

SEPARATING INNER SKIN FROM *JATROPHA CURCAS* SEED KERNEL TO REDUCE THE PHORBOL ESTERS CONTENT IN SEED CAKE

FIELD OF THE INVENTION

The present invention relates to processes for reducing the amount of phorbol esters in the oil and cake produced from *Jatropha curcas* seed kernels. The invention also relates to uses of the products of the process for oil, animal feed and anti-microbial, anti-mollusc, anti-tumor or bio-pesticide agents.

BACKGROUND

The present invention is to be understood in light of what has previously been done in the field. However, the following discussion is not an acknowledgement or an admission that any of the material referred to was published, used or part of the common knowledge of the skilled person as at the priority date of the application.

Jatropha (*Jatropha curcas* L.) is a tropical shrub yielding high quality non-edible oil suitable as feedstock for biodiesel and bio jet fuel. In addition to the oil, an almost equal amount of high protein-containing seed cake (>50% crude protein) can be produced from its kernels which has potential as animal feed. However, phorbol esters (PEs) and some anti-nutrients in seeds make it unsuitable for animal nutrition despite a good amino acid profile. Phorbol Esters also raise safety and environmental concerns on the *Jatropha* plantation and in the processing of seed and other products. Phorbol esters are present at high level in seed kernel, but much higher concentrations (6-10 times concentration as in total kernel, as high as 14,449 ppm or 1.4% dry weight) are found in the maternally derived inner skin of the seed, referred to as the tegmen, which envelopes the endosperm (He, W., A.J. King, et al., *Plant Physiol. Biochem.* 49 (10): 1183-1190 (2011)). There is almost no phorbol ester in the seed coat (testa), which is removed from the seed through a decortication process to yield a kernel. Processing of kernels yields phorbol ester-containing oil and cake. The cake produced also has fibers from the inner skin. Removing the high phorbol ester- and fiber-containing inner skin from kernels before processing endosperm would be expected to decrease the amount of phorbol ester in both the oil and cake. In addition, removing the inner skin should also decrease the fiber and increase the protein content of the cake. However, it is difficult to peel the inner skin (tegmen) from the kernels of *Jatropha* because it is tightly bound. Manual or mechanical force peeling is not feasible, especially in the context of handling and processing on an industrial scale tens of tonnes of seeds per day. In addition, attempts at soaking the seeds in water overnight and then trying to peel off the

inner skin have proven to be inefficient because less than 5% of the kernel skins were removed (data not shown).

In view of the above problems with the presence of phorbol esters in *Jatropha* seeds, it is desirable to provide *Jatropha* seed oil and cake with reduced phorbol ester content and an improved process therefor.

SUMMARY OF THE INVENTION

Described herein is a method of using chemicals to remove the inner skin of *Jatropha* seeds. The phorbol ester-containing tegmen can be used to produce bio-pesticides and in various medical applications. The method allows peeling of the inner skins of *Jatropha* seeds, by chemically treating the *Jatropha* kernels. Suitable chemicals may be selected from the group comprising hydrogen peroxide, sodium bicarbonate and dilute alkaline solutions, including sodium hydroxide and potassium hydroxide. The phorbol ester content of the kernel may be reduced by up to about 25% w/w, and the resulting endosperm can be processed to further reduce the phorbol ester content in the cake to make it more suitable as animal feed.

In one aspect of the invention there is provided a process to reduce the phorbol ester content of *Jatropha curcas* seed kernel, which kernel comprises an endosperm and a tegmen, by removing the tegmen from the endosperm. Preferably, the process is an industrial scale process.

In a preferred embodiment of the invention the process comprises the steps of:

- a) contacting at least one *Jatropha curcas* seed kernel with a chemical solution;
- b) separating the tegmen from the endosperm; and
- c) collecting the separated endosperm and optionally the tegmen.

Preferably the chemical solution comprises one or more chemicals that act to loosen the tegmen around the endosperm.

In another preferred embodiment, the one or more chemicals are selected from the group comprising, alkalis, including alkali salts such as sodium hydroxide, magnesium hydroxide, potassium hydroxide and calcium hydroxide; acids, salts, such as sodium chloride, sodium carbonate and sodium bicarbonate; and oxidizing agents, such as hydrogen peroxide.

In another preferred embodiment, the one or more chemicals are selected from the group comprising potassium hydroxide, sodium hydroxide, sodium bicarbonate and hydrogen peroxide.

5 In another preferred embodiment of the process, in a) the contacting includes mixing of the seed kernel with the chemical solution, preferably by rotation or shaking.

In another preferred embodiment the process further comprises a step of rinsing the kernels from step a) prior to step b).

In another preferred embodiment of the process, the contacting in step a) is for a period of at least 1, 5, 10, 15, 20, 25 or 30 minutes.

10 In another preferred embodiment of the process, step a) is performed at room temperature.

In another preferred embodiment of the process, the chemical solution comprises sodium bicarbonate and/or hydrogen peroxide.

15 In another preferred embodiment of the process, the chemical solution comprises at least 0.005% sodium bicarbonate and/or at least 0.025% hydrogen peroxide.

In another preferred embodiment there is provided an industrial scale process comprising the steps of:

a) contacting at least one *Jatropha curcas* seed kernel with a chemical solution by continuous mixing for about 10-15 minutes;

20 b) separating the tegmen from the endosperm by washing with water over a sieve; and

c) collecting the separated endosperm and optionally the tegmen.

