Heliyon 5 (2019) e01689

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.heliyon.com

Comparative study between chemical, physical and enzymatic methods for *Jatropha curcas* kernel meal phorbol ester detoxification

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ARTICLE INFO

Keywords:
Food science
Food analysis
Chemistry
Natural product chemistry

ABSTRACT

Detoxification of protein rich *Jatropha* kernel cake to eliminate the phorbol esters is a great challenge for its industrial utilization in food processing. Several methods either chemical or physical have been previously applied trying to degrade phorbol esters in *Jatropha curcas* seed cake, which are the main toxic compound with thermo-stable properties. The objective of this study was to compare the different methods to get rid of phorbol esters (PE) including, chemical treatments (0.1 M NaOH/90 % methanol, 85 % ethanol, 90 % methanol & 85 % ethanol (50:50) for 8 h), physical treatments (microwave, ultrasonic and microwave + ultrasonic) and enzymatic treatment by crude germinated *Jatropha* seed lipase (CGJS). The results showed that the elimination of phorbol esters content chemically treated either by 0.1 M NaOH/90 % methanol or ethanol 85% had effectively decreased by 98.04 % and 98.17 %, respectively compared to control. The treatment by methanol 90% and ethanol 85% (50:50) gave degradation percentage of 95.43% of phorbol esters. The enzymatic elimination of phorbol esters by crude germinated *Jatropha* seed (CGJS) lipase proved high efficiency of detoxification by reducing the percentage to 98.43%. On the other hand, physical detoxification of *Jatropha* seed kernel showed good results with microwave treatment by reducing phorbol esters content to 86.29 %. Treatment by ultrasound has detoxified the phorbol esters content by 87.60 % in *Jatropha curcas* seed. While, combination between microwave & ultrasound increased the percentage of phorbol esters degradation to 88.38 %. In conclusion, enzymatic degradation of phorbol esters is a safe method for degradation as it is both an un-expensive and easy way for detoxification.

1. Introduction

Jatropha curcas L. (*J. Curcas*) which belongs to the *Euphorbiaceae* family is developed in tropical and sub-tropical locales like Central and South America, Africa, India and South East Asia (Makkar and Becker, 2009a). It is a multipurpose plant with several industrial and medicinal applications.

A considerable quantity of seed cake is left as a by-product after oil extraction from the *Jatropha curcas* seed. One ton of seed yields approximately 615 kg of kernels of a potential oil recuperation of roughly 51 % using solvent extraction method (Makkar and Becker, 2009b).

The seeds contained approximately 50–60 % oil and hence could be considered as a very rich source of oil (Saetae and Suntorasuk, 2010). The *Jatropha curcas* seed cake, after extraction of oil, is rich in protein (50–64 %). All essential amino acids in Jatropha cake has been reported to have higher concentrations than those stated by the FAO recommendations, with the exception of lysine (Xiao and Zhang, 2011).

However, the *Jatropha curcas* seed cake has been reported to be toxic to animals and human, which significantly restricted its utilization (Xiao and Zhang, 2011). The major toxic components in the cake are phorbol esters (PEs) besides other anti-nutrients such as lectin, trypsin inhibitor, phytic acid and saponin (Malviya *et al.*, 2011). They exhibited cytotoxic activity towards bovine kidney cells (Oskoueian *et al.*, 2019).

Phorbol esters are widely distributed in various parts of the Jatropha plant, and they are concentrated in the seed kernel (Ahmed and Soliman, 2009). Phorbol ester molecule is a hydrophobic, oil soluble tetracyclic diterpenoid with a tigliane skeletal structure, with thermo-stable properties in both seed cake and meal. Around 70–75% of phorbol esters are separated from the pressed cake after the extraction of oil, while 25–30% remains in the pressed cake despite its lipophilic properties (Makkar and Becker, 2009a; Devappa *et al.*, 2010).

Jatropha phorbolesters comprise a group of six compounds, with non-identical structures as the commonly known PEs of croton oil, for example 12-O-tetradecanoyl phorbol-13-acetate (EFSA CONTAM Panel,

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https://doi.org/10.1016/j.heliyon.2019.e01689
Received 4 December 2018; Received in revised form 13 March 2019; Accepted 7 May 2019

2015).

