Degradation of *Jatropha curcas* phorbol esters derived from Jatropha oil cake
and their tumor-promoting activity.

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Abstract

Large amount of oil cake is generated during biodiesel production from *Jatropha* seeds. Although *Jatropha* oil cake is rich in plant nutrients, presence of toxic phorbol esters restricts the usage of oil cake as a fertilizer. The objective of this study is to evaluate the components and tumor promoting activity of phorbol esters in *Jatropha* oil cake-supplemented soil and plants grown in the treated soil. Contents and their biological activity of *Jatropha* phorbol esters in soil and plants were sequentially analyzed by high-performance liquid chromatography (HPLC) and *in vitro* cell transformation assay, respectively. Disappearance of *Jatropha* phorbol-ester-specific peaks were followed with HPLC during incubation of *Jatropha* oil cake with soil for 5 weeks. Along with the degradation of *Jatropha* phorbol ester in soil, tumor-promoting activity in the sample was also attenuated and ultimately disappeared. *Jatropha* phorbol esters and tumor promoting activity were not detected from mustard spinach grown in the *Jatropha* oil cake-supplemented soil. In addition, the esterase KM109 degrades DHPB (see definition below; *Jatropha* phorbol ester) and reduced its tumor-promoting activity. From these data, we conclude; (1) components and tumor promoting activity of *Jatropha* phorbol esters in the oil cake disappeared completely by incubation with soil for 5-week, (2) *Jatropha* phorbol esters did not transfer into plants grown in the *Jatropha* oil cake-supplemented soil, and (3) DHPB can be degraded by esterase from soil bacterium. These observations are useful for utilization of *Jatropha* oil cake as a fertilizer.

Keywords: *Jatropha curcas*, phorbol ester, esterase, soil, biodegradation

1. Introduction

Jatropha curcas L. is an oil seed plant widely distributed in tropical and subtropical regions. *Jatropha* oil is obtained by mechanical press of the seed, and large amount of oil cake is generated as by-product. *Jatropha* seed is consisted by 43-59% of oil and 41-57% of other solid contents, such as proteins, fiber and ash on dry matter mass basis (Makkar, 1997). Although *Jatropha* oil cake is rich in nitrogen, presence of toxic phorbol esters restricts the usage of oil cake as a fertilizer. Phorbol esters are tetracyclic diterpines containing tiglane skeleton as a fundamental structure. Hydroxylation and esterification of this tiglane skeleton in various positions characterize the large number of phorbol ester compounds and are mainly responsible for their toxicity (Goel, 2007). Phorbol esters mimic a biochemical effect of diacylglycerol and cause prolonged stimulation of protein kinase C (PKC). Activation of PKC by phorbol esters induces many biological processes, such as cell proliferation, skin irritation and tumor promotion. *Jatropha* seed contains 6 kinds of phorbol esters (Haas, 2002). An intramolecular 13, 16-diester of 12-deoxy-16-hydroxyphorbol, 12-deoxy-16-hydroxyphorbol-4'-[12'-14'-butadienyl]-6'-[16', 18', 20'-nonatrienyl]-bicyclo [3. 1. 0] hexane-(13-*O*)-2'-[carboxylate]-(16-*O*)-3'-[8'-butenoic-10'] ate (DHPB), is the most abundant phorbol ester in *Jatropha* seed (Figure 1). However, the time course of the changes in the contents and tumor promotion activity of phorbol esters in *Jatropha* oil cake has not been well documented with respect to its use as a fertilizer. It is also unclear whether *Jatropha* phorbol esters in the oil cake can transfer into plants. The objective of this study is to monitor the

changes in the contents and tumor-promoting activity of phorbol esters in *Jatropha* oil cake-supplemented soil and plants grown in the treated soil. Contents and their biological activity of *Jatropha* phorbol esters in soil and plants were sequentially analyzed by high-performance liquid chromatography (HPLC) and studied *in vitro* cell transformation assay, respectively.

2. Materials and methods

2.1 Materials

Jatropha seed was obtained from Myanmar. *Jatropha* oil cake was obtained from *Jatropha curcas* seeds from Philippines after processing the seeds with a mechanical press. The pressed oil cake was powdered using mortar and pestle, and stored in a deep-freezer at -80°C until usage.

