

Original Article

## Identification and quality of four varieties of adlay

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### Abstract

Simple methods were used to identify and evaluate the quality of adlay, which is currently in focus for its nutraceutical aspect and functional food. Four varieties of adlay, *Coix lachryma-jobi* L., Poaceae, were investigated for their physicochemical properties, DNA fingerprints, antioxidant activities, and the content of coixenolide. They were called waxy adlay (AD1), stone adlay (AD2), grey adlay (AD3), and normal adlay (AD4). Morphology of intact seeds of the four varieties can be distinguished visually. Iodine stained on starch granules of all varieties, except AD4, were blue. The gelling temperatures ranged from 67.92-73.27°C. TLC-fingerprints of acetone and methanol were documented. DNA fingerprints were established to differentiate the varieties. AD1 showed distinct properties over the others in the antioxidant activities, acetone extractive and coixenolide content.

**Keywords:** adlay, Job's tears, physicochemical properties, DNA fingerprint, antioxidant activity, coixenolide

### 1. Introduction

Adlay, *Coix lachryma-jobi* L., Poaceae, is a grass crop that has long been used in traditional Chinese medicine and as a nourishing food. Adlay is believed to be beneficial to the gastrointestinal tract and may be used as a prebiotic due to its modifying effect on some intestinal bacteria (Chiang *et al.*, 2000). Tokuda *et al.* (1990) found that a methanolic extract of adlay exhibited an anti-tumor promoting activity. Numata *et al.* (1994) showed that an acetone extract of adlay inhibited transplantable mouse tumors. Shih *et al.* (2004) reported that adlay, a natural anti-inflammatory agent, could affect colorectal carcinogenesis through modulation of COX-2 expression. The main components in the acetone extract were four free-fatty acids (palmitic, stearic, oleic, and linoleic acids), coixenolide [1-methyl-2-(cis-9-hexadecenoyloxy) propyl trans-11-octadecenoate, C<sub>38</sub>H<sub>70</sub>O<sub>4</sub>]. Coixenolide ex-

hibited antitumor activity towards Ehrlich ascites sarcoma in mice (Tanimura *et al.*, 1961). Wu *et al.* (2003) found that feeding coarse *Coix lachryma-jobi* seed reduced plasma TBARS values. Serum and liver lipids in hamsters were also lowered after feeding with adlay (Lin *et al.*, 2008). The polyphenolic substances contained in *Coix lachryma-jobi* provide good anti-oxidation capability and good free-radical-capturing activity (Kuo *et al.*, 2001; 2002).

In this study, simple methods were employed to identify and evaluate the quality of adlay, which is now in focus for its nutraceutical aspect and as a functional food. In this work, four varieties of adlay, *Coix lachryma-jobi* L., Poaceae, were investigated to compare the physicochemical properties, DNA fingerprint, antioxidant activity, and the content of coixenolide, so that their qualities can be comparatively evaluated and differentiated. These specimens were waxy adlay (AD1), stone adlay (AD2), grey adlay (AD3), and normal adlay (AD4).

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## 2. Experimental

### 2.1 Plant Materials

Two varieties of intact fruits of adlay were purchased from a wholesaler in Loei Province; one was commercially called waxy adlay (AD1) and the other normal adlay (AD4). These two varieties were the main commercial adlays in Thailand. Stone adlay (AD2) were bought from Chiang Rai Province, the grey adlay (AD3) from Sakon Nakhon Province. All varieties of seed were cultivated and the plants, flowers, and seeds were dried and deposited in the Pharmaceutical Sciences Faculty, Voucher Nos. KL1-KL4. Samples were dehulled then blended into powder and passed through a 20 mesh sieve. A dehulled and polished sample of AD5 was purchased from a grocery in Khon Kaen Province.