The chemical structures of phorbol ester show that it has an ester bond with its fatty acid, which is a simple to diacyl glycerol (Li *et al.*, 2010). That bond might be hydrolyzed using lipase (Bose and Keharia, 2013). However, enzymatic hydrolysis data are very limited. In general lipase activity is absent in ungerminated seeds, but it could be produced during germination of seeds (Abigor *et al.*, 2002).

Consequently, the presence of phorbol ester limits the utilization of the seed cake for food and feed consumption. So, its degradation is an important issue to make it safe for consumption. Many physical and chemical methods have been tested for its efficiency for the degradation of PE (Devappa and Swamylingappa, 2008). Most of the treatments use a combination of solvent extraction, alkaline, and high temperature (Zimila *et al.*, 2018) which are neither specific nor environmentally friendly and expensive.

Since, most of the phorbol ester detoxification methods that had been studied on defatted *Jatropha curcas* seed used solvent extraction, so, the objective of this study is to investigate the degradation of phorbol esters (PEs) from *Jatropha curcas* seeds by different methods to obtain a safe and secure method that only effectively removes phorbol esters compounds while the crude protein content is retained.

2. Materials and methods

2.1. Materials

Jatropha species (*Jatropha curcas L.)* was collected during two successive seasons of 2016 and 2017 of *Jatropha curcas* trees (4 years old) grown in South Sinai Governorate which irrigated with saline fresh water. The selected Jatropha trees were uniform in vigor and size, planted at 2 × 4 m apart. All trees received the same horticultural practices.

2.2. Sample preparation

Sample was cleaned manually to remove all foreign materials, small branches and immature seeds. The cleaned and graded seeds were dehulled to gain access to a cream-colored endosperm, which is the sample material. The portions of sample materials were blended to powder form (0.5 mm) in a high-speed blender (Braun KMM 30 mill, type 3045, CombiMax, Germany), and oil was extracted by soxhlet apparatus, using diethyl ether (boiling point of 40–60 °C), for 16 h (fat <1%) (Martinez-Herrera *et al.*, 2006). The defatted seeds were air dried at room temperature (25 ± 2 °C) and stored in the air tight polyethylene bags and kept in the refrigerator at 4 °C until required for further analysis.

2.3. Chemicals and reagents

All used chemicals and reagents were obtained from Sigma, Aldrich and Fluka. Standard phorbol esters (phorbol-12- myristate13-acetate) were purchased from Sigma- Aldrich Co. (St. Louis, Mo, USA).

2.4. Methods

In this study, three kinds of treatments were used in degradation of phorbol ester. **Control sample** without any treatments was prepared by milling defatted kernels, then phorbol esters were extracted as described below in the part of extraction and estimation of phorbol ester by HPLC.

2.4.1. Chemical treatments

A) Treatment with 0.1 NaOH & 90 % methanol

Defatted kernel meal was washed with 0.1 M NaOH in 90 % methanol. The procedures were carried out by putting 80 g of defatted meal into round bottom flask and adding 800 mL of alkali in methanol solution into the flask. The heating was operated under reflux and constant stirring at 65 °C for 30 min. Then, the solution was removed by vacuum filtration. The residue was washed again with 100 mL of 90 % methanol. After that, the residue was spread to dry at room temperature (Vittaya and Rayakorn, 2013).

B) Treatment with 85 % ethanol

The samples were only washed by 85 % ethanol. The procedures were carried out by putting 80 g of defatted meal into round bottom flask and adding 800 mL of 85 % ethanol solution into the flask. The heating was operated under reflux and constant stirring at 40 °C for 30 min. Then the solution was removed by vacuum filtration. The residue was washed again with 100 mL of 85 % ethanol. After that, the residue was spread to dry at room temperature. Phorbol esters in the residue were further extracted and analyzed by HPLC (Vittaya and Rayakorn, 2013). .

C) Treatment with methanol 90 % and ethanol 85 % (50:50)

Defatted kernel meal was extracted with 90 % methanol and 85 % ethanol (50:50) for 8 hrs at room temperature (25 ± 2 °C) with constant stirring. The solvent was removed by filtration and residue was dried in hot air oven at 40 °C and powdered to pass through 60 mesh sieves for analysis. Phorbol esters in the residue were further extracted and analyzed by HPLC.