2.2.1 Blending of soil samples and cultivation of mustard spinach

Jatropha oil cake was homogenously blended with a soil (1.8 kg) from the loamy layer of the Kanto District in Japan. The formulated soil was divided into an amount of the single dose (8.38 g), double dose (16.76 g) and quadruple dose (33.52 g) on the day of testing. The soil was allowed to settle in a Neubauer pot according to the guideline by Japan Fertilizer and Feed Inspection Association (JFFIA). Moisture of slightly acidic soil from Kanto loamy layer was maintained between 50 – 60% of maximum water retention capacity. We have used a single lot of soil sample. Soil samples were supplemented with ammonium sulfate (21.10 g/1.8 kg of soil), superphosphate (17.98 g/1.8 kg soil) and potassium chloride (63.10 g/1.8 kg soil). Seven days after blending *Jatropha* oil cake with the soil, mustard spinach seeds were planted (day 7).

Mustard spinach was grown for 28 days (until day 35) and the plant was then harvested. Soil sample was collected on days 0, 21 and 35 to evaluate the contents and tumor promoting activity associated with *Jatropha* phorbol esters. The schedule for the cultivation and sampling is shown in Figure 2.

2.2.2 Extraction of phorbol esters from soil and mustard spinach

The soil (22.5 g) was blended thoroughly with 50 ml methanol and the mixture was allowed to stand without mixing overnight at room temperature.

Supernatant of the extract was collected and concentrated under nitrogen stream and residual solid was resuspended in a small amount of methanol for use in HPLC analysis and cell transformation assay.

Mustard spinach was dried and powdered on day 35. Dried mustard spinach (1 g) was added to 10 ml 100% methanol and was incubated at room temperature for 2 days. The extract was concentrated under nitrogen stream, and the concentrate was then resuspended in 90% acetonitrile. Chlorophyll was removed by passing the samples through ODS cartridge. Flow-through eluates and the fraction eluted with 90% acetonitrile were collected and concentrated under nitrogen gas stream for HPLC analysis and cell transformation assay.

2.2.3 Detection of *Jatropha* phorbol esters by HPLC

Jatropha phorbol esters in soil and mustard spinach were analyzed by HPLC. The soil extract samples (equal to 1.4 g of soil) were loaded to Inertsil ODS-4 column (4.6 x 150 mm; GL Sciences Inc., Tokyo, Japan) equilibrated with 77% acetonitrile. The column was maintained at 40°C. The following program

was used at a flow rate of 1 ml/min: 77% acetonitrile (23% distilled water) (0-10 min), 77-100% acetonitrile (23-0% distilled water) (10-13 min), 100% acetonitrile (13-17 min), and 100-77% acetonitrile (0-23% distilled water) (17-18 min). *Jatropha* phorbol esters were detected by the absorbance of 282 nm.

The mustard spinach extract samples (equal to 5 µg of dried mustard spinach) were loaded to Inertsil ODS-4 column (4.6 x 150 mm) equilibrated with 10 mM HCl:acetonitrile:methanol (20:45:35). The column was maintained at 40°C. *Jatropha* phorbol esters were separated by isocratic solvent system at a flow rate of 1 ml/min, and peaks were detected by absorbance at 282 nm.

2.2.4 Purification of DHPB from Jatropha seed

Jatropha seed (1 kg) was grounded by a food processor and immersed in methanol (2 L) overnight at room temperature. The extract was collected and the residue was re-extracted with methanol overnight at room temperature again. This procedure was repeated three times. Collected extract was concentrated with rotary evaporator. The concentrate was resuspended with acetonitrile and the acetonitrile-soluble fraction was applied to preparative HPLC equipped with TSK-gel ODS-80TM (20 mm x 300 mm, Tosoh Corporation, Tokyo, Japan) equilibrated with 100% acetonitrile (flow rate: 5 ml/min, column temperature: 40°C). The peak containing DHPB was collected and concentrated with centrifugal evaporator (SpeedVac SAVANT SP-D 1010, Thermo Fischer Scientific Inc., Waltham, MA, USA). The concentrate was resuspended with 77% acetonitrile and fractionated in HPLC equipped with Inertsil ODS4 (20 mm x 150 mm, GL Science Inc., Tokyo, Japan) equilibrated with 77% acetonitrile (23% distilled water) (flow rate: 5 ml/min, column

temperature: 40°C). The peak containing DHPB was collected and concentrated with centrifugal evaporator. The concentrated powder residue was resuspended with 1 ml methanol and the quantity of DHPB was determined by spectrophotometer (U5100, Hitachi High Technologies Corporation, Tokyo, Japan) calculated using molar absorbance coefficient of DHPB (48977 L.mol⁻¹cm⁻¹ at 280 nm in methanol).