In another preferred embodiment of the process, at least 10, 20, 30, 40, 50, 60, 70, 80 or 90% of the tegmen is removed.

25 In another preferred embodiment of the process, step a) and/or b) is performed in a rotary drum.

In another preferred embodiment of the process, the phorbol ester content of the separated endosperm in step c) is at least 5, 10, 15, 20, 25 or 30% w/w reduced compared to the kernel in step a).

In another preferred embodiment of the process, the separated endosperm from step c) is further processed to extract oil and produce seed cake.

In another preferred embodiment of the process, the separated tegmen from step c) is further processed into phorbol ester-containing products selected from the group
5 comprising anti-microbial, anti-viral, anti-mollusc, anti-tumor and bio-pesticide agents.

Another aspect of the invention provides at least one *Jatropha curcas* seed kernel with reduced phorbol ester content, wherein the tegmen has been removed and the phorbol ester reduction is at least 5, 10, 15, 20, 25 or 30% w/w.

In a preferred embodiment, the at least one *Jatropha curcas* seed kernel with
10 reduced phorbol ester content is produced by a process according to any aspect of the invention.

Another aspect of the invention provides a *Jatropha curcas* seed cake with a phorbol ester content reduced by at least 5, at least 10, at least 15, at least 20, at least 25 or by at least 30% w/w, wherein the cake has been produced using at least one seed kernel without
15 its tegmen.

In a preferred embodiment, the *Jatropha curcas* seed cake is produced by a process of the invention in which the phorbol ester content of the separated endosperm in step c) is at least 5, 10, 15, 20, 25 or 30% reduced compared to the kernel in step a).

Another aspect of the invention provides at least one isolated *Jatropha curcas* seed
20 tegmen, obtained by the process according to any aspect of the invention.

In a preferred embodiment, the tegmen has further been dried and powdered.

Another aspect of the invention provides at least one isolated *Jatropha curcas* seed tegmen obtained according to any aspect of the invention for use as an anti-microbial, anti-viral, anti-mollusc, anti-tumor or bio-pesticide agent.

25 Another aspect of the invention provides the use of the at least one *Jatropha curcas* seed kernel with reduced phorbol ester content for the production of oil and seed cake.

A preferred embodiment provides the use of *Jatropha curcas* seed cake with reduced phorbol ester content for the production of animal feed.

30 Another preferred embodiment provides the use of *Jatropha curcas* seed tegmen with highly concentrated phorbol ester content for the production of an anti-microbial, anti-viral, anti-mollusc, anti-tumor or bio-pesticide agent.

Another aspect of the invention provides *Jatropha curcas* oil, isolated from at least one *Jatropha curcas* seed kernel with tegmen removed and having a phorbol ester content reduced by at least 5, 10, 15, 20, 25 or 30%.

BRIEF DESCRIPTION OF THE FIGURES

5 Figure 1A-E shows the gross structure of a *Jatropha* seed. Shown is an endosperm without inner skin (tegmen) (A); an inner skin removed from a kernel (B); a kernel (endosperm with inner skin) (C); an intact *Jatropha* seed (D) and a cut-away view of a seed showing the arrangement of the outer testa, inner skin and endosperm (E).

10 Figure 2A-E shows the visual effects of various chemical treatments on *Jatropha* seed kernels. Kernels were treated for 30 mins with (A) 0.05 N NaOH; (B) 0.2 M KOH; (C) 0.125% NaHCO₃; (D) 5% Hydrogen Peroxide; and (E) Separated inner skins after 30 min treatment of seeds with 5% hydrogen peroxide.

15 Figure 3A-B shows a machine for shelling *Jatropha* seeds. The machine operates with a grooved conical roller (A), which is enclosed in a casing (B). The bottom part of the casing has sieves, which measure 8.5 mm in width. Soaked seeds are fed through a hopper into the casing, where the seeds are pressed by the grooved roller against the sieved wall of the casing, thereby breaking the outer shell and separating the kernel.

20 Figure 4 shows a machine for processing *Jatropha* kernels. The machine comprises a stainless drum with a stirrer operated by a 1 HP Motor. The stirrer is fitted with paddles to ensure uniform stirring of kernels and solution to loosen the tegmen and has the capacity to process 200 kg of kernels per hour. The machine has a tilting facility which facilitates easier discharge of the output for further processing.

25 Figure 5A-D shows different types of sieves used in the manufacture process for grading and sorting of seeds, kernels and peeled skins. The 8.5 mm sieve (A) is used to retain seeds that are above 8.5 mm in width for shelling and further processing. The shelling machine output after winnowing is passed through the 8.0 mm sieve (B), which separates the unbroken whole seeds and kernels. The kernels pass through the 8.0 mm sieve whereas the whole unbroken seeds are retained in the sieve. The 6 mm sieve (C) is used during the washing of the treated kernels to retain the whole and half kernels and allow the peeled skins and small broken bits of kernels pass through and be collected in the 1.0 mm fine sieve (D).

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Figure 6 shows a flow chart which includes an industrial scale process according to a preferred embodiment of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Bibliographic references mentioned in the present specification are for convenience listed at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference but are not to be assumed to form part of the common
 5 general knowledge.

Definitions

For convenience, certain terms employed in the specification, examples and appended claims are collected here.

10 The term "chemicals that loosen the tegmen" is herein intended to refer to chemicals that weaken the tight association between tegmen and kernel endosperm such that the tegmen separates or floats away from the endosperm in the composition or may be simply separated from the endosperm by, for example, mechanical means such as in a rotary drum if on an industrial scale. Suitable chemicals may include, for example, potassium hydroxide, sodium hydroxide, sodium bicarbonate and hydrogen peroxide.