2.4.2. Enzymatic treatment

A) Stimulation of the production of lipase enzyme by germination

The germinated *Jatropha curcas* seeds were prepared according to the method of Hidayat *et al.* (2014a), with some modifications. Seeds were soaked in phosphate buffer at pH 7 at room temperature for 12 hrs, air-dried, and dehydrated at room temperature for 24 hrs. The soaked seeds were spread onto tray which was covered with cotton. They were allowed to germinate. The seeds were harvested when the length of germinated seeds was approximately 2–2.5 cm. The germinated seeds were carefully cracked to remove the shells. The seed kernels (20 g) were crushed using a homogenizer at 5200 rpm for 10 min, then dried in hot air oven at 40 °C as crude germinated Jatropha seed lipase (CGJS), which was further kept at -18 °C.

B) Utilization of crude germinated *Jatropha* seed lipase powder

One g of CGJS lipase powder was added to Erlenmeyer containing 5 g defatted *Jatropha* seed meal and 40 mL phosphate buffer pH 7.0. The suspension was incubated in a shaking water bath at 30 °C for 12 hrs. The reaction was terminated by placing the Erlenmeyer into ice bath. Seed cake residue was obtained by filtering the suspension. Phorbol esters in the residue were further extracted and analyzed by HPLC (Avita *et al.*, 2016).

2.4.3. Physical treatment (microwave and/or ultrasonic)

A) Ultrasonic

Ten g of *Jatropha* powder was extracted with 100 ml of methanol 80 % in an ultrasonic device (200 W, 59 kHz, Shanghai Kudos Sonication Machine Company Ltd., China) for 60 min at room temperature. The mixture was then filtered through Whatman no. 4 filter paper and the filtrate was evaporated under reduced pressure at 30 °C until its volume was about 40 ml. The final volume of the extract was completed to 50 ml with the extraction solvent and then, it was used for analysis by HPLC (Abou-Arab *et al.*, 2016).

B) Microwave

Ten g of *Jatropha* powder was treated with microwave for 6 min. Microwave oven used in the present study was Samsung, Model MF245 (Korea) with oven cavity dimensions of 419 × 245 × 428 mm and operation frequency of 2.450 MHz, with a power source of 230 V-50 Hz. The nominal microwave power was 900 Ws (Mahmoud *et al.* 2015). Phorbol esters in the treated powder were further extracted and analyzed by HPLC.

C) Microwave and ultrasonic

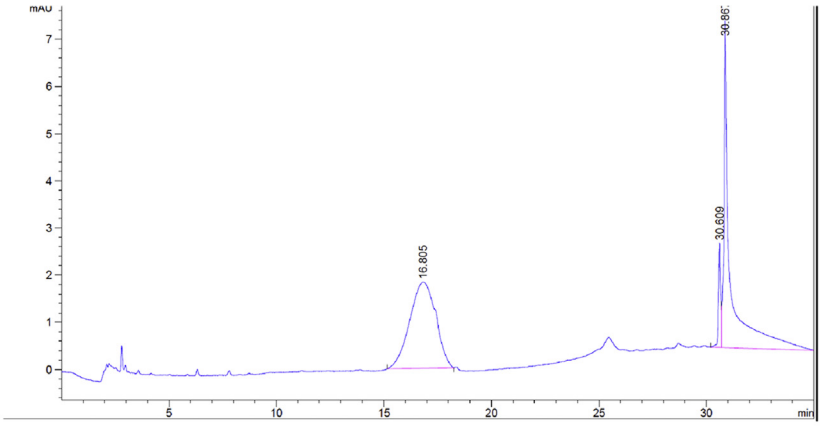
Ten g of *Jatropha* powder was treated with microwave for 6 min. Then it was extracted with 100 ml of methanol 80 % in an ultrasonic device for 60 min at room temperature. The mixture was then filtered and evaporated and the final volume was made to 50 ml, then it was used for the analysis by HPLC (Abou-Arab *et al.*, 2016).