### 2.2 Chemical reagents and Equipments

All chemicals were analytical grade. Ascorbic acid was obtained from Ajax Chemicals, thiobarbituric acid (TBA), 1, 5-pentanediol and ferric chloride were obtained from Sigma-Aldrich, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH),  $\alpha$ -tocopherol, sodium methoxide, vanillin and 2, 3-butanediol were obtained from Fluka. A Hewlett Packard gas chromatograph, 6890 series, U.S.A. equipped with a DB-wax (0.2  $\mu$ m) capillary column, 30m $\times$ 0.25 mm, (J&W Scientific, USA) was used. The spectrophotometer was a Shimadzu's 1700, Japan, the light microscope was Olympus BH-2, Japan, Differential Scanning Calorimeter, DSC, Mettler-Toledo, Switzerland, TLC pre-coated plates, silica gel 60 F254, were purchased from Merck, Germany.

### 2.3 Physicochemical characterisation

#### 2.3.1 Morphology of seeds with seed coat and dehulled

Intact and dehulled seeds of each variety were observed and recorded.

#### 2.3.2 Microscopic characterisation Characterisation of starch granules

Starch granules were prepared from the white part of the dehulled seeds, by grinding into fine powder, soaked with distilled water, precipitated, and dried at 45°C, then ground into fine powder. Starch granules were examined unstained and stained with 1% iodine solution under a light microscope.

#### 2.3.3 Determination of gelling temperatures of starch granules

Starch powder (2 mg) was sealed in an aluminum DSC pan and allowed to stand for 30 min. A DSC profile

was recorded at the temperature range of 25-120°C and the increasing rate of 10°C.min<sup>-1</sup>. An empty sealed pan served as a control. The gelatinised temperature was determined from the enthalpy change.

#### 2.3.4 TLC fingerprints

A portion of 5  $\mu$ L of a 10% of coix powder sample in solvents (acetone and methanol) was loaded on a precoated TLC plate (silica gel 60 F254) and developed in a mixture of dichloromethane:ethanol (95:5). The plate was dried and sprayed with a 2% vanillin in ethanol and followed by a solution of 10% sulfuric acid in ethanol, then heated at 105°C for 5 min.

Detection was also done by spraying with a 0.6 mM DPPH solution, and visually observed to monitor the antioxidative spots.

### 2.4 Establishment of DNA fingerprints

#### 2.4.1 Preparation of genomic DNA

DNA was extracted from 2-5 dried seeds (depended on their sizes) using the method of Tai and Tanksley (1990) with some modifications.

#### 2.4.2 RAPD-PCR amplification

Approximates of the genomic DNA were used as a template for PCR with three random primers OPA10 (GTGATCGCAG), OPA15 (TTCCGAACCC), and OPA20 (GTTGCGATCC). Amplification was performed in a reaction mixture containing 5.32  $\mu$ L of sterilised distilled water, 1  $\mu$ L of 10  $\times$  PCR buffer, 0.6  $\mu$ L of 25 mM MgCl<sub>2</sub>, 1  $\mu$ L of 2 mM dNTP, 0.08  $\mu$ L of 0.625 U AmpliTaq Gold DNA polymerase, 1  $\mu$ L of 10 pM primer, and 1  $\mu$ L DNA in a total volume of 10  $\mu$ L PCR programmed were as follow: 1 cycle of 2 min at 95°C, then 45 cycles each of 30 sec at 94°C, 30 sec at 40°C, 1.30 min at 72°C followed by one final extension cycle of 5 min at 72°C, to ensure that the primer extension reaction was completed. For reaction of specific primers, 5SrRNA (5S-P1 (GGATCCGTGCTGGGGCGAG AT) and 5S-P2 (GGAGTCTTAGTGCTGGTATGA)) performed as RAPD-PCR amplification, except using 4.32  $\mu$ L of sterilised distilled water.

#### 2.4.3 Agarose gel electrophoresis

The amplification products were size separated by electrophoresis in 1.5% and 1% (w/v) agarose (FMC) gel for random and specific primers reaction mixtures, respectively, with 5X TBE buffer at 100 V, stained with ethidium bromide, and photographed under UV light. The 100 bp DNA Ladder Plus (Gene Ruler TM) was used as a molecular size marker.

