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Development and evaluation of an effective process for the recovery of oil and detoxification of meal from jatropha curcas

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DEVELOPMENT AND EVALUATION OF AN EFFECTIVE PROCESS FOR THE RECOVERY OF OIL AND DETOXIFICATION OF MEAL FROM JATROPHA CURCAS

by

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ABSTRACT

There has been significant research on biofuels to ensure national security and economic stability of the US. Jatropha curcas, a hardy plant, holds tremendous potential as a biodiesel source and cattle feed-stock largely due to its high triglyceride and crude protein content. However, its viable economic utilization is limited due to the presence of important toxic compounds, phorbol esters.

A novel and efficient process has been developed that obtains high yields of jatropha oil and detoxifies the defatted (oil free) jatropha meal. Principles of solid-liquid extraction were utilized to detoxify the meal and then liquid chromatography was employed to analyze it.

In the proposed approach, various polar and nonpolar solvents were utilized for several extraction cycles. The highly nonpolar solvents were experimented for extracting the triglycerides. Due to the polar nature of phorbol esters attributed to the presence of hydroxyl functionalities, high-polarity solvents like methanol, ethanol, and isopropyl alcohol were experimented to selectively remove the phorbol esters from defatted meal. All the extractions were carried out in an ultrasonic bath (sonicator) or extraction tubes (soxhlets). Phorbol esters in raw meal, extracted oil, and defatted meal were characterized using high performance liquid chromatography (HPLC). Analysis showed that extraction of ground seed kernels in soxhlets involving a sequential combination of hexanes, followed by methanol was highly efficient in detoxifying the meal. Results showed that the phorbol ester content was reduced by 99.63% from 6.05 mg/g in untreated meal to about 0.06 mg/g in solvent-treated meal, which is well below the value of 0.11 mg/g found in the nontoxic Mexican accession of jatropha seed.

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

1. INTRODUCTION

1.1. JATROPHA PLANT

Jatropha curcas L is a hardy plant and belongs to the euphorbiaceae family. It is a drought-resistant bush or small tree with spreading branches that can grow to 20 feet high under favorable conditions. The species occur naturally in the parts of tropical America (central and southern regions), and many tropical and sub-tropical parts of Africa and Asia, specifically India. It is believed that the species were introduced into other regions from the Caribbean, where it was used during the Mayan period[1], by the Portuguese ships via the Cape Verde islands and Guinea Bissau[2]. One major trait associated with the plant is its hardiness and sustainability in warm and arid climates. Also, the plant is drought resistant and can survive in high temperature conditions. Since the plant can be grown on "bad lands", and prefers well-drained alkaline soil (pH 6-9) for its growth, it holds tremendous potential to be cultivated on wastelands, specifically in the tropical and sub-tropical regions of the world. Moreover, the plant does not compete with conventional food crops which makes it an ideal choice in order to utilize the vast land resources which are underutilized. Since the jatropha plant is not a forage crop, it plays an important role in keeping out the cattle and protects other valuable food crops or cash crops by serving as a hedge. It is also a fast growing plant; it can achieve a height of about three meters within three years under a variety of growing conditions. The seed production from the plants propagated from seeds can be expected within 3-4 years of planting[3]. The seed resembles the castor seed in shape, but is dark brown in color and smaller in size. It has been reported that seed yield is 5 tons/hectare [2]. The kernel to shell ratio in the seed has been reported to be about (62.7±1.5): (37.3±1.5) by Makkar et al.[6], and about (68.66±0.4): (31.33±0.39) by Martínez-Herrera et al.[7], respectively.

1.2 PROPERTIES OF JATROPHA

Table 1.1 summarizes the major physical properties of jatropha seed as reported by various authors. Of particular importance are the oil content, the energy content, and the nutritional content of seeds.

Table 1.1. Oil content, gross energy, and nutritional composition of jatropha seeds.

Property (units)	Seed	Shell	Kernel	Seed Oil	Defatted Cake	Source
Oil Content (%w/w)	37.4	-	46-48.6	-	-	[8]
	33-39.1%	-	46.2-58.1	-	-	[9]
	-	-	48.5	-	-	[10]
	-	-	21-74	-	-	[11]
	-	-	40-60	-	-	[4]
Gross Calorific Energy (MJ/kg)	-	16.9	-	-	-	[12]
	-	17.2	29.8	-	-	[13]
	-	-	31.1-31.6	-	-	[7]
	-	-	-	30.1	-	[4]
	-	-	-	37.8	-	[14]
	-	-	-	40.7	-	[13]
	-	-	-	41.8	-	[10]
Nutritional Composition (% w/w)		N-0.7 P-0.047 K- 1.58	N-2.53 P-0.37 K-1.25			[15]
					N-6.4 P-2.8 K-0.95	[16]

*N-Nitrogen
P-Phosphorus
K-Potassium

From this table, it is clearly evident that the oil content of jatropha kernel is fairly high and constitutes about 55% of the total weight of kernel[9]. Another important thing to note is the high value of the gross energy content of oil from the kernel. A chart illustrating the biodiesel yield based on data obtained from [17] is shown in Figure 1.1.

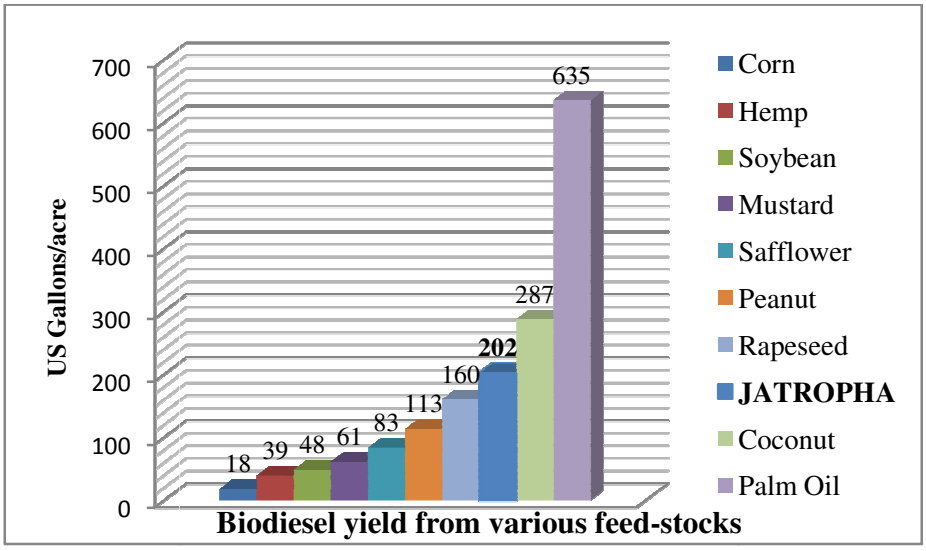

Figure 1.1. Biodiesel yield (gallons/acre) from different feedstocks.

Another Figure 1.2 from an article originally published on August, 24, 2007 in the Wall Street Journal [18] illustrates the price per barrel of biofuels from various feedstocks.

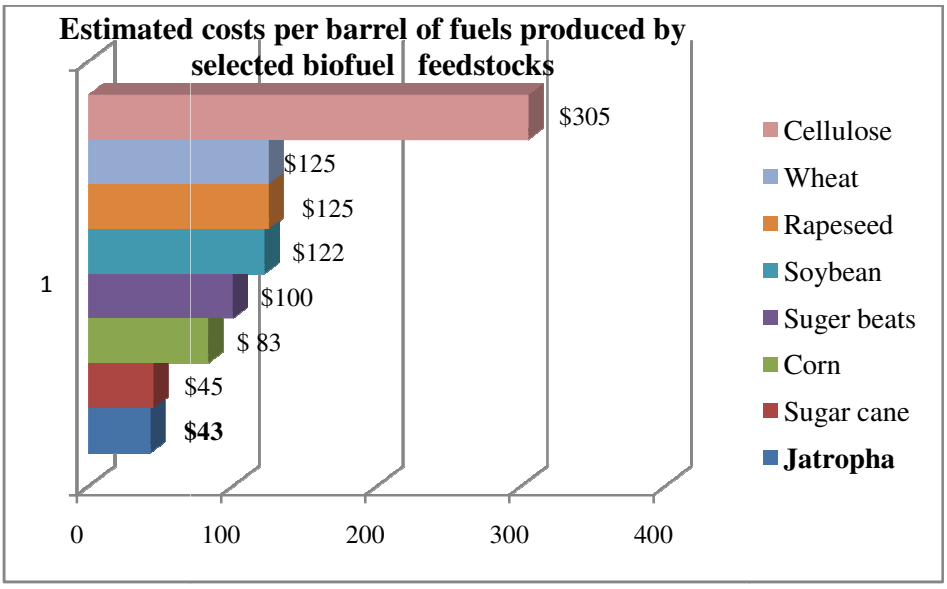

Figure 1.2. Costs (in USD) per barrel of fuel from commonly used biofuel feedstocks.

These data clearly indicate that jatropha is a highly suitable candidate for biodiesel production mainly due to high yields and low cultivation costs. In fact, the suitability of jatropha seed oil for trans-esterification into biodiesel has also been demonstrated [19 - 21]. But, apart from its primary usage as a biodiesel feedstock, there are various other uses of jatropha. The seed cake (defatted meal) is nutrient-rich but at present is used only as fertilizer or as feedstock for biogas production [12], [22], [23].

The plant has also been used to prevent soil erosion and as a hedge [13], [16]. Certain medicinal uses of jatropha have also been reported. Jatropha can be used for the treatment of a variety of ailments due to its purgative effect. The latex of the jatropha plant has been believed to have an alkaloid, jatrophine, which has anti-cancerous properties. It is also used to treat sores on livestock. The roots of the plant are also used as an antidote to treat snake-bite. Several researchers have also isolated and characterized the substances responsible for wound healing and anti-inflammatory effects [24], [25]. Seed oil from jatropha has also been used to treat eczema and skin diseases. Linoleic acid present in the oil is also of considerable interest in skincare. Apart from this, jatropha seed oil has also been shown to possess an insecticidal property [26]. The glycerin obtained as a result of the trans-esterification process is added to soap in some countries. However, the production costs are high and the quality of soap is poor. But in developing countries, soap production has been very conducive where this business generates employment. Oil from seeds has also been used as lubricant and as leather softener. Very recently, jatropha derived biodiesel has also been mixed with jet fuel and used as an aviation fuel. Various uses of jatropha are summarized in Figure 1.3.

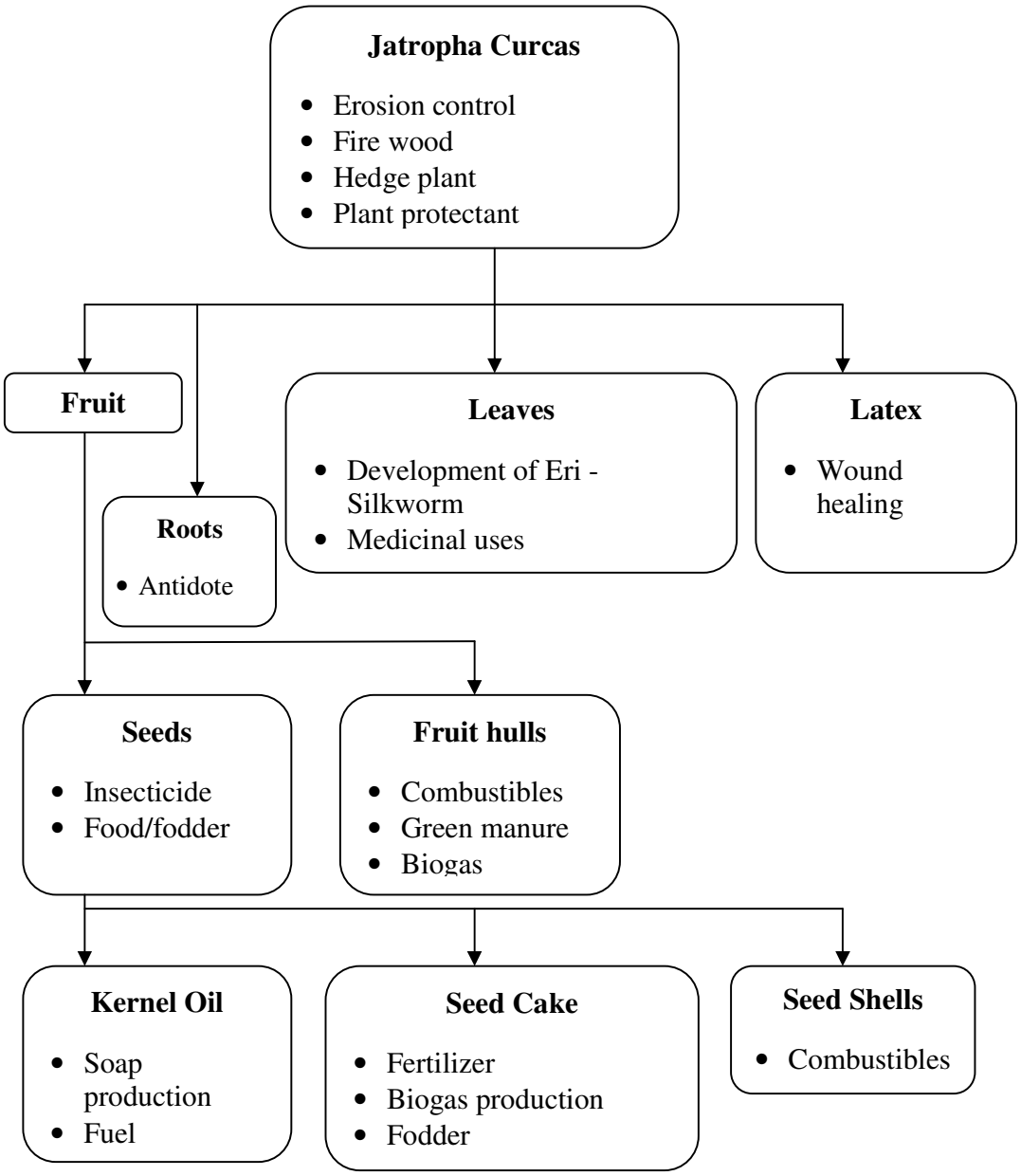

Figure 1.3. Uses of the components of *jatropha curcas* [5].