2.4.4. Extraction and estimation of phorbol esters by HPLC

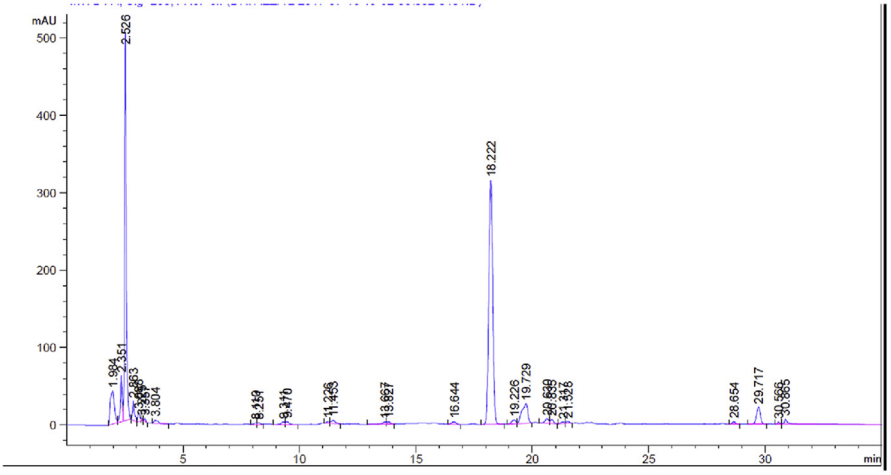
The phorbol ester concentration was estimated in the untreated and treated samples. A weight of 2.5 g sample was ground with a small amount of sand using a pestle and mortar, then 20 ml dichloromethane was added. The mixture was again ground for about 5 min with the mortar. The material was allowed to settle and the liquid phase was filtered. The residues on the filter paper and in the pestle were pooled using about 20 ml dichloromethane and then ground for about 5 min using the mortar. The liquid phase was again collected. The extraction procedure was repeated three more times and the filtrate from all five extractions were pooled. The filtrate was dried under vacuum at 40 °C. The dried residue was dissolved in 5 ml tetrahydrofuran, passed through a 0.2 μm glass filter and injected (20 μl) into the HPLC (Makkar *et al.*, 1997). A water HPLC system consisting of a 600-controller pump, a temperature control Module, a tunable absorbance detector Water 486. Chromatographic separation was achieved on phenomonex (Luna 5 μ C18 (2) column (250 × 4.6 mm). The separation was performed at room temperature (23 °C) and the flow rate was 1.3 ml min^{-1}. The phorbolesters peaks were identified and integrated at 280 nm. The results are expressed as equivalent to a standard, phorbol-12-myristate 13- acetate (PMA), which appeared between 16 and 30 min (Fig. 1). Concentrations of each PE derivative were calculated from peak areas with reference to the internal standard (PMA) peak area, whereas the total PE contents were calculated by summarizing PE derivative contents.

2.4.5. Statistical analysis

The data obtained from the present study was statistically subjected to analysis of variance (ANOVA) according to Snedecor and Cochran (1980) by the computerized program SPSS software, version "15" for Windows. The least significant difference (LSD) value was used to determine significant difference between means. Data was represented as Mean ± SD. Values were considered significant at $P < 0.05$, otherwise were considered non-significant.



a. Standard (phorbol-12-myristate 13- acetate)



b. Control sample

Fig. 1. HPLC Chromatogram of Phorbol esters (phorbol-12-myristate 13- acetate).

Table 1
Detoxification of Phorbol ester by chemical treatments of defatted *Jatropha curcas* seeds (on dry weight basis).

Treatments	Content (mg/g)	Degradation %
Control (untreated)	$7.66^{a} \pm 1.0$	-
0.1 M NaOH/90 % methanol	$0.15^{b} \pm 0.02$	98.04
85 % ethanol	$0.14^{b} \pm 0.02$	98.17
90 % methanol+85 % ethanol (50:50)	$0.35^{b} \pm 0.02$	95.43

-All values are means of triplicate determinations ±standard deviation (SD).
- Means within columns with different letters are significantly different ($P < 0.05$).

3. Results and discussion

3.1. Effect of chemical treatments on the degradation of phorbol esters

A) Treatment with 0.1 NaOH & 90 % methanol

Alkaline degradation of phorbol esters using methanol 90% is shown in Table 1. It is obvious that the phorbol esters content was significantly decreased ($P < 0.05$) from 7.66 mg/g in untreated (control) sample to 0.15 mg/g after alkaline treatment. That degradation represented about 98.04 % degradation of total phorbol esters in defatted kernel seed meal. 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 that it was an effective method for reducing the phorbol esters by up to 90 %.

