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SCIENTIFIC OPINION

Risks for human and animal health related to the presence of phorbol esters in Jatropha kernel meal[1]

EFSA Panel on Contaminants in the Food Chain (CONTAM)[2,3]

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ABSTRACT

Following a request from the European Commission, the risks for human and animal health related to the presence of phorbol esters (PEs) in Jatropha kernel meal were assessed by the EFSA Panel of Contaminants in the Food Chain (CONTAM). *Jatrophacurcas* (Jatropha) seeds contain substantial amounts of extractable oil utilised for biodiesel production. The remaining protein-rich products (seed meal or kernel meal)may be used as a protein source in animal feed after removal of anti-nutritive factors and toxic PEs.The available dataonabsorption of Jatropha PEs afteroral ingestion, biotransformation, elimination, and dose-dependent toxic effects are very limited, and only for pigs a no observed adverse effect level (NOAEL) of 0.4 mg PEs/kg bw per day (12-*O*-tetradecanoylphorbol-13-acetate (TPA) equivalent),based on decreases in body weight gain and feed intake, could beidentified from short-term feeding studies. No health based guidance value for humans could be established.Processes that almost completely remove or degrade toxic PEs in Jatropha products are available, resulting in levels below the limit of detection of 3 mg Jatropha PEs/kg (TPA equivalent).Replacement of 50% of the protein in compound feedswith treated Jatropha materials would result in animal exposures that are still 10 to 200-fold lower than the NOAEL for pigs. The CONTAM Panel concluded that such use of Jatropha material would not pose a health risk to pigsandthat the risk to other species is likely to be low. The transfer of Jatropha PEs to animal derived products is unknown. In a human exposure scenario using a 50% transfer rate from feed to milk, a daily intake of 1 µg Jatropa PEs/kg bw per day was calculated. The CONTAM Panel concluded that more data are needed to draw firm conclusions on human risks.

KEY WORDS

Jatropha curcas, Jatropha kernel meal, seed cake, seed meal, protein isolate, protein replacement, phorbol esters, Jatropha factors

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SUMMARY

Jatropha curcas (Jatropha) is a member of the Euphorbiaceae family. It originated in Central America but is now widely grown in many tropical and sub-tropical countries, predominantly as a source of seed oil that is increasingly usedfor biodiesel production. Following oil extraction from the seeds, the remaining cakes or meals have a high protein content (approximately 60–65% in the case of kernel meal), making them potentially valuable as an animal feed ingredient.Untreated Jatropha kernel meal contains, however, toxic phorbol esters (PEs)in concentrations varying between 600 and3,700 mg/kg fresh weight (FW)and also anti-nutritional substances, making it– and products derived from it– unsuitable for use as a feed ingredient. Non-toxic genotypes of Jatrophahave been identified, but their distribution is restricted to limited regions in Central America and they are not used for oil extraction for biodiesel production or as a feed material.

Becauseof their well-documented toxicity, Jatropha seeds are currently listed as a harmful botanical impurity in the Annex to Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed.

The increasing availability of by-products from Jatropha oil production, their high protein content and, hence, their potential use as a feed material, has stimulated the development of various methods of extraction or degradation of PEsin Jatropha products. This resulted in the mandate to the Panel on Contaminants in the Food Chain (CONTAM Panel) to assess the toxicity of PEs, the effectiveness of the detoxification processes and the safety of the detoxified Jatropha kernel meal when used as a protein source in animal diets. In this context, the CONTAM Panel has not identified any previous exposure or risk assessments on Jatropha kernel meal in Europe or elsewhere.

Toxic PEs are diesters of the pentahydroxylated tetracyclic diterpene tigliane with saturated or unsaturated fatty acids. PEs fromJatropha comprise a group of at least six compounds (denoted Jatropha factors C1 to C6), with similar but not identical chemical structures as the commonly known PEs from croton oil, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

Analytical procedures to measure Jatropha PEs have been developed. Following extraction with methanol,separation of Jatropha PEs can best be achieved by high-performance liquid chromatography (HPLC) on reverse phase columns. Ultraviolet (UV) absorbance at 280 nm and tandem mass spectrometry (MS/MS) after electrospray ionization (ESI) in positive or negative mode are used for detection and quantification. Up to now no fully validated analytical procedures are available, which is explained by the lack of commercial availability of reference standards.As yet, analytical results are expressed as equivalents of TPA, with a detection limit of 0.4–0.8 mg PEs (TPA equivalent)/kg feed for HPLC-UV and 0.07 mg PEs (TPA equivalent)/kg feed for liquid chromatography-mass spectrometry (LC/MS).

Concerning the mode of action, Jatropha PEs, which show a high degree of similarity to other PEs including TPA, act at the cellular level as potent inducers of protein kinase C, due to their structural similarity with the endogenous second messenger diacylglycerol. Protein kinases are involved in various signal transduction pathways of many neurotransmitters and hormones, as well as in the regulation of the cell cycle and apoptosis.

For a toxicological assessment of the potential human and animal health risks associated with the oral exposure with food and feed to Jatropha PEs only a very limited database is available. For example, the toxicokinetics of the six known Jatropha PEs have not been studied to date and even their oral bioavailability remains unknown. *In vivo* and *in vitro* studies with TPA, which has a similar chemical structure as Jatropha PEs, show that hydrolysis of the ester groups constitutes the major if not sole metabolic route as demonstrated in various rodent tissues. When the rates of metabolic hydrolysis of analogues of TPA with different saturated acyl groups were compared, a clear influence of the structure and position of the acyl groups was noted. Although cytochrome P450-mediated metabolism appears not to occur with TPA, it cannot be ruled out entirely for the Jatropha PEs, dueto the structural

differences. In the absence of toxicokinetic data in target animal species, including a lack of data on the oral availability, the potential transfer of Jatropha PEs into animal derived products is unknown.

TPA has been recognised as a tumour promoter in a mouse skin bioassay and in the mouse forestomach as well as in *in vitro* cell proliferation assays. However, there was no evidence for tumour-initiating properties of TPA. Similarly to TPA, Jatropha PEs act as tumour promoters in mice skin. As Jatropha PEs are similar but not identical to TPA, a read-across analysis following the Organisation for Economic Co-operation and Development (OECD) guidance documents was conducted, whichsuggested similar, but also additional structural alerts, relevant to genotoxicity when compared to TPA.This analysis identified potential differences in the biotransformation and bioactivation of Jatropha factors.However, these hypothetical alerts have not been tested in any experimental investigations.

The toxicity of Jatropha plant products (seeds and leaves)has been documented in experimental and farm animals afteroral application. Symptoms resulting from the (forced) ingestion of non-treated Jatropha seeds or kernel meal include reduction in feed intake and reduced weight gain, erosions of the mucosal membranes and haemorrhage in the gastro-intestinal tract,diarrhoea, anaemia, acute necrotic lesions in the liver and proximal renal tubule cells, and congestions in cardiac blood vessels and death. Fish, and particularly carp, also appear to be sensitive to Jatropha PEs. The threshold at which carp exhibited adverse effects (reduction in growth rate and anorexia) has been estimated to be 15 mg PEs/kg feed. No studies on horses or companion animals could be identified.For untreated Jatropha products, the available data do not allow the establishment of no-observed-adverse-effect-levels (NOAELs) or lowest-observed-adverse-effect-levels (LOAELs) for individual animal species.

Intoxications in humans have been described as a result of accidental ingestion of Jatrophaseeds, particularly by children. Clinical symptoms include burning and pain in the mouth and the upper digestive tract. Following ingestion of larger amounts, a shock-like syndrome with increased pulse rate and neurological symptoms, including delirium and loss of vision, has been observed. However, the immediate and strong vomiting that usually follows ingestion makes most intoxications self-limiting.

Considering the toxicity of Jatropha PEs, Jatropha kernel meal, seed cake, seed meal and protein isolates have been subjected to various physical (e.g. heat), chemical (alkaline hydrolysis and solvent extraction) and biological (enzymatic degradation by microorganisms) treatments with the aim of reducing concentrations of PEs. From initial concentrations of Jatropha PEs of 50–6,070 mg/kgdry matter (DM) in expeller cake and 600–3,700 mg/kg FW in kernel meal, a number of treatment processeshave been reported to substantially reduce(up to 99%) the level of PEs in the treated Jatropha materials. However,all these data refer to analytical values expressed as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) equivalents, as currently no standards for Jatropha esters are commercially available. Moreover, the nature of the degradation products has not been identified, and many of the described processing methodsare not supported by analytical data or animal feeding studies to confirm the efficacy of the processes.

From a short-term feeding study in pigs, in which45% of the feed protein was replaced by treated Jatropha kernel meal,a NOAEL of 0.4 mg PEs (TPA equivalent)/kg body weight (bw) per day was identified, based on decreases in feed intake and body weight gain. Rainbow trout, carp and shrimp tolerated feed in which 50% of the protein was replaced with treated Jatropha kernel meal containing a non-quantified concentration of PEs which was below 3 mg PEs/kg. Due to the limitations of the available studies, no NOAEL could be identified for ruminants, horses, poultry species, aquatic species and companion animals. For ruminants, there is no evidence that rumen microorganisms degrade PEs, and therefore there is no reason to consider these species as less sensitive than monogastric animals to dietary exposure to PEs from Jatropha products.

Assuming a residual PE concentration in treated Jatropha material of 3 mg/kg (the analytical limit of detection for the reference compound TPA in most currently available experimental studies on detoxification), and a 50% replacement of the ‘conventional’ vegetable or animal proteins in

compound or complementary feed for livestock species, fish and companion animals with Jatropha kernel meal protein, exposure estimates ranged from 0.002 mg PEs/kg bw for ruminants (fattening beef cattle on a forage based diet) to 0.04 mg PEs/kg bwfor rabbits.Considering the identified NOAEL of 0.4 mg PE (TPA equivalent)/kg bw per day in pigs(based on decreases in body weight gain and feed intake), and the estimated exposure of up to 0.026 mg PEs/kg bw per day in pigs, the CONTAM Panel concluded that replacing 50% of feed protein with treated Jatropha material with ≤3 mg PEs/kg DM would not pose a health risks to pigs.Ruminants may be at least as sensitive as monogastric animal species. However, under the condition that Jatropha products replace up to 50% of the feed proteins, the CONTAM Panel considers that a 10-fold lower exposure to Jatropha PEs than the NOAEL in pigs would be associated with a low risk for adverse effects also in other farm animals (including farmed aquatic species) or companion animals. The CONTAM Panel noted that for all species, the estimated exposure is 10–200-fold lower than the NOAEL in pigs, indicating that the risk to other species is also likely to be low under these conditions.

The CONTAM Panel was unable to establish a health based guidance value for humans due to lack of toxicological information on Jatropha PEs.Exposure to humans from Jatropha products could only occur from residues of Jatropha PEs in animal derived products, originating from animals given treated Jatropha kernel meal. However, the transfer of Jatropha PEs to animal derived products is unknown. Using a conservative scenario, the CONTAM Panel estimated a daily intake of about 1 µg PEs/kg bw from milk, assuming that 50% of Jatropha PEs and its metabolites are transferred to milk from cows fed with Jatropha material. The margin of exposure (MOE) between the human daily intake and the NOAEL of 0.4 mg PEs (TPA equivalent)/kg bw per day identified in pigs, is about 400. Due to the limitations of the study in pigs from which the NOAEL was identified, and the ability of PEs to activate PKC, as well as the structural alerts for genotoxicity, this MOE is not sufficient to conclude that human health risk is low. Therefore, no firm conclusions can be drawn on human health risks in the absence of sufficient data on toxicity and transfer from feed to animal derived foods.

The CONTAM Panel therefore concluded that the uncertainties associated with the assessment of Jatropha products are substantial, due to the lack of qualifying studies.

The CONTAM Panel recommendsthe production of standards for individual Jatropha PEs (Jatropha factors) and the validation of the analytical methods for the control of the presence of toxic Jatropha factors in feed materials. The availability of reference materials/standards would also allow studies onthe tolerance of detoxified Jatropha kernel meal in all animal species, and on the possible transfer of Jatropha PEs into edible animal tissues, milk and eggs.

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1. Introduction

1.1. Background and Terms of Reference as provided the European Commission

1.1.1. Background

*Jatropha curcas*is a tree belonging to the *Euphorbiaceae* family. It originated in Central America, but is now found in many tropical and sub-tropical countries in Africa and Asia. The de-shelled[4] seeds contain 55–60% oil. For many years the oil was used predominantly in the manufacture of soaps and candles, but more recently Jatropha oil has become of significant economic importance as a result of its potential as a source of biodiesel.

Jatropha seedcake contains toxins, making it unsuitable for animal feed, with phorbol esters being the major class of toxins.[5] Jatropha seedcake also contains amounts of anti-nutritional constituents (trypsin inhibitors, lectins and phytate).*J. curcas*is therefore listed as a harmful botanical impurity in the Annex to Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed.[6] Seeds and fruit of *J. curcas* as well as their processed derivatives may only be present in feed in trace amounts not quantitatively determinable.

Nevertheless, the kernel meal obtained after oil extraction is an excellent source of nutrients and contains 60–66% crude protein. Jatropha protein isolate obtained from Jatropha seed cake (residue obtained after mechanical pressing of the whole seeds) has about 81–85% crude protein. The contents of essential amino acids (EAAs) (except lysine) are higher in Jatropha kernel meal than in soyabean meal (SBM), and higher in Jatropha protein isolate than soy protein isolate.

Detoxification processes have been demonstrated to reduce the presence of phorbol esters in Jatropha kernel meal by more than 95%. In addition, the anti-nutritional constituents have been shown to be inactivated or significantly reduced by the detoxification process. Therefore the detoxified Jatropha kernel meal could be possibly suitable as feed material. If so the listing as a harmful botanical impurity in the Annex to Directive 2002/32/EC would no longer be needed for the detoxified *J. curcas* kernel meal and might eventually be replaced by a maximum level on phorbol esters, providing also a high level of animal health and public health protection.

Another *Jatropha* species, *J. platyphylla*, is free of phorbol esters. However, its seed kernels and kernel meal still contain the anti-nutritional constituents trypsin inhibitors, lectins and phytate.

Therefore, it is appropriate for EFSA to assess the toxicity of phorbol esters, the effectiveness of the detoxification process and the safety of the detoxified Jatropha kernel meal.

1.1.2. Terms of referenceas provided by the European Commission

In accordance with Art. 29 (1) (a) of Regulation (EC) No 178/2002 the Commission asks EFSA for a scientific opinion on the risks for animal and human health related to the presence of phorbol esters in Jatropha kernel meal used in feed.

The scientific opinion should, *inter alia*, comprise the:

a) evaluation of the toxic exposure levels (daily exposure) of phorbol esters for the different animal species of relevance (taking into account differences in sensitivity between animal species), above which

   - signs of toxicity can be observed (animal health/impact on animal health)

[4] The terms ‘de-shelling’ or ‘dehulling’ are used to describe the same process.
[5] Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on ricin (from *Ricinus communis*) as undesirable substances in animal feed.*The EFSA Journal* (2008) 726, 1-38.
[6] OJ L 140, 30.5.2002, p. 10.

- transfer/carry over of phorbol esters from the feed results in unacceptable levels of phorbol esters and/or their toxic metabolites in the products of animal origin, in view of providing a high level of public health protection.

b) evaluation of the effectiveness of the detoxification processes to reduce the level of phorbol esters to safe levels and to inactivate or reduce the presence of anti-nutritional constituents.

c) evaluation of the safety for animal and public health of the detoxified Jatropha kernel meal.

1.2. Interpretation of the Terms of Reference

One of the main focuses of the mandate is the effectiveness of the detoxification processes used to reduce the presence of phorbol esters. With regards to the anti-nutritional constituents, these will only be addressed generally, and given particular reference if detoxification processes result in their decrease. The different substances used in the detoxification processes will not be evaluated and environmental risks will not be addressed.

Considering the use of *J. curcas* as a potential animal feed, not only the kernel meal but also the seed cake and protein isolate will be considered.

1.3. Additional information

1.3.1. Previous assessments

No previous risk assessments on *J. curcas* phorbol esters in animal feed materials could be identified.

1.3.2. Legislation

J. curcas seeds are listed as a harmful botanical impurity in the Annex to Directive 2002/32/EC on undesirable substances in animal feed. Seeds and fruits and their processed derivatives may only be present in feed in trace amounts not quantitatively determinable.

1.3.3. Physical characteristics of plants, seeds and seed fractions

The genus Jatropha, found within the Euphorbiaceae family, is a large family of flowering plants with 321 genera and around 7,550 species (Devappa et al., 2010a). Members of the *Jatropha* genus are succulent plants, shrubs or trees where *Jatropha curcas* is the most commonly available species. The name *J. curcas* is derived from the Greek word 'iatros' (doctor) and 'trophe' (food), which refers to its traditional use as a medicinal plant (Sharma et al., 2012). The most widely used common names in English are Physic nut and Purging nut, the latter indicating the strong purgative effect following the oral intake of this plant (Heller, 1996). It grows in tropical or subtropical regions around the world and is cultivated in South and Central America, SouthEast Asia, India and Africa (Gübitz et al., 1999). The plant is well adapted to dry and semiarid conditions and it has been planted to prevent soil erosion, but more importantly it is used as a living fence since it is not grazed by cattle and wildlife. Despite the diversity of the subgenera of Jatropha and curcas species, *J. curcas* remains the most prevalent and most cultivated species.In this opinion, the term 'Jatropha' refers to '*J. curcas*' unless otherwise specified.

The size of the Jatropha plant under normal circumstances is between 3 and 5 metres in height, but can under favourable conditions become up to 10 metres high (Kumar and Sharma, 2008). Jatrophais a monoecious species and its flowers are unisexual. Insects pollinate the flowers, and after pollination a green fruit is formed.

Each fruitusuallycontains three ellipsoidal seeds, which are about 2 cm long and have a blackish thin shell around a whitish kernel (see Figure 1). Seed weights ranging from 0.69 to 0.86 g have been reported for various toxic genotypes of Jatropha(Aderibigbe et al.,1997; Liberalino et al., 1988).The kernel to shell ratio is about 63:37 (Aderibigbe et al., 1997).

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Figure 1: Jatrophaseeds

Other uses for this plant include traditional medicines (seed, leaves, bark), soap production (seed oil) and fuel (wood, oil).

The seed kernels contain a large percentage of oil (55–60%), and there has been an increasing interest in the use of Jatropha oil as a source of bioenergy in the form of biodiesel. It is possible to grow this crop in areas unsuitable for food production and to produce CO_2 neutral fuel at a low cost. A co-product after seed oil extraction is a seed cake or kernel meal with high protein content. Furthermore, the protein has a high proportion of EAAs making it potentially useful as a feed for livestock. However, the raw seed cake or kernel meal should not be fed to animals without first being detoxified due to the presence of toxic and anti-nutritive substances.

The major toxic constituents are phorbol esters (abbreviated to PEs in this opinion). Although concentrations are highest in the seeds, PEsare also found in the leaves, stems and flowers (Devappa et al., 2011a). Incidental intoxications following the ingestion of Jatropha seeds by children have been reported, but comprehensive records about human toxicity have not been identified. In large-scale production units for Jatropha oil, the potential occupational exposure remains of concern, as the native oil contains substantial amounts of PEs, which act as skin irritants and potential tumour promoters (Pelletier et al., 2015).

The use of Jatropha plant products in animal nutrition is also limited by a number of anti-nutritional substances, notably phytates, trypsin inhibitors and lectins, including curcin (Makkar et al., 2012). Lectin and trypsin inhibitors can be neutralised by heat treatment. Phytate can be inactivated by adding phytase to feed to mitigate its adverse effects.For the removal or inactivation of PEs, a variety of methods have been developed in an attempt to detoxify the protein-rich seed cake and kernel meal. The validation of such processes by means of chemical analysis of the residual amounts of PEs and/or by feeding experiments in target animal species varies considerably.Therefore in this Opinion the term ‘treated’material is used in the description of such processes, while the term ‘detoxified material’ is reserved for methods that have been validated by chemical analyses and feeding experiments.

For detoxification, the first step is either de-shelling of seeds to yield the kernels, or mechanical pressing of seeds to yield ‘seed cake’ and oil (Figure 2). Seed cake has almost 50% shells and therefore high fibre and lignin contents, which make it a poor livestock feed. In some studies, shells have been physically removed from Jatropha seed cake using a sieve to obtain a ‘seed meal’. Also ‘protein isolates’ have been prepared from seed cake by dissolving protein at high pH followed by precipitation at low pH. Oil from kernels can be obtained by mechanical pressing and/or by solvent extraction. Pressing of kernels yields ‘kernel cake’, whereas solvent extraction leads to ‘kernel meal’,

which can also be obtained by solvent extraction of the kernel cake (Figure 2). Kernel cake and kernel meal are free of shells, low in fibre, and after complete detoxification could be a potential feed.

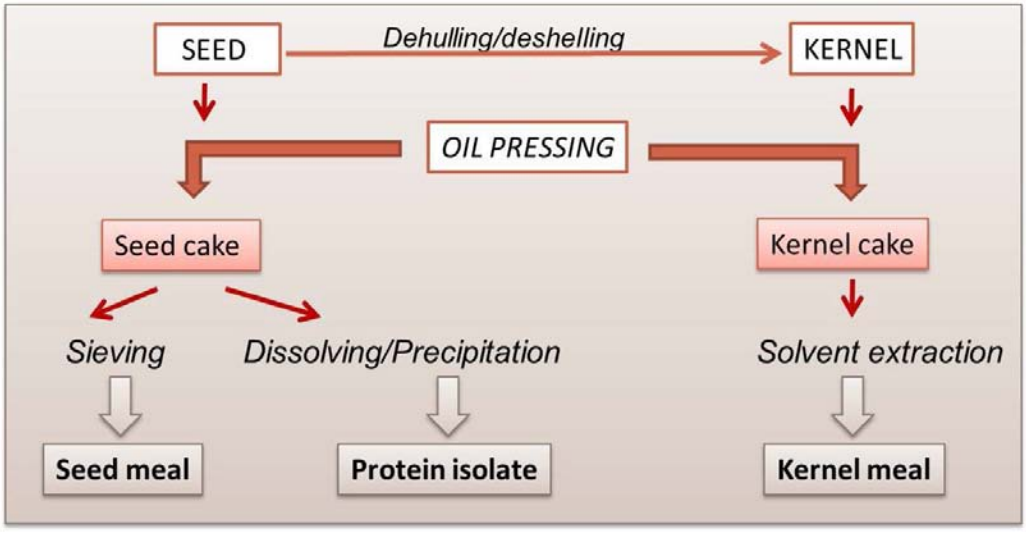


Figure 2: Different products obtained from Jatrophaseeds by using various processes

As described above, the commonly available Jatrophais toxic, but there is also a non-toxic genotype which originates from Mexico, where its seeds are even used for human consumption after roasting (Makkar et al., 1998a,b). This non-toxic Jatropha genotypelooks similar to the toxic one but does not produce PEs. Kernel meal from the non-toxic genotype has been successfully used in feeding trials with fish and rats and could be considered as a suitable animal feed ingredient (Makkar et al., 2012). One study investigated the short-term toxicity of seed oil and seed meal from a non-toxic genotype of Jatropha(grown in the Veracruz region of Mexico) and found no indications for toxicity when a diet containing up to 14% of this material was fed to Wistar rats for 5 weeks (Panigrahi et al., 1984).However, the non-toxic genotype of Jatropha has a very limited distribution even in Mexico and the toxic genotype is most prevalent at a global level and mainly used for oil extraction and biodiesel production (Maghuly et al., 2015). Because of its very limited distribution and availability of feed by-products derived from it, feed materials derived from the non-toxic genotype are not included in this assessment.

1.3.4. Chemistry

The Jatropha PEs, also called Jatropha factors, have similar but not identical chemical structures to the more commonly known PEs from croton oil, which have been widely studied as tumour promoters. Both classes of PEs are diesters of pentahydroxylated tigliane, which is a tetracyclic diterpene with the systematic name (1aS,1bR,3S,4aS,6R,7aR,7bR,8R,9aR)-1,1,3,6,8-pentamethyltetradecahydro-1H-cyclopropa[3,4]benzo[1,2-e]azulene ($C_{20}H_{34}$, CAS number 67707-87-3), carrying an additional keto group at C-3. However, whereas PEs from croton oil are derived from phorbol ($C_{20}H_{32}O_6$, with the hydroxyl groups at C-4β, 9α, 12 β, 13α and 20, Figure 3), Jatropha factors are derived from the isomeric 12-deoxy-16-hydroxy-phorbol (Figure 3). The major PE from croton oil is 12-*O*-tetradecanoylphorbol-13-acetate (TPA, CAS number 16561-29-8). TPA does not occur in Jatropha, but isgenerally used as a reference compound in the analysis of Jatropha materials because no authentic reference compounds are commercially available for Jatropha PEs.

