



Identification of best detoxification strategies for sustainable valorization of waste from *Jatropha*-based biodiesel industry: Compounding the benefits of plant-based vehicular fuel

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ABSTRACT

Bioenergy generated from plants is one of the best potential energy supplies. Recently, the non-edible *Jatropha curcas* has been considered one of the world's most sustainable biofuel crops. Protein rich defatted *Jatropha curcas* kernel meal (DJKM) is toxic which contains phorbol esters (PEs, 1307 $\mu\text{g g}^{-1}$) as a major toxic component, therefore it was detoxified through physical, chemical and solid-state fermentation treatments. Water soaking and moist heat treatment (autoclaving) had no significant effect on the PEs removal compared to control. Exposure of DJKM to electron beam radiation (2.5, 5, 10, 15 and 20 kGy) had a significant effect on PEs degradation and most efficient was at 25 kGy dose. Solvent extraction with either 90% methanol or 80% ethanol in combination with moist heat treatment was the most efficient method compared to other concentrations of methanol or ethanol. Solvent extraction of kernel meal through 0.2 or 0.3 M NaOH in 90% methanol and 0.1, 0.2 or 0.3 M NaOH in 80% ethanol, followed by moist heat treatment led to the removal of PEs completely. The PEs content in DJKM through highly alkaline methanol plus moist heat treatment (AM3.0) and ferric chloride & hydrogen peroxide plus moist heat treatments significantly ($p < 0.05$) decreased to 13.0 and 53.6 $\mu\text{g g}^{-1}$, respectively. Non-alkaline solvent extraction of DJKM using either 90% methanol (M90H) or 80% ethanol (E80H) in addition to heating while magnetic stirring reduced PEs content significantly ($p < 0.05$) compared to control exhibiting 99.77 and 99.32% removal, respectively. The DJKM was free of PEs when it was treated via solid-state fermentation through *Aspergillus niger* fungus. Conclusively, chemical detoxification treatments are cumbersome, un-economical, and their incorporation in the diet may affect animal performance, whereas, the fermentation approach is cost economical, environmentally sustainable and offers greater efficiency.

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1. Introduction

The demand for conventional fossil fuels has been growing consistently which has posed serious threats to the environment, human health and global energy security (Hussan et al., 2013; Sanjid et al., 2014). However, the currently available liquid fuel reserve can fulfill only 50% of global energy demand by 2023 (Owen et al., 2010; Sanjid et al., 2014). The wide fluctuations in the price of crude oil, the huge gap in demand and supply and threats of climate change have stimulated concerted efforts across the globe to explore suitable alternative energy sources which are readily available, economically viable and environmentally acceptable (Rahman et al., 2014). The use of biofuels as an alternative to fossil-based fuels received major impetus after the signing of Paris Agreement on global, coordinated response framework for climate change in 2015. The production of biodiesel in 2020 has been forecasted to increase sharply to 33.2 billion liters (Hajjari et al., 2017; Teo et al., 2019). As per a projection by International Energy Agency (IEA) (2011), around 27% of transport fuel will be completely replaced by biofuels by 2050. Since last decade, biodiesel has gained considerable attention as a renewable source of energy (Devappa et al., 2010; Xiao et al., 2011) that has promising potential to reduce global dependence on fossil-based diesel fuels and emissions of environmental pollutants (Silalertruksa et al., 2012; Rahman et al., 2014). Currently, biodiesel is being produced from first generation feedstocks such as soybean, rapeseed, palm fruit, corn starch and sugarcane (Devappa et al., 2013) but the use of these edible feedstocks for biodiesel production has raised serious concerns regarding their likely shortage for human and livestock consumption. Therefore, much emphasis has been paid to explore non-edible feedstuffs that can be potentially used for biodiesel production. *Jatropha curcas* is one of such non-edible crop that has been perceived with great potential as a source of biofuel (L.) (Devappa et al., 2010; Xiao et al., 2011). It can withstand drought conditions and is distributed in several tropical and sub-tropical countries (Gübitz et al., 1999). This plant has been found to be growing on degraded soils with minimal requirement of nutrients and water (Singh et al., 2008) and hence, the propagation of *Jatropha* is no threat to existing arable land (Sanjid et al., 2014). The capsulated fruits produced by this plant bears seeds having an average weight of 0.53–0.86 g (Makkar et al., 1998). The seeds and kernels contain 27%–40% and 60% oil, respectively (Makkar et al., 1998). This golden yellow color oil can be trans-esterified into biodiesel meeting the European and American biodiesel standards (Azam et al., 2005). Oil can be extracted from *Jatropha* seeds or kernels through mechanical or solvent extraction generating a huge amount of seed cake or kernel meal as by-products. Approx. 500 gallons of biodiesel is produced from one hectare plantation in a year along with the generation of more than 3500 kg of by-products (Sathishkumar et al., 2012). One ton of *Jatropha* seeds can yield up to 270 kg of kernel meal (Guedes et al., 2014). The Govt. of India in its biofuel policy has aimed to substitute 20% of the diesel consumption with biodiesel, primarily from *Jatropha* beginning with 2011–2012 (Biswas et al., 2010). Various countries around the world have been promoting the *Jatropha* plantation programs (Siang, 2009). These initiatives will assure the availability of an enormous amount of raw material in the future for the *Jatropha* based biodiesel industry. But the unsuitability of this agro-industrial waste as agro-fertilizer owing to low nutritive value and high contents of lignin, cellulose and hemi-cellulose may lead to disposable problems in the environment (Tongpoothorn et al., 2011; Sathishkumar et al., 2012). Therefore, the sustainability and feasibility of the *Jatropha* based biofuel industry can be ensured through optimum utilization of the de-oiled *Jatropha* by-products.

The *J. curcas* kernel meal is a rich source of crude protein (58%–62%) (Makkar et al., 2007, 2008) and has an amino acid composition superior to soybean meal (except lysine) making it a potential protein supplement for livestock feed. However, its direct utilization in animal feeds is precluded due to the presence of inherent antinutritional factors (e.g. trypsin inhibitor, phytate, lectin, saponin and tannin) and extremely toxic components such as phorbol esters (PEs) (Makkar and Becker, 1997; Martínez-Herrera et al., 2006; Xiao and Zhang, 2011; Devappa et al., 2013). Though heat labile antinutritional factors (e.g. lectin and trypsin inhibitor) can be easily deactivated by moist heating/autoclaving (Aderibigbe et al., 1997) and negative effect of phytate can be overcome either by dietary supplementation of phytase enzyme or phosphorus containing mineral mixture (Sardar et al., 2007). In *J. curcas* kernel meal, saponins are reported to be harmless and tannins are present in negligible amounts. But the PEs are not degraded even by heat treatment at higher temperature (Makkar et al., 1997). PEs have exhibited toxicity in rats, mice, rabbits, goats, calves, chicks, pig, fish and humans (Adam and Magzoub, 1975; Gandhi et al., 1995; Li et al., 2010; Aregheore et al., 2003; El-Badwi and Adam, 1992; Ahmed and Adam, 1979; Makkar and Becker, 1997; Becker and Makkar, 1998; Mampane et al., 2006; Singh et al., 2010). Accidental ingestion of *Jatropha* seeds by human beings has inflicted symptoms such as abdominal pain, vomiting and diarrhoea (Rai and Lakhanpal, 2008; Singh et al., 2010). Becker and Makkar (1998) have reported that PEs concentration as low as 15 $\mu\text{g g}^{-1}$ caused adverse effects in carp (*Cyprinus carpio* L.). A concentration of 0.13 mg g^{-1} PEs in the *J. curcas* seed meal exhibited significant adverse effect on food intake and growth rate of rats (Aregheore et al., 2003). In a previous study in our laboratory, Angel et al. (2015) also reported that untreated defatted *Jatropha* kernel meal cannot be incorporated in the diet of *Labeo rohita* fish fingerlings. Therefore, it is imperative to detoxify kernel meal prior to its optimum utilization in animal feeds. Three different techniques have been used by researchers to detoxify the toxic compounds in *Jatropha* seed cake or kernel meal (Phengnuam and Suntornsuk, 2013) such as physical methods by heat treatment and irradiation (Makkar and Becker, 1997; Sanap et al., 2018; Diwani et al., 2011; Gogoi et al., 2014), chemical treatment (Martínez-Herrera et al., 2006; Chivandi et al., 2006; Aregheore et al., 2003; Makkar and Becker, 2010; Brooker, 2011; Fawole et al., 2018) and microbial fermentation (solid state fermentation) using fungi (Shamna et al., 2015; Belewu and Sam, 2010; de Barros et al., 2011; Kasuya et al., 2012; Najjar et al., 2014) and bacteria (Joshi et al., 2011; Phengnuam and Suntornsuk, 2013). A varying degree of success has been reported through these detoxification approaches. The overarching goal of this study was to compare the efficacy of various approaches for the removal of PEs from defatted *J. curcas* kernel meal.