15 The term "comprises/comprising" and grammatical variations thereof when used in this specification are to be taken to specify the presence of stated features, integers, steps or components or groups thereof, but do not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof. Accordingly, the term "comprising" not only encompasses but can be replaced by or used interchangeably with the
 20 more restrictive terms "consisting essentially of" and "consisting of."

The terms "inner skin" and "tegmen" are used interchangeably herein to relate to the seed's inner protective layer covering the seed endosperm.

The term "isolated" is herein defined as a biological component (such as; kernel; tegmen; endosperm; cake; oil) that has been substantially separated, produced apart from,
 25 or purified away from other biological components in the plant in which the component naturally occurs, i.e., other proteins and/or macromolecular structures.

The term 'kernel' as used herein is the portion of the seed that remains after the outer layer or testa is removed, and comprises the endosperm with covering inner skin (tegmen) layer. For the purpose of the invention whole kernel or pieces or fragments thereof
 30 may be chemically treated to remove the tegmen, thereby leaving tegmen-free endosperm that are reduced in PEs compared to kernels. In a preferred embodiment whole kernels are treated according to the method of the invention.

A person skilled in the art will appreciate that the present invention may be practiced without undue experimentation according to the disclosures given herein.

In one aspect of the invention there is provided a process to reduce the phorbol ester content of *Jatropha curcas* seed kernel, which kernel comprises an endosperm and a tegmen, by removing the tegmen from the endosperm.

In a preferred embodiment of the invention the process comprises the steps of:

- a) contacting at least one *Jatropha curcas* seed kernel with a chemical solution;
- b) separating the tegmen from the endosperm; and
- c) collecting the separated endosperm and optionally the tegmen.

In another preferred embodiment of the process of the invention the chemical solution comprises one or more chemicals that act to loosen the tegmen around the endosperm.

In another preferred embodiment of the process of the invention the one or more chemicals are selected from the group comprising, alkalis, including alkali salts such as sodium hydroxide, magnesium hydroxide, potassium hydroxide and calcium hydroxide; acids, salts, such as sodium chloride, sodium carbonate and sodium bicarbonate; and oxidizing agents, such as hydrogen peroxide or other compounds containing an oxygen–oxygen single bond or peroxide anion. It would be understood by a skilled person in the art that it would be apparent from the nature of the chemicals disclosed as suitable in the Examples that the working concentrations may be empirically determined and vary from those disclosed in the Examples and that other similar chemicals may be acceptable for the process of the invention, without departing from the invention.

In another preferred embodiment of the process of the invention the one or more chemicals are selected from the group comprising potassium hydroxide, sodium hydroxide, sodium bicarbonate and hydrogen peroxide. Preferably the chemical solution comprises sodium bicarbonate. More preferably, the chemical solution comprises hydrogen peroxide or a combination of hydrogen peroxide and sodium bicarbonate.

In another preferred embodiment of the process of the invention the chemical solution comprises at least 0.005% sodium bicarbonate. In another preferred embodiment the sodium bicarbonate in the solution is at least 0.025%. In another preferred embodiment the sodium bicarbonate in the solution is at least 0.125%. In another preferred embodiment the sodium bicarbonate in the solution is in the range of about 0.025% to about 0.125%.

Preferably the chemical solution comprises at least 0.025% hydrogen peroxide. In another preferred embodiment the hydrogen peroxide in the solution is at least 1%. In another preferred embodiment the hydrogen peroxide in the solution is at least 5%. In another preferred embodiment the hydrogen peroxide in the solution is in the range of about 1% to about 5%. The amount of chemical required in the solution to loosen the inner skin may be determined for temperature and/or contact time.

In another preferred embodiment of the process of the invention the contacting includes mixing of the seed kernel with the chemical solution, preferably by rotation or shaking. The mixing may be continuous or discontinuous. Preferably the mixing is continuous. The steps of contacting and/or removal of the tegmen from the kernel may be, for example, performed in a rotary drum. The contacting may be performed in a mixer similar to that described in Example 2 and shown in Figure 4.

In another preferred embodiment the process of the invention further comprises a step of rinsing the kernels from step a) prior to step b). Rinsing or washing of the kernels from step a) to essentially remove the chemical solution may be with water or other suitable liquid. The rinsing or washing may be performed on one or more sieves such as those described in Example 2 and shown in Figure 5. The rinsing may preferably be performed with a water jet to assist separation of chemically-loosened tegmen from the kernel; and may be performed on a 6 mm sieve to retain only the whole and half kernels.

In another preferred embodiment of the process of the invention the contacting in step a) is for a period of at least 1, 5, 10, 15, 20, 25 or 30 minutes. It would be understood that the contact time can be empirically determined depending on parameters such as contact temperature, scale of treatment, concentration of chemical solution, strength of the mixing/shaking. Intermediate contact times, between the recited amounts, are intended to fall within the scope of the invention. Preferably step a) is performed at room temperature.

In another preferred embodiment of the process of the invention at least 10, 20, 30, 40, 50, 60, 70, 80 or 90% of the tegmen is removed. Intermediate amounts of tegmen removal, between the recited amounts, are intended to fall within the scope of the invention.

In another preferred embodiment of the process of the invention the phorbol ester content of the separated endosperm in step c) is at least 5, 10, 15, 20, 25 or 30% w/w reduced compared to the kernel in step a). Intermediate values, between the recited amounts, are intended to fall within the scope of the invention.

