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Chapter 6

ELIMINATION OF TOXIC PHORBOL ESTERS IN *JATROPHA CURCAS* SEED OIL BY ADSORPTION TECHNIQUE

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ABSTRACT

Nowadays *Jatropha curcas* is one of the important alternative oil plants to produce biodiesel. But because of toxic substance especially phorbol esters are dangerous compounds for human who working with this oil. And so it need to eliminate this substance before utilization.

Phorbol esters are a natural toxic ester found in tropical plant in the family of Euphorbiaceae. It is main toxic compounds in seed oil of Jatropha curcas. The biological effects of phorbol esters are tumor promotion or cocarcinogen when taken and inflammation when contacted. At least 5 types of phorbol esters are detected in *J. curcas* oil. The major chemical structure of detected phorbol ester is 12-Deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl]-6'-[16',18'20-nonatrie-nyl]-

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bicyclo[3.1.6]hexane-(13-0)-2'-[carboxylate]-(16-0)-3'-[8'-butenoic-10'] ate or DHPB.

Many researchers tried to detoxify phorbol esters in seed oil by the extraction with ethanol or methanol but this experiment is difficult to apply for industrial scale because of the immense solvent consumption. Some researcher studied on tradition oil refining process by using deacidification followed bleaching step. The result of experiment showed only 55% of phorbol esters were removed. So in our experiment, the adsorption technique using bentonite was applied to adsorp phorbol esters compounds. The result showed that the optimum adsorption condition on *J. curcas* oil was 3.2%(w/v) of bentonite, 15 min of adsorption time, 100 rpm of stirring rate at room temperature. The phorbol esters can be removed up to 98% for one time of adsorption. This technique is recommended for detoxification *J. curcas* oil in large scale production.

In addition, our study also develop a technique to confirm the presence of phobol esters left in oil after adsorption using liquid chromatography-tandem mass spectrometry with multiple reaction monitoring mode that detects the ionization of parent molecule with mass 711 to precursor and product ion with mass 311 and 293 respectively. This technique is useful technique to confirm phorbol esters left in oil.

Keywords: Phorbol esters, Adsorption, Biodiesel, *Jatropha curcas*

1. INTRODUCTION

Nowadays, the demand and supply gap of vegetable oil has been widening all over the world because of the oil price is increased. Globally, the usage of friendly environmentally fuels is encouraged.

The energy extracted from biomass and tree based materials are perhaps the oldest source of renewable energy. Biomass can be generated from various sources, such as edible and non-edible seed oils, algae and bacteria, forest residues, waste from food and processing, kitchen wastes, etc. The most important biofuels generated from biomass are biodiesel and bioethanol.

Thailand is not rich in petroleum reserves and crude oil, petroleum products must be imported to meet growing energy needs. These fuel and products are usually high prices.

The seeking alternative energy is urgently needed for biodiesel production. Plant species which can be processed to provide a diesel fuel substitute have captured the interest of Thai scientists.

Most of these plant species are such as palm, coconut, soy bean, sunflower, *Jatropha curcas* L. (Saboodum), etc. Ministry of Thai Energy has a policy on renewable energy strategy in the year 2004 that the use of renewable energy in Thailand will increase about 8% of the total energy or 6,540,000 tons within the year 2011 which biodiesel is the one purpose of renewable energy.

Thai government has a policy to support *J. curcas* L. plantation for farmers mainly for renewable energy. *J. curcas* L. is a drought-resistant shrub. It is a member of Euphobiaceae family which is cultivated in Central and South America, South-east Asia, India and Africa. This plant came to Thailand about 200 years ago by Portuguese. Seed oil of *J. curcas* L. is used for soap making and lighting for lamps. The plants grow quickly, survive in poor stony soil and resist to drought. The height of the plant is 2-7 meters and the lifetime is about 50 years. In Thailand the name Saboodam is usually used for *J. curcas* L. The plant can be used in many ways, such as to prevent erosion, reclaim land, grown as a live fence, etc. The seed kernels contain 40-60% oil (Makkar et al., 1997) in which its fatty acid composition is similar to the oil used for human nutrition (Gübitz et al. 1998). A total of 19-27% crude protein can be obtained from press cake (Makkar et al., 1997) which can be a protein source for animal feed. The kernels also contain a number of several toxic and antinutritional compounds. These compounds are trypsin inhibitors, lectins, saponins, phytate and phorbol esters which might cause or at least aggravate the adverse effects in the long term contact, except phorbol esters affect on the short term contact (Makkar et al., 1997).

Phorbol esters are toxic substances that found in plant species of Euphobiaceae and Thymelaceae families. Their structures are based on tetracyclic carbon skeleton known as tigliane. They are known to cause a wide range of biological effects including tumor promotion, cell proliferation, activation of blood plateles and inflammation (Aitken, 1986). These effects are closely related to the structure of several compounds.

Therefore, detoxification of these phorbol esters from the seed oil is required, even when it is industrially used because of the possibility to direct contact of persons with the seed oil. Many experiments eliminate phorbol esters in seed oil by the extraction with ethanol (Gross et al., 1997).

This experiment is difficult to apply for industrial scale because of the immense solvent consumption. Experiment on traditional oil refining process that examines the effects on the phorbol esters content from *J. curcas* oil was performed by Hass (Hass, 2000). It showed that deacidification step and bleaching step could reduce the content of phorbol esters up to 55%.

In addition, phorbol esters are heat stable and can withstand roasting temperature as high as 160°C for 30 min (Makkar and Becker, 1997).

In this experiment, the adsorption process of phorbol esters from *J.* curcas seed oil is examined. In seed oil, bleaching steps in refining of edible oil process can be replaced by the adsorption process.

2. LITERATURE REVIEW

2.1. *Jatropha Curcas* Linn

2.1.1. Botanical Description

Jatropha curcas L., as known as 'physic nut, purging nut, big purging nut, American purging nut, black vomit nut, saboodum, etc.', is a member of the Euphobiaceae family.

It is a tropical plant which can reach a height of 2-7 meters. It is cultivated mainly as a hedge in many Latin America, Asia and African countries. It can be grown in low and high rainfall areas either in the farms as a commercial crop or on the boundaries as a hedge to protect fields from grazing animals and to prevent erosion.

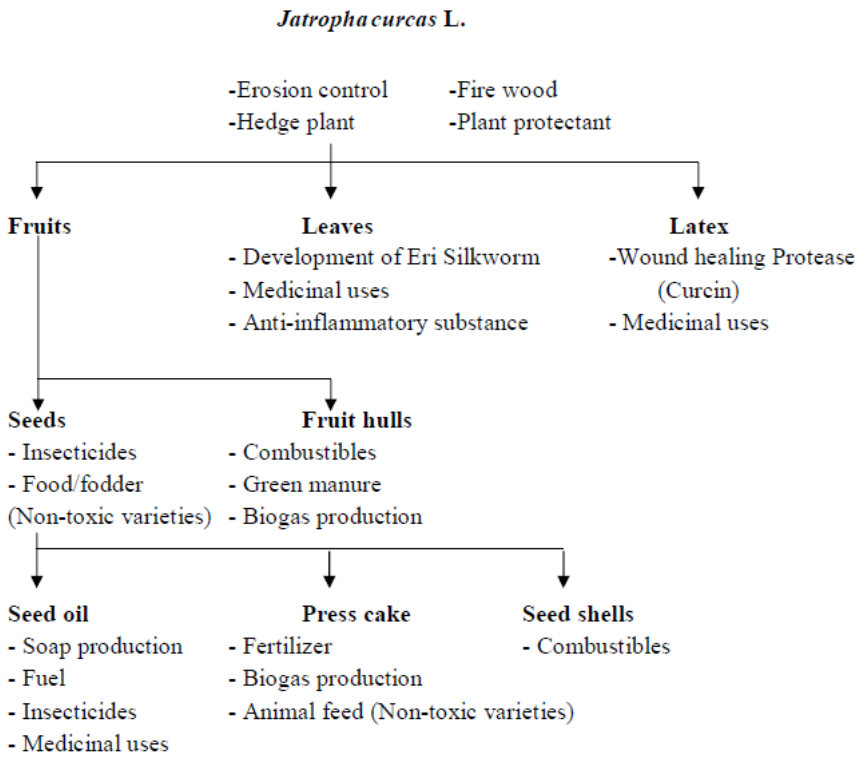
2.1.2. Utilization of Various Parts of Jatropha curcas L.