2. Materials and methods

2.1. Preparation of defatted *Jatropha* kernel meal

J. curcas seeds were procured from a commercial supplier Genius Herbs, Coimbatore, Tamil Nadu, India. These seeds were deshelled manually to obtain kernels which were then ground with the help of a kitchen grinder and defatted in a soxhlet extraction apparatus (Socsplus, SCS-08-As, Pelican Equipments, Chennai) using diethyl ether (boiling point 40–60 °C). Defatted *Jatropha* kernel meal (DJKM) was oven dried at low temperature (50 °C) and later packed in airtight polythene bags and stored at –20 °C for further use.

2.2. Detoxification approaches

Various physical, chemical and microbial fermentation approaches were employed for the detoxification of DJKM.

2.2.1. Physical approaches

2.2.1.1. Water soaking. The DJKM was soaked by dipping in water with a ratio of 100 g/300 ml (1:3 w/v) in a plastic container for 12 h at room temperature. After soaking, the meal was oven dried in an oven at 60 °C for 24 h, ground and stored at –20 °C for further use.

2.2.1.2. Moist heat treatment. DJKM was given heat treatment through autoclaving at 121 °C and 1.1 atm. for 30 min. Autoclaved samples were then dried in an oven at 60 °C for 24 h and stored at –20 °C for further use.

2.2.1.3. Electron beam irradiation. The DJKM was subjected to electron beam irradiation at different dose levels of 2.5, 5, 10, 15, 20 and 25 KGy at Electron Beam Centre, BARC, Kharghar, Navi Mumbai, Maharashtra. The irradiated samples were stored at –20 °C for further use.

2.2.2. Chemical approaches

2.2.2.1. Solvent extraction by methanol/ethanol. This treatment was carried out as per the method suggested by [Makkar and Becker \(2010\)](#) with some modifications. Various concentrations (70, 80, 90 and 100%) of methanol (designated as M70, M80, M90 and M100, respectively) and ethanol (designated as E70, E80, E90 and E100, respectively) were used separately for extraction of PEs from DJKM using specially designed refluxing unit. One hundred and fifty ml of each concentration of methanol/ethanol was added to 10 g DJKM (15:1 v/w) in the round bottom flask of refluxing unit with proper mixing. Methanol and ethanol extraction was done respectively at 65 °C and 78 °C for 30 min. After the extraction was over, the mixture was cooled and filtered through Buchner funnel with suction arrangements and using Whatman filter paper No. 1 to obtain residue, which was retreated by the similar solvent and similar process as mentioned above and finally the residue was given repeated washings by hot water to make it solvent free. The residue was then lyophilized and the dried sample was subjected to PEs estimation through HPLC (Waters, Massachusetts, USA) using UV detector at 280 nm. Based on the PEs removal efficacy, the best concentration of methanol (designated as M_b) and ethanol (designated as E_b) were selected for the next treatment.

2.2.2.2. Solvent extraction by alkaline and non-alkaline methanol/ethanol plus moist heat treatment. In this detoxification approach, 10 g fresh DJKM sample was treated with 150 ml M_b/E_b solvent with 0.1, 0.2, and 0.3 M NaOH (designated as AM0.1, AM0.2, AM0.3 and AE0.1, AE0.2, AE0.3, respectively) through same refluxing method at same temperature and time as mentioned earlier. However, while filtering in the Buchner funnel, the residue was washed with 120 ml of M_b/E_b solvent without NaOH. The residue was then re-treated with 150 ml M_b/E_b solvent (without NaOH) through same refluxing method as mentioned earlier. Finally, the residue was given hot water washings for complete removal of solvent and alkali. The residue was then given moist heat treatment through autoclaving (121 °C, 30 min) followed by freeze drying using a lyophilizer. The freeze dried samples were stored at –20 °C for further analysis of PEs.

For non-alkaline method, 10 g DJKM was mixed with 50 ml of E_b/M_b solvent (1:5, w/v) in a 150 ml beaker. The mixture was heated on a magnetic stirrer at 78 °C /65 °C, respectively for 5 min. After heating, the mixture was filtered using Whatman filter paper No. 1. The filtered residue was retreated (three times) in the same way as mentioned earlier. The residue was then given moist heat treatment through autoclaving (121 °C, 30 min) followed by freeze drying using lyophilizer. The freeze dried samples (designated as M90H and E80H, respectively) were stored at –20 °C for further analysis of PEs.

2.2.2.3. Highly alkaline methanol plus moist heat treatment. In another detoxification approach using methanol and alkali, 10 g DJKM was mixed with 30 ml 90% methanol (1:3, w/v) in a 150 ml beaker. The mixture was sonicated for 5 min and then incubated at 65 °C for 15 min in a water bath. Then the mixture was centrifuged (4500 g; 15 min) and the supernatant was carefully discarded. The meal was lyophilized and mixed with 3% NaOH (0.75 M) solution (1:1, w/v) and incubated in a water bath at 65 °C for 5 min. The alkali treated meal was further washed with hot water in a muslin cloth to make it alkali free and autoclaved (121 °C, 30 min). The sample was then lyophilized and stored at –20 °C for further PEs estimation. This treatment was designated as AM3.0.

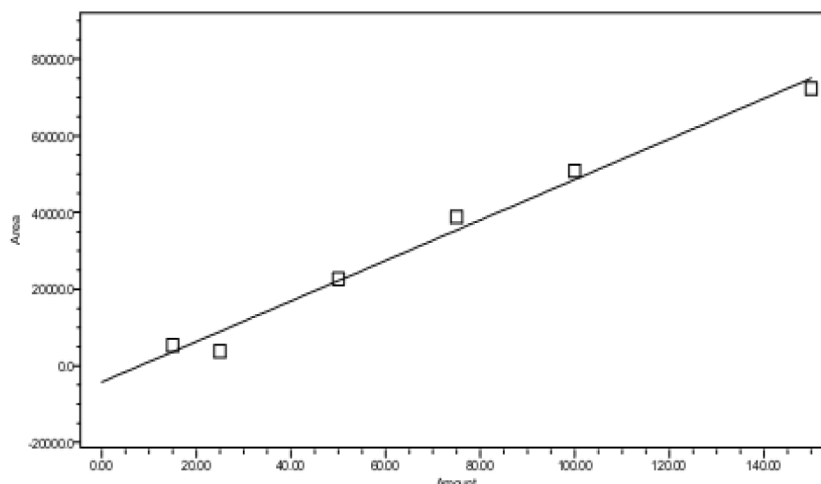


Fig. 1. The calibration curve of Phorbol-12-myristate-13-acetate (PMA) of various concentrations range from $15 \mu\text{g mL}^{-1}$ to $150 \mu\text{g mL}^{-1}$.