The preceding clearly demonstrates that jatropha plant holds a huge potential as a biodiesel feedstock. However, despite high nutrient content, its potential as an animal feed has remained at best only marginal. Enhancement in its use as a feed can bring about a dramatic change in the farm economy by providing a rich source of additional income for farmers.

1.3. CHEMICAL COMPOSITION OF JATROPHA SEEDS

The shell and the kernel form approximately 60 and 40 percent of the total mass of the seed. Primarily, the seed has been reported to be rich in crude protein (CP), lipids, neutral detergent fibers, and ash. Table 1.2 summarizing the values reported in the literature for nutritional compounds and other components of jatropha seeds is shown below.

Table 1.2. Principal constituents of jatropha seeds.

Property (units)	Seed	Shell	Kernel	Seed Cake	Source
Crude Protein (% w/w)	38.38	7.8			[27]
		3.7-4.1	14.1-19.6		[28]
		4.3-4.5	22.2-27.2	56.4-63.8	[29]
			24.6		[30]
			26		[4]
Lipid (% w/w)		0.7-1.4	56.8-58.4	1-1.5	[29]
			47.3		[31]
			53		[4]
			56.88		[27]
Crude Fiber (%w/w)	22.88				[27]
	10.6-11.5	28.8-32	1.9-3		[28]
		83.9-89.4	22.2-27.2	8.1-9.1	[29]
		53.52	4.33		[27]
			10.1		[30]
			5		[4]
Ash (% w/w)		4			[12]
		5	3		[13]
			3.6-4.3		[5]

Composition of jatropha oil is given in Table 1.3 which summarizes the data from experiments conducted by Adebowale and Adedire [26]. The data shows that jatropha oil is rich in triglycerides. These are easily trans-esterified with methanol to yield a mixture of fatty acid methyl esters (biodiesel).

Table 1.3. Percentage oil composition and lipid classes of jatropha curcas seed kernel.

Composition	Percentage
Unsaponifiable	3.8
Hydrocarbons	4.8
Tryacylglycerols	88.2
Free fatty acids	3.4
Diacylglycerols	2.5
Sterols	2.2
Monoacylglycerols	2.7
Polar lipids	2.0

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The oil content of jatropha seeds is higher than most of the other oil seeds [31], [32]. As with most oil seed crops, tryacylglycerol forms the major component of the jatropha oil. Unsaponifiable lipids including sterols and triterpene alcohols are believed to impart insecticidal properties to the jatropha oil. Fatty acid composition of jatropha triglycerides has been reported by many researchers. Oleic and linoleic acids are the two most abundant fatty acids in jatropha oil. A comparison of fatty acid composition of jatropha oil and other oil seeds is shown in Table 1.4 [33].

Table 1.4. Fatty acid compositions of selected biodiesel feedstock.

Fatty Acid	Jatropha Curcas Oil	Palm Oil	Sunflower Oil	Soybean Oil	Palm Oil
Oleic 18:1	44.7	15.4	21.1	23.4	39.4
Linoleic 18:2	32.8	2.4	66.2	53.2	10.2
Palmitic 16:0	14.2	8.4	-	11.0	44.0
Stearic 18:0	7.0	2.4	4.5	4.0	4.5
Palmitoleic 16:1	0.7	-	-	-	-
Linolenic 18:3	0.2	-	-	7.8	0.4
Arachidic 20:0	0.2	0.1	0.3	-	-
Margaric 17:0	0.1	-	-	-	-
Myristic 14:0	0.1	16.3	-	0.1	1.1
Caproic 6:0	-	0.2	-	-	-
Caprylic 8:0	-	3.3	-	-	-
Lauric 12:0	-	47.8	-	-	0.2
Capric 10:0	-	3.5	-	-	-
Saturated	21.6	82.1	11.3	15.1	49.9
Monosaturated	45.4	15.4	21.1	23.4	39.2
Polysaturated	33	2.4	66.2	61	10.5

1.4. LIMITATIONS FOR THE COMMERCIAL EXPLOITATION OF JATROPHA CURCAS

There are two major constraints for the commercial utilization of jatropha curcas. They are: 1) poor oil extraction efficiency and 2) toxicity of the seed cake.

1.4.1 Poor Oil Extraction Efficiency. There exist two most common methods for extracting oil from jatropha seeds: mechanical presses and chemical methods. Mechanical presses are traditional methods of oil extraction. These involve pressing the seeds with high pressure to extract the oil. The commonly used presses are screw type

expeller presses where the raw material (seeds) are squeezed under high pressure in a single step. Although these expellers are convenient to use, the oil extraction efficiency of these presses is low 30-38% (w/w) [34]. Moreover, the oil obtained from the expellers requires filtering and degumming. The other disadvantage with these presses is that their design is suited only for a particular seed variety (like rapeseed or soybean) and the yield varies if used with more than one kind of seeds. On the other hand, the solvent extraction method that involves crushing of the seeds and extraction of crushed seeds with an organic solvent is considerably more efficient. It has been reported that the solvent *n-hexane* can extract about 98% of the oil present in the jatropha seed [35]. But there are many factors which influence the rate of oil extraction. The predominant ones are particle size of seeds, shape selectivity of the solvent to crushed kernel, the viscosity of solvent, temperature of the solvent, and agitation of the solvent during extraction. Hence a prudent selection of the optimum conditions becomes tedious.

1.4.2. Toxicity of the Seed Cake. Even though the seeds are rich in oil and crude protein, these are highly toxic and unsuitable for human or animal consumption. The toxic nature of oil and meal has been demonstrated in a number of studies [36 - 39]. It has also been reported that humans who had accidentally consumed seeds showed signs of giddiness, vomiting and diarrhea [40, 41]. The toxic and anti-nutritive compounds from jatropha seeds which have been isolated include curcin, a lectin [42], flavonoids, vitexine, and 12-deoxyl-16-hydroxyphorbol [43]. Lectin was thought to be responsible for the toxicity of jatropha [44], however, Aderibigbe et al.[45] and Aregheore et al.[46] have shown that lectin is not the major toxic compound in jatropha meal. Furthermore, lectin and trypsin inhibitor activity can be suppressed through heat treatments [45]. The higher concentration of phorbol esters (PE) present in jatropha curcas seed have been reported as the primary substances responsible for the seed's toxicity [4, 43]. Phorbol esters have also been reported even in the non-toxic Mexican accessions of jatropha seeds [7, 28]. Anti-nutritive compounds in 18 provenances of jatropha have been examined and it has been reported that the trypsin inhibitor activity in the defatted meal varied from 18.4 to 27.5 mg of trypsin inhibited per gram of dry meal [4]. The saponin content can vary from 1.8-3.4% (reported as diosgenin equivalent), phytate content from 6.2-10.1% (reported as phytic acid equivalent), and lectin activity from 51 to 204 mg per gram of dry meal. Anti-

nutritional compounds found in jatropha meal and soybean meal are listed in Table 1.5 [6, 29].

Table 1.5. Anti-nutritive components in the jatropha meal and soybean meal.

Toxic and Anti-nutritive compound	Seed Variety			
	Cape Verde (Highly toxic)	**Nicaragua (Highly toxic)**	**Mexican (Non-toxic)**	**Soybean meal**
Phorbol ester (mg/g kernel)	2.70	2.17	0.11	--
Lectin (mg/g kernel)	102	102	51	0.32
Trypsin inhibitor activity (mg inhibition/ g meal)	21.3	21.1	26.5	3.9
Phytate (% in meal)	9.4	10.1	8.9	1.5
Saponin (% diosgenin eqv. in meal)	2.6	2.0	3.4	4.7

Viable and successful utilization of jatropha meal cannot be achieved without the removal of all of the anti-nutritional compounds. As discussed above, a number of anti-nutritionals can be eliminated/reduced by heat treatment. However, phorbol esters, the major toxic compounds are highly stable; they cannot be eliminated with heat even with treatment for 30 minutes at temperatures of 160^0 C [4, 47]. Aregheore et al. [48] have reported that apart from the heat treatment, several chemical treatment methods can be used to reduce the concentration of phorbol esters.

It is clear that phorbol esters are indeed the major impediment to the wide commercial use of jatropha seeds as a feedstock. Hence, the following sections shall discuss phorbol esters and the detoxification experiments conducted by various researchers to eliminate them.

1.5. PHORBOL ESTERS

Phorbol esters are naturally-occurring compounds which are widely distributed in the plant species of the Euphorbiaceae and Thymelaeceae. They are tetracyclic diterpenoids and esters of tigliane diterpenes [49]. Tigliane, a tetracyclic diterpene, constitutes the major alcoholic moiety of this family of compounds. As shown in Figure 1.4, tigliane consists of 4 rings – A, B, C, and D.

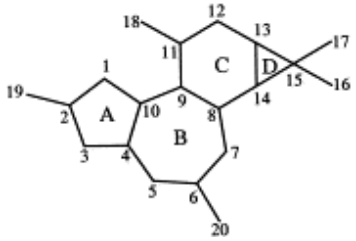

Figure 1.4. Tigliane.

Hydroxylation at various positions and connection to various acid moieties characterized by ester bonding constitutes a large number of compounds collectively known as phorbol esters. The parent diterpene of phorbol esters, phorbol, consists of five hydroxyl groups and each one of them has different reactivity towards acylation [50]. Figure 1.5 shows a phorbol (12-Deoxy-16-hydroxyphorbol) with the 4 rings. On the extreme left is ring A which is trans-linked to ring B at the fourth carbon atom. The ring B is a 7 membered ring. Connected to ring B is ring C which is a 6 membered ring. Ring C is cis-connected to the cyclopropane ring D.

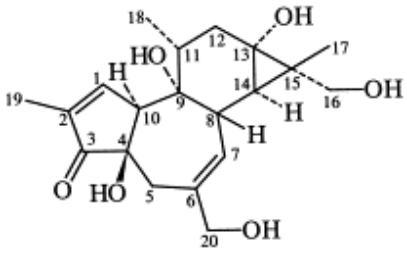

Figure 1.5. 12- Deoxy-16-hydroxyphorbol (β phorbol).

The position of the -OH group on ring C results in two kinds of phorbols– phorbol (∝) or phorbol(β). Among them, phorbol (β) is an active phorbol with the –OH group at the C-13, and phorbol (∝) is an inactive phorbol with the –OH group at C-12. The activity of these phorbols depends on the relative position of hydroxyl group. Although both the active and inactive forms have the same lipophilicity and physiochemical properties, the inactive ∝ phorbol esters are not able to activate the Protein Kinase C (PKC) due to conformational shifts [51].

The most widely known and accepted phorbols are tetradecanoyl phorbol-13 acetate, TPA (4ß-12-*O*-tetradecanoylphorbol-13-acetate) and PDBu (4ß-phorbol-12,13-dibutyrate) which differ only by their substitutions at positions 12 and 13 of ring C. The molecular arrangement of TPA is shown in Figure 1.6.

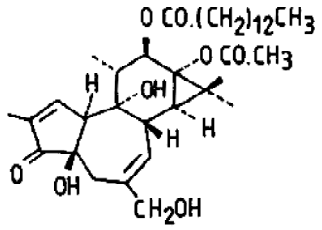

Figure 1.6. Tetradecanoyl phorbol-13 acetate.

It has been reported that the jatropha curcas kernel has at least four different phorbol esters and the predominant among these is 12-deoxy-16-hydroxyphorbol-4′-[12′,14′-butadienyl]-6′-[16′,18′,20′-nonatrienyl]-bicyclo[3.1.0]hexane-(13-*O*)-2′-[carboxylate]-(16-*O*)-3′-[8′-butenoic-10′]ate (DHPB) [52] which is shown below in Figure 1.7.

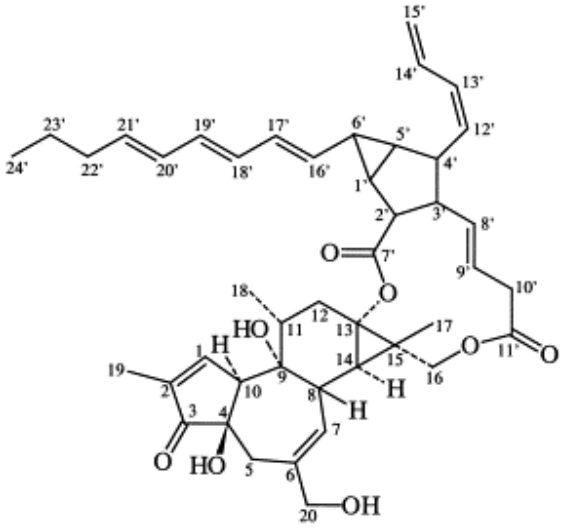

Figure 1.7. DHPB.

