diacetate, phorbol-12,13,20-triacetate, phorbol-13-acetate [56] and phorbol-12-tetradecanoate [50]. However, phorbol-13,20-diacetate and 4-*O*-tetradecanoylphorbol-13-acetate were unable to bind to PKC; thus, were declared as non-promoters [57]. While phorbol-13-acetate was reported as effective, the closely related 12-deoxyphorbol-13-acetate exhibited inhibitory or antitumor properties [58].

It has been observed that shorter chain derivatives of phorbol are irritant but not promoting, these being two biological effects that could be considered as independent [59]. The presence of a C_{20} hydroxyl group has been seen to be important for the irritant and tumor promoting activities of phorbol esters. Introduction of an acetyl group in the 20-position gives rise to lower irritancy [60]. Therefore, it may be postulated that the spatial position of this function, as determined by their particular carbon skeleton and relative to specifically binding cellular structures, may play a delicate role in the hydrophilic interaction of the esters with membranes and receptors [41]. One way to weaken or abolish tumor promoting efficacy of a phorbol ester without impairing its mitogenic and irritant activities is to introduce double bonds into the long chain fatty acid residue at position 12 of the phorbol moiety [61].

Although many biological activities of phorbol esters are mediated through their direct interaction and activation of PKC, there are some phorbol esters that are active without being tumor promoters, such as ostodin and prostratin [62,63]. Studies by El-Mekkawy *et al.* [63,64] reported that 12-*O*-acetylphorbol-13-decanoate potently inhibits the cytopathic effects of human immunodeficiency virus (HIV) type 1 without appreciable activation of PKC.

Phorbol, the parent diterpenoid of phorbol esters, contains five hydroxyl groups with different reactivities towards acylation (Hecker, 1987). The three most widely used, biologically active phorbol esters, TPA (4β-12-O-tetradecanoylphorbol-13-acetate), PDBu (4β-phorbol-2,13-dibutyrate) and PMA (phorbol 12-myristate-13-acetate), differ only by their substitutions at positions 12 and 13 of the 6-membered ring. Their epimeric α-phorbol esters, which are often used as inactive controls, differ on the OH group at position 4, thus affecting the spatial arrangement of the cyclopentenone ring. [65]

Information about structure of PKC has been limited by its large size, which makes nuclear magnetic resonance (NMR) structural analysis quite difficult. It should be emphasized that the competitive binding to PKC, exhibited by diacylglycerol and the different phorbol esters, does not require that they possess similar recognition subunits. They could be interacting differently with functionality in the receptor domain. [40]

5. DETOXIFICATION

The *J. curcas* seed cake is a by-product of oil extraction from the seed. Although the seed cake has been found to be protein-rich, its utilization as animal feed is hindered by the presence of toxic phorbol esters and the antinutritional factors that include lectin (curcin), saponins, phytic acid, and trypsin inhibitor [6,12,66]. According to Makkar *et al.* [4], lectin and trypsin inhibitor can be deactivated by heat treatment, whereas phytic acid can be diminished by addition of phytase. However, phorbol esters must be removed from the seed cake to ensure complete detoxification. Several biological, chemical and physical detoxification methods have been suggested.

Makkar et al. [12] showed that roasting of seeds inactivates trypsin inhibitor and reduces lectin activity by 50%, on average. However, saponins, phytic acid and phorbol esters are not affected by roasting. In Mexico, Quintana Roo state, non-toxic *J. curcas* seeds are consumed by humans, after roasting. The consumption of raw seeds is known to produce cramps and uneasy feeling in the stomach [12]. It is not possible to destroy phorbol esters by heat treatment because they are heat stable and can withstand roasting temperature as high as 160ºC for 30 minutes [67].

Complete degradation of phorbol esters by stripping or deodorization of *J. curcas* oil at 260°C, with 3 mbar pressure and 1% steam injection has been reported by Makkar *et al.* [68].

El Diwani et al. [69] claimed a 75% removal of phorbol esters in *J. curcas* seed cake by treatment with an equal portion of 0.2M $NaHCO_3$, followed by addition of 50mg/l ozone for 3 minutes.

Devappa and Swamylingappa [70] reported total elimination of phorbol esters during preparation of protein isolates from *J. curcas* kernel meal. They suggested that the effectiveness of phorbol-ester removal could be due to the processing steps such as steam injection, washing and removal of oil in the whey.

Solvent extraction is among the most successful means of detoxifying *J. curcas* seed cake. Chivandi et al. [71] reported that a hexane/ethanol solvent system reduced phorbol ester content in the seed meal by 70%. Sequential extraction involving non-polar solvent followed by polar solvent has been recommended by Gaur et al. [72]. Approximately 80% of phorbol esters present in Jatropha oil were extracted using methanol, in a single extraction of 1:2 (w/v) oil-methanol ratio [73]. Aqueous ethanol (1:5 w/v) was reported by Makkar and Becker [16] to have removed 93% phorbol esters in Jatropha seed cake, after four extractions. Saetae and Suntornsuck [26] claimed that 90% ethanol was just as effective as 99.5% methanol for complete phorbol ester extraction from *J. curcas* seed cake. They preferred ethanol since it is safer for humans and other animals than methanol. Ethanol was also found to be efficient in removing lectin and reducing saponin, phytic acid and trypsin inhibitor in the seed cake [74]. Research done by Martínez-Herrera et al. [75] indicated that extraction with ethanol, followed by treatment with 0.07% $NaHCO_3$ decreased phorbol ester content by 98% in Jatropha seeds; there was also a considerable decrease in lectin activity. Organic solvents (alkaline methanol) were used by Makkar and Becker to reduce phorbol esters in *J. curcas* kernel meal to undetectable levels (< 5μg/g); their procedure was patented in 2010 [76].