## 2.5 Determination of antioxidant activities

### 2.5.1 Sample Preparation

Stock solution of a 10% w/v of each sample in methanol was prepared separately in a volumetric flask. They were sonicated for 1 hr and allowed to stand overnight at room temperature, mixed and filtered. The filtrate of each sample was diluted as appropriate to 1-4 times for DPPH assay. For TBARS assay, 50% (w/v) of stock solutions of samples were prepared.

### 2.5.2 DPPH assay

The methanolic solution containing 0.06 mM DPPH radicals, 1.5 ml were mixed with 0.5 ml of the extract solutions. The mixtures were allowed to stand for 15 min in the dark, and the absorbance was then measured at 517 nm against a blank (methanol). The ascorbic acid (Vit C) and  $\alpha$ -tocopherol (Vit E) were used as positive controls. The scavenging ability was calculated as follows:

$$\text{Scavenging ability (\%)} = \frac{(\text{A}_{517\text{nm}} \text{ of blank} - \text{A}_{517\text{nm}} \text{ of sample}) \times 100}{\text{A}_{517\text{nm}} \text{ of blank}}$$

### 2.5.3 TBARS assay

The mixture of 0.45 mL of 50 mM potassium phosphate buffer, pH 7.4, 0.125 mL of 0.1 mM  $\text{CuSO}_4$ , 65 ml of 3 % (v/v)  $\text{H}_2\text{O}_2$ , 50 mL of sample extract and 0.25 mL of 50% (w/v) homogenate (50% (w/v) pig brain in phosphate buffer, pH 7.4). The mixture was incubated at 37°C for 2 hrs. Then, 25 ml of 0.1 mM BHT, 0.25 mL of 5 mM EDTA and 0.25 mL of 35 % (v/v) perchloric acid were added. The solution was mixed and centrifuged at 5,400 rpm for 15 min. Mixed 0.25 mL of supernatant and 0.5 mL of 0.67% TBA, was incubated at 100°C for 1 h; then the mixture was cooled and measured at 532 nm. The scavenging ability was calculated as follows:

$$\text{Inhibition of lipid peroxidation (\%)} = \frac{(\text{A}_{532\text{nm}} \text{ of blank} - \text{A}_{532\text{nm}} \text{ of sample}) \times 100}{\text{A}_{532\text{nm}} \text{ of blank}}$$

Antioxidant activities were assessed as  $\text{EC}_{50}$  values (% w/v), the effective concentration of extracts to scavenge DPPH radicals or inhibit lipid peroxidation at 50%, were estimated by interpretation from linear regression analysis.

## 2.6 Determination of coixenolide

Coixenolide was determined by measuring 2, 3-butanediol liberated from coixenolide by acid-catalyzed hydrolysis as described by Yang *et al.* (2004), and using 1, 5-

pentanediol as an internal standard. The treated samples were determined by a GC-FID. The GC conditions were as follows: a DB-Wax capillary column (30 m $\times$ 0.25 mm $\times$ 0.2mm) and a flame ionization detector (FID). The carrier gas was nitrogen with the flow rate of 2.7 mL.min<sup>-1</sup>. The column temperature was programmed from 50-120°C at 10°C.min<sup>-1</sup>, 120-200°C at 8°C.min<sup>-1</sup>, and 200-250°C at 20°C.min<sup>-1</sup>. Both injector and detector temperatures were 250°C. Coixenolide content was stoichiometrically calculated from the 2, 3-butanediol detected.

## 3. Results and Discussion

### 3.1 Physicochemical characterisation

#### 3.1.1 Morphology of seeds with seed coat and dehulled

The intact seeds of adlay can be distinguished visually. The seed coat was shiny but different in colour and size. The average weight of seeds of AD1-AD4 was 0.20 $\pm$ 0.05, 0.12 $\pm$ 0.08, 0.31 $\pm$ 0.05, and 0.20 $\pm$ 0.05 g/seed, respectively. The sizes of waxy adlay (AD1) were slightly smaller than normal adlay (AD4), 8 $\times$ 7 $\times$ 12 and 9 $\times$ 8 $\times$ 11 mm, respectively. The colour of AD1 was very dark purple (Figure 1a), whereas the normal adlay was light brown, as shown in Figure 1d. Seeds of AD3 were dark gray; they were the largest amongst all, the width, length and thickness were 11 $\times$ 9 $\times$ 11 mm as can be seen in Figure 1c. The colour of the seed coat of stone adlay (AD2), was light bluish gray, the smallest compared to others, 6 $\times$ 5 $\times$ 5 mm (Figure 1b). The tegmens of the seeds of