The phorbol esters have been reported to be potential tumor promoters. They are responsible for skin irritant effects and tumor promotion because they stimulate PKC [53], which is involved in signal transduction and developmental processes of most of the cells and tissues, producing a variety of biological effects in a wide range of organisms. Phorbol esters also contribute to the formation of cancer. These phorbol esters do not cause the tumor formation alone, but can also lead to an increased risk of tumor formation when there is a co-exposure to the chemical carcinogen [54].

1.6. DETOXIFICATION EXPERIMENTS WITH JATROPHA KERNEL TO ELIMINATE PHORBOL ESTERS

A few studies aimed at detoxification of the jatropha oil and meal by reducing phorbol esters concentration have been reported. Haas and Mittelbach reported that traditional oil refining methods like degumming, deacidification, bleaching, and deodorization can decrease the phorbol content of the seed by about 50% [55]. They concluded that the treatment with alkali hydroxides during acidification as well as bleaching with traditional bleaching earth had the most influence on decreasing the amount of phorbol esters in the oil. On the other hand, degumming and deodorization was unable to significantly reduce the quantity of phorbol esters in the oil.

Devappa and Swamilingappa have reported that up to 90% of the phorbol esters can be removed by treating the meal with 20 g/L of calcium hydroxide {$Ca(OH)_2$}[56].

Makkar and Becker have reported that ethanol (80%) or methanol (92%) [1:5 w/v] reduced both the saponins and phorbol esters by 95% after four extractions [47].

E. Chivandi, et al.[58] reported that laboratory scale petroleum ether extraction reduced the phorbol ester content in the jatropha curcas seeds by 67.69% [6.5 mg/g in the raw kernels to 2.10 mg/g], double solvent extraction followed by moist heat treatment reduced phorbol esters by 70.77% to 1.90 mg/g. Double solvent extraction accompanied with wet extrusion, re-extraction with hexane and moist-heat treatment reduced phorbol ester content to 0.80 mg/g, an 87.69% decrease. Rakshit and Bhagya have shown that the amount of phorbol esters can be eliminated by about 90% by chemical treatment [57].

A process is thus needed that efficiently extracts the oil and detoxifies the meal.

2. RESEARCH OBJECTIVES

A novel and efficient process for obtaining high yields of oil from Indian accession of jatropha curcas meal and detoxifying the seed cake to a phorbol ester content of less than 0.11 mg g^{-1} (Mexican non-toxic level) was developed. The overall objective was accomplished through a set of sub-objectives which were sequentially linked and each sub-objective was accomplished with a set of experiments. The sub-objectives were:

1) To set-up lab-scale experimental apparatus for carrying out solid liquid extractions involving the jatropha meal and various solvents.
2) To carry out the leaching operation of the meal utilizing pure solvents and mixed solvents using ultrasonic waves in a sonicator.
3) To carry out the leaching operation utilizing single (pure) solvents and a cycle of sequential solvents.
4) To assess the extraction efficiency with various solvents based on their polarity index.
5) To carry out a comprehensive quantitative and qualitative analysis of the treated oil and meal for characterizing phorbol esters with HPLC.

3. EXPERIMENTAL

3.1 EXTRACTION APPARATUS

3.1.1. Extraction Using Soxhlets. The solvent extraction was performed with soxhlet extractors. The soxhlet extractor is typically used to extract the lipids from solid material. Oil and fat from solid material is extracted by washing the solid repeatedly with the organic solvent under reflux in a glass tube. The solid material is made to come in contact and equilibrate repeatedly with the solvent. After an equilibrium period, solvent is then siphoned out from the solid / solvent suspension and fresh solvent is allowed to accumulate and equilibrate with the solid till it is siphoned out. The assembly consists of three major apparatuses: a round bottom flask, an extraction tube, and a condenser. A schematic of this assembly is shown in Figure 3.1.

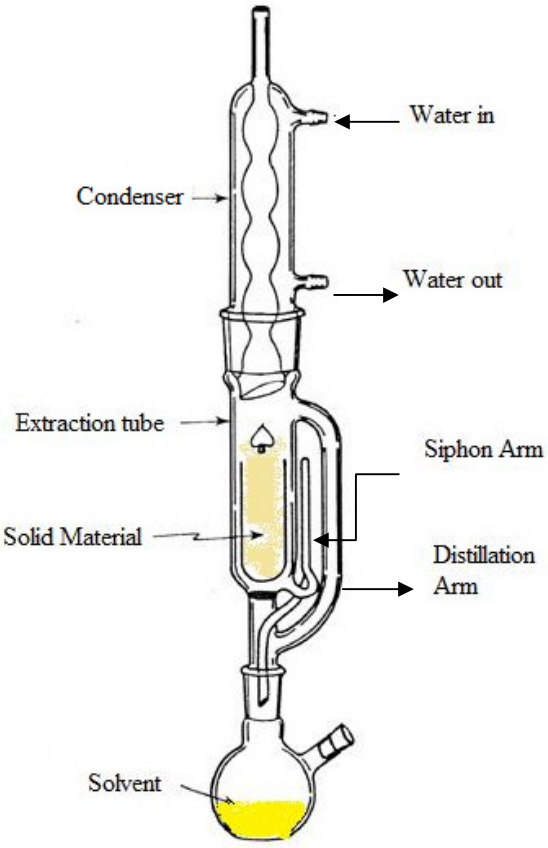

Figure 3.1. An assembly consisting of a condenser, soxhlet extractor, and round bottom flask.

Finely divided solid material to be extracted is packed and placed in the extraction tube. Soxhlet extraction tube is connected to a condenser and a solvent reservoir (round bottom flask - RBF). It was ensured that the joints at the top and bottom were vapor tight. The solvent in the RBF was heated with a thermostated heating mantle to bring to a low boil. The solvent vapors were carried to the water cooled condenser through the distillation arm. The RBF and the distillation tube were wrapped in a ceramic blanket to ensure efficient transfer of solvent vapors from the RBF to the condenser. The extraction tube was also wrapped in the ceramic blanket to ensure that the condensed solvent remained at elevated temperature thus permitting faster cycling rate, faster equilibration, and higher solvent flow through the extractor. A photograph of the unwrapped extractor assemblies used during this study is given in Figure 3.2.

Figure 3.2. A pictorial representation of soxhlet assembly.

Two different size soxhlet apparatuses were used during the study; one had a 50 g capacity, while the other had a 125 g capacity. A 500 mL RBF was used with the former while a 1000 mL RBF was used with the latter.

3.1.2 Ultrasound Assisted Extraction. Ultrasound assisted extraction was carried out in 8.5 cm x 7.5 cm closed aluminum containers, Figure 3.3. Finely divided jatropha meal and solvents were put in the containers. The containers were placed in an ultrasonic water bath. The meal extraction was carried for time periods varying between 30 and 60 minutes.

Figure 3.3. Aluminum containers for sonication.

3.2. ULTRASOUND ASSISTED EXTRACTIONS WITH PURE SOLVENT AND SOLVENT MIXURES

Ultrasound assisted extractions of jatropha meals were carried out with four different solvents. Solvents used during the extractions were:

1) Hexanes (H, n-hexane and 4.2% methyl pentanes)

2) Methanol (M)

3) Ethanol (E)

4) Isopropyl alcohol (I)

All of the solvents were of ACS reagent grade purity and were purchased from Fisher Scientific.

3.2.1. Extractions with Single Solvents. Hexanes and methanol were used for extractions individually. Four 10 gram aliquots of ground kernel were placed in four aluminum containers along with 100 mL of solvent. The containers were placed in an ultrasonic bath and the contents were sonicated for 30 min. After sonication, the extract was gravity-drained, centrifuged, and filtered. The filtered extract was then transferred to a 250 ml RBF. The extract was then rotavaporated to recover nearly all of the solvent. Residue in the RBF was transferred to a 10 ml glass vial and its weight was noted. The meals were extracted three more times with 100 mL of the solvent for half an hour and extract was removed. The amount of oil recovered during each extraction was recorded.
The hexane extracted meal aliquots were allowed to dry in a fume hood at room temperature for 24 hours. The dried (solvent free) meals were then extracted four times with 100 mL of methanol. The methanol extracts were weighed individually and then pooled in vials.

3.2.2. Extractions with Solvent Mixtures. In these experiments, jatropha meal was extracted with different solvent mixtures. Ten gram aliquots of the meal were extracted with 100 mL of solvent mixtures. The meal was washed with 100 mL of solvent to extract the residual oil trapped in meal. The weight of oil recovered after each extraction was recorded individually. The oils were then pooled. Solvent mixtures used during the extractions were:

1. Hexanes : Methanol [(9:1), (8:2) and (7:3)]
2. Hexanes : Ethanol [(9:1), (8:2) and (7:3)]
3. Hexanes : Isopropanol [(9:1), (8:2) and (7:3)]

A flowchart of the steps involved in extraction with the solvent mixtures is shown in Figure 3.4.

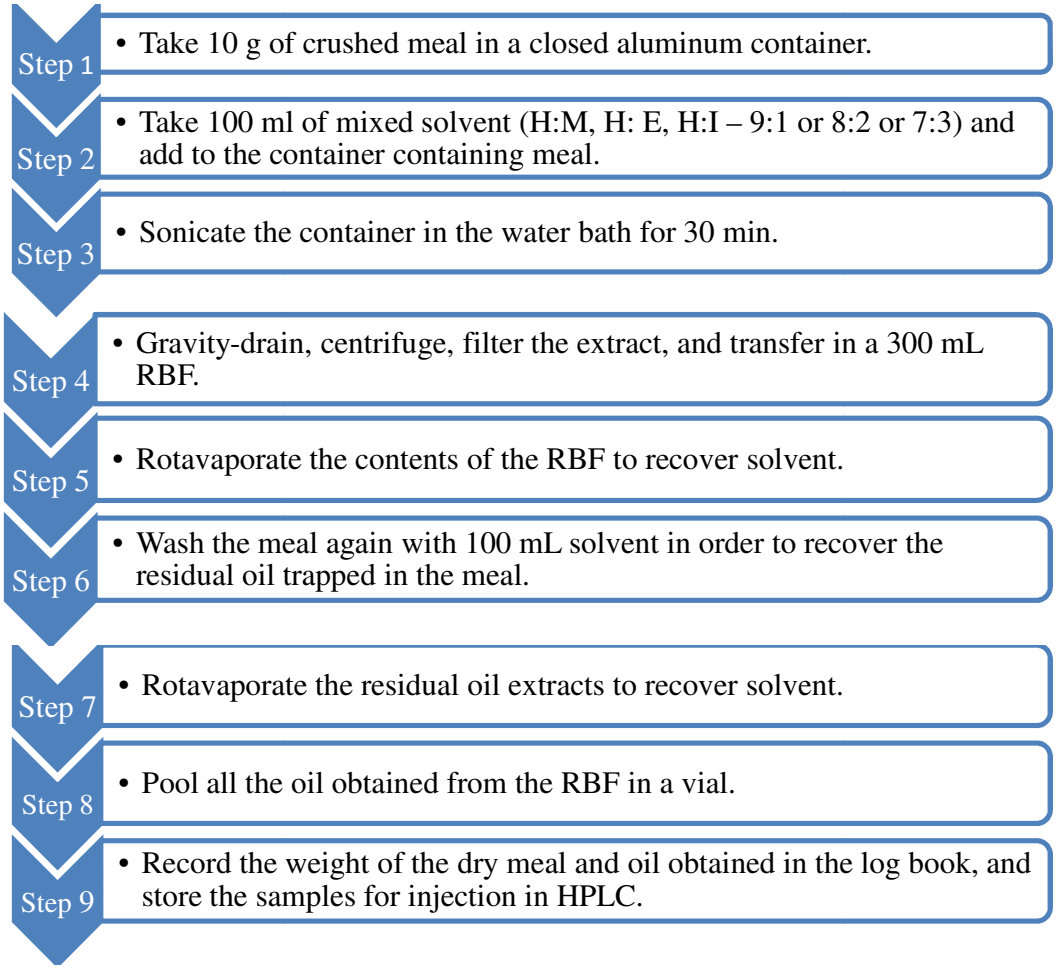

Fig 3.4. A block diagram of the ultrasound-assisted extraction process with mixed solvents.

3.3. JATROPHA MEAL EXTRACTIONS WITH SOXHLET APPARATUS

Jatropha meal was also extracted in a soxhlet apparatus. Sequential extractions were performed with solvents of different polarity.

1. Extraction with hexanes for 24 hours followed by methanol for 24 hours followed by fresh methanol for 24 hours.
2. Extraction with hexanes for 24 hours followed by isopropyl alcohol for 24 hours followed by fresh isopropyl alcohol for 24 hours.
3. Extraction with hexanes for 24 hours followed by ethanol for 48 hours.
4. Extraction with hexanes for 24 hours followed by methanol for 72 hours.

As mentioned earlier, the reflux flask, distillation arm, and the extraction tube were wrapped in ceramic blanket insulation. The insulation maintained temperatures near the boiling point in the flask and the distillation arm. It also helped in maintaining the extractor temperature near the boiling point of the solvent. The elevated temperature helped maintain a higher reflux rate and enhanced extraction efficiency. The solvent drop rate was maintained at about 10 drops per minute. After the desired extraction period the heating mantle was turned-off and the solvent in the flask along with the extracted oil was allowed to cool down to room temperature. For sequential extractions, first extraction was carried out with the nonpolar solvent followed by extraction with a more polar solvent. In these cases, flasks with non-polar solvent and extracted materials were removed and replaced with flasks containing the more polar solvent and the soxhlet apparatus was reassembled. Extraction with polar solvent was carried out for the desired period.