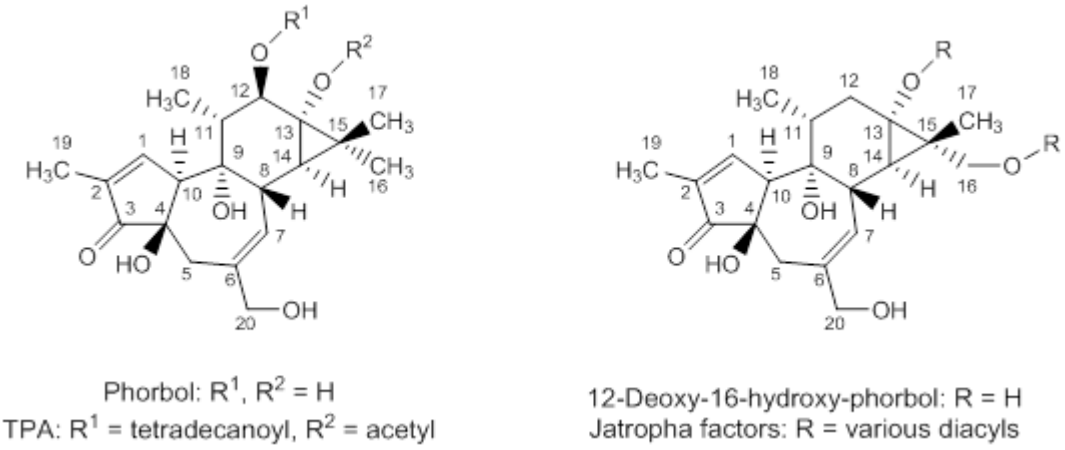
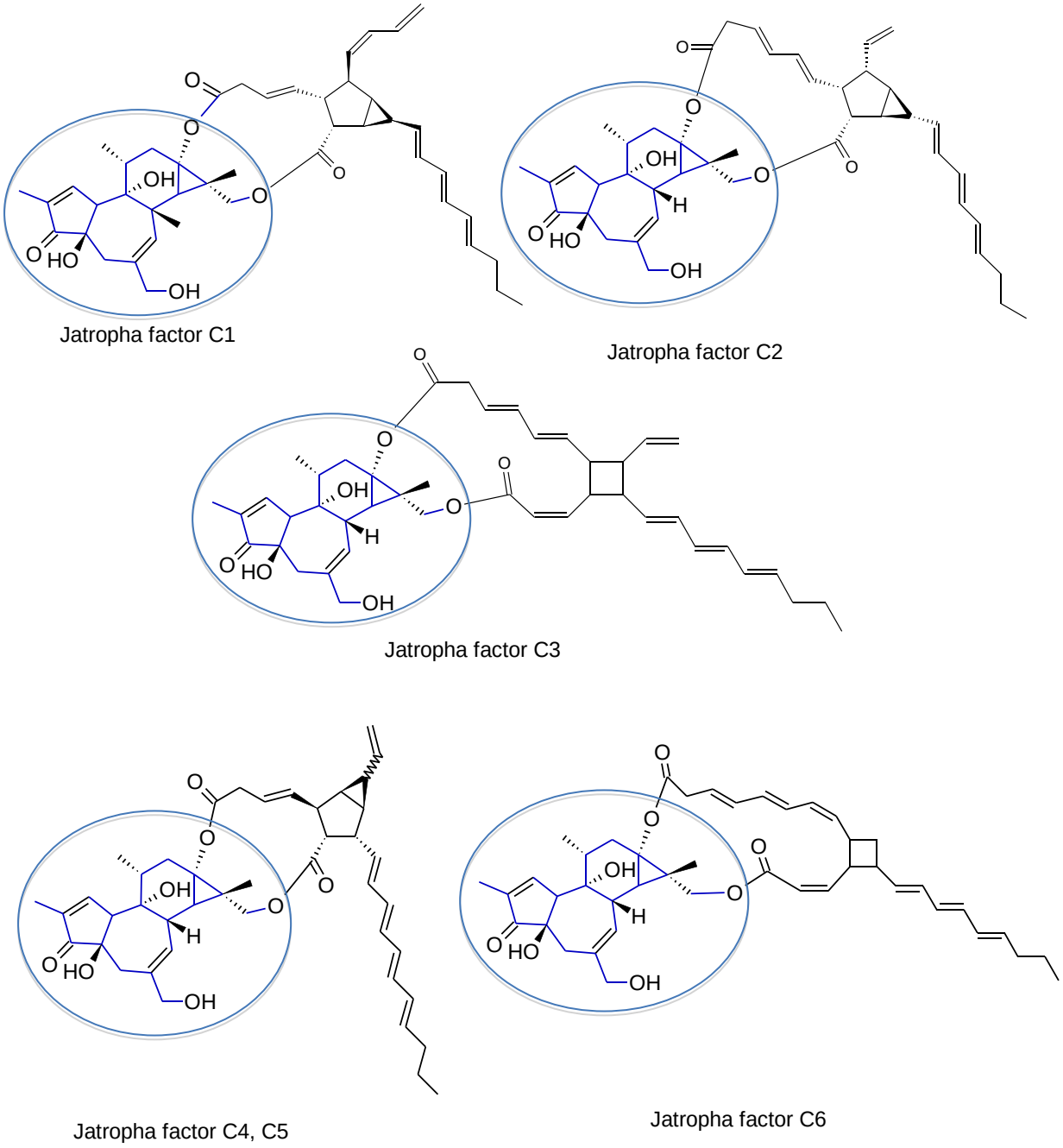


Figure 3: Phorbol esters, from croton oil frome.g. TPA (left), and from Jatropha(right)

Esters of phorbol and 12-deoxy-16-hydroxyphorbol are constituents of certain plant families. Their biological activity depends on the stereochemistry of the hydroxyl group at C-4, which strongly affects the overall conformation of these compounds (Driedger and Blumberg, 1980; Goel et al., 2007; Devappa et al., 2011b). Jatropha PEs are characterised by a 4ß-hydroxyl group and are biologically active. They constitute a group of at least six compounds, commonly referred to as Jatropha factors C1 to C6 (Haas et al., 2002; Goel et al., 2007; Hua et al., 2015), and differ in the ester functions at positions 13 and 16 (Figure 4). In contrast to the PEs from croton oil, which carry separate acyl groups at the two ester functions (e.g. tetradecanoyl and acetyl in TPA), Jatropha factors are cyclic diesters of complex dicarboxylic acids containing bicyclo[3.1.0]hexane (factors C1, C2, C4, C5) or cyclobutane (factors C3 and C6) moieties. The most abundant derivative is Jatropha factor C1 (Roach et al., 2012). Factors C4 and C5 are in general isolated as mixture of epimers differing in the C-8' configuration (Haas et al., 2002; Goel et al., 2007).



The diterpene moiety common to all analogues is circled.

Figure 4: Structures of the ester groups of Jatropha factors C1–C6

Jatropha PEs are considered moderately polar compounds, having affinity for solvents such as dichloromethane (Makkar et al., 1997), methanol or ethanol (Martínez-Herrera et al., 2006; Devappa et al., 2010b). Jatropha PEs are also well soluble in oil. The type of solvent has a profound impact on the chemical stability of PEs (see below).

1.3.5. Methods of analysis

Several methods of analysis have been proposed for the analysis of PEs in Jatropha oils or cakes. Some simple methods have been reported for screening; however, methods with sufficient sensitivity such as high-performance liquid chromatography with diode array detection (HPLC-DAD) and more recently high-performance liquid chromatography with mass spectrometry (HPLC-MS) are required

which allow for the analysis of trace amounts of these toxic compound even in treated feed materials. Because Jatropha PEs are not commercially available, TPA is generally used as a reference compound for the quantitative determination of Jatropha PEs (Makkar et al., 1998a; Liu et al., 2013; Devappa et al., 2011a, 2013a,b). Recently Hua et al. (2015) reported the use of ultra performance liquid chromatography – mass spectrometry (UPLC-MS) method for the analysis of PE-rich crude extracts showing the presence of more than 15 different compounds with similar mass spectrum, being homologues to known Jatropha factors.

1.3.5.1. Stability of Jatropha PEs

PEs are chemically unstable and prone to photodegradation, isomerisation, oxidation and hydrolysis (Schmidt and Hecker, 1975; Dimitrijevic et al., 1996; Vogg et al., 1999; Haas et al., 2002; Goel et al., 2007; Roach et al., 2012; Devappa et al., 2013b). These features make their isolation in purified form challenging (Haas et al., 2002). To date, the degradation products of Jatropha PEs have not been identified. Devappa et al. (2013b) studied the stability of pure Jatropha PEs, showing that the main degradation pathway is related to auto-oxidation and suggested the need for low temperature storage of such compounds. PE instability also needs to be considered during analytical procedures, calling for gentle extraction and separations methods (Vogg et al., 1999). Jatropha PEs in fractions containing oil and methanol are in general more stable than the pure compounds (Devappa et al., 2010b; Roach et al., 2012; Devappa et al., 2013b). Storage at low temperatures further reduces the degradation of PEs. A dimethyl sulfoxide (DMSO) solution of TPA from croton oil (Figure 3) has been reported to be stable for 6 months when stored in the dark at −20°C, but it decomposed slowly in the dark at 4°C during 3 months, and extensively at 25°C when stored for 3 months in diffused daylight (Schmidt and Hecker, 1975). Due to their instability, the storage of Jatropha extracts and purified Jatropha PEs is recommended in methanol or ethanol, in the dark, and preferably at −20°C or even lower temperatures (Roach et al., 2012). Addition of antioxidants could increase stability (Roach et al., 2012; Devappa et al., 2013b).

1.3.5.2. Extraction of Jatropha PEs

The instability of Jatropha PEs due to oxidation, heat, hydrolysis and light requires gentle extraction conditions (Vogg et al., 1999). PEs are moderately polar compounds, and their extraction can be achieved using different solvents. Makkar et al. (1997, 1998a, 2009) extracted Jatropha PEs from seeds using dichloromethane. More recently, a mixture of methanol and tetrahydrofuran (99/1, v/v) was used for PE extraction from Jatropha kernel meal or defatted kernel (Devappa et al., 2011a). Soxhlet methods using methanol as solvent are suitable for PE quantification except for oil samples (Devappa et al., 2013a,b). The same authors evaluated different solvent mixtures and extraction procedures, employing magnetic stirrer or ultraturrax apparatus (Devappa et al., 2010b).Methanol is considered the solvent of choice, and it can be used for performing liquid-liquid partition of PEs from Jatropha oil as well as extraction of PEs from Jatropha seeds, tissues or other biological samples. Extraction can also be performed at low temperature in an ultrasonic bath (Baldini et al., 2014). In general, in a container, oil, kernel meal, ground seeds or seed cake can be placed in a volume of methanol approximately 5-fold compared to the material mass, and the container placed in an ultrasonic bath maintained at room temperature or at low temperature. The methanol layer is then separated from the oil and concentrated under reduced pressure or under a flow of nitrogen at temperatures below 40°C to a desired volume (Roach et al., 2012; Devappa et al., 2013a,b; Baldini et al., 2014).

1.3.5.3. Analysis

Screening methods

Simple qualitative approaches use thin layer chromatography (TLC) or spectrophotometry measuring absorbance at 280 nm of a methanol extract of kernel after passing through a solid phase extraction (SPE) cartridge. These qualitative methods were proposed for the rapid screening of toxic or non-toxic Jatropha samples (Devappa et al., 2011a).

Quantitative analysis of PEs in Jatropha samples

As reported by several authors (Dimitrijevic et al., 1996; Makkar et al., 2009; Devappa et al., 2013a, Baldini et al., 2014), HPLC coupled with a UV detector (HPLC-UV), λ max 280 nm, is a well-established method to detect and quantify the PEs contents in Jatropha seeds and related products (kernel meal, seed cake and oil). In general, separations can be achieved on reverse phase (RP) columns (C-18) using different mobile phases and gradient elutions (Makkar et al., 1997, 1998a; Vogg et al., 1999; Ichihashi et al., 2011; Roach et al., 2012; Devappa et al., 2013a;Liu et al., 2013; Baldini et al., 2014).

HPLC methods using UV detection at 280 nm have been widely used for measuring Jatropha PEs and allow the compounds determination also in low concentrations (mg/kg); however, the limitsof detection (LOD) and of quantification (LOQ) have not been reported in most publications. Devappa et al. (2013a) described improved HPLC methods (on 50 mm column) for C1 determination (as TPA equivalents) with LOD of 50 ng while LOQ was 125 ng (injecting 50µL), translating to LOD of 0.4–0.8 mg/kg and LOQ of 1.0–2.0 mg/kg (Devappa et al., 2013a; Baldini et al., 2014).

As mentioned earlier, the Jatropha factors C1 to C6 are not commercially available as references or standard compounds for analytical purposes. Therefore, TPA (Figure 3) has been commonly used as a reference compound due to its commercial availability and structural similarity to Jatropha PEs. Roach et al. (2012) and Devappa et al. (2013a) compared the quantitative results obtained using TPA or Jatropha factor C1 as reference compounds and reported that the ratio of TPA to factor C1 (at 280 nm) was in the range 40.5–42.7. The use of DAD detectors allowed the recording of Jatropha factors UV spectra (Devappa et al., 2013a,b). Compared to HPLC-UV or HPLC-DAD, the HPLC-tandem MS based methods (Vogg et al., 1999; Ichihashi et al., 2011; Liu et al., 2013; Baldini et al., 2014) are more sensitive and specific.

Among the available methods, the HPLC-MS method of Baldini et al. (2014) has the highest sensitivity (LOD of 0.07 mg/kg; LOQ of 0.21 mg/kg). Any of these methods (DAD- or MS-based), using TPA as a standard, are useful for evaluating the degree of detoxification of Jatropha products. They can also be applied to measure PEs in biological fluids and tissues.

Bioassays

In the absence of certified reference materials for individual Jatropha factors, biological tests may provide an estimate of difference in the toxicity of individual substances, and the effect of detoxification methods.For Jatropha PEs bioassays using snails, crustaceous or isolated cells have also been reported (Devappa et al., 2012).

For example, Roach et al. (2012) observed differences in the biological activities of Jatropha factors in various bioassays (snails, Artemia and platelet aggregation bioassays). Authors evaluated Jatropha factors C1 (purified to homogeneity), factor C2 (purified to homogeneity), factor C3 mixture (majority factor C3 and negligible amount of factor 4), and factors (C4+C5) mixtures. However, ratio of impurity to purified Jatropha factors was considered to be minute and taken as it is for further studies. In snail bioassay, the order of potency based on EC_{50} (µg/mL, equivalent to Jatropha factor C1) was: factor C3 mixture (6.78) > factor C2 (6.54) > factor C1 (4.12) > factors (C4+C5) mixture (2.18). In Artemia bioassay, the order of potency based on EC_{50} (mg/kg, equivalent to Jatropha factor C1) was: factor C2 (11.8) > factor C3mixture (1.08) > factor C1 (0.43) > factors (C4+C5) mixture (0.043). In platelet aggregation assay, the order of potency was compared between Jatropha factors and commonly used TPA. The order of potency based on the ED_{50} (µM, factor C1 equivalent) for Jatropha factors was: factor C2 (0.19) > factor C3 mixture (0.15) > factor C1 (0.11) > factors (C4+C5) mixture (0.04). In comparison, the TPA induced platelet aggregation at 0.5 µM concentration with an ED_{50} of 0.012 µM (factor C1 equivalent) (Devappa, 2012; Roach et al., 2012).

2. Data and methodologies

2.1. Data

2.1.1. Current occurrence data

Jatrophaseeds are listed as a harmful botanical impurity in the Annex to Directive 2002/32/EC on undesirable substances in animal feed. Seeds and fruits and their processed derivatives may only be present in feed in trace amounts not quantitatively determinable. Therefore,no data could be identified from the EU Member States.

2.1.2. Toxicokinetic and toxicological data

All data were identified as described in Section 2.2.3.1.

2.2. Methodologies

2.2.1. Collection and appraisal of previous occurrence results

A comprehensive literature search was conducted in September–October 2014 and has since been updated in April 2015 focusing on research and reports related to occurrence of PEs in Jatrophamaterial. The references obtained were screened using title and abstract to identify the relevant literature. All information retrieved has been reviewed and used for the present assessment using expert judgement.

2.2.2. Exposure assessment

2.2.2.1. Animal exposure assessment

Exposure to PEs by livestock is a function of the concentration of PEs in Jatropha kernel meal, and the amount of the meal consumed.Currently, the seeds of Jatropha, together with their processed derivatives, may only be present in feed materials and compound feeds for livestock and companion animals in the EU in amounts that are not quantitatively determinable. Since it is not possible to estimate exposure to Jatropha PEs based on current occurrence data, potential future exposure has been estimated where 50% of the protein provided in compound feeds or complementary feeds is replaced by protein from treated Jatropha kernel meal in diets that might be indicative of those fed to livestock in the EU. In the absence of a comprehensive database on the amount or type of feeds consumed by livestock in the EU, estimates of feed consumed for each of the main categories of farm livestock and companion animals are based on published guidelines on nutrition and feeding (e.g. AFRC, 1993; Carabano and Piquer, 1998; NRC, 2006, 2007a,b; Leeson and Summers, 2008; EFSA Scientific Committee, 2009; McDonald et al., 2011), and data on EU manufacture of compound feeds (FEFAC, 2009), together with expert knowledge of production systems in Europe. Details of the intakes and composition of diets used in estimating animal exposure to PEs are given in Appendix C.

2.2.3. Hazard assessment

2.2.3.1. Strategy for literature search

For the present evaluation the CONTAM Panel considered literature made publicly available until April2015. A comprehensive search for literature was conducted for peer-reviewed original research and reviews, pertaining to Jatropha PEsadverse health effects on animals and humans. The search strategy was designed to identify scientific literature dealing with chemistry, analysis, detoxification treatments, exposure, toxicokinetics, toxicity, and mode of action. Additionally, theses and patents were considered.

The literature search was not restricted to publications in English language; however, literature in other languages was only considered if an English abstract was available. A first literature search was performed in September–October 2014 and has since been updated in November 2014, December

2014, January 2015, March 2015 and April 2015. Web of Science[7] and Pubmed[8] were identified as databases appropriate for retrieving literature for the present evaluation.

2.2.3.2. Appraisal of studies

Information retrieved has been reviewed by the CONTAM Panel working group on PEs in Jatropha kernel meal and used for the present assessment using expert judgement. The information assessed included human data on accidental ingestions of Jatropha kernels and all available data on animal studies with various Jatropha products (treated and untreated materials). Any limitations of the information used are clearly documented in this opinion.

2.2.4. Methodology applied for risk assessment

The CONTAM Panel applied the general principles of the risk assessment process for chemicals in food as described by WHO/IPCS (2009), which include hazard identification and characterisation, exposure assessment and risk characterisation. Additionally to the principles described by WHO/ICPS (2009), EFSA guidance pertaining to risk assessment (EFSA Scientific Committee, 2012) has been applied for the present assessment. In brief, the EFSA guidance documents cover the procedures currently used within EFSA for the assessment of dietary exposure to different chemical substances and the uncertainties arising from such assessments (EFSA Scientific Committee, 2006). For details on the specific EFSA guidance applied see Appendix A.

3. Assessment

3.1. Occurrence of phorbol esters in untreated Jatropha seeds and seed fractions

As mentioned above, no occurrence data of PEs in seeds and seed fractions are available from Europe, as Jatropha is not commercially cultivated in Europe and its use as feed material is not currently permitted. Studies from non-EU countries have involved mainly the toxic genotypes of Jatropha.Jatrophais cultivated in almost all tropical and subtropical countries and seeds from 18 different countries (West and East Africa, North and Central America, and Asia) were investigated by Makkar et al. (1997). PEs were not detected in the one sample from Mexico containing seeds of the non-toxic genotype (Kingsbury, 1964; Dias et al., 2012). Levels of PEs in the remaining 17 samples ranged from 870 to 3,302 mg/kgof kernel (see Table 1).

Liu et al. (2013) investigated PE derivatives in Chinese Jatrophaseeds by HPLC-MS from six geographic locations in southern China. Oil was extracted using ethanol, and total PE contents ranged from 1,100 to 2,420 mg/kgfresh weight (FW), with large regional differences in the concentrations of the six Jatropha factors.

Pasha et al. (2013) also examined the presence of PEs in Jatropha seeds, seed cakes, and oil collected in India from different regions. The oil was physically extracted, by screwpressing, in contrast to solvent extraction used in the study reported above. The average JatrophaPE content in whole seeds was 7,700 mg/kg FW. In contrast to other study reported here, the average PE concentrations in Jatrophaseed cake following oil extraction (4,240 mg/kg FW) was higher than in the oil (2,900 mg/kg FW), which probably reflects the method of oil extraction used, resulting in higher levels of residual oil in the seed cake, although levels of the oil content are not given.

In order to study the distribution of toxic and non-toxic genotypes within Mexico, Martínez-Herrera et al. (2006) collected seed kernels of Jatrophafrom four regions. While no Jatropha PEs were detected in kernel meal from three of the four regions, Jatropha PEs were present in high concentrations in the

[7] Web of Science (WoS), formally ISI Web of Knowledge, Thomson Reuters. Available online: http://thomsonreuters.com/thomson-reuters-web-of-science/

[8] PubMed, Entrez Global Query Cross-Database Search System, National Center for Biotechnology Information (NCBI), National Library of Medicine (NLM), Department of the National Institutes of Health (NIH), United States Department of Health and Human Services. Available online: http://www.ncbi.nlm.nih.gov/pubmed/

kernels from one region (Coatzacoalcos) at up to 3,850 mg/kgdry matter (DM) in kernel meal with an average of 1,640 mg/kg DM in five samples. This data confirm previous reports of a non-toxic genotype in Mexico,which is restricted to certain areas (Kingsbury, 1964; Dias et al., 2012).

Pradhan et al. (2011) obtained whole Jatropha seeds, which were dehulled in order to separate the kernel and shells. Oil was extracted from the kernels by either mechanically pressing the whole seeds (seed cake) or by using petroleum ether (solvent extracted kernel meal). In this study, the Jatropha PE content was higher in the solvent extracted meal (1,100 mg/kg FW) than in the seed cake (800 mg/kg FW). Furthermore, the level of Jatropha PEs in the solvent extracted oil was higher (2,800mg/kg FW) than in expeller oil (2,100mg/kg FW). The authors noted that esters are heat sensitive and are degraded at high temperature, and since heat is generated during the expelling process this may degrade the Jatropha PEsand account for the lower levels in expeller oil and cake.

In another study involving Jatrophaof Indian origin, seeds were collected from Chattishgarh and oil was extracted from the kernels using petroleum ether. Most (82%) of the Jatropha PEs were extracted in the oil fraction, while the Jatropha PE content in the meal was 600 mg/kg FW (Prasad et al., 2012).

Chikpah and Demuyakor (2013) analysed seeds of Jatrophaobtained from four agro-environmental regions of Ghana. The seeds were processed into either kernel meal (by solvent extraction) or seed cake (mechanically defatted) from each region. Jatropha PE levels were 2,600–3,700 mg/kg FW for the kernel meal and 4,870–6,070 mg/kg FW for the seed cake. Again, these data suggest that the Jatropha PE levels are reduced as more of the oil is removed.

From a study designed to examine oil extraction and detoxification methods of Jatropha seed meal, Nokkaew and Punsuvon (2015) reported Jatropha PE contents in oil and 'de-oiled' meal of 3,070 and 65.5 mg/kg FW,respectively, where oil was extracted using hexane. Subsequent treatment of the 'de-oiled' meal with ethanol resulted in a Jatropha PE concentration of 122.8 mg/kg.

In pressed seed cake obtained from India, a Jatropha PE content of 460 mg/kg DM was reported. However, following oil extraction by petroleum ether, a lower JatrophaPE concentration (240 mg/kg DM) was observed (Makkar et al., 2008).

Saetae and Sunornsuk (2010) examined the PE content in Jatropha seed cake produced from four provinces in Thailand. The oil was extracted using a screw press, and levels of Jatropha PEs in the resulting seed cake, analysed by HPLC, ranged from 50 to 140 mg/kg FW. It should be noted that these levels are markedly lower than those observed by other authors, although in a subsequent study by the same authors, levels of Jatropha PEs of 730 mg/kg DM were reported (Saetae and Sunornsuk, 2011).

Table 1 provides a summary of the studies described above with respect to the different products, processes and levels of Jatropha PEs.