2.2.5 Cell transformation assay

To investigate tumor-promoting activity of *Jatropha* phorbol esters, Bhas 42 cell transformation assay was performed (Hasegawa, 2012; Ohmori, 2004). Bhas 42 cells were established from BALB/c 3T3 cells transfected with v-Ha-ras oncogene, and are regarded as tumor-initiated cells in the two-stage carcinogenesis model. Bhas 42 cells were cultured in DMEM/F12 medium supplemented with 5% fetal bovine serum at 6-well plates (4 x 10⁴ cells/well, day 0). After cultivation for 3 days, medium was replaced with fresh medium containing soil extracts (0-86 mg soil) or mustard spinach extracts (0-3 mg dry weight). Supplementation of soil extract more than 86 mg and mustard spinach extracts more than 3 mg interfered with viability of Bhas 42 cells (data not shown). The culture medium was changed on day 7 and day 10 with fresh medium containing soil extracts or mustard spinach extracts, and was then changed on day 14 and day 17 with fresh medium without soil extracts or mustard spinach extracts. On day 21, cells were fixed with methanol for 30 min and stained with 2.5% Giemsa solution for 20 min. Transformed colonies were defined by morphological characteristics: deep basophilicity, dense multi-layering of cells, random orientation of cells at the outer edge of colonies

and more than 50 cells within a focus.

2.2.6 Degradation of DHPB by esterases

DHPB (500 ng/ 10 μ l) was mixed with 90 μ l of 50 mM potassium phosphate buffer (pH 7.5) containing 100 μ g of esterase in three tubes. Each tube contains one of each of the 3 esterases (lipase from *Aspergillus niger* (*A. niger*), Sigma-Aldrich, St. Louis, MO, USA; SNSM87 derived from *Klebsiella oxytoca*, Nagase & Co, ltd., Tokyo, Japan; KM109 derived from *Acinetobacter calcoaceticus*, Nagase & Co., ltd.). Distilled water was added instead of enzyme solution as a buffer control. Prior to the incubation, all reagents were sterilized by filtration (pore size: 0.22 μ m, Millex-GP syringe-driven filter unit, EMD-Millipore Corporation., Billerica, MA, USA) and stored on a clean bench top under an aseptic condition to avoid the bacterial contamination. The reaction mixture was incubated in 37°C for 0, 7, 14, and 28 days. Acetic acid (10 μ l) was added to the mixture to stop the enzyme reaction and the reaction mixture was evaporated with SpeedVac. The residue was extracted with methanol (40 μ l). The methanol extract was centrifuged to remove insoluble salts. Supernatant was used for HPLC analysis and cell transformation assay.

2.2.7 Data analysis

All data are expressed as mean $\pm$ standard deviation. Two-way ANOVA with Fisher's protected least employed to compare mean values between two groups. A *P* value of 0.05 was considered to be statistically significant difference.

3. Results

3.1 *Degradation of Jatropha phorbol esters in soil*

Contents of Jatropha phorbol esters in soil sampled on days 0, 21 and 35 were evaluated by HPLC analysis (Figure 3). *J. curcus* contains 6 types of phorbol esters (Haas, 2002), and these Jatropha phorbol esters were detected in the range of the retention time as indicated by bold line in Figure 3. The peak of DHPB, the most abundant Jatropha phorbol ester, is indicated by an arrow. On day 0, Jatropha phorbol esters were detected in soil in a Jatropha oil cake in a dose-dependent manner. A similar dose-dependency of Jatropha phorbol esters in Jatropha oil cake-supplemented soil was no longer observed on day 21. As compared with the HPLC chromatogram from the control soil, Jatropha phorbol ester-specific peaks disappeared on day 35, suggesting that Jatropha phorbol esters in the oil cake were completely degraded by incubation with soil sample for 5 weeks.