The non-polar solvent and extract materials were transferred from the flasks to the centrifuge tubes. The centrifuge tubes were placed in a centrifuge and spun at 3500 rpm for about 30 minutes to remove small meal particles that had been transported into the flask during extraction. The particle free solvent layer in the centrifuge tube was filtered and transferred into a clean round bottom flask, solvent in the flask was removed with a rotary evaporator. Residual oil in the meal was extracted by washing the meal with the solvent, and processing the extract to recover the solvent leaving behind oil. The weight of solvent free extracted oil was determined gravimetrically and the percentage extracted oil was calculated. A similar procedure was followed with extract obtained with the polar solvent/s. Once the extractions had been completed, the extracted meal (defatted meal) was placed in an open tray and placed in the fume-hood and the residual solvent was allowed to evaporate till a “dry” i.e. solvent free meal was obtained. Weight of the extracted dry meal was obtained.

3.4. DETERMINATION OF PHORBOL ESTERS WITH HIGH PRESSURE LIQUID CHROMATOGRAPHY WITH A DIODE ARRAY DETECTOR

The defatted meal and oil extracts from each cycle were back extracted with methanol. Methanol extracts were filtered through 0.2 µm filters and analyzed for phorbol esters with an HPLC system equipped with a diode array detector (DAD). The procedure used during the analysis is outlined in the following section.

3.4.1. Sample Preparation.

1. Approximately 1 g (±0.1 g) of the sample was weighed and transferred to 15 mL centrifuge tube.
2. 5 mL of methanol (HPLC grade) was added to the tube.
3. The contents of the tube were sonicated for 30 minutes in a water bath.
4. The tube was placed in a centrifuge and spun at 3500 rpm for about 20 minutes.
5. Supernatant was carefully transferred to a 15 mL glass tube.
6. Steps 2-5 were carried out three times with each sample. The methanol extracts were pooled.
7. The volume of the pooled extract was brought down to 1 mL by rotavaporation.
8. The concentrated extract was filtered through a 0.22 µm filter into a LC autosampler vial.
9. Vials were placed in the autosampler rack and analyzed for phorbol esters. A phorbol ester standard (100 µg/l) for each of the four phorbol esters was included with each set of samples.

3.4.2. Instrument Parameters. The liquid samples were injected into a bench top liquid chromatograph, Model LaChrom Elite, Hitachi High-Technologies Corporation, Schaumburg, Illinois, USA, consisting of a Column Oven, Model L-2300; Autosampler, Model L-2200; Pump, Model L-2100/2130, and a Diode Array Detector, Model L-2450. LC parameters used for separation of phorbol esters are listed in Table 3.1.

3.4.3. Chemicals. High purity standards (~99%) of the phorbol esters were collected as pure fractions in the fraction collector in prep LC. Identification of constituents was based on their retention time, and the information obtained from their

mass-spectra. This mass-spec information was obtained from the LC-MS characterization of PE by a fellow student.

Table 3.1. LC parameters used during quantification of phorbol esters.

Instrument Parameters	
Column	Microsorb –MV 100-5 C18 column 15 cm long x 4.6 mm ID x 5μm particle size of packing
Mobile Phase	A (20% Nanopure Water) B (80% Acetonitrile)
Flow Rate	1 mL/min
Injection Volume	10 μL
Detection Wavelength	DAD / 280 nm
Oven Temperature	25^0 C
Run Time	15 minutes
Elution	Isocratic

3.4.4. Sampling Procedure and Instrument Operation. The following protocol for operating the instrument was used during determination of phorbol esters:

1. DAD detector was turned on, followed by the column oven, and then the pump.
2. The mobile phase line was purged for about 5 minutes with solvents to remove air-bubbles or previously used solvents.
3. LC parameters were in accordance with those listed in Table 3.1.
4. Mobile phase was allowed to flow through the system for 10 minutes.

5. Autosampler sequence was started; the first sample in the sequence was set to solvent blank.
6. Switch-off the lamp in the detector after the sequence is injected.

4. RESULTS AND DISCUSSIONS

4.1. DETERMINATION OF PHORBOL ESTERS IN FRESH MEAL

The first step in the experiment was to determine the amount of phorbol esters in fresh meal. This served as the bench-mark in comparing the phorbol esters in untreated (fresh) meal and solvent-treated meal. Six aliquots of fresh meals were prepared and the pick-backs of extract samples were injected into the LC instrument according to steps discussed in Section 3.4.1. The instrument was operated as discussed in Section 3.4.5. Figure 4.1 shows a chromatogram which has been obtained from one of the six aliquots.

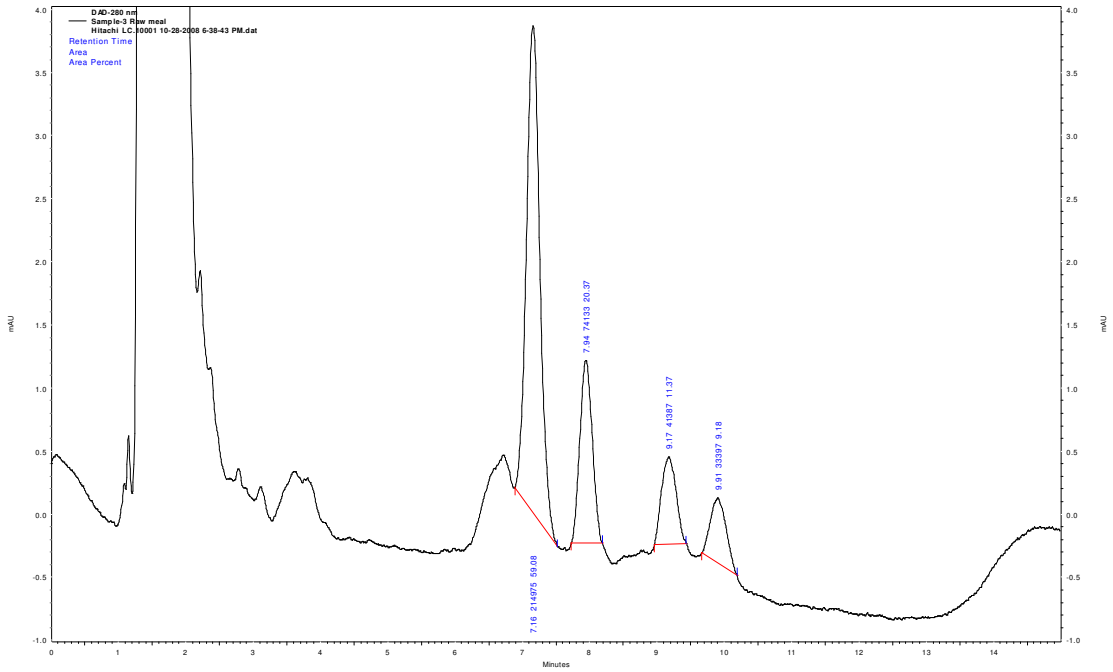

Figure 4.1. Chromatogram of fresh (unextracted) meal.

Four major phorbol ester peaks were detected in the methanol extract of the raw jatropha meal. The retention times (R_t) of the phorbol ester peaks under the LC parameters used during this work were found to be 7.16, 7.94, 9.17, and 9.81 min respectively. The peak areas were used for determining phorbol ester concentration.

The phorbol ester (PE) concentrations in samples were determined by comparing the area of PE in the samples with those in the standards.

$$\frac{\text{Unknown concentration of PE peak in sample (U)}}{\text{Known area of PE peak in sample (A1)}} = \frac{\text{Known concentration of PE peak in standard}}{\text{Known area of PE peak in standard (S2)}}$$

The concentration of PE peak in standard was 100 µg/mL.

Hence, unknown concentration of PE in sample, U $(\mu g/mL) = (A1 * 100\ \mu g/mL)/S2$

$$\text{Concentration of PE in sample (mg/g)} = \frac{\text{U } (\mu\text{g/mL}) * \text{Dilution Factor}}{\text{Sample Weight (g)} * 1000}$$

The dilution factor was 15 since the samples were extracted thrice with 5 mL of methanol (Section 3.4.1). Thus, the concentration of phorbol ester was determined for each and every extraction, and this concentration was expressed in milligrams of PE present per gram of meal. PE concentrations in the six raw jatropha meals aliquots are given in Table 4.1.

Table 4.1. Phorbol ester concentration in mg/g of treated meal for 6 replicates.

Raw Meal Aliquot #	**Phorbol Ester (mg/g)**
1	6.98
2	5.44
3	5.25
4	5.89
5	6.31
6	6.46
Average	**6.05**
Standard Deviation	**0.65**

The average PE concentration in the raw jatropha meal was found to be 6.05 mg g^{-1}, this value was found to be in agreement with the values reported in the literature.

4.2. ULTRASOUND ASSISTED EXTRACTION (SONICATION) OF JATROPHA MEAL

Ultrasound in the 20 – 40 kHz range is routinely used in chemical laboratories for varied purposes including cleaning and mixing. Ultrasound has also been used during extraction of a solid material with solvent/s. Ultrasound waves are believed to break the intra-molecular interactions of the solid matrix with the solute by providing them kinetic energy. Propagation of ultrasound through the liquid leads to cavitation which in turn produces heat which helps in a better extraction.

Jatropha meal samples were sonicated with hexanes and methanol over eight extraction cycles each of 30 minute duration. Oil extracted after each cycle was recovered and weighed to check extraction efficiency of each cycle. Results obtained from the extractions are listed in Table 4.2.

Table 4.2. Sonication-assisted extraction with hexane and methanol.

Cycle # and Duration	Sonication-Set 1	Sonication-Set 2	Sonication-Set 3
	Starting material: 10 g crushed and dehulled Jatropha meal and 100 mL hexanes.		
1 – 30 minutes	Wt. of Oil: 3.6 g	Wt. of Oil: 4.0 g	Wt. of Oil: 4.2 g
2 – 30 minutes	Wt. of Oil: 0.8 g	Wt. of Oil: 1.1 g	Wt. of Oil: 1.0 g
3 – 30 minutes	Wt. of Oil: 0.3 g	Wt. of Oil: ND	Wt. of Oil: ND
4 – 30 minutes	Wt. of Oil: 0.1 g	Wt. of Oil: ND	Wt. of Oil: ND
	Starting material: Extracted as well as dried meal (after cycle 4) and 100 mL methanol; fresh methanol, extracted as well as dried meal (from previous cycles) used in sequential cycles.		
5 – 30 minutes	Wt. of Oil: 0.2 g	Wt. of Oil: 0.6 g	Wt. of Oil: 0.4 g
6 – 30 minutes	Wt. of Oil: 0.2 g	Wt. of Oil: ND	Wt. of Oil: ND
7 – 30 minutes	Wt. of Oil: 0.2 g	Wt. of Oil: ND	Wt. of Oil: ND
8 – 30 minutes	Wt. of Oil: ND	Wt. of Oil: ND	Wt. of Oil: ND
Total oil extracted	5.4 g	5.7 g	5.6 g

Oil extracted in third and fourth cycles was not detected due to the very small amount of oil present in RBF (<0.1 g) which was below the minimum detectable limit of the analytical balance in our lab.

Also, the experimental results justify the inherent assumption of the sonication procedure of complete extraction of oil in four cycles. This is evident from the fact that four cycles produced a non-detectable level of oil (with the only exception of Set #1).

Results showed that oil averaged 56% of the total mass of the raw meal and most of the oil can be extracted from the meal through four extraction cycles.

Statistical analysis of the extraction data showed that the oil yield from ground kernels varied from 48% - 52 % for extractions of the meal with hexanes and 54-57% for extractions of meal with hexanes followed by ethanol.

In addition to extraction with single solvents, aliquots of ground jatropha meal were also extracted with binary solvent mixtures consisting of Hexanes : Methanol, Hexanes : Ethanol, and Hexanes : Isopropyl alcohol. The ratios of hexanes and alcohols in the mixtures were varied from 9:1, 8:2, to 7:3. During each extraction cycle 10 g ground jatropha meal was extracted with 100 mL of solvent mixture in sonic bath for 30 minutes. The extraction results obtained with solvent mixtures are given in Table 4.3.

Table 4.3. Sonication-assisted mixed-solvent experiments.