2.2.2.4. Ferric chloride & hydrogen peroxide plus moist heat treatment. In this detoxification approach, 10 g DJKM was mixed with 16 ml ferric chloride solution ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $53 \mu\text{g mL}^{-1}$, w/v) in a 100 ml beaker. The mixing was done properly until the meal absorbed the ferric chloride solution and then the mixture was incubated in the water bath at 70°C for 30 min. Thereafter, 8 ml diluted hydrogen peroxide (1:1, v/v) was added to this mixture with proper mixing to get a homogeneous paste, which was incubated (70°C , 30 min) in the water bath. The treated meal was further autoclaved (121°C , 30 min) followed by freeze drying. The dried sample was stored at -20°C for further estimation of PEs.

2.2.3. Solid state fermentation (SSF)

For the detoxification of DJKM through solid state fermentation approach (Belewu and Sam, 2010), the *Aspergillus niger* fungus (MTCC 281) was procured from IMTECH (Institute of Microbial Technology), Chandigarh, India. The fungus was sub-cultured in Czapek Yeast Extract Agar (CYA) as suggested by IMTECH. For this, first CYA was autoclaved and poured in sterilized petri plates and were left overnight. Then the plates were checked for contamination, if any on the subsequent day. The plates without contamination were inoculated with *A. niger* fungus. Once the fungus were grown fully on these plates, the fungal spores were harvested in Tween-80 solution and diluted to achieve a concentration of 10^7 – 10^8 spores mL^{-1} through serial dilution. The dried, cooled and autoclaved DJKM was fermented aerobically at 30°C for three weeks using fungal spores suspension. The fermented product was then autoclaved to inhibit the further growth of fungus. The fermented meal was cooled and lyophilized to obtain dried fermented *Jatropha* kernel meal (designated as FJKM), which was stored at -20°C for further analysis of PEs.

2.3. Extraction and estimation of phorbol esters (PEs)

The PEs was extracted from all detoxified DJKM samples following the method of Saetae and Suntornsuk (2010) as described in Shamna et al. (2015). The PEs was estimated as per the method of Makkar et al. (2007) through HPLC (Waters, Massachusetts, USA) using a UV detector at 280 nm. A calibration curve (Fig. 1) was prepared using Breeze™ 2 software of HPLC by running different concentrations of working standards ($15, 25, 50, 75, 100$ and $150 \mu\text{g mL}^{-1}$) prepared by dissolving Phorbol-12-myristate-13-acetate (PMA) standard (Sigma) in HPLC grade methanol (Merck). After that, untreated and treated DJKM samples were run in HPLC and concentrations of PEs were calculated against calibration curve and expressed as $\mu\text{g g}^{-1}$.

2.4. Statistical analysis

Statistical analysis of the data was done using Sigma Plot (V 12.5; Systat Software, Inc., Point Richmond, CA, USA). The normality of each data set and homogeneity of variance was assessed with the use of Shapiro–Wilk and Levene's test, respectively. The treatment effects were analyzed using One-Way ANOVA, followed by Tukey's post-hoc, all pairwise multiple comparison procedure. All data are presented are mean $\pm$ standard error of the mean (SEM). Significant differences of mean values were observed at less than 5% probability level ($p < 0.05$).

Table 1

Comparative efficacy of moist heat treatment, water soaking and electron beam irradiation for removal of phorbol esters (PEs) from defatted *Jatropha* kernel meal (DJKM).

Treatments	PEs ($\mu\text{g g}^{-1}$)	Removal (%)
DJKM	1307.0 ^a $\pm$ 15	–
Moist heat treatment	1240.0 ^a $\pm$ 10	5.13
Water soaking	1265.3 ^a $\pm$ 10	3.19
Electron beam irradiation (kGy)		
2.5	1160.0 ^b $\pm$ 10	11.25
5	1080.0 ^{bc} $\pm$ 20	17.37
10	1010.0 ^{cd} $\pm$ 20	22.72
15	970.0 ^d $\pm$ 20	25.78
20	950.0 ^d $\pm$ 30	27.31
25	760.0 ^e $\pm$ 30	41.85

Data are expressed as Mean $\pm$ SEM, ($n = 3$).

Mean values in the same column with different superscripts differ significantly ($p < 0.05$).

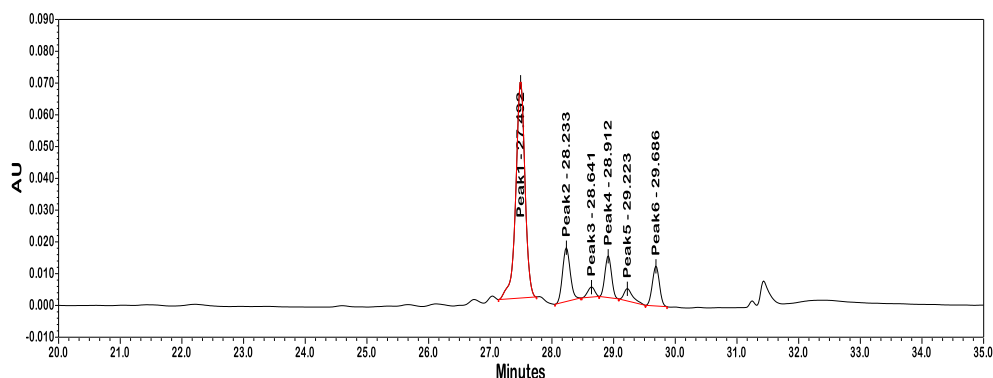


Fig. 2. HPLC chromatogram of untreated DJKM showing six distinct peaks.

3. Results and discussion

The term phorbol refers to the family of triterpene diterpenes, which are naturally occurring compounds (Evans, 1986) and are known to mimic the action of diacyl glycerol (activator of protein kinase C) leading to activation of a cascade of signal transduction reactions causing tumor generation (Goel et al., 2007). Phorbol esters are defined as polycyclic compounds in which two hydroxyl groups on the neighboring carbon atoms are esterified to fatty acids (Goel et al., 2007). The HPLC chromatogram of untreated DJKM (control) in the present study is presented in Fig. 2, which showed six distinct peaks at retention times 27.49, 28.23, 28.64, 28.91, 29.22, and 29.68 min, respectively, indicating six different types of phorbol esters. Four to six phorbol esters or its derivatives have been reported in *Jatropha* sp. (Joshi et al., 2011). Similarly, Haas and Mittelbach (2000) and Haas et al. (2002) detected six different derivatives of phorbol esters in *J. curcas* oil possessing basic triterpene diterpene moiety, out of which phorbol-12-myristate 13-acetate (PMA) was suggested to be the major phorbol ester in *J. curcas* (Makkar and Becker, 1997). The PEs concentration of untreated DJKM in the present investigation was found to be 1307 $\mu\text{g g}^{-1}$ or 1.3 mg g^{-1} (Table 1) and the value was within the range (0.62–3.32 mg g^{-1}) of the earlier report (Goel et al., 2007). The type and individual amount of PEs depend on the variety and growing conditions (Martínez-Herrera et al., 2006). Since the existing literature regarding the efficiency of different detoxification approaches for removal of PEs is scanty, the current manuscript provides a comparative analysis of the efficacy of existing strategies for removal of PEs from defatted *J. curcas* kernel meal.