All parts of *J. curcas* L. have been used in traditional medicine and for various purposes. The oil has been used as a purgative, to treat skin diseases and to soothe pain such as rheumatism. Decoction of the leaves has been used against coughs or as antiseptics after birth, and the branches as chewing sticks (Heller, 1996).

Various extracts from *Jatropha* seeds and leaves show molluscicidal, insecticidal and fungicidal properties (Nwosu and Okafor, 1995; Liu et al., 1997; Solsoloy et al., 1997). The utilization of various parts of *J. curcas* L. is reviewed in Figure 1 (Gübitz et al., 1999).

2.1.3. Chemical and Physical Properties of Jatropha curcas L.

The seed kernels, which seem to be the part of the plant with the highest potential for utilization, contain 40-60% oil (Makkar et al., 1997) with a fatty acid composition similar to oils used for human nutrition (Gübitz et al., 1999).

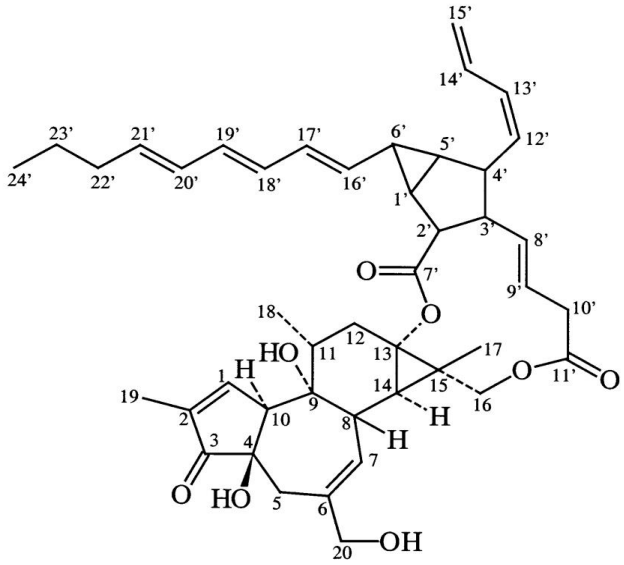


Source: Gübitz et al. (1999).

Figure 1.Exploitation of *Jatropha curcas* L.

2.2. Phorbol Esters

Phorbol esters have been identified as the major toxic principal in *J. curcas* L. (Makkar and Becker, 1997). Phorbol esters were first isolated in 1934 as the hydrolysis product of *Croton tiglium* oil and its structure was determined in 1967. Later, phorbol esters analogues are found in several members of the plant family Euphorbiaceae and *J. curcas* L. is also the plant in family Euphorbiaceae. Phorbol esters in *Jatropha* kernels content at least four different types which can cause the short term toxicity (Makkar et al., 1999). The main chemical structure of phorbol esters in *Jatropha* kernel is 12-Deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl]-6'-[16',18',20'-nonatrienyl]-bicyclo[3.1.0]hexane-(13-*0*)-2'-[carboxylate]-(16-*0*)-3'-[8'-butenoic-10']ate (DHPB) as shown in Figure 2.



Source: Hass and Mittelbach (2000).

Figure 2. Structure of 12-Deoxy-16-hydroxyphorbol-4'-[12', 14'-butadienyl]-6'-[16', 18', 20'-nonatrienyl]- bicyclo [3.1.0]hexane-(13-*0*)-2'-[carboxylate]-(16-*0*)-3'-[8'-butenoic-10']ate; (DHPB).

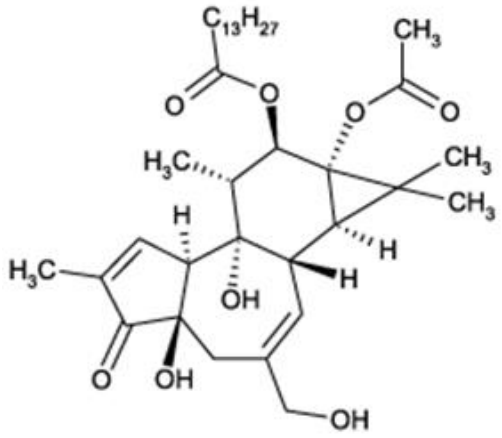


Figure 3.Structure of phorbol-12-myristate 13-acetate or 12-*O*-tradecanoylphorbol13-acetate; (TPA).

Figure 3 shows the chemical structure of phorbol-12-myristate 13-acetate (TPA). It is the phorbol esters standard found in the commercial market and

used as phorbol esters standard in this experiment. Mitsuru et al. (1988) reports the tumors-promoting activity of DHPB. It is weaker than TPA because the application of 2.5 μg of TPA induces tumors nearly 100% in mice within 12 weeks. DHPB results in 46.7% incidence of tumors within 30 weeks. The weaker activity of DHPB might be explained by the structural difference between DHPB and TPA. They contain (a) the alcohol moiety that is 12-deoxy-16-hydroxy phorbol of DHPB and TPA, (b) the acid moieties that is the unsaturated acid of DHPB and saturatured acid of TPA.

2.2.1. Definition of Phorbol Esters

The fundamental substance of phorbol esters is the alcohol moiety, of this family of compounds is tigliane, a tetracyclic diterpene. Hydroxylation of this fundamental substance in various positions and connection to various acid moieties by ester bonding characterize the large number of compounds termed as phorbol esters (Evans, 1986), as shown in Figure 4.