Tumor promoting activity of Jatropha phorbol esters in Jatropha oil cake-supplemented soil was evaluated by *in vitro* Bhas 42 cell transformation assay (Figure 4).

Transformation activity was observed in all samples (single, double and quadruple dose) of Jatropha oil cake-supplemented soil extracts obtained on day 0, although transformation activity was not detected in the soil without the addition of Jatropha oil cake (control). The number of transformed colonies of Bhas42 cells was increased in the presence of Jatropha oil cake to soil in dose-dependently. After the incubation of Jatropha oil cake with soil for at least 21 days, transformation activity in Jatropha oil cake-supplemented soil was significantly decreased. Transformation activity was significantly decreased by 75, 40 and 31% on the day 21, and by 91, 100 and 96% on the day

35 for quadruple, double and single dose soil samples, respectively, as the number of the background colonies was subtracted from the observed values. The transformation activity of *Jatropha* oil cake-supplemented soil disappeared on day 35 from the incubation mixture in all dosages of *Jatropha* oil cake supplementation to soil, indicating that *Jatropha* phorbol esters in the oil cake were completely degraded by incubation with soil for 5 weeks.

3.2 Evaluation of Jatropha phorbol esters in mustard spinach

In order to evaluate the contents of *Jatropha* phorbol esters in mustard spinach, HPLC analysis was performed by using mustard spinach extracts (Figure 5A). Although mustard spinach-derived peaks were present, *Jatropha* phorbol ester-derived peak was not observed in the extracts from mustard spinach grown in soil supplemented with the single, double or quadruple dose of *Jatropha* oil cake-supplemented soil.

The tumor promoting activity characteristic of *Jatropha* phorbol esters was also evaluated in mustard spinach extracts (Figure 5B). Similar to the results of HPLC analysis, the transformation activity was not detected in the extracts from mustard spinach grown in soil supplemented with the single, double or quadruple dose of *Jatropha* oil cake-supplemented soil. In summary, *Jatropha* phorbol ester was not detected in mustard spinach grown in *Jatropha* oil cake-supplemented soil.

3.3 Degradation of Jatropha phorbol esters by esterase

To examine modes of destruction of *Jatropha* phorbol esters in soil, several esterases and a lipase were incubated with DHPB for several weeks. Residual

DHPB amount was analyzed with HPLC. 93.7% of DHPB was degraded by an esterase KM109 in 4 weeks while 57.6% of DHPB was degraded in buffer control (Figure 6A). Degradation of DHPB by SNSM87 and lipase from *A. niger* was not significantly different from the buffer control on days 14 and 21, respectively. Degradation of DHPB was the same rate in pH range between pH 6.0 to 8.0 (data not shown). Different elution profiles were observed with the sample incubated with the buffer control and that treated with KM109 (Figure 6B).

The tumor-promoting activity of DHPB incubated with KM109 was also examined. After one-week incubation with KM109, the tumor-promoting activity of DHPB was significantly attenuated compared to that in buffer control (Figure 6C). These results indicated that esterase degrade DHPB though the water (in the buffer control) also degraded DHPB to some extent.

4. Discussion

The contents and tumor promoting activity of phorbol esters in *Jatropha* oil cake-supplemented soil and plants were evaluated. The results of this study demonstrate biodegradation of phorbol esters and disappearance of the tumor promoting activity in *Jatropha* oil cake following incubation with soil. *Jatropha* phorbol esters in the oil cake were not transferred into plants grown in the treated soil. DHPB, the main component of *Jatropha* phorbol esters were degraded by an esterase activity.