Table 1: Summary of reports of PEs in Jatrophawhole seed and seed fractions after oil extraction

Reference	**Origin of samples**	**Material**	**Process**	**Mean PE content (mg/kg FW unless otherwise stated)**(±SD where reported)
Makkar et al. (1997)	18 countries[(a)]	Whole seed	-	870–3,320
Liu et al. (2013)	Southern China	Whole seed		1,100–2,420
Pasha et al. (2013)	India	Whole seed	-	7,700(±200)
Martínez-Herrera et al. (2006)	Mexico	Kernel meal	Defatted (by solvent)	1,640 (DM)[(b)]

Reference	Origin of samples	Material	Process	Mean PE content (mg/kg FW unless otherwise stated)(±SD where reported)
Pradhan et al. (2011)(c)	India	Kernel meal	Solvent extraction	1,100
Prasad et al. (2012)	India	Kernel meal	Solvent extraction	600
Chikpah and Demuyakor (2013)	Ghana	Kernel meal	Solvent extracted	2,600–3,700
Nokkaew and Punsuvon (2015)	Thailand	Kernel meal	Solvent extraction (hexane) followed by treatment with ethanol	3,070
Makkar et al. (2008)	India	Seed cake	Expeller ('pressed cake') Solvent extraction	460 (±20) (DM) 240 (±20) (DM)
Saetae and Sunornsuk (2010)	Thailand	Seed cake	Expeller	50–140
Saetae and Sunornsuk (2011)	Thailand	Seed cake	Expeller	730 (± 60) (DM)
Pradhan et al. (2011)(c)	India	Seed cake	Expeller ('pressed cake')	800
Pasha et al. (2013)	India	Seed cake	-	4,240
Chikpah and Demuyakor (2013)	Ghana	Seed cake	Expeller	4,870–6,070

DM: dry matter; FW: fresh weight; PE: Phorbol ester.
(a): West and East Africa, North and Central America, and Asia. Jatropha PEs were not detected in all seeds from Mexico.
(b): Only for toxic seeds; not detected in non-toxic seeds.
(c): There were more Jatropha PEs in the oil following solvent extraction (2,800mg/kg) compared to that of expeller oil (2,100mg/kg).

Gámez-Meza et al. (2012) investigated the PE content in kernels of other toxic *Jatropha* species, such as *J. cordata* and *J. cardiophylla* seeds from Mexico. Concentrations varied between 2,730 and 1,460 mg/kg, respectively. These results indicate that other *Jatropha* species are also able to synthesise PEs, but these species are of minor economic importance.

3.2. Hazard identification and characterisation

In the absence of toxicokinetic and toxicodynamic studies on individual Jatropha PEs, the well-known phorbol ester TPA has been used as a surrogate for hazard identification. TPA has a diterpene moiety, phorbol very similar to the 12-deoxy-16-hydroxyphorbol moiety of Jatropha PEs but differs in the long-chain fatty acid part of the molecule (Figures 3 and 4). Both Jatropha PEs and TPA activate protein kinase C (PKC), a common mode of action. TPA is the major PE of croton oil but is not present among the PEs of Jatropha.

3.2.1. Mode of action

Jatropha seeds and products thereof contain numerous biologically active substances, of which the group of PEs is considered to be the most toxic. As described in Section 1.3.4. (Chemistry), PEs found in Jatropha comprise a diverse group of esters called Jatropha factors. Common toxic effects described in various animal species following the ingestion of non-treated Jatropha seeds containing these Jatropha factors resulted in severe irritation of the entire intestinal tract followed by extensive haemorrhages in the intestines and congestions in other organs such as kidneys, liver and lungs, focal necroses in the liver and heart. The actual toxic principle, however, has not been clearly defined, but as cooking of seeds (which would destroy the heat-labile enzymes in Jatropha) only marginally reduced the toxicity in rodents (Liberalino et al., 1988), it can be assumed that most of these lesions originate from Jatropha PEs. PEs have both hydrophilic and hydrophobic domains and may disrupt

cellular membranes by direct interaction with membrane phospholipids (Li et al., 2010), which could explain the mucosal lesions in the gastro-intestinal tract.

3.2.1.1. Activation of protein kinase C by phorbol esters

The mechanism of toxicity of Jatropha PEs has not been studied in detail, as Jatropha factors have only recently been purified and are not commercially available. However, Jatropha PEs, like TPA, activate PKC *in vitro*and *in vivo*(Oskoueian et al., 2012a,b; León-López et al., 2015) (see Section 3.2.1.2 for details)). Therefore, activation of PKC by TPA is used as a reference in the present section. It needs to be reiterated, however, that TPA is not present in Jatropha seeds (and products thereof) and that the Jatropha PEs are derivatives of 12-deoxy-16-hydroxyphorbol, whereas the structure of TPA, found generally in croton oil, is derived from phorbol (see Figure 3). Moreover, the acyl groups of TPA and Jatropha PEs are different. Considering the substantial differences between various esters of phorbol, differences in the potency of the Jatropha esters are likely.

TPA is a well-known activator ofPKC, a multigene enzyme family of related serine/threonine kinases that occurs virtually in every cell. PKCs are involved in general signal transducing pathways for proliferation, differentiation, and metabolism, and have also more cell type-specific functions. Individual isoforms have specific phosphorylation targets, and individual isoforms show cell- or tissue-specific expression. In early publications it has been described that PKC activation is measurable for at least the following PEs: phorbol-12,13-didecanonate, phorbol-12,13-dibutyrate, phorbol-12,13-dibenzoate, phorbol-12,13-diacetate, phorbol-12,13,20-triacetate, phorbol-13-acetate, and phorbol-12-tetradecanoate, whereas phorbol-13,20-diacetate and 4-*O*-tetradecanoylphorbol-13-acetate are apparently unable to bind to PKC, and were also declared as non-tumour promoters (Yuspa et al., 1976; Dunphy et al., 1980; Kikkawa et al., 1983).

The ability of TPA (and other PEs) to activate PKC is associated with the structural similarity of TPA with the endogenous second messenger diacylglycerol (DAG) that activates PKC (Garg et al., 2014; Steinberg, 2015). DAG is a key second messenger formed after activation of phospholipase C by several G-protein-dependent receptors which are activated by binding of ligands to extracellular membrane receptors.

PKC enzymes are divided into subclasses based on their structural features in their regulatory domains and their role in cellular responses (originally identified by Nishizuka, 1995, and recently reviewed by Steinberg, 2015). The conventional PKC isoforms (cPKCs; α, βI/βII, and γ) contain two discrete membrane-targeting modules harbouring binding sites for DAG and Ca^{++} which are responsible for their activation by DAG and calcium.

Novel PKCs(nPKCs, δ, θ ε, and η) are activated by DAG, in a calcium independent way, as they lack calcium binding sites. Some of the isoenzymes in this group have different domains that facilitate various protein-protein interactions (Benes et al., 2005).

The earliest experiments with TPA were conducted in neuronal cells, in which DAG is a key second messenger in the signal transduction of adrenergic, m-cholinergic and the central amino acid-regulated receptors. Experimental activation of PKC by different PEs in neuroblastoma, glioblastoma and other neuronal cells has been used as tool to study the individual functions of neurotransmitters (for recent reviews see Rosse et al., 2010; Ludeman et al., 2015; Thangsunan et al., 2015).

PKCβ plays an important role in the activation of immune cells and is essential for the development and maturation of B-1 lymphocytes and their immunoglobulin production. The mitogenic effects of TPA on B-lymphocytes are even used as a diagnostic tool in the monitoring of chronic leukaemias. Activation of immunoreceptors by antigens results in PKCβ activation, which in turn, for example in T-lymphocytes, activates the NFκB pathways and initiates the expression of cytokines as mediators in inflammation.

PKCβ is also expressed in pancreatic islet cells (together with other PKC isoforms) and plays a crucial role in the (*myc*-dependent) regulation of the transcription of the insulin gene and hence potentially in the development and severity diabetes. PKCβ is also involved in the cellular processes associated with the secondary signs of diabetes such as retinopathy and diabetic nephropathy (Kawakami et al., 2002).

Moreover, PKCs are involved in cellular oxidative stress.Cells generatereactive oxygen species (ROS) in response to a variety of conditions, including exposure to toxic agents and inflammatory stimuli. Oxidative stress and cellular growth factor receptors activate different pathways that result in an activation of PKCs. There is limited evidence that free radicals (including ROS) can directly oxidise membrane phospholipids and disrupt cell membranes; the observed phospholipase C (PLC)-dependent cleavage of phospholipid hydroperoxides seems to be associated with the formation of a DAG hydroperoxide which acts as a potent stimulator of PKC in inflammatory neutrophils (Kambayashi et al., 2007) contributing to the overall clinical signs of inflammation after tissue injury.

In many cases it remains to be elucidated if the changes in PKC expression observed under certain disease conditions are the cause or just a symptom within the pathogenesis (Garg et al., 2014).

These examples of the regulatory functions of the PKC enzyme family may illustrate that many of the clinical symptoms associated with the ingestions of Jatropha PEs, such as membrane damage and irritation of the mucosa of the intestinal tract, and haemorrhages as well as changes in lymphocyte population (see mitogenic effects on different lymphocyte subsets), necrotic organ lesions (see ROS pathways) and even the effect on glucose levels (which may be associated to diarrhoea but also to modulated insulin production) can be linked to known PKC-dependent effects.

3.2.1.2. Activation of PKC by Jatropha Phorbol Esters

Oskoueian et al. (2012a) treated human hepatocytes (Chang cell line) and African green monkey kidney cells (Vero cell line) with concentrations of 50, 100, 150 and 200 mg/L of isolated Jatropha PEs (PE1, PE2, PE3 and PE4 representing the PEs present in Jatrophameal) or with TPA that served as positive control. Exposure to PEs resulted in a 50% cell proliferation inhibition, at concentrations of 125.9 mg/L and 110.3 mg/L, in Chang and Vero cells respectively (corresponding concentrations were similar with TPA and were 124.5 mg/L and 106.3 mg/L, respectively). Microscopic evaluation of cells incubated at these concentrations for 24 h, revealed cell damage suggestive of apoptosis in both cell lines. These findings were corroborated by observations of increased numbers of apoptotic cells and DNA fragmentation seen upon Jatropha PE and PMA treatment in both cell lines and were paralleled by increased expression of protein kinase – δ (PKCδ) and activation of caspase-3 proteins in Jatropha PE and TPA treated cells. Based on their results the authors conclude that toxicity of Jatropha PEs seen in the study is caused by apoptotic cell death mediated by induction of over-expression of PKCδ and activation of caspase-3 proteins.

In a further investigation by the same authors (Oskoueian et al., 2012b), following a very similar study design, breast cancer (MCF-7) and cervical cancer cells (HeLa) were treated with PEs and TPA as a positive control at the same dose levels as in the previous experiment. Isolated Jatropha PEs and TPA inhibited proliferation of both MCF-7 and HeLa cells with similar effectivity,resulted in microscopic changes suggestive of apoptosis, increases in apoptotic cells and DNA fragmentation in both cell lines and led to down-regulation of proto-oncogenes (c-Myc, c-Jun, c-Fos) and over-expression of PKCδ and activation of caspase-3 proteins in both cell lines. The authors concluded that both TPA and isolated Jatropha PEs behaved similarily with regard to down-regulation of proto-oncogens, activation of Caspase-3 proteins and induction of apoptosis.

León-López et al. (2015) reported increases in serum glucose, insulin, triglycerides and cholesterol levels, in rats fed diets containing 20% Jatropha protein concentrate (possible Jatropha PE presence was confirmed, although concentration was not reported) compared to control rats receiving casein or soy protein. Western blot analysis of liver samples from rats fed with Jatropha protein concentrate revealedhigher protein expression levels in relation to various pathways including Akt, the mTOR

pathway,SREBP1 and LXRα. Furthermore, PKCα protein expression in the liver of rats fed Jatropha protein concentrate was increasedcompared to the control. There were no differences in PKCδ expression between the treated and control groups. The study also demonstrated the activation of the transcription factors AP1 and NF-kB (known targets of PKC) by liver nuclear extracts from rats fed with Jatropha protein concentrate.

3.2.2. Toxicokinetics

No studies on the toxicokinetics, i.e. on absorption, distribution, metabolism or excretion, could be identified for Jatropha PEs. This is probably due to the fact that these compounds are not commercially available and have only been isolated in small amounts in few laboratories. In the absence of data on Jatropha PEs, a summary of the kinetic data on TPA is given below.

3.2.2.1. Laboratory animals

No *in vivo* studies on the absorption, metabolism,distribution, and excretion of TPA after oral administration have been identified. The lack of such data is probably due to the fact that TPA is a tumour promoter (see Section 3.2.1 for further details) predominantly for the skin, which has focused the interest on the fate of TPA in the skin.

The biotransformation studies with TPA are briefly summarised here but are described in more detail in Appendix B. In essence, these studies have shown that the major pathway in the metabolism of TPA is the hydrolysis of the two ester groups, and that in the rodent skin model all hydrolytic products lack tumourpromoting activity, the major toxicological effect of TPA. The metabolic hydrolysis requires the activity of esterases,the activity of which differs between tissues and species.

Kreibich et al. (1971, 1974) were the first to disclose that both ester groups of TPA can be hydrolysed in mouse skin and in cultured cells, giving rise to the monoesters 12-tetradecanoylphorbol and phorbol-13-acetate, as well as the product of complete hydrolysis, i.e. phorbol. Reduction of the keto group at C-3 was identified as a further metabolic pathway in mouse skin by Segal et al. (1975). Berry et al. (1978) confirmed the hydrolysis of the ester groups of TPA as the major metabolic route in mouse skin and also in mouse liver microsomes. Noteworthy, no other metabolites were detected in the microsomal incubations, suggesting that cytochrome 450-mediated oxidative metabolism is not involved in TPA metabolism. Ester group hydrolysis was also the only metabolic reaction observed in various cultured cells (O'Brien and Diamond 1978a). In the same study, the hydrolysis of TPA paralleled the loss of activity for induction of ornithine decarboxylase (ODC). As ODC is a marker for tumour promotion, these findings suggest that all three hydrolytic metabolites of TPA (the two monoesters and phorbol) are devoid of tumour promoting activity. Marked differences in the rate of hydrolysis of TPA and a structural analogue, phorbol-12,13-didecanoate (PDD) were observed between cultured fibroblasts from various animal species, suggesting that the hydrolytic metabolism of phorbol diesters depends on the cell type and on the chemical structure of the diester (O'Brien and Saladik, 1980).

In 1981, Shoyab et al. reported the isolation of an esterase capable of hydrolysing TPA-like phorbol esters from mouse liver cytosol, and disclosed that this enzyme was lacking in mouse skin but was highly expressed in the skin of several other species, e.g. hamsters, not sensitive to the tumour promoting activity of TPA. However, Barrett et al. (1982) showed that TPA was not hydrolysed in hamster skin *in vivo*. Esterases capable of hydrolysing TPA were also isolated from the serum of mice, rats, guinea pigs, rabbits and goats (Lachey and Cabot, 1983; Saito and Egawa, 1984) and rat liver endoplasmic reticulum (Mentlein, 1986).

The ability of mouse liver microsomes to hydrolyse TPA as shown by Berry et al. (1978) was confirmed by Müller et al. (1990). Hydrolysis was also observed for nine TPA-like compounds, i.e. esters of phorbol with different fatty acids, although the rate of hydrolysis differed considerably. Like in the study of Berry et al. (1978), no products other than those resulting from hydrolysis were observed, again suggesting that oxidative metabolism, e.g. hydroxylation, did not occur.

In 1991, Roeser et al. studied the metabolism of radiolabeled TPA in the back skin of mice *in vivo*. In addition to hydrolytic metabolites, several novel lipophilic metabolites were detected and identified as TPA esterified with long chain fatty acids at the C-20 hydroxyl group. These TPA-20-acylates appeared to be devoid of tumourpromoting activity but were partly hydrolysed back to TPA in mouse skin (Roeser et al., 1991).

In extrapolating from these studies with TPA to the metabolism of Jatropha PEs, hydrolysis of the ester groups at C13 and C16, as well as esterification of the hydroxyl group at C20 may be expected as potential pathways. However, these metabolic reactions depend on the nature and position of the acyl groups, as well as on the structure of the diterpene moiety. Moreover, unlike TPA, Jatropha PEs, have highly unsaturated acyl groups which may be prone to cytochrome P450-mediated metabolism. In an *in silico* simulation of the metabolism of TPA and Jatropha PEs by rat liver post-mitochondrial supernatant ('S9') using the OECD Toolbox (see Appendix D), almost three times as many hypothetical metabolites where found for each of the Jatropha PEs C1 to C5 (34–35 metabolites) as for TPA (13 metabolites), and many of the metabolites of Jatropha PEs were epoxides of the unsaturated acyl groups. However, the metabolism of Jatropha PEs needs to be verified by experimental studies.

For a full description of the studies see Appendix B.

3.2.2.2. Humans

No data on the toxicokinetics of Jatropha PEs and TPA in humans after oral ingestion have been identified. Some studies were identified in which patients with haematological or tissue malignancies were treated intravenously (slow infusion) with TPA. The initial results of a formal phase I clinical trial in the US were reported by Strair et al. (2002) and the final results by Schaar et al. (2006). In this clinical study, in the absence of an analytical method with appropriate sensitivity, blood TPA levels were measured with a biological assay,expressed as TPA-like activity (sensitivity about 0.1 ng TPA/mL). The biological assay, as described in Cui et al. (2002), involved the determination ofethyl acetate-extractable differentiating activity of TPA in blood, by measuring formation of adherent HL-60 (Human promyelocytic leukemia) cells.In the first part of the study 14 patients of either sex were treated with a single TPA infusion (1 h duration) at dosages of 0.063 or 0.125 mg/m^2 (corresponding to approximately 0.11 and 0.22 mg TPA/person). In some patients, the treatment was repeated 7 days later. TPA-like activity in blood was detected in all patients at the end of the administration (range 0.31–5.3 ng/mL), and in eight patients 2 hours later (up to 3.6 ng/mL), with an average TPA-like activity of 0.47±0.26 ng/mL calculated from 13 infusions in six patients. A terminal half-life of 11 ± 3.9 hours was calculated (from five infusions in four patients) (Strair et al., 2002). Schaar et al. (2006) described the completion of the phase I clinical study, in which 35 patients of either sex underwent TPA treatment at dosages of 0.063, 0.125 or 0.188 mg TPA/m^2 (corresponding to approximately 0.11, 0.22 or 0.33 mg TPA/person). TPA-like activity was measured in blood before dosing, at the end of the infusion and at 1 and 3 h post-infusion. Patients receiving the highest dosage had blood measurements at 1, 2, 5, and 11 h after the end of the infusion. At the end of the infusion, levels of TPA equivalents (mean ± SD) were 1.09 ± 0.24, 1.66 ± 0.20, and 4.93 ± 1.06 ng/mL in patients receiving 0.063, 0.125 or 0.188 mg TPA/m^2, respectively. In seven subjects receiving the highest dosage, a blood half-life of about 3–4 hours could be calculated considering the levels measured between 5 and 11 h after infusion.

The few *in vitro* metabolism studies of TPA involving human cells (O'Brien and Diamond, 1978a,b; O'Brien and Saladik, 1980) indicate that many human cell lines in culture do not metabolise TPA to an appreciable extent (Appendix B).

3.2.2.3. Livestock

No data on the toxicokinetics of Jatropha PEs in livestock have been identified.

3.2.2.4. Companion animals

No data on the toxicokinetics of Jatropha PEs in companion animals have been identified.

3.2.2.5. Transfer rate

In pig and goat feeding studies with Jatropha material by Li et al.(2015) and Baldini et al.(2014), (see Sections 3.2.4.1 and 3.2.4.3, respectively, for further details), Jatropha PEs were not detected in liver samples from either species.

In the absence of toxicokinetic data in target animal species, including a lack of data on the oral availability, the potential transfer of Jatropha PEs into animal derived products is unknown.

3.2.3. Toxicity in laboratory animals

In contrast to the toxicokinetic studies given above, which have only been conducted with TPA, the toxicity studies described in this section have used Jatropha material, thus allowing an appropriate clinical and pathological description.

3.2.3.1. Acute and short-term toxicity

So far, the isolated Jatropha PE fraction has been tested for toxicity in only a few studies. In most cases, test materials were seed cake, or kernel meal or oil (see Figure 2). Table 2 provides an overview of studies on the acute and short-term toxicity of Jatrophaseed fractions from toxic genotypes. Both studies using 'native' Jatropha material (i.e. materials not subjected to treatment aiming at detoxification) and studies using treated material are discussed in this chapter. As PEs were not known to be the cause of Jatrophatoxicity until 1998 (Makkar et al., 1998a, Makkar and Becker, 1998), their levels were not determined in the earlier studies.

Table 2: Summary of feeding studies in laboratory animals on the acute and short-term toxicity of non-treated and treated Jatrophamaterials

Test material	Origin ofJatropha	PE measured (Y/N) (concentration)	Test animals	Duration of feeding	Major toxic effects	Reference
Non-treated material						
Oil	India	N	Rats	Single oral gavage	Lethality with diarrhoea and inflammation of the gastro-intestinal tract	Gandhi et al. (1995)
PE fraction isolated from oil	India	Y (21–36 mg/kg bw)[(a)]	Mice	Single oral gavage	Lethality, gastro-intestinal haemorrhage, microscopic lesions in liver, spleen, lung, kidney and heart	Li et al. (2010)
Seed powder	Sudan	N	Mice	14–75 days	Reduced feed intake, diarrhoea, damage of intestine, liver, kidney, heart, and lung, lethality	Adam (1974)
Kernel powder	Nigeria	N	Mice	2 days	Reduce feed intake and motor activity, intestinal bleeding, haemorrhagic colon, congested livers and lungs, lethality	Abdu-Aguye et al. (1986)
Kernel powder	Brazil	N	Rats	16 days	Lethality with haemorrhagic and necrotic livers and hearts, degeneration of kidney tubular cells	Liberalino et al. (1988)
Kernel meal		N				
Oil		N				
Seed cake	Nigeria	N	Rats	21 days	Lethality, increased weight of heart and lung	Annongu et al. (2010)
Seed powder	India	N	Rats	21 days	Changes in biochemical parameters in blood plasma, lethality at higher dose	Awasthy et al. (2010)
Oil	Unknown	N	Rats	Daily oral dose for 28 days	Depressed growth, decreased white blood cell count	Poon et al. (2011)
Treated material						
Kernel meal	Nicaragua	N	Rats	10 days	Higher feed intake and body weight gain compared to rats fed non-treated material.	Makkar and Becker (1998)
Kernel meal	Nicaragua	Y (20 µg/g feed)	Rats	7 days	Reduced feed intake	Aregheore et al. (2003)
Kernel meal	India	Y (25–240 µg/g feed)	Rats	12 days	Reduced feed intake, diarrhoea, impaired motor function, lethality, no effect on organ weights and histology	Rakshit et al. (2008)
Seed meal		Y				
Kernel meal	Unknown	N	Rats	28 days	Increases in heart and kidney weights and decreases in lung weight	Rahma et al. (2013)

bw: body weight; N: no; PE:phorbol ester; Y: Yes.
(a): single dose given by oral gavage.

In an acute study by Gandhi et al. (1995), Jatrophaoil (ratanjyot oil) from an Indian genotype was given by oral gavage to groups of four Haffkine Wistar rats (two males and two females) as single doses of 4, 6, 9 and 13.5 mL/kg bw, while four control animals received ground-nut oil at 13.5 mL/kg bw. Animals dosed with Jatrophaoil at 9 and 13.5 mL/kg bw exhibited diarrhoea, haemorrhagic eyes, and inflammation of the gastro-intestinal tract, and all of them died. Two of the four rats dosed with 6 mL/kg bw died, but none of the group receiving 4 mL/kg bw.

Li et al. (2010) isolated the PE fraction from Indian Jatrophaoil and studied its acute toxicity in male Swiss Hauschka mice. Six groups of 10 mice each received a single dose of the PE fraction diluted in corn oil by intragastric administration, while one group received only corn oil. The dosage of PEs ranged from 21.3 to 36.0 mg/kg bw. The animals were observed for 19 days, after which the surviving mice were sacrificed, and all mice were examined for gross and microscopic changes. The death of mice due to the dosed PEs, occurred in a dose-dependent manner, with one dead animal in the lowest and nine in the highest dose group. An LD_{50} value of27.3 mg PEs/kg bw was calculated for the mixture of Jatropha PEs. All treated mice exhibited a transient reduction in body weight gain during the first week, and their stool in rectum consisted of dry beads. Both small and large intestines contained black digesta, supposedly due to gastro-intestinal haemorrhage. No histopathological changes were observed in the liver, kidney, lung, heart, spleen and brain at the lowest dose. At doses of 26.2 and 29.3 mg PEs/kg bw, congestion of sinus hepaticus and of pulmonary alveolar capillaries, haemorrhage of spleen, and glomerular atrophy were noted. At higher doses, diffuse haemorrhage and exudate in lung, glomerular necrosis, abruption of cardiac muscle fibres, and fatty vacuoles in liver cells appeared.