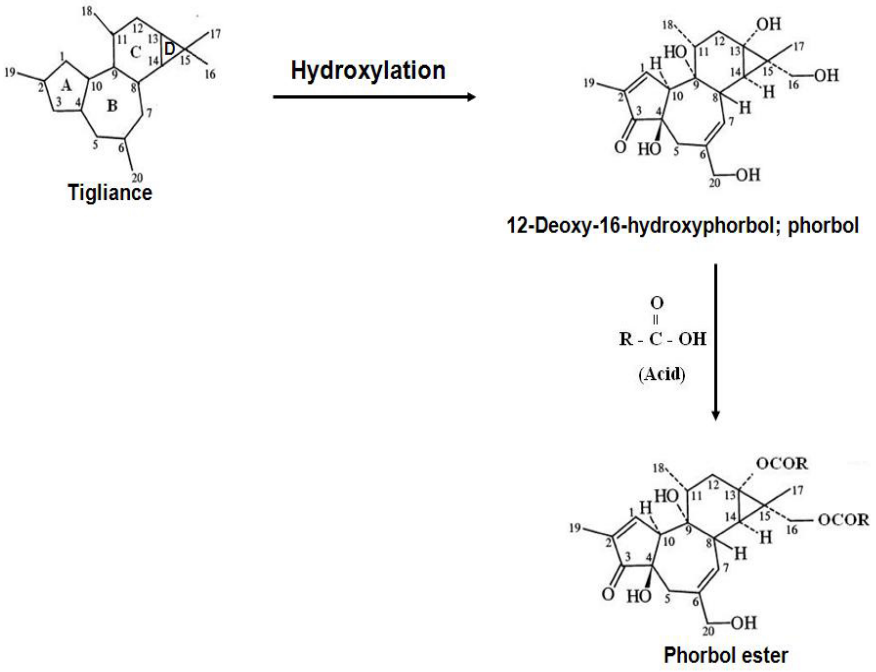
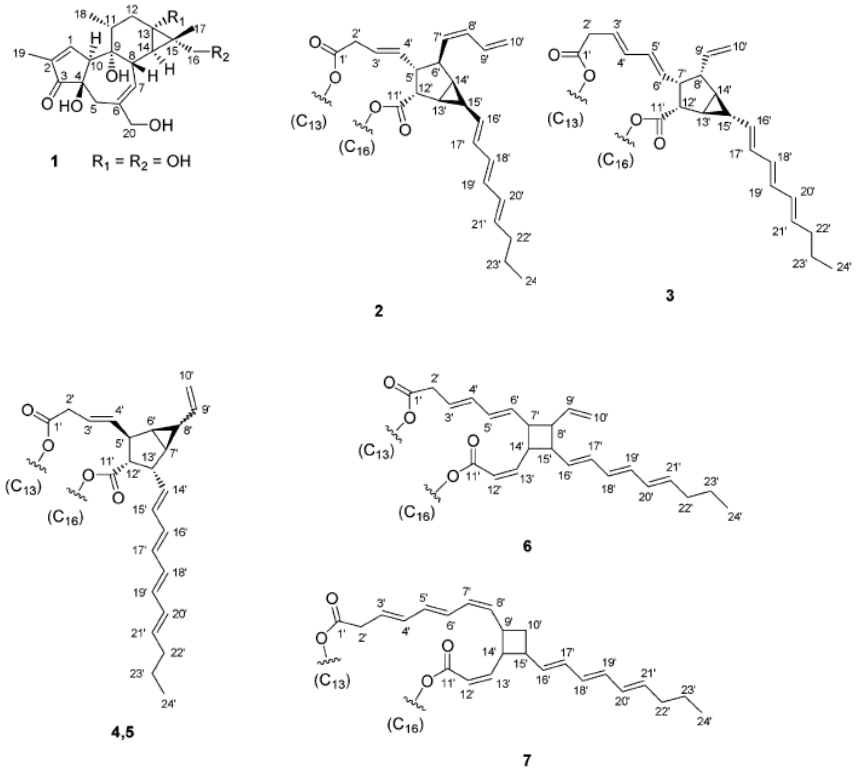


Figure 4.Occurring of phorbol esters.



Source: Hass et al. (2002).

Figure 5. Different chemical structures of phorbol esters named DHPB.

Phorbol esters in *Jatropha* seed oil have six forms that are the isomers of chemical structures (Hass et al., 2002). They have a main structure of 12-deoxy-16-hydroxyphobol (Figure 5(1)) and also contain different side chains R_1 and R_2 to form six different isomers of phorbol esters (Figures 5(2-7)).

Figures 5(4) and 5(5) are actually epimer and could not be separated by chromatography technique. All of these phorbol esters structures are named as DHPB.

2.2.2. Physical and Chemical Properties of Phorbol Esters

2.2.2.1. Description

Phorbol esters are isolated as white crystals or powders. When isolated from volatile organic solvents (ether, methylene dichloride) during

fractionation of oil, they form brittle foams which change to amorphous which are soften at temperature below 100°C. Phorbol-12-myristate 13-acetate (TPA) is like phorbol, strongly retains solvent molecules which it forms addition compounds. The same probably applies to other phorbol esters as well. They are soluble in water and polar organic solvents.

Anhydrous phorbol (crystallized from water) has a melting point of 250-251°C. Phorbol crystallized from ethanol and methanol retains solvent molecules tenaciously and these "alcohol phorbols" have sharp melting points in the region of 230-240°C.

2.2.2.2. Stability

Phorbol esters are very sensitive to acid, alkali, elevated temperatures, light and atmospheric oxygen. Solid TPA appears to be stable when stored in the dark at -20°C. It shows slow decomposition at 4°C within 3 months in the dark and more extensive decomposition at 25°C in diffuse daylight within 3 months. The solution of TPA in dimethyl sulfoxide may be kept at -20°C in the dark for 6 months. Solution of TPA in ethanol may be kept in the dark under nitrogen at -4°C in the dark for 5 months. At -4°C there are only traces of decomposition, while at 25°C (in acetone, ethyl acetate or methylene chloride) autoxidation is extensive. The main products have been identified and consist mainly of oxidation products at the double bonds (Schimdt and Hecker, 1975; Jacobson et al., 1975; Ohuchi and Levine, 1978).

2.2.2.3. Chemical Reactivity

Hecker and Schmidt (1974) review phorbol esters and its esters. Phorbol esters reduce Fehling's and Tollen regents, and form esters and ethers. The C_5 carbonyl group shows weak activity in the reaction with carbonyl agents but is reduced by sodium borohydride. The double bonds are subjected to reduction and to autoxidation. The primary alcohol group at C_{20} is oxidized to the aldehyde with MnO_2 or CrO_3.

2.2.2.4. Biological of Phorbol Esters

The phorbols themselves do not induce tumors but promote tumor growth following exposure to a subcarcinogenic dose of a carcinogen. They are rapidly absorbed through the skin and probably the intestinal tract. They may cause severe irritation of tissues (skin, eyes, mucous membranes and lungs) and induce sensitivity. Laboratory operations should be conducted in a fume

hood and glove. If phorbol esters contact skin, wash with soap and cold water, avoid washing with solvents.

Highly irritant factors to skin are isolated from the seed oil of four *Jatropha* species (Adolf et al., 1984). These irritant factors are determined and that one is new polyunsaturated esters of 12-deoxy-16-hydroxyphorbol. The seed oil of *J. curcas* L. in Thailand is intended to produce in large amounts for the use as a substitute of a biodiesel and an ingredient in commercial printing ink. The irritant factors are tumor promoters, therefore its widely use might result in exposure of a large population to tumor promoters. In 1987 the irritant factors were partially purified from the seed oil of *J. curcas* L. in Thailand (Horiuchi et al., 1987). It shows the tumor-promoting activity in 12-Deoxy-16-hydroxyphorbol-4'-[12' ,14'-butadienyl]-6'-[16',18',20'-nonatrienyl]-bicyclo [3.1.0] hexane-(13-*0*)-2'- [carboxylate] -(16-*0*)-3'-[8'-butenoic-10']ate (DHPB) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) when it is experimented on mouse skin. The results showed that DHPB (unsaturated acid) has slightly weaker biological effect than TPA (saturated acid). TPA is widely used as standard phorbol esters in biochemical experiment.

2.2.2.5. Experimentation on Phorbol Esters

Many researches study and try to detoxify the phorbol esters substances in oil of *J. curcas* L. as follows.