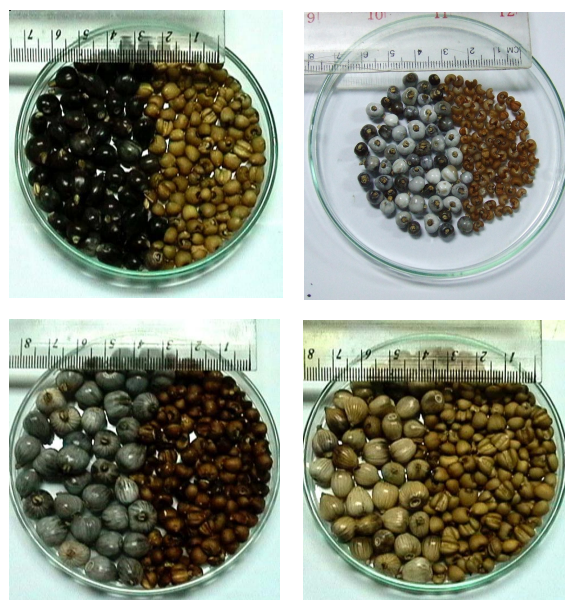


Figure 1. Seeds of various adlays:

- (a) Waxy adlays from Loei Province (AD1)
- (b) Stone adlays from Chiang Rai Province (AD2)
- (c) Grey adlay from Sakon Nakhon Province (AD3)
- (d) Normal adlay from Loei Province (AD4)

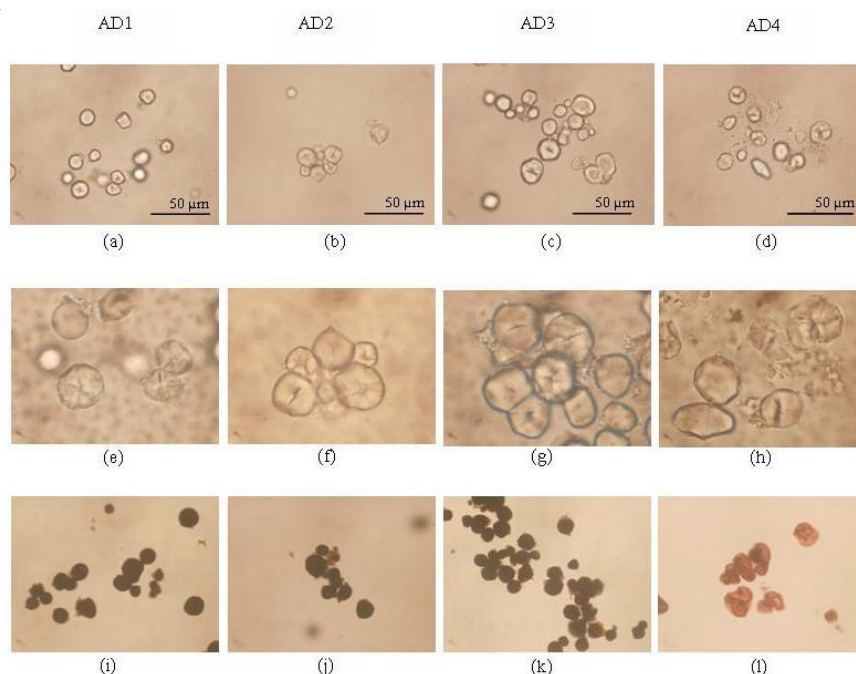


Figure 2. Starch granules of four varieties of *Coix lachryma jobi* AD1-AD4 (a-d) 1.25×2.5×40; (e-h) 1.25×2.5×100; (i-l) 1.25×2.5×40 and iodine stained.