3.1. Effect of physical detoxification methods on the removal of PEs

The effect of different physical detoxification methods (water soaking, moist heat treatment and electron beam irradiation) on the PEs content in DJKM along with percent removal is presented in Table 1. The soaking of DJKM in water for 12 h and the moist heat treatments had no significant effect ($p > 0.05$) on the removal of PEs as compared to untreated DJKM (control). The water soaking and moist heat treatments reduced PEs content in DJKM only by 3.19 and 5.13%, respectively. PEs are highly heat stable compounds and heating of *J. curcas* cake as a single approach even at 160 °C for 30 min have been reported to be ineffective in reducing PEs levels significantly (Aregheore et al., 2003; de Castro et al., 2010). Makkar et al. (1998) reported no reduction in PEs content as a result of roasting of *J. curcas* seeds. In corroboration to our results, Makkar and Becker (1997) also reported only 5% reduction in PEs levels in the *Jatropha* kernel meal through

Table 2

Comparative efficacy of different methanol concentrations for removal of PEs from DJKM.

Treatments	PEs ($\mu\text{g g}^{-1}$)	Removal (%)
DJKM	1307.0 ^a $\pm$ 15	–
M70	13.3 ^b $\pm$ 0.29	98.98
M80	5.8 ^c $\pm$ 0.17	99.63
M90	4.5 ^d $\pm$ 0.12	99.66
M100	14.1 ^b $\pm$ 0.21	98.92

Data are expressed as Mean $\pm$ SEM, ($n = 3$).Mean values in the same column with different superscripts differ significantly ($p < 0.05$).PEs- Phorbol Esters, DJKM- Defatted *Jatropha* Kernel Meal (control), M70- 70% Methanol, M80- 80% Methanol, M90- 90% Methanol, M100- 100% Methanol.

autoclaving (121 °C, 30 min). The electron beam irradiation of DJKM at different doses had a significant effect ($p < 0.05$) on PEs removal in DJKM (Table 1). The exposure of DJKM at 25 kGy reduced PEs content significantly ($p < 0.05$) by 41.85%, whereas, the 2.5 kGy treatment had no significant effect ($p > 0.05$) on the removal of PEs in comparison to the untreated DJKM. The PEs removal due to electron beam irradiation corresponds to the HPLC chromatograms (Fig. 3a–3f) of DJKM treated at different doses of radiation indicating degradation of PEs as evident by the variation in PEs peaks in comparison to the chromatogram of untreated DJKM (Fig. 2). The γ radiation is effective in the degradation of phorbol esters as these are susceptible to oxidation and carbon–carbon double bonds are degraded converting the ester into a nontoxic compound (Gogoi et al., 2014; Gomes et al., 2018). Similar to our results, Gogoi et al. (2014) reported 33.4% reduction in the concentration of PEs in *J. curcas* cake exposed to 30 kGy gamma radiation. The same study exhibited 96% reduction in PEs at 125 kGy dose of gamma radiation. Similarly, exposure of Egyptian *Jatropha* oil seed cake to gamma radiation at 50 kGy resulted in 71.4% removal of PEs (Diwani et al., 2011).

3.2. Effect of chemical detoxification methods on the removal of PEs

3.2.1. Solvent extraction by methanol

The effect of treatment of DJKM with various concentrations of methanol on the PEs level and percent removal are presented in Table 2. The PEs concentration in DJKM as a result of treatment with different concentrations of methanol varied significantly ($p < 0.05$). The 90% methanol concentration exhibited the significantly lowest ($p < 0.05$) PEs value showing 99.66% reduction in comparison to untreated DJKM followed by 80% methanol showing 99.63% reduction. There was no significant difference ($p > 0.05$) between 70% and 100% methanol treatments on the PEs concentrations in DJKM. These results of PEs removal are substantiated by the chromatograms (Fig. 4a–4d) of different treatments indicating a decrease in number and height (representing area) of PEs peaks in treated samples in comparison to the chromatogram of untreated DJKM (Fig. 2) signifying the breakdown and degradation of the PEs in treated DJKM samples. Our finding is in corroboration to Makkar and Becker (2010), wherein they achieved maximum PEs removal through 90% methanol when *Jatropha* kernel meal was extracted through different aqueous concentrations of methanol. In another study, Makkar and Becker (1998) observed that extraction (four times) of heat treated (121 °C, 30 min, 66% moisture) kernel meal containing < 1% oil [1:5 w/v; kernel meal:solvent] with 92% aqueous methanol treatment resulted in 95% removal of *Jatropha* PEs. Similarly, heat treatment (121 °C, 30 min) and four times washing with 92% aqueous methanol exhibited 95% reduction of PEs content in *Jatropha* kernel meal (Aregheore et al., 2003). Pighinelli et al. (2012) subjected *Jatropha* seed cake to methanol (100%) and to 40% aqueous methanol treatment for six hours soxhlet extraction and reported PEs reduced to undetectable levels. However, PEs were reduced by only 39% when *Jatropha* seed cake was treated with 40% methanol and stirred for 2 h at room temperature. Guedes et al. (2014) reported 97.30% reduction in the concentration of PEs in *Jatropha* seed cake following an 8 h soxhlet extraction process with a mixture of methanol (50%) and ethanol (50%) having solute:solvent ratio of 1:10 (w/v).

3.2.2. Solvent extraction by ethanol

The PEs content in DJKM treated with different concentrations of ethanol and their percent reduction level is given in Table 3 and the HPLC chromatograms of treated samples are represented through Fig. 5a–5d. The E80 treatment (extraction of DJKM with 80% aqueous ethanol) showed the lowest PEs value and was significantly ($p < 0.05$) different to PEs values in control and other treatments (E70, E90 and E100). Amongst other ethanol concentrations, absolute ethanol (E100) exhibited the least PEs removal (97.02%), followed by E70 (99.61%) and E90 (99.45%). In agreement to our results, Makkar and Becker (1998) reported 95% reduction in PEs in *Jatropha* kernel meal extracted four times with 80% aqueous ethanol and heat treated at 121 °C for 30 min. Similarly, treatment of DJKM with 90% aqueous ethanol followed by treatment with 0.07% NaHCO₃ plus autoclaving (121 °C, 20 min) reduced PEs content by 98%, while a reduction of 96% was reported after using 90% aqueous ethanol only (Martínez-Herrera et al., 2006). In a detoxification approach adapted from Martínez-Herrera et al. (2006), Pighinelli et al. (2012) reported only 54% reduction in PEs level in *Jatropha* seed cake