The demand and supply gap of vegetable oil in the world because of the oil price increasing. *J. curcas* L. as an energy crop and *J.* seed oil is produced for biodiesel. In 1996, Foidl et al. developed *J. curcas* L. They study a technical process to produce methyl ester and ethyl ester from seed oil. The results shows that the fuel properties of both esters are followed the standard properties of biodiesel. Shweta et al. (2005) illustrate that the combination of sonication and enzyme treatment with a commercial preparation of pH 9 ledds to 97% oil yield within 2 hours.

J. curcas L. has a large number of potential utilizations. The seed weighs about 0.75 g, contains 30-32% protein and 60-66% lipid (Liberalino et al., 1988) indicating a good nutritional value. However, the seed or oil is found to be toxic to mice (Adam, 1974), rat (Liberalino et al., 1988), calves, sheep and goats (Ahmed and Adam, 1979), human (Mampane et al., 1987) and chickens (Samia et al., 1992). Hence, it is restricted to use as a food or feed source. The biological effects of phorbol esters are found by Aitken et al. in 1986. The biological effects are tumor promotion, cell proliferation, activation of blood platelets, lymphocyte mitogenesis, inflammation, prostaglandin production and stimulation of degranulation in neutrophils.

So, phorbol esters substances are interested and in 1988 Mitsuru et al. find a new type of phorbol esters which has a macrocyclicdicarboxylic acid diester structure. It is isolated from the seed oil of *J. curcas* L. and its structure is proposed as an intramolecular 13, 16 diester of 12-deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl] -6'- [16',18',20'-nonatrienyl] -bicyclo [3.1.0] hexane-(13-*0*) -2'-[carb-oxylate] -(16-*0*)-3'- [8'-butenoic-10'] ate (DHPB). The results show that DHPB is tumor promotion with weaker biochemical activity than 12-*o*-tetradecanoylphorbol-13-acetate (TPA).

In 1995, Gandhi et al. provide data on toxicity of *Jatropha* seed oil which contains phorbol esters. A toxic fraction of the phorbol esters is isolated from the oil and LD_{50} is tested in rats. The acute oral LD_{50} of the oil is 6 mg/kg body weight in rats. Gross et al. (1997) suggest a method for detoxification of oil by extraction phorbol esters using ethanol. This method is in economic effort because of a lot of solvent consumption.

The toxic of phorbol esters substances have different biochemical activities depending on species of *J. curcas* L. In 1997, Makkar et al. evaluated the non-toxic and toxic varieties of *J. curcas* L. They describe that *Jatropha* meal contains high protein, high energy and low fiber. The amino acids composition of meals from the non-toxic and toxic varieties is also similar. The meal contains significant level of trypsin inhibitor, lectin and phytate. Their levels do not differ much between the non-toxic and toxic varieties. The differences between non-toxic and toxic varieties are the amount of phorbol esters content. The amount of phorbol esters in non-toxic from Mexico is 0.11 mg/g of kernel whilst toxic varieties content about 3.45 mg/g of kernel.

The biological effects of phorbol esters are necessary to find routes for detoxification of the oil. In 2000, Hass et al. experiment the edible oil processing steps on phorbol esters detoxification. They find that deacidification step and bleaching step are efficient for phorbol esters removal by 55% whereas degumming step and odor removal step are not effective on phorbol esters removing. In the same year, Rug and Ruppel (2000) also find phorbol esters to be an effective biopesticide against diverse fresh-water snails. Extracts from *J. curcas* L. are found to be toxic against snails transmitting *Schistosomamansoni* and S. *haematobium*. When compared with aqueous extract, methanol extract shows the highest toxicity against all organisms that are tested with values 25 ppm for cercariae and the snail *Biomphalariaglabrata* and 1 ppm for the snails *Bulinustruncates* and B. *natalensis*. Attenuation of cercariae leading to reduced infectivity in mice could be achieved in concentration below those exporting acute toxicity.

Jatropha oil or methanol extract of *Jatropha* oil containing phorbol esters has also been shown to have strong insecticidal effects against *Busseolafusca* and *Sesamiacalamistis* larvae (Mengual, 1997) and pesticidal effects against *Sitophiluszeamays* and *Callosobruchuschinesis* and deterred their oviposition on sprayed corn and mungbeans seeds (Solsoloy and Solsoloy, 1997).

2.3. Adsorption

Deacidification and bleaching steps of the traditional refinery oil process can reduce phorbol esters content in seed oil of *J. curcas* L. up to 55% (Wilhelm et al., 2000). This research is interested to select the method of phorbol esters elimination in seed oil of *J. curcas* L. The bleaching agent can adsorb color of oil and may also adsorb phorbol esters.

Therefore, the adsorption process is selected a method to eliminate phorbol esters from seed oil.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. *Jatropha curcas* Seed Oil from KU Biodiesel Project, Kasetsart University

3.1.2. *Jatropha curcas* Press Cake from KU Biodiesel Project, Kasetsart University

3.1.3. Reagents

- Methanol (Analytical grade, Merck, Germany)
- Acetronitrile (HPLC grade, Merck, Germany)
- Hexane (Analytical grade, Merck, Germany)
- Sodium chloride (Analytical grade, APS, Australia)
- Sodium hydroxide (Analytical grade, J.T. Baker, US)
- Potassium hydroxide (Analytical grade, J.T. Baker, US)
- Heptane (Analytical grade, Merck, Germany)
- Boron trifluoride in methanol (BF_3,14%v/v, Supelco Analytical, US)

3.1.4. Chemical Standards

- 4β, 9α, 12β, 13α, 20-pentahydroxytiglia-1, 6-dien-3-on-12β-myristate-13- acetate (tetradeca-noylphorbolacetate, TPA) (Sigma, US)
- Mehylheptadecanoate
- Fatty acid methyl esters mixture (C_8-C_{24}) (Supelco Analytical, US)

3.1.5. Adsorbent Agents

- Activated carbon from Patum Vegetable Oil Co., Ltd., Thailand
- Bentonite 150 mesh from Patum Vegetable Oil Co., Ltd., Thailand
- Bentonite 200 mesh from Patum Vegetable Oil Co., Ltd., Thailand
- Chitosan (Seafresh chitosan (lab), Thailand)
- Chitin (Seafresh chitosan (lab), Thailand)

3.2. Equipments

3.2.1. Balance 4 digit (Percisa, 120A, US)
3.2.2. Soxhlet Extraction Instrument (BÜchi, B811, Switzerland)
3.2.3 Gas Chromatography Instrument (Agilent Technique, 6890N, US)
3.2.4. High Performance Liquid Chromatography with UV detector (Shimadzu, LC-10AC, Japan)
3.2.5. High Performance Liquid Chromatography with diode array and mass spectrometry detector (Agilent Technique, US)
3.2.6. Surface area analysis (Quanta Chrome, Atosorpb-1)
3.2.7. Platfrom Shaker (Inonva 2100, Japan)
3.2.8. Centrifugation (Mermle, Z323, Germany)
3.2.9. Autoclaving (Dectra, US)
3.2.10. Rota evaporator (BÜCHI, R114, Switzerland)
3.2.11. Overhead Stirrer (Ingenieurbüro, CAT R17, Germany)
3.2.12. Hot air oven (Binder, German)
3.2.13. Fourier transform infrared spectrophotometer (Perkin Elmer System 2000, US)
3.2.14. Kjeldakl-digestion and distillation system (C. Gerhardt GmbH and Co. KG, VAP30, Germany)
3.2.15. Water bath (Memmert, WB14, Germany)

3.3. Methods

3.3.1. Elimination of Phorbol Esters from Seed Oil by Adsorption Process

3.3.1.1. Selection of the Most Suitable Adsorbent

About 25 ml of seed oil were mixed with 0.8 g of each adsorbent (activated carbon, bentonite150, bentonite200, chitin and chitosan) into a 250 ml Erlenmeyer flask. Adsorption was experimented at room temperature for 45 min of stirring time and 200 rpm of stirring rate. After that adsorbent and seed oil were separated by filtration with filter paper No.1. Extracted phorbol esters from 10 g of the seed oil with methanol. Content of phorbol esters was analyzed by HPLC. The best adsorbent was selected from maximum adsorbed phorbol esters from seed oil.