The first study on the short-term toxicity of Jatrophaseeds appears to have been conducted in mice of the A.S.1. strain by Adam (1974). Ground seeds of a toxic Jatrophagenotype from southern Sudan were offered as 50% of the basic diet to 15 mice for 14 days (group 1), 40% to 15 mice for 18 days (group 2), 20% to 15 mice for 24 days (group 3), 10% to 10 mice for 27 days (group 4), 5% to 10 mice for 28 days (group 5), 1% to eight mice for 75 days (group 6), and 0% to six mice for 75 days (group 7, control). All animals in groups 7 and 6 survived, while 13 and 10 of the 15 mice of groups 1 and 2, respectively, died between day 3 and 16. Groups 3, 4, and 5 exhibited mortality of 40–50% during days 10–26. Mice of groups 1–5 had a much lower feed consumption than groups 6 and 7. From the fourth day of the study, animals of the two high dose groups (1 and 2) showed impaired appetite, diarrhoea, accelerated respiration and difficulty in keeping their normal posture. In the intermediate dose groups (3–5), these symptoms began during day 7 and 14, while no clinical signs were observed in groups 6 and 7. Macroscopic organ damage was most frequently observed in the intestine, liver, kidneys, and heart and less frequently in the lungs. Intestinal lesions of the high dose groups 1 and 2 included acute catarrhal enteritis with extravasation of blood in the lumen, swollen mucous membranes of the small intestine and superficial focal erosions of the intestinal mucosa. In groups 3–5, scattered areas of mild inflammation were present along the small intestine. Mice in groups 1–4 had congested and fatty livers with focal necrosis, and kidneys with cortical haemorrhage and pale brown medulla. The hearts of mice of groups 1–3 exhibited congestion and petechial haemorrhages in the endocardium. Pulmonary congestion was observed in a dose-dependent manner in groups 1–4, while groups 5–7 showed no gross changes in the lung. These macroscopic alterations were confirmed by histopathological findings. In summary, this study demonstrates that Jatrophaseedis toxic to mice with a clear correlation between the concentration of seed material in the diet and the toxic response. Only at a concentration of 1% seed in the dietwere clinical disturbances and pathological changes absent after 75 days of feeding. Higher concentrations gave rise to severe organ damage, mostly in the small intestine, liver, kidneys and lungs, and caused mortality.

Abdu-Aguye et al.(1986) mixed 25, 50, 75 or 100% (w/w) of powdered kernels from a Nigerian Jatrophagenotype withground pellets and fed the mixture to groups of 10 mice of unspecified age, strain and sex for 48 h. The mice were then kept on their normal pellet diet for another 12 days. All mice receiving a feed containing 50%or more of the Jatropha material died (100%- and 75%-groups during days 4–7, 50%-groups during days 6–9 of the study), whereas 3 of the 10 mice of the 25%-group died on day 11, and all of the control group survived. Animals of the 50%- and higher dosed

groups avoided their feed and exhibited reduced motor activity. All mice dying had blood clots in their faeces, and most of them exhibited a dilated and haemorrhagic ascending colon and infarcts of the intestinal mucosa upon dissection. In addition, some had congested livers and lungs. None of the control mice showed any abnormalities upon *post mortem*examination.

Liberalino et al. (1988) mixed powdered kernels (37%) or kernel meal (17%) or oil (20%) of a Jatrophagenotype from the Brazilian state of Minas Gerais in a corn starch diet; the control diet contained casein and corn oil instead of the Jatropha materials. Feeding of the diets to groups of six male weanling Holtzman rats caused the death of all rats exposed to Jatropha materials (kernel powder after 2–3 days, kernel meal or oil after 6–8 days), while the rats on the control diet grew normally until the end of the study after 16 days. Cooking the seeds had no effect on the lethality of the materials, whereas cooking followed by roasting delayed dying to 14–16 days. Feed consumption was not measured. Histopathological examination revealed haemorrhages and necrosis in liver and heart, as well as degeneration of renal tubular cells. The histological lesions were milder in rats fed the cooked plus roasted material.

Annongu et al. (2010) studied the toxicity of treated Jatropha seed cake in male and female Albino rats. Dried Jatropha seeds were boiled, fermented, and soaked in hexane and ethanol for 24 h. The extracted seeds were then milled and included at levels ranging from 5% to 25% in a diet based on corn starch and soya bean. This diet was fed to groups of rats of six each for 21 days, and feed intake, body weight gain, survival rate, and the weight of liver, intestine, heart, and lung were determined. All rats dosed at 20% and 25% of the treated seed cake died within one week, while no mortality was observed for the rats at 15% or less. Moreover, the rats at the latter dose level exhibited a normal feed intake and even a slight increase in body weight over controls. Organ weights of these lower dose groups were also not affected. The authors conclude that the treated Jatropha seed cake had no deleterious effects on rats if included in the diet at up to 15%. However, as the Jatropha PE content, which was 2.8 mg/g in the 'native'Jatropha seed cake, was not determined in the treated product, no conclusions can be drawn from this study.

Awasthy et al. (2010) studied the effects of powdered Jatrophaseeds from an Indian genotype on several biochemical parameters in the blood of young weaned Wistar rats after short-term oral exposure to sub-lethal doses. Three groups, each consisting of eight male and eight female rats, were fed a maize/soya bean diet where 0% (group I, control), 32% (group II) or 63% (group III) were substituted by Jatrophaseed powder for 21 days. On day 0, 7, 14 and 22, blood samples were analysed for glucose, creatinine, total protein, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and alkaline phosphatase (ALP). All rats of groups I and II survived, while four rats of group III died on day 13 and another two on day 16. Therefore, blood of rats from group III could not be analysed on day 22. Changes were observed for all biochemical blood plasma parameters in the treated groups II and III compared to the control group I:glucose was significantly lower in group II on day 22, and in group III on day 14;plasma protein was decreased while creatinine and ALP were markedly elevated in groups II and III from day 7 onward; GOT and GPT were significantly increased in group II on day 14 and 22, and in group III on day 7 and 14. No concentration of the PE content of the fed Jatropha meal was given and hence no conclusion can be identified from this study.

Poon et al. (2011) conducted a 28-day oral toxicity study of Jatrophaoil in Sprague–Dawley rats. The PE content of the oil has not been determined in this study nor has the geographical origin of the Jatropha genotype been given. Five groups of male and female rats (six animals each) were administered doses of 0, 0.5, 5, 50 and 500 mg/kg bw of Jatropha oil diluted in corn oil for 28 consecutive days by oral gavage.A reduction in body weight gain compared to controls was observed for male (10.6%) and female rats (11.7%) at the highest dose, although weekly feed intake was not significantly decreased in any treatment group. No overt signs of toxicity were observed other than a consistent production of watery stools by one female of the 500 mg/kg bw treatment group. Organ weights of liver, kidney, heart, brain, thymus, spleen and testis were not affected, and gross examination did not reveal any abnormalities. Haematological analysis exhibited a mild decrease of haemoglobin levels in males and females in the 500 mg/kg bw dose group and a slight reduction of red

blood cell counts in females of this dose group. White blood cell and lymphocyte counts were substantially decreased in the 50 and 500 mg/kg bw females and in the 500 mg/kg bw males. At this highest dose, blood urea nitrogen was slightly reduced in male and inorganic phosphate in female rats, while all other serum clinical values were not affected. The effects of Jatrophaoil on lymphocyte counts were corroborated by histopathological findings in the spleen, where the volume of the periarteriolar lymphoid sheath was reduced in the 50 and 500 mg/kg bw females and in the 500 mg/kg bw males. Mild histological changes, which were not dose-related, were also observed in the liver (periportal vacuolation in females, increased portal cytoplasmic density in males and females) and mammary gland (increased acinar proliferation). No indication of inflammatory response in the tissues and organs examined, and no changes in the neutrophil, monocyte and eosinophil counts were observed. Serum C-reactive protein, which is a sensitive indicator of systemic inflammation, was not affected. Thus, the most prominent effects of oral administration of Jatrophaoil in this study were the depressed growth in male and female rats dosed with 500 mg oil/kg bw, and the decreased white blood cell counts in the 50 and 500 mg oil/kg bw females.

Treated Jatropha kernel meal (see Section 3.3.1 for treatment method) fed to rats for 10 days (at inclusionof 16% in the diet), resulted in a greaterfeed intake and weight gain in rats fed the treated meal compared with therats fed with non-treated meal(Makkar and Becker, 1998).

Aregheore et al. (2003) studied the effect of feeding a treated Jatrophakernel meal on food intake and growth rate of male weanling Sprague–Dawley rats with an initial body weight of about 85 g. The treated Jatropha kernel meal (see Section 3.3.1 for treatment method) had a PE concentration of 0.13 mg/g and was added to the diet at a level of 16%, resulting in an approximate Jatropha PE concentration in the diet of 20 µg/g. Feeding of this diet for 7 days gave rise to a pronounced reduction in daily feed intake and subsequent failure to increase body weight, indicating that Jatropha PEs at the level of 20 µg/g in the feed have strong adverse effects.

Rakshit et al. (2008) compared the effects of various methods aiming at mitigating the adverse effect of Indian Jatropha kernel and seed meal (Sections 3.3.1 and 3.3.3 for treatment methods) on mortality, food intake, body weight, various clinical signs, organ weights, and histopathological changes in vital organs of male weanling Wistar/IND/CFT rats. Forty-two male rats were divided into seven groups of six rats/group and fed diets containing either non-treated or treated Jatropha kernel meal or seed meal.[9]A control group fed with casein was also included.The Jatropha PE content of the non-treated kernel meal or non-treated seed meal was 1.35 (Group 2) and 0.74 mg/g (Group 5), respectively, and the PE content of the treated Jatropha material was markedly lower, ranging from 0.08 to 0.16 mg/g. Diets were prepared containing corn starch, groundnut oil, a vitamin and salt mixture, and contained the following concentrations of Jatropha PEs: Group 2 diet, 240 µg PE/g; Group 5 diet, 240 µg PE/g;Group 3 diet, 30 µg PE/g; Group 4 diet, 25 µg PE/g; Group6 diet, 30 µg PE/g; Group 7 diet, 50 µg PE/g. These diets were fed for 12 days, resulting in an estimated daily dose of 24 mg PEs/kg bw for Group 2 and 2.4 mg PEs/kg bw for Group 4. All Jatrophafed groups gave rise to a marked reduction of feed intake (ranging from 0.9 to 2.5 g/day) as compared to the control group (5.1 g/day) and to a severe loss of body weight (ranging from 8 to 14 g), during the 12-day feeding study, while the control rats gained 14 g. The weight loss did not correlate with the amount of Jatropha PEs consumed (which ranged from 9.0 mg/rat (Group 2 diet)to 0.65 mg/rat (Group 6 diet)). All rats in all the Jatrophagroups died between day 8 and 12, while all rats of the casein control group survived. Mortality was noted one or two days earlier with the non-treated Jatropha materials.All rats receiving Jatrophamaterial had severe diarrhoea and difficulties in motor function. However, no distinct effects of the Jatropha materials on organ weights and histology of liver, lung, kidney, heart, testis and brain were observed. No no-observed-adverse-effect level (NOAEL) could be determined from this study.

[9] Group 1 – casein (control); Group 2 – non-treated ground kernel meal; Group 3 – treated kernel meal (2% aqueous $Ca(OH)_2$, Group 4 – treated kernel meal (2% aqueous NaOH); Group 5 – non-treated seed meal; Group 6 – treated seed meal (2% aqueous $Ca(OH)_2$); Group 7 – treated seed meal (2% aqueous NaOH).

Rahma et al. (2013) fed a diet containing 22.8% of treated Jatropha kernel meal (see Section 3.3.1 for treatment method by Martínez-Herrera et al.(2006)) to rats for 28 days. Changes in organ weights were noted compared to the control, consisting of increases in heart and kidney weights and decreases in lung weight.

In conclusion, most feeding studies with Jatropha material containing PEs showed severe clinical observations and pathological lesions in rats and mice. Among the prominent effects was a loss of body weightand mild to severe macroscopic and microscopic changes in the lung, kidney, liver, heart and spleen.The toxic effects were more severe at higher concentrations of Jatropha material in the diet. Only a single study used a mixture of purified Jatropha PEs (isolated from Jatropha oil) and could be used to derive an LD_{50} of 27 mg Jatropha PEs/kg bw in Swiss Hauschka mice (Li et al., 2010). The study by Rakshit et al. (2008) showed severe adverse effects in rats with treated Jatropha material, containing a PE level (in TPA-equivalents) that would lead to an exposure of 2.3 mg PEs/kg bw perday. Due to the lack of quantitative data on the level of PEs in the administered Jatropha material in most studies, and/or the absence of studies conducted with non-toxic concentrations of PEs, no quantitative dose-response relationshipand no NOAEL could be established from the rodent studies.

3.2.3.2. Long term toxicity

No studies on the long-term toxicity of materials derived from Jatropha seeds could be identified.

3.2.3.3. Genotoxicity

No studies on the genotoxicity of Jatropha PEs could be identified.In experimental studies, TPA was not demonstrated to be a genotoxicant even though structural alerts for genotoxicity have been identified by using read-across (OECD toolbox; Appendix D). Clastogenic, mutagenic and sister chromatid exchange-inducing effects of TPA have been shown in some experimental systems but are mediated by secondary products (possibly from arachidonic acid) formed by the cell, only under culture conditions with low antioxidant content in culture media and sera,in response to the tumour promoter (Emeritand Lahoud-Maghani, 1989).

Based on the read-across analysis described in Appendix D,it could be concluded that the six Jatropha PEs cannot be considered entirely similar to TPA in terms of their genotoxic potentials. Based on the potential difference between TPA and Jatropha factors, some additional structural alerts relevant to genotoxicity (DNA binding for α, β-unsaturated esters and protein binding for polarised alkene esters) were identified in parent molecules (factors C3 and C6) as well as after metabolic activation (for all 6 factors) (see Appendix D for further details). However, none of these hypothetical alerts could be confirmed by experimental data using standard protocols for the assessment of genotoxic effects, as Jatropha factors are not commercially available. The available data on carcinogenicity are summarised below.

3.2.3.4. Carcinogenicity

No studies on the carcinogenicity of Jatropha materials, using oral or other routes of administration, could be identified.

A number of studies, however, reported the tumour promoting effects in model experiments, which is inline with the well-known tumour promoting effects of PEs such as TPA (in mouse skin and forestomach). These studies with TPA provided no evidence for any tumour initiating properties.The outcome of a clinical trial using TPA as an anti-tumour agent for the treatment of human malignancies is described in Section 3.2.5.

Goerttler et al. (1979), investigated tumour initiation and promotion in the epithelium of the forestomach of micetreated intragastrically with a single dose of 7,12-dimethylbenz [a]anthracene(DMBA)at 50 mg TPA/kg bw followed by repeated dosing(twice per week) for 35 weeks of TPA at 10 mg/kg bw. Forty-five out of 50 mice which received this treatment had tumours (papillomas) in the forestomach. There were no forestomach tumours noted for mice in the untreated

control and the TPA-only groups, although in the DMBA-only group, papillomas were observed in the forestomach of 10 mice.

Horiuchi et al. (1987) reported that a partially purified fraction from a methanol extract of Jatropha seed oil from Thailand induced ornithine decarboxylase (ODC, a marker for tumour promotion) in mouse skin and inhibited the specific binding of ^{3}H-labelled TPA to a particulate fraction of mouse skin, suggesting tumour promoting activity of the Jatropha seed oil fraction with a similar mode of action as TPA. In an initiation-promotion experiment (15 mice/group), skin tumours were observed in 36% of female CD-1 mice 30 weeks after a single local dermal application of 50 µg DMBA, followed by local dermal treatment with the methanol fraction from the Jatropha oil twice a week for 30 weeks. Control mice treated with DMBA alone or the methanol fraction alone exhibited tumour incidences of 7%(1/15) and 13%, (2/15) respectively, in week 30. No non-treated mice were included. The CONTAM Panel noted the elevated incidence in the case of the methanol fraction alone, as compared to the DMBA-treated group, but concluded that the study is too poor to conclude on initiating properties of the methanol extract.

In a subsequent study, Hirota et al. (1988) isolated a phorbol ester from the methanol fraction of the Jatropha seed oil from Thailand. Based on spectroscopic data and chemical derivatisation, the structure of an intramolecular 13,16-diester of 12-deoxy-16-hydroxyphorbol was proposed. The dicarboxylic acid moiety was the same as later identified by Haas et al. (2002) for Jatropha factor C1 (see Figure 4), but proposed by Hirota et al. (1988) to be inversely esterified with the hydroxyl groups at C13 and C16. Thus, the phorbol ester isolated in this study was Jatropha factor C1. It induced ODC in mouse skin, inhibited the binding of ^{3}H-TPA to specific phorbol ester binding sites, and activated protein kinase C *in vitro*. Using essentially the same protocol as Horiuchi et al. (1987), Jatropha factor C1 acted as a promoter of skin tumours in CD-1 mice: after 30 weeks, 47% of the mice initiated with 100 µg DMBA and subsequently promoted with Jatropha factor C1 exhibited tumours of the skin, whereas 7% of the mice treated with DMBA alone and none of the mice treated with Jatropha factor C1 alone developed skin tumours. Horiuchi et al. (1987) and Hirota et al. (1988) concluded from their studies that Jatropha PEs act as tumour promoters after local dermal application. The activity of Jatropha factor C1 was assessed to be weaker than that of TPA by Hirota et al. (1988).

3.2.3.5. Developmental and reproductive toxicity

Marneesh et al. (1963) observed a complete reproductive failure in female rats fed a diet containing the seeds of Jatropha at a concentration of 3.3% and mated with untreated males. Feeding was started 10 days prior to mating and continued for a total of 25 consecutive days. Treated females exhibited slightly depressed feed intake and body weight gain and produced soft faeces but not diarrhoea. Males and control females received the normal diet. The contraceptive principle present in the seeds was not identified.

Goonasekera et al. (1995) prepared various extracts from fresh and dried Jatropha fruits by using methanol, petroleum ether, and dichloromethane. No chemical analysis of the extracts is provided. The residues of the extracts were solubilised in water with the help of polyvinylpyrrolidone or tween 80, and administered daily by oral gavage to groups of 10 female Sprague–Dawley rats from the first day of pregnancy for up to 10 days. Doses of the extracted material ranged from 0.1 to 3.1 g/kg bw. Animals were sacrificed and autopsied on the 16th day of pregnancy. Body weights were determined during the whole study but data were not shown in the publication. During autopsy, the number of implantation sites, corpora lutea, normal and degenerated fetuses, and the state of liver, lung and kidney were noted. The authors reported a loss of body weight in the treated rats during the dosing period with all the extracts, but the animals gained weight after cessation of treatment. Mortality was observed with several but not all the extracts. The major findings for the methanol extracts were a high incidence of absorbed and degenerated fetuses, which may be due to maternal toxicity. Similar observations were made with the dichloromethane extract and the hot petroleum ether extract, but the composition of the extracts was not clarified by chemical analysis.

Non-treated Jatropha seed cake was also subjected to testing in the well-established zebrafish embryotoxicity test (Hallare et al., 2014). The test material (JatrophaPE ester concentration not reported) was added at different concentrations (ranging between 1.0 and 2.15 g seed cake/L medium) to the incubation chambers filled with the watery medium containing per assay 20 zebra fish embryos. Embryonic development was assessed over 72 hours. At the highest concentration of Jatropha (2.15 g seed cake/L medium), a 100% mortality was observed within 24 hours of exposure and a lethal concentration of 1.61 g extruded seed meal/L calculated. No lethality was found at the lowest tested concentration of 1.0 g/L in the same assay. Other endpoints measured embryo coagulation, non-formation of somites and non-detached tails. For all parameters, a dose-dependent increase in abnormalities could be observed at the concentrations of 1.2, 1.47 and 1.78 g seed cake/L medium. No alterations were seen at the lowest dose of 1.0g/L. In addition, pericardial oedemas in surviving embryos we observed in the two highest concentrations (1.78 and 1.47 g/L), whereas yolk sac oedemas were observedin a concentration-dependent manner in all test animals. These findings confirm the *in vitro* embryotoxicity of Jatropha PEs in extruded Jatropha kernel meal, but due to the absence of analytical measurements, these data cannot be further interpreted.

Overall there is insufficient evidence to conclude ondevelopmental and reproductive toxicity of JatrophaPEs.

3.2.3.6. Immunotoxicity

No data on Jatropha PEs and immuotoxicity were identified.

3.2.3.7. Neurotoxicity

No specific studies on the neurotoxicity of Jatropha PEs could be identified. The reduced motor activity, which was observed in short-term toxicity studies, occurred only at PE exposure concentrations that also induced severe distress and an inflammatory reaction and hence are regarded as signs of general depression rather than an indication for specific neurotoxic effects (see Section 3.2.3.1).Besides these PKC-dependent mechanisms described in *in vitro* experiments, no specific toxic effects on the central or peripheral nervous system could be identified for PEs. This observation is confirmed by a human clinical study in which TPA was given as constant rate infusion or bolus injection to patients (see Section 3.2.5) and in which no specific neurological signs were observed.

3.2.4. Adverse effects of PEs in farm animals

In animal husbandry, *Jatropha* species are known as toxic plants, and have historically been used as natural fences because animals do not consume the plants. Nevertheless, some feeding experiments, particularly with small ruminants, have attempted to identify potential non-toxic levels and describe the dose-dependent signs of toxicity. It should be noted that these experimental studies applied a forced feeding approach to achieve an intake of Jatropha material. Recent data focus on the potential use of treated material, as non-treated Jatropha products are too toxic to be used as animal feed material.

3.2.4.1. Effects in pigs

No studies with non-treated Jatropha material could be identified.

Chivandi et al. (2006) reported a comprehensive4-week study with pigs given commercially treated kernel meal(see Section 3.3.1 for treatment method) at different inclusion rates, replacing between 6.25 and 25% of the crude protein fraction. These concentrations are equivalent to dietary inclusion rates of kernel meal of0, 1.3, 2.5, 3.7, and 5.0% of the total feed ration. Treated animals (three male and three females per dietary treatment) showed a persistent diarrhoea and a decrease in packed cell volume and serum glucose levels, while serum cholesterol and triglyceride as well as alpha-amylase activity were only moderately impaired. Other clinical signs were anaemia, haemorrhage in the gastro-intestinal tract and skin irritation especially around the ears with these effects being observed at the lowest treated kernel meal group (with an inclusion rate of 1.3%). The authors showed that the treated

kernel mealcontained some residual amounts of toxic PEs of 0.8 mg/g kernel meal (described in Chivandi et al.(2004)in more detail) equivalent to 10.4, 20.0, 29.6 and 40.0 mg/kg feed,[10] respectively), and that these can be toxic to pigs, even at the lowest concentration tested. Considering the lowest level of 10.4 mgPEs per kg feed, a feed intake (restricted feeding) of at maximum 1.3 kg feed (controlled feeding) per animal of a body weight of 16.2 kg(only the average body weight at the beginning of the feeding trial is given),this would result in an exposure of 0.83 mg/kg bw, which might be considered as a lowest-observed-adverse-effect-level (LOAEL). This conclusion is supported by the fact that almost all parameters measured in the study (with the exception of serum cholesterol concentrations) showed a clear (linear) dose-response at higher exposure rates.

In a 28-daystudy conducted by Wang et al. (2011),treated kernel meal (briefly mentioned in Section 3.3.1) was used to replace the soya protein fraction in the diet of growing pigs (18 male and 18 females in total, with three replicates per treatment regime and four pigs per replication, initial body weight approximately 21 kg). Inclusion rates amounted to 0%,25%and 50% of the soya bean protein fraction, respectively (denoted as 'DJM' 0, 25, or 50by the author), equivalent to an inclusion rate of 0, 54 and 102 g treated material/kg diet, respectively. In this study no major adverse effects were observed in feed intake or weight gain, and no pathological alterations were noted during the *postmortem* analyses. Feed intakewas only decreased in the DJM25 animals, but not in animals of DJM50. No significant differences were observed in the serological parameters tested, including total protein, albumin, urea nitrogen, glucose, triglyceride, superoxide dismutase, LDH, lysozyme,GOT, GPT, ALP, acid phosphatase. Only the animals of DJM50 showed increased levels of total protein and superoxide dismutase. The authors concluded from this study that with additional lysine added to the diet, the treated Jatropha kernel meal can replace up to 50% of the protein fraction of a balanced diet for growing pigs without adverse effects. The major difference with the Chivandi et al. (2006) study is that these authors (Wang et al., 2011) used the procedure byMakkar and Becker (2010a) for the treatment of the kernel meal (see Section 3.3.1 for treatment method).In this study by Wang et al. (2011),the concentration of JatrophaPEs in the untreated material were 0.98 g/kgwhile in the treated material used in the pig diet PEs were not detected.[11]With the aim of providing a quantitative estimate of the PEexposure levels in this study,the following assumptions were made: as in the treated material no PEs could be detected, the concentration seems to be below 3 mg PEs/kg kernel meal, as this is the common limit of detection for analytical methodsdescribed in studies on the treatmentof kernel meal. Considering the maximal inclusion rate of 102 g treated kernel meal/kg diet (DJM50), a feed consumption of 1.15 kg feed (0–14 days of the trial) and a body weight of the animal of 21.44 kg (initial body weight), this would result in a maximal exposure of 0.35 mg PEs per day or 0.016 mg/kg bw.In turn, using the data from the second phase of the experiment (days 15–28), a feed consumption of 1.68 kg results in an intake of 0.51 mg PEs per day or 0.013 mg/kg bw.