B) Treatment with ethanol 85 %

The effect of 85 % ethanol treatment on degradation the phorbol esters content in defatted kernel seeds are presented in Table 1. Fig. 1 shows the chromatogram of phorbol esters content of standard and control sample which had two peaks of phorbol esters that appeared at 30.59 and 30.86 min. Phorbol ester concentrations were calculated by summation the area of these peaks and they were compared to the concentration of TPA (phorbol-12-myristate 13- acetate) as standard. The results proved that the phorbol esters content was affected significantly ($P < 0.05$) by that treatment. As shown clearly in the chromatogram of the treated samples (Fig. 2), the disappearance of the two main peaks of phorbol esters and the appearance of other small peaks with a different retention time reflect the breakdown and degradation of the phorbol ester into another compounds. The phorbol esters content decreased from 7.66 mg/g in untreated (control) sample to 0.14 mg/g in treated sample with a percentage of degradation of 98.17 % in defatted kernel seed meal. That highly significant degradation of phorbol esters may be due to its solubility in ethanol as reported by Makkar and Becker (1997), who found that treating defatted meal with aqueous ethanol was promising as it removed phorbol esters by about 93 %. Herrera *et al.* (2006) demonstrated that, solvents like methanol and ethanol have a real affinity in detoxification of Jatropha meal whereas phorbol esters are tolerably polar.

C) Treatment with methanol 90% and ethanol 85% (50:50)

The results given in Table 1 showed the phorbol esters content in defatted kernel seed meal after and before treatments. As shown in the table, the phorbol esters content decreased ($P < 0.05$) by methanol 90% & ethanol 85% (50:50) treatment which was 0.35 mg/g compared with control (7.66 mg/g). This treatment decreased the phorbol esters content by 95.43%. These results are in accordance with those mentioned by Vittaya and Rayakorn (2013) who reported a reduction percentage by 97% of phorbol esters.

By comparing the reduction in phorbol ester percentage between chemical treatments, it was found that they have a similar efficiency in phorbol ester removing. On the other hand, the advantage of utilizing ethanol that is nontoxic when compared to methanol is that it could be exploited in detoxification of nutritive Jatropha meal which is used in food production.

3.2. Effect of crude germinated Jatropha curcas seed (CGJS) lipase enzyme as biocatalyst

The results as shown in Table 2 and the chromatogram in Fig. 3 display phorbol esters content of crude germinated *Jatropha curcas* seed which was decreased significantly ($P < 0.05$) from 7.66 mg/g control sample to 0.12 mg/g after treatment with lipases enzyme. The degradation percentage of phorbol esters was 98.43%. The results indicated that enzymatic degradation of PE could be good and safe as a processing method for the degradation of PE in Jatropha seed meal. PE has an ester bond between phorbol moiety and its fatty acid, which is an analogue to diacyl glycerol. Ester bonds of PE may be hydrolyzed by lipase (Goel *et*

Table 2
Effect of treatment of defatted *Jatropha curcas* seeds with lipases enzymes on the detoxification of Phorbol ester (on dry weight basis).

Treatments	Content (mg/g)	Degradation %
Control (untreated)	$7.66^{a} \pm 1.0$	-
Lipases enzymes	$0.12^{b} \pm 0.03$	98.43

-All values are means of triplicate determinations ±standard deviation (SD).
- Means within columns with different letters are significantly different ($P < 0.05$).

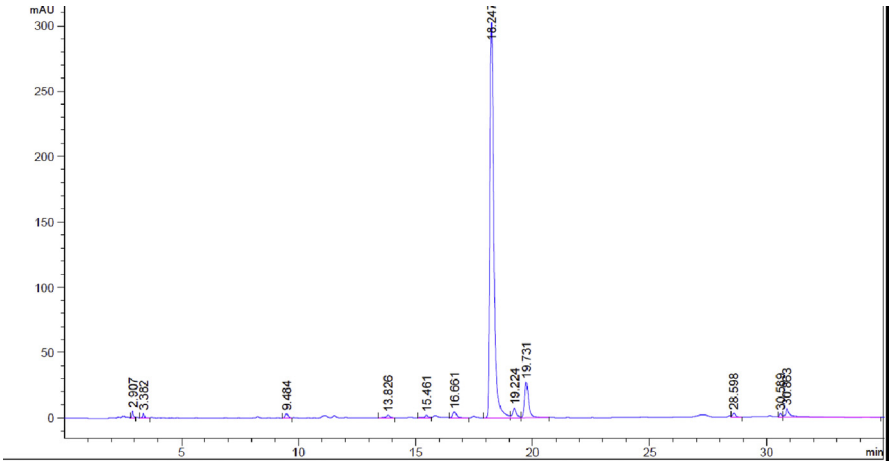


Fig. 2. HPLC Chromatogram after treatments by 85 % ethanol of defatted *Jatropha curcas* seeds achieved 98.17 % Phorbol ester removal.