3.3.1.2. Optimization of the One-Time Adsorption

About 25 ml of seed oil were mixed with the most suitable adsorbent from experiment 3.3.1.1 in a 250 ml Erlenmeyer flask. The experiments were continued in order to find the optimum conditions of adsorption in terms of the following factors:

a. Amount of adsorbent: 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 2.0 g.
b. Stirring time: 15, 30, 45, 60, 120 and 180 min.
c. Temperature: 32, 45, 65, 85 and 120°C.
d. Stirring rate: 0, 100, 150, 200, 250 and 300 rpm.

After each experiment, the adsorbent and seed oil were separated by filtration with filter paper no.1. Phorbol esters substance was extracted from the seed oil and the amount of phorbol esters was analyzed by HPLC.

3.3.1.3. Optimization of Two-Time Adsorption

About 25 ml of seed oil from the one-time adsorption were mixed with the most suitable adsorbent from experiment 1.1 in a 250 ml Erlenmeyer flask. The experiments were continued in order to find the optimum conditions of adsorption in terms of the following factors:

a Amount of adsorbent: 0.2, 0.4, 0.6, 0.8 and 1.0 g.
b Stirring time: 0, 15, 30 and 45 min.

After each experiment, the adsorbent and seed oil were separated by filtration with filter paper No.1. Phorbol esters substance was extracted from the seed oil and amount of phorbol esters was analyzed by HPLC.

3.4. Analytical Methods

3.4.1. Phorbol Esters Extraction

3.4.1.1. Phorbol Esters Extraction in *Jatropha* Seed Oil

Phorbol esters in seed oil were extracted from 10 g of seed oil with 10 ml of methanol for 4 times using funnel separation. The combined extracts were centrifuged to separate the extracts from the oil residue at 3000 rpm for 15 min. Then, the extracts were concentrated with rotaevaporator at 45°C and 200 mmHg. After that, the concentrated extracts were transferred into a 25 ml volumetric flask and filled up to 25 ml with methanol. The extracts were stored at - 20°C for HPLC analysis.

3.4.2. Analysis of Phorbol Esters Content

3.4.2.1. Preparation of Sample

About 1.5 ml of the extracts were filtered through 0.45 µl membrane prior to the measurement of the phorbol esters by HPLC.

Figure 6. Phorbol esters extraction from *Jatropha* seed oil with funnel separation.

The operation condition was 1 ml/min flow rate, 35°C thermal control column, 280 nm UV detector and 20 µl samples were injected. The mobile phase was acetronitrile and deionized water (80:20, v/v) with isocratic mode.

3.4.2.2. Calibration Curve of Phorbol Esters Standard

The standard tetradecanoylphorbolacetate (TPA) was dissolved in methanol. TPA standard concentrations were prepared at 10, 20, 30, 40 and 50 ppm, respectively. After that, phorbol esters content in the form of TPA was measured as previously described in 3.2.1. Area peak and phorbol esters concentration were plot on y-axis and x-axis, respectively. The calibration curve was a straight line that passed through the origin point. The external standard technique was used to quantify phorbol esters content according to the standard curve.

3.4.3. Conformation of Phorbol Esters by LC-MS/MS Using Multiple Reaction Monitoring (MRM) Mode

Chromatographic separation of phorbol of phorbol esters was preformed on C18 water Atlantis (5µm 2.7 × 50 mm). Isocratic program was used with mobile phase, consisted of solvent (50 mmol ammonium acetate + acetonitrile, 9 + 1 v/v). The flow rate was 0.2 ml/min, the injection volum was 40 µl MS/ MS condition: MS/MS was performed on a Micromass Quattro Ultima triple-quadrapole spectrometer equipped with ESI source. The parameters used for the mass spectrometry under ESI^+ mode were as follows: capillary voltage 3.00 KV, cone voltage 50 V, source block temperature 120 °C, cone gas 52 l/ h, desolvation temperature 350 °C, desolvation gas 593 l/h.

4. RESULTS AND DISCUSSION

4.1. Raw Material

The seed oil was pressed from *Jatropha* seed by screw press and then the seed oil and the press cake were separated. After that, the seed oil was filtered through filter paper No.1. Phorbol esters content in seed oil analyzed by HPLC was approximately 3-6 mg/g. However, phorbol esters content of seed oil depended on the region culture of *J. curcas* L. For example, phorbol esters content of *Jatropha* varieties from Mexico was about 0.11 mg/g while phorbol esters content of *Jatropha* varieties from Thailand contained about 3-6 mg/g.

The adsorbents used in this study were activated carbon, bentonite 150, bentonite 200, chitin and chitosan as presented in Figure 7. Activated carbon was a general term covering carbon material mostly derived from charcoal. Bentonite was special clay and usually formed from weathering of volcanic ash. Bentonite 150 and bentonite 200 are the same material with different particle sizes. The number '150' and '200' behind the word 'bentonite' present the mesh bentonite particle size in mesh. Chitin and chitosan are co-polymers of carbohydrates and included the derivative of Nitrogen-Glucose combination cation molecules. Chitin is a natural organic compound which is insoluble in water and general organic solvents but dissolved in concentrate organic acids. Chitosan can dissolve in various organic acids and form gel, granule and fiber and is used in surface coating. We can find the hard-shelled of shellfish which have many profits for plants, animals and humanity like these.

The Physical Properties of Adsorbents

The physical properties of 5 adsorbents in this experiment were presented in Table 1. Among all adsorbents, the activated carbon has the highest surface area and is strong alkaline which the activated carbon has 923.80 m^2/g and pH equal of 9.84, respectively. Particle sizes of bentonite 150 and 200 were 150 and 200 mesh, respectively. Bentonite 150 and 200 are strong acidic condition which pH of 3.05 and 2.50, respectively.

Figure 7. The adsorbents of Activated carbon (a), Bentonite 150 (b), Bentonite 200 (c), Chitin (d) and Chitosan (e).

Table 1. The physical properties of 5 adsorbents

Types of adsorbent	Particle size (mesh)	Surface area (m^2/g)	Pore volume (cc/g)	Pore size (°A)	pH
Activated carbon	150	923.80	0.4818	60.1060	9.84
Bentonite 150	150	190.40	0.0885	101.1500	3.05
Bentonite 200	200	327.30	0.1488	101.4000	2.50
Chitin	40	1.13	$5.856E^{-4}$	83.8200	5.60
Chitosan	60	-	-	-	7.90

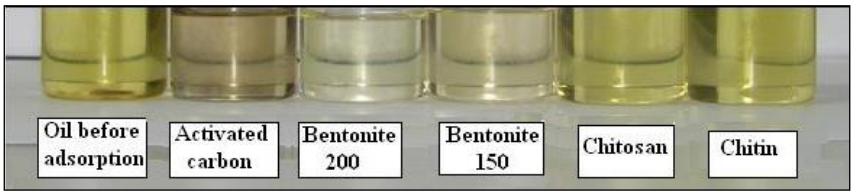


Figure 8.Comparison of *Jatropha* seed oil before and after adsorption with adsorbents.