4.1 Degradation of *Jatropha* phorbol esters in soil

Jatropha oil cake-derived phorbol esters were degraded time-dependently upon incubating with soil (Figure 2). Peaks corresponding to those of Jatropha phorbol esters were detected in HPLC profile of the oil cake-supplemented soils on day 0, but Jatropha phorbol ester-derived peaks which were significantly attenuated on days 21 and 35. The peak attributable to DHPB, the most abundant phorbol ester in Jatropha seed, was only detected in Jatropha oil cake-supplemented soil only on days 0 and 21. The elution profile of Jatropha oil cake-supplemented soil on day 35 was similar to that of control soil, suggesting that Jatropha phorbol esters in the oil cake-supplemented soil were totally degraded. Although the mechanism of degradation of Jatropha oil cake-derived phorbol esters in soil is unclear, it has been reported recently that *Pseudomonas aeruginosa* degrades Jatropha phorbol esters in solid state (Joshi, 2011). Jatropha phorbol esters are also degraded by *Bacillus subtilis* var. *natto* (He, 2011). Hydrocarbon moieties which contain polyenes and connected with which forms intramolecular 13,16-diester bonds in Jatropha phorbol esters is reported to influence its toxicity (Goel, 2007). *Pseudomonas aeruginosa* produces lipases which prefer long carbon chain fatty acid ester substrates (Gaur, 2008; Mahanta, 2008), suggesting that bacterial lipases hydrolyze C-13 and C-16 ester linkages and detoxify Jatropha phorbol esters. Detail mechanism of degradation of Jatropha phorbol esters in soil will be investigated further.

Detection of Jatropha phorbol esters has been usually conducted only by HPLC analyses. We employed *in vitro* Bhas 42 cell transformation assay to evaluate tumor promoting activity of Jatropha phorbol esters. Bhas 42 cell transformation assay is highly sensitive method to detect tumor-promoting

activity (Ohmori, 2004). This assay can detect DHPB at the concentrations of the order of pg/ml (data not shown). Along with the degradation of Jatropha phorbol esters in soil, tumor promoting activity in Jatropha oil cake-supplemented soil also diminished and finally disappeared on day 35 (Figure 3). The number of transformed colonies in Bhas 42 cells decreased to the baseline level on day 35, indicating that Jatropha phorbol esters in the oil cake was completely degraded by incubation with soil for 5 weeks. This is the first report to evaluate tumor-promoting activity of Jatropha phorbol esters by *in vitro* Bhas 42 cell transformation assay. Although small HPLC peaks derived from Jatropha phorbol esters were difficult to quantitate due to various soil-derived baseline peaks. The results of the *in vitro* Bhas 42 cell transformation assay were more evident to estimate the presence of Jatropha phorbol esters in the oil cake-supplemented soil. Since the tumor-promoting activity can be performed in 3 weeks, this assay is effective for detection and estimation of biological activity of Jatropha phorbol esters.

4.2 Transfer of Jatropha phorbol esters into plants

Concerning with the application of Jatropha oil cake for fertilizer, we investigated whether Jatropha oil cake-derived phorbol esters in soil were absorbed by plant. As shown in Figure 5A, Jatropha phorbol esters were not detected from mustard spinach extract grown in the soil supplemented with Jatropha oil cake. Moreover, tumor-promoting activity was not detected from mustard spinach extract from the plant grown in the Jatropha oil cake-supplemented soil (Figure 5B). Taken together these data, Jatropha

phorbol esters were not absorbed by plant. Evaluation of the transfer of Jatropha phorbol esters into plants cultured with Jatropha oil cake-supplemented soil is reported here for the first time to the best of our knowledge.

Jatropha seeds are mechanically pressed in order to obtain oil, and generating a large amount of oil cake (41-57% on dry matter basis) as a by-product (Makkar, 1997). Jatropha oil cake is rich in nitrogen, phosphorus and potassium (Kumar, 2008), thus it is thought to be an excellent candidate for use as a fertilizer for plant nutrient source. However, toxic Jatropha phorbol esters restrict its utilization as fertilizer. Degradation of Jatropha phorbol esters has been usually conducted by various chemical and physical methods. It has been reported that high heat and pressure treatments (260°C, 3 mbar) with moisture degraded Jatropha phorbol esters in oil completely (Makkar, 2009). Combination of alkali and autoclave treatments (121°C, 30 min) decreased Jatropha phorbol esters up to 89% in seed meal (Rakshit, 2008). An autoclave treatment of Jatropha seed meal followed by four repeated washes with methanol degraded 95% of phorbol esters (Aregheore, 2003). Although these chemical and physical treatments decreased Jatropha phorbol esters efficiently, our detoxification method of Jatropha oil cake by incubation with soil for only 5 weeks is milder and more cost-effective than other methods.