Makkar and Becker [77] reported that rumen microbes cannot degrade phorbol esters and ruminants, like cattle, are as prone to phorbol ester toxicity as monogastric animals. Hydrolytic enzymes such as phorbol-diester hydrolase in mouse plasma [78] and liver carboxyesterase [79] have been reported to degrade phorbol esters.

Devappa et al. [80] have demonstrated that Jatropha phorbol esters are biodegradable in the soil, within nine days, at 42ºC and 230g/kg moisture. The degraded products are innocuous. Degradation rates of phorbol esters in the soil are influenced by temperature and moisture levels. The occurrence of storage fungi in *J. curcas* seeds points to the use of these microorganisms in detoxifying the seed meal. Different species of Aspergillus, Penicillium, Mucor and Rhizopus were isolated from stored Jatropha seeds [81]. Solid-state fermentation of *J. curcas* seed cake, using various microorganisms is a technique that has received attention from researchers. Here, the method constitutes a fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in the absence of free flowing liquid [82]. The same technique had been employed to eliminate or reduce antinutritional factors in other plants, such as saponin from whole soybean tempe by *Rhizopus oligosporus*, caffeine and tannins from coffee husk by *Aspergillus* sp. and gossypol from cotton seed by *Candida tropicalis* [83].

Belewu and Akande [84] studied the effect of *Aspergillus niger* and *Penicillium* sp. treated *J. curcas* kernel cake, as stock feed for West African dwarf goat. They concluded that fungi-treated seed meal can partially replace soybean cake for growing goat by as much as 50%. Other work by Belewu and Sam [85] showed that solid-state fermentation of *J. curcas* kernel cake with *Aspergillus niger,* for 7 days, achieved a 77% reduction in phorbol esters. Rosa *et al.* [86] reported that *Aspergillus niger* is not only a detoxifier but a nutritional enrichment additive. Joshi *et al.* [83] have claimed that phorbol esters can be completely degraded in 9 days by *Pseudomonas aeruginosa* PseA, under optimized solid-state fermentation of deoiled *J. curcas* seed cake. Studies by de Barros *et al.* [87] showed that incubation of *J. curcas* seed cake with the white-rot fungi, *Bjerkandera adusta* and *Phlebia rufa*, for 30 days, decreased phorbol esters by 91% and 97%, respectively. Phengnuam and Suntornsuck [88] performed submerged fermentation experiments of *J. curcas* seed cake with *Bacillus licheniformis (GRAS)*, in distilled water, for 5 days and observed a reduction in phorbol esters by 62%.

6. FUTURE RESEARCH

The role of chemists in finding the most appropriate detoxification method for the *J. curcas* seed cannot be overlooked. Research on structure-toxicity relationships and detoxification mechanisms of phorbol esters is important. Investigations on possible chemical detoxification methods are also useful.

6.1 Structure-Toxicity Relationship

The active sites that are responsible for phorbol ester toxicity are still unknown. Conflicting suggestions have been made about the polarity [41,54], conformation [40,65] and critical groups [41,60,61] being contributory for toxicity in these compounds. However, the universally agreed configuration and functional groups of phorbol esters that make them toxic have not yet been determined, considering that the Jatropha molecules are larger than those previously studied. More work is required to characterize the appropriate structure or particular groups that give rise to toxic properties of phorbol esters.

6.2 Detoxification Mechanisms

Biological methods of detoxification, where microorganisms are used, imply that reduction of phorbol esters occurs by degradation. The same applies to the physical detoxification methods, such as the deodorization process described by Makkar et al. [68] and chemical methods that involve digestion with reagents like those proposed by El Diwani et al. [69]. Despite their various degrees of success, such detoxification processes should be used to determine the mechanisms of degradation of phorbol esters. Solvent extraction techniques do not involve phorbol ester degradation.

6.3 Chemical Detoxification Methods

Detoxification of the *J. curcas* seed by chemical digestion can be quite fast and economically efficient. The major challenge, however, could be the generation of secondary toxins. Another option would be to identify and isolate an enzyme that quickly degrades the phorbol esters; this protein can then be produced in bulk.

7. CONCLUSION

The toxicity of *J. curcas* seeds emanates mainly from phorbol esters. Their structure-toxicity relationships have not been well studied. Biological, physical and some chemical detoxification methods of Jatropha seed have been proposed, but further research on detoxification mechanisms and chemical detoxifying methods is necessary.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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