AD1 and AD4 were light brown, AD2 was brown, whereas the AD3 was reddish brown.

### 3.1.2 Microscopic characterisation Characteristic of starch granules

Starch granules of the four varieties were round, the size ranged from 6.25 to 21.25  $\mu\text{m}$ , exists in either single or the compounds of 4 to 5, as can be seen in Figure 2. The starch granules of all varieties were blue-violet when stained with iodine, except AD4, which was reddish brown. The staining indicated that starches of AD1, AD2, and AD3 were normal starch, which was high in amylose, and AD4 was waxy starch which high in amylopectin.

### 3.2 Gelling temperatures of starch granules

The gelling temperatures of all species were around 69-73°C, thus gelling temperature could not be used to distinguish the four varieties.

Li and Corke (1999) found that the gelling temperature of normal adlay was 71.9-79.5°C and the waxy adlay was 71.1-71.4°C, whereas that of rice starches were 61-78 and 55-65°C for normal and waxy, respectively (Belitz, 1999). The temperatures and thermograms of gelatinisation shown in Table 1 and Figure 3, respectively. Iodine staining is a superior mean to distinguish the type of starch found in adlays.

### 3.3 TLC fingerprints

For the chromatograms obtained from all adlay extracts, acetone and methanol exhibited similar compositions, but were different in quantity (Figure 4a). All extracts contained the  $\beta$ -sitosterol, thus it was used as a marker. The content of constituents of AD1 of both extracts was higher than others, especially spot at  $R_f$  0.59, whereas the content of AD2, stone adlay, showed the lowest values.

The detection by DPPH spray reagent (Figure 4b)

Table 1. Gelling temperature, antioxidant activities, acetone extract and coixenlide contents of various adlays, all data are given in mean values with standard deviation.

Varieties Properties	AD1	AD2	AD3	AD4	AD5
Gelling temperature (°C)	70.51±0.29	67.92±0.23	71.82±0.15	71.58±0.11	73.27±0.11
EC <sub>50</sub> of DPPH assay (% w/v)	1.50±0.01	2.95±0.12	1.93±0.05	1.89±0.05	2.72±0.01
EC <sub>50</sub> of TBARS assay (% w/v)	12.33±3.56	17.50±1.25	17.29±2.64	24.39±3.94	18.58±3.59
Acetone extract (% w/w)	8.51±0.03	4.80±0.01	7.23±0.01	7.40±0.02	7.89±0.01
Coixenolide content (ppm)	1,048.25±12.06	774.31±7.10	623.26±9.32	844.94±8.36	455.44±8.88



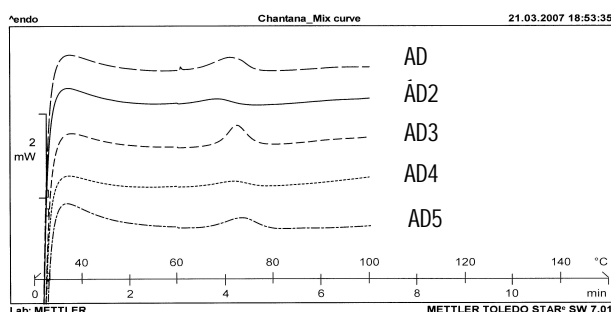


Figure 3. Differential scanning calorimetry profiles, thermogrammes of gelatinisation of various adlays.