4.3 Esterase, KM109 degrades DHPB in the buffer

To explore the mechanism involved in the degradation of DHPB in soil, we examined several esterases to degrade DHPB. As mentioned above, some soil bacteria produce enzymes which degrade Jatropha phorbol esters (Joshi, 2011;

He, 2011; Gaur, 2008, Mahanta, 2008). We tested three-kinds of esterases, a lipase from *A. niger*, SNSM87 from *Klebsiella oxytoca*, and KM109 from *Acinetobacter calcoaceticus* (*A. calcoaceticus*).

Lipase from *A. niger* has typical lipase activity. SNSM87 is a heat-resistant esterase (Chikusa, 2003). KM109 can degrade bulky side chain unlike ordinary esterase which only degrades acetic ester or phosphate ester (Mitsuhashi, 1999). In this study, only KM109 degrades nearly all DHPB in 4 weeks (Figure 6A). Others including buffer control also degrades 43 – 67% of DHPB in the same period, indicating that DHPB could be degraded non-enzymatically by the water. However, we observed different pattern of changes in elution profiles monitored at 242 nm and 282 nm for the sample treated with KM109 as compared with those of the buffer control (Figure 6B). Phorbol ring such as 4 α -phorbol has absorbance peak at 242 nm. The major degradation product peak of DHPB was eluted at 1.6 min. This peak was suggested to be a phorbol ring structure because as the same peak was present at 1.6 min under the identical condition for 4 α -phorbol as an authentic standard (data not shown). In the KM109 digested sample, the area of the peak at 1.6 min reached maximum on day 7 and then decreased thereafter at day 28. The peak eluted at 1.9 min was increased on day 28 instead of the peak eluted at 1.6 min in KM109 sample. The 1.9 min peak was increased time-dependently in the buffer control. In addition, the peak eluted at 1.1 min showed same changes in the elution pattern as that at 1.6 min. The 1.1 min peak was uniquely found in KM109 treated sample, suggesting that this peak was also the product of the enzymatic degradation of DHPB. These results suggested

that KM109 degrades DHPB in a different manner from the hydrolysis of DHPB by the water. The detailed mechanism will be studied in the near future. Moreover, KM109 degrades significantly more DHPB than buffer control and other esterase. However, even with KM109, it took some time to degrade *Jatropha* phorbol esters presumably because *Jatropha* phorbol esters were not optimal substrate for this enzyme. From those data, we speculate that esterases like KM109, which can hydrolyze bulky esters, in the soil degrade *Jatropha* phorbol esters slowly but steadily because the bacterium such as *A. calcoaceticus* producing KM109 is ubiquitously distributed in the environment including soil.

To examine the tumor-promoting activity of the degradation product of DHPB by KM109, methanol extracts of the reaction mixture (day 0 and 7) were added to Bhas42 cells for the cell transformation test. The number of colony of transformed cells was significantly low in the KM109 sample when compared with the buffer control (Figure 6C). When the number of background colony was subtracted from the colony formed in the presence of sample, there were $64.6 \pm 16.5\%$ less colonies for the KM109 treated sample, while the $22.4 \pm 10.0\%$ decreased colonies in the buffer control. The results of the cell transformation test were consistent with those of the HPLC analyzes (Figure 6A) which showed $57.7 \pm 4.8\%$ of DHPB was degraded by treatment with KM109 while hydrolysis of DHPB by water (buffer control) was $36.9 \pm 21.1\%$.

4.4 Conclusion

Our observations that (1) components and tumor promoting activity of *Jatropha* phorbol esters in the oil cake completely disappeared by

5-weeks-incubation with soil and that (2) *Jatropha* phorbol esters did not transfer into plants grown in *Jatropha* oil cake-supplemented soil, which are useful information for utilization of *Jatropha* oil cake as a fertilizer. (3) Additionally, *Jatropha* phorbol esters can be degraded by esterase KM109 from *A. calcoaceticus* widely distributed in the environment including soil.