In another preferred embodiment of the invention the process is an industrial scale process comprising the steps of:

a) contacting at least one *Jatropha curcas* seed kernel with a chemical solution by mixing for about 10-15 minutes;

5 b) separating the tegmen from the endosperm by washing with water over a sieve; and

c) collecting the separated endosperm and optionally the tegmen.

10 Preferably the mixing in step a) is performed at a rate of about 2 litres of solution per kg of kernels. Preferably the washing in step b) is performed with a water jet, which assists removal of tegmen, small fragments of kernels and powdered kernels. In step b) the separating may also comprise drying of the washed seeds followed by winnowing to further separate processed kernels from remaining tegmen.

In another preferred embodiment of the invention the process comprises the steps of:

15 a) contacting at least one *Jatropha curcas* seed kernel with a chemical solution, at a rate of 0.5 kg kernels per litre of solution, by mixing for about 10-15 minutes;

b) (i) separating the tegmen from the kernels by washing with water over a 6 mm sieve,

(ii) drying the washed kernels;

(iii) winnowing the dried kernels to remove remaining tegmen; and

20 c) collecting the separated endosperm and optionally the tegmen.

In a preferred embodiment the chemical solution comprises 3% H₂O₂.

A more preferred embodiment of process of the invention is as shown in Figure 6.

Preferably the separated endosperm from step c) is further processed to extract oil and produce seed cake.

25 Preferably the separated tegmen from step c) is further processed into phorbol ester-containing products selected from the group comprising anti-microbial, anti-viral, anti-mollusc, anti-tumor and bio-pesticide agents.

Another aspect of the invention provides at least one *Jatropha curcas* seed kernel with reduced phorbol ester content, wherein the tegmen has been removed to leave only the

endosperm wherein the phorbol ester reduction is at least 5, 10, 15, 20, 25 or 30% w/w. Intermediate values, between the recited amounts, are intended within the scope of the invention. The reduced amount is relative to unprocessed seed kernel.

5 A preferred embodiment provides at least one *Jatropha curcas* seed endosperm produced by the process of the hereinbefore described invention.

Another aspect of the invention provides the use of the at least one *Jatropha curcas* seed kernel with reduced phorbol ester content, i.e. an endosperm, for the production of oil and seed cake. The seed endosperm according to any aspect of the invention may be processed to obtain oil and seed cake. The seed cake may be further processed to remove
10 sufficient remaining phorbol esters to be suitable as animal feed.

A preferred embodiment provides a *Jatropha curcas* seed cake with reduced phorbol ester content, wherein the cake has been produced using at least one seed kernel, in which the tegmen has been removed thereby reducing the phorbol ester content in the cake by at least 5, 10, 15, 20, 25 or 30% w/w. Intermediate values, between the recited amounts, are
15 intended within the scope of the invention.

Another preferred embodiment provides *Jatropha curcas* seed cake produced by the process of the invention.

A preferred embodiment provides the use of *Jatropha curcas* seed cake with reduced phorbol ester content for the production of animal feed.

20 Another preferred embodiment provides at least one isolated *Jatropha curcas* seed tegmen, obtained by the process of the invention. The tegmen may be in a dried and powdered form.

Another preferred embodiment provides the use of *Jatropha curcas* seed tegmen with highly concentrated phorbol ester content for the production of an anti-microbial, anti-mollusc, anti-tumor or bio-pesticide agent. The tegmen may further be dried and powdered
25 in the production of these agents. The tegmen may be suitable for use in the production of anti-microbial, anti-viral, anti-mollusc, anti-tumor or bio-pesticide agents.

Another aspect of the invention provides *Jatropha curcas* oil, isolated from at least one *Jatropha curcas* seed kernel having reduced phorbol ester content. In a preferred
30 embodiment the tegmen has been removed according to the invention and the phorbol ester reduction in the kernel is at least 5, 10, 15, 20, 25 or 30% w/w.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

A person skilled in the art will appreciate that the present invention may be practised without undue experimentation according to the method given herein.

EXAMPLES

Materials and Methods

Jatropha fruits were harvested and decorticated from the fruits from JOil farm in Coimbatore, India. Figure 1 shows the gross structure of a *Jatropha* seed. Shown is an endosperm without inner skin (tegmen) (A); an inner skin removed from a kernel (B); a kernel (endosperm with inner skin) (C); an intact *Jatropha* seed (D) and a cut-away view of a seed showing the arrangement of the outer testa, inner skin and endosperm (E). For Example 1 the seed coat (testa) was removed manually to obtain kernels attached to inner skin.

EXAMPLE 1

Small scale production of *Jatropha curcas* seed kernel

Treatment of kernels to loosen tegmen

Hydrogen peroxide (1%, 2%, 3%, 4% and 5% by volume), sodium bicarbonate 0.025%, 0.05%, 0.075%, 0.1% and 0.125% by volume), potassium hydroxide (0.025M, 0.05M, 0.1M and 0.2M) and sodium hydroxide of 0.05N solutions were prepared and kept ready. Twenty grams of kernels for each treatment were placed in a one liter glass beaker. 100 ml of various solutions were added to the beaker and the contents shaken at 125 rpm in an orbital shaker for 10, 20 or 30 min at room temperature.

