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Determination of toxic phorbol esters in biofertilizer produced with *Jatropha curcas* seed cake

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ABSTRACT: Seeds of *Jatropha curcas* contain oils, essential amino acids, and toxic phorbols that are found to be carcinogenic. Application of *J. curcas* seed cake for producing biofertilizer for growing sweet potato was studied in this work. The *J. curcas* seed cake, soil, and sweet potato were examined to determine the contamination of phorbol esters residue after the plant was treated with *J. curcas* seed cake fertilizer. Our study develops a technique to confirm the presence of phorbol esters residue by 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 recommended to confirm phorbol esters residue. The results showed that both sweet potato and soil after harvesting did not have contamination with toxic phorbol esters.

KEYWORDS: residue, ionization, sweet potato, toxic substance

INTRODUCTION

Jatropha curcas is a drought-resistant plant which belongs to the Euphobiaceae family. It is widely cultivated in central and south America, south-east Asia, India, and Africa. Since it is a multipurpose tree, it has been promoted for planting in Thailand. It can be grown in low to high rainfall areas either in farms as a commercial crop or as a hedge to protect fields and prevent erosion. Its seeds contain a high amount of oil content approximately 50–60% which is a good source of biodiesel fuel [1]. After extraction of oil, the *J. curcas* seed cake is rich in protein between 50–64%. Except for lysine, all other essential amino acids in the cake have been reported to have higher concentrations than those referenced by the Food and Agriculture Organization. However, the *J. curcas* seed cake was found to be toxic to mice, rats, calves, sheep, goats, human, and chickens, and its use has been restricted. Some antinutritional components, such as saponin, phytate, trypsin inhibitor, glucosinolateds, amylase inhibitors, flavonoids, vitexin, isovitexin, and cyanogenic, as well as toxic irritant compounds, such as curcin, β-D-glycosides of sitosterol, and 12-deoxy-16-hydroxy phorbol were reported in *J. curcas* seed cake. These phorbol esters (PEs) present at high levels in the cake have been identified as the main toxic agent. If these toxic agents can be removed, *J. curcas* seed cake could be used as a protein source for livestock feeds [2].

The term PE is used today to describe a naturally occurring family of compounds widely distributed in plant species of the Euphobiaceae family. These compounds are esters of tigliane diterpenes. The fundamental substance, the alcohol moiety of this family compounds, is a tetracyclic diterpene (Fig. 1).

The hydroxylation of this fundamental substance in various positions and concentrations to various acid moieties by ester bonding characterize the large number of compounds termed PE which have the main chemical structure shown in Fig. 1.

The biological effect of this compound is tumour promotion and cell proliferation. The determination of PE content is usually analysed by high performance liquid chromatography with UV detector (HPLC-UV) [3]. There is little literature on PE confirmation. The first work identifying PE in *J. curcas* oil by ESI-MS/MS found that the parent ion of PE has molecular mass 711 and its daughter ions have mass 693, 383, 311, and 293, respectively [4].

The second method was liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique to

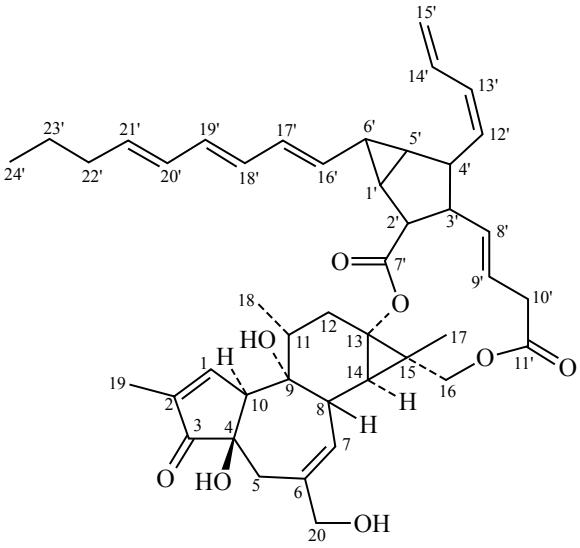

Fig. 1 Chemical structure of the main phorbol esters and tigliane.

detect tumour-promoting diterpene esters of tigliane within plant extracts[5]. The experiment employed fractionation on a C18 high-performance liquid chromatography (HPLC) column followed by MS-MS multiple reaction monitoring (MRM) with collision induced dissociation. The result showed a characteristic precursor ion at molecular mass 311 which was monitored simultaneously with one of its fragmentation products at mass 293.

The objective of this study is to determine PE residues in *J. curcas* seed cake, sweet potato, and soil after harvesting by using LC-MS/MS with MRM mode for PE.