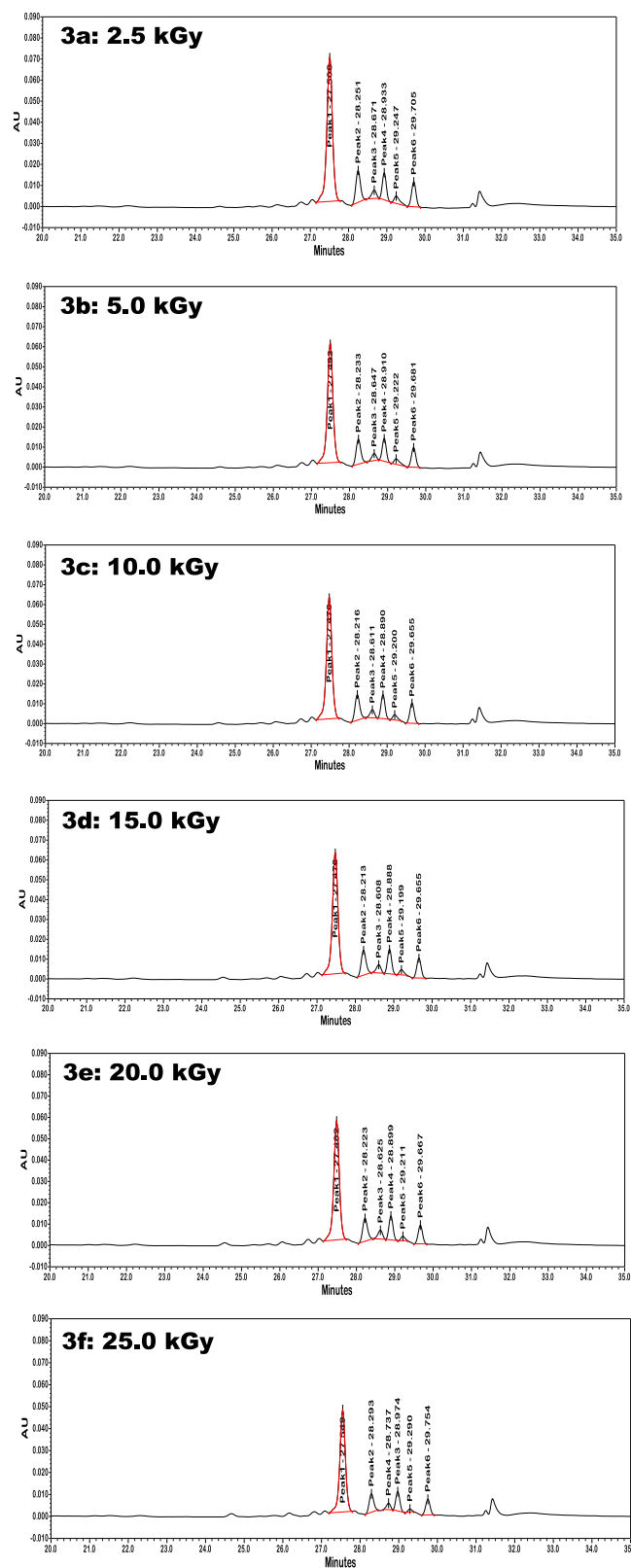


Fig. 3a–3f. Chromatograms of DJKM treated at different dose of electron beam radiations.

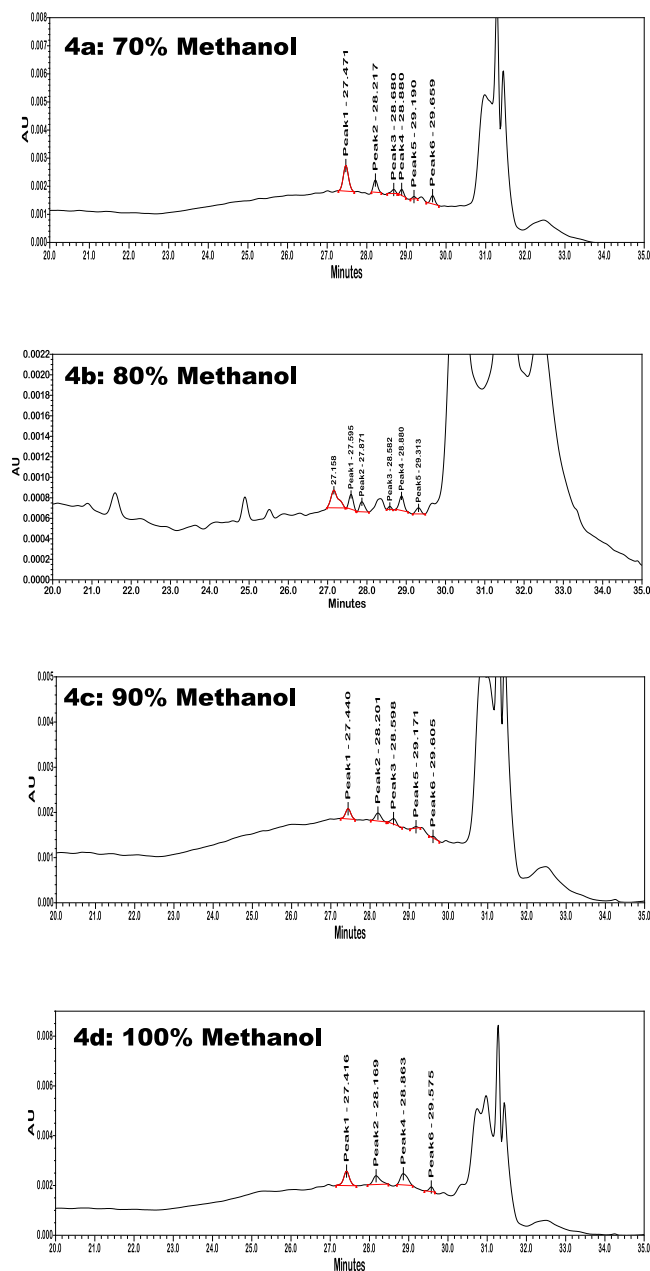


Fig. 4a–4d. Chromatograms of DJKM treated with different concentrations of methanol.

treated with ethanol (99.5%) and stirred at room temperature for 2 h, whereas, treatment with 99.5% ethanol followed by treatment with 0.07% NaHCO_3 plus autoclaving (121 °C, 20 min) reduced PEs content by 64%.

The PEs are moderately polar and have major affinity towards either ethanol or methanol. Being relatively non-toxic, the application of ethanol has advantage over methanol and in terms of the presence of any residues in the treated meal (even though not probable because of more volatility) may not negatively affect animals consuming the treated meal (Martínez-Herrera et al., 2006).

3.2.3. Solvent extraction by alkaline and non-alkaline methanol plus moist heat treatment

The effect of solvent extraction of DJKM with non-alkaline 90% methanol (M90H) and with 90% alkaline methanol having different molar concentrations of alkali (NaOH) followed by moist heat treatment (121 °C, 30 min) is presented in Table 4. The PEs concentration in M90H (Fig. 6a) and AM0.1M (Fig. 6b) treatments decreased significantly ($p < 0.05$)

Table 3

Comparative efficacy of different ethanol concentrations for removal of PEs from DJKM.

Treatments	PEs ($\mu\text{g g}^{-1}$)	Removal (%)
DJKM	1307.0 ^a $\pm$ 15	–
E70	5.0 ^d $\pm$ 0.35	99.61
E80	3.8 ^e $\pm$ 0.17	99.71
E90	7.2 ^c $\pm$ 0.40	99.45
E100	38.9 ^b $\pm$ 0.46	97.02

Data are expressed as Mean $\pm$ SEM, ($n = 3$).Mean values in the same column with different superscripts differ significantly ($p < 0.05$). PEs- Phorbol Esters, DJKM- Defatted *Jatropha* Kernel Meal (control), E70- 70% Ethanol, E80- 80% Ethanol, E90- 90% Ethanol, E100- 100 Ethanol.**Table 4**

Comparative efficacy of alkaline and non-alkaline methanol plus moist heat treatments for removal of PEs from DJKM.

Treatments	PEs ($\mu\text{g g}^{-1}$)	Removal (%)
DJKM	1307.0 ^a $\pm$ 15	–
M90H	3.9 ^e $\pm$ 0.17	99.70
AM0.1	2.2 ^c $\pm$ 0.06	99.83
AM0.2	N.D.	$\approx$ 100.00
AM0.3	N.D.	$\approx$ 100.00
AM3.0	13.0 ^b $\pm$ 0.04	99.01

Data are expressed as Mean $\pm$ SEM, ($n = 3$).Mean values in the same column with different superscripts differ significantly ($p < 0.05$). PEs- Phorbol Esters, DJKM- Defatted *Jatropha* Kernel Meal (control), M90H- 90% methanol plus moist heat treatment, AM0.1- 90% methanol with 0.1 M NaOH plus moist heat treatment, AM0.2- 90% methanol with 0.2 M NaOH plus moist heat treatment, AM0.3- 90% methanol with 0.3 M NaOH plus moist heat treatment, AM3.0- 90% methanol with 3% (0.75M) NaOH plus moist heat treatment.