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References

- Aregheore EM, Becker K, Makkar HP. 2003. Detoxification of a toxic variety of *Jatropha curcas* using heat and chemical treatments, and preliminary nutritional evaluation with rats. *South Pac J Nat Appl Sci* 21: 51-56, doi: 10.1071/SP03010.
- Chikusa Y, Hirayama Y, Ikunaka M, Inoue T, Kamiyama S, Moriwaki M, Nishimoto Y, Nomoto F, Ogawa K, Ohno T, Otsuka K, Sakota AK, Shirasaka N, Uzura A, Uzura K. 2003. There's No Industrial Biocatalyst Like Hydrolase: Development of Scalable Enantioselective Processes Using Hydrolytic Enzymes. *Org Process Res Dev*, 7: 289-296. doi: 10.1021/op034014b.
- Gaur R, Gupta A, Khare SK. 2008. Purification and characterization of lipase from solvent tolerant *Pseudomonas aeruginosa* PseA. *Process Biochem* 43: 1040-1046. doi: 10.1016/j.procbio.2008.05.007.
- Goel G, Makkar HP, Francis G, Becker K. 2007. Phorbol esters: structure, biological activity, and toxicity in animals. *Int J Toxicol* 26: 279-288. doi: 10.1080/10915810701464641.
- Haas W, Sterk H, Mittelbach M. 2002. Novel 12-deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *Jatropha curcas*. *J Nat Prod* 65: 1434-1440. doi: 10.1021/np020060d.
- Hasegawa G, Shimonaka M, Ishihara Y. 2012. Differential genotoxicity of chemical properties and particle size of rare metal and metal oxide nanoparticles. *J Appl Toxicol* 32: 72-80. doi: 10.1002/jat.1719.
- He Y, Kikutsugi H. 2011. Method for removing phorbol ester from organic material, method for producing organic material having high protein content, organic material having high protein content, method for producing feed, and

431 feed. E.P. Patent No. 2397240A1.

432 Joshi C, Mathur P, Khare SK. 2011. Degradation of phorbol esters by
433 *Pseudomonas aeruginosa* PseA during solid-state fermentation of deoiled
434 *Jatropha curcas* seed cake. *Bioresour Technol* 102: 4815-4819. doi:
435 10.1016/j.biortech.2011.01.039.

436 Kumar A, Sharma S. 2008. An evaluation of multipurpose oil seed crop for
437 industrial uses (*Jatropha curcas* L.): A review. *Ind Crops Prod* 28: 1-10,
438 doi:10.1016/j.indcrop.2008.01.001.

439 Mahanta N, Gupta A, Khare SK. 2008. Production of protease and lipase by
440 solvent tolerant *Pseudomonas aeruginosa* PseA in solid-state fermentation
441 using *Jatropha curcas* seed cake as substrate. *Bioresour Technol* 99: 1729-1735.
442 doi: 10.1016/j.biortech.2007.03.046.

443 Makkar HP, Becker K, Sporer F, Wink M. 1997. Studies on Nutritive Potential
444 and Toxic Constituents of Different Provenances of *Jatropha curcas*. *J Agric*
445 *Food Chem* 45: 3152-3157. doi: 10.1021/jf970036j.

446 Makkar HP, Maes J, De Greyt W, Becker K. 2009. Removal and degradation of
447 phorbol esters during pre-treatment and transesterification of *Jatropha curcas*
448 Oil. *J Am Oil Chem Soc* 86: 173-181. doi: 10.1007/s11746-008-1327-6.

449 Mitsuhashi K, Yamashita M, Hwan YS, Ihara F, Nihira T, Yamada Y. 1999.
450 Purification and Characterization of a Novel Extracellular Lipase Catalyzing
451 Hydrolysis of Oleyl Benzoate from *Acinetobacter nov. sp.* Strain KM109. 1999.
452 *Biosci Biotechnol Biochem* 63 (11): 1959-1964. doi: 10.1271/bbb.63.1959.

453 Ohmori K, Sasaki K, Asada S, Tanaka N, Umeda M. 2004. An assay method for
454 the prediction of tumor promoting potential of chemicals by the use of Bhas 42
455 cells. *Mutat Res* 557: 191-202. doi: 10.1016/j.mrgentox.2003.10.014.

456 Rakshit KD, Darukeshwara J, Rathina Raj K, Narasimhamurthy K, Saibaba P,
457 Bhagya S. 2008. Toxicity studies of detoxified Jatropha meal (*Jatropha curcas*)
458 in rats. Food Chem Toxicol 46: 3621-3625. doi: 10.1016/j.fct.2008.09.010.