Chitin and chitosan have large particle sizes with 40 and 60 mesh, respectively, indicating that they contain low surface area and are neutral. However, the surface area of chitosan could not be detected because the temperature of surface area test was 300°C where the chitosan cannot stand for. Figure 8 showed the *Jatropha* seed oil after the adsorption experiment. It demonstrated that all adsorbents improved the clarity of *Jatropha* seed oil. However, bentonite 150 and 200 showed the best adsorption capability as indicated by the clearest of *Jatropha* seed oil after adsorption, followed by activated carbon, chitin and chitosan. The highest adsorption capability of bentonite could be because bentonite is usually applied as a bleaching agent in a traditional edible oil refining.

4.2. Phorbol Esters Chromatogram

Analysis of phorbol esters by an isocratic mixture of 80% acetronitrile and 20% deionized water showed the retention time of phorbol esters about 8-12 min as referred with the method of Wink et al. (1997). Within 8-12 min, the phorbol esters chromatogram contained 5 peaks and therefore the total area of the 5 phorbol esters peaks were used for quantification (Figure 9).

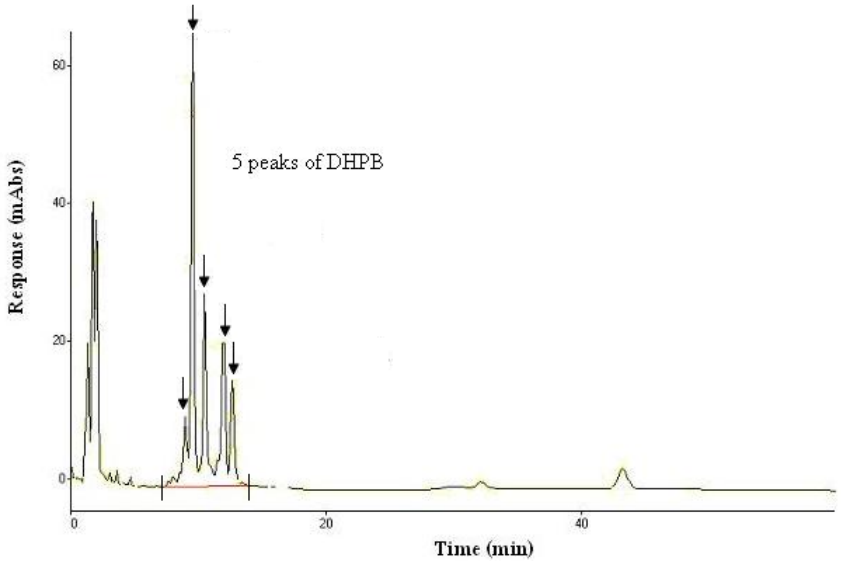


Figure 9. Chromatogram of phorbol esters (DHPB) in *Jatropha* seed oil.

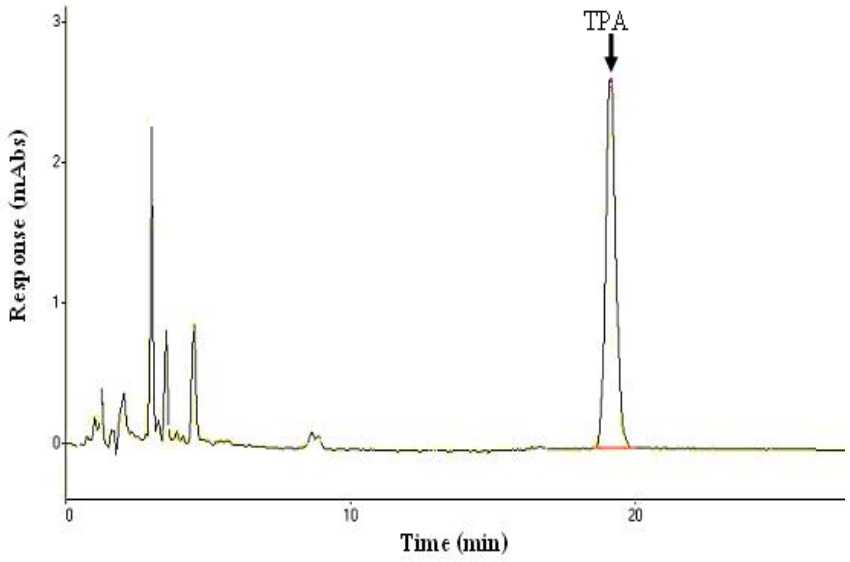


Figure 10. Chromatogram of phorbol-12-myristate-13-acetate (TPA) standard.

Although the phorbol esters found in *Jatropha* seed was DHPB (12-Deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl]-6'-[16',18',20'-nonatrienyl]-bicyclo[3.1.0] hexane-(13-*0*)-2'-[carboxylate]-(16-*0*)-3'-[8'-butenoic-10']ate, Hass and Mittelbach, 2000) but it is not commercially available. TPA was used as external standard for quantification of phorbol esters according to Wink et al. (1997). However, this could lead to far higher values than when using DHPB in this experiment, only the relative decrease of phorbol esters was interesting thus this difference was neglected (Gläser, 1991).

The chromatograms of standard phorbol esters (phorbol-12-myristate 13-acetate, TPA), phorbol esters obtained from *J. curcas* oil and *Jatropha* wood were presented in Figures 10.

4.3. Elimination of Phorbol Esters from *Jatropha* Seed Oil

4.3.1. Selection of the Most Suitable Adsorbent

The phorbol esters adsorption by different adsorbents was presented in Figure 11.

All experiments were performed under the same condition which was 3.2% adsorbents (w/w) at room temperature with 200 rpm stirring rate. When increased the stirring time of adsorption, the phorbol esters adsorption was also increased. The results illustrated that the highest % phorbol esters obtained from activated carbon, bentonite 150, bentonite 200, chitosan and chitin were 18.01% (15 min stirring time), 96.09% (45 min stirring time), 98.38% (45 min stirring time), 8.12% (300 min stirring time) and 12.28% (300 min stirring time), respectively.

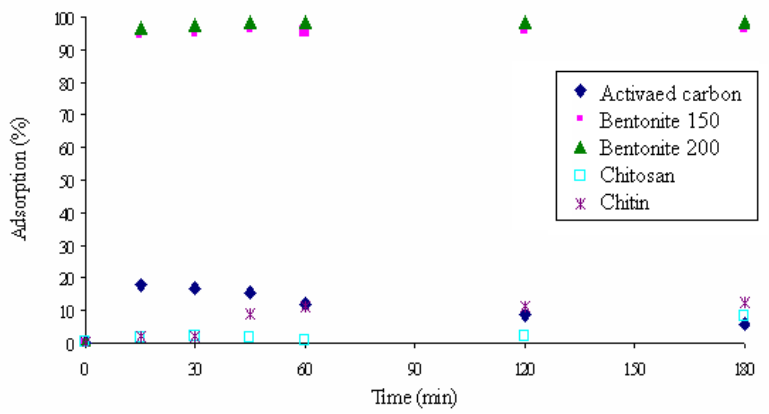


Figure 11. Phorbol esters adsorption capability in *Jatropha* seed oil by 5 adsorbents.