as compared to untreated DJKM indicating 99.70% and 99.83% removal, respectively. The PEs was not detected in AM0.2 (90% methanol plus 0.2 M NaOH and autoclaving) and AM0.3 (90% methanol plus 0.3 M NaOH and autoclaving) treatment groups. These results are substantiated by the HPLC chromatograms of AM0.2 (Fig. 6c) and AM0.3 (Fig. 6d) treatments, which did not show any of the PEs peaks indicating 100% removal of PEs from DJKM. However, the AM0.1 treatment group having 2.2 $\mu\text{g g}^{-1}$ PEs level exhibited two small peaks of PEs at retention time 27.68 and 28.61 min (Fig. 6b). Contrary to our finding, Makkar and Becker (2010) found that JKM treated by 90% aqueous methanol with 0.1 M NaOH could able to eliminate PEs completely. The disparity in results might be because of the difference in sensitivity of the instrument used for extraction or due to procedural variation. Makkar and Becker (2010) used solvent to JKM ratio 10:1 (v/w) in contrast to our study where it was 15:1 (v/w). However, our results are in conformity with Abou-Arab et al. (2019), wherein they reported 98.04% decline in PEs content in *Jatropha* kernel meal treated with 0.1M NaOH in 90% methanol (w/v, 1:10) by reflux heating at 65 °C for 30 min. These results are in agreement with those reported by Rakshit and Bhagya (2007) who studied the effect of alkaline treatment (NaOH-treated) of defatted meal, and concluded that it was an effective method for reducing the phorbol esters by up to 90%. In comparison to the highly significant reduction (99.70%) in PEs level reported through non-alkaline methanol treatment in this study, Xiao et al. (2011) reported 65.97% PEs reduction through washing the kernel meal with 90% methanol at 50 °C with constant stirring for 2 h. The difference in the findings might be due to procedural variation as Xiao et al. (2011) treated kernel meal only once with methanol whereas, in our study, kernel meal was treated thrice.

3.2.4. Solvent extraction by alkaline and non-alkaline ethanol plus moist heat treatment

The treatment of DJKM by 80% aqueous ethanol with 0.1, 0.2 and 0.3 M NaOH followed by autoclaving (AE0.1, AE0.2 and AE0.3) exhibited complete removal of PEs (Table 5). These results are also supported by the chromatograms (Fig. 7b–7d) of different treatments showing no PEs peaks indicating total removal. Degradation of PEs by alkaline ethanol extraction has not been used before, to the best of our knowledge.

The E80H treatment (non-alkaline extraction of DJKM using 80% ethanol followed by moist heating) reduced PEs level to 3 $\mu\text{g g}^{-1}$ showing 99.77% removal (Table 5, Fig. 7a). In corroboration to our observation, a two-stage extraction (1:3, w/v) of hexane de-oiled meal with aqueous ethanol (concentration not given) through magnetic stirring (1000 rpm) at 50 °C for 30 min reduced the PEs by 100% (Nokkaew and Punsuvon, 2015). Saetae and Suntornsuk (2010) extracted (four times) *Jatropha* seed cake with 90% ethanol (w/v, 1:3) by shaking at 150 rpm for 5 min at room temperature and PEs level was non-detected in detoxified seed cake. Abou-Arab et al. (2019) reported 98.17% reduction in PEs in *Jatropha* kernel

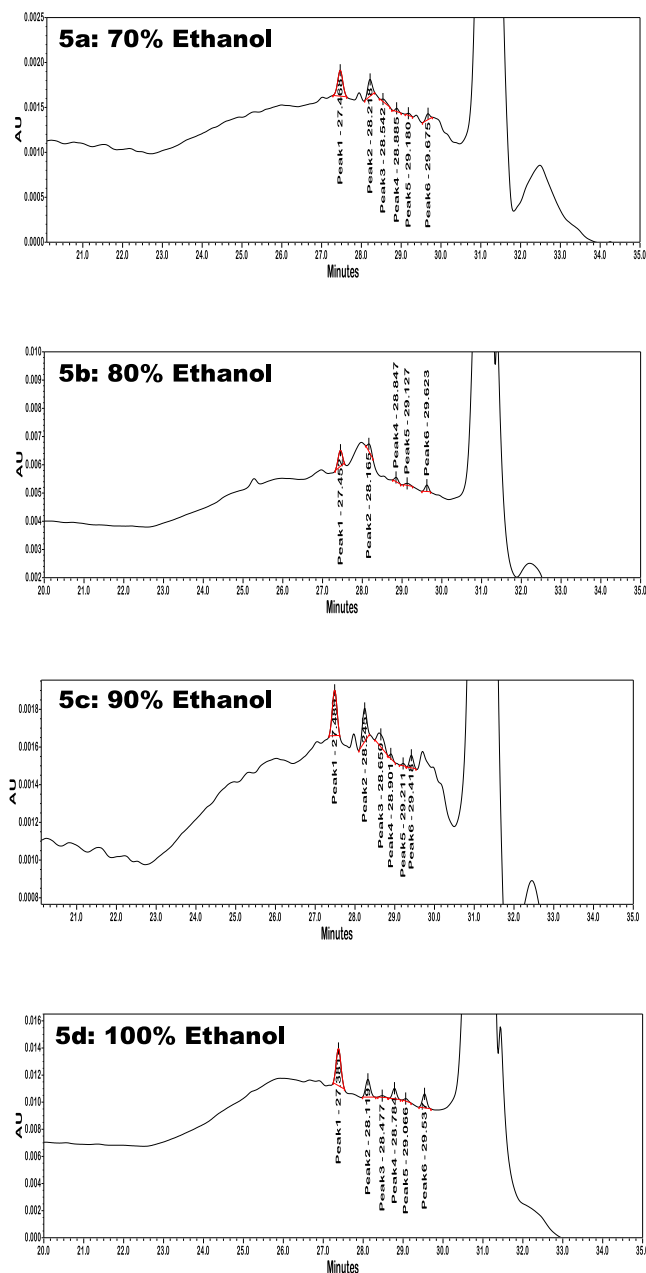


Fig. 5a–5d. Chromatograms of DJKM treated with different concentrations of ethanol.

meal treated with 85% ethanol by heating at 40 °C for 30 min. [Xiao et al. \(2011\)](#) observed 100% removal of PEs when kernel meal is treated 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. In the same study, washing of *Jatropha* kernel meal with 90% ethanol (10:1, v/v) at 50 °C with constant stirring for 2 h, reduced PEs by 59.7%.

3.2.5. Highly alkaline methanol plus moist heat treatment

The PEs content in AM3.0 treatment (i.e. treatment of DJKM using 90% aqueous methanol plus 3% NaOH followed by autoclaving) reduced significantly ($p < 0.05$) compared to control but the PEs value was significantly higher ($p < 0.05$) than M90H (Fig. 6a) and AM0.1 (Fig. 6b) treatment groups (Table 4). In close similarity to our results, [Rakshit et al. \(2008\)](#) achieved an 89% degradation of PEs when *Jatropha* kernel meal was treated through 2% NaOH and heat treatment (autoclaving at 121 °C for 30 min). [Aregheore et al. \(2003\)](#) reported reduction in PEs to the tune of 92% in *Jatropha* kernel