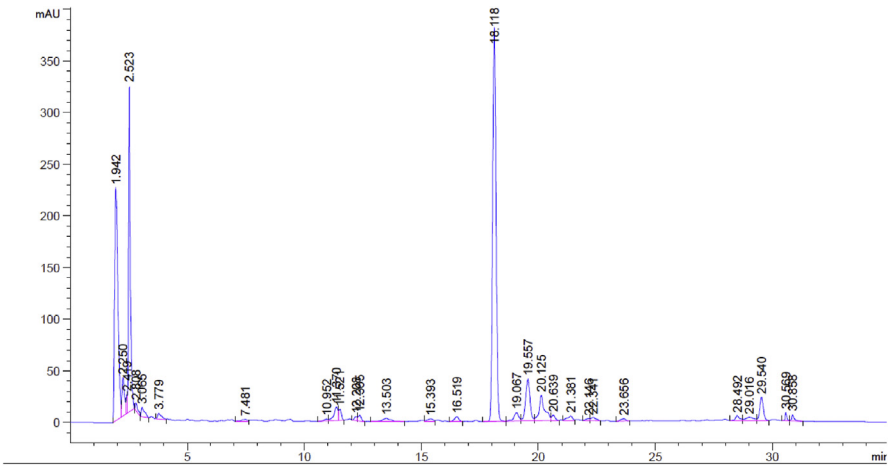


Fig. 3. HPLC Chromatogram after treatments by lipases enzymes of defatted *Jatropha curcas* seeds achieved 98.43 % of Phorbol ester removal.

Table 3
Effect of microwave and ultrasonic/80 % methanol treatment of defatted *Jatropha curcas* seeds on the detoxification of Phorbol ester (on dry weight basis).

Treatments	Content (mg/g)	Degradation %
Control (untreated)	7.66[a] ±1.0	-
Microwave	1.05[b] ± 0.02	86.29
Ultrasonic	0.95[b] ± 0.02	87.60
Microwave & ultrasonic	0.89[c] ± 0.02	88.38

-All values are means of triplicate determinations ±standard deviation (SD).
- Means within columns with different letters are significantly different ($P < 0.05$).

al., 2007). Seed germination has been previously mentioned as the most important treatment based on inducing lipase secretion and consequently shortening the germination time (Hu *et al.*, 2009). Moreover, Hidayat et al. (2014b) reported that PE could be hydrolyzed by rice bran lipase and dried germinated Jatropha seed lipase. Thus, it is recommended that the produced lipase during *Jatropha* seed germination is more effective for hydrolyzing PE that other lipase sources (Hidayat *et al.*, 2014b). These results are in accordance with those reported by Avita *et al.* (2016) who mentioned that the predicted degradation of phorbol esters by germination was 98.96 %.

3.3. Effect of physical treatments on the degradation of phorbol esters

It seems that degradation of phorbol esters by physical methods as the microwave and ultrasonic treatments nearly has not been used before, so no reference was found in this respect.

A) Microwave

The results given in Table 3 demonstrated that the phorbol esters content was affected significantly ($P < 0.05$) by microwave treatment that was extracted by methanol 80 %. Phorbol esters content was reduced from 7.66 mg/g in control sample to1.05 mg/g in defatted kernel seeds. So, this treatment achieved a success rate in degradation of phorbol esters by 86.29 %.

B) Ultrasonic

The effect of extraction procedure using ultrasound with methanol 80% treatment on the degradation of phorbol esters of defatted kernel seeds was shown in Table 3. The phorbol esters content was affected significantly by ultrasound ($P < 0.05$) with 80 % methanol extraction treatment. The phorbol ester content was7.66 mg/g in control sample that was reduced in to 0.95 mg/g in ultrasound treatment with 80 % methanol extraction. This treatment was able to detoxify Jatropha meal by reducing the phorbol ester content into 87.60%.