Figure legends

Figure 1. Structure of 12-deoxy-16-hydroxyphorbol-4'-[12'-14'-butadienyl]-6'-[16', 18', 20'-nonatrienyl]-bicyclo [3. 1. 0] hexane-(13-*O*)-2'-[carboxylate]-(16-*O*)-3'-]8'-butenoic-10'] ate (DHPB)

Figure 2. Scheme of the schedule of mustard spinach cultivation and soil sampling using Jatropha oil cake-supplemented soil. Jatropha oil cake was blended with a soil and additional fertilizers on day 0. The seeds of mustard spinach were planted on day 7. After cultivation for 4 weeks, mustard spinach was harvested on day 35. Soil sample was collected on days 0, 21 and 35.

Figure 3. Detection of Jatropha phorbol esters in soil by HPLC. Jatropha oil cake was blended with a soil at the amount of the single dose (8.38 g/1.8 kg soil), double dose (16.76 g/1.8 kg soil) or quadruple dose (33.52 g/1.8 kg soil) on day 0. Jatropha phorbol esters were extracted from soil sampled on days 0, 21 and 35. The control soil was sampled on days 0 and 35. Jatropha oil cake, without blending with a soil (oil cake), was also extracted. Crude extract from Jatropha seeds was used for positive control (Jatropha phorbol esters). Retention time zone of Jatropha phorbol esters are indicated by bold bar in HPLC chart. Peak derived from DHPB is indicated by arrow.

Figure 4. Tumor-promoting activity of soil extracts in the Bhas 42 cell transformation assay.

Number of transformed colonies of Bhas42 cells induced by soil extracts sampled on day 0 (open circle), 21 (shaded circle) and 35 (filled circle) are shown. Shaded area indicates the range for mean $\pm$ 2SD of the numbers of colonies in the control group. Data are expressed as means $\pm$ SD of six experiments in duplicate. *: $P < 0.05$, (vs 0 mg/well).

Figure 5. (A) Detection of Jatropha phorbol esters in mustard spinach by HPLC.

Mustard spinach grown in Jatropha oil cake-supplemented soil (single, double or quadruple dose) or control soil was harvested on day 35, and phorbol esters were extracted from mustard spinach. The crude extract from Jatropha seeds was used as a positive control (Jatropha phorbol esters). Retention time zone of Jatropha phorbol esters is indicated by bold bar. (B) Tumor-promoting activity of mustard spinach extracts in the Bhas 42 cell transformation assay. Mustard spinach was grown in the soil supplemented with single (open square), double (shaded square) or quadruple (filled circle) dose of Jatropha oil cake, or control soil (open circle). Data are expressed as mean $\pm$ SD of six experiments in duplicate. The variation in reference to the concentration of mustard spinach extracts was analyzed by two-way ANOVA ($P = 0.0297$ (mustard spinach), $P < 0.0001$ (dose), $P = 0.8438$ (mustard spinach x dose)).

Figure 6. (A) Degradation of DHPB by esterase. DHPB incubated with esterase (lipase from *A. niger* (open square), SNSM87 (filled square), and KM109 (filled circle)) or buffer as a control (buffer control; open circle) were analyzed with HPLC. Data are presented as mean $\pm$ SD ($n = 3 - 9$). The

509 variation in reference to the experimental day was analyzed by two-way ANOVA
510 ($P < 0.0001$ (day), $P < 0.0001$ (group), $P < 0.0001$ (day x group)). *: $P < 0.01$ vs.
511 buffer control at the day. (B) Chromatogram of DHPB samples at 282 and 242
512 nm. DHPB incubated with KM109 and buffer control were analyzed on day 0
513 (dotted line), 7 (gray line), and 28 (solid line). (C) Tumor-promoting activity of
514 methanol extracts of the reaction mixture including DHPB. The methanol
515 extract from reaction mixture was prepared at experimental day 0 and 7. The
516 numbers of transformed colonies are shown (white bar, buffer control; black bar,
517 KM109). The data were presented as mean $\pm$ SD ($n=4$) and were analyzed by
518 t -test. * $P < 0.01$ vs. buffer control at the day.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

B

Figure 6