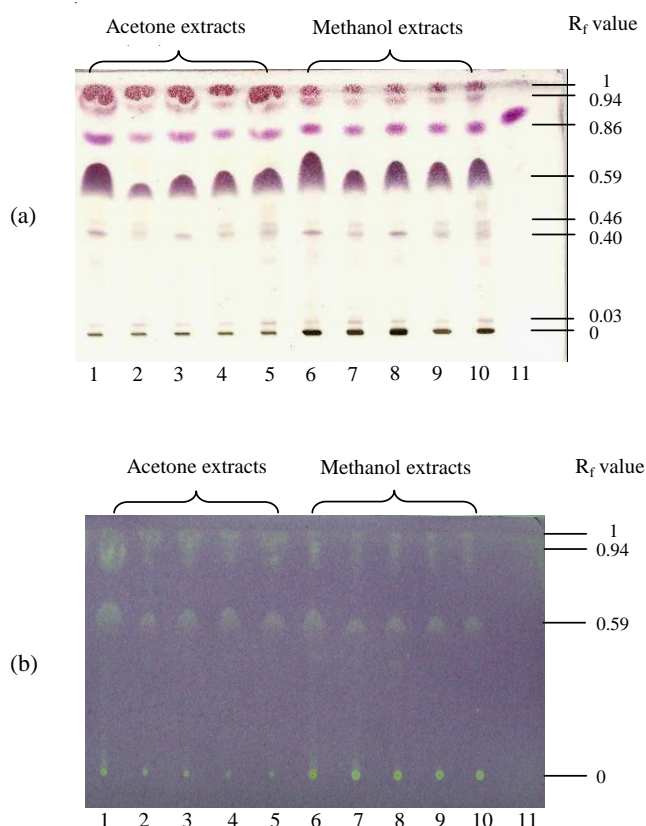


Figure 4. Chromatograms of acetone and methanolic extracts of various adlays which developed on TLC precoated plates (silica gel 60 F<sub>254</sub>, Merck, Germany) using a mixture of dichloromethane: ethanol (95:5) as a mobile phase.

a) The developed TLC was sprayed with 2% vanillin in ethanol and followed by a 10% sulfuric acid in ethanol, heated at 105°C for 5 min.

b) The developed TLC was sprayed with 0.6 mM DPPH solution.

Each picture, lanes 1-5 were acetone extracts of AD1, AD2, AD3, AD4 and AD5, respectively. Lanes 6-10 were methanolic extracts of AD1, AD2, AD3, AD4 and AD5, respectively. Lane 11 was β-sitosterol.

could locate the antioxidative components of the extracts. The high polar compounds ( $R_f$  value, 0) are contained more in methanol extracts, gave rapid action to DPPH radicals,

followed by spot at  $R_f$  0.94 and 1. The least active compounds,  $R_f$  0.59, changed color after 45 min. With the TLC method, methanol and acetone extracts of AD1 showed higher antioxidant activities than other varieties (Figure 4b).

### 3.4 DNA fingerprints

For the discrimination of adlay varieties identified by RAPD markers, accessions were preliminary assayed with OPA10, OPA15, and OPA20, which were random decamer oligonucleotide primers. The RAPD amplified patterns are shown in Figure 5 (a-c). OPA10 yielded more products than OPA20 and OPA15, respectively, with a range from 180 to 1,800 bp. OPA10 (Figure 5a) and OPA20 (Figure 5c) gave several polymorphic bands with some putative varieties-specific bands. In the OPA15 reactive (Figure 5b), all samples showed 1,100 and 1,700 bp products, which were monomorphic. The 350 and 1,200 bp PCR products of OPA10 were specific of the AD4 variety. In addition, a 350 bp fragment produced from RAPD-PCR of AD2 variety is specific for this sample. While using OPA20, two monomorphic bands were discovered across all tested varieties. The putative various specific fragments were also discovered as show in Figure 5c. In this work, we made a preliminary study of adlay varieties identification. As expected, the pattern of DNA bands in our study varied with the used primers. The monomorphic DNA exhibited when used specific primer, 5SrRNA (Figure 5 (d)).

### 3.5 Determination of antioxidant activities

#### 3.5.1 DPPH assay

The  $EC_{50}$  of AD1, AD2, AD3, AD4, and AD5 are shown in Table 1. AD1 gave the highest activity ( $1.50 \pm 0.01$  % w/v of adlay powder).  $EC_{50}$  of ascorbic acid (vit C) and  $\alpha$ -tocopherol (vit E) were  $8.56 \times 10^{-4}$  and  $13.97 \times 10^{-4}$  % (w/v), respectively. Kuo *et al.* (2002) found that methanolic extract of adlay hull showed the highest activity on DPPH scavenging, followed by that of testa, bran, and polished adlay. From our study, the activity depended on the variety and condition of the seed being treated. The polished adlay gave the lowest activity ( $2.72 \pm 0.01$  %), whereas those non-polished gave higher activity, but differently depended on the variety of the adlay. The methanol extract was directly used to determine the activity without drying into residue and redissolved, which is convenient, so that the activity can be quickly estimated and compared.