Solvent Mixture	Sonication-Set 1	Sonication-Set 2	Sonication-Set 3
Hexane: Methanol Volume: 100 mL Ratio: 9:1	Wt. of oil: 3.4 g Wt. of dry meal: 6.2 g	Wt. of oil: 2.91 g Wt. of dry meal: 6.6 g	Wt. of oil: 3.8 g Wt. of dry meal: 6.2 g
Hexane: Methanol Volume: 100 mL Ratio: 8:2	Wt. of oil: 3.67 g Wt. of dry meal: 5.8 g	Wt. of oil: 3.44 g Wt. of dry meal: 5.4 g	Wt. of oil: 3.32 g Wt. of dry meal: 6.0 g
Hexane: Methanol Volume: 100 mL Ratio: 7:3	Wt. of oil: 3.14 g Wt. of dry meal: 4.0 g	Wt. of oil: 3.20 g Wt. of dry meal: 3.6 g	Wt. of oil: 3.14 g Wt. of dry meal: 4.0 g
Hexane: Ethanol Volume: 100 mL Ratio: 9:1	Wt. of oil: 4.8 g Wt. of dry meal: 6.1 g	Wt. of oil: 4.2 g Wt. of dry meal: 6.6 g	Wt. of oil: 5 g Wt. of dry meal: 6.0 g
Hexane: Ethanol Volume: 100 mL Ratio: 8:2	Wt. of oil: 3.2 g Wt. of dry meal: 7.2 g	Wt. of oil: 4.2 g Wt. of dry meal: 6.9 g	Wt. of oil: 3.9 g Wt. of dry meal: 6.6 g
Hexane: Ethanol Volume: 100 mL Ratio: 7:3	Wt. of oil: 3.6 g Wt. of dry meal: 6.8 g	Wt. of oil: 3.8 g Wt. of dry meal: 7.0 g	Wt. of oil: 3.4 g Wt. of dry meal: 7.0 g
Hexane: 2-propanol Volume: 100 mL Ratio: 9:1	Wt. of oil: 3.4 g Wt. of dry meal: 6.0 g	Wt. of oil: 3.6 g Wt. of dry meal: 5.6 g	Wt. of oil: 4 g Wt. of dry meal: 6.4 g
Hexane: 2-propanol Volume: 100 mL Ratio: 8:2	Wt. of oil: 3.0 g Wt. of dry meal: 6.0 g	Wt. of oil: 3.6 g Wt. of dry meal: 6.0 g	Wt. of oil: 3.6 g Wt. of dry meal: 6.2 g
Hexane: 2-propanol Volume: 100 mL Ratio: 7:3	Wt. of oil: 3.2 g Wt. of dry meal: 6.6 g	Wt. of oil: 3.6 g Wt. of dry meal: 6.0 g	Wt. of oil: 3.4 g Wt. of dry meal: 6.2 g

It was observed that in certain experiments the total weight of the extracted oil and the weight of defatted solvent free meal was less than 10 g. The most likely reason for this is loss of oil and fine meal particles during sample transfer steps. In several other experiments, the weight of defatted solvent free meal and extracted oil was greater than 10 g, which is most likely due to the solvent trapped in the meal.

The results of ultrasound assisted extraction with hexanes and hexanes – alcohol mixtures showed that the amount of oil extracted with hexanes was greater than the amount of oil extracted with the solvent mixtures.

4.2.1. Liquid Chromatographic Analysis of Solvent-Treated and Untreated Meal. Aliquots of raw jatropha meal and defatted meals obtained after extraction with hexanes and hexanes – alcohol mixtures were analyzed for phorbol ester (PE) content with liquid chromatography. Sample preparation and chromatographic separation – quantification were performed according to steps described in the experimental section. The HPLC system was calibrated with phorbol ester standards. A representative chromatogram for one of the four PE standard is shown in Figure 4.2. The peak eluted after 7.7 minutes.

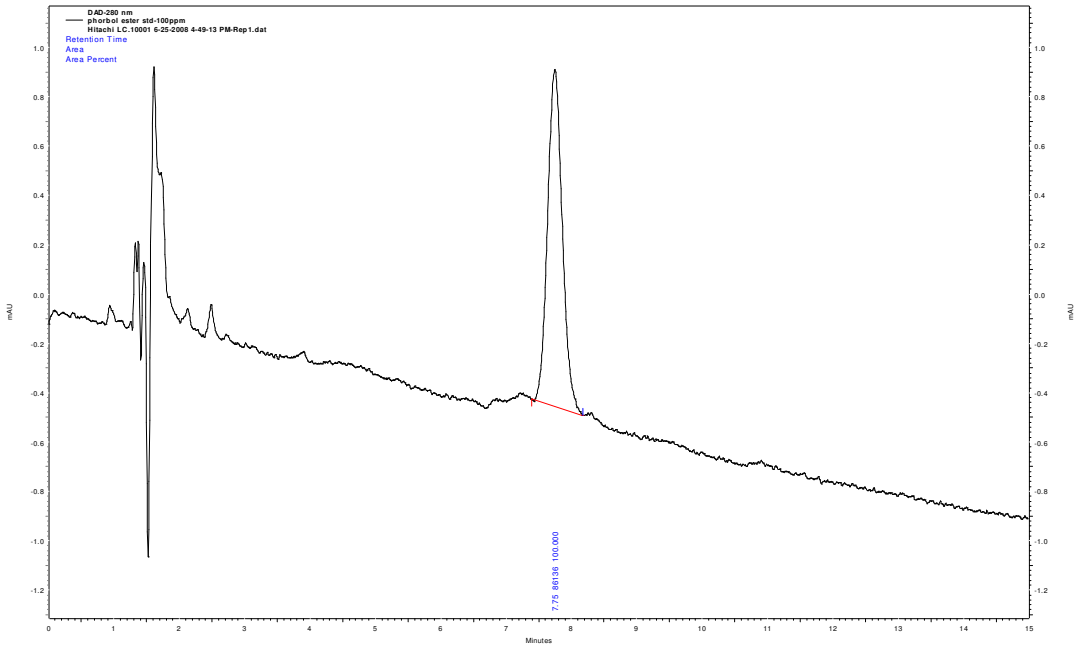

Figure 4.2. Phorbol ester standard – 100 µg/mL.

A representative chromatographic output for PEs present in methanol extracts of meals obtained after ultrasound assisted extraction with hexanes – alcohol mixtures is shown in Figure 4.3. The chromatogram shows the residual amounts of the four PEs in the meal extracted with hexanes – methanol mixture with a ratio of 9:1. Similar residual amounts of PEs were detected in the meals extracted with hexanes – methanol mixtures

containing eight parts hexanes – two part methanol and seven parts hexanes – three parts methanol.

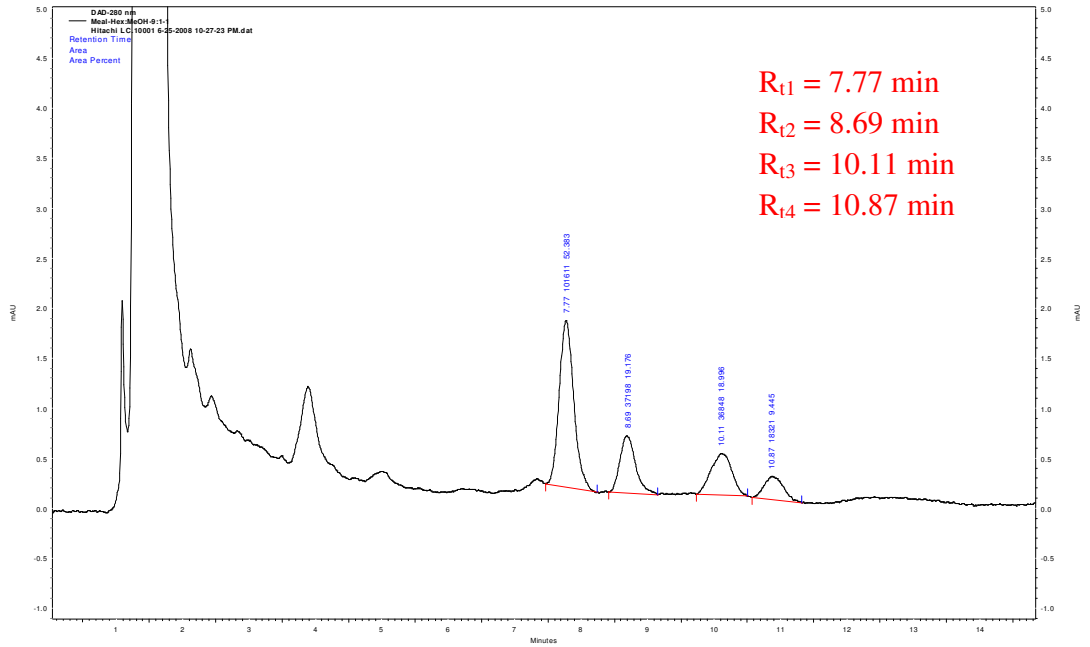

Figure 4.3. Chromatogram obtained from meal treated with hexanes - methanol mixture (volumetric ratio of 9:1).

The retentions times of PEs peaks observed in the extracts were similar to the PE peaks in jatropha meals reported by Haas et al.[59] and Makkar et al.[60]. Similar PE residues were observed in the meals extracted with hexanes - isopropyl alcohol mixtures. A representative chromatogram of PE residues in these meals is shown in Figure 4.4.

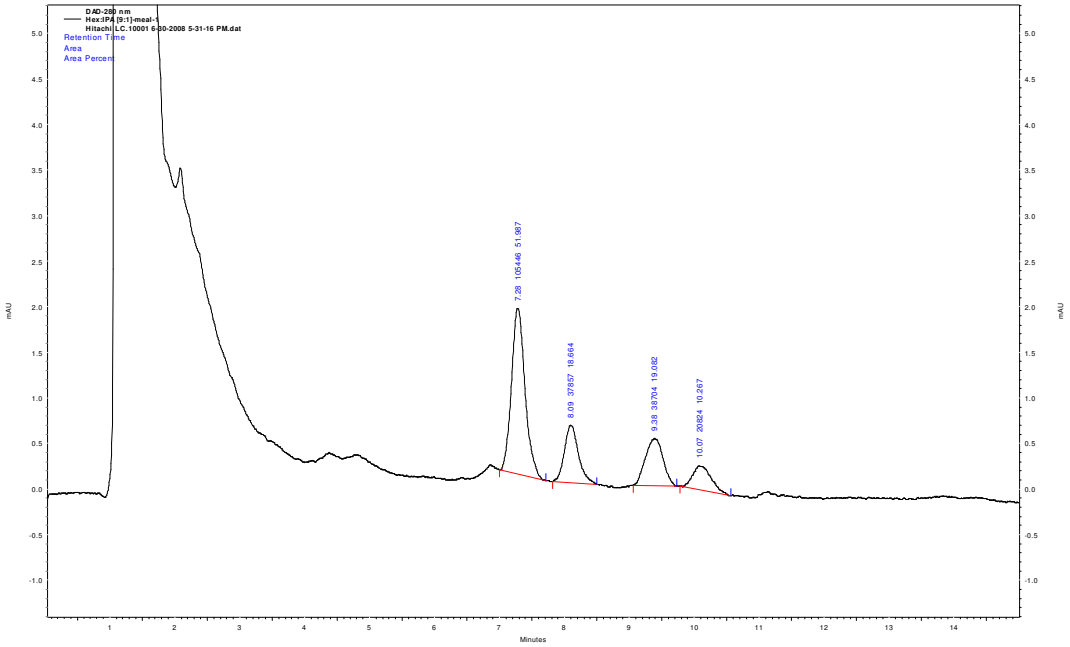

Figure 4.4. Chromatogram obtained from meal treated with hexanes – isopropyl alcohol mixture (volumetric ratio of 9:1).

Similar PE residues were detected in meals extracted with hexanes – isopropyl alcohol mixtures with hexanes – isopropyl ratios of 8:2 and 7:3; as well as the meals extracted with the hexanes – ethanol mixtures. The UV-Vis spectra for the peaks were very similar indicating a similarity in structure. The absorption maxima [$\lambda_{max.}$] of all the peaks were found to lie at 280 nm. The absorption spectra were nearly identical to those reported by Makkar et al.[60].

4.2.2. Quantification of Phorbol Ester Content in Meals Extracted with Hexanes and Hexanes – Alcohol Mixtures. Concentrations of PEs in meals obtained after ultrasound assisted extraction with hexanes are given in Table 4.4. Results showed that average concentration of PEs in these meals was 4.3 mg g^{-1}. The value was approximately 31 % lower than the average concentration of PEs in the raw jatropha meal.

Table 4.4. Phorbol ester content in hexane extracted jatropha meal.

Determination #	PE (mg/g) in meal treated with hexane
1	4.2
2	4.5
3	4.2

Concentrations of PEs in meals obtained after ultrasound assisted extraction with hexanes – alcohol mixtures are given in Table 4.5. Results showed that average residual concentration was lowest in meals extracted with hexanes – alcohol mixtures with the volumetric ratio of 9:1. Most effective mixture was the hexane – methanol mixture. The residual PEs concentration extracted with this mixture was 2.1 mg g^{-1}. The value was approximately 63 % lower than the average concentration of PEs in the raw jatropha meal.

Table 4.5. Phorbol ester content in solvent-treated and dried jatropha meal.

Solvent		PE (mg/g) in Mixed Solvent 9:1	PE (mg/g) in Mixed Solvent 8:2	PE (mg/g) in Mixed Solvent 7:3
Hexane:Methanol	Det. # 1	**2.9**	-	3.3
	Det. # 2	**2.1**	4.1	2.9
	Det. # 3	**2.1**	5.5	2.4
Hexane: 2-Propanol	Det. # 1	2.8	2.7	2.9
	Det. # 2	3.2	3.2	3.4
	Det. # 3	2.8	3.3	2.8
Hexane: Ethanol	Det. # 1	**2.3**	3.0	2.4
	Det. # 2	**2.4**	2.8	2.6
	Det. # 3	**2.1**	2.8	3.1

Hence, these results showed that ultrasound assisted extraction with hexanes and the hexanes – alcohol mixtures was not very effective in extracting PEs from the jatropha meal.

4.3. EXTRACTION WITH SOXHLET APPARATUS

4.3.1. Sequential Extraction of Jatropha Meal with Hexanes and Methanol.

These extractions were carried out in accordance with the procedure described in the experimental section. Meal was extracted first with hexane for 24 hours and then with methanol for 24 – 72 hours. The rationale for the sequential extraction lay in the fact that PEs are more polar than triacyl glycerols, the major lipids present in the jatropha meal, and as a result cannot be efficiently extracted with non-polar solvents such as hexanes that have a polarity index of 0.01 [61]. However, PEs are bound in the lipid fraction of the meal and are not efficiently extracted with polar solvent such as the methanol, polarity index 5.1, because of poor wetability of non-polar meal surface with polar solvents. The non-polar oil therefore needs to be extracted prior to efficient extraction of PEs. The amounts of oil extracted with sequential hexanes - methanol extractions are given in Table 4.6. Results showed that oil fraction extracted with hexanes represented ~ 52 % of the total initial mass of the meal while oil fraction extracted with methanol represented ~ 6.7 % of the mass of the meal and the defatted meal accounted for ~42% of the initial mass of the meal.