C) Combination of microwave and ultrasonic

The combined effect of microwave and ultrasonic treatments on the degradation of phorbol esters is presented in Table 3 and Fig. 4. The

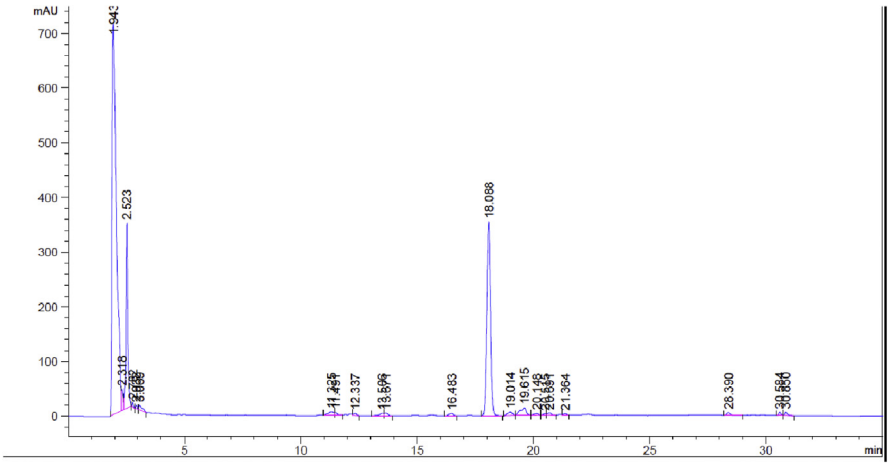


Fig. 4. HPLC Chromatogram after treatments by microwave & ultrasound/80 % methanol of *Jatropha curcas* seeds achieved 88.38 % of Phorbol ester removal.

chromatogram shows that the phorbol esters content was affected significantly ($P < 0.05$) by microwave and ultrasonic treatment extracted with 80 % methanol. The phorbol esters content was 7.66 mg/g in control sample which was reduced in to 0.89 mg/g for defatted kernel seeds microwave and ultrasound treatment extracted with 80 % methanol. The degradation of phorbol esters was 88.38 % by this treatment.

The results showed that the elimination of phorbol esters content chemically treated either by 0.1 M NaOH/90 % methanol or by ethanol 85% had effectively decreased by 98.04 % and 98.17 %, respectively compared with control. While, the treatment by methanol 90% and ethanol 85% (50:50) gave degradation percentage 95.43% of phorbol esters. On the other hand, the enzymatic elimination of phorbol esters by crude germinated Jatropha seed (CGJS) lipase proved high efficiency of detoxification by reducing the percentage to 98.43 %. Moreover, physical detoxification of Jatropha seed kernel showed good results with microwave treatment by reducing phorbol esters content to 86.29 %. Treatment by ultrasound detoxified the phorbol esters content by 87.60 % in *Jatropha curcas* seed. While, combination between microwave and ultrasound increased the percentage of phorbol esters degradation to 88.38 %.

4. Conclusion

Utilization of *Jatropha curcas* seed cake is limited by the presence of phorbol esters (PE), which are the main toxic compounds with thermo stable properties. Detoxification methods for phorbol esters degradation of defatted *Jatropha curcas* meal are very important since the meal is a co-product of biofuel industry. Therefore, a simple and inexpensive detoxification technique of phorbol esters is necessary. On the basis of the results of this research, it could be concluded that the 85 % ethanol washing was more economic and effective method than alkali in 90 % methanol washing. That method was a promising way to detoxify *Jatropha curcas* deoiled meal. Also, the degradation of phorbol esters by crude germinated Jatropha seed (CGJS) lipase treatment might be a recommended method as a safe, inexpensive and easy way of detoxification. These observations show that acceptance of Jatropha seeds as a food processing ingredient is influenced by the content of phorbol ester and the lower phorbol esters, the higher the acceptance of Jatropha seeds.

Declarations

Author contribution statement

Marwa H. Mahmoud, Azza A. Abou-Arab: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dorria M. M. Ahmed, Ferial M. Abu-Salem: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by the National Research Centre, Giza, Egypt (Grant No:11090407/2016–2019 National Research Centre) as a part of an ongoing project.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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