After the incubation period 100 ml of water was added to the mixture and the mixture was gently stirred for 2-3 minutes to allow the seeds to contact each other to assist the inner skins to peel off. The seeds and skins were transferred to a beaker which contained water, rinsed once again, and the seeds and peeled inner skins were separated. Subsequently, the kernels were dried at 65°C in an oven overnight, weighed and the kernels with and without skin were counted to calculate the percentage of the kernels from which the inner skin were removed. The amount of inner skin removed from kernels using varying concentration of chemicals and incubation times is shown in Table 1.

Table 1. Treatment at Room Temperature with shaking

Reagents	Concentration	Starting Weight In grams (gm)	Treatment time									
			10min.			20min			30min			% of skin removed
			Weight after treat	Seeds with / without skin	% of skin removed	Weight after treat	Seeds with / without skin	% of skin removed	Weight after treat	Seeds with / without skin	% of skin removed	
Potassium hydroxide	0.025M	20	19.56	41/36	46.75%	19.52gm	38/37	49.33%	19.36	40/36	47.36%	
	0.05M	20	18.95	45/31	40.78%	18.79gm	39/36	48.00%	18.72	38/34	47.22%	
	0.1M	20	18.80	38/32	45.71%	18.70gm	36/37	50.68%	18.70	35/41	53.94%	
	0.2M	20	18.80	30/38	55.88%	18.72gm	30/40	57.14%	18.70	32/41	56.16%	
Sodium bicarbonate	0.025%	20	18.95	30/41	57.74%	18.90gm	28/41	59.42%	18.88	25/42	62.68%	
	0.05%	20	18.85	28/40	58.82%	18.80gm	27/40	59.70%	18.70	25/44	63.76%	
	0.1%	20	18.70	27/41	60.29%	18.60gm	22/48	68.57%	18.50	20/48	70.58%	
	0.125%	20	18.73	25/48	65.75%	18.53gm	21/51	70.83%	18.48	15/54	78.26%	
Hydrogen peroxide	1%	20	18.51	16/50	76.92%	18.48gm	15/55	78.57%	18.42	13/52	80.00%	
	2%	20	18.51	15/56	78.87%	18.43gm	14/53	79.01%	18.36	11/50	81.96%	
	3%	20	18.48	14/58	80.55%	18.36gm	14/54	79.41%	18.30	12/52	81.25%	
	4%	20	18.47	14/59	80.82%	18.33gm	12/55	82.08%	18.25	8/55	84.30%	
	5%	20	18.38	10/60	85.72%	18.27gm	8/59	86.76%	18.24	6/60	90.90%	
Sodium hydroxide	0.05N	20	19.10	57/13	18.57%	18.95	51/18	26.08%	18.85	40/30	42.85%	

Phorbol ester analysis

The oven-dried kernels were powdered for PE analysis as follows:

Extracted samples for PE quantitation: 4 g of kernel powder was extracted with 20 ml methanol twice by sonication at 60°C for 1 hr and the final volume adjusted to 37.5 ml. 0.4 g of skin powder was extracted with 2 ml methanol twice by sonication at 60°C for 1 hr, and the final volume adjusted to 4 ml. 100 µl of filtered sample was injected into a HPLC system for standard PE analysis according to the method of Bu et al., (*Ecotoxicol. Environ. Saf.* 84: 268 – 273 (2012)). Five micrograms of PMA5 (phorbol-12-myristate-13-acetate) was used as the reference for PE quantitation.

10 **Results**

The removal of inner skin or tegmen of *Jatropha* kernels was carried out with the aim of keeping the operation low cost and uncomplicated when scaled up to industrial processing of hundreds of tons of kernels at one time. The testa or the seed coats from the decorticated *Jatropha* seeds were removed by breaking the seeds, and the kernels were collected.

15 Twenty grams of the seeds were found to contain an average of 70 to 75 kernels.

These were placed in solutions of various concentrations of the chemicals according to the Materials and Methods section. The treatments were carried out at room temperature keeping in mind an aim of minimizing the costs of operating an industrial scale process. The kernels in various chemical solutions were incubated in a shaker for ten, twenty or thirty minutes (for industrial scale, rotary drums or other suitable devices can be used for this purpose). The treated seeds were processed, the percentage of skin removed was calculated and the phorbol ester content was analyzed as mentioned in Materials and Methods.

Treatment with 0.025 M potassium hydroxide for 10 minutes showed 47% skin removal. This percentage increased to 49% maximum skin removal when treated for 20 minutes. As the concentration was increased stepwise from 0.025 M to 0.2 M the percentage of skin removal was also increased at every time point (Table 1). A maximum of 57% of kernels were devoid of the inner skin when the treatment was at 0.2 M for 20 minutes duration. On the other hand, treatment with 0.05 N sodium hydroxide at different time points showed a lower percentage of skin removal, compared to the potassium hydroxide. When treated for 10 to 30 minutes the percentage skin removal varied from 19% to 43%, respectively (Table 1). However, treatment of kernels with potassium hydroxide reduced kernel quality, with browning of kernels after drying overnight at 65°C compared to the

sodium hydroxide treatment which had no effect on kernel quality after drying at 65°C (Figure 2A and 2B).