When considered the physical properties of adsorbents, pH and pore size, the results indicated that pH of adsorbent affected the adsorption capability more than their surface areas and pore sizes. It demonstrated that bentonite 150 and 200 had the adsorption capability more than those of activated carbon, chitin and chitosan. This might be due to stronger acidity of bentonite 150 and 200 (pH 3.05 and 2.50, respectively) compared with the basicity of activated carbon and chitosan.

Therefore, it resulted in the hydrolysis reaction simultaneously with the adsorption of phorbol esters. The comparison between bentonite 150 and 200 showed that bentonite 200 had smaller size, stronger acidity and more contact surfaces area and therefore showed higher efficiency for phorbol esters adsorption. As shown that the adsorption capability was higher under the acidic condition. When bentonite 150 and 200 were applied, they contained the same pore size (101°A).

Therefore, these results indicated that pH affected of adsorbent the capability more than the surface area and pore size of adsorbents. The phorbol esters adsorption of bentonite 200 reached equilibrium after 15 min and also reached the highest adsorption capability at 96.72%. The removal of phorbol esters from our study was far better than that obtained from Hass et al. (2000) which phorbol esters were removed only by 55%.

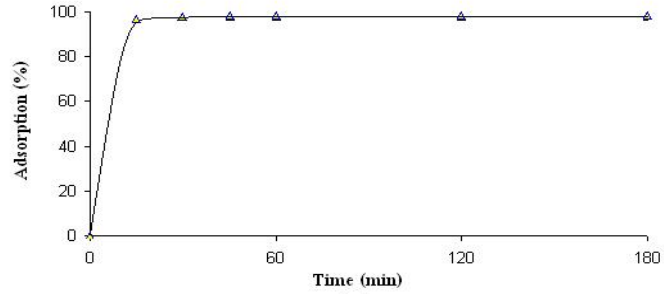
As a result, bentonite 200 was the most suitable adsorbent for phorbol esters adsorption from the *Jatropha* seed oil and applied for further experiment.

4.3.2. Optimization of One-Time Adsorption

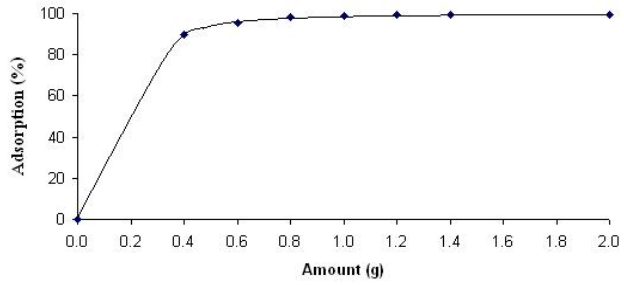
The optimum phorbol esters adsorption condition by bentonite 200 (the most suitable adsorbent from previous section) was summarized as follows. The stirring time, amount of bentonite 200, temperature and stirring rate of adsorption were optimized. The results were showed in Figure 12.

Figure 12(a) showed the effect of stirring time on adsorption. The optimum stirring time was 15 min where the phorbol esters adsorption was 96.72%. When increasing the stirring time, % adsorption was also increased until at 45 min where the adsorption became flatten out. In Figure 12 (b), the results indicated an increase in adsorption capability with increasing the bentonite amount with the maximum adsorption at 99.63% when 2.0 g of bentonite 200 were applied. However, the optimum adsorption with 0.8 g bentonite 200 was selected because at the amount of bentonite 200 more than 1.0 g, the adsorption became flattened out.

The effect of temperature on adsorption was shown in Figure 12(c).



a



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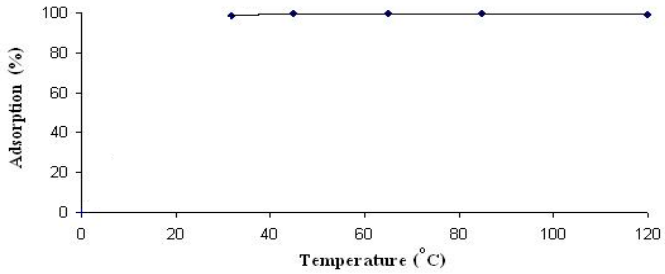
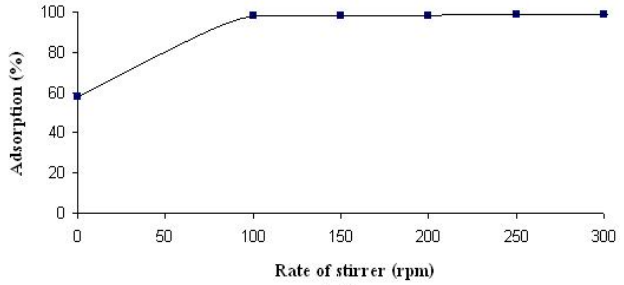


c



d

Figure 12. The effect of stirring time (a), amount of bentonite 200 (b), temperature (c) and stirring rate (d) on one-time adsorption of phorbol esters in *Jatropha* seed oil.

The adsorption capability of all tested temperatures was about 99 % with remaining phorbol esters content as small as 0.11 mg/g (equivalent to that in the non-toxic of *Jatropha curcas* L.). The effect of stirring rate on adsorption capability of bentonite 200 was shown in Figure 12(d). As the stirring rate increased, the adsorption capability also increased. However, the stirring rate faster than 100 rpm gave constant adsorption about 98%, therefore, 100 rpm was selected as the most optimum stirring rate for adsorption by bentonite 200.

The result also showed that the equilibrium condition of adsorption was obtained under the condition: 3.2% (w/v) bentonite 200 at room temperature, 100 rpm of stirring rate and stirring time for 15 min, illustrating high phorbol esters adsorption efficiency at 96.72%, 98.45%, 98.25% and 98.38%, respectively. Temperature and stirring rate of adsorption almost did not affect the phorbol esters adsorption with bentonite 200, whereas stirring time and amount of bentonite 200 highly affected the adsorption.

4.3.3. Optimization of the Two-Time Adsorption

Even though one-time adsorption of *Jatropha* seed oil with bentonite 200 showed high efficiency of phorbol esters removal up to 98.44% or 0.0928 mg/g phorbol esters remained in *Jatropha* seed oil, it would be of more advantageous if there was no remaining phorbol esters at all. As a result, the two-time adsorption was experimented when the seed oil after the one-time adsorption was selected to the adsorption again with new bentonite 200 under the new adsorption condition. Stirring rate and temperature of the two-time adsorption were fixed at 100 rpm and 32°C, respectively, according to the previous results. The results were demonstrated in Tables 2 and 3.

Tables 2 and 3 showed the effect of bentonite 200 amount and stirring time on the second time adsorption, respectively. Phorbol esters content in *Jatropha* seed oil was 5.9670 mg/g. After one-time of adsorption, the remaining phorbol esters in *Jatropha* seed oil was 0.0928 mg/g.