DPPH is a simple method to determine antioxidant by radical scavenging activity. Antioxidant activity is commonly found in herbs and natural products. This finding would be a source of information for those who consume these cereals. In the view of investigators we truly believed that antioxidant activity reflects ageing and storage conditions of products as well as the nature of the herb by itself.

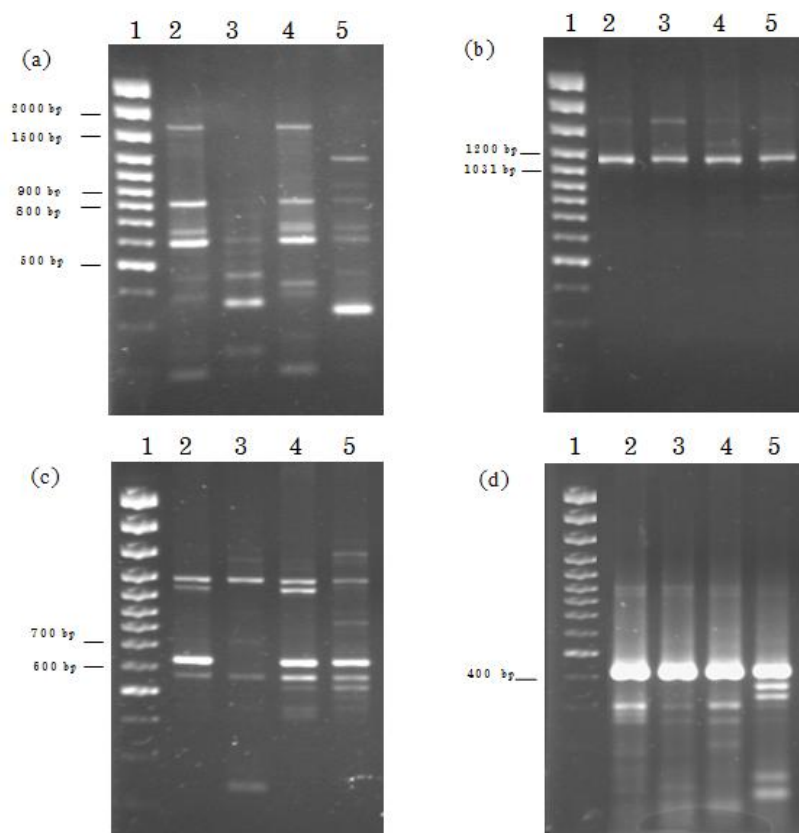


Figure 5. (a-c) PCR products were generated by random decamer oligonucleotide primers; OPA10 (a), OPA15 (b) and OPA20 (c). (d) PCR products were generated by specific primers, 5S-P1 and 5S-P2. Each picture lane 1 signifies the size of the markers. Lanes 2-5 shows PCR products from AD1, AD2, AD3 and AD4, respectively.

### 3.5.2 TBARS assay

Inhibition effects of methanolic extracts of four varieties was shown in Table 1. AD1 also gave the highest activity,  $EC_{50}$   $12.33 \pm 3.56$  % w/v. Lipid peroxidation activity indicate the inhibition ability of coix on the lipid preoxidation. AD1 gave the highest activity to prevent to peroxidation of lipid in pig brain tissue activity ( $12.33 \pm 3.56$  % w/v) compared to other varieties. In any reason, from the discussion with the wholesaler, this variety is in great demand from overseas.