A similar trend of increasing percentage of skin removal was observed with increasing sodium bicarbonate and hydrogen peroxide concentration and longer incubation time. However, the actual percentage of skin removal varied between the treatments and time points. For example, treatment with 0.025%, 0.05%, 0.1% or 0.125% by volume sodium bicarbonate showed a minimum skin removal percentage of 58% at 0.025% by volume when treated for 10 minutes. However, the largest skin removal percentage of 78% was at 0.125% sodium bicarbonate concentration for 30 minutes. When kernels were treated with 1%, 2%, 3%, 4% or 5% by volume hydrogen peroxide a similar trend of increasing skin removal with increased concentration was observed and, surprisingly, the treatment removed up to 91% of the inner skin from the kernels at 5% concentration for 30 minutes. The minimum percentage of inner skin removal was 77% with 1% hydrogen peroxide for 10 minutes (Table 1). The quality of the dried kernels after skin removal was normal and did not show any color change observed with the potassium hydroxide treatment (Figure 2C and 2D). At the end of the treatment times the inner skin of the kernels that had peeled off floated on the top of the solution (Figure 2E). Of the chemicals tested, hydrogen peroxide was the most efficient at loosening and removing the inner skin of the *Jatropha* kernels, with the sodium bicarbonate treatment being the next best.

The oven-dried kernels (at 65°C) were powdered and the phorbol ester content was analyzed as described in the Materials and Methods. The analysis of the PE content of selected samples per treatment showed a reduction in phorbol ester content of up to about 25% w/w (Table 2). On the other hand, inner skin samples isolated contained very high level of phorbol esters (9,525 ppm, 8,904 ppm and 9,437 ppm respectively).

Table 2. Average crude fat content and percentage of PE reduction in treated samples

Treatment	Ave. Crude fat content (%)	Ave. PE content (mg/g of sample)	Ave. Reduction in PE (mg/g of sample)	Ave.% PE reduction
Control	50.1	2.55	0	0
Hydrogen peroxide 3%	50.2	1.91	0.64	25.1
Potassium hydroxide 0.05%	48.7	2.0	0.55	21.6
Sodium hydroxide 0.05%	51.6	2.27	0.28	11

Hydrogen peroxide and potassium hydroxide treatment reduced the phorbol ester content by a similar amount. However, treatment of the kernels with potassium hydroxide caused browning effects on the kernels which may lower the quality of the seed cake after

processing. A reduction by as much as 25% w/w is a significant removal of PE by introducing an additional processing step, and may be further reduced in the defatted kernel meal by other chemical and physical methods.

EXAMPLE 2

5 Large scale processing of *Jatropha curcas* seed kernel

The following is an example of machinery and methods to separate the inner skin from the *Jatropha* seed kernel and reduce the phorbol ester content of the seeds.

Machinery employed

1. *Jatropha* seed sheller

Figure 3A shows a seed shelling machine that operates with a grooved conical roller, which is enclosed in a casing (Figure 3B). The bottom part of the casing has sieves, which measure 8.5 mm in width. Soaked seeds are fed through a hopper into the casing, where the seeds are pressed by the grooved roller against the sieved wall of the casing, thereby breaking the outer shell and separating the kernel. The output which consists of whole kernels, shells and whole seeds is sundried, which makes it amenable for further processing. The gap between the grooved roller and the casing can be adjusted by moving the roller along with the shaft. In addition, the bottom casing can be replaced with casings having different sieve sizes. This enables processing of seeds of different sizes.

Machinery specifications:

20	Shelling Capacity	10 kg per hour
	Shelled Seeds	90%
	Whole Kernels	90 – 95%
	Shelling Percentage	45 – 50%
	Motor	3 HP

25 2. Kernel processing machine

Figure 4 shows a kernel processing machine that is made up of a 175 litres capacity stainless drum with a stirrer operated by a 1 HP Motor. The stirrer is fitted with paddles to ensure uniform stirring of kernels and chemical solution as described in Example 1 to loosen the tegmen. The machine has the capacity to process 200 kg of kernels per hour. The

machine has a tilting facility which facilitates easier discharge of the output for further processing.

3. Sieves

Figure 5A-D shows various types of sieves used in the manufacture process for grading and sorting of seeds, kernels and peeled skins. An 8.5 mm sieve (Figure 5A) is used to grade the seeds prior to processing. Seeds that are above 8.5 mm in width are retained in the sieve and used for shelling and further processing. The shelling machine output after winnowing is passed through an 8.0 mm sieve (Figure 5B), which separates the unbroken whole seeds and kernels. The kernels pass through the 8.0 mm sieve whereas the whole unbroken seeds are retained in the sieve. A 6 mm sieve (Figure 5C) is used during the washing process. The treated kernels are spread over the sieve and washed with a water jet. Only the whole and half kernels are retained in the sieve, whereas the peeled skins and small broken pieces of kernels pass through the sieve which gets collected in a 1.0 mm fine sieve (Figure 5D).

15 **Process steps**

A large scale processing scheme according to the invention is shown in Figure 6. *Jatropha* seeds are graded using sieves (Figure 5A-D) such that only those with a width of at least 8.5 mm are selected for further processing. The selected seeds are soaked in water for 10 minutes to soften the seed coat and make it amenable for shelling. The seeds may be shelled in a rotary cylindrical sheller such that shown in Figure 3. The sheller output comprises about 90 - 95% whole kernels, broken shells, powdered shell, whole seeds and partly broken seeds. The product from the sheller is dried to remove surface moisture and then winnowed to remove the shells. Sunshine or other sources of heat, such as an oven, may be utilized for the drying step. For example, the product may be dried under bright sunshine for about 3-4 hours; whereas under moderate sunshine the drying step may require about 6-7 hours.

After the shells are removed the seeds are sieved through an 8 mm sieve to separate the whole seeds and the kernels. Kernels are then processed by mixing them with 3% H₂O₂ in a mixer such as that shown in Figure 4, at a rate of 2 litres per kg of kernels and stirred continuously for about 10-15 minutes. Longer mixing times are generally not required because the reaction is normally completed within 10-15 minutes but, nevertheless, the reaction and peeling of tegmen may be monitored visually as floating inner skins. Alternatively, or in addition, samples may be taken and visualized on a petri dish.