MATERIALS AND METHODS

Seed cake

Two *J. curcas* seed cakes were obtained after oil recovery by pressing extraction. The oil content in these cakes was about 1 w% and 10 w%, respectively. Sweet potato and soil were obtained after harvesting from an experiment on growing sweet potato with *Jatropha* seed cake as biofertilizer during December, 2008 to March, 2009 at a plantation in Ayutthaya province. The amount of seed cake in each experiment was 1600 kg/rai (10 t/ha). Phorbol-12-myristate-13-acetate (TPA) was obtained from sigma chemical Co., Ltd (St. Louis, MO). All other chemical solvents used were of analytical grade.

Determination of phorbol esters content

Phorbol esters were extracted from 10 g of *J. curcas* seed cake, dry sweet potato, or soil after harvesting. Extraction used 200 ml of methanol as solvent in Soxhlet extractor running for 4 h. After extraction, methanol was further evaporated by vacuum rota-evaporator until 10 ml of solution was obtained. One portion of solution was used to determine the PE concentration by HPLC-UV. Another portion of solution was analysed by LC-MS/MS MRM mode for confirmation. PE concentration was determined as described[6]. An aliquot was loaded on an HPLC-UV reverse phase C18 LiChrophere 100, 5 µm (250×4 mm ID. from Merck, Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at room temperature (35 °C) and the flow rate was 1 ml/min using isocratic elution of 1:1 (v/v) deionized water mixed with acrylonitrile as mobile phase. The group of PE peaks was detected at 280 nm and appeared at 8–12 min of chromatogram. The results were expressed as equivalent to an external standard, phorbol-12-myristate-13-acetate. The detection limit for phorbol esters quantification was 0.03 mg/g.

Confirmation of PE LC conditions

Chromatographic separation of PE was performed on C18 water Atlantis (5 µm, 2.7×50 mm). Isocratic program was used with mobile phase, consisted of solvent (50 mm ammonium acetate + acetonitrile, 9 + 1). The flow rate was 0.2 ml/min, the injection volume was 40 µl MS/MS conditions. MS/MS was performed on a Micromass Quattro Ultima triple-quadrupole 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.

RESULTS AND DISCUSSION

The results of phorbol esters analysis by HPLC-UV in seed cake with low content of oil (1 w%) and high content of oil (10 w%) from two chromatograms show a group of peaks indicating low-, and four peaks indicating high-oil content occurring at the retention time from 8–12 min. The concentrations of PE in both chromatograms were calculated by the summation of all area peaks which appeared between 8–12 min and their concentrations were compared with the concentration of TPA used as external standard. The concentrations of PE in seed cake with low and high content

of oil content were 0.3281 mg/g and 0.9295 mg/g, respectively. This result indicated that seed cake with low content of oil had less PE concentration than seed cake with high content of oil.

The confirmation of PE by LC-MS/MS with MRM mode of both seed cakes (low and high PE concentration) showed two chromatograms of ionization peaks of PE. Both chromatograms had one peak of ionization that occurred at the same time (1.54 min), one ionization peak that represented parent molecule with mass 711 ionized to precursor ion with mass 311 and another ionization peak ionized from precursor ion to produce an ion with mass 293. This result could be explained assuming that PE was fragmented by eliminating its ester groups (C_{13} and C_{16} of Fig. 1) and alcohol group (C_{20} of Fig. 1) to diterpene ester of tigliane type (molecular formula = $C_{20}H_{23}O_{23}$) resulting in a 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 PE residue in sweet potato and soil after harvesting as confirmed by the results that follow.

The three samples of sweet potato were analysed by HPLC-UV to give three chromatograms, i.e., PE in control (without seed cake), low, and high PE content in seed cake. No PE peak was detected at the retention time between 8–12 min for sweet potato of control, but PE peaks for sweet potato of seed cake with low and high PE content were observed. The PE content in sweet potato grown by seed cake was 0.1285 mg/g and 0.1664 mg/g. Chromatograms showed no ionization peaks at the corresponding time (1.54 min) as shown in both ionization peaks of PE in seed cake as observed previously in this study. This suggests that both sweet potato grown by seed cake were not contaminated with toxic PE compounds from seed cake fertilizer.

In the case of soil sample analysis, the result of HPLC-UV from low and high PE content in seed cake was obtained from two chromatograms. The PE confirmation by LC-MS/MS was obtained from two chromatograms of ionization peaks of PE. The result from HPLC-UV chromatogram of both soil samples had a small peak occurring between 8–12 min but after confirmation they did not show the two ionization peaks occurring at 1.54 min, indicating that no PE was left in the soil after harvesting.

CONCLUSIONS

It can be concluded that LC-MS/MS with multiple reaction monitoring mode can be used to confirm the presence of PE residues in sweet potato and soil after harvesting. In addition, *Jatropha* seed cake with low and high PE content can be applied for biofertilizer with environmental friendly and safety.

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