In the most recently reported study, Li et al. (2015) treated Jatropha kernel meal (see Section 3.3.1 for treatment method)and evaluated the effects of its incorporation at different levels in pig diets when given to 144 pigs (six dietary treatmentswith 12 males and 12 females per treatment, for a 79-day period). PEs were still present in the treated Jatropha material as determined by an HPLC-UV based analytical procedure according to Makkar et al. (1997, 2007). The amount of PEs measured was 0.11 mg PEs/g Jatropha kernel meal. Subsequently, different rates of incorporation of the treated kernel meal were selected such that 15%, 30%, 45%, 60%or 75% of the soybean meal protein was replaced by kernel meal protein. This replacement resulted in a concentration of PEs of 0, 2.75, 5.50, 8.25, 11.00, 13.75 mg PEs/kg diet, respectively. Parameters monitored were feed intake, weight gain and feed conversion efficiency, as well as some whole blood analysis (red blood cells and white blood cells), the alkaline phosphatase and serum alanine transferase activities. At the end of the feeding experiments, animals were sacrificed, organ weights determined and histological investigations of liver and kidneys conducted. The adverse effects observed at levels equal to or higher than 8.25 mg

[10] Table 1 of the article by Chivandi et al. (2006) contains an error in the dimension given for the calculated concentration (x^{-5}). This is clarified later in the text of the discussion, where the residual concentration of 0.8 mg/g treated material as also described in Chivandi et al. (2004) is confirmed.

[11] The limit of detection (LOD) is not stated by Wang et al. (2011), but the method for treating the kernel meal used in this study reported an LOD of 3 mg/kg.

PEs/kg diet included decreases in average daily (body weight) gain (ADG), average daily feed intake (ADFI) and gain-to-feed ratio. The effects on growth performance (based on ADG and ADFI) were found to be reversible when six male and six female pigs which received 13.75 mg PEs/kg diet for the first 29 days were then given a control diet for the remaining 50 days of the feeding trial. In addition, activities of serum alkaline phosphatase increased while that of serum alanine transaminase decreased in some treatment groups, but the changes were inconsistent and not related to any clinical findings. The authors reported pathological alterations in the liver consisting of mild leucocyte infiltration and steatosis/hepatic lipidosis from5.5 mg PEs/kg diet, and cell disorder, degeneration and necrosis from8.25 mg PEs/kg diet. However, the CONTAM Panel was unable to conclude on these histological findings, as pictures of the liver lesions presented for each treatment group in Figure 1 of the paper, are not clearly in support of these diagnoses, but possibly represent artefacts from tissue fixation/processing. Additional evidence supporting a lack of reliability of the morphological description, is the absence of indication of pathology from the biochemical markers.Furthermore, no indication of the incidence of the lesionsper treatment group was reported.PEs were not detected in the liver samples, however no details on the applied methods to measuring tissue levels are given. No adverse effects were observed at levels of up to 5.50 mg PEs/kg of diet. Using the values of 5.50 and 8.25 mg PEs/kg in the diet, and the average body weight of pigs (20.47 kg) as well as an average daily intake of the diets in these two groups (1.59 and 1.47 kg/d respectively), an apparent NOAEL and LOAEL (based on decreases in body weight gain and feed intake) would be 0.4 and 0.6 mg PEs/kg bw per day, respectively, which is in line with the previous study of Chivandi et al. (2006). Li et al. (2015) also showed that discontinuation of the diet containing 13.75 mg PEs/kg diet and feeding of the control diet free of PEs alleviated the adverse effects of PEs, demonstrating their reversibility.

Based on these data the CONTAM Panel identified a NOAEL for pigs of 0.4 mg PE/kg bw per day (based on decreases in body weight gain and feed intake). It should be noted, however, that this value is based on analytical measurements of in-feed concentrations of Jatropha PEs expressed as TPA-equivalents.

3.2.4.2. Effects in poultry species

El Badwi et al. (1995) studied the effects of 0.5%ground Jatropha seeds(non-treated),given in the diet to nine 7-day-old Brown Hisex chicks for up to 4 weeks. Blood analyses revealed a decrease in haematocrit values and erythrocyte counts. Serum analyses showed an increase in transaminases and changes in the electrolyte levels, particularly a decrease in serum potassium concentrations. *Post mortem* histology of the main organs showed necrotic lesions in the liver and proximal renal tubule cells, as well as erosions in the mucosal membranes of the intestines and congestions in cardiac blood vessels. In a previous study (El Badwi et al.,1992*)* the same group of authors showed an increase in toxicity of a combined exposure,when ground Jatropha and Ricinus seeds (0.5% each) were given in the diet to 12, 7-day-old Brown Hisex chicks,for 2 weeks.

Recent investigations from Wang et al. (2012) revealed that dietary exposure to non-treated Jatropha kernel meal (produced by pressing a mix of the kernels and shells in 9:1 ratio) at inclusion rates of 3-12% for up to 21 days to day-old male Arbor Acres chicks (875 chicks in total, divided into five groups with seven replicates of 25 chicks),resulted in immune-suppression or immune-depression in young broilers in a dose dependent manner. A dose dependent increase in mortality was observed during the 1st week, reaching 56% in the highest dose group. Substantial lesions were observed in all lymphatic organs, immunoglobulin A(IgA) and IgG levels decreased, whereas IgM levels increased dose-dependently. Moreover, total blood T-lymphocyte counts and T-subset distribution changed significantly. The authors concluded that non-treated Jatropha kernel meal exerts strong immunotoxic effects in broilers and pointed out that the alterations in T-lymphocyte subpopulations reflect the histological changes observed in the thymus.

Research from Ojo et al. (2013) indicated that supplementation of diet with 0, 4, 8 and 12% non-treated Jatropha seeds when given to 40 broilers randomly allocated to the four treatment groups, for

4 weeks,was hepatotoxic and nephrotoxic, as indicated by increased serum levels of liver transaminases (AST, ALP), total bilirubin, urea and creatinine.

Due to the lack of precise analytical data on the concentration of PEs in the diets used in these experiments, and hence the lack of information about the actual intake of JatrophaPEs, these studies remain descriptive and cannot be used to identify a NOAEL. They confirm, however, the general assumption that non-treated seeds (and products thereof) should be avoided in animal feeds.

3.2.4.3. Effects in ruminants

In an early experiment conducted by Ahmed and Adam (1979a), the toxic effects of non-treated seed meal fed to 6–18 months old calves (two calves per treatment group) was described. This study revealed that this crude seed meal is highly toxic and lethal to calves fed at a single dose of 2.5 g meal per kg bw within 4 h. Even the lowest doses tested (0.025 g meal/kg feed) given over a period of 2 weeks resulted in mortality. Clinical signs included acute tympani, abdominal pain, salivation, inappetence, respiratory distress and finally recumbence and death. *Post mortem* findings included large haemorrhages in the entire gastro-intestinal tract as well as in all major organs, fatty degeneration in the liver and the kidneys, and extensive exudation in the peritoneal and pleural cavity.

Recently, Sudake et al. (2013), in an 80-day study, showed that a mixture of feed with 4% of lime-treated Jatropha cake resulted in adverse effects on growth performance of young crossbred calves (14 animals randomly allocated to one control group and one treatment group). Rumen fermentation was not affected but treated animals lost weight, and almost all blood and biochemical parameters were changed with a significant decrease in the white blood cell counts and a significant increase in hematocrit as well as serum creatinine values. The authors concluded that lime treatment is ineffective to detoxify kernel meal.

The sensitivity of ruminants to Jatropha seeds is in line with experiments of Makkar and Becker (2010b), demonstrating that rumen microorganisms are unable to efficiently degrade PEs. Therefore, ruminants have to be considered to be as sensitive as monogastric animal species to the dietary exposure to PEs in Jatropha seeds.

In line with the experiments described above for calves, Adam and Magzoub (1975) used the same experimental approach with goats, feeding different concentrations of non-treatedkernel meal for a maximum period of 21 days to 11 goats at concentrations between 0.25 and 10 g kernel meal/kg of feed. A high rate of mortality was observed in all groups, which was time- and concentration-dependent. Even in the lowest inclusion group mortality occurred. Clinical signs and *postmortem* findings with extensive haemorrhages were comparable to those observed in calves.

Abdel Gadir et al.(2003) demonstrated in a study with Nubian goat kids(three per treatment group) that even 0.25 g ofnon-treated Jatropha kernel meal per kg feed resulted in deaths after 11 days and *postmortem* investigations showed large haemorrhages along the entire intestines and in all major organs.

Comparable signs of intoxication were also observed in sheep and goats(two/threeanimals per treatment group) (Ahmed and Adam, 1979b) showing again haemorrhages in rumen, reticulum, intestines, lung and kidney as major *postmortem* findings. This study indicated that feeding the animals with even lower doses of non-treated powdered or ground Jatropha seeds in the diet(0.05% for goats or 0.5%for sheep) could lead to death within 19 days in goats and within 7 days in sheep.

Katole et al. (2011) confirmed that treated seed cake (see Section 3.3.2 for treatment method) fed for 90 days at5 or 10 g/kg bwto adult sheep(five per treatment), resulted in an increase in hepaticLDHand AST.

In a study by Elangovan et al. (2013), non-treated and treated Jatropha seed cake (see Section 3.3.2 for treatment method) when fed to Deccani lambs(12 per treatment) for up to 11 days, at 25%inclusionthe

concentrate mixture resulted in clinical observations, deaths, alterations in clinical chemistry and histological changes (gastro-intestinal tract, heart, kidney and liver). The content of PEs by the applied treatment methods resulted in a 55% reduction of PEs, but the remaining PE concentration of 0.58 mg PEs/kg kernel meal at the lowest intake of 25.75 g treated Jatropha, resulted in an exposure of approximately 1.16 mg PEs/kg bw. This exposure level evoked severe signs of intoxication, and the animals needed more than one month to recover clinically.

Shukla and Singh (2013)reported that the oral administration of non-treated Jatropha seed oil at a dose of 1 mL/kg bw to three goats (aged 16–18 months old), for 28 days, resulted in moderate diarrhoea, dullness, depression and lethargy along with significant increase in serum creatinine.

Kasuya et al. (2012) fed fermented seed cake (*Pleurotus ostreatus* fermentation; see Section 3.3.2 for treatment method)included at levels of0, 7, 14 and 20% in the diet, to 24 Alpine goats allocated to four dietary treatments,for 72 days. The residual amount of Jatropha PEs was estimated to be on average 1.8 mg/kg DM. The authors reported that no symptoms of poisoning or changes in blood parameters were observed when up to 20% of treated Jatropha material was incorporated into the diet.

In a study by Baldini et al. (2014), one young male goat was dosed for 15 days with Jatropha seed cake, corresponding to 1.2 mg PEs/kg per bw. Only liver samples were analysed and the authors reported clear histopathological lesions in the liver linked to effects of PEs; however, no description of the lesions was given. No PE-related peaks could be detected by LC-MS/MS in liver samples from both the control and the treated animal.

From these studies with ruminants it was not possible to identify a NOAEL but the various studies suggest that ruminants are at least as sensitive as pigs.

3.2.4.4. Effects in horses

No data could be identified.

3.2.4.5. Effects in companion animals

No data could be identified.

3.2.4.6. Effects in aquatic species

Becker and Makkar (1998) described for the first time that carp (*Cyprinus carpio)* are highly sensitive to PEs from Jatropha seeds. The threshold at which carp showed adverse effects was 15 µg PEs/g feed and higher doses resulted in a reduction of growth rate and anorexia.

In a more recent study, Fernandes (2010) reported that physic nut meal of Jatropha (non-treated Jatropha seed meal) in the diet of fingerlings ofNile tilapia resulted in death. Moreover, Kumar et al. (2011a) indicated that even supplementation of partially purified phytate from Jatropha in fish diets at 1.5% and 3% would affect the growth performance and digestive physiology in tilapia.

In a recent comprehensive study with rainbow trout (Kumar et al., 2011b), the tolerance of treated Jatropha kernel meal was described. In a feeding trial, treated Jatropha kernel meal (see Section 3.3.1 for treatment method (Makkar and Becker, 2010a))was used to replace the fishmeal protein fractions of the diet by 50 and 62.5%. A comparative analysis of the major nutritional components (amino acids, crude protein, non-starch polysaccharides) is included in the manuscript. A 50% replacement resulted in no differences with the control group (fishmeal protein fraction set to 100%), the Jatropha diets were supplemented with phytase and lysine to balance the difference in amino acid composition between the two protein sources. PEs were not detectable in the treated Jatropha material (according to the authors the LOD was 3 µg/g). This study suggests that 50% of the fishmeal protein in trout diets can be replaced by treated Jatropha kernel meal (see Section 3.4 for further details), provided that extra phytase and lysine are added to meet nutrient requirements.

Treated Jatropha kernel meal material, prepared using the same method as described aboveand fed to carp and shrimp, replacing 50% fish meal protein in the diet (Kumar et al., 2010; Harter et al., 2011) did not produce adverse effects on growth performance. Biochemical and histological parameters in fish species, even after feeding for a longer term (12 weeks), also remained in the normal range.

Feeding of treated Jatropha protein isolate (see Section 3.3.3 for treatment method) (Makkar and Becker, 2010c) to Common Carp (*Cyprinus carpio*) fingerlings for 8 weeks, with diets in which the protein isolate replaced up to 75% of the fish meal protein, did not result in any alterations in haematological and clinical chemistry parameter or histological changes or in body mass, compared to the control (Kumar et al., 2011c; Makkar et al., 2012).

Growth and feed utilisation parameters in carp fingerlings were similar to those of the control when treated Jatropha protein isolate (see Section 3.3.3 for treatment method) was added to diets(up to 200 g/kg) (replacing the same amount of soya protein concentrate) in a 45-day trial (Shamna et al.,2015).

In conclusion, studies in fish and particularly carp, indicated the high sensitivity of these animal species to Jatropha PEs. Hence, experiments with carp have been used to demonstrate the efficacy of detoxification procedures.Although the available data do not allow the identification of a NOAEL for individual aquatic species, it can be deduced from the reported studies that anon-quantified concentration of PEs in Jatropha kernel meal which could maximally be the LOD of3 mg PEs/kg (expressed as TPA equivalent) used at inclusion ratesof up to 50% of the protein in feed are tolerated by all aquatic species tested (rainbow trout, carp and shrimp).

3.2.5. Observations in humans

Intoxications in humans have been described following the accidental ingestion of Jatropha seeds, particularly by children. Clinical symptoms include burning and pain in the mouth and the upper digestive tract, as well as vomiting. After ingestion of larger amounts, a shock-like syndrome with increased pulse rate, and neurological symptoms including delirium and loss of visionwas observed.

Most of the published data refer to case reports in which the actual exposure is incompletely described. For example, Shah et al. (2010) described five cases of Jatropha poisoning occurring in one family. All family members ingested between one andthree seeds, and signs of intoxications occurred within 10–15 minutes (min) with abdominal pain, vomiting, and increased pulse rates (which might be attributable partly to the pain and stress). Chomchai et al. (2011) described incidents of Jatropha intoxication in Thai children reported to the Poison control centre. Seventy-five cases were recorded over a period of 40 months, involving children in the age group between 2 and14 years who had ingested Jatropha seeds. The most common signs of intoxication were nausea, vomiting, diarrhoea and abdominal pain. The immediate and strong vomiting makes most of the intoxications self-limiting, as the ingested material is expelled from the stomach. In severe cases, symptomatic therapy in the form of fluid substitution might be indicated. In all cases intoxicated patients recovered spontaneously and uneventfully. The actual ingested amount of Jatropha PEs was not determined in the case reports.

A high incidence of oesophageal cancer among populations in Curaçao has been epidemiologically well-documented and is partly due to the high consumption of tea made from the leaves of the bush *Croton flavens L*, which belong to the family Eurphoriaceae and which are known to contain croton factors (diterpene esters of tigliane). The amounts of croton factors present in the tea are considered sufficient to maintain chronic irritation of the oesophagus, important for co-carcinogenesis and in particular tumour promotion (Hecker et al., 1983).

Some 10 years ago, TPA was used in clinical trials in humans suffering from recurrent malignancies, particularly haematological malignancies including severe forms of leukaemia (Strair et al., 2002; Schaar et al., 2006). The objective of this trial was the use of TPA as an agent to induce, at low doses,apoptosis and cell differentiation. The TPA application was based on current protocols for cytostatic agents, and involved 35 patients givena low dose constant rate infusion over a defined

period (here treatments on day 1–3 and 8–12 with a 2 weeks rest period until re-treatment, dose rate 0.063 mg/m^2 body surface). Various patients developed severe side effects following the treatment, such as transient fatigue, anaemia, neutropenia and thrombocytopenia, mild dyspnoea, nausea fever, rigor and cardiovascular effects with syncope and hypertension (the latter limiting the dose to 0.188 mg/m^2 body surface area), but only one patient exhibited a tumour response, consisting in a reduction in mass dimensions.Under conditions of daily administration for 5 consecutive days on 2 consecutive weeks, the maximum tolerated dose was 0.125 mg TPA/m^2, corresponding to approximately 0.22 mg TPA/day.

3.3. Treatments used for detoxification

As it is well-known that Jatropha seeds and kernels contain toxic Jatropha PEs and that these PEs are also present in de-oiled Jatropha kernel meal, the use of the kernel meal as a feed ingredient requires extraction or degradation of JatrophaPEs. Therefore, the TOR provided by the EC also requested an evaluation of the effectiveness of the various treatments described in the literature aiming to reduce the concentration of PEs and other anti-nutritional constituents to safe levels.An overview of the technical processes is given in the Sections 3.3.1, 3.3.2 and 3.3.3 and in Tables 3 to 5. Where feeding studies have been performed with the treated material, these have been indicated in the tablesand the findings of these studies are reported in Sections3.2.3.1, 3.2.4.1, 3.2.4.3 and 3.2.4.6.

The kernel meal, seed cake, seed meal and protein isolate (see Figure 2) have been subjected to various chemical, physical and biological treatments with the aim to reduce PE concentrations in the Jatropha material. In some studies 100% removal of PEs has been claimed. In all studies aiming at detoxification, Jatropha PEs have been measured by the HPLC-UV methods. However, in the absence of appropriate standards for Jatropha factors, their concentration has been expressed as equivalent of TPA. The LOD of the method used for PE determination has not been reported in most of these studies.

The following section reviews treatments used for the detoxification of Jatropha materials.

3.3.1. Jatropha kernel meal

Different methods for the reduction of Jatropha PEs have been evaluated and are summarised in Table 3, which also contains information on initial and end concentrations of PEs and whether feeding studies have been conducted.

Initial studies have shown that heat treatment alone is not effective in reducing the PE content. For example, Makkar and Becker (1997) observed a 5% reduction of PE levels in the kernel meal following heat treatment at 121°C for 30 min. Thereafter, Makkar and Becker (1998)reported that extraction (four times) with 80% aqueous ethanol or 92% aqueous methanol treatments of the heat treated (121°C, 30 min, 66% moisture) kernel meal containing < 1% oil [1:5 w/v; kernel meal: solvent] reduced Jatropha PEs by 95%.

Aregheore et al. (2003) observed a 95% reduction ofJatrophaPE content in kernel meal after heat treatment at 121°C for 30 min and washing with 92% aqueous methanol (four times). A reduction of 92% PE content was noted after alkali treatment with 4% sodium hydroxide and 10% sodium hypochlorite followed by heat treatment (at 121°C for 30 min).

Chivandiet al. (2004) reported that double solvent extraction (hexane-ethanol system) coupled with wet extrusion (126°C, 2 atmospheres for 10 min) and re-extraction with hexane and moist heat at 121°C, for 30 min, reduced PE levels by 87.7%.

Martínez-Herreraet al. (2006) found that extraction with 90% aqueous ethanol, followed by treatment with 0.07% $NaHCO_3$ and autoclaving at 121°C for 20 min reduced PE content in kernel meal by 98%, while a reduction of 96% was observed using 90% aqueous ethanol only.

Rakshit et al. (2008) treated kernel meal with aqueous solutions of either 2% sodium hydroxide or 2% calcium hydroxide in the ratio 1:1 (w/v), autoclaved it at 121°C for 30 min, dispersed in water in a ratio 1:5 (w/v) for 1 h, filtered and finally dried. This resulted in 90% and 88% reduction in PE content.

Gaur (2009) applied the principle of solid-liquid extraction in the treatment of ground Jatropha seed kernels. By using a Soxhlet extractor, and involving a sequential combination of hexane followed by methanol, PE content was reduced by 99.6%.

Makkar and Becker (2010a), reported a method involving extraction and inactivation of PEs in Jatropha kernel meal using 70–90%aqueous methanol containing 0.05 to 0.2 M sodium hydroxide at 50–70°C for 1 h, followed by washing with organic solvent. The PE concentration of the resultant material was <3 mg/kg.

Li et al. (2015) reduced the concentration of PEs in the kernel meal by 85.5%, using steam treatment and extraction with ethyl alcohol (55°C for 2 h).

Nokkaew and Punsuvon et al. (2015) used aqueous ethanol (concentration not given) to remove Jatropha PEs from the hexane-de-oiled kernel meal. A two-stage extraction at 1:3 (w/v) of de-oiled meal to aqueous ethanol at 50°C for 30 min, reduced the PEs by 96.6%.

Xiao et al. (2011) treated kernel meal with enzymes (cellulase plus pectinase; 50°C and pH 4.5–5.0 for 1 h) followed by washing with aqueous ethanol (65%) or aqueous methanol (60%) with stirring at 50°C for 1 h and reported a reduction in PE level by 100% (LOD not reported).

Najjar et al. (2014) studied degradation of the extracted PE-rich fraction from Jatropha kernels in broth cultures by fermentation with non-pathogenic fungi (*Trichoderma harzianum* JQ350879.1, *T. harzianum* JQ517493.1, *Paecilomyces sinensis* JQ350881.1, *Cladosporium cladosporioides* JQ517491.1, *Fusarium chlamydosporum* JQ350882.1, *F. chlamydosporum* JQ517492.1 and *F. chlamydosporum* JQ350880.1). At day 30 of incubation, two *T. harzianum* spp., *P. sinensis* and *C. cladosporioides* removed PEs with percentage losses of between 92 and 97.8%, while *F. chlamydosporum* strains showed percentage losses of between 86 and 90%.

Table 3: A summary of the results on detoxification of Jatropha kernel meal

Treatment	PE concentration before treatment (mg/g)[(a)]	PEconcentration after treatment (mg/g) [(a)]	Reduction in PE (%)	Feeding studies conducted (Y/N)	Reference
Heat treatment (121°C, 30 min, 66% moisture)	1.81	1.72[(b)]	5	Y (rats, chicken, fish)	Makkar and Becker (1997)
80% aqueous ethanol or 92% aqueous methanol	1.81	0.09[(b)]	95	Y (rats)	Makkar and Becker (1998)
Heat treatment (121°C for 30 min and 92% aqueous methanol wash per 4 times)	1.78	0.09	95	Y (rats)	Aregheoreet al.(2003)
Alkali treatment with 4.0% sodium hydroxide (w/w) and 10% sodium hypochlorite followed by heat treatment (121°C for 30 min)	1.78	0.13	92	Y (rats)	Aregheore et al. (2003)
Double solvent extraction (hexane-ethanol) coupled with wet extrusion (at 126°C, 2 atmospheres, 10 min) and re-extraction with hexane and moist heat (121°C for 30 min)	6.50	0.80	88	N Y (pigs)	Chivandi et al. (2004) Chivandi et al. (2006)
Extraction with 90% aqueous ethanol	3.85	0.16	96	N	Martínez-Herrera et al. (2006)
Extraction with 90% aqueous ethanol, followed by treatment with 0.07% sodium bicarbonate followed by autoclaving (121°C for 30 min)	3.85	0.08	98	N Y (rats)	Martínez-Herrera et al. (2006) Rahma et al. (2013)
Alkali treatment (2% sodium hydroxide or 2% calcium hydroxide) combined with heat treatment (autoclaved at 121°C for 30 min), followed by washing with water	1.35	0.136 (sodium hydroxide) 0.16 (calcium hydroxide)	90 (sodium hydroxide) 88 (calcium hydroxide)	Y (rats)	Rakshit et al. (2008)
Sequential (solid-liquid) extraction: hexane and then methanol	6.05	0.06	99.6	N	Gaur (2009)
Extraction using 70–90% methanol containing 0.05–0.2 M sodium hydroxide, (at 50–70°C for 1 h),followed by treatment to inactivate trypsin inhibitor and lectins.	2.79[(c)]	< LOD 3 µg/g[(c)]	>99	Y (carp, trout, shrimp and growing pigs)	Harter et al. (2011); Kumar et al. (2010, 2011b); Makkar and Becker (2010a); Wang et al.(2011)
Steam treatment and extraction with ethyl alcohol (concentration not given), (at 55°C for 2 h).	0.76	0.11	85.5[(b)]	Y (pigs)	Li et al. (2015)
Aqueous ethanol (concentration not given), 2 stage extraction at 1:3 (w/v) (50°C for 30 min)	0.6555	0.0228	96.5	N	Nokkaew and Punsuvon et al. (2015)

Treatment	PE concentration before treatment (mg/g)[a]	PEconcentration after treatment (mg/g) [a]	Reduction in PE (%)	Feeding studies conducted (Y/N)	Reference
Enzyme treatment, followed by extraction with 65% aqueous ethanol or 60% aqueous methanol (50°C for 1 h)	2.88	Undetectable[d]	Close to 100[d]	N	Xiao et al. (2011)
Submerged fermentation with non-pathogenic fungi:					
Trichoderma harzianum JQ350879.1	2.78	0.06	97.8	N	Najjar et al. (2014)
Trichoderma harzianum JQ517493.1	2.78	0.11[b]	96.0		
Paecilomyces sinensis JQ350881.1	2.78	0.16[b]	94.0		
Cladosporium cladosporioides JQ517491.1	2.78	0.22[b]	92.0		
Fusarium chlamydosporum JQ350882.1	2.78	0.28[b]	90.0		
Fusarium chlamydosporum JQ517492.1	2.78	0.30[b]	89.0		
Fusarium chlamydosporum JQ350880.1	2.78	0.39[b]	86.0		

h: hour; min: minutes; N: no; PE: Phorbol ester; Y: Yes, if yes, animal species in parentheses.
(a): as TPA (12-*O*-tetradecanoylphorbol-13-acetate) equivalents and measured by HPLC-UV.
(b): Calculated value.
(c): Obtained from author's laboratory.
(d): Limit of detection not reported.