Table 4.6. Amount of oil extracted with sequential extraction with hexanes and methanol.

Solvent and duration	**Soxhlet #1**	**Soxhlet #2**
Hexanes, 24 hrs	Weight of Ground meal 50.1 g	Weight of Ground meal 50.0 g
	Wt. of extracted oil 26.0 g	Wt. of extracted oil 26.8 g
Methanol, 24 hrs	Wt. of extracted oil 2.0 g	Wt. of extracted oil 2.4 g
Methanol, 48 hrs	Wt. of extracted oil 1.3 g	Wt. of extracted oil 1.3 g
-	Wt. of dry meal 21.0 g	Wt. of dry meal 21.5 g
Total weight of substance extracted	50.4 g	52.0 g

4.3.1.1 PEs in hexane, methanol extracted oils and meals. Oil fractions obtained after hexanes and methanol extractions were analyzed for PEs with HPLC. All oil samples showed the presence of PEs. A representative chromatogram of PEs in oil extracted with hexanes is shown in Figure 4.5. The chromatogram shows the presence of four PEs peaks. The few co-eluting peaks observed are other lipids extracted with hexanes.

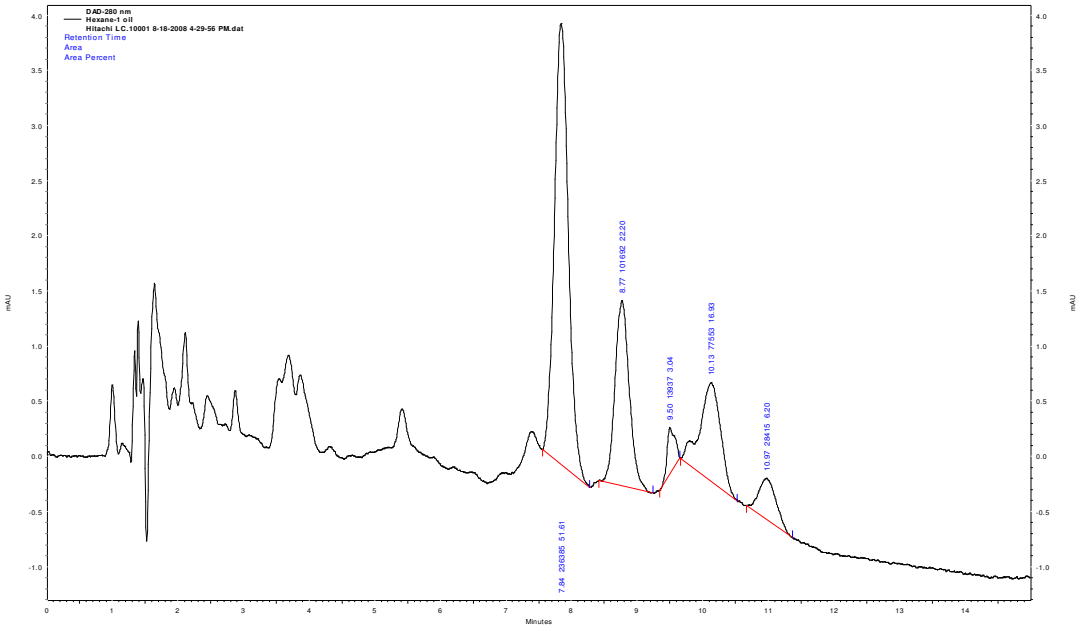

Figure 4.5. Chromatogram of PE residues in hexanes extracted jatropha oil.

Chromatography results were obtained with oil fractions extracted with methanol showed the presence of the same PEs peaks, Figure 4.6. However, the chromatogram contained fewer co-eluting components than observed in the hexanes extracted oils.

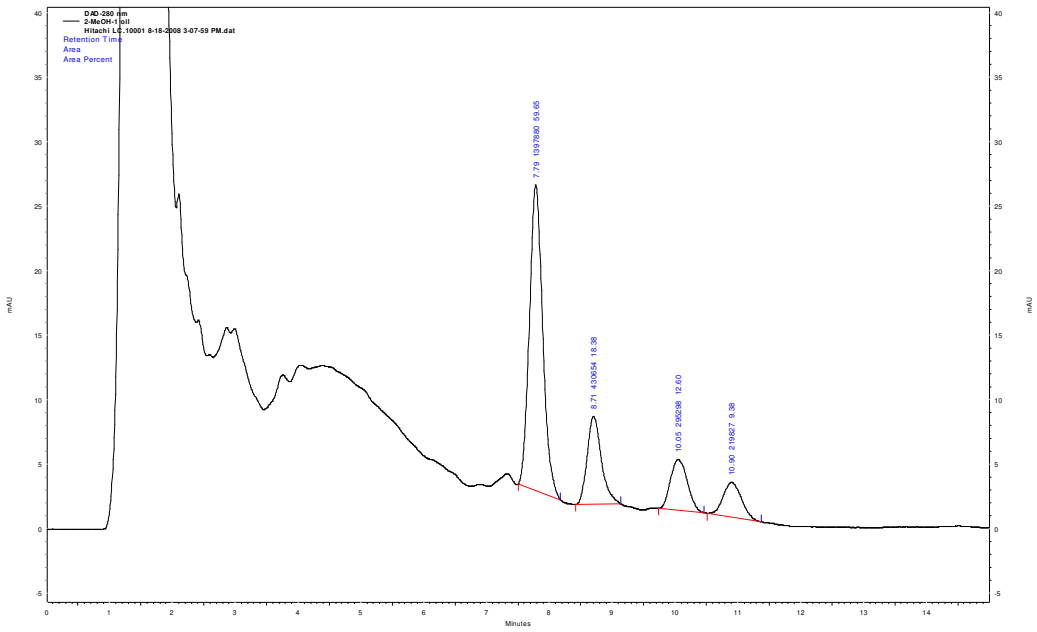

Figure 4.6. Chromatogram of oil fraction obtained after the first methanol extraction.

Chromatogram for the PE present in the oil obtained after the second methanol extraction is shown in Figure 4.7. Peak areas were markedly lower in this oil fraction indicating that most of PEs had been extracted by the hexanes and the first cycle of extraction with methanol.

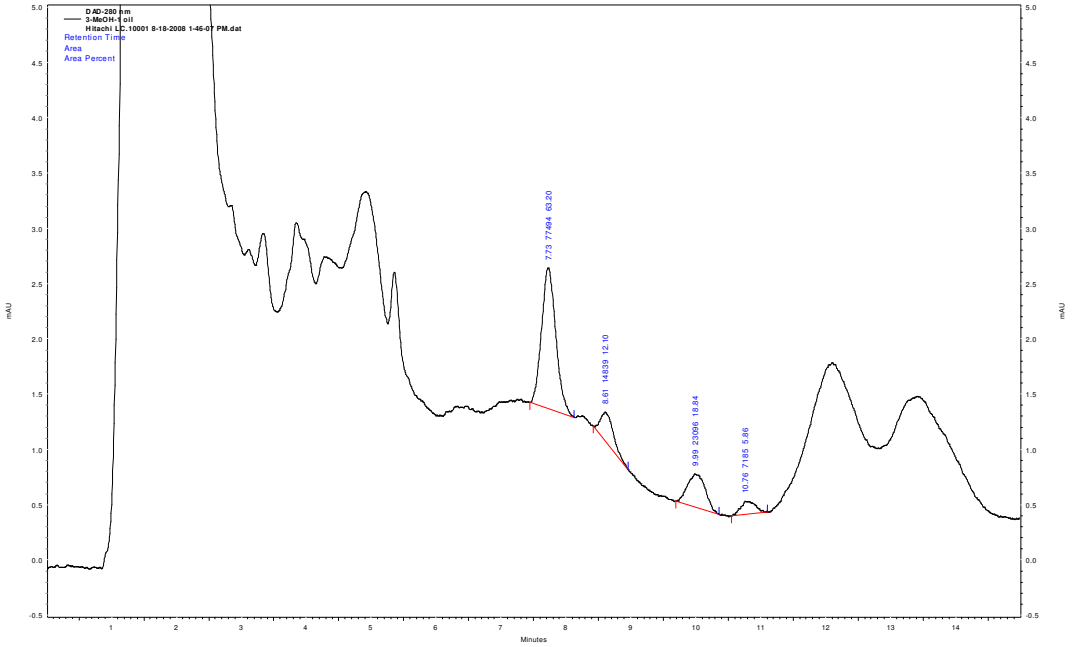

Figure 4.7. Chromatogram of oil fraction obtained after the second methanol extraction.

A chromatogram of the PEs residues in the meal obtained after sequential extraction with hexanes and methanol is shown in Figure 4.8. Peaks with retention similar to the PE peaks were detected but the peak area was smaller clearly indicating that hexanes and methanol can be suitable as a solvent sequence for detoxifying the meal.

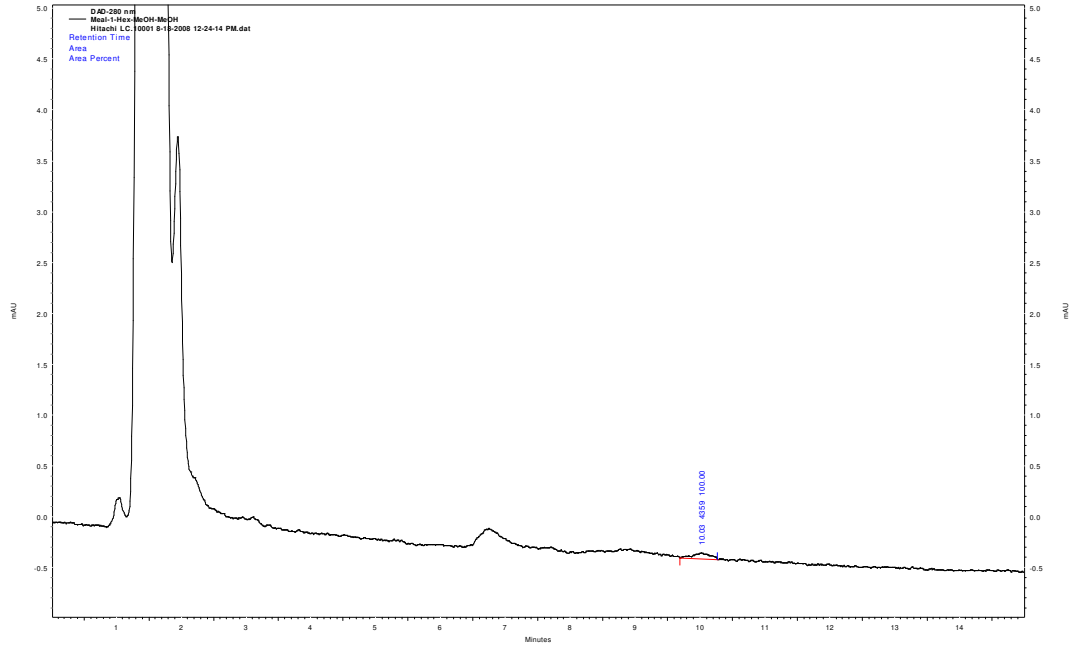

Figure 4.8. Chromatogram of PE residues in jatropha meal sequentially extracted with hexanes and methanol.

4.3.1.2 Quantification of phorbol esters in hexanes, methanol extracted oils and meal. Quantitative results for PEs in the hexanes, methanol extracted oils and meal are given in Table 4.7. The final PE (mg of total PE output) has also been calculated based on the values obtained in Table 4.6 and Table 4.7.

Table 4.7. Phorbol ester concentration in hexane extracted oil, methanol extracted oil and meal.

Oil and Meal	PE in soxhlet # 1 (mg/g)	PE in soxhlet # 2 (mg/g)
Hexane extracted oil	6.5	6.8
Methanol extracted oil -I	35.0	41.3
Methanol extracted oil -II	2.9	4.3
Meal	0.07	0.23
PE Input (6.05 mg/g@ 50 g)	302 mg	
Final PE (mg)	245	293
Average PE in meal	0.15 mg/g	

The results showed sequential extraction of jatropha meal represents an efficient procedure for removing PEs in the meal thereby reducing its toxicity. The level of PEs approached that of the non-toxic Mexican variety.

4.3.2. PEs in Hexane, Isopropyl Alcohol Extracted Oils and Meals. Sequential extractions were also carried out with hexanes and isopropyl alcohol. The results from the extraction have been shown in Table 4.8.

Table 4.8. Amount of oil extracted with sequential extraction with hexanes and isopropyl alcohol.

Solvent and duration	Soxhlet #1	Soxhlet #2
Hexanes, 24 hrs	Weight of Ground meal 50.0 g	Weight of Ground meal 50.1 g
	Wt. of extracted oil 27.2 g	Wt. of extracted oil 25.3 g
Isopropyl 24 hrs	Wt. of extracted oil 0.9 g	Wt. of extracted oil 1.5 g
Isopropyl 48 hrs	Wt. of extracted oil 0.2 g	Wt. of extracted oil 0.2 g
-	Wt. of dry meal 21.0 g	Wt. of dry meal 25.4 g

Results show that the amount of oil extracted with isopropyl alcohol was less than the amount of oil extracted with methanol. The difference most likely results from the fact that isopropyl alcohol [polarity index 3.8] is less polar than methanol and therefore less efficient in extracting polar lipids from the meal including PEs.