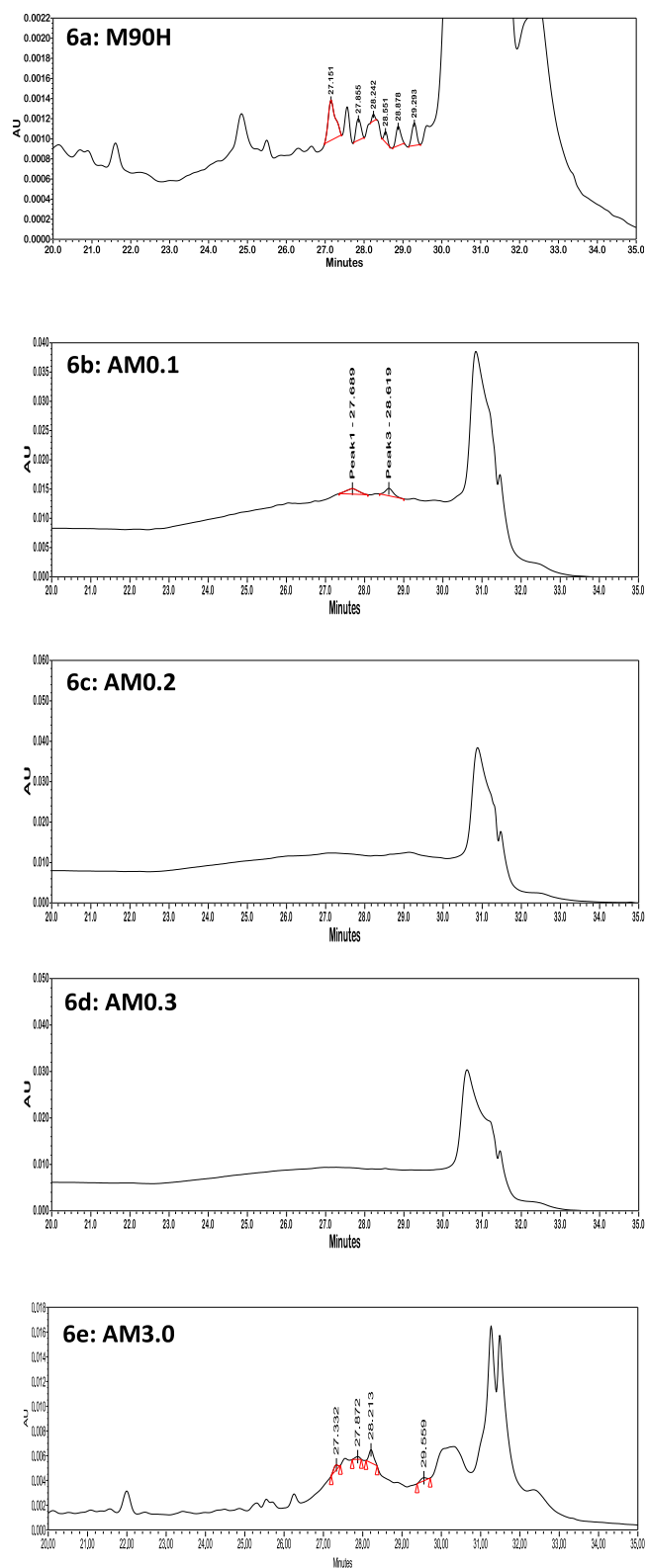


Fig. 6a–6e. Chromatograms of DJKM treated with different non-alkaline and alkaline methanol concentrations.

Table 5

Comparative efficacy of alkaline and non-alkaline ethanol plus moist heat treatments for removal of PEs from DJKM.

Treatments	PEs ($\mu\text{g g}^{-1}$)	Removal (%)
DJKM	1307.0 ^a $\pm$ 15	–
E80H	3.0 ^b $\pm$ 0.06	99.77
AE0.1	N.D.	≈100.00
AE0.2	N.D.	≈100.00
AE0.3	N.D.	≈100.00

Data are expressed as Mean $\pm$ SEM, ($n = 3$).

Mean values in the same column with different superscripts differ significantly ($p < 0.05$). PEs- Phorbol Esters, DJKM- Defatted *Jatropha* Kernel Meal (control), E80H- 80% ethanol plus moist heat treatment, AE0.1- 80% ethanol with 0.1 M NaOH plus moist heat treatment, AE0.2- 80% ethanol with 0.2 M NaOH plus moist heat treatment, AE0.3- 80% ethanol with 0.3 M NaOH plus moist heat treatment.

Table 6

Comparative efficacy of chemical plus moist heat treatments and solid state fermentation for removal of PEs from DJKM.

Treatments	PEs ($\mu\text{g g}^{-1}$)	Removal (%)
DJKM	1307.0 ^a $\pm$ 15	–
Ferric chloride plus hydrogen peroxide plus moist heat treatment	53.6 ^b $\pm$ 0.23	95.89
Solid state fermentation by <i>Aspergillus niger</i>	N.D.	≈100.00

Data are expressed as Mean $\pm$ SEM, ($n = 3$).

Mean values in the same column with different superscripts differ significantly ($p < 0.05$). PEs- Phorbol Esters, DJKM- Defatted *Jatropha* Kernel Meal (control).

meal given alkali treatment (4% sodium hydroxide and 10% sodium hypochlorite) followed by autoclaving (121 °C, 30 min). On the contrary, [Elangovan et al. \(2013\)](#) reported a reduction of only 55% in PEs content in *Jatropha* kernel cake using chemical treatment with 3% sodium hydroxide or sodium bicarbonate.

3.2.6. Ferric chloride & hydrogen peroxide plus moist heat treatment

The PEs concentration in DJKM treated with FeCl_3 and hydrogen peroxide followed by autoclaving was found to be $53.6 \mu\text{g g}^{-1}$ showing 95.89% reduction ([Table 6](#), [Fig. 8](#)). PEs of *Jatropha* are reported to be highly susceptible to oxidative degradation either in purified form or when present in the oily extract and their degradation products were found to be innocuous when tested in sensitive snail bioassay ([Roach et al., 2012](#)). Therefore, in this study, PEs present in the kernel meal was degraded through oxidation approach by treating with hydrogen peroxide (oxidizing agent) and ferric chloride (as a catalyst) ([Munter, 2001](#)). [Schmidt and Hecker \(1975\)](#) have reported similar susceptibility of purified PEs towards oxidation, wherein 7-hydroperoxide was formed as a major degradation product. [Baocai et al. \(2014\)](#) reported >99.8% reduction of PEs after treatment of *Jatropha* seed cake by hydrogen peroxide followed by alkali (sodium hydroxide or potassium hydroxide or sodium carbonate) treatment. PEs were reduced to a non-detectable level in *J. curcas* seed cake when oxidized with hydrogen peroxide under optimized conditions ([Zhang et al., 2014](#)).

3.3. Solid state fermentation (SSF)

Solid state fermentation of DJKM using *A. niger* could able to eliminate PEs by 100% in the present investigation ([Table 6](#), [Fig. 9](#)). The microbial approach represents a promising strategy for the degradation of PEs ([de Barros et al., 2011](#); [da Luz et al., 2014](#)). The microbial treatment supports sustainable or green processing by eliminating the need for hazardous and costly chemicals or organic solvents. Solid state fermentation of *J. curcas* kernel meal using various microorganisms is a technique that has received great attention among researchers. SSF constitutes a fermentation process performed on a non-soluble material that acts as both physical support and a source of nutrients in the absence of free flowing liquid ([Pandey, 1992](#)). Many microorganisms including bacteria (*Pseudomonas aeruginosa* PseA strain, *Bacillus subtilis* and *B. licheniformis*) ([Joshi et al., 2011](#); [Phengnuam and Suntornsuk, 2013](#)) and fungi (*A. niger*, *A. versicolor*, *Bjerkandera adusta*, *Phlebia rufa*, *Trichoderma longibrachiatum*, *T. harzianum*, *Paecilomyces sinensis*, *Cladosporium cladosporioides*, *Fusarium chlamydosporum*, *Neurospora sitophila*, *Pleurotus ostreatus*, and *Cunninghamella echinulata*) ([Belew and Sam, 2010](#); [Veerabhadrapa et al., 2014](#); [Belew et al., 2010](#); [de Barros et al., 2011](#); [Kurniati, 2012](#); [Kasuya et al., 2012](#); [Najjar et al., 2014](#); [da Luz et al., 2014](#); [Sharath et al., 2014](#)) have been studied for detoxification of *Jatropha* seed cake or kernel meal. [Veerabhadrapa et al. \(2014\)](#) have reported 81.1% reduction in PEs level in *Jatropha* seed cake through solid-state fermentation using *Aspergillus versicolor*. In our study, SSF of DJKM at 30 °C over a period of three weeks using *A. niger* resulted in the total removal of PEs. In corroboration to our result, [Belew and Sam \(2010\)](#) also demonstrated that SSF