In addition to the above studies, treatments for Jatropha kernel meal have also been applied in studies by Gross et al. (1997), Belewu et al. (2010), Brooker (2011), Wang et al. (2013), and in some cases feeding studies have also been performed. However, as the PE concentration (before and/or after treatment) is not given, the treatment details have not been included in this section.

3.3.2. Jatropha seed cake

Different methods for the reduction of PEs in Jatropha seed cake have been evaluated and are summarised in Table 4, which also contains information on initial and end concentrations of PEs and whether feeding studies have been conducted.

El Diwani et al. (2011) evaluated a number of chemical treatments using sodium bicarbonate, ozonation, and ethanol extraction. The maximum Jatropha PE removal (76.5%) was with 0.075%sodium bicarbonate treatment when combined with heat treatment (121°C for 30 min), while with 0.075%sodium bicarbonate moist treatment combined with 3 min of ozone flushing at an ozone dose of 50 mg/L, reduced PE concentration by 75.3%.

Katole et al. (2011) after treating Jatropha seed meal with sodium chloride at 10 g/kgDM or calcium hydroxide at 5 g/kg DM, together with roasting at 100°C for 30 min reported reductions inPE concentrations by 85% and 83.2%, respectively.

Phasukarratchai et al. (2012) treated Jatropha seed cake with surfactant solutions (non-ionic and anionic) and observed reductions in Jatropha PE levels of between 78% and 82%.

Pighinelli et al. (2012) subjected Jatropha seed cake to various treatments with aqueous methanol or ethanol, with and without heat treatment. Two of the methods applied, namely (i) methanol (100%) treatment for 6 h in a Soxhlet with heating, and (ii) 40% aqueous methanol extraction for 2 h at room temperature, reduced Jatropha PEs to undetectable levels (level of detection not reported).

Elangovan et al. (2013) found that treatment with 3% sodium hydroxide or sodium bicarbonate reduced Jatropha PEs by 55%.

Baocai et al. (2014) , reported reductions of Jatropha PEs of $\geq$ 99.8% (LOD not reported) after treatment of Jatropha seed cake with hydrogen peroxide, followed by alkali (sodium hydroxide, potassium hydroxide or sodium carbonate) treatment to bring pH between 7.5 and 8.5 and then stirring at 40–70°C for 2 to 12 h.

Guedes et al. (2014), using a mixture of 50% of aqueous methanol (extraction time of 8 h and solute/solvent ratio of 1:10 w/v), observed a reduction in Jatropha PEs of 97.3%.

de Barros et al. (2011) used solid state fermentation (SSF) with the fungi, *Bjerkandera adusta* or *Phebia rufa* (at 28°C for 30 days) and showed reduced Jatropha PE content in the seed cake by 91% and 97%, respectively).

Joshi et al. (2011) applied SSF to seed cake using *Pseudomonas aeruginosa* PseA strain, and found that Jatropha PE levels were undetectable (LOD not reported) after 9 days under optimised conditions (30°C, pH 7.0 and relative humidity 65%). Jatropha PE contents were not reported for the treated or the untreated Jatropha seed cake.

De Oliveira et al. (2012) applied the technique of ensiling to Jatropha seed cake, by the addition of soluble carbohydrates and inoculants comprising of *Lactobacillus plantarum* and *Propionibacterium*, for 60 days at room temperature. Jatropha PEs levels were reduced (by 47%).

Kasuya et al. (2013) reported a 99% reduction in PE levels following 45 days of incubation with the fungi *Pleurotus ostreatus.*

Kurniati (2012) observed that fermentation of the seed cake with a combination of *Aspergillus niger* and *Neurospora sitophila* reduced PE concentration by 79.7%.

Phengnuam and Suntornsuk (2013) used submerged fermentation (5 days) with *Bacilluslicheniformis* and found that PE levels were decreased by 62%.

Bose and Keharia (2014) evaluated 10 different fungi and found that following incubation at 30°C for 20 days *Ganoderma lucidum* and *Trametes zonata* degraded PEs in the seed cake to undetectable levels (LOD not reported).

da Luz et al. (2014) observed that after 60 days of incubation with the fungus *Pleurotus ostreatus*, PE concentration in Jatropha seed cake was reduced by 99%.

Hidayat et al. (2014) used rice bran lipase to degrade PEs. The addition of 0.82 g of the lipase into 5 g defatted seed cake in a pH 7 buffer at 30°C resulted in a decrease in PEs of about 99.4% over a period of 16–20 h of incubation.

Sharath et al. (2014) used fungal culture *Cunninghamella echinulata* CJS-90 in a SSF with Jatropha seed cake and noted a 75% reduction in PE levels, following 12 days fermentation at 30°C.

Veerabhadrappa et al. (2014) used *Aspergillus versicolor* CJS-98 in a SSF with Jatropha seed cake and observed an 81% reduction in Jatropha PE levels.

El Diwani et al. (2011) evaluated treatment of Jatropha seed cake with gamma irradiation at 50 kGy. A reduction in PEs of 71.4% was observed.

Gogoi et al. (2014) showed that exposure of seed cake to gamma irradiation between 30 kGy to 125 kGy, decreased Jatropha PE levels by 33.4% to 95.8%, respectively.

A range of treatments of Jatropha seed cake were examined by Sadubthummarak et al. (2013) with the following results: (a) sunlight (40°C) or heating in an oven at temperature varying from 80–220°C reduced Jatropha PEs by 1.81–28.18%; (b), heating of the seed cake mixed with 10% bentonite at 220°C for 1 h reduced Jatropha PEs levels by 69.7%; (c). the application of zinc oxide nanoparticles (100 ppm) in combination with varying temperatures of 80–220°C reduced Jatropha PEs by 2.43–20.98%; (d) the addition of 300 ppm of zinc oxide nanoparticles in combination with heat (220°C), together with alkaline (4% sodium bicarbonate), resulted in 51.7% removal of PEs, and (e) heating at 120°C or 220°C for 1 h mixed with 10% bentonite, and 100 ppm of zinc oxide and 4% sodium bicarbonate followed by a 4-week incubation, reduced Jatropha PEs by 97.5–98.0%.

Masten et al. (2015) using an ozone dose of 8.14 mg/g of seed cake reduced the Jatropha PEs by 82.5%. In addition, the effect of sunlight exposure (solar radiation) at different durations of up to 72 h was explored, and achieved a reduction in Jatropha PEs of 77.9%.

Table 4: A summary of the results on detoxification of Jatropha seed cake

Treatment	PE concentration before treatment(mg/g)[a]	PE concentration after treatment (mg/g)[a]	Reduction in PE (%)	Feeding studies conducted (Y/N)	Reference
Alkaline treatment (moistened with 0.075%sodium bicarbonate) combined with heat treatment (121°C, 30 min)	0.3766–1.193 (average 0.637)	Not reported	76.5[d]	N	El Diwani et al. (2011)
Alkaline treatment (moistened with 0.075%sodium bicarbonate), followed by 3 min of treatment with ozone at a dose of 50 mg/L	0.3766–1.193 (average 0.637)	Not reported	75.3[d]	N	El Diwani et al. (2011)
Treatment with either: sodium chloride at 10 g/kg DM)	2.1[e]	0.315[e]	85	Y (sheep)	Katole et al. (2011)
calcium hydroxide at 5 g/kg DM)		0.355[e]	83.2		
Treatment with nonionic and anionic surfactants:					
40 mmol/L Tween 80	1.45	0.27	81.4		
40 mmol/L Tween 80 and 5 mmol/L AOT at 100 mmol/Lsodium chloride	1.45	0.27	81.2	N	Phasukarratchai et al. (2012)
40 mmol/L Dehydol LS9	1.45	0.26	81.9		
40 mmol/L Dehydol LS9 and 5 mmol/L AOT at 100 mmol/Lsodium chloride	1.45	0.31	78.8		
Methanol (100%) treatment for 6 h in a Soxhlet with heating	1.28	Undetectable[c]	Close to 100[c]	N	Pighinelli et al. (2012)
40% aqueous methanol extraction for 2 h at room temperature	1.28	Undetectable[c]	Close to 100[c]		
3% sodium hydroxide or sodium bicarbonate	1.29	0.58	55	Y (lambs)	Elangovan et al. (2013)
Treatment with hydrogen peroxide, followed by alkali (sodium hydroxide, potassium hydroxide or sodium carbonate) treatment (pH between 7.5 and 8.5) and then stirring at 40–70°C for 2 to 12 h:					Baocai et al. (2014)[e]
Sodium hydroxide/pH 8.0/stirring 50°C for 10 h	3.12	≤0.01	≥99.7	N	
Sodium hydroxide/pH 8.0/stirring 55°C for 8 h	4.05	≤0.01	≥99.8	N	
Sodium hydroxide/pH 8.0/stirring 60°C for 8 h	1.13	≤0.01	≥99.1	N	

Treatment	PE concentration before treatment(mg/g)[a]	PE concentration after treatment (mg/g)[a]	Reduction in PE (%)	Feeding studies conducted (Y/N)	Reference
Potassium hydroxide/pH 8.0/stirring 40°C for 12 h	Not reported	Not reported	≥99.5	N	
Sodium carbonate/pH 8.0/stirring 70°C for 2 h	Not reported	Not reported	≥99.3	N	
50% of aqueous methanol (extraction time of 8 h and solute/solvent ratio of 1:10 w/v)	3.60	0.10	97.3	N	Guedes et al. (2014)
Solid state cultivation with fungi:					
Bjerkandera adusta	0.66	0.06	91	N	de Barros et al. (2011)
Phebia rufa	0.66	0.02	97		
Solid state fermentation (9 days, 30°C, pH 7.0 and relative humidity 65%) using *Pseudomonas aeruginosa* PseA strain	Not reported[d]	Undetectable[c]	Close to 100[c]	N	Joshi et al. (2011)
Ensiling (60 days) by adding soluble carbohydrates in the cake and inoculant of *Lactobacillus plantarum* and *Propionibacterium*	0.424	0.223	47	N	De Oliveira et al. (2012)
Fermentation (45 days) using fungi *Pleurotus ostreatus*	1.09	0.0018	99	Y (goats)	Kasuya et al. (2013)
Fermentation (96 h) using combination of *Aspergillus niger* and *Neurospora sitophila*	7.19	0.0015	79.7	N	Kurniati (2012)
Submerged fermentation with *Bacilluslicheniformis* for 5 days	119.9	0.0394	62	N	Phengnuam and Suntornsuk (2013)
Fermentation (30°C for 20 days) using fungi, *Ganoderma lucidum* and *Trametes zonata*	1.07	Undetectable[c]	Close to 100[c]	N	Bose and Keharia (2014)
Solid state fermentation (60 days) using fungi *Pleurotus ostreatus*	1.07	0.002[c]	99	N	da Luz et al. (2014)
Treatment with rice bran lipase (0.82 g) at 30°C for 16–20 h	0.98[b]	0.006	99.4	N	Hidayat et al. (2014)
Solid state fermentation with *Cunninghamella echinulata* CJS-90 (12 days at 30°C).	0.83	0.2[b]	75	N	Sharath et al. (2014)
Solid state fermentation using *Aspergillus versicolor* CJS-98	0.832	0.158	81.1	N	Veerabhadrappa et al. (2014)
Gamma irradiation 50 kGy	0.3766	0.1077	71.4	N	El Diwani et al. (2011)
Gamma irradiation:					
30 kGy	0.29	0.19	33.4		
50 kGy	0.29	0.064	78.0		
70 kGy	0.29	0.057	80.5	N	Gogoi et al. (2014)
100 kGy	0.29	0.024	92.0		
125 kGy	0.29	0.012	95.8		
Sunlight (40°C) or heating in an oven at temperatures varying from 80–220°C:				N	Sadubthummarak et al. (2013)
40°C for 1h	2.20	2.16	1.81		
80°C for 1/2h	2.20	2.07	5.9		

Treatment	PE concentration before treatment(mg/g)[(a)]	PE concentration after treatment (mg/g)[(a)]	Reduction in PE (%)	Feeding studies conducted (Y/N)	Reference
80°C for 1h	2.20	1.93	12.3		
120°C for 1/2h	2.20	1.74	2.1		
120°C for 1h	2.20	1.66	24.5		
220°C for 1/2h	2.20	2.03	7.7		
220°C for 1h	2.20	1.58	28.2		
Heating mixed with 10% bentonite at 220°C for 1 h	2.18	0.66	69.7	N	Sadubthummarak et al. (2013)
Zinc oxide nanoparticles (100 ppm) treatment at temperature varying from 80–220°C for 1 h:					
80°C	2.05	2.00	2.4	N	Sadubthummarak et al. (2013)
120°C	2.05	1.92	6.3		
220°C	2.05	1.62	21.0		
Zinc oxide nanoparticles (300 ppm) in combination with heat (220°C) and 4% sodium bicarbonate)	2.05	0.99	51.7	N	Sadubthummarak et al.(2013)
Heating at 120°C or 220°C for 1 h mixed with 10% bentonite, and 100 ppm of zinc oxide and 4% sodium bicarbonate (4-week incubation)	2.01	0.05–0.04	97.5–98.0	N	Sadubthummarak et al. (2013)
Ozonation, 8.14 mg ozone/g seedcake	0.078	0.014	82.5	N	Masten et al. (2015)
Solar radiation, 5 cm thickness of seed cake, turned 3-times daily at 4 h interval and treatment time 72 h	0.078	0.017	77.9	N	Masten et al. (2015)

AOT: sodium bis (ethylhexyl) sulfosuccinate; h: hour(s); min: minutes; PE: Phorbol ester; N: no; Y: Yes, if yes, animal species in parentheses.
(a): as TPA (12-*O*-tetradecanoylphorbol-13-acetate); equivalents and measured by HPLC-UV.
(b): calculated value;
(c): limit of detection not reported;
(d): PE reduction determined from the areas of peaks obtained using HLPC;
(e): calculated from graph.

In addition to the above studies, treatments for Jatropha seed cake have also been applied in studies by Chandrasekar et al. (2009) and Okukpe et al. (2012), where feeding studies have also been performed. However, as PE concentrations before and/or after treatment are not given, the treatment details have not been included in this section.

3.3.3. Jatropha seed meal and protein isolate

Different methods for the reduction of PEs in Jatropha seed meal and protein isolate have been evaluated and are summarised in Table 5, which also contains information on initial and end concentrations of PEs and whether feeding studies have been conducted.

Rakshit et al. (2008) treated defatted seed meal with aqueous solutions of either 2% sodium hydroxide or 2% calcium hydroxide in the ratio 1:1 (w/v), autoclaved it at 121°C for 30 min, dispersed in water in a ratio 1:5 (w/v) for 1 h, filtered and finally dried. This resulted in 71% and 89% reduction in PE.

Devappa and Swamylingappa (2008) obtained the protein isolate by subjecting the solubilised proteins obtained from both the seed cake (atpH 10.5) and the kernel meal, followed by steam treatment at 92°C for 10 min and dropping the pH to 5.5 and then washing the protein isolate with water. Following this treatment Jatropha PEs were not detectable in protein isolate obtained from both the seed cake and kernel meal (LOD not reported).

In Makkar and Becker (2010c), a procedure for the preparation of treated protein isolate is described. The method involves bringing a warm (approximately 60°C) aqueous mixture of Jatropha seed cake or kernel meal to pH 11 by adding sodium hydroxide, separating solubilised proteins from the insoluble fraction using a centrifuge, bringing the pH of the solubilised proteins to 8.0, adding to it ethanol to bring ethanol level to 80% to precipitate the proteins and finally washing the protein isolate using ethanol. Using this procedure Jatropha PEs were not detected in the protein isolate (LOD 3 mg/kg).

Shamna et al.(2015) subjected protein isolate prepared by iso-electric precipitation to SSF with *Aspergillus niger* for 7 days. Jatropha PEs were not detected in the fermented protein isolate (LOD not reported).

Table 5: A summary of the results on detoxification of Jatropha seed meal and protein isolate

Treatment	PE concentrationbefore treatment (mg/g)[(a)]	PE concentration after treatment (mg/g)[(a)]	Reduction in PE (%)	Feeding studies conducted (Y/N)	Reference
Seed meal Alkali treatment (2% sodium hydroxide or 2% calcium hydroxide) combined with heat treatment (autoclaved it at 121°C for 30 min), followed by washing with water	0.74	0.14 (sodium hydroxide) 0.081 (calcium hydroxide)	71 (sodium hydroxide) 89 (calcium hydroxide)	Y (rats)	Rakshit et al. (2008)
Protein isolate Steam treatment (92°C, 10 min) of alkali solubilised proteins followed by protein precipitation at pH 5.5	0.72 (seed cake) 1.35 (kernel meal)	Undetectable[(b)]	Close to 100[(b)]	N	Devappa and Swamylingappa (2008)
Protein isolate Alkali solubilisation of proteins followed by protein precipitation at pH 8 using ethanol	1.48[(c)]	<LOD 3 µg/g	>98	Y (Carp fingerlings)	Kumar et al. (2011c); Makkar and Becker (2010c); Makkar et al. (2012)
Protein isolate Solid state fermentation of protein isolate using *Aspergillus niger* for 7 days	1.4	Undetectable[(b)]	Close to 100[(b)]	Y (Indian major carp fingerlings)	Shamna et al. (2015)

h: hour(s); LOD: limit of detection; min: minutes; N: no; PE: Phorbol ester; Y: Yes, If yes, animal species in parentheses.
(a): as TPA equivalents and measured by HPLC-U;
(b): limit of detection not reported;
(c): Source: Makkar et al. (2008).

3.3.4. Summary of treatments used for detoxification

Detoxification treatments used on Jatropha products to remove, degrade or inactivate PEs fall in three main categories: chemical treatments, biological treatments and physical treatments. The chemical treatments involve the use of a number of aqueous alkalis and organic solvents, alone or in combination, resulting in substantial lower PEs in the treated material. In some studies the PEs in the treated materials were undetectable. Biological treatments have used a number of fungi and other microorganisms in submerged or solid-state fermentation systems. Some microbial treatments alone resulted in products in which PEs were not detectable or were present at very low levels. The comparison of the different methods is hampered by the fact that in many studies in which PEs were undetectable after treatment, the exact analytical procedure and the limit of detection have not been reported. The most commonly applied HPLC-UV method for the quantification of PEs in Jatropha feed materials reaches an LOD of 3 mg PEs/kg (expressed as TPA equivalent). Therefore it seems necessary to include the outcome of feeding trials in the final assessment of the efficacy of detoxification methods. Such feeding trials are also needed, as the nature and chemical composition of the degradation products of PEs remains unknown and in order to assess if the treatments also reduce the presence of anti-nutritional constituents.

3.4. Feed consumption and exposure to Jatropha PEs

Currently the seeds of Jatropha, together with their processed derivatives, may only be present in feed materials and compound feeds for livestock and companion animals in the EU in trace amounts that are not quantitatively determinable. This measure was taken, because Jatropha seeds and kernels can occur as botanical impurities in other feed materials. As non-treated seeds and kernels are highly toxic, such botanical impurities needed to be avoided and an assessment of non-treated Jatropha seeds and kernels is not relevant.

3.4.1. Potential exposure to residual amounts of Jatropha PEs present in treated materials

In accordance with the TOR, a quantitative assessment of the potential exposure to residual amounts of PEs after a treatment/detoxification steps has been undertaken, using the approach outlined in Section 2.2.2 (details given in Appendix C). In estimating potential exposure, the CONTAM Panel noted that feed materials derived from the Jatropha seed contain relatively high levels of crude protein. Concentrations of up to 65% have been reported, which compares to other protein-rich feed materials widely used in diets for livestock and companion animals, such as soya bean meal (SBM), rapeseed meal and fish meal which contain 40–45%, 35–39% and 60–65% crude protein in the DM, respectively Furthermore, with the exception of lysine, the levels of essential amino acids in treated Jatropha meal are even higher than in SBMs (Makkar and Becker, 2009).

Compared to the more widely used protein-rich feeds in animal diets, there is relatively little information on the maximum or optimal inclusion rates of treated Jatropha products in livestock diets. Most research has been undertaken with aquatic species (carp, trout and shrimp) with some limited studies on pigs (Makkar et al., 2012; Wang et al., 2011; Li et al., 2015). Kasuya et al. (2012) reported a study in which goats were fed diets containing up to 20% treated Jatropha seed cake (see Section 3.2.4.3), with no apparent adverse effects on feed intake or any of the blood parameters examined. In this study the maximum feed dry matter intake was observed in the control group (receiving no Jatropha seed cake), but this was only1.8% of body weight. One of the effects of Jatropha intake by livestock is a reduction in feed intake, but the levels of feed intake in this study may have been too low (in all groups) for the Jatropha meal to have this effect. Therefore caution is needed in extrapolating the results of this study to more productive animals with higher levels of feed intake.

Makkar et al. (2012) concluded from studies with fish (rainbow trout) that treated Jatropha meal (containing < 3 mg PEs/kg) could replace 50% of fishmeal protein in fish diets without adversely affecting growth, nutrient utilisation, and physiological or haematological parameters. Similarly, Wang et al. (2011) showed that treated Jatropha kernel meal could replace 50% of SBM protein in the

diets of growing pigs with no significant differences in growth rate or feed conversion efficiency compared to the control treatment. However, in many livestock diets – particularly for ruminants – SBM or fishmeal protein may not be commonly used.

Based on these considerations, estimates of exposure by livestock have been made where 50% of the protein provided by compound or complementary feeds in 'conventional' diets is replaced by protein from non-toxic Jatropha kernel meal. In making these estimates it has been assumed that the treated material contains 3 mg PEs/kg dry matter (DM) and that the diets are appropriately supplemented with lysine. This resulted in the potential total intake of Jatropha kernel meal and estimates of exposure to PEs as given in Table 6. Based on the assumptions given above, the highest estimated daily exposure to PEs is 0.04 mg PEs/kg bw for rabbits. For poultry and pigs, daily exposure levels of 0.031mg PEs/kg bw (broilers) and 0.026 mg PEs/kg bw (pig starters), respectively, are predicted. For ruminantsand horses, where forages represent a major part of the ration, maximum daily exposuresare lower (0.017 mg PEs/kg bw for goats and 0.004 mg PEs/kg bw for horses) (Table 6).

Table 6: The amounts of Jatropha meal (JM) required to replace 50% of the protein supplied by the compound feeds in livestock diets and the effect on PE intake (or exposure), where PE content of JM is 3.0 mg/kg dry matter (DM)

Livestock	Protein supplied by compound feed (g/day)	Replacing 50% of the protein provided in the compound feed			
		Amount of JM DM required to replace 50% protein (kg/day)[(a)]	PE intake mg/day[(a)]	PE intake mg/kg DM[(a)]	PE intake mg/kg bw per day[(a)]
Dairy: high yielding	1694	1.30	3.91	0.189	0.006
Beef: cereal-based diet	1352	1.04	3.12	0.312	0.008
Beef: forage-based diet	349	0.27	0.81	0.084	0.002
Lactating sheep	286	0.22	0.66	0.236	0.011
Lactating goats	452	0.35	1.04	0.307	0.017
Fattening goats	109	0.08	0.25	0.168	0.006
Horses	818	0.63	1.89	0.210	0.004
Pig starters	227	0.17	0.52	0.524	0.026
Pig finishers	477	0.37	1.10	0.367	0.011
Lactating sows	1159	0.89	2.67	0.446	0.013
Broilers: growers	27	0.02	0.06	0.524	0.031
Laying hens	26	0.02	0.06	0.498	0.030
Turkeys: growers	91	0.07	0.21	0.524	0.017
Ducks: growers	29	0.02	0.07	0.472	0.022
Salmonids	9	0.01	0.02	0.524	0.010
Rabbits	34	0.03	0.08	0.524	0.039
Cats	19	0.01	0.04	0.734	0.011
Dogs	102	0.08	0.24	0.656	0.009

bw: body weight; PE: Phorbol ester.
(a): JM crude protein(CP) content=650 g/kg DM; PE content=3 mg/kg DM.