4.3.2.1. PEs in hexane, isopropyl alcohol extracted oils and meals. Oil fractions obtained after hexanes and isopropyl alcohol extractions were analyzed for PEs with HPLC. All oil samples showed the presence of PEs. Chromatographic results that were obtained with oil fractions extracted with isopropyl alcohol showed the presence of the four PEs peaks, Figure 4.9. The overall appearance of the chromatogram was similar

to the chromatogram of methanol extracted oil. However, the intensity of the PE peaks was higher than that in the methanol extracted oils.

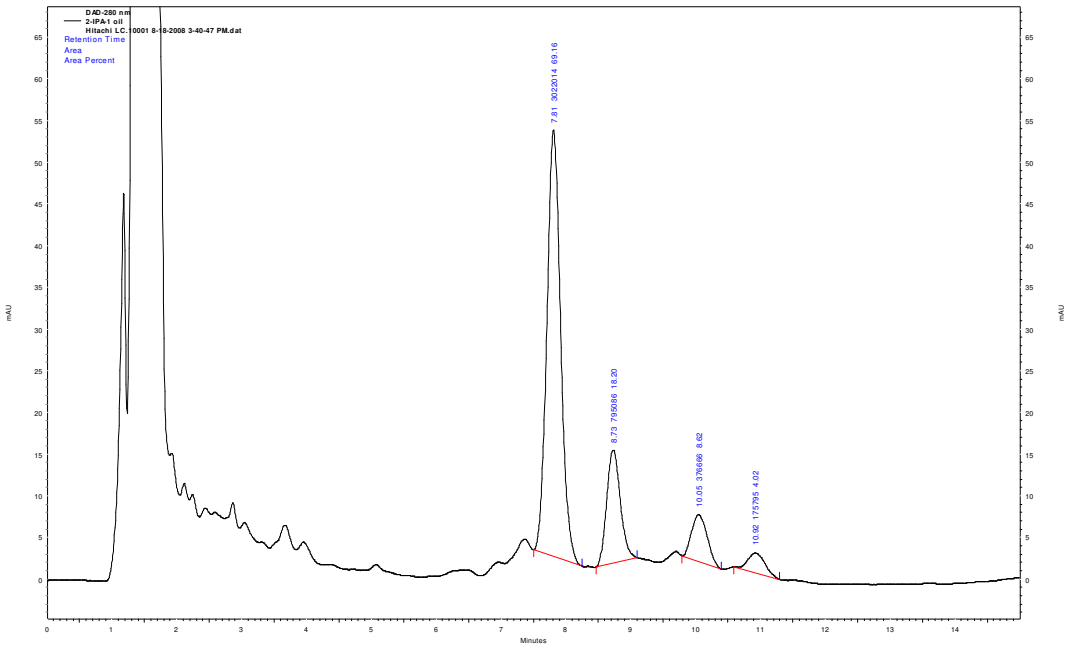

Figure 4.9. Chromatogram of oil fraction obtained after the first isopropyl alcohol extraction.

Chromatogram for the PE present in the oil obtained after the second isopropyl alcohol extraction is shown in Figure 4.10. The peak intensity of the fourth PE peak was markedly lower than the other PE.

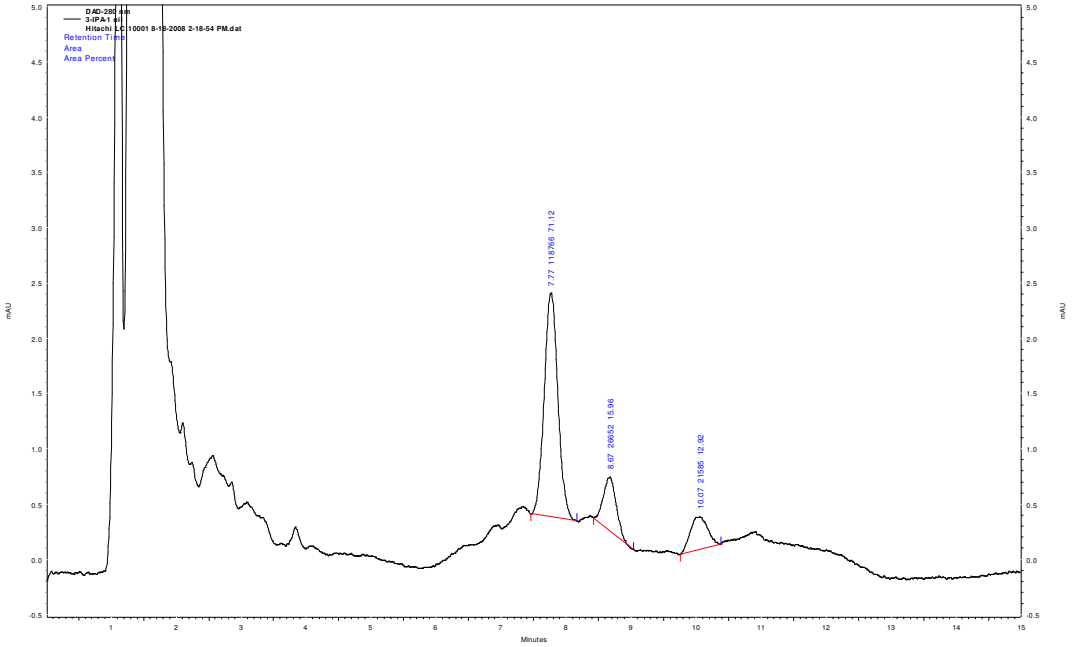

Figure 4.10. Chromatogram of oil fraction obtained after the second extraction with isopropyl alcohol.

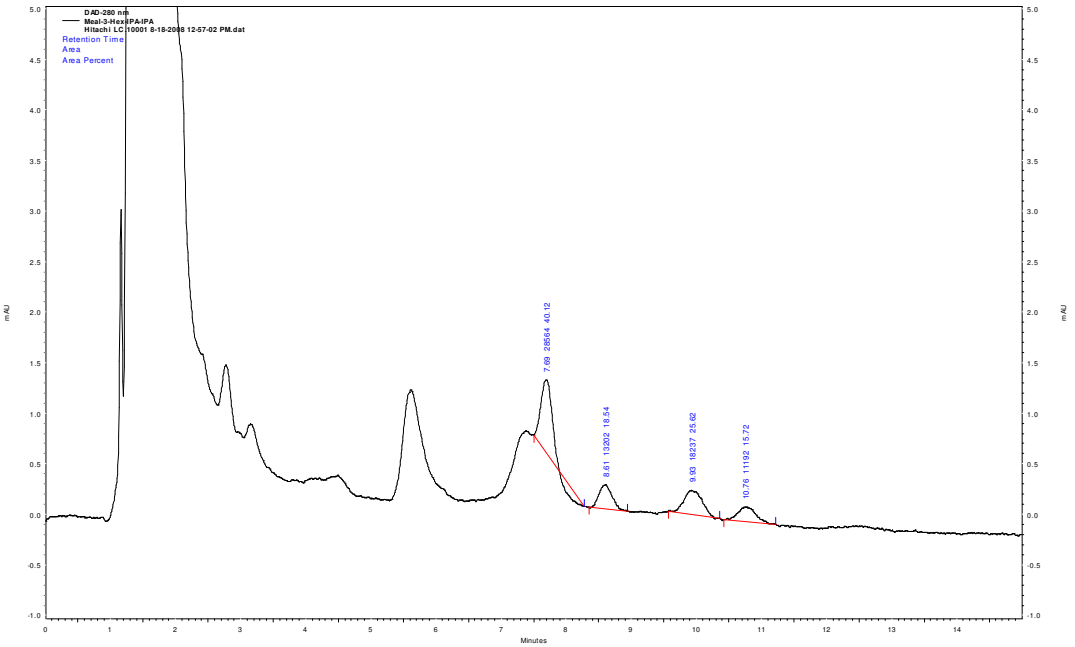

Figure 4.11. Chromatogram of PE residues in jatropha meal sequentially extracted with hexanes and isopropyl alcohol.

The chromatogram obtained for the meal extracted with hexanes and isopropyl alcohol suggested that a significant amount of phorbol ester was still present.

4.3.2.2 Quantification of phorbol esters in hexanes, isopropyl alcohol extracted oils, and meal. Quantitative results for PEs in the hexanes, isopropyl alcohol extracted oils, and meal are given in Table 4.9.

Table 4.9. Phorbol ester concentration in hexane extracted oil, isopropyl alcohol extracted oil and extracted meal.

Extraction solvent	**Amount of phorbol esters in soxhlet # 1 (mg/g)**	**Amount of phorbol esters in soxhlet # 2 (mg/g)**
Hexane extracted oil	5.8	7.5
Isopropyl Alcohol extracted oil - I	56.3	29.6
Isopropyl Alcohol extracted oil - II	12.3	5.6
Meal obtained after sequential extraction	2.0	1.0
Final PE (mg)	257	263
PE Input (6.05 mg/g@ 50 g)	305 mg	

The results obtained with hexanes and isopropyl alcohol extracted meal show that extraction with isopropyl alcohol was less exhaustive than extraction with methanol. Jatropha meal obtained after sequential extraction with hexanes and isopropyl alcohol contained 1.5 mg g^{-1} of PEs.

It was also observed that methanol extracted several derivatives of PEs which IPA could not extract. This is evident from chromatograms 4.6, 4.7, 4.9, and 4.10, respectively. This may be due to the affinity of those polar derivatives to the highly polar methanol, which resulted in their elution with third peak.

4.3.3. Sequential Extraction with Hexanes and Ethanol. Sequential extraction of jatropha meal with hexanes and ethanol yielded extraction efficiencies that were

between those obtained with methanol and isopropyl alcohol. Results from the analysis have been shown below in Table 4.10.

Table 4.10. Amount of oil extracted with sequential extraction with hexanes and ethanol.

Solvent and duration	**Soxhlet #1**	**Soxhlet #2**
Hexanes 24 hours	Initial Amount of Meal 50.2 g	Initial Amount of Meal 50.2 g
	Wt. of oil 27.6 g	Wt. of oil 27.8 g
Ethanol 72 hours	Wt. of oil 0.2 g	Wt. of oil 1.8 g
-	Wt. of defatted meal 20.1 g	Wt. of defatted meal 17.9 g
Weight of extracted material	47.3 g	46.7 g

All the oil samples including oil, bottoms, and dry meal were prepared according to steps in Section 3.4.4 and analyzed in the LC.

Phorbol ester was determined in all the samples, the oil, bottoms from ethanol extracts, and the defatted and treated meal. Table 4.11 summarizes the phorbol ester content.

Table 4.11. Phorbol ester concentration in oil, bottoms from ethanol extracts, and meal.

Extraction solvent, Substance analyzed		**Amount of phorbol esters in soxhlet # 1 (mg/g)**	**Amount of phorbol esters in soxhlet # 2 (mg/g)**
Hexane, Oil		7.4	7.4
Ethanol, Bottoms		1.3	2.2
Meal	Det. # 1	0.4	0.3
	Det. # 2	0.4	0.2
	Average	0.4	0.25
Final PE (mg)		214	216
PE Input (6.05 mg/g@ 50 g)		305 mg	

4.3.4. Extraction of Jatropha Kernel with Hexanes (One Day) Followed by Methanol (Three Days). The crushed kernels were extracted with hexanes for a day and then with methanol for one, three-day cycle. The rationale behind this extraction was to check whether a three day extraction with methanol can significantly reduce the amount of phorbol esters in meal. Table 4.12 shows the results from the extractions.

Table 4.12. Extraction of kernel with hexane (one day) sequentially followed by methanol (three days).

Solvent and duration	Determination #1	Determination #2	Determination #3	Determination #4
Hexanes, 1 day 300 mL	Ground kernel – 50.4 g	Ground kernel – 50.1 g	Ground kernel – 50.7 g	Ground kernel – 50.3 g
	Wt. of oil = 28.6 g	Wt. of oil = 28.0 g	Wt. of oil = 28.0 g	Wt. of oil = 27.8 g
Methanol, 3 days	Wt. of bottoms = 3.8 g	Wt. of bottoms = 4.1 g	Wt. of bottoms = 3.2 g	Wt. of bottoms = 4.3 g
-	Wt. of dry meal = 18.0 g	Wt. of dry meal = 18.2 g	Wt. of dry meal = 18.0 g	Wt. of dry meal = 17.6 g
Total extracted material	50.4 g	50.3 g	49.2 g	49.7 g

4.3.4.1. Liquid chromatographic analysis of jatropha oil, bottoms from methanol extracts, and treated meal. Analysis was performed on meal, oil, and bottoms from the methanol extracts. Sample preparation was done as discussed in Section 3.4.1. The results of the analysis are shown in Figures 4.12 and 4.13, respectively.