The results remonstrated that an increase in bentonite 200 amount increased a little of adsorption capability with the remaining phorbol esters content in *Jatropha* seed oil about 0.0213 mg/g or 99.64% of adsorption. Increasing of stirring rate also increased a little of adsorption capability with the remaining phorbol esters content in *Jatropha* seed oil about 0.0216 mg/g or 99.64% of adsorption. Thus, it was concluded that the maximum adsorption efficiency of phorbol esters from the second time was about 99.60% with remaining phorbol esters in the seed oil of approximately 0.02 mg/g.

Figure 13 summarized the adsorption efficiency of bentonite 200 on phorbol esters for one and two times.

Table 2.The effect of bentonite 200 amount on the two-time adsorption

Weight of BT200 (g)	PEs content (mg/g)		Adsorption for one time (%)	Weight of BT200 (g)	PEs content (mg/g)	Adsorption for two-time (%)
	Before adsorption	After one-time adsorption				
0.8	5.9670	0.0928	98.44	0.0	0.0928	98.45
				0.2	0.0213	99.64
				0.4	0.0186	99.689
				0.6	0.0204	99.66
				0.8	0.0215	99.64
				1.0	0.0212	99.64

Table 3. The effect of stirring time on the two-time adsorption

Weight of BT200 (g)	PEs content (mg/g)		Adsorption for one time (%)	Weight of BT200 (g)	PEs content (mg/g)	Adsorption for two-time (%)
	Before adsorption	After one-time adsorption				
0.8	5.9670	0.0928	98.44	0	0.0928	98.44
				15	0.0216	99.64
				30	0.0213	99.64
				45	0.0174	99.71

PEs = Phorbol esters.
BT200 = Bentonite200.

The equilibrium condition was 0.2 g bentonite 200 (0.8%, w/v), 15 min stirring time at 32°C (room temperature) and 100 rpm stirring rate. The adsorption of phorbol esters was up to 99%.

Figure 14, the comparison between one and two-time adsorption showed the phorbol esters content remained in *Jatropha* seed oil and percentage of adsorption. The two-time adsorption could increase adsorption about 1.20% from the one-time adsorption and the remaining phorbol esters were about 0.0213 mg/g. When increased amount of bentonite 200 more than 0.8% (w/v) and increased stirring time longer than 15 min, it showed almost no effect on the adsorption capability. As a result, bentonite 200 had limited to adsorb phorbol esters in *Jatropha* seed oil in the two-time adsorption.

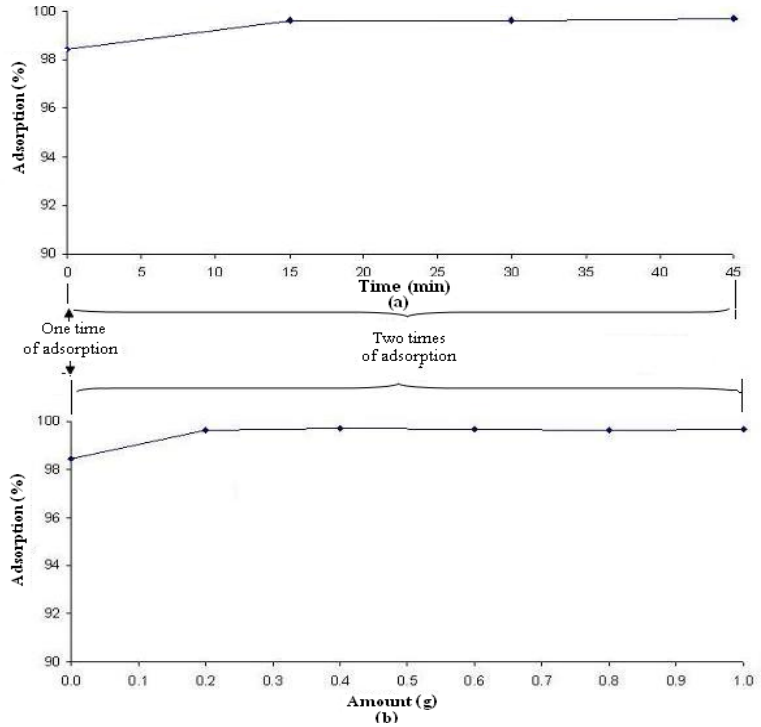
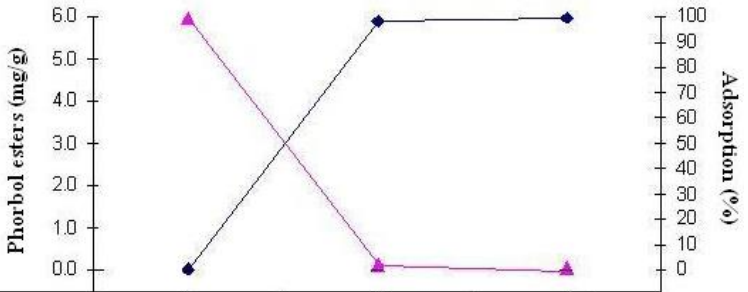


Figure 13. The effect of stirring rate (a) and bentonite 200 amount (b) on the two-time adsorption capability of phorbol esters.



Time of adsorption	0	1st	2nd
Phorbol esters (mg/g)	5.9670	0.0928	0.0213
Adsorption (%)	0.0000	98.4448	99.6430

Figure 14. One-time and two-time of adsorption capability of phorbol esters in seed oil.

4.4. Confirmation of Phorbol Ester by LC-MS/MS

The confirmation of phorbol ester by LC-MS/MS with MRM mode of *Jatropha curcas* oil before adsorption showed two ionization peaks. The first one ionization peak represented parent molecule with mass 711 ionized to precursor ion with mass 311 and the second ionization peak ionized from precursor ion to produce an ion with mass 293.

This result could be explained assuming that phorbol ester fragmented by eliminating its ester groups (C_{13} and C_{16} of figure 2) and alcohol group (C_{20} of figure 2) to diterpene ester of tigliane type (molecular formula = $C_{20}H_{23}O_{23}$) resulting in precursor molecule with mass 311.

The skeleton was further fragmented by losing H_2O (molecular mass = 18) to produce ion with mass 293. Hence this characteristic pattern could be used to establish specific detection of phorbol ester residuce in *Jartropha curcas* oil after adsorption.

In the case of oil after adsorption. The result from HPLC chromatogram revealed a small peak occurring between 8-12 min (the amount of phorbol ester about 0.02 mg/g) but after confirmation it did not show the two ionization peaks, indicating that phorbol esters were not left in the oil after adsorption. In addition, the concentration of residue phorbol esters in oil after adsorption is lower than 0.11 mg/g phorbol esters that reported by Makkar and Becker (1997) in the nontoxic Mexican varieties, too.

CONCLUSION

Bentonite 200 was the most suitable adsorbent for phorbol esters adsorption from seed oil when compared among the activated carbon, bentonite 150, chitin and chitosan. The optimum condition was 15 min adsorption time, 3.2% (w/v) bentonite 200, 32°C temperature and 100 rpm stirring rate with maximum removal up to 98.00% or 0.09 mg/g phorbol esters remained in seed oil.

The 2^{nd} adsorption showed the optimum condition at 0.8% (w/v) bentonite 200, 15 min stirring time at 32°C temperature and 100 rpm stirring rate with maximum removal up to 99.50% or 0.02 mg/g phorbol esters remained in seed oil. Liquid chromatogram-tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM) mode is useful technique to confirm phorbol esters left after adsorption by bentonite 200.

The results of our study show no two ionization peaks appear that indicate the adsorption technique by bentonite 200 is useful technique for phorbol esters elimination from *J. curcas* oil.

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