3.5. Derivation of health based guidance values

3.5.1. Health based guidance value in humans

Only limited information is available on the toxicity of Jatropha PEs. Most of the studies in experimental animals have been carried out for Jatropha-derived products without information on the doses of PEs administered. When available, dose information is expressed as TPA-equivalents in the lack of standards for analysis. An acute oral LD_{50} of 27 mg PEs (TPA equivalent)/kg bw in mice was derived by Li et al. (2010). Effects including severely reduced feed intake and body weight, diarrhoea and difficulties in motor function were observed following short-term exposure in rats at doses as low as 2.4 mg PEs(TPA equivalent)/kg bw (Rakshit et al., 2008). The CONTAM Panel identified an NOAEL of 0.4 mg PEs (TPA equivalent)/kg bw per day(based on decreases in body weight gain and feed intake) from a 79-day study in pigs (Li et al., 2010). There is insufficient evidence to conclude on possible effects of Jatropha PEs on reproduction and development and there is no information on long-term effects of Jatropha PEs. In addition, no genotoxicity studies are available for Jatropha PEs. A read-across comparison with the structural analogue TPA, a well-known non-genotoxic tumour promoter, indicated similar but also additional structural alerts for genotoxicity, which suggests that more data are needed to conclude on the possible genotoxic potential of Jatropha PEs.

Overall, the CONTAM Panel concluded that it is not possible to derive a health based guidance value for humans for individual Jatropha factors due to the aforementioned limitations in datasets.

3.5.2. No-Observed-Adverse-Effect Levels in farm animals

Only one study could be identified that allowed the identification of a no-observed-adverse-effect level (NOAEL) in farm animals. In the 79-day study in pigs of Li et al. (2015) only a limited number of haematological and blood chemistry parameters were tested in addition to feed intake, weight gain and feed conversion and was presented together with histological findings with insufficient quality. The clear dose-effect relationship noted for the feed intake and body weight gain data justifies its use for hazard characterisation. Using these data the CONTAM Panel identified a NOAEL of 0.4 mg PEs (TPA equivalent)/kg bw per day for pigs based, calculated from feed consumption and body weight at the start of the study.

Rainbow trout, carp and shrimp tolerated feed in which 50% of the protein was replaced with treated Jatropha kernel meal containing a non-quantified concentration of PEs which was below3 mg PEs/kg(again expressed as TPA equivalent, the LOD for the method of analysis used).

It was not possible to identify NOAELs for ruminants, horses, poultry species, aquatic species and companion animals. For ruminants, there is no evidence that rumen microorganisms degrade PEs, and therefore there is no reason to consider these species as less sensitive than monogastric animals to dietary exposure to PEs from Jatropha products. In lambs, severe effects were observed at an exposure of 1.2 mg PEs/kg bw per day (in TPA-equivalents), indicating a possible higher sensitivity than in other species.

3.6. Risk characterisation

3.6.1. Human health risk characterisation

As Jatropha products are not intended for human consumption, exposure to humans could only occur from residues of PEs in animal derived products, originating from animals given treated Jatropha kernel meal. However, the transfer of Jatropha PEs to animal derived products isunknown (see Section 3.2.2.5).

In a hypothetical scenario, considering a daily intake of 3.9 mg PEs per day for a high-yielding cow fed with a diet where 50% of proteins were replaced by Jatropha material containing 3 mg PEs/kg DM (see Table 6), and assuming, as a conservative approach, a transfer rate of 50% for Jatropha PEs from feed

into cow milk and a daily milk production of 40 L, the estimated Jatropha PE concentration in milk would be approximately 49 µg/L. Assuming a daily milk consumption of 1.5 L by a 70 kg adult, this would correspond to a daily intake of about 1 µg PEs/kg bw per day, i.e. about 400 times lower than the NOAEL of 0.4 mg PEs (TPA equivalent)/kg bw per day identified in pigs. Due to the limitations of the study in pigs from which the NOAELwas identified, and the ability of PEs to activate PKCas well as the structural alerts for genotoxicity,this MOE is not sufficient to conclude that human health risk is low.Therefore, no firm conclusions can be drawn on human health risks in the absence of sufficient data on toxicity and transfer from feed to animal derived foods.

3.6.2. Animal health risk characterisation

The CONTAM Panel estimated animal exposure levels in a scenario in which 50% of the 'conventional' vegetable or animal proteins in compound or complementary feeds is replaced by Jatropha material containing 3 mg/kg DM,equal to the limit of detection for the reference TPA in analytical methods used in most studies on detoxification. Under this scenario, exposure estimates ranged from 0.002 mg PEs/kg bw for ruminants (fattening beef cattle on a forage-based diet) to 0.04 mg PEs/kg bwfor rabbits (see Table 6).

Considering the identified NOAEL of 0.4 mg PEs (TPA equivalent)/kg bw per dayin pigs(based on decreases in body weight gain and feed intake), and the estimated exposure of up to 0.026 mg PEs/kg bw per day in pigs,the CONTAM Panel concluded that replacing up to 50% of feed protein with treated Jatropha material with 3 mg PEs/kg DM or less would not pose a health risk to pigs.

Ruminants may be at least as sensitive as monogastric animal species, also based on effects observed in lambs exposed to 1.2 mgPEs/kg bw per day. No adverse health effects were identified in aquatic species (carp, trout, and shrimp) when Jatropha kernel meal with a maximum of 3 mg PEs/kg meal (equal to the LOD for TPA) was used as protein replacement in animal diets with a maximum inclusion rate of 50% of the total protein content.

Under the condition that Jatropha products replace up to 50% of the feed proteins, the CONTAM Panel considers that a 10-fold lower exposure to Jatropha PEs than the NOAEL in pigs would be associated with a low risk for adverse effects also in other farm animals (including farmed aquatic species) or companion animals.

The CONTAM Panel noted that for all species, the estimated exposure is 10-to 200-fold lower than the NOAEL in pigs, indicating that the risks to other species (including farmed aquatic species) is likely to be low under these conditions.

3.7. Uncertainty analysis

3.7.1. Assessment objectives

The objectives of the assessment were clearly specified in the terms of reference. There was no uncertainty in addressing these objectives.

3.7.2. Exposure scenario/Exposure model

There is considerable variation in both the feeds used and the feeding systems adopted throughout Europe for farm livestock, companion animals and fish. This variation is largely due to the availability of feeds and market demands for specific animal products, together with variations in the nutritive value of the feed and the nutritional requirements of the animal. As a result there is uncertainty in estimates of feed intake by the different livestock species and therefore potential animal exposure.

3.7.3. Other uncertainties

Due to the lack of authentic reference materials for Jatropha PEs, the analysis of PEs is currently expressed in TPA-equivalents, creating a high level of uncertainly about the true concentrations. Also,

the lack of knowledge about the chemical stability of Jatropha PEs during extraction from feed products or tissues adds to the uncertainty of the analytical values. The absorption and excretion of Jatropha PEs and TPA after oral ingestion have not been studied and thiscreates a high level of uncertainty. The levels of Jatropha PEs in animalderived food products are considered to be limited due to the low exposure of food-producing animals when levels in detoxified materials are below 3 mg/kg. However, as only in two studies an attempt was made to measure PEs in the liver of pigs and goats (not detectable), a high level or uncertainty remains. This includes the lack of information on potential metabolites. In addition, in treated materials, the chemical nature of the degradation products and their potential toxicity is unknown. The NOAEL value in pigs was based on body weight gain and feed intake data from a 79 day study and the NOAEL was considered to be conservative because it was calculated using initial body weight measurements. Moreover, toxicity testing with treated materials hasonly been conducted in a limited number of species for a few endpoints, leaving their toxicity to other species uncertain. In addition, no genotoxicity studies are available for Jatropha PEs.

3.7.4. Summary of uncertainties

In Table 7, a summary of the uncertainty evaluation is presented, highlighting the main sources of uncertainty and indicating an estimate of whether the respective source of uncertainty might have led to an over- or underestimation of the exposure or the resulting risk.

Table 7: Summary of qualitative evaluation of the impact of uncertainties in this risk assessment

Sources of uncertainty	Direction[(a)]
Use of TPA as a surrogate for Jatropha PEsin the chemical analysis of feed material and animal derived products.	+/–
Use of TPA as a surrogate for Jatropha PEs in kinetic and biotransformation studies.	+/–
Lack of studies describing the transfer rate of Jatropha PEs and their metabolites into farm animal derived products.	+/–
Limited number of feeding studies with treated Jatropha seed products supported by analytical measurements.	+/–
No studies with treated Jatropha seed products in dairy and beef cattle, laying hens, horses, or companion animals.	+/–
No studies with treated Jatropha seed products on the effect on animal reproduction.	+/–
Representativeness of feed consumption data in livestock is limited.	+/–
No information on potential degradation products formed during current treatment methods are available.	+/–
Available data to establish a dose response for pigs are limited.	+/–
The NOAEL value for pigs is based on body weight gain and feed intake data and derived using initial body weight measurements.	+
Lack of long term studies in experimental animals, farm animals and companion animals	+/–
A lack of data from feeding studies in farm animals other than pigs and aquatic species.	+/–

(a): +: uncertainty with potential to cause over-estimation of exposure/risk; –: uncertainty with potential to cause under-estimation of exposure/risk.

Overall the CONTAM Panel considers that the uncertainties associated with the assessment are substantial due to the lack of qualifying studies.

4. Conclusions

General

- *Jatropha curcas*(Jatropha)contains phorbol esters (PEs), which are considered to be the main toxic principle occurring in all parts of the plant, with the highest concentrations in the seeds.

- Because of the high toxicity of PEs, untreated seeds of Jatropha plants and products derived from them may not be used as animal feed. Therefore Jatrophais listed as a harmful botanical impurity in the Annex to Directive 2002/32/EC of the European Parliament and of the Council

of 7 May 2002 on undesirable substances in animal feed. Seeds and fruit of Jatropha as well as their processed derivatives may only be present in feed in trace amounts not quantitatively determinable.

- *Jatropha curcas* seeds are being increasingly used as a source of biodiesel. The remaining kernel meal contains a high concentration of proteins and may be used as an animal feed material. However, as Jatropha kernel meal retains considerable amounts of toxic Jatropha PEs, it cannot be used as a feed ingredient without further processing.

- Genotypes of Jatrophathat do not containtoxic PEs are known to occur in Central America, but these genotypes are not widely distributed and are not used for oil extraction for biodiesel production.

- At present at least six PEs from Jatrophahave been identified but none of them are commercially available as references for analytical purposes. Given its structural similarity, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is used as a reference compound and PE quantities are expressed as TPA equivalents.

- Currently, there are no analytical methods,fully validated in collaborative trials, available for JatrophaPEs as no certified standards are available. Analytical methods currently applied have limits of detection (LODs) of 0.4–0.8 mg PEs (TPA equivalent)/kg feed (high-performance liquid chromatography – ultraviolet, HPLC-UV) and 0.07 mg PEs (TPA equivalent)/kg feed (liquid chromatography mass spectrometry, LC/MS).

Occurrence data

- Published reports give PE concentrations (expressed as TPA equivalents) of 870–7,700 mg/kg fresh weight (FW)in whole Jatropha seeds, 50–6,070 mg/kg FW in expeller cake and 600–3,700 mg/kg FW in solvent-extractedkernel meal producedfrom toxicgenotypes of Jatropha.

- Because Jatropha products are not used as animal feeds in the EU,no occurrence data of PEs in seeds and seed fractions are available from Europe.

Hazard identification and characterisation

Mode of action

- Jatropha phorbol esters show a high degree of similarity to other PEs, including TPA, and activate protein kinase C (PKC*)*, as shown *in vitro*.

- The main mechanisms of action of TPA is the activation of PKC,since it resembles the structure of the endogenous second messenger diacylglycerol (DAG). PKC activation is involved in numerous cell functions including the release of neurotransmitters, hormones and other signalling molecules as demonstrated *in vitro*.

- Higher PKCα protein expression and activation of transcription factors AP1 and NF-kB (specific targets of PKC)have been observed in livers of rats fed with Jatropha protein concentrate.

Toxicokinetics

- There are no data on the absorption, distribution, metabolism, and excretion of Jatropha PEs. Given its structural similarity, TPA is used as a model for toxicokinetics, despite the fact that

also no data are available on the absorption, metabolism,distribution and excretion of TPA after oral administration.

- Biotransformation studies with TPA revealed that the only metabolic pathway is hydrolysis of the ester groups, resulting in biologically non-active metabolites. Ester hydrolysis can be assumed to be also the major biotransformation pathways in Jatropha PEs, but the rate of ester hydrolysis of PEs depends on the chemical structure and position of the acyl (fatty acid) groups and the chemical structure of the diterpene moiety.

- From feeding studies with Jatropha materials, Jatropha PEs were not detected in pig or goat liver samples, but no LOD was mentioned.

- In the absence of toxicokinetic data in target animal species, including a lack of data on the oral availability, the potential transfer of Jatropha PEs into animal derived products is unknown.

Toxicity in experimental animals

- Jatropha seed products containing PEs have been studied in acute and sub-chronic rodent bioassays showing as major effects, reduced feed intake, loss of body weight, diarrhoea, haemorrhage and necrosis in multiple organs. Along with these findings alterations in haematological parameters and blood biochemistry have been reported.

- No experimental data are available on genotoxicity of Jatropha PEs. A read-across approach suggested similar but also additional structural alerts when compared to TPA.

- TPA acts as a tumour promoter in a mouse skin model after local application and in mouse forestomach, but exhibits no genotoxicity.

- Mouse skin models indicate that Jatropha PEs arealso tumour promoters.The tumourpromoting activity was mechanistically confirmed in *in vitro* experiments in cell cultures.

Adverse effects in farm andcompanion animals

- Untreated Jatropha products are not voluntarily consumed by animals. In forced feeding experiments with untreated Jatropha products, spontaneous mortality and severe symptoms, comparableto those described for experimental animals,have been reported in several farm animal species, including ruminants. No data were available for horses and companion animals.

- Feeding studiesin pigs and fish with treated Jatropha kernel meal(Jatropha PEs lower than 3 mg (TPA equivalents)/kg meal and substituting up to 50% of the protein in feed) showedno or only mild alterations of organ functions (diagnostic enzymes and metabolites). However, a study with lambs fed with treated Jatropha seed cake, resulting in an exposure of 1.2 mg PEs/kg bw per day, showed severe effects.

- In pigsnegative effects on growth performance were reversible when the treated meal was removed from the diet.

Observations in humans

- Observations in humans confirmed the acute oral toxicity of accidentally ingested Jatropha seeds.Symptoms observed in humans include a burning sensation on the mucosa of the upper intestinal tract and vomiting.All clinical symptoms are reversible.

Treatments used for detoxification

- A number of treatment processes substantially reducing (up to 99%) the amount of Jatropha PEs in kernel meal, seed cake, seed meal and protein isolate have been reported. However, the effectiveness of these detoxification processes are only in part supported by reliable analytical data and appropriate bioassays.

- Feeding studies in which up to 50% of the protein in the diet was replaced with treated Jatropha products,have confirmed the efficacy of certain detoxification processes.

Feed consumption and exposure of animals

- Assuming a residual PE concentration in treated Jatropha kernel meal of 3 mg/kg (the analytical limit of detection of TPA in most currently available experimental studies), and a 50% replacement of the non-forage proteins in feed for livestock species, fish and companion animals with Jatropha kernel meal protein, exposure estimates ranged from 0.002 mg PEs/kg bw for ruminants (beef, forage based diet) to 0.04 mg PEs/kg bwfor rabbits.

Health based guidance values in humans

- The limitations of the dataset do not allow the derivation of a health based guidance value for humans, especially regarding the lack of studies with pure compounds.

No observed adverse effect levels in animals

- From a feeding study in pigs with treated Jatropha kernel meal, a NOAEL of 0.4 mg PEs/kg bw per day, was identified based on decreases in body weight gain and feed intake and using exposure data based on the measurement of PEs as TPA equivalent.

- Rainbow trout, carp and shrimp tolerated feed in which 50% of the protein was replaced with treated Jatropha kernel meal containing Jatropha PEs at concentrations below the limit of detection in those studies (below3 mg PEs/kgexpressed as TPA equivalent).

- Due to the limitations of the available studies, no NOAEL could be identified for ruminants, horses, poultry species, aquatic species and companion animals. In lambs however, an exposure of 1.2 mg PEs/kg bwper day resulted in severe effects, indicating that a NOAEL is at least as low as that for pigs.

Risk characterisation

Human health risk characterisation

- Exposure to humans from Jatropha products could only occur from residues of Jatropha PEs in animal derived products, originating from animals given treated Jatropha kernel meal. However, the transfer of Jatropha PEs to animal derived products is unknown.

- Using conservative scenario, the CONTAM Panel estimated a daily intake of about 1 µg PEs/kg bw from cow milk,assuming that 50% of Jatropha PEs and its metabolites are transferred to milk from cows fed with Jatropha material.Themargin of exposure (MOE), between the human daily intake and the NOAEL of 0.4 mg PEs (TPA equivalent)/kg bw per dayin pigs, is about 400.

- This MOE is not sufficient to conclude that human health risk is low, due to limitations in the pig study and the ability of PEs to activate PKC, as well as the structural alerts for

genotoxicity.Therefore, no firm conclusions can be drawn on human health risks in the absence of sufficient data on toxicity and transfer from feed to animal derived foods.

Animal health risk characterisation

- Considering the identified NOAEL of 0.4 mg PE (TPA equivalent)/kg bw per day in pigs (based on decreases in body weight gain and feed intake), and the estimated exposure of up to 0.026 mg PEs/kg bw per day in pigs, the CONTAM Panel concluded that replacing 50% of feed protein with treated Jatropha material with ≤3 mg PEs/kg DM (expressed as TPA equivalent) would not pose a health risks to pigs.

- Ruminants may be at least as sensitive as monogastric animal species.This conclusion is supported by a study with lambs, showing severe effects at 1.2 mg PEs/kg bw per day.

- Under the condition that Jatropha products replace up to 50% of the feed proteins, the CONTAM Panel considers that a 10-fold lower exposure to Jatropha PEs than the NOAEL in pigs would be associated with a low risk for adverse effects also in other farm animals (including farmed aquatic species) or companion animals.

- The CONTAM Panel noted that for all species, the estimated exposure is 10-to 200-fold lower than the NOAEL in pigs, indicating that the risks to other species (including farmed aquatic species) is likely to be low when 50% of the protein in the compound or complementary feed is replaced by protein from treated Jatropha kernel meal containing a maximum of 3 mg PEs/kg (expressed as TPA equivalent).

5. Recommendations

- There is a need for standards for individual Jatropha PEs (Jatropha factors)and for analytical methods validated in collaborative trials for the quantification of Jatropha PEs.

- The toxicokinetics, including metabolism of Jatropha PEsneed to be elucidated in experimental and farm animalsand more data are needed to confirm the assumption that the transfer rate of PEs and their metabolites from Jatropha materials fed to animals is low.

- There is a need for studies to define the NOAEL in target animals after oral administration, ideally based on pure standards.

- The structural alerts from read-across studies on genotoxicity need to be investigated by experimental studies.

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APPENDICES

Appendix A. EFSA guidance documents applied in the assessment

- EFSA Scientific Committee, 2006. Guidance of the Scientific Committee on a request from EFSA related to uncertainties in Dietary Exposure Assessment. EFSA Journal 2007;4(1):438, 54 pp. doi:10.2903/j.efsa.2007.438

- EFSA Scientific Committee,2009. Guidance of the Scientific Committee on transparency in the scientific aspects of risk assessments carried out by EFSA. Part 2: General principles. EFSA Journal 2009;7(5):1051, 22 pp. doi:10.2903/j.efsa.2009.1051

- EFSA Scientific Committee,2012. Scientific Opinion on Risk Assessment Terminology. EFSA Journal 2012;10(5):2664, 43 pp. doi:10.2903/j.efsa.2012.2664

Appendix B. Toxicokinetic of TPA – laboratory animals full text

No studies on the absorption, metabolism, distribution, and excretion of TPA after oral administration have been identified.

Studies by Kreibich et al. (1971, 1974) have shown that [20-^{3}H]-labelled TPA is virtually not metabolised in the skin of NMRI mice *in vivo* within 12 h after dermal administration. In contrast, radiolabelled TPA was rather extensively metabolised upon incubation with skin explants of embryonic mice *in vitro*: In addition to unchanged TPA, a metabolite which migrated like synthetic 12-tetradecanoylphorbol in thin layer chromatography was detected both in the tissue extract and in the incubation medium after 12 h. The same putative deacetylated metabolite of TPA was found in the medium of cultured mouse skin fibroblasts (L-cells) after a 2- and 4-h incubation with TPA, together with small amounts of phorbol and phorbol-13-acetate. Both monoesters and phorbol are no longer biologically active as tumour promoters (Kreibich et al., 1974).

Segal et al. (1975) identified TPA with the carbonyl group at C3 reduced to an alcohol group, as a metabolite of [20-^{3}H]-labelled TPA in the skin of female ICR/Ha Swiss mice 5 h after dermal application. Identification was achieved by thin layer chromatographic comparison with a synthetic reference compound. The reductive metabolite was shown to have an inflammatory effect equal to or slightly less than TPA (Segal et al., 1975).

Berry et al. (1978) reported that the skin of adult female CD-1 and new-born BALB/c mice metabolise [20-^{3}H]-labelled TPA, after dermal application, to the hydrolytic products 12-*O*-tetradecanoylphorbol, phorbol-13-acetate and phorbol, as determined by HPLC and comparison with authentic reference compounds. The predominant dermal metabolite was 12-*O*-tetradecanoylphorbol. Traces of the oxidative product, 20-oxo-TPA, which has been identified as an autoxidation product of TPA (Schmidt and Hecker, 1975), were also found in mouse skin, while the reductive metabolite 12-*O*-tetradecanoylphorbolol-13-acetate (reported by Segal et al., 1975) was not observed in this study.12-*O*-Tetradecanoylphorbolbut not phorbol-13-acetate was also formed in a time-dependent manner when [20-^{3}H]-TPA was incubated with epidermis homogenates (Berry et al.1978).

In incubations with liver microsomes from adult female CD-1 mice, 12-*O*-tetradecanoylphorbol was formed much more rapidly than phorbol-13-acetate and phorbol, and the liver microsomes were about 15 times more active than the epidermal homogenate in converting TPA into its monoesters and phorbol (Berry et al., 1978). Noteworthy, no other metabolites were detectable in the incubations with liver microsomes, suggesting than cytochrome P450 (CYP) is not involved in the metabolism of TPA. This notion was supported by the observation that the profile of microsomal metabolites was the same in incubations conducted in the presence or absence of nicotinamide adenine dinucleotide phosphate (NADPH) (a cofactor of CYP-mediated monooxygenation reactions), and the absence or presence of carbon monoxide (an inhibitor of CYP). In contrast, the presence of α-naphthyl acetate in the microsomal incubations markedly reduced the metabolism of TPA, probably by competing with esterases essential for the hydrolytic cleavage of TPA. The authors therefore concluded that esterases but not CYP contributed to the metabolism of TPA (Berry et al., 1978).

Hydrolysis of [20-^{3}H]-TPA was also the only metabolic reaction observed by O'Brien and Diamond (1978a) in cultures of primary Syrian hamster embryo fibroblasts (HEF) and in a human fibroblast cell line (HC-4). In contrast to the study of Berry et al. (1978) with mouse epidermis homogenate and mouse liver microsomes discussed earlier, phorbol-13-acetate was the only metabolite and no 12-*O*-tetradecanoylphorbol could be detected by thin-layer chromatography in the media of the HEF cultures after 3 and 7 days. Little or no metabolism of [20-^{3}H]-TPA was observed in the cultured HC-4 human fibroblasts under the same conditions (O'Brien and Diamond, 1978a). When cultured hamster fibroblasts were exposed to [20-^{3}H]-TPA for various time periods up to 3 days and the culture media subsequently tested for their ability to induce ornithine decarboxylase (ODC) as a marker for tumour promoting activity, a rapid and progressive loss of ODC-inducing activity was noted, which paralleled

the formation of phorbol-13-acetate (O'Brien and Diamond, 1978b). Neither pure phorbol-13-acetate nor 12-*O*-tetradecanoylphorbol were able to induce ODC. When the loss of ODC-inducing activity was used as a bioassay to analyse the metabolism of TPA or other phorbol diesters, cells from several other rodent species, but none of four human cell lines were able to metabolize TPA. Moreover, it was disclosed that phorbol-12,13-diacetate was metabolized in HEF cells whereas phorbol-12,13-didecanoate (PDD) was not (O'Brien and Diamond, 1978b). Marked differences in the hydrolytic metabolism of the two phorbol diesters [20-^{3}H]-TPA (rapid hydrolysis) and [20-^{3}H]-PDD (slow hydrolysis) were observed in cultured hamster, rat, chick and mouse fibroblasts and also in a human hepatoma cell line, whereas human HC-4 fibroblasts virtually did not metabolise either PE over a 3-day period (O'Brien and Saladik, 1980). While phorbol-13-acetate was the major if not only metabolite of TPA in all cultured cells, both phorbol-12-decanoate and phorbol-13-decanoate were formed from PDD, although at varying amounts. These data suggest that the hydrolytic metabolism of phorbol diesters depends on the cell type and on the chemical structure of the diester.