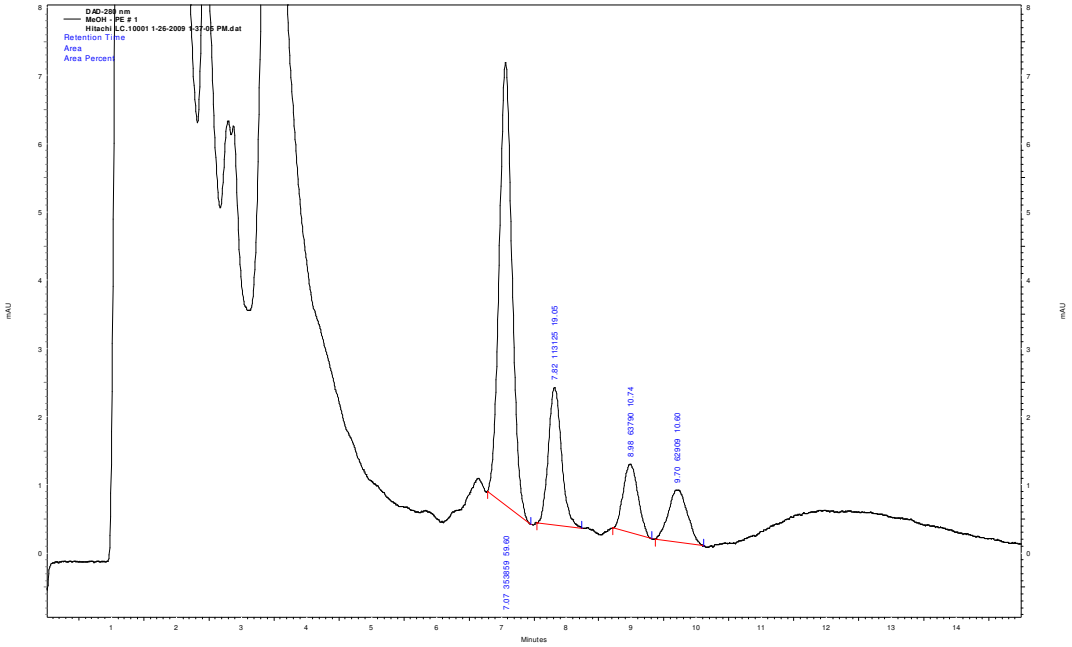

Figure 4.12. Chromatogram of bottoms from methanol extract (after three days).

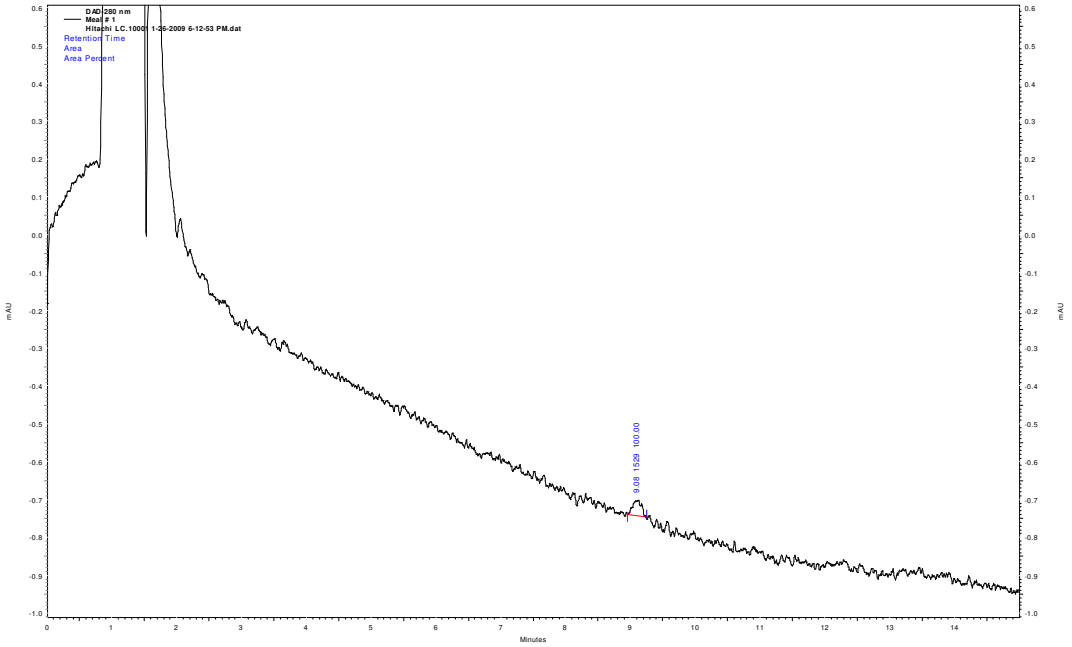

Figure 4.13. Chromatogram of dried and extracted meal after 4-day cycle.

The one thing which is apparent in Figure 4.13 is the absence of three phorbol ester peaks. The similar trend was also observed in 3 replicate determinations which were performed.

4.3.4.2 Quantification of phorbol esters in jatropha oil, bottoms from methanol extracts, and defatted and treated meal. Phorbol ester was quantified from the chromatograms. Table 4.13 summarizes the amount of phorbol esters in all the samples from four replicate determinations.

Table 4.13. Phorbol ester in oil, methanol extracts, and treated meal.

Substance Analyzed	Phorbol ester (mg/g) in Soxhlet # 1	Phorbol ester (mg/g) in Soxhlet # 2	Phorbol ester (mg/g) in Soxhlet # 3	Phorbol ester (mg/g) in Soxhlet # 4
Jatropha oil (first day)	6.4	7.4	7.1	7.9
Bottoms (after 3 days)	7.6	-	7.5	6.9
Defatted meal	0.02	0.07	0.06	0.07
Average PE (mg/g)	Average PE concentration in meal- 0.06 mg/g Standard Deviation of PE concentration in meal: 0.02 mg/g			

4.4. DETERMINATION OF PHORBOL ESTERS IN JATROPHA SEED SHELL

Unextracted and ground jatropha shell was analyzed in LC for PE content. The shell was also extracted for a day with hexanes to determine the oil content in the shell. Table 4.14 summarizes the experimental observations with three determinations.

Table 4.14. Extraction of jatropha kernel with hexanes.

Solvent and duration	Soxhlet # 1	Soxhlet # 2	Soxhlet # 3
Hexanes (300 mL); 1 day	55.1 g of shell	55.1 g of shell	55.0 g of shell
	Wt. of bottoms = 1.0 g	Wt. of bottoms = 0.6 g	Wt. of bottoms = 0.6 g
--	Wt. of dry shell: 53.6 g	Wt. of dry shell: 54.2 g	Wt. of dry shell: 54.0 g

4.4.1. Liquid Chromatographic Analysis of Unextracted Jatropha Shell and Jatropha Shell Extracts. Sample preparation for injection in the LC was done according to the steps in Section 3.4.1. Figures 4.14 and 4.15 show the chromatograms obtained.

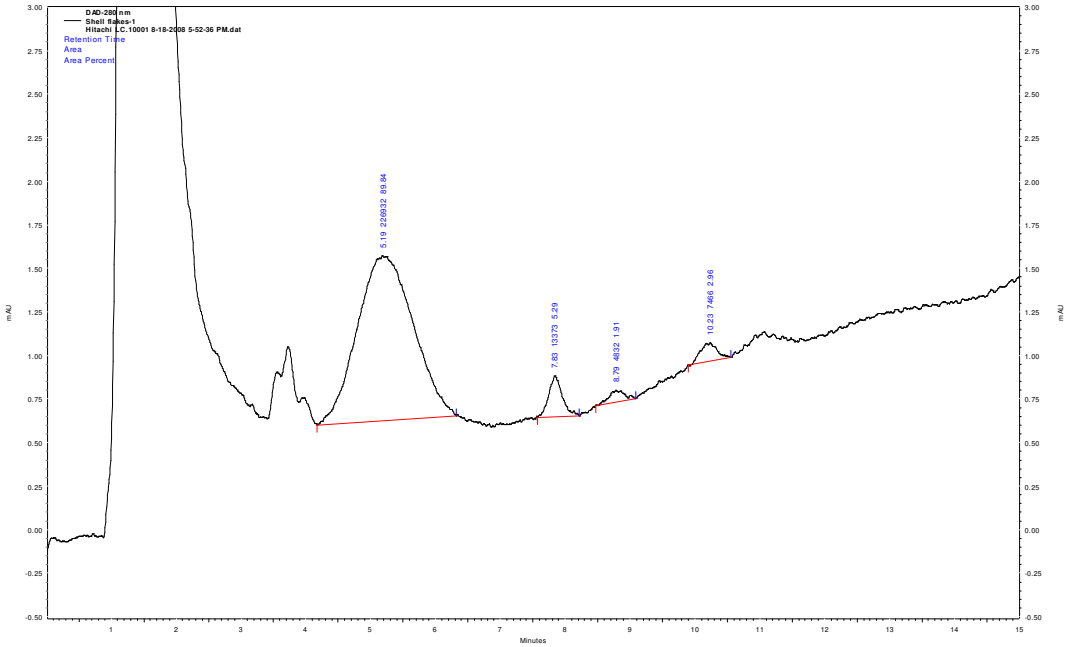

Figure 4.14. Chromatogram for unextracted jatropha shell.

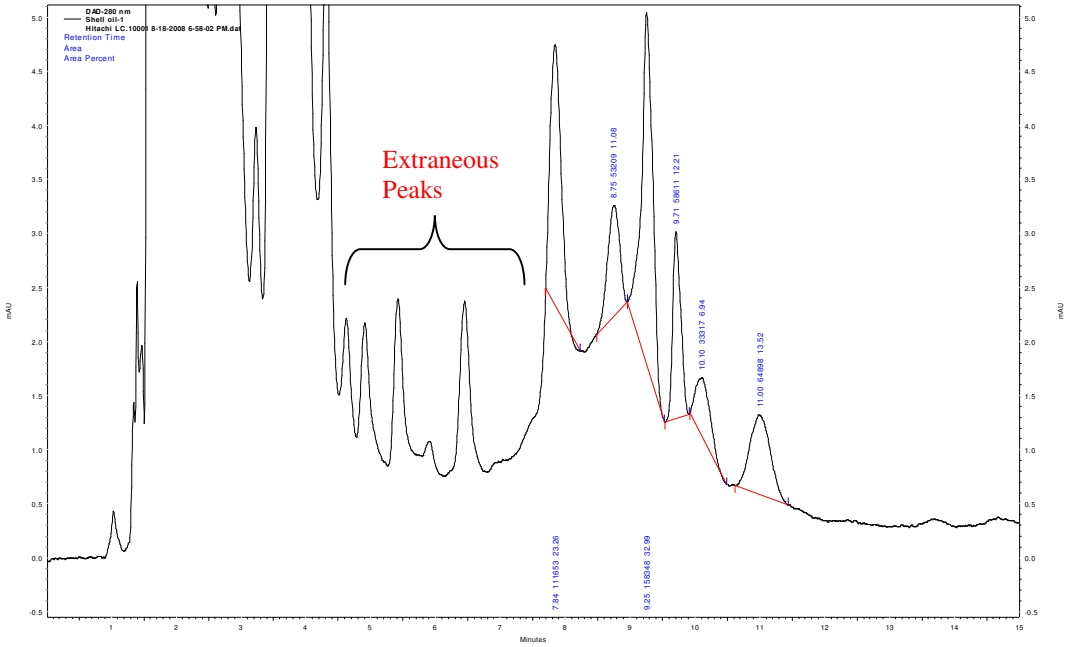

Figure 4.15. Chromatogram of bottoms from hexane extracts.

The chromatogram in Figure 4.14 suggests that even though there is some amount of PE present in the unextracted shell, the quantity is quite less than in the kernel which is apparent from the small peak areas. Also, the chromatogram from Figure 4.15 suggests that there is some PE in the extracts which is evident in the four peaks, but there are certainly other compounds which elute as well, which is evident from the extraneous peaks shown in the chromatogram.

4.4.2. Quantification of Phorbol Esters in Unextracted Jatropha Shell and Bottoms from Hexane-Extracts from Shell. Phorbol esters from jatropha shell, and hexane extracts from the shell were determined from the chromatograms obtained from LC. Two more determinations were performed and PE quantities were determined as discussed in Section 3.4.4. Table 4.15 summarizes the phorbol ester content.

Table 4.15. Phorbol ester content in jatropha shell and hexane-extracts from shell.

Substance characterized	Phorbol esters (mg/g) in Soxhlet # 1	Phorbol esters (mg/g) in Soxhlet # 2	Phorbol esters (mg/g) in Soxhlet # 3
Jatropha shell prior to extraction	0.3	0.2	0.1
Hexane–extracts from shell	8.6	11.6	9.9

The data in Table 4.15 suggests that the amount of phorbol esters in hexane-extracts from jatropha shell is quite high. On the other hand, PE concentration is comparatively smaller in raw jatropha shell.

5. CONCLUSION

- Extraction of jatropha meal with hexanes for 24 hours followed by methanol for 72 hours: 1) recovers the oil and 2) detoxifies the PEs in the meal to a concentration which is well below the nontoxic Mexican variety. The average amount of oil extracted with hexanes was nearly 56% (% weight of the kernel) and the average material extracted from the meal treated with hexanes followed by methanol was about 63%. The average value of phorbol esters detected in meal treated with the above solvent sequence was 0.06 mg/g which was well below the value of 0.11 mg/g; phorbol ester content in nontoxic Mexican variety of jatropha seeds.
- The oil extraction efficiency of soxhlet extractor is slightly better than sonicator. This might be due to the higher temperature of solvents used in soxhlets. The oil extraction using hexanes in the sonicator varied from 48% - 52% (w/w of jatropha kernel) and 54% -56% (w/w of jatropha kernel) for hexanes followed by methanol for pure solvents and 33%-42% (w/w of jatropha kernel) for mixed solvents.
- Jatropha shell contains little phorbol esters, the values of which varied from about 0.18 -0.35 mg/g of shell.

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