### 3.6 Quantitative analysis of coixenolide

The acetone extracts obtained by Soxhlet apparatus of the four varieties were shown in Table 1. AD1 gave the highest acetone extract  $8.51 \pm 0.03$  % w/w, whereas the stone adlays gave the lowest yield,  $4.80 \pm 0.01$ , however the coixenolide content was not the lowest. The coixenolide content from acetone extract of each variety is shown in Table 1. AD1 gave the highest content of coixenolide ( $1,048 \pm 12$  ppm) and the polished adlay's (AD5) the lowest,  $455 \pm 9$  ppm. Coixenolide is an active constituent in adlay, which had been shown to have antitumor and anticancer activities (Tanimura *et al.*, 1961; Tokuda *et al.*, 1990).

## 4. Conclusions

Two varieties of adlay, AD1 and AD4, were largely grown in the northeastern region of Thailand and sold to a larger agricultural sole distributor in the area. According to the distributor, the AD1 was mainly for export, whereas the AD4 are those distributed as polished adlay and mainly consumed in Thailand. AD3 is domestically growth and the availability is limited to the community. AD2, the stone adlay, usually is used as an ornament grown in the hill-tribe villages. In this study, AD1 showed superior quality due to the high content of acetone extract, coixenolide, and antioxidant activities. AD1 can be distinguished by others from the seed coat, which is very dark purple, the dehulled seeds can be identified by the DNA fingerprint. Iodine stain can be used to distinguish the two main commercial varieties, AD1 (blue stained) and AD4 (reddish brown).

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## References

- Belitz, H.D. and Grosch, W. 1999. Food Chemistry, 2<sup>nd</sup> ed Springer, Germany.
- Chiang, W., Cheng, C.Y., Chiang, M.T. and Chung, K.T. 2000. Effects of dehulled adlay on the culture count of some microbiota and their metabolism in the gastrointestinal tract of rats. *Journal of Agricultural and Food Chemistry*. 48, 829–832.
- Kuo, C.C., Chiang, W.C., Liu, G.P., Chien, Y.L., Chang, J.Y. Lee, C.K., Lo, J.M., Huang, S.L., Shih, M.S. and Kuo, Y.H. 2002. 2, 2-Diphenyl-1-picrylhydrazyl radical-scavenging active components from adlay (*Coix lachryma-jobi* L. var. *mayuen* Stapf) hulls. *Journal of Agricultural and Food Chemistry*. 50, 5850-5855.
- Kuo, C.C., Shih, M.C., Kuo, Y.H. and Chiang W.C. 2001. Antagonism of free-radical-induced damage of adlay seed and its antiproliferative effect in human histolytic lymphoma U937 monocytic cells. *Journal of Agricultural and Food Chemistry*. 49, 1564-1570.
- Lin, Y. and Tsai, C.E. 2008. A study of adlay on lowering serum and liver lipids in hamsters. *Journal of Food Lipids*. 15, 176-189.
- Numata, M., Yamamoto, A., Moribayashi, A. and Yamada, H. 1994. Antitumor components isolated from the Chinese herbal medicine *Coix lachryma-jobi*. *Planta Medica*. 60, 356-359.
- Tai, T.H. and Tanksley, S.D. 1990. A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. *Plant Molecular Biology Reporter*. 8, 297-303.
- Tanimura, A. 1961. Studies on the anti-tumor component in the seeds of *Coix lachryma-jobi* L. var. *mayuen* (Roman.) Stapf. II. The structure of coixenolide. *Chemical & Pharmaceutical Bulletin* 9, 47-53.
- Tokuda, H., Matsumoto, T., Konoshima, T., Kozuka, M. Nishino, H. and Iwashima, A. 1990. Inhibitory effects on Epstein-Barr virus activation and anti-tumor promoting activities of coix seed. *Planta Medica*, 56, 653-654.
- Wu, I.C., Chiang, W.C., Yao, H.T. and Chiang, M.T. 2003. Studies of dehulled adlay its processing food on plasma cholesterol and lipid peroxidation. *Taiwanese Journal Agriculture Chemistry and Food Science*. 41, 254-262.
- Yang, J.H., Tseng, Y.H., Chang, H.L., Lee, Y.L. and Mau, J.L. 2004. Storage stability of monascal adlay. *Food Chemistry*. 90, 303-309.