After the shells have been stirred in H_2O_2 they are placed on a 6 mm sieve and washed thoroughly with water. Use of a water jet advantageously clears away the skins, small fragments of kernels and powdered kernels. The kernels are then dried, conveniently sun-dried as described above or by other means.

- 5 The dried kernels are winnowed to remove any remaining skins and sorted to remove any foreign particles.

The processed seeds can then be packed for shipping according to requirements.

- 10 *Jatropha curcas*, a bio-diesel feedstock plant has gained importance over the past decade as its seeds can be crushed to obtain oil which can be used as fuel either directly or as a bio-diesel blend after trans-esterification. The cake obtained after oil extraction from kernels of *Jatropha* seeds is considered to be of high potential nutritional value in terms of protein and fiber and could be used as an animal feed. However, the presence of PEs makes the seed cake unusable for animal feed as they are considered a co-carcinogen and also have other detrimental health effects (NIH Phorbol esters, safety data sheet, National Institute of Health (2007)). PEs are enriched in the inner skin or tegmen (6-10 times the concentration of the whole kernel; as high as 14,449 ppm or 1.4% dry weight) as well as in the endosperm of the kernel. It is difficult to peel the tightly bound inner skin or tegmen from the kernels of *Jatropha*, thereby creating processing problems if wanting to reduce the PE content of the seed cake. Manual or mechanical force peeling is not possible to any great extent especially in the context of handling industrial scale quantities of seeds (tens of tonnes) daily.

- 25 It was imperative to find an alternative method to loosen and remove the tightly bound inner skin of the *Jatropha* kernel that is feasible commercially in cost, time and efficiency to suit the industrial scale needs. It is also highly important to select a chemical that will be used in removal of the inner skin that will not modify the nutritional quality of the seed cake or the quality and quantity of the oil produced. These parameters were considered and chemicals such as sodium bicarbonate, potassium hydroxide, sodium hydroxide and hydrogen peroxide were selected that were relatively inexpensive and/or commonly used in the food industry.

- 30 Though the cost is cheaper and around 50% of the inner skin was removed from the kernels after treatment with potassium hydroxide and sodium hydroxide, the quality of the seeds might be compromised, particularly with potassium hydroxide (Figure 1B). The sodium hydroxide treatments may not be an optimal method to remove the inner skin of

Jatropha, although a combination with another chemical such as hydrogen peroxide may improve performance and lessen the potential negative effects of the hydroxide.

On the other hand, hydrogen peroxide treatment and the sodium bicarbonate treatment worked most effectively in removing the inner skin of *Jatropha* kernels, with removal percentage varying from 77 to 91% and 58 to 78% for the varying concentrations of hydrogen peroxide and sodium bicarbonate, respectively. As soon as the treatment was over, the skins floated on the surface and could be easily removed without rubbing or force. Between sodium bicarbonate and hydrogen peroxide, sodium bicarbonate is the more cost-effective although it did not perform as well. Lower concentrations of hydrogen peroxide should be further tested with longer incubation times, or in combination with the sodium bicarbonate to make it more cost-effective.

The increased removal of the inner skin was reflected in the reduction of PE content, whereby treatment with hydrogen peroxide and potassium hydroxide showed higher reduction (up to 25% w/w reduction) of the PEs. However, in light of the browning effects of the potassium hydroxide treatment and the potential effect on nutritional value of the seed cake, the hydrogen peroxide treatment was found to be the most effective of those tested for the industrial processing of seed cake to remove PE. This treatment along with other physical or chemical methods can be used in combination to reduce the PE content of the defatted meal to safer levels for use as animal feed.

The inner skins which are removed can be dried and powdered to use directly as a bio-pesticide to control pests or the PEs can be isolated to be used as bio-pesticides. Phorbol esters are not only bio-pesticidal, they have also been shown to have anti-microbial, anti-viral, anti-molluscidal and anti-tumor properties (Antal Corina E., et al., *Cell* 160 (3):489-502, doi:10.1016/j.cell.2015.01.001; El-Mekkawy S., et al., *Phytochemistry* 53 (4): 457-464 (2000); Goel G., et al., *International Journal of Toxicology* 26 (4): 279-288 (2007); Zhong Y., et al., *Antivir Chem Chemother* 16 (5): 303-313 (2005)). Thus the inner skin of *Jatropha* can be processed and sold into the Agri-industry to generate additional revenue.