In 1981, Shoyab et al. reported the isolation and partial characterisation of an enzyme from mouse liver cytosol, which exclusively hydrolyses the C12 ester group of phorbol-12,13-diesters, thereby converting a biologically active diester into an inactive phorbol-13-monoester. This enzyme was not present in cytosol from mouse skin, but had high concentrations in cytosol from hamster, rat, guinea pig, and rabbit skin. The promotion of skin tumours by TPA in mice but not in the other four animal species may be due to this enzyme activity, with cells expressing high levels of the enzyme not responding to TPA (Shoyab et al., 1981). However, there is a discrepancy with the study by Berry et al. (1978) discussed above with respect to the ester group preferred for hydrolysis: Whereas Berry et al. (1978) observed preferential hydrolysis of the ester group at C13 with the microsomal enzyme, Shoyab et al. (1981) reported specific hydrolysis of the C12 ester group by the cytosolic enzyme.

In 1984, Saito and Egawa isolated an esterase converting TPA to phorbol-13-acetate and tetradecanoic acid, i.e. hydrolyzing the C12 ester group, from murine serum. Of five esterases isolated from rat liver endoplastic reticulum, only two were able to hydrolyse TPA, and the predominant product was phorbol-13-acetate (Mentlein, 1986).

The hydrolysis of [20-^{3}H]-TPA and [20-^{3}H]-PDD was studied in hamster cells in culture and hamster skin *in vivo* by Barrett et al. (1982). TPA was more rapidly metabolised (predominantly to phorbol-13-acetate and trace amounts of 12-*O*-tetradecanoylphorbol and phorbol) than PDD (with phorbol-12-decanoate as major and phorbol-13-decanoate and phorbol as minor products) in cultured Syrian hamster embryo fibroblasts and preneoplastic and neoplastic derivatives of these cells. In contrast to the observations in cultured cells, no hydrolysis of TPA and PDD was detected in intact hamster skin for up to 48 h. These findings do not support the hypothesis of Shoyab et al. (1981) that the lack of tumour promotion in hamster skin is due to metabolic inactivation of TPA.

Müller et al. (1990) studied the metabolism of eight phorbol diesters and two phorbol monoesters with different acyl groups at C12 and C13 in incubations with NADPH-fortified liver microsomes from female NMRI mice. The products of ester hydrolysis were found for each of the ten compounds, and no product of other metabolic pathways was observed. Some of the 12,13-diesters, including TPA, were readily hydrolysed by the microsomes, while others, e.g. 'inverse TPA', i.e. 12-*O*-acetylphorbol-13-tetradecanoate, but also the TPA stereoisomer 12-*O*-tetradecanoyl-4α-phorbol-13-acetate were much more slowly hydrolysed. The authors concluded that metabolism of diterpene esters depends on the nature and position of the acyl group, as well as on the structure of the diterpene moiety.

Roeser et al. (1991) conducted a toxicokinetic study of [20-^{3}H]-TPA in the back skin of female NMRI mice, providing a quantitative account of virtually all metabolites and autoxidation products of TPA formed in the skin up to 72 h after dermal administration. In addition to the products arising from TPA hydrolysis, which are more polar than TPA, a large number of more lipophilic metabolites were disclosed by normal phase HPLC. Co-chromatography with authentic reference compounds in argentation and reverse phase HPLC revealed the structures of numerous esters at C20 of TPA with

long-chain saturated and unsaturated fatty acids for these novel metabolites. The chain length of the fatty acids ranged from 16 to 26 carbons in the group of saturated TPA-20-acylates, and from 16 to 24 carbons for cis-mono-unsaturated TPA-20-acylates. In the groups of di- and tetra-unsaturated TPA-20-acylates, linoleate and arachidonate were the major components. TPA-20-acylates represented the major TPA metabolites found in the surface lipids, epidermis and dermis of mouse skin, e.g. accounting for 30% of the applied radioactivity in the dermis fraction after 72 h. Together with unchanged TPA, its hydrolytic metabolites and several autoxidation products, the total recovery of radioactivity was between 92.6% and 98.8% in all experiments. Several of the TPA-20-acylates were tested for irritant activity and TPA-20-tetradecanoate for tumour promoting activity on mouse skin, and proved to be much less active than TPA itself. Because TPA-20-acylates are partly hydrolysed to TPA in mouse skin, their low activity may result from the metabolically formed TPA. Therefore, the authors concluded that TPA-20-acylates may be considered products of metabolic deactivation of TPA.

Appendix C. Intakes and composition of diets used in estimating animal exposure to phorbol esters

This Appendix gives feed intakes for different livestock and companion animals used in this Scientific Opinion to estimate exposure to phorbol esters (PEs). The composition of diets for each of the major farm livestock species are based on generally accepted guidelines on nutrition and feeding (e.g. AFRC, 1993; Carabano and Piquer, 1998; NRC, 2006, 2007a,b; Leeson and Summers, 2008; EFSA Scientific Committee, 2009; McDonald et al., 2011). In the absence of a database of feed consumption by livestock, fish and companion animals in the EU, these estimates have been used by the Panel on Contaminants in the Food Chain (CONTAM Panel), and are in agreement with common practice. Since detoxified feeds derived from *Jatropha curcas*(Jatropha) are likely to be used principally as ingredients of compound or complementaryfeeds, only exposure via compound feeds has been estimated.

C1. Feed intake

C1.1. Ruminants and horses

The diets of cattle, sheep, goats and horses consist predominantly of forages, but their daily ration may be supplemented with feed materials and/or compound feedingstuffs where the nutritional need of the animal cannot be met from forages alone. Forages may be fed fresh or conserved, e.g. as hay or silage. In some beef production systems, where rapid rates of liveweight gain are required, cereals (predominantly barley) constitute the main ingredient in the ration.

Live weights, feed intakes and growth rates/productivity are from AFRC (1993) and NRC (2007a,b). The live weights, feed intakes, the proportion of the daily ration that is non-forage feed and growth rates/productivity for cattle, sheep and goats used in this Scientific Opinion are given in Table 8.

Table 8: Live weights, growth rate/productivity, dry matter intake for cattle, sheep and goats, and the proportions of the diet as non-forage

	Live weight (kg)	Growth rate or productivity	Dry matter intake (kg/day)	% of diet as compound feed	Reference
Dairy cows, lactating[(a)]	650	40 kg milk/day	20.7	40	AFRC (1993)
Beef: cereal-based diet	400	1.4 kg/day	10.0	85	AFRC (1993)
Beef: forage-based diet	400		9.6	20	AFRC (1993)
Lactating sheep	60		2.89	50	AFRC (1993)
Lactating goats	60		3.4	65	NRC (2007a)
Fattening goats	40		1.5	40	NRC (2007a)
Horses	452	n.a.	9.0	50	NRC (2007b)

n.a.: not applicable.
(a): Months 2–3 of lactation.

C1.2. Pigs, poultry and rabbits

Data for feed intake and live weight of pigs and poultry are from EFSAScientific Committee(2009) and of ducks from Leeson and Summers (2008). The live weights and feed intakes these animal species are presented in Table 9. A daily intake of 75 g/kg bw for a 2 kg rabbit is used in this Scientific Opinion to estimate exposure (derived from Carabano and Piquer, 1998).

Table 9: Live weights and feed intake for pigs and poultry and ducks

	Live weight (kg)	Feed intake (kg dry matter/day)	Reference
Pigs: piglets	20	1.0	EFSA Scientific Committee (2009)
Pigs: fattening pigs	100	3.0	EFSA Scientific Committee (2009)
Pigs: lactating sows	200	6.0	EFSA Scientific Committee (2009)
Poultry: broilers[(a)]	2	0.12	EFSA Scientific Committee (2009)
Poultry: laying hens	2	0.12	EFSA Scientific Committee (2009)
Turkeys: fattening turkeys	12	0.40	EFSA Scientific Committee (2009)
Ducks: fattening ducks	3	0.14	Leeson and Summers (2008)

(a): chickens for fattening.

In the calculations that follow it is assumed that all the feed is consumed as compound feed.

C1.3. Companion animals (dogs and cats)

The amount of food consumed is largely a function of the mature weight of the animal, level of activity, physiological status (e.g. pregnancy or lactation) and the energy content of the diet. In this Scientific Opinion the CONTAM Panel estimated daily intake of dogs and cats based on NRC (2006). Intakes for a 25 kg dog and a 4 kg cat given below in Table 10have been used to estimate exposure.

Table 10: Estimates of total food and intake, derived from NRC (2006)

	Dogs	Cats
Body weight (kg)	25	4
Feed intake (g/day)	360	60

C2. Diet composition and concentration estimates

Most livestock in the European countries are fed proprietary commercial compound feeds, often as the sole feed. The following table provides estimates of the amount of protein provided by conventional proteins in livestock diets, and the amounts of Jatropha mealrequired to replace 50% of that protein.

Table 11: Estimates of Jatropha meal required to replace 50% of conventional proteins in livestock diets

Livestock	Compound feed intake (kg/ DM per day	Protein content of compound feed (g/kg FW)	Protein content of compound feed (g/kg DM)	Protein supplied by compound feed (g/day)	Amount of JM (kg DM) required to replace 50% protein
Dairy: high yielding	8.28	180	204	1,694	1.30
Beef: intensive cereal	8.5	140	159	1,352	1.04
Beef: fattening	1.92	160	182	349	0.27
Sheep - lactating	1.4	180	204	286	0.22
Goats - lactating	2.21	180	204	452	0.35
Goats - fattening	0.6	160	182	109	0.08
Pig starters	1	200	227	227	0.17
Pig finishers	3	140	159	477	0.37
Lactating sows	6	160	193	1,159	0.89
Broilers: growers	0.12	200	227	27	0.02
Laying hens	0.12	190	216	26	0.02
Turkeys: growers	0.4	200	227	91	0.07
Ducks: growers	0.14	200	204	29	0.02
Rabbits	0.15	200	227	34	0.03

Livestock	Compound feed intake (kg/ DM per day	Protein content of compound feed (g/kg FW)	Protein content of compound feed (g/kg DM)	Protein supplied by compound feed (g/day)	Amount of JM (kg DM) required to replace 50% protein
Cats	0.06	180	318	19	0.01
Dogs	0.36	180	284	102	0.08

DM: dry matter; FW: fresh weight; JM: Jatropha meal.

Appendix D. Genotoxicity profiling of TPA and the six Jatropha phorbol esters by OECD Toolbox

Aim: To use OECD Toolbox in order to study the similarity in terms of genotoxic potential, between TPA and the six different Jatropha phorbol esters.

End points studied: Both endpoints gene mutation and chromosomal aberrations should be evaluated for TPA and the six Jatropha phorbol esters.

There are in general two aspects when the similarity between substances is studied in order to perform a read-across: the first one is structural similarity preferably to be based on a working hypothesis which is related with molecular initiating events important for the studied endpoint; and the second one toxicokinetic similarity e.g. metabolism.

Profilers used: Molecular initiating events of relevance for this assessment are interaction with DNA and/or proteins. The profilers included in the OECD Toolbox which codified the structural alerts that are important for these two types of interactions are mechanistic profilers - DNA binding by OASIS v.1.3, DNA binding by OECD, Protein binding by OASIS v 1.3, Protein binding by OECD and endpoint specific profilers- DNA alerts for AMES, MN and CA by OASIS v1.3, *In vitro* mutagenicity (AMES test) alerts by ISS, *In vivo* mutagenicity (Micronucleus) alerts by ISS, Protein binding alerts for Chromosomal aberrations by OASIS v1.1.

Above mentioned profilers have been applied to the six Jatropha phorbol esters as chemicals of interest and to TPA as a 'known' substance.

Rat liver S9 metabolism simulator has been used to simulate the metabolism for TPA and the six Jatropha phorbol esters.

Results

No structural alerts for genotoxicity in the TPA and the 6 Jatropha phorbol esters were found by the profiler Protein binding alerts for Chromosomal aberrations by OASIS v1.1.

The alerts found by DNA binding by OASIS v.1.3, DNA binding by OECD, Protein binding by OASIS v 1.3, Protein binding by OECD and endpoint specific profilers – DNA alerts for AMES, MN and CA by OASIS v1.3, *In vitro* mutagenicity (AMES test) alerts by ISS and *In vivo* mutagenicity (Micronucleus) alerts by ISS are presented in the Table 12.

Table 12: Genotoxicity profiling of TPA and the six Jatropha phorbol esters by OECD Toolbox

profilers	**DNA Binding OASIS**	**DNA Binding OECD**	**Protein binding by OASIS**	**Protein binding by OECD**			**DNA alerts for AMES, MN, CA by OASIS**	***In vitro* mutagenicity (AMES) by ISS**	***In vivo* mutagenicity (Micronucleus) by ISS**	
Structural alerts	**Specific acetate esters**	**α,β-unsaturated esters**	**α,β-Carbonyl compounds with polarized double bonds**	**Acetates**	**Polarised alkene - ketones**	**Polarised alkene - esters**	**Specific acetate esters**	**α,β-unsaturated carbonyls**	**α,β-unsaturated carbonyls**	**H-acceptor-path3-H-acceptor**
TPA	x		x	x	x		x	x	x	x
C1			x	x	x			x	x	x
C2			x	x	x			x	x	x
C3		x	x	x	x	x		x	x	x
C4, C5			x	x	x			x	x	x
C6		x	x	x	x	x		x	x	x

CA: chromosomal aberration; ISS: Istituto Superiore di Sanità; MN: micronucleus ; OECD: Organisation for Economic Co-Operation and Development.

Three structural alerts in TPA were recognised by different profilers – specific acetate esters, esters and α, β – carbonyl compounds with polarized double bound (Figure 5). The alert H-acceptor-path3-H-acceptor, identified by *In vivo* mutagenicity (Micronucleus) alerts by ISS, refers also to the same mentioned above structural alerts.

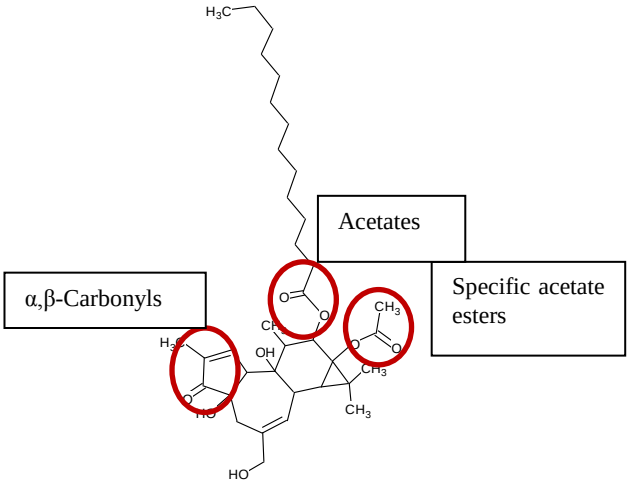

Figure 5: TPA - Structural alerts for genotoxicity

The alerts – acetates and α, β – carbonyls were identified also in all Jatropha phorbol esters. The alert - Specific acetate esters (identified by DNA binding by OASIS v.1.3) disappeared, since the functional group is not present any longer in the Jatropha phorbol esters. In Jatropha factors C3 and C6 a new alert - α, β – unsaturated esters, for DNA and protein binding was identified by two of the profilers (DNA binding by OECD and Protein binding by OECD)(Figure 6, the new alert is highlighted in blue).

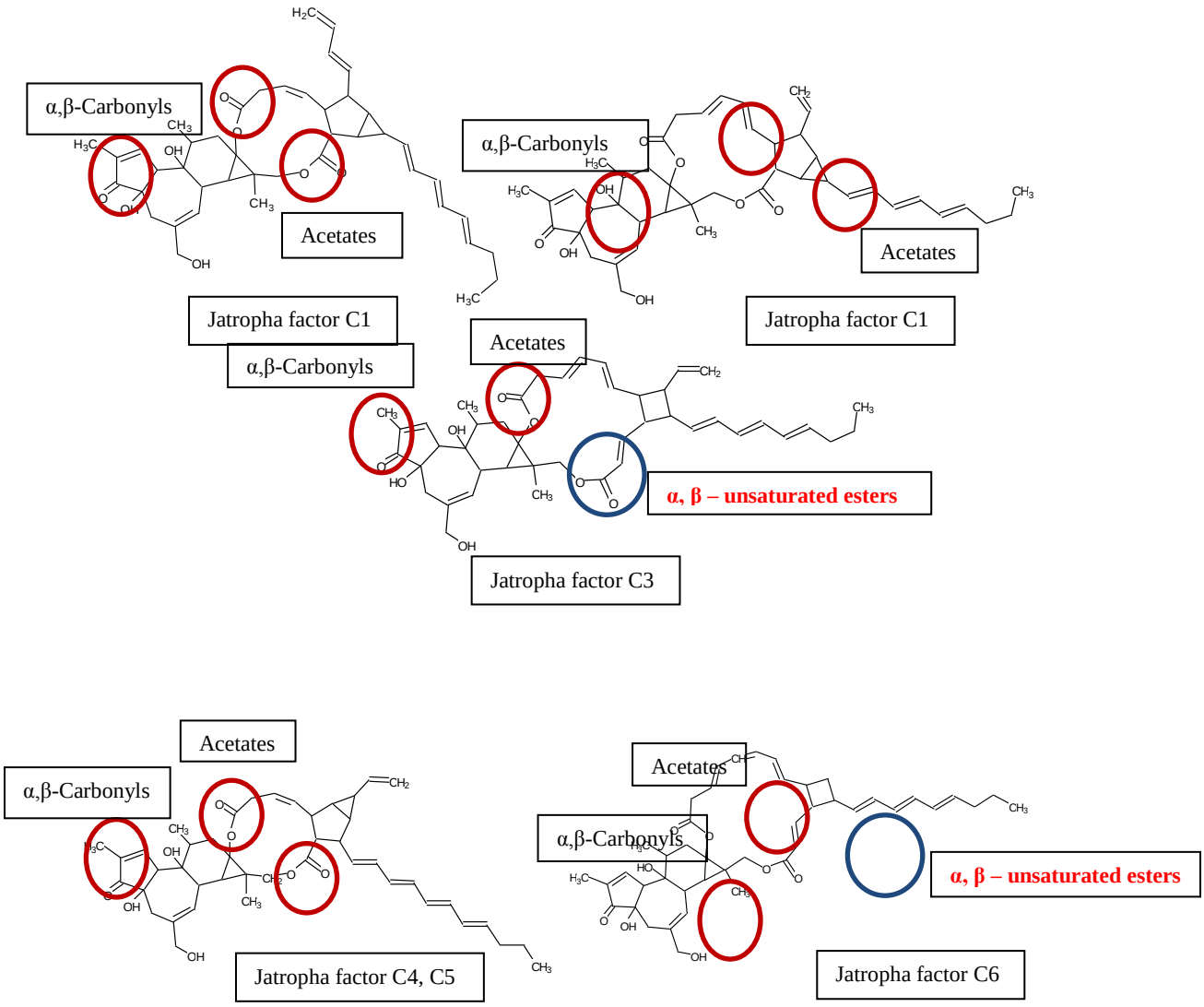

Figure 6: The Jatropha phorbol esters – structural alerts for genotoxicity

Rat liver S9 metabolism simulator has been used to simulate the metabolism for TPA and the six Jatropha phorbol esters

13 metabolites of TPA were generated by the metabolic simulator. To all of them the same profilers relevant for genotoxicity were applied. A new alert appears - α, β - unsaturated aldehydes as a result of oxidation of the OH group in C_{20} position. The group is present also in the six Jatropha phorbol esters and the new alert is also appeared in all of them (Figure7).

Figure 7: Formed α, β - unsaturated aldehyde after metabolic activation

The metabolic simulator has been also applied to the six Jatropha factors, to all generated metabolites (factor C1 – 35 metabolites, C2 – 34 metabolites, C3 – 34 metabolites, C4,5 – 35 metabolites, C16 – 16 metabolites) the same profilers relevant for genotoxicity were applied. A new alert - direct acting epoxides and related, appeared as a result of metabolism of the double bounds at different position in the parts of the molecules which are different than TPA (Figure 8). A mono aldehyde is also recognised as an alert for DNA and protein binding, formed after opening of the fused unsaturated heterocycle (Figure 9). These two alerts are new and not present neither in TPA nor in any of its metabolites. Should be mentioned that in factor C3 and C6 the new alert identified in the parent molecule (α, β – unsaturated esters) is still present in some of predicted metabolites.

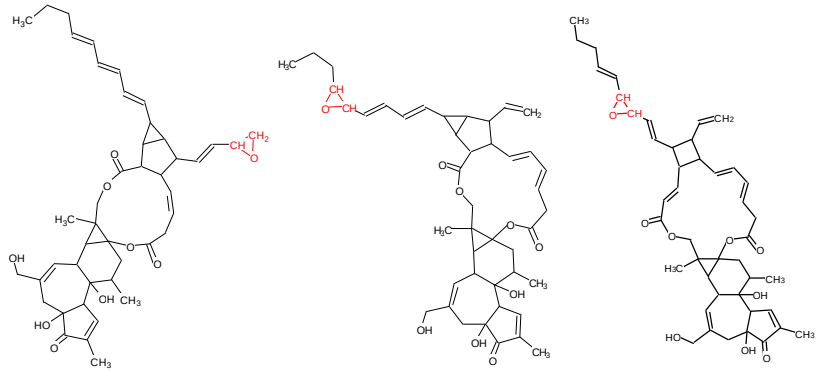

Figure 8: A few examples for forming of epoxides as result of metabolic activation

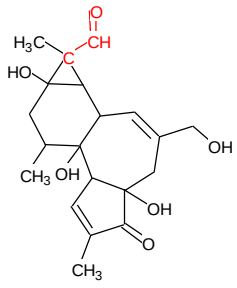

Figure 9: Mono aldehyde formed as an result of metabolic activation

Conclusion

Based on the analysis described above it could be concluded that the six Jatropha phorbol esters cannot be considered similar to TPA in terms of structural alerts for genotoxicity. Additional structural alerts relevant to genotoxicity, as compared to TPA, were identified in parent molecules (factors C3 and C6) as well as after metabolic activation (for all six factors).

ABBREVIATIONS

ADFI	average daily feed intake
ADG	average daily (body weight) gain
ALP	alkaline phosphatase
AOT	sodium bis (ethylhexyl) sulfosuccinate
AST	aspartate aminotransferase
bw	body weight
CA	chromosomal aberration
CONTAM Panel	EFSA Panel on Contaminants in the Food Chain
CP	crude protein
CYP	cytochrome P450
DAD	diode array detector
DAG	diacylglycerol
DM	dry matter
DMBA	7,12-dimethyl[a]anthracene
DMSO	dimethyl sulfoxide
EEA	essential amino acid
EC	European Commission
ER	estrogen receptor
ESI-MS	electrospray ionization mass spectrometry
FW	fresh weight
GOT	glutamic oxaloacetic transaminase
GPT	glutamic pyruvic transaminase
h	hour
HEF	hamster embryo fibroblasts
HPLC-DAD	high-performance liquid chromatography with diode-array detection
HPLC-MS	high-performance liquid chromatography with mass spectrometry
HPLC-UV	HPLC coupled with a UV detector
ISS	Istituto Superiore de Sanità
JM	Jatropha meal
LC	liquid chromatography
LDH	Lactate dehydrogenase
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOQ	limit of quantification
min	minute
MOE	margin of exposure
MN	micronucleus
MS	mass spectrometry
MS/MS	tandem mass spectrometry
n.a.	Not applicable
NADPH	nicotinamide adenine dinucleotide phosphate
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
ODC	ornithine decarboxylase
OECD	Organisation for Economic Co-operation and Development
PDD	phorbol-12,13-didecanoate
PE(s)	phorbol ester(s)
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
ROS	Reactive oxygen species
RP	reverse phase

SBM	soya bean meal
SPE	solid phase extraction
SSF	solid state fermentation
TLC	thin layer chromatography
TPA	12-*O*-tetradecanoylphorbol-13-acetate
UPLC-MS	ultra performance liquid chromatography–mass spectrometry
UV	ultraviolet