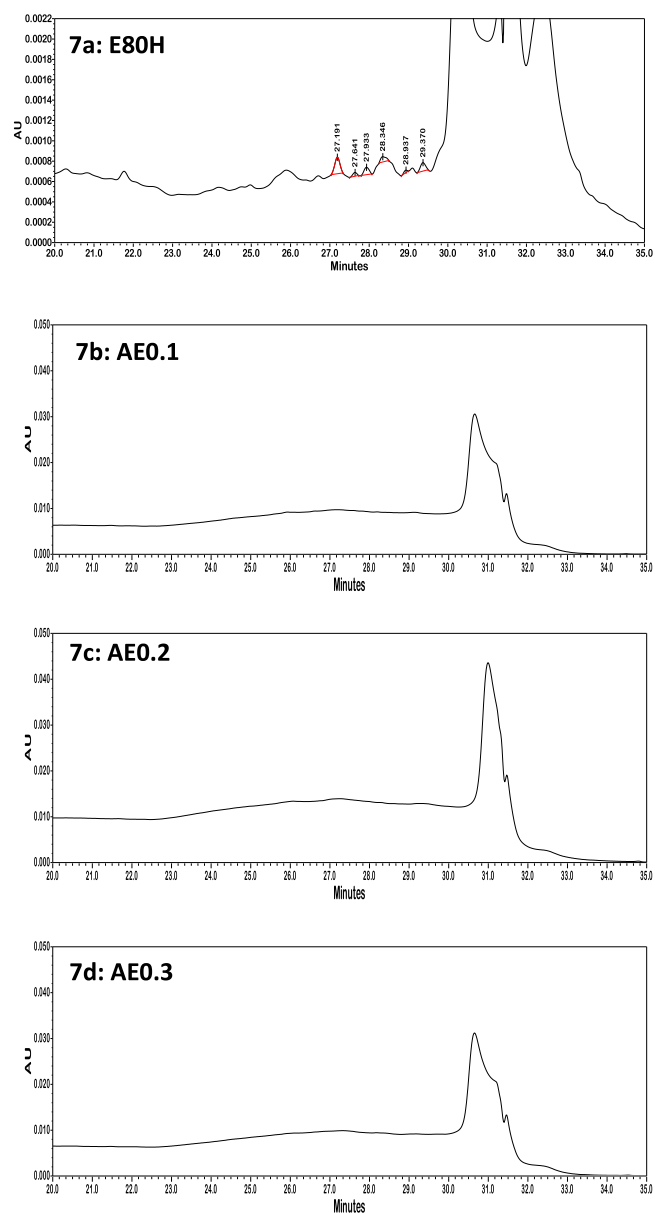


Fig. 7a–7d. Chromatograms of DJKM treated with different non-alkaline and alkaline ethanol concentrations.

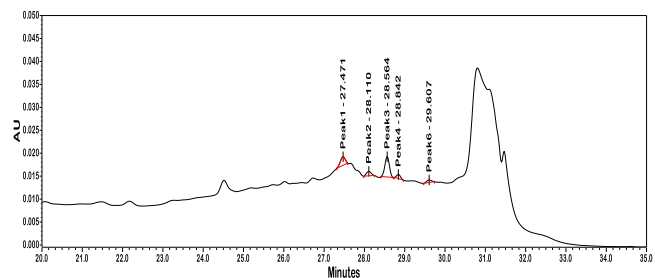


Fig. 8. Chromatogram of DJKM treated with ferric chloride & hydrogen peroxide plus moist heat treatment.

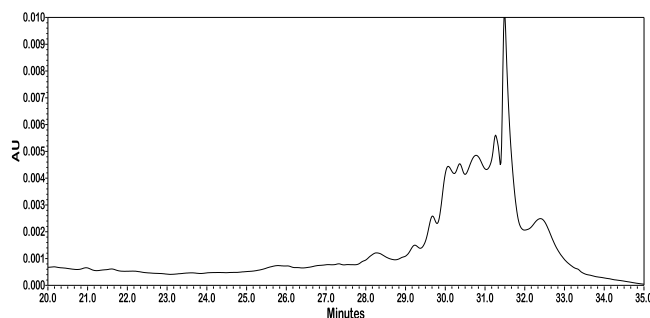


Fig. 9. Chromatogram of DJKM treated by solid state fermentation using *Aspergillus niger*.

of *Jatropha* seed cake for 7 days using *A. niger* could able to remove PEs by nearly 100%. In a previous study in our laboratory, Shamna et al. (2015) found that SSF of *Jatropha* protein concentrate for two weeks using *A. niger* fungus produced detoxified product, which was completely free from PEs. In contrary to our results, de Barros et al. (2011) found the reduction of PEs by 91% and 97% in *Jatropha* seed cake fermented over 30 days with white rot fungi, *B. adusta* and *P. rufa*, respectively. Incubation of kernel meal with *P. ostreatus* fungus for 60 days exhibited 99% reduction in PEs levels (da Luz et al., 2014). SSF of deoiled *Jatropha* seed cake by *P. aeruginosa* PseA strain after 9 days at 30 °C and 65% relative humidity exhibited complete removal of PEs in (Joshi et al., 2011). During SSF, the degradation of PEs in DJKM can be attributed to the production of various enzymes during the vegetative and reproductive phase of fungi (Jacqueline and Visser, 1996). As PEs are found to be cytotoxic (*in situ*) even at nano molar concentrations (Devappa et al., 2011), the complete (100%) removal of PEs from JKM is inevitable as confirmed through feeding trial in *Labeo rohita* (Angel et al., 2015; Shamna et al., 2015). In our present study, SSF by *A. niger* and some chemical treatments (AM0.2, AM0.3, AE0.1, AM0.2 and AM0.3) could able to remove PEs in DJKM by 100%.

4. Conclusion

The complete removal of phorbol esters in defatted *Jatropha* kernel meal is necessary prior to its utilization in animal diets. Based upon the results of various treatments in terms of removal of phorbol esters, it can be concluded that although combinations of physical and chemical treatments require less time in detoxification of kernel meal, their cumbersome nature, operational complexity and the high cost involved, limit their widespread acceptance. Further, the use of organic solvents and alkali may change odor affecting the feed intake, degrade proteins and certain amino acids diminishing nutrition qualities of the detoxified product. The fermentation approach on the other hand, though time consuming, is environmentally sustainable and cost economical. It offers greater efficiency and may also improve the nutritional value of the rendered product by adding microbial protein and decreasing crude fiber. However, prior to the application of detoxified *Jatropha* kernel meal in animal feeds, in-vivo and in-vitro studies are suggested. The bio-diesel industry is already considered to be eco-friendly and the effective utilization of its waste will further enhance the sustainability of this industry.

CRediT authorship contribution statement

Vikas Phulia: Investigation, Methodology, Data curation, Writing – original draft. **Parimal Sardar:** Conceptualization, Methodology, Project administration, Writing – review & editing. **Ankur Jamwal:** Formal analysis, Writing – review & editing. **Vikas Kumar:** Methodology, Writing – review & editing. **Bhushan N. Sanap:** Investigation. **N.P. Sahu:** Resources, Visualization, Writing – review & editing. **Subodh Gupta:** Methodology, Supervision, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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