References

- Antal Corina E, Hudson Andrew M, Kang E, Zanca C, Wirth C, Stephenson Natalie L, Trotter Eleanor W, Gallegos Lisa L, Miller Crispin J, Furnari Frank B, Hunter T, Brognard J, Newton Alexandra C, Cancer-Associated Protein Kinase C Mutations Reveal Kinase's Role as Tumor Suppressor. *Cell* 160 (3): 489-502. doi:10.1016/j.cell.2015.01.001.
- Bu, Y.P., B.T. Ha, et al., "Light induced degradation of phorbol esters" *Ecotoxicol. Environ. Saf.* 84: 268 – 273 (2012).
- El-Mekkawy S., Meselhy MR, Nakamura N, Hattori M, Kawahata T, Otake T, Anti-HIV-1 phorbol esters from the seeds of *Croton tiglium*. *Phytochemistry* 53 (4): 457-464 (2000).
- 10 He, W., A.J. King, et al., "Analysis of seed phorbol-ester and curcumin content together with genetic diversity in multiple provenances of *Jatropha curcas* L. from Madagascar and Mexico". *Plant Physiol. Biochem.* 49 (10): 1183-1190 (2011).
- Goel G., H.P.S. Makkar, et al., "Phorbol Esters: Structure, Biological Activity, and Toxicity in Animals". *International Journal of Toxicology*, 26 (4): 279-288 (2007).
- 15 NIH (2007) Phorbol esters, safety data sheet. National Institute of Health.
- Zhong Y., Matsuya Y, Nemoto H, Mori M, Saito H, Yamamoto N., Novel phorbol esters exert dichotomous effects on inhibition of HIV-1 infection and activation of latent HIV-1 expression. *Antivir Chem Chemother* 16 (5): 303-313 (2005).

CLAIMS:

1. A process to reduce the phorbol ester content of *Jatropha curcas* seed kernel, which kernel comprises an endosperm and a tegmen, by removing the tegmen from the endosperm.

5

2. The process of claim 1, comprising the steps of:

- a) contacting at least one *Jatropha curcas* seed kernel with a chemical solution;
- b) separating the tegmen from the endosperm; and
- c) collecting the separated endosperm and optionally the tegmen.

10

3. The process of claim 1 or 2, wherein the chemical solution comprises one or more chemicals that act to loosen the tegmen around the endosperm.

15

4. The process of claim 3, wherein the one or more chemicals are selected from the group comprising, alkalis, including alkali salts such as sodium hydroxide, magnesium hydroxide, potassium hydroxide and calcium hydroxide; acids, salts, such as sodium chloride, sodium carbonate and sodium bicarbonate; and oxidizing agents, such as hydrogen peroxide.

20

5. The process of claim 4, wherein the one or more chemicals are selected from the group comprising potassium hydroxide, sodium hydroxide, sodium bicarbonate and hydrogen peroxide.

6. The process of any one of the previous claims, wherein the contacting includes mixing of the seed kernel with the chemical solution, preferably by rotation or shaking.

25

7. The process of any one of the previous claims, further comprising a step of rinsing the kernels from step a) prior to step b).

8. The process of any one of the previous claims, wherein the contacting in step a) is for a period of at least 1, 5, 10, 15, 20, 25 or 30 minutes.

5 9. The process of any one of the previous claims, wherein step a) is performed at room temperature.

10. The process of any one of the previous claims, wherein the chemical solution comprises sodium bicarbonate and/or hydrogen peroxide.

10 11. The process of any one of the previous claims, wherein the chemical solution comprises at least 0.005% sodium bicarbonate and/or at least 0.025% hydrogen peroxide.

12. The process of any one of the previous claims, being an industrial scale process comprising the steps of:

15 a) contacting at least one *Jatropha curcas* seed kernel with a chemical solution by mixing for about 10-15 minutes;

b) separating the tegmen from the endosperm by washing with water over a sieve; and

c) collecting the separated endosperm and optionally the tegmen.

20

13. The process of any one of the previous claims, wherein at least 10, 20, 30, 40, 50, 60, 70, 80 or 90% of the tegmen is removed.

25 14. The process of any one of the previous claims, wherein step a) and/or b) is performed in a rotary drum.

15. The process of any one of the previous claims, wherein the phorbol ester content of the separated endosperm in step c) is at least 5, 10, 15, 20, 25 or 30% reduced compared to the kernel in step a).

5 16. The process of any one of the previous claims, wherein the separated endosperm from step c) is further processed to extract oil and produce seed cake.

17. The process of any one of the previous claims, wherein the separated tegmen from step c) is further processed into phorbol ester-containing products selected from the group
10 comprising anti-microbial, anti-viral, anti-mollusc, anti-tumor and bio-pesticide agents.

18. At least one *Jatropha curcas* seed kernel with reduced phorbol ester content, wherein the tegmen has been removed and the phorbol ester reduction is at least 5, 10, 15, 20, 25 or 30%.

15

19. The at least one *Jatropha curcas* seed kernel of claim 18, produced by the process of any one of claims 1 to 15.

20. *Jatropha curcas* seed cake with a phorbol ester content reduced by at least 5, at least 10, at least 15, at least 20, at least 25 or by at least 30% w/w, wherein the cake has been
20 produced using at least one seed kernel without its tegmen.

21. The *Jatropha curcas* seed cake of claim 20, produced by the process of claim 15.

22. At least one isolated *Jatropha curcas* seed tegmen, obtained by the process of any one of claims 1 to 14.
25

23. The at least one isolated *Jatropha curcas* seed tegmen of claim 22, wherein the tegmen has further been dried and powdered.

5 24. The isolated *Jatropha curcas* seed tegmen of claim 22 or 23 for use as an anti-microbial, anti-viral, anti-mollusc, anti-tumor or bio-pesticide agent.

25. Use of the at least one *Jatropha curcas* seed kernel of claim 18 or 19 for the production of oil and seed cake.

10 26. Use of the *Jatropha curcas* seed cake of claim 20 or 21 for the production of animal feed.

27. Use of the *Jatropha curcas* seed tegmen of claim 22 or 23 for the production of an anti-microbial, anti-viral, anti-mollusc, anti-tumor or bio-pesticide agent.

15 28. *Jatropha curcas* oil, isolated from the kernel of claim 18 or 19.

29. The process substantially as hereinbefore described with